

Applications of Enzymes to Lignocellulosics

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About the Cover

The cover art shows the structure of *Cellulomonas fimi* GH10 cellulase/xylanase. The structure displays the 8-fold α/β barrel. E127 (the proton donor) and E233 (the nucleophile) are positioned to catalyze the double displacement reaction with retention of the anomeric configuration in the product.

The structure is reproduced from “Microbial Strategies for the Depolymerization of Glucuronoxylan: Leads to Biotechnological Applications of Endoxylanases” by James F. Preston, Jason C. Hurlbert, John D. Rice, Anuradha Ragunathan, and Franz J. St. John, Chapter 12 in this book.

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Applications of Enzymes to Lignocellulosics

Shawn D. Mansfield, Editor
University of British Columbia

John N. Saddler, Editor
University of British Columbia

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Foreword

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ACS Books Department

Preface

During the past decade, a radical shift in the way society views forestry has occurred. A new paradigm is emerging with an emphasis on conservation of natural forestlands and recognition of the inherent value of trees as carbon sinks, wildlife habitats, scenic vistas, and recreational areas. However, with the growing human population, the world's forests are experiencing increasing pressures to meet demands for wood products, fuel, and agricultural land. A dichotomy is emerging between the need for more wood fiber and the increasing societal pressure to preserve our environment. Biotechnology, or more specifically the manipulation of biocatalysts, has the potential to significantly improve traditional pulp and paper manufacturing processes and to minimize the ecological footprint that this industry leaves on our environment.

The forest products biotechnology scientific community has devoted decades of research to elucidate the fundamental microbiology, biochemistry, genetic and enzymology of microorganisms as well as their extracellular enzymes, while concurrently manipulating these biocatalysts to alter lignocellulosic processing in an environmentally benign manner. Consequently, new technologies or applications to complement traditional pulp and paper manufacture (such as xylanase-aided biobleaching, direct bleaching with oxidative enzymes, biopulping with microorganisms, enzyme-induced modifications to inherent fiber morphology, improved paper recycling, enzymatic deinking, mill whitewater remediation, reduction in energy consumption during processing, to name but a few) have evolved. Clearly, the number of applications in the pulp and paper industry has grown incrementally over the past few years and, while some concepts have yet to move beyond the laboratory scale, several biotechnology-based approaches have been successfully implemented at a commercial scale.

As research in this area has not yet reached its full potential, the field of forest products biotechnology remains an expanding, exciting, and promising field of study for both research and commercial development. Although advances in molecular genetics and engineering have already facilitated the generation and production of several industrial enzymes, it is likely that this scientific arena will generate several new and improved enzyme preparations in the very near future. Additionally, the enormous gains currently evident in the field of tree biotechnology have the potential to make some very significant short- and long-term improvements in pulp and paper processing, and *opens the door* to several other potential high-value products and processes.

The symposium on which this book is based was sponsored by the American Chemical Society (ACS) Cellulose and Renewable Materials (formerly the Cellulose, Paper, and Textiles Division) at the 223rd ACS National Meeting in Orlando, Florida, from April 7–11, 2002.

Shawn D. Mansfield

Canada Research Chair
Department of Wood Science
Faculty of Forestry
Vancouver, British Columbia V6T 1Z4
Canada

John N. Saddler

Department of Wood Science
Faculty of Forestry
University of British Columbia
Vancouver, British Columbia V6T 1Z4
Canada

Chapter 1

Applications of Biotechnology in the Forest Products Industry

Shawn D. Mansfield¹ and Ali R. Esteghlalian²

¹Canada Research Chair in Wood and Fibre Quality, Department of Wood Science, University of British Columbia, 4030–2424 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada

²Diversa Corporation, 4955 Directors Place, San Diego, CA 92121

Biotechnology, or more specifically the employment of biological agents, such as fungi, bacteria and more exclusively extracellular enzymes offers a wide range of potential applications, and consequently benefits for the forest products industry. Biotechnological manipulations have been shown to complement and/or enhance conventional wood processing operations, such as pulp and paper manufacture and paper recycling, and may provide many economic and environmental benefits through waste reduction, energy savings and reductions in toxic chemicals released into the environment. The following review is intended to highlight progress in the field over the last decade, and has been compiled to include significant fundamental and industrially relevant findings. Although extensive, it by no means represent a comprehensive review of the plethora of work that has been instrumental in developing this scientific research area.

Introduction

During the past decade, biological agents, such as microorganisms and proteins, have been evaluated for a wide range of applications in the forest products industry. Modifications to virgin and recycled wood fibre, biobleaching, mill effluent remediation, and the production of chemicals and fuels from wood-derived lignocellulosics are some of the technologies currently under investigation and use. Biological treatments, as compared to chemical reagents, offer many processing and environmental advantages, such as ease of degradation and disposal, as well as reaction by-products that are environmentally benign. They also have very low, if any, corrosive or toxic properties, which in turn eliminates the need for expensive specialized equipment (alloys, polymers, etc...). More importantly, enzyme treatment operations can be easily implemented and do not need highly sophisticated technology. With the rapid advancement in the industry over the last decade, the price of enzymes has, and continues to diminish, hence making these attractive technologies more economically viable. Currently, interdisciplinary research in microbiology, molecular biology and enzymology is underway to further improve enzyme production efficiencies and consequently lower commercial pricing, to optimise process parameters and conditions for enzymatic treatment operations, and to effectively recover and reuse enzymes.

This paper provides an overview of how the forest products sector is utilising and fostering biotechnological research to produce more value-added products from wood residues and enhance the profitability of forest industry. These applications are described in three general categories: wood processing, paper manufacturing and wood residue utilisation.

Wood Processing

Biopulping

Pulping is the process by which the macroscopic structure of raw wood fibre is broken apart, rendering the fibre much more pliable and thereby suitable for papermaking. Biopulping is the direct application of fungal cultures onto chip piles to enhance both the production yield and processing of the pulp manufacture. This application has been directed by the need to alter and/or modify two separate target substrates: lignin for mechanical pulp production which improves both pulping and bleaching, and the lipophilic extractives to limit pitch accumulation during processing (1-20). Globally, mechanical pulping

processes are rapidly growing primarily because of their high yields, relatively low capital investment, and the fact that these processes yield pulps with properties advantageous to certain products. However, there are also disadvantages, as they generate pulps of lower product strength and require greater input of electrical energy during processing. Most importantly, in mechanical pulping, pitch problems are often encountered during paper manufacture. Colloidal pitch can condense to form larger droplets that are subsequently deposited on the paper machine or found directly on the ensuing paper. These pitch deposits can cause large economic losses, from shutdown of equipment to contamination of the pulp, as well as the added cost of chemicals such as pitch control agents. In addition, the compounds that form pitch (resin and free fatty acids, sterols and glycerides released from parenchyma cells and resin canals) can represent the primary source of toxicity in totally chlorine-free (TCF) mill effluents, and have a severe detrimental effect on the environment. The nature and chemical composition of the lipophilic extractives varies from mill to mill, and also between pulping and bleaching processes.

It has been well recognised that many microorganisms, particularly the white-rot fungi attack and degrade lignin in wood. One of the first attempts to exploit this natural phenomenon and enhance the pulping process was conducted in 1975 at STFI, Sweden (21,22). This investigation revealed that the application of a cellulase-negative mutant of *Phanerochaete chrysosporium* onto birch and pine wood only slightly delignified the wood, but demonstrated 25-30% less energy requirement during refining, and the ensuing pulps gave better strength properties than the corresponding controls. Since these incipient discoveries, a substantial amount of work has gone into this research area. In 1987, a consortium was established at the Forest Products Laboratory in Madison, WI, involving the aforementioned institution, universities and several pulp and paper companies (23). Comprehensive studies by this collective uncovered other fungal species (*Ceriporiopsis subcermispora*), which generated superior results (5). This initiative, after several years of work, conducted a 50-ton outdoor chip pile experiment. The results appeared to be very attractive based on an economic feasibility study, which has demonstrated savings of more than \$40 US per ton, and a 30% increase in mill productivity per year (23,24).

Traditionally, the seasoning of logs or chips is used as a measure to reduce pitch. During the storage period some extractives are lost through volatilisation, oxidative processes, and enzymatic hydrolysis by microorganisms. Recognition that naturally occurring microorganisms could degrade these undesirable wood constituents prompted the quest for isolates that could generate significant reductions in short periods of time. Work by Farrell *et al.* (25) revealed that after only a 2-week incubation period several strains of *Ophiostoma piliferum* could effectively reduce the extractives content by as much as 50%. This technology was developed into commercial scale and has been marketed under Cartapip®

97, in which a lyophilized fungal inoculum is applied immediately after chipping to reduce the extractive content of wood chips prior to pulping.

Most recently, the development of a company dedicated to researching, establishing and implementing biopulping units (Biopulping International) has been conceived from the technologies developed from the Biopulping Consortium. It has been shown that this technology can successfully employ biopulping to generate chips suitable for sulfite, kraft and biomechanical pulping. Furthermore, it has been suggested that improvements include up to 50% reductions in kappa following pulping, 15-20% reductions in cooking time, a significant reduction in pulping rejects, as well as the generation of superior quality dissolving pulp. Occasionally, improvements in yield and bleachability are also observed. More information can be obtained at: <http://www.ecw.org>.

Debarking

Hydrolases, including cellulases and hemicellulases, have been successfully employed for the retting of non-woody herbaceous fibres such as flax, jute and coconut hull for years (26-31). Pectinases have also been shown to participate in this process, but other than in the retting process, have been limited in their application in the forest products sector. However, recently, pectinases have been shown to be an effective aid in debarking wood logs (32).

Extensive and efficient debarking is required both in mechanical and chemical pulping, as even small amounts of bark residue can cause severe problems with pulp colour. The cambium layer, an interface between the wood and bark, is rich in both pectin and protein. The use of pectinases prior to debarking has been investigated to determine their efficacy as a debarking aid and/or reduce the energy required for this process (32,33). Research has demonstrated, with a commercially available preparation, energy savings as high as 80%, as well as substantial savings in raw material by direct application onto logs after felling (32).

Paper Manufacturing

Fibre Modification

Fibre modification by definition is the alteration in ultrastructural and/or chemistry of wood fibres to improve their papermaking properties. Generally,

finer fibres (thinner cell wall) are considered more desirable for papermaking than coarse fibres, because finer fibres inherently undergo greater collapse without the need for chemicals or extensive mechanical processing (refining). It has been shown that the specific surface area of bonding can be determined solely by the thickness of the fibre wall, which are inversely related (34,35). Thus, the development of mechanisms to effectively reduce coarseness (mass per unit length of fibre) would be a valuable tool to the papermaking industry, since coarseness affects numerous optical (sheet opacity) and mechanical properties (relative bonded area) of paper (34-37).

It has been conclusively demonstrated that low-dose hydrolytic treatments can alter the coarseness of pulp fibres by weakening the fibres' natural integrity. Consequently, fibres are more susceptible to collapse and exhibit a greater degree of conformability during subjection to the mechanical pressures of papermaking. It has been suggested that cellulases either "peel off" the subsequent cell wall layers (38-43) or disrupt the fibres at their weak points, hence increasing their swelling capacity and flexibility (44). Therefore, alterations in paper properties can be facilitated only at the expense of yield loss, as it has been shown that coarseness is proportional to pulp yield (45). Thus, to achieve beneficial modifications one must successfully maintain the balance between fibre strength, bonding strength and yield loss.

It has been observed that cellulases cause greater decreases in strength than other hydrolytic enzymes, such as xylanases or mannanases (46). Furthermore, it has also been shown that even small amounts of contaminating cellulases can significantly compromise the beneficial results that xylanase applications can offer in prebleaching (47,48). It has also been shown that chemical and mechanical pulps respond differently to these treatments, with kraft pulps being more susceptible at comparable enzyme loadings (49). To date, it has proven an arduous task to adjust all the experimental parameters and find a balance point between increased fibre flexibility and fibre degradation. In enzymatic fibre modification, achieving this balance is the key to success.

It has been shown (43,50) that enzymatic treatments (cellulase) of softwood-derived kraft pulp could decrease fibre coarseness, while concurrently maintaining pulp strength and allowing minimum yield loss. Both unfractionated and individual fibre length fractions responded in a similar manner to cellulase treatments, resulting in improvement in handsheet density and smoothness, increased pulp freeness and reductions in fibre coarseness. However, the response of the same fibres to xylanase treatments differed, indicating that fibre composition plays a role in determining the efficacy of the treatments. Having established the effect of cellulase treatment, enzyme treated coarse fibres were combined with the untreated fibres and refined, or alternatively, first refined and then recombined with the untreated fibres. This approach resulted in

improvements in both the tensile and burst indices with minimal enzyme addition and only minimal yield loss (51,52).

Treatments of Douglas-fir kraft pulp with an enzyme preparation containing cellulase and xylanase activities, as well as individual endoglucanase and xylanase components were shown to enhance both pulp characteristics and paper properties (53). The use of cellulase mono-components greatly reduced both yield and strength losses when compared to treatments with the complete cellulase preparations. Xylanase treatments enhanced fibre flexibility, resulting in denser paper sheets with a lower light scattering coefficient. Although treatments with the endoglucanases decreased individual fibre integrity, fibre collapsibility was enhanced. In contrast, when the xylanase and endoglucanase preparations were used in combination, the action of the xylanase increased fibre accessibility to the endoglucanase. The combined action of the two enzymes resulted in enhanced bonding, as indicated by as much as 10% increase in burst index over the level obtained by xylanase alone (53).

Similarly, enzymatic treatment of Subalpine-fir fibres (50) increased handsheet density. However, the inherent fibre coarseness of the original pulps significantly influenced the magnitude of response to enzymatic treatments. While the tensile index of the coarser pulp was improved by treatment with the cellulase preparation, a similar trend was not evident with the pulps of lower initial coarseness. In contrast, the tensile strengths of all pulps, irrespective of the inherent fibre coarseness, were improved by the endoglucanase treatments. In contrast, xylanase treatments did not significantly alter the handsheet properties of any of the pulps, regardless of the nature of the starting furnish.

Polysaccharide degrading enzymes of the hemicellulolytic nature (xylanases and mannanases) have conventionally been employed in the pulp and paper industry as a means to improve the bleachability of kraft fibres, and thus reduce the amount of bleaching chemicals required to achieve target brightness (54). Recently, a substantial amount of work has been conducted to investigate the use of xylanases to alter the response of kraft pulp to refining (55). This approach has previously been explored by other workers (56), and more recently has shown that the selective removal of xylan from unbleached radiata pine kraft pulp yields a furnish that is more easily refined. Both sheet density and tensile strength of the pulp developed quicker with PFI refining after less than 0.5% of the pulp carbohydrate, by weight, (approximately 5% of the available xylan) was enzymatically solubilised.

Monocomponent cellulases (endoglucanases and cellobiohydrolases) from both fungi and bacteria, and their core proteins have recently been compared for their affect on kraft pulp (57,58). It was shown that the presence of cellulose binding domains (CBDs) in the intact enzymes did not affect their action against soluble substrates. However, in the case of insoluble isolated cellulose and chemical pulp, the presence of CBD enhanced the enzymatic hydrolysis of

cellulosic polysaccharides. The effect of CBDs was more pronounced with cellobiohydrolases, hydrolysing mainly crystalline cellulose, than in the endoglucanases, which primarily attack the amorphous cellulose. It was therefore concluded that the presence of CBDs in intact cellulases, influences the accessibility of enzymes to cellulose during saccharification. It was also shown that treatment with intact endoglucanases improved beatability of bleached pulp, when compared to the corresponding core proteins (catalytic domains), suggesting that the presence of CBDs in endoglucanases could result in beneficial effects to pulp properties (57). The paper properties derived from *C. fimi* cellobiohydrolase (Cel48A) treated pulp also demonstrated significantly improved strength after PFI refining, a result attributed to altered (reduced) cellulose crystallinity (58).

In addition to the extensive amount of work that has investigated carbohydrate degrading enzymes for fibre modification, laccase treatment of mechanical pulp fibres seems to activate pulp lignin and enhance bonding strength in a manner similar to that, which increases auto-adhesion of fibres in the manufacture of medium density fibreboard (59). Sheets made from laccase treated mechanical pulp have been shown to exhibit superior tensile strength through the calendering process (60). Pilot scale treatment of mechanical fibres with a laccase enzyme also demonstrated slightly improved paper strength, with concurrent reductions in refining energy (61). Similarly, an increase in strength properties has also been reported after the treatment of chemithermomechanical pulp with manganese peroxidase (62). The potential of using oxidative enzyme systems, as well as lipases, clearly warrants further investigation.

Altering Refining Energy

Refining, or the development of papermaking fibres, is an energy intensive operation. Only a small fraction of the energy spent is consumed during the separation and development of the fibres, while the majority is transformed into heat. Thus, the development of innovative processes that beneficially reduce energy consumption, while maintaining pulp quality is an important objective of the pulp and paper industry.

The biomechanical pulping process, in which wood chips are treated with white-rot fungi prior to mechanical pulping, has successfully demonstrated reductions in refining energy demands (12) and improvements in pulp properties (13), as has been previously discussed (63,64). However, generally, the biopulping process is still considered too slow and difficult for commercial application. Furthermore, this application can also be restricted by geographic location (*i.e.* northern climates). A technically more attractive alternative is the direct application of concentrated enzyme preparations on chip/pulp furnishes.

It has been shown that individual components of the cellulolytic enzyme system (*i.e.* CBH I) can selectively reduce the crystallinity of the cellulose, and subsequently produce more amorphous material with a higher affinity for water. The retention of water by fibres during the refining process reduces the softening temperature of hemicellulose and lignin between the fibres and simultaneously weakens inter-fibre bonding, consequently improving the separation of fibres from one another (65,66). Treatment with CBH I was shown to be able to reduce the refining energy demands by 40%, and prompted a pilot-scale investigation, where 900 kg of TMP rejects were subjected to CBH I prior to refining. An energy saving of 10-15% were obtained during a two-stage secondary refining of the rejects, while maintaining both strength and optical properties (65).

For a number of years it has been suggested that hydrolytic enzymes could be used to decrease the energy consumed during the refining of chemical pulps (67,68). For example, in 1968 a patent was granted for a process where cellulases were applied to kraft pulp fibres to reduce refining time (69). Although similar effects were thought to be achievable using xylanase treatments (70), this was only recently verified with unbleached kraft pulp using cellulase-free xylanases (56,71). The advantage of using xylanase over cellulases is that there is little or no loss in intrinsic fibre strength, and only minor changes to handsheet properties. Moreover, it was suggested that the beneficial effects seemed to be more prominent during low consistency refining, which is more representative of mill-scale refining (55). However, the effectiveness of xylanase-aided refining varies with pulp type, with fully bleached pulps being less responsive in contrast to a high kappa pulp (72).

Release papers, used as backings to hold sticky labels, are high density papers made by extensively refining chemical pulp. Mill trials have shown that treatment with a commercial cellulase reduces the refining energy required for sheet making by 7.5% (73). This cellulase application has been implemented in some mills, and its success may be partly due to a greater tolerance for losses in fibre strength that are often associated with cellulase treatments. Other product grades in this category include high-density papers used in the food industry, condenser papers and glassine, the refining of which all have been shown to be enhanced by cellulase treatments (74). Cellulase treatment of hardwood pulp has also been reported to reduce vessel picking, a problem associated with vessel protrusions that are torn out of paper or yield poor ink-fibre contacts (46).

In addition to using enzymes that alter the cellulose and hemicellulose fractions of woody material, research has also targeted other non-structural minor components such as proteins, as it has been suggested that some of the cell wall proteins (*i.e.* hydroxyproline-rich glycoproteins, arabinogalactan proteins, and glycine- and proline-rich proteins) contribute to the integrity of the microfibrillar architecture of the primary cell wall. A recent study (75) evaluated the use of a commercial proteinase to modify the refinability, and consequently, the

papermaking properties of a radiata pine mechanical pulp. The results indicated that an inter-stage proteinase treatment could not attain any obvious energy savings during secondary refining, however, 10% energy savings were achieved when de-structured wood chips were impregnated with proteinase prior to primary refining, without any deleterious effects to paper properties. Similarly, a laccase preparation was employed in an attempt to alter the refinability of mechanical pulp by oxidizing the lignin moieties (61), and demonstrated approximately 8% savings in refining energy. Further consideration should be given to the use of proteinases and/or laccases for the modification of mechanical pulping, particularly their use in combination with carbohydrate-solubilising enzymes for improving fibre processing and paper properties.

Oxidative enzymes have also been evaluated on high yield pulps in other studies. For example, a manganese peroxidase preparation isolated from *Phanerochaete chrysosporium* was used to treat an alkaline peroxide pulp derived from poplar following secondary refining. The authors (76) demonstrated that the manganese peroxide treatment induced both internal and external fibrillations to the fibres, and consequently resulted in improvements in pulp quality. It was suggested that the enzymatic treatment following secondary refining resulted in 25% energy savings during fibre development, and maintained or improved the physical properties of the ensuing paper (76).

Enhancing Drainage and Machine Speed

Efficient drainage of pulp furnishes on the wire of paper machines is desired to maximise machine speed. Since the initial report in 1988 (77), many researchers have explored the use of enzymatic treatments for increasing pulp freeness. Most of this past work has shown that cellulases, particularly endoglucanases, yield the largest increases in freeness (41,78,79). Pilot plant trials (42) and mill trials (73,80,81) have since confirmed the improvements to machine runnability. Cellulases have also been successfully used in combination with drainage-aid polymers to improve drainage properties (82,83).

Since the presence of fines and highly fibrillated fibres are associated with low pulp freeness, several theories have been proposed to explain the freeness increases occurring after enzymatic treatments. The enzymatic attack may involve a peeling mechanism (41), which removes fibrils and fibre bundles that naturally have a high affinity for water, and leaves the fibres less hydrophilic and easier to drain. Alternatively, it has been suggested that enzymes act preferentially on fines (46) which have a propensity to block up interstices in the fibre network. The increase in drainage has also been attributed to the cleaving of amorphous cellulose on the surface of fines (84). All of these proposed

mechanisms seem plausible, and their relevance may depend on the type of furnish.

During the processing of recycled furnishes, other contaminants may also hinder stock drainage. The use of amylase to degrade starch in old corrugated containers has been shown to improve the drainage of this recycled furnish, and this concept has been successfully tested in a mill (85). The amylase treatment has the advantage of avoiding the loss in intrinsic fibre strength that often occurs with the application of cellulases.

Bleaching

The kraft pulping process successfully removes a large percentage of the lignin, and dissolves and degrades the hemicelluloses leaving the cellulose relatively intact. However, simultaneously, this process generates chromophoric material and recalcitrant lignaceous moieties that need to be removed with additional bleaching agents. The removal of these materials is difficult as they are physically entrapped in, and covalently bound to, the carbohydrate moieties in the pulp matrix (87,88). Biobleaching, the use of microorganisms or their extracellular enzymes for the bleaching process, has enabled the pulp and paper industry to reduce the use of chlorine-based bleaching chemicals, and consequently minimise the adverse environmental impacts of mill effluents. Different groups of enzymes, including xylanases, mannanases and oxidative enzymes, have been shown to remove or alter chromophoric compounds, and increase the brightness of the resulting pulp.

Xylanases

The xylan-degrading enzymes, xylanases, can be used as an indirect bleaching agent, as they do not directly attack the residual lignin moieties associated with the pulp. It has been suggested that the lignin in both wood and pulp is covalently bound to the wood hemicellulose (88), and therefore cleavage of the accompanying carbohydrate moieties enhances lignin removal. Viikari and co-workers (89) first demonstrated that by cleaving these bonds with hemicellulolytic enzymes that lignin could effectively be removed from both pine and birch kraft pulps. This study reported a 25% reduction in the consumption of active chlorine by the application of xylanase, or for the same charge of active chlorine charge, more extensive delignification to lower kappa numbers occurred when compared to the reference pulps. This groundbreaking investigation paved the path for several other successes, where xylanases have been shown to modify pulp properties and reduce the chemical requirements for bleaching both

softwood and hardwood kraft pulps (90-99). However, the major effects of xylanase treatments are only apparent after subsequent bleaching (100), indicating that the enzyme works by enhancing the reaction mechanisms of the other bleaching chemicals.

Xylanases have been shown to be more effective on hardwoods, which can be attributed to the fact that xylan constitutes over 90% of the hemicellulose in hardwood kraft pulps, but only 50% of that in softwood pulp (101). There are several theories as to how xylanases aid the bleaching of kraft pulp: one hypothesis suggests that the xylanases partially hydrolyse the reprecipitated xylan on the pulp without degrading the cellulose portion. As solubilisation of the hemicellulose proceeds, a substantial increase in fibre porosity increases the accessibility to the remaining lignin molecules, and therefore during subsequent bleaching stages more lignin can be extracted from the fibres (102,103). Ultimately, reducing the quantities of bleaching chemicals required to obtain the desired paper brightness (100,104-115). Another common hypothesis suggests that the xylan backbone, which has lignin moieties bound to it via lignin-carbohydrate complexes (LCC), is hydrolysed by the action of the enzyme and made water-soluble. Consequently, the increased solubility allows for enhanced diffusion away from the pulp fibres (102,103).

While several groups have demonstrated success with the inclusion of xylanases in the bleaching sequence, others have not met the same success. This may be a function of the specificity of the enzymes employed, as it has been shown that different hemicellulases, even those produced by the same organism, can display different behaviours on pulp fibres (109,116-119). Furthermore, these modifications have demonstrated differing results with regards to the strength properties of the ensuing pulp and paper, as increases (120), decreases (121) and no change in strength (122) have been observed. Presently, there are several mills worldwide employing xylanases in their bleaching sequences to enhance the bleaching of kraft pulps (48,123).

Mannanases

The use of other hemicellulases, such as mannanases, as a prebleaching aid has not received the attention that xylanases have. However, this application has had limited success (119, 124-127). It is possible that the limited success is due to the location of the target substrates. The solubilisation of hemicellulose during the chemical pulping process results in altered distribution and content of xylan and glucomannans in pulp fibres compared to those found in the native wood. Furthermore, it has been shown that in softwood fibres the xylan concentration is

generally higher in the outer layers, while the glucomannan is more concentrated in the inner layers of the fibres (128).

Mannanases, as a general rule, exhibit greater specificity with respect to their substrate, and it has been shown that their effectiveness is dependent on both the degree of substitution as well as the distribution of the substituents (129). Furthermore, the hydrolysis of glucomannans has clearly been shown to be affected by the glucose:mannose ratio (130). Only a few purified mannanases without xylanase activity, such as those from *Trichoderma reesei*, have been reported to significantly increase the bleachability of softwood pulps (131). A comprehensive investigation of the prebleaching efficiency of a number of mannanases indicated that some mannanases work better than others (125). It has also been shown that the effects of mannanase prebleaching is strongly dependent on the cooking method and the bleaching sequences employed (125). Although both mannanases and xylanases have been shown to improve the bleachability of pulps independently, a synergism between the two has also been detected (132,133).

Oxidative Enzymes

In order to drive their catalytic cycles, oxidative enzymes such as laccases require low molecular weight electron carriers (mediators) or a slight pressure of oxygen, while manganese peroxidases requires a low and controlled dosage of H₂O₂ together with Mn²⁺ (134,135). Several investigations have conclusively demonstrated that the laccase-mediator system (LMS) can effectively facilitate the direct removal of lignin from kraft pulp (135-138). Similarly, manganese peroxidase (MnP) has also shown significant improvements in biobleaching (134), and more recently been shown to work synergistically with hemicellulotic enzymes, such as xylanase (139). In contrast to prebleaching with hemicellulolytic enzymes, oxidative enzymes require the addition of expensive co-factors, which is currently a technological barrier. However, a recent investigation has shown that transition metal complexes can be used as co-factors to facilitate the catalytic delignification and bleaching of kraft pulps. The advantage of these metal complexes over the organic mediators is that they can be recycled and reused multiple times, due to their ability to undergo reversible, successive electron-exchange reactions without being consumed (140).

Another class of oxidative enzymes, the oxidoreductases, such as cellobiose dehydrogenase (CDH) and cellobiose quinone oxidoreductase (CBQ) have also been investigated for their ability to contribute to pulp bleaching (141). These enzymes have the ability to reduce quinone, phenoxyradicals, as well as cation radicals (142), suggesting that these enzymes could play a role in lignin

degradation by preventing re-polymerisation reactions, and consequently reduce the amount of bleaching chemicals required to attain target brightness. Conversely, these enzyme preparations could potentially be used in combination with lignin degrading enzyme, which cause direct depolymerisation, and thus prevent the re-polymerisation of the reaction byproducts.

Whitewater Treatment

Growing economic and environmental concerns have resulted in a move to reduce fresh water utilisation in mechanical pulp and paper mills. This involves retaining and reusing process waters, which consequently provides benefits such as lower effluent treatment costs, energy savings, and improved environmental performance. However, mills experience major problems when attempting water system closure, such as the accumulation of dissolved and colloidal substances (DCS). The buildup of DCS can lower paper quality, increase rates of corrosion, and reduce paper machine runnability. Consequently, efficient and cost effective treatment strategies are required for the removal of these contaminants in order for mills to achieve effective water system closure. Unfortunately, existing treatment technologies are far from ideal, and as a result, fresh water usage at mills continues to remain high.

Emerging biotechnological treatment strategies have demonstrated that the direct employment of enzymes, such as hemicellulases, pectinases, lipases and some oxidative enzymes, can successfully target the reduction of dissolved compounds accumulating in the mill whitewater (143-148). However, a more interesting concept has been to employ fungi, rather than expensive enzymes, to effectively remove and/or degrade the whitewater contaminants (149-151). Zhang *et al.* (152) have conclusively demonstrated that *Trametes versicolor* could successfully act as a biofilter to control DCS buildup, a treatment that would require little or no capital or operating cost, and no additional nutrients (fungal growth and enzyme secretion has been optimised to grow directly on mill whitewater). These authors have further demonstrated, with purified enzyme preparations, that the degradation of the lipophilic extractives found in the whitewater was a result of a combined lipases and laccases effect. Lipases were shown to only degrade and remove 50% of the triglycerides, while the laccase enzymes were shown to degrade the lipophilic extractives, as well as resin acids and lignin-derived materials. It was clearly shown that laccases could initiate fatty acid oxidation reactions (153).

Recycled Fibre

Deinking

Several key factors necessitate the use of safe, efficient means of deinking office waste paper. Growing environmental awareness and laws have streamlined paper recycling, and these same factors drive the development of environmentally benign fibre processing. Basically, the use of industrial enzymes for deinking secondary fibres reduces the large amounts of chemicals employed in conventional deinking operations, and makes pulp and paper practices more environmentally friendly. The use of waste paper in pulp and paper manufacture has increased significantly. For example, as early as 1992, it was suggested that 72% of the furnish for German newsprint production came from waste paper (154).

While success has been achieved using a single homogeneous wastepaper source, when two or more sources are combined this application becomes more problematic. Some of the variables include toner quality and type, the type and amount of sizing, and the presence of other contaminants (155,156). However, recent developments using enzyme/surfactant combinations have been successfully applied on a variety of wastepapers, such as mixed office, sorted white ledger, sorted coloured ledger, computer printout, coated book sections, sulphated bleached sections, groundwood sections and ultraviolet printed papers in various proportions (157-159).

There have been published reports of mill-scale trials using enzymatic deinking applications, the results from which confirmed the laboratory data (160). The improved enzyme/surfactant technologies have facilitated investigations using enzyme-enhanced deinking of mixed office wastes (MOW) in three industrial-scaled mill trials (161). The results demonstrated both increased toner removal and pulp brightness with the application of low levels (0.04% dry weight) of a commercially available enzyme preparation in combination with a readily available surfactant (0.125% dry weight). The enzyme-enhanced deinking trials also displayed improved drainage and preserved fibre integrity when compared with the corresponding controls. Furthermore, there were no significant differences in the quality and treatability of the process water from the trials (161). Further mill scale trials would appear to be imminent.

More recently, it has been shown that enzymatic treatments can also improve the deinking capacity of waste paper during magnetic deinking trials, a new technology being developed (162). In general, enzymatic deinking results in little or no loss in fibre strength (49,163,164). However, the significant

hydrolysis of the fines (46,49,165-167) has resulted in a reduction in bondability of fibre networks (168-172). Although strength properties have not been compromised substantially in many instances, care must be taken not to overload enzyme to the fibre (49). These authors have demonstrated strength reductions with elevated cellulase loadings on different types of primary pulps, and shown that the strength properties of recycled pulps with a high content of chemical pulp fibres are more drastically reduced compared to secondary fibres consisting mainly of mechanical pulps (49).

Mechanistically, improvements in both dewatering and deinking of the various pulps is presumed to be accomplished from the peeling of individual fibrils and bundles, which have a high affinity for the surrounding water and ink particles (40). It appears that cellulase treatments can release ink particles, both bound to the fines and separately from the fibre, thereby enhancing the removal of ink by flotation. Therefore, the inclusion of enzymes in these processes results in decreased ink counts, allowing for higher brightness values, or alternatively decreases in the loading of the deinking chemicals (173). Although it is clear that cellulases enhance these processes, the mechanical agitation accompanying the enzymatic treatments plays a critical role in the efficiency of ink removal (173-175). These claims are consistent with similar findings concerning enzymatic stone washing of cotton fabrics, which indicated that enzymatic treatments in combination with mechanical agitation improve the efficacy of the process (176). In textile processes, small fibre ends protruding from the yarn are weakened by the action of the enzymes (176), while the concerted mechanical action completes the process by releasing the short fibres from the surface of the fabric (177).

Secondary Fibre Processing

The pulp and paper industry recognises that it is increasingly necessary to utilise recovered fibre, although, doing so decreases pulp drainability and compromises the strength characteristics of many of the end products. As recycled fibres become more widely utilised, especially for high quality paper production, the necessity of maintaining desired pulp properties through numerous life cycles is of paramount importance. Treatment of recycled fibres with commercial cellulases has been shown to reduce the refining energy required to achieve specific freeness. At equivalent levels of refining, cellulase treated recycled pulp yielded increases in freeness, although average fibre lengths were shown to decrease (178). These treatments have indicated that the end products of such applications are superior to non-enzymatic treated product (179,180). Mill trials have revealed that the freeness of the refined stock could be increased to allow greater incorporation of the recycled fibres into a corrugating

medium (80). Other mill trials using recycled kraft fibres and old corrugated container pulp have also successfully demonstrated savings in refining energy (181).

Similarly, it has been shown (182) that the treatment of the waste pulp component stream with a cellulase, prior to mixing with the other fibre constituents of a linerboard furnish, may serve as a means to increase the freeness of the entire pulp stock, with minimal yield loss. These treatments also demonstrated positive changes in the short span compression strength of the resultant linerboard at both 50% and 95% relative humidity, while other strength parameters were not adversely affected. Similar modifications were not attained by xylanase treatments.

Monocomponent cellulases have also been recently evaluated (183) for their potential to upgrade recycled furnishes. It is well known that the drying operation decreases the accessibility of cellulose and hemicellulose due to losses in fibre wall porosity. Interestingly, the increased solubilisation of amorphous cellulose mediated by endoglucanase treatments (EG I and EG II) improved water retention values, and consequently improved drainability. Furthermore, combinations of endoglucanases and hemicellulases acted synergistically to improve drainage beyond that accomplished by single enzymes alone. In contrast, cellobiohydrolase treatments (CBH I) failed to improve fibre drainage, and only in combination with hemicellulases were slight positive effects observed. Tensile and tear indices were decreased by endoglucanases alone and endoglucanases supplemented with hemicellulases, while very little strength loss was recorded after CBH I and CBH I/hemicellulase treatment. It was concluded that drying and refining have important, but unique effects on recycled fibre properties, and enzymatic treatment can substantially improve drainage, but the strength losses that accompanied endoglucanases treatment must be addressed.

Wood Residue Utilisation

Biotechnological production of chemicals and fuels from lignocellulosic-derived materials constitutes a substantial research effort of the forest and agricultural sectors. In a typical bioconversion process, the polysaccharide fraction of wood is 'isolated' through pretreatment, delignification and washing, and then depolymerised to its monomeric constituents using cellulose-degrading enzymes. The ensuing monomeric sugars can then be converted to ethanol or other products, such as furfural, lactic acid or other pharmaceuticals, using different strains of bacteria and yeast (*i.e. Saccharomyces cerevisiae, Escherichia coli*). Alternatively, these sugars can be used as the basic growth nutrients for microorganisms (*Trichoderma, Aspergillus, Humicola*) for the

production of commercial enzymes employed in a wide range of applications in the textile, chemical, and pulp and paper industry.

The conversion of lignocellulosic biomass to ethanol promises to provide an environmentally benign alternative fuel that can reduce the consumption of petroleum-based gasoline, and improve the quality of urban air. Combustion of gasoline in the transportation sector generates significant amounts of greenhouse gases, ozone, and volatile organic compounds, all of which have been shown to have a discernible impact on global climate and human health. In addition to providing a cleaner fuel, the biomass-to-ethanol conversion process offers a viable waste management strategy without the problems associated with wood waste incineration, landfilling and stockpiling that result in air pollution and groundwater contamination (184).

Both hardwoods and softwoods can potentially be used for bio-ethanol production. Short-rotation hardwood trees, such as poplar and willow, have been considered as the likely feedstock candidates for bioconversion operations due to their fast growth rate and relatively high yields (5-20 dry ton biomass/acre/year). The waste residues generated by the vast coniferous forests of Canada, US, and Scandinavia are also regarded as renewable sources of feedstock for the bioconversion industry (185,186).

The major steps in a biomass-to-ethanol process include feedstock preparation (cleaning and size reduction), pretreatment, enzymatic hydrolysis and ethanologenic fermentation. The pretreatment step is an acid or alkali hydrolysis process that removes, or otherwise alters, the hemicellulose and lignin fractions producing a highly digestible cellulose-rich substrate, and a soluble hemicellulose fraction known as the *prehydrolysate*. The solubilisation of the hemicellulose renders the remaining cellulose more porous, and therefore, more accessible to cellulolytic enzymes, which depolymerise cellulose to monomeric glucose (187).

The differences in the chemistry of softwoods and hardwoods require discrete sets of processing conditions for each type of wood. Guaiacyl lignin, the predominant subunit (99%) in softwood lignin, is generally more resistant to chemical modification than the syringyl-based lignin found in hardwoods and grasses (183). Also, solubilisation of galactoglucomannan, the principal hemicellulose in softwoods, produces a mixture of hexoses that can be relatively easily metabolised by yeasts, such as *Saccharomyces cerevisiae*. However, the mixed pentose-hexose prehydrolysates resulting from the hydrolysis of hardwood hemicelluloses, contains a large percentage of arabinoxylan, which requires the use of either two different species or a genetically modified microorganism (*i.e.* *Zymomonas mobilis* or *E. coli*) to metabolise both pentose and hexose sugars simultaneously (188).

Pretreatment conditions must be optimised to minimise the formation of sugar degradation products, such as furfural or hydroxymethyl furfural, which are

known to inhibit the growth and metabolism of microorganisms during fermentation (189). Currently, dilute-sulphuric acid hydrolysis and acid-catalysed steam explosion are among the most promising pretreatment processes (190) being evaluated and considered. Both operations require high temperatures (170-250 °C), low acid concentrations (1-5% w/w), and short residence times (a few seconds to 5 min). Using various acid-catalysed pretreatment regimes, it has been shown possible to recovery 80-90% of the carbohydrate (185,190).

Hydrolysis of cellulose to glucose can be achieved using either inorganic acids or cellulolytic enzymes. Despite its relatively slow rate, enzymatic hydrolysis has major advantages, as it does not require complex equipment and involves no corrosive reagents. Additionally, unlike acid hydrolysis reactions, the enzymatic breakdown of cellulosic polymers to monomeric glucose does not cause any sugar degradation.

Conclusion

Currently, biotechnology offers a wide range of alternative processes for the forest products industry to complement and/or enhance their conventional operations, such as xylanase-aided bleaching and enzymatic deinking technologies. As research in this area has not yet reached its full potential, the field of forest products biotechnology remains an expanding, exciting, and promising field of study for both academic research and industrial development. Clearly, the forest products industry can potentially take advantage of these opportunities to improve the efficiency of pulp and paper processes, reduce the adverse environmental impacts of wood utilisation operations, and generate a range of new value-added products, which penultimately could aid in the economic viability of their enterprises.

Predictions for Future Applications

Clearly, biotechnology in the pulp and paper industry is still very active, and continuing to make scientific advances. However, these are largely fundamental. Additionally, it is apparent that presently research in biopulping and bioconversion is making a resurgence, and will likely continue to make

inroads into their respective disciplines for the next few years, especially with the recent establishment of Biopulping International and the startup of Iogen's bioconversion demonstration plant in Ottawa, Canada.

There are several potential applications for new and novel enzymes in pulp and paper, including: lignin modification (reduction or activation), fibre surface modification (strength, forming, sizing and printing), fibre reactivity (glue and resins), and tree biotechnology to alter inherent fibre attributes. The enzyme preparations required for the aforementioned modifications include a range of traditional enzymes, such as cellulases, cellulase monocomponents and xylanases, but the future will likely see these enzymes genetically engineered to alter their specific activities, binding capacities and reactivities towards target substrates. Additionally, enzyme preparations will likely be engineered by mixing catalytic and binding domains from a variety of origins, including constructs designed to contain multiple binding domains. Another possibility includes the development/engineering of "mega enzymes", such as a combined cellulase-xylanase-mannanase preparation, which would be extremely large and unable to penetrate past the fibre surfaces, and therefore facilitate a true "surface modification". Similarly, plant- and microorganism-derived transferases for specifically modifying the surface properties of fibres, and making value-added products from wood/pulp and paper by-products (*i.e.* generating extremely high-value cello-oligosaccharides from waste stream sugars via transglycosylation) will likely emerge in the near future.

It is fair to say that the future will likely see a shift from the traditional enzymes to new classes of enzymes for lignocellulose modification, such as expansins, which are involved in a number of aspects of plant development, including: longitudinal growth, cell wall flexibility and degradation. Although expansins clearly influence the properties of plant cells, no hydrolytic activity has been observed. More specifically, this class of enzyme does not demonstrate any liberation in reducing sugars or change in viscosity, but does induce modifications in ultrastructural flexibility. It has been suggested that expansins can bind preferentially at cellulose-hemicellulose interfaces, and have the capacity to disrupt inter- and intra-molecular hydrogen bonding. In a similar light, the use of cellulose binding domains (CBD) from various organisms, has the potential to induce a number of very exciting and promising modifications to lignocellulosics (refer to accompanying chapters published in this book).

In these authors opinion, currently, the area with the greatest amount of potential for making short-term significant improvements in the pulp and paper industry is the application of tree biotechnology (genetically modified trees). However, public acceptance of such modifications will be slow coming, and therefore may actually be long-term benefits.

References

1. Akamatsu, I.; Yoshihara, K.; Kamishima, H.; Fuji, T. *Mokuzai Gakkaishi*. **1984**, *30*, 697-702.
2. Blanchette, R.A.; Burnes, T.A.; Leatham, G.F.; Effland, M.J. *Biomass*. **1988**, *15*, 93-101.
3. Blanchette, R.A.; Burnes, T.A.; Eerdmans, M.M.; Akhtar, M. *Holzforschung*. **1992**, *46*, 109-115.
4. Akhtar, M. *Holzforschung*. **1994**, *48*, 199-202.
5. Akhtar, M.; Attridge, M.C.; Myers, G.C.; Blanchette, R.A. *Holzforschung*. **1993**, *47*, 36-40.
6. Chen, Y.-R.; Schmidt, E.L. *Wood Fibre Sci*. **1995**, *27*, 198-204.
7. Dawson-Andoh, B.E.; Morrell, J.J.; Biermann, C.J.; Hull, J.L. *Tappi J*. **1991**, *74*, 187-189.
8. Fujita, K.; Kondo, R.; Sakai, K.; Kashino, Y.; Nishida, T.; Takahara, Y. *Tappi J*. **1991**, *74*, 123-127.
9. Fujita, K.; Kondo, R.; Sakai, K.; Kashino, Y.; Nishida, T.; Takahara, Y. *Tappi J*. **1993**, *76*, 81-84.
10. Fukui, H.; Abuhassan, J.M.; Chang, H.-M.; Joyce, T.W. *Cellulose Chem. Technol*. **1992**, *26*, 701-711.
11. Kohler, L.J.F.; Dinus, R.J.; Malcolm, E.W.; Rudie, A.W.; Farrell, R.L.; Brush, T.S. *Tappi J*. **1997**, *80*, 135-140.
12. Leatham, G.F.; Myers, G.C.; Wegner, T.H. *Tappi J*. **1990**, *73*, 197-201.
13. Leatham, G.F.; Myers, G.C.; Wegner, T.H.; Blanchette, R.A. *Tappi J*. **1990**, *73*, 249-254.
14. Messner, K.; Srebotnik, E. *FEMS Microbiol. Rev*. **1994**, *13*, 351-364.
15. Pilon, L.; Barbe, M.C.; Desrochers, M.; Jurasek, L.; Neumann, P.J. *Biotechnol. Bioeng*. **1982**, *24*, 2063-2076.
16. Sachs, I.B.; Leatham, G.F.; Meyers, G.C. *Wood Fibre Sci*. **1989**, *21*, 331-342.
17. Thornton, J.; Tenkanen, M.; Ekman, R.; Holmbom, B.; Viikari, L. *Holzforschung*. **1994**, *48*, 436-440.
18. Gutierrez, A.; del Rio, J.C.; Martinez, M.J.; Martinez, A. *Trends in Biotechnol*. **2001**, *19*, 340-348.
19. Breen, A.; Singleton, F.L. *Curr. Opin. Biotechnol*. **1999**, *10*, 252-258.
20. Chen, Y.-R.; Schmidt, E.L.; Olsen, K.K. *Wood Fibre Sci*. **1999**, *31*, 376-384.
21. Ander, P.; Eriksson, K.-E. *Svensk Papperstidning*. **1975**, *78*, 641-642.
22. Ander, P.; Eriksson, K.-E. *Physiol. Plant*. **1977**, *41*, 239-248.
23. Kirk, T.K.; Akhtar, M.; Blanchette, R.A. *Tappi Biological Sci. Symp*. **1994**, *57-66*.

24. Akhtar, M.; Kirk, T.K.; Blanchette, R.A. In *Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research* Srebotnik, E.; Messner, K. Eds.; Facultas-Universitätsverlag, Vienna, Austria, 1996, 187-192.
25. Farrell, R.L.; Blanchette, R.A.; Brush, T.S.; Gysin, B. In *Biotechnology in Pulp and Paper Industry* Kuwahara, M.; Shimada, M. Eds.; UNI Publishers Ltd. Tokyo, Japan, 1992, 27-32.
26. Kobayashi, Y.; Matsuo, R. *Agricul. Biolog. Chem.* **1984**, *48*, 1333-1335.
27. Kobayashi, Y.; Matsuo, R.; Hamada, Y.; Ohkawa, A.; Ebuchi, E. *Mokuzai Gakkaishi.* **1984**, *30*, 848-856.
28. Nazareth, S.; Mavinkurve, S. *Internat. Biodeter.* **1987**, *23*, 343-355.
29. Sharma, H.S.S. *Internat. Biodeter.* **1987**, *23*, 181-186.
30. Sharma, H.S.S. *Internat. Biodeter.* **1987**, *23*, 329-342.
31. Sreenath, H.K.; Shah, A.B.; Yang, V.W.; Gharia, M.M.; Jeffries, T.W. *J. Ferm. Bioeng.* **1996**, *81*, 18-20.
32. Rättö, M.; Kantelinen, A.; Bailey, M.; Viikari, L. *Tappi J.* **1993**, *76*, 125-128.
33. Viikari, L.; Kantelinen, A.; Rättö, M.; Sundquist, J. In *Enzymes in Biomass Conversion* Leatham, G.F.; Himmel, M.E. Eds.; American Chemical Society Symposium Series, Washington, DC, 1991; Vol. 460, 12-21.
34. Seth, R. In *Material Interactions Relevant to Pulp, Paper, and Wood Industries* Caulfield, D.F.; Passaretti, J.D.; Sobczynski, S.F. Eds.; Material Research Society, Pittsburgh, PA, 1990, 125-141.
35. Seth, R. In *Material Interactions Relevant to Pulp, Paper, and Wood Industries* Caulfield, D.F.; Passaretti, J.D.; Sobczynski, S.F. Eds.; Material Research Society, Pittsburgh, PA, 1990, 143-161.
36. Seth, R. *Tappi J.* **1995**, *78*, 99-102.
37. Seth, R.S.; Page, D.H. *Tappi J.* **1988**, *71*, 103-107.
38. Bhat, G.R.; Heitmann, J.A.; Joyce, T. *Tappi J.* **1991**, *74*, 151-157.
39. Chanzy, H.; Henrissat, B. *FEBS.* **1985**, *184*, 285-288.
40. Lee, S.B.; Kim, I.H. *Biotechnol. Bioeng.* **1983**, *25*, 33-51.
41. Pommier, J.-C.; Fuentes, J.-L.; Goma, G. *Tappi J.* **1989**, *72*, 187-191.
42. Pommier, J.-C.; Goma, G.; Fuentes, J.-L.; Rousset, C.; Jokinen, O. *Tappi J.* **1990**, *73*, 197-202.
43. Mansfield, S.D.; Wong, K.K.Y.; de Jong, E.; Saddler, J.N. *Tappi J.* **1996**, *79*, 125-132.
44. Stone, J.E.; Scallan, A.M.; Donefer, E.; Ahlgren, E. *Adv. Chem. Ser.* **1969**, *95*, 219-241.
45. Scallan, A.M.; Green, H.V. *Wood Fibre* **1975**, *7*, 226-233.
46. Jackson, L.S.; Heitmann, J.; Joyce, T. *Tappi J.* **1993**, *76*, 147-154.
47. Tolan, J.S. *J. Pulp Paper Sci.* **1995**, *21*, J132-J137.
48. Tolan, J.S.; Olson, D.; Dines, R.E. *Pulp Paper Can.* **1995**, *96*, 107-110.

49. Stork, G.; Pereira, H.; Wood, T.; Düsterhöft, E.; Toft, A.; Puls, J. *Tappi J.* **1995**, *78*, 79-88.
50. Lumme, M.S.; Mansfield, S.D.; Saddler, J.N. *Wood Fibre Sci.* **1999**, *31*, 385-393.
51. Mansfield, S.D.; Saddler, J.N. *J. Pulp Paper Sci.* **1999**, *25*, 84-89.
52. Mansfield, S.D.; Swanson, D.J.; Roberts, N.; Olson, J.A.; Saddler, J.N. *Tappi J.* **1999**, *82*, 152-158.
53. Edgar, C.D.; Mansfield, S.D.; Gübitz, G.M.; Saddler, J.N. In *Enzyme Applications in Fibre Processing* Eriksson, K.-E.L.; Cavaco-Paulo, A.M. Eds.; American Chemical Society Symposium Series, Washington, DC, 1998; Vol. 687, 75-87.
54. Wong, K.K.Y.; Martin, L.A.; Gama, F.M.; Saddler, J.N.; de Jong, E. *Biotechnol. Bioeng.* **1997**, *54*, 312-318.
55. Dickson, A.R.; Wong, K.K.Y.; Mansfield, S.D. *Tappi J.* **2000**, *83*, 1.
56. Bhardwaj, N.K.; Bajpai, P.; Bajpai, P.K. *J. Biotechnol.* **1996**, *51*, 21-26.
57. Suurnäkki, A.; Tenkanen, M.; Siika-Aho, M.; Niku-Paavola, M.-L.; Viikari, L.; Buchert, J. *Cellulose.* **2000**, *7*, 189-209.
58. Mansfield, S.D.; Gilkes, N.R.; Warren, R.A.J.; Kilburn, D.G. In *Biotechnology in the Pulp and Paper Industry* Viikari, L.; Lantto, R. Eds.; Progress in Biotechnology, Elsevier, Amsterdam, The Netherlands, 2002, Vol. 21, 301-310.
59. Felby, C.; Pedersen, L.S.; Nielsen, B.R. *Holzforchung.* **1997**, *51*, 281-286.
60. Wong, K.K.Y.; Richardson, J.D.; Mansfield, S.D. *Biotechnol. Prog.* **2000**, *16*, 1025-1029.
61. Mansfield, S.D. *Appita J.* **2002**, *55*, 49-53.
62. Sigoillot, J.-C.; Petit-Concil, M.; Ruel, K.; Rest of Authors *Horzforchung.* **1997**, *51*, 549-556.
63. Scott, G.M.; Akhtar, M.; Lentz, J.; Kirk, T.K.; Swaney, R. *Tappi J.* **1998**, *81*, 220-225.
64. Scott, G.M.; Swaney, R. *Tappi J.* **1998**, *81*, 153-157.
65. Pere, J.; Siika-Aho, M.; Viikari, L. Canadian Patent 2,157,213, 1994.
66. Pere, J.; Siika-Aho, M.; Viikari, L. *Tappi J.* **2000**, *83*, 1.
67. Bolaski, W.; Gallatin, A.; Gallatin, J.C. United States Patent 3,041,246, 1962.
68. Diehm, R.A. Process United States Patent 2,280,307, 1942.
69. Yerkes, W.D. United States Patent 3,406,089, 1968.
70. Noé, P.; Chevalier, J.; Mora, F.; Comtat, J. *J. Wood Chem. Technol.* **1986**, *6*, 167-184.
71. Wong, K.K.Y.; Kibblewhite, R.P.; Signal, F.A. *J. Wood Chem. Technol.* **1999**, *19*, 203-212.

72. Mansfield, S.D.; Wong, K.K.Y.; Dickson, A.R. *Wood Fibre Sci.* **2000**, *32*, 105-115.
73. Freiermuth, B.; Garrett, M.; Jokinen, O. *Paper Technol.* **1994**, *35*, 21-23.
74. Yamaguchi, H.; Yaguchi, T. *Proc. 50th Appita Ann Gen. Conf.* 1996, Vol. 1, 91-98.
75. Mansfield, S.D.; Wong, K.K.Y.; Richardson, J.D. *Appita J.* **1999**, *52*, 436-440.
76. Sigoillot, J.C.; Petit-Conil, M.; Herpoel, I.; Joseleau, J.P.; Ruel, K.; Kurek, B.; de Choudens, C.; Asther, M. *Enzyme Microb. Technol.* **2001**, *29*, 160-165.
77. Fuentes, J.L.; Robert, M. European Patent 262,040, 1988.
78. Bhardwaj, N.K.; Bajpai, P.; Bajpai, P.K. *Appita J.* **1995**, *48*, 378-380.
79. Oksanen, T.; Pere, J.; Buchert, J.; Viikari, L. *Cellulose.* **1997**, *4*, 329-339.
80. Moran, B.R. *Pulp Paper.* **1996**, *70*, 119-121.
81. Rutledge-Cropsey, K.; Klungness, J.H.; Abubakr, S.M. *Tappi J.* **1998**, *81*, 148-151.
82. Caram, F.C.; Sarkar, J.M.; Didwania, H.P.; Espinoza, E.; Benavides, J.C. *Tappi Papermakers Conf. Proc.* Tappi Press, Atlanta, GA, 1996 Vol. 2, 481-498.
83. Moran, B.R. In *Wastepaper VII*. Miller Freeman Inc, Chicago, IL, 1996, 10.
84. Stork, G.; Pereira, H.; Wood, T.; Düsterhöft, E.; Toft, A.; Puls, J. *Tappi J.* **1995**, *78*, 79-88.
85. Lascaris, E.; Mew, L.; Forbes, L.; Mainwaring, D.; Lonergan, G. *Appita J.* **1997**, *50*, 51-55.
86. Gierer, J.; Wännström, S. *Holzforschung.* **1984**, *38*, 181-184.
87. Jiang, J.-E.; Chang, H.-M.; Bhattacharjee, S.S.; Kwoh, L.W. *J. Wood Chem. Technol.* **1987**, *7*, 81-96.
88. Tenkanen, M.; Tamminen, T.; Hortling, B. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 241-248.
89. Viikari, L.; Ranua, M.; Kantelinen, A.; Sundquist, J.; Linko, M. *3rd Internat. Conf. Biotechnol. Pulp Paper Ind.* Stockholm, Sweden, 1986, 67-69.
90. Puls, J.; Poutanen, K.; Lin, J.J. In *Biotechnology in the Pulp and Paper Manufacture*. Kirk, K.T.; Chang, H.-M. Eds. Butterworth-Heinemann Inc., Boston, USA, 1990, 183-190.
91. Chen, C.C.; Adolphson, R.; Dean, J.F.D.; Eriksson, K.E.L.; Adams, M.W.W.; Westpheling, J. *Enzyme Microb. Technol.* **1997**, *20*, 39-45.
92. Christov, L.P.; Prior, B.A. *Appl. Microb. Biotechnol.* **1994**, *42*, 492-498.
93. Hamilton, J.; Senior, D.J.; Rodriguez, A.; Santiago, D.; Szwec, J.; Ragauskas, A.J. *Tappi J.* **1996**, *79*, 231-234.

94. Leduc, C.; Daneault, C.; Jaspers, C.; Penninckx, M. *8th Internat. Symp. Wood Pulping Chem.* Helsinki, Finland, 1995, 403-408.
95. Senior, D.; Hamilton, J. *J. Pulp Paper Sci.* **1992**, 18, J165-J169.
96. Vicuña, R.; Escobar, F.; Osses, M.; Jara, A. *Biotechnol. Let.* **1997**, 19, 575-578.
97. Yang, J.L.; Eriksson, K.E.L.; Law, S.E.; Sacon, V.M. *Tappi J.* **1993**, 76, 91-96.
98. Yang, V.W.; Zhuang, Z.; Elegir, G.; Jeffries, T.W. *J. Ind. Microbiol.* **1995**, 15, 434-441.
99. Mansfield, S.D.; Wong, K.K.Y.; de Jong, E.; Saddler, J.N. *Appl. Microbiol. Biotechnol.* **1996**, 46, 319-326.
100. Tolan, J.S.; Canovas, R.V. *Pulp Paper Can.* **1992**, 93, 39-42.
101. Tenkanen, M.; Buchert, J.; Puls, J.; Poutanen, K.; Viikari, L. In *Xylans and Xylanases*. Visser, J.; Beldman, G.; Kusters-van Someren, M.A.; Voragen, A.G.J. Eds. Elsevier, Amsterdam, 1992, 547-550.
102. Wong, K.K.Y.; de Jong, E.; Saddler, J.N.; Allison, R.W. *Appita J.* **1997**, 50, 415-422.
103. Wong, K.K.Y.; de Jong, E.; Saddler, J.N.; Allison, R.W. *Appita J.* **1997**, 50, 509-518.
104. Ragauskas, A.J.; Poll, K.M.; Cesternino, A.J. *Enzyme Microb. Technol.* **1994**, 16, 492-495.
105. Sinner, M.; Preselmayr, W. *Pulp Paper Internat.* **1992**, 34, 87-89.
106. Tolan, J.S.; Foody, B.E. *J. Pulp Paper Sci.* **1995**, 21, J191-J196.
107. Vidal, T.; Torres, A.L.; Colom, J.F.; Siles, J. *Appita J.* **1997**, 50, 144-148.
108. Wakai, M.; Takahashi, C.; Kai, K.; Ohira, Y. *Japan Tappi J.* **1993**, 47, 111-115.
109. Wong, K.K.Y.; Nelson, S.L.; Saddler, J.N. *J. Biotechnol.* **1997**, 48, 137-145.
110. Yang, J.L.; Eriksson, K.-E.L. *Holzforschung.* **1992**, 46, 481-488.
111. Sun, Y.P. *Appita J.* **1999**, 52, 45-50.
112. Baraznenok, V.A.; Becker, E.G.; Ankudimova, N.V.; Okunev, N.N. *Enz. Microb. Technol.* **1999**, 25, 651-659.
113. Shah, A.K.; Cooper, D.; Adolphson, R.; Eriksson, K.-E.L. *J. Pulp Pap. Sci.* **2000**, 26, 8-11.
114. Shah, A.K.; Cooper, A.; Adolphson, R.B.; Li, X.-L.; Chen, H.; Ljungdahl, L.G.; Eriksson, K.-E.L. *Tappi J.* **2000**, 83, 1-12.
115. Torres, A.L.; Roncero, M.B.; Colom, J.F.; Pastor, F.I.J.; Blanco, A.; Vidal, T. *Biores. Technol.* **2000**, 74, 135-140.
116. Elegir, G.; Sykes, M.; Jeffries, T.W. *Enzyme Microb. Technol.* **1995**, 17, 954-959.
117. Elegir, G.; Szakács, G.; Jeffries, T.W. *Appl. Environ. Microb.* **1994**, 60, 2609-2615.

118. Gübitz, G.M.; Haltrich, D.; Latal, B.; Steiner, W. *Appl. Microb. Biotechnol.* **1997**, *47*, 658-662.
119. Gübitz, G.M.; Schnitzhofer, W.; Balakrishnan, H.; Steiner, W. *J. Biotechnol.* **1996**, *50*, 181-188.
120. Clark, T.; Steward, D.; Bruce, M.E.; McDonald, A.; Singh, A.P.; Senior, D.J. *Appita J.* **1991**, *44*, 389-404.
121. Roberts, J.C.; McCarthy, A.J.; Flynn, N.J.; Broda, P. *Enzyme Microb. Technol.* **1990**, *12*, 210-213.
122. Paice, M.G.; Gurnagul, N.; Page, D.H.; Jurasek, L. *Enzyme Microb. Technol.* **1992**, *14*, 272-276.
123. Thibault, L.; Tolan, J.; White, T.; Yee, E.; April, R.; Sung, W. *Proc. 85th Ann. CPPA Meeting.* **1999**, Vol. B, 263-266.
124. Grant, R. *Pulp Paper Internat.* **1994**, *36*, 20-21.
125. Suurnäkki, A.; Clark, T.A.; Allison, R.W.; Viikari, L.; Buchert, J. *Tappi J.* **1996**, *79*, 111-117.
126. Suurnäkki, A.; Kantelinen, A.; Buchert, J.; Viikari, L. *Tappi J.* **1994**, *77*, 111-116.
127. Viikari, L.; Kantelinen, A.; Sundquist, J.; Linko, M. *FEMS Microbiol. Rev.* **1994**, *13*, 335-350.
128. Viikari, L.; Suurnäkki, A.; Buchert, J. In *Enzymes for Pulp and Paper Processing* Jeffries, T.W.; Viikari, L. Eds. American Chemical Society Symposium Series, Washington, DC 1996, Vol. 655, 15-24.
129. McCleary, B.V. 1991. In *Enzymes in Biomass Conversion*. Leatham, G.F.; Himmel, M.E. Eds. American Chemical Society Symposium Series, Washington, DC 1991, Vol. 460, 437-449.
130. Tenkanen, M.; Buchert, J.; Viikari, L. *Enzyme Microb. Technol.* **1995**, *17*, 499-505.
131. Cuevas, W.A.; Kantelinen, A.; Tanner, P.; Bodie, B.; Leskinen. In *Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research*. Srebotnik, E.; Messner, K. Eds. Facultas-Universitätsverlag, Vienna, Austria, 1996, 123-126.
132. Buchert, J.; Salminen, J.; Siika-aho, M.; Ranua, M.; Viikari, L. *Holzforschung.* **1993**, *47*, 473-478.
133. Clarke, J.H.; Davidson, K.; Rixon, J.E.; Halstead, J.R.; Fransen, M.P.; Gilbert, H.J.; Hazlewood, G.P. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 661-667.
134. Call, H.-P.; Strittmatter, G. *Das Papier.* **1992**, *46*, 32-37.
135. Paice, M.G.; Bourbonnais, R.; Reid, I.D.; Archibald, F.S.; Jurasek, L. *J. Pulp Paper Sci.* **1995**, *21*, J280-J284.
136. Sealey, J.; Ragauskas, A.J.; Elder, T.J. *Holzforschung.* **1999**, *53*, 498-502.
137. Balakshin, M.; Chen, C.-L.; Gratzl, J. S.; Kirkman, A.; Jakob, H. *Holzforschung.* **1999**, *54*, 390-396.

138. Poppius-Levlin, K.; Wang, W.; Tamminen, T.; Hortling, B.; Viikari, L.; Niku-Paavola, M.-L. *J. Pulp Paper Sci.* **1999**, *25*, 90-94.
139. Bermek, H.; Li, K.; Eriksson, K.-E.L. *Tappi J.* **2000**, *83*, 69.
140. Bourbonnais, R.; Rochefort, D.; Paice, M.G.; Renaud, S.; Leech, D. *Tappi J.* **2000**, *83*, 68-78.
141. Roy, B.P.; Dumonceaux, T.; Koukoulas, A.A.; Archibald, F.S. *Appl. Environ. Microbiol.* **1996**, *62*, 4417-4427.
142. Samejima, M.; Eriksson, K.-E.L. *Eur. J. Biochem.* **1992**, *207*, 103-107.
143. Zhang, T.; Wada, S.; Yamagishi, T.; Hiroyasu, I.; Tatsumi, K.; Zhao, Q.-X. *J. Environ. Sci. China.* **1999**, *11*, 480-48.
144. Van Haute, E. *53rd Appita Ann. Gen. Conf.* **1999**, Vol. 2, 575-579.
145. Reid, I.; Ricard, M. *Enz. Microbiol. Technol.* **2000**, *26*, 115-123.
146. Chen, S.; Lin, Y.; Zhang, Y.; Wang, X.H.; Yang, J.L. *Tappi J.* **2001**, *84*, 44-47.
147. Wagner, M.; Nicell, J. A. *Water Res.* **2001**, *35*, 485-495.
148. Suurnäkki, A.; Mustranta, A.; Siika-Aho, M.; Holmbom, B.; Viikari, L.; Buchert, J. *Pap.* **2001**, *45*, 51-55.
149. Stebbing, D. *Pulp Paper Can.* **1999**, *100*, T72-75.
150. Nagarathnamma, R.; Bajpai, P. *Appl. Environ. Microbiol.* **1999**, *65*, 1079-1082.
151. Dorado, J.; Claassen, F.W.; van Beek, T.A.; Lenon, G.; Wijnberg, J.; Sierra-Alvarez, R. *J. Biotechnol.* **2000**, *80*, 231-240.
152. Zhang, X.; Stebbing, D.W.; Soong, G.; Saddler, J.N.; Beatson, R. *Tappi J.* **2002**, *1*, 26-32.
153. Zhang, X.; Eigendorf, G.; Stebbing, D.W.; Mansfield, S.D.; Saddler, J.N. *Arch. Biochem. Biophys.* **2002**, *405*, 44-54.
154. Stork, G.; Puls, J. In *Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research* Srebotnik, E.; Messner, K. Eds. Facultas-Universitätsverlag, Vienna, Austria, 1996, 145-150.
155. Jeffries, T.W.; Sykes, M.S.; Rutledge-Cropsey, K.; Klungness, J.H.; Abubakr, S. In *Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research* Srebotnik, E.; Messner, K. Eds. Facultas-Universitätsverlag, Vienna, Austria, 1996, 141-144.
156. Ow, S.K.; Park, J.M.; Han, S.H. In *Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research* Srebotnik, E.; Messner, K. Eds. Facultas-Universitätsverlag, Vienna, Austria, 1996, 163-168.
157. Franks, N.E.; Munk, N. *Tappi Pulping Conf.* **1995**, 343-347.
158. Morkbak, A.L.; Degn, P.; Zimmerman, W. *J. Biotechnol.* **1999**, *67*, 229-236.
159. Elegir, G.; Panizza, E.; Canetti, M. *Tappi J.* **2000**, *83*, 1-9.
160. Kim, T.-J.; Ow, S.S.-K.; Eom, T.-J. *Tappi Pulping Conf.* **1991**, 1023-1030.

161. Heise, O.U.; Unwin, J.P.; Klungness, J.H.; Fineran, W.G.; Sykes, M.J.; Abubakr, S. *Tappi J.* **1996**, *79*, 207-212.
162. Gübitz, G.M.; Mansfield, S.D.; Böhm, D.; Saddler, J.N. *J. Biotechnol.* **1998**, *65*, 209-219.
163. Sarkar, J.; Cosper, D.; Hartig, E. *Tappi J.* **1995**, *78*, 89-95.
164. Sarkar, J.M. *Appita J.* **1997**, *50*, 57-60.
165. Prasad, D.Y. *Appita J.* **1993**, *46*, 289-292.
166. Prasad, D.Y.; Heitmann, J.; Joyce, T. *Nordic Pulp Paper Res. J.* **1993**, *8*, 284-286.
167. Prasad, D.Y.; Heitmann, J.A.; Joyce, T. *Prog. Paper Recycl.* **1992**, *1*, 21-30.
168. Beatson, R.P.; Bichard, W.; Muradali, N. *Pap. ja Puu.* **1988**, *70*, 66-72.
169. Karnis, A. *Pap. ja Puu.* **1995**, *77*, 491-497.
170. Kibblewhite, R.P. *Pap. ja Puu.* **1975**, *57*, 519-526.
171. Kibblewhite, R.P.; Bawden, A.D.; Brindley, C.L. *Appita J.* **1995**, *48*, 367-377.
172. Paavilainen, L. *Pap. ja Puu.* **1990**, *72*, 516-526.
173. Welt, T.; Dinus, R.J. *Prog. Paper Recycl.* **1995**, *4*, 36-47.
174. Zeyer, C.; Joyce, T.; Heitmann, J.A.; Rucker, J.W. *Tappi J.* **1994**, *77*, 169-177.
175. Zeyer, C.; Joyce, T.; Rucker, J.W.; Heitmann, J.A. *Prog. Paper Recycl.* **1993**, *2*, 36-44.
176. Tyndall, R.M. *Text. Chem. Color.* **1992**, *24*, 23-26.
177. Chikkodi, S. *Ind. Text.* **1995**, *1265*, 29-31.
178. Eriksson, K.-E.L. In *Enzyme Applications in Fibre Processing*. Eriksson, K.-E.L.; Cavaco-Paulo, A.M. Eds. American Chemical Society Symposium Series, Washington, DC, Vol. 687, 2-50.
179. Park, J.; Park, K. *Biores. Technol.* **2001**, *79*, 91-94.
180. Pala, H.; Lemos, M.A.; Mota, M.; Gama, F. M. *Enzyme Microb. Technol.* **2001**, *29*, 274-279.
181. Caram, F.C.; Sarkar, J.M.; Didwania, H.P.; Espinoza, E.; Benavides, J.C. *Tappi Papermakers Conf.* **1996**, 481-498.
182. Mansfield, S.D.; Wong, K.K.Y. *Prog. Paper Recycl.* **1999**, *9*, 20-29.
183. Oksanen, T.; Pere, J.; Paavilainen, L.; Buchert, J.; Viikari, L. *J. Biotechnol.* **2000**, *78*, 39-48.
184. Saddler, J.N.; Ramos, L.P.; Breuil, C. In *Bioconversion of Forest and Agricultural Plant Residues*. Saddler, J.N. Ed. CAB International Wallingford, UK, 1993, 73-91.
185. Boussaid, A.; Esteghlalian, A.R.; Gregg, D.J.; Lee, K.H.; Saddler, J.N. *Appl. Biochem. Biotechnol.* **1999**, *84-86*, 693-705.
186. Nikolov, T.; Bakalova, N.; Petrova, S.; Benadova, R.; Spasov, S.; Kolev, D. *Biores. Technol.* **2000**, *71*, 1-4.

187. Fan, L.T.; Lee, Y.-H.; Beardmore, D.H. *Biotechnol. Bioeng.* **1980**, *22*, 177-199.
188. Wyman, C.E. In *Handbook on Bioethanol*. Wyman, C.E. Ed. Taylor and Francis, Washington, D.C., 1996, 1-18.
189. Robinson, J.; Keating, J. D.; Bousaid, A.; Mansfield, S.D.; Saddler, J. N. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 443-448.
190. Esteghlalian, A.R.; Hashimoto, A.G.; Fenske, J.; Penner, M.H. *Biores. Technol.* **1997**, *59*, 129-136.

Chapter 2

Lignocellulose Modifying Enzymes for Sustainable Technologies

Liisa Viikari

VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Finland

The versatile microbial systems in nature degrade vast amounts of biomass into carbon dioxide, and are partially responsible for the carbon cycle in the hemisphere. Different types of micro-organisms produce a variety of enzymes which depolymerize organic polymers and complex compounds into small metabolizable molecules that serve as energy and nutrient source for these organisms. These enzymes may also be useful in industrial processes, where they can be employed as isolated biocatalysts to develop environmentally benign processes and products. Today, enzymes are increasingly used in many sectors, such as feed, textile, pulp and paper, and chemical industries. Enzymes have already been shown to be efficient and environmentally sustainable tools in reducing the consumption of energy and chemicals *e.g.* in the pulp and paper sector, and their importance in biomass conversion processes is growing. Understanding the degradative and metabolic reactions can also be exploited in several other ways; to enhance and monitor the biodegradation of organic molecules in the field of environmental biotechnology or, on the other hand, to prevent these reactions in the fields of wood protection or process hygiene. The cost efficiency of enzyme based processes can be improved with the new tools of modern biotechnology.

Industrial sustainability can be defined by lower relative consumption of energy and raw materials, as well as the reduction or elimination of waste. Modern biosciences offer tools which may cause revolutionary changes in traditional industries and help fulfill the needs of sustainable technologies. A range of biotechnical methods and processes are becoming available and offer the prospect of reducing raw material and energy consumption, as well as pollution. Biotechnology is considered to be a powerful enabling technology for developing cleaner industrial products and processes. Although the perception of biotechnology being inherently cleaner is simplistic, biocatalysts offer cleaner products and processes because they are non-toxic, more specific and more selective than their non-biological competitors. Biotechnology also offers tools for increased utilization of renewable raw materials for chemical and energy production. The world's fossil fuel reserves are rapidly being depleted and new ways of producing chemicals and energy need to be explored. Biotechnology is key to developing new, alternative and clean energy and chemical sources for the next decades. There are three major drivers of sustainable technology; economic competitiveness, government policies which enforce changes in manufacturing practises, and public pressure which takes on strategic importance as companies seek to improve their environmental performance. Government policies enhancing the cleanliness of industrial products and processes may be decisive factors in the development and industrial use of biotechnical processes, but to be commercialized, they need to be economically competitive. Market forces can also provide very powerful incentives for achieving environmental objectives. Environmental concerns, such as increasing energy consumption and chlorine crisis have been driving forces in identifying biotechnical solutions and for developing the technologies needed. This paper summarizes research activities in biomass enzymology, revisited from the point of view of environmental drivers.

THE FIRST ENERGY CRISIS IN 1973

The first energy crisis began in 1973, after Arab oil-producing states embargo oil to the United States. The world consumption of oil rose between 1965 and 1973, and fell until 1982 when it started to increase again. The 1970's energy crisis resulted in growing research activities focussed on developing alternative fuels. Also in Finland, a research programme on the enzymatic hydrolysis of lignocellulose to sugars, and their further conversion to ethanol was initiated. After intensive exploratory research, final mill scale trials on enzymatic hydrolysis were carried out in the late 1970s in a sulphite mill, which normally produced ethanol from spent sulphite liquor. Meanwhile, the price of oil had decreased to a third, and further development of the concept was therefore deemed economically unviable. The energy crisis was, however, one of the initial driving forces for focusing enzymatic research on the basic and applied aspects of polysaccharide degrading enzymes, especially cellulases and

hemicellulases. Major part of the cellulase studies have concentrated on secreted fungal enzymes, which are also currently used in many industrial applications (1). Especially the cellulase system from the filamentous fungus *Trichoderma reesei* has been intensively studied at a molecular level. Genes coding for two cellobiohydrolases, five endoglucanases and two β -glucosidases have been isolated and characterised from *T. reesei*. It has been shown that the activities of all these three classes of enzymes are needed for efficient hydrolysis of crystalline cellulose into glucose. Fungal cellulases (*i.e.* cellobiohydrolases and endoglucanases) have usually a two-domain structure composed of a catalytic domain and a cellulose-binding domain (CBD) linked via a heavily glycosylated linker peptide (2). Both domains are needed for the efficient hydrolysis of insoluble substrates. During the last 10-15 years, the three-dimensional structures of these domains have been determined either by X-ray crystallography or NMR spectroscopy (3-12), which has enabled the detailed structure-function studies of the fungal cellulases and cellulase domains (13-21). This growing understanding will ultimately help to improve the performance of cellulases in the hydrolytic processes of lignocellulosic materials.

Research on lignocellulose enzymology also included the identification and characterisation of all major hemicellulolytic enzymes participating in the hydrolysis of hemicellulosic substrates (22-35). *Trichoderma reesei* has been used as the model organism for the characterization of the enzymatic system of lignocellulose degradation. The three dimensional structures of the *T. reesei* family 11 xylanases have also been determined (36, 37), as well as those of mannanase and acetyl xylan esterase (38, 39). The properties of xylanases of the glycosyl hydrolase families 10 and 11 have been recently reviewed (40). Other, interesting organisms have also been studied as sources of xylanolytic enzymes, potentially capable of acting under extreme conditions (41-46). The production of various lignocellulose modifying enzymes has been studied and occasionally optimized to enable their production in sufficient amounts for characterization, evaluation and testing (47-51).

Cellulolytic and hemicellulolytic enzymes have been studied for total or partial hydrolysis of lignocellulosic materials (52-65). While the total hydrolysis seemed to be economically unfavourable, the focus has been in elucidating the chemical and practical significance of specific modifications, *i.e.* partial hydrolysis of fibres using tailored or monocomponent enzymes on the fibre-bound substrates. The detailed mechanisms of enzymatic modifications of isolated and fibre-bound carbohydrates have been studied (60, 66-70).

After the first energy crisis, the aim of hydrolysing cellulose and hemicellulose in lignocellulosic residues was to provide building blocks for the production of ethanol (52, 71, 72) or chemicals, such as xylonic acid (73) or xylitol (74-77). Today, the focus is in understanding and directing the metabolic fluxes to achieve more efficient production of the desired metabolite (78, 79).

THE CHLORINE CRISIS IN 1985

Pressure resulting from public awareness is a potential force for changing production systems. This was clearly observed especially in Europe when dioxins turned the consumers against chlorine bleached pulps. In 1985, the U.S. Environmental Protection Agency (EPA) issued its first formal cancer risk assessment of dioxins and in 1986, the EPA then proposed controls on dioxin emissions from the pulp and paper industry. This study, conducted jointly with the American Paper Institute, collected and analyzed wastewater and sludge samples from five mills across the country. Dioxins were detected in effluent wastewaters at three of the mills, in waste-treatment sludge at all five mills and in bleached pulps at four of the mills. Later, it was observed that dioxins can be eliminated by using less elemental chlorine and more chlorine dioxide in the first bleaching stage. The search for alternative bleaching methods limiting these emissions, but still maintaining the high quality pulp, had started. Today, in Europe, the majority of pulp is bleached by chlorine dioxide in the ECF (Elemental Chlorine Free) process or by using TCF (Totally Chlorine Free) bleaching processes, where oxygen, ozone and hydrogen peroxide are used.

Since the discovery of lignin peroxidase (80) and manganese peroxidase (81), direct enzymatic delignification became a worldwide focus of intensive research. The key enzymes of the white-rot fungus *Phlebia radiata*; three lignin peroxidases, one Mn-peroxidase and one laccase, were produced, purified, characterised and studied *in vivo* and *in vitro* (82-86). Laccases were for the first time heterologously expressed in *Trichoderma* (87) and later in *Pichia pastoris* (88). The characterisation of the isolated laccase gene showed homology to the plant ascorbate oxidase and to the laccases of *Trametes hirsutus* and *Neurospora crassa* (83, 89). Interest in more thermo- or alkali stable laccases has resulted in isolation of a laccase from *Melanocarpus albomyces* with unique properties (90). The molecular structure of this laccase, containing all four copper atoms was recently solved (91).

The concept of using hemicellulases in bleaching was first introduced in 1986, and raised interest after the growing dioxin problem (92, 93). Xylanases were originally studied to replace or to significantly reduce the consumption of chlorine gas. Due to the indirect effect of xylanases on lignin, the method was not found efficient enough to replace chlorine chemicals totally. However, in chlorine bleaching, a significant average reduction of 25% in active chlorine consumption in the first stage or a reduction of about 15% in total chlorine consumption was obtained in laboratory and subsequent mill scale trials. As a result, the concentration of chlorinated compounds, measured as AOX (absorbable organic halogen), in the bleaching effluents was reduced by 15-20% (94). Today, xylanases are industrially used both in ECF and TCF sequences. In ECF sequences, the enzymatic step is often implemented due to the limitations in chlorine dioxide production capacity. The use of enzymes facilitates bleaching to higher brightness values when chlorine gas is not used. In TCF sequences, the application of an enzymatic step improves brightness, maintains the fibre

strength and allows savings in bleaching costs. Presently, about 20 mills in Northern America and Scandinavia use enzymes (95). The approximate price of xylanase treatment in 2000 was less than 2 USD per ton of pulp. Calculations of the economic benefits in an ECF sequence reveal that reductions in the chlorine dioxide consumption lead to savings of at least 2 USD per ton of pulp (96). The costs of oxygen based chemicals (ozone, peroxide) are even higher and the respective savings even more pronounced. Thus, the potential economic benefits of enzyme-aided bleaching are significant to the user.

The effect of xylanases in bleaching is based on the modification of pulp surface, and has been shown to result in enhanced extractability of lignin in subsequent bleaching stages (97-102). Several alternative and obviously concurrent mechanisms seem to be involved in xylanase-aided bleaching (94, 103, 104). Extensive modification of hemicellulose polymers take place during pulping processes; hemicelluloses are solubilized, the side groups are partially cleaved off, the majority of the 4-O-methylglucuronic acid side groups are converted to hexenuronic acid (105), and part of the solubilized xylan is reprecipitated onto the cellulose fibres. Relatively high amounts of xylan and lignin have been observed on the fibre surfaces and xylanase treatments have been found to uncover surface lignin (102, 104) and increase the pore size distribution (106). Swelling was expected to be an important parameter in the action of enzymes on fibres (107), but it was found to be affected more by the Donnan effect (108). Although glucomannan is the main hemicellulose in softwoods, the bulk of glucomannans are dissolved and degraded during kraft pulping without significant reprecipitation or relocation. The enhanced leachability of lignin in the fibre wall has been suggested to be due to the hydrolysis of reprecipitated xylan or to the removal of xylan from the lignin-carbohydrate complexes (LCC) in fibres. The action of xylanases on both reprecipitated and LC-xylan suggests that it is probably not only the type, but also the location of the xylan that is important in xylanase-aided bleaching (109, 110). The role of hexenuronic acid in the effect of xylanase treatments on kappa number can be estimated to be relatively small. Hexenuronic acid, containing a double bond, gives rise to the consumption of bleaching chemicals and permanganate, increasing the apparent kappa number of pulp (111, 112).

Xylanases seem to be efficient on all types of kraft fibers, whereas the effect by mannanases depends on the type of fibers used (97, 113, 114). Usually less uniform results have been obtained on sulphite pulps. The effect of xylanases on bleachability has in most cases been independent of the origin of the enzyme, although modest differences between different xylanases, such as the major *Trichoderma* xylanases (115) have been observed. Comparison of different enzymes is complicated because the basic parameters usually vary (enzyme dosed based on protein, activity on a certain substrate at a defined pH or temperature, different bleaching sequences, etc.). Although differences between fungal and bacterial xylanases have been reported, few systematic studies have been carried out with comparable methodologies. It has been proposed that xylanases from family-11 could be more effective in bleach boosting than the family-10 xylanases (116), which is in accordance with previous results obtained

with *Trichoderma* and *Aspergillus* xylanases. The effects of cellulose and xylan binding domain (CBD and XBD) of xylanases on bleaching efficiency have also been investigated, but so far neither XBD nor CBD has been reported to have any significant role in bleach boosting efficiency (117). Cellulases have neither been found to affect the extractability of lignin, with the exception of EG I of *T. reesei* which also acts as a xylanase (118).

Most recently, the revolutionary laccase-mediator concept was shown to efficiently delignify with high specificity (119-121). The degree of delignification achieved by the laccase-NHA system after alkaline extraction is reported to be high, up to 40 % in low-kappa number softwood and hardwood kraft pulps (122). In addition, the LMS has been found to be efficient on Eucalyptus kraft pulp (123) and in the bleaching of high yield pulp (124). In spite of the considerable amount of research carried out on model compounds (120, 125, 126), fairly few results have been published on the actual mechanisms of the LMS on natural lignin substrates (127). In addition to the metabolite, 3-hydroxyanthranilate (128) of a white rot fungus, siderophores (129) have been shown to act as potential natural mediators in the delignification process *in vitro*. Hydroxamate siderophores, having structural similarities with the synthetic mediators, were found to act as mediators in laccase-aided delignification processes (130). Combinations of xylanases and LMS have been shown to further improve delignification (131).

SUSTAINABLE PULP AND PAPER MANUFACTURE IN THE 1990s

Mechanical pulps, such as pressurized ground wood (PGW) or thermomechanical pulp (TMP) have a high yield (up to 95%) and can be used to produce paper with high bulk, good opacity, and excellent printability. The drawbacks of these processes are the required high energy intensity and the resulting fiber and paper quality with lower strength, higher pitch content, and higher color reversion rate as compared to chemical pulps. Energy consumption is thus a key issue when developing new technical processes for high yield pulping. The demand for improving paper quality has been the main reason for the increased specific energy consumption in thermomechanical pulping during the last few years. One way of reducing the high energy consumption of mechanical pulping is to modify the raw material by biotechnical means prior to refining.

The incorporation of an enzymatic step in the mechanical pulping process was found to be successful after a primary refining step, where the accessibility of the raw material is improved towards the enzymatic action. A new process concept, based on the treatment of coarse mechanical pulp fibers, using monocomponent cellulases was developed (132). Experiments with different enzymes demonstrated that a minor modification in the cellulose by the *T. reesei* cellobiohydrolase I (CBH I) results in 20 % energy saving in laboratory-scale

disk refiner. Interestingly, when an unoptimized cellulase mixture was used, no positive effect on energy consumption was detected. Based on the fibrillation index analyses, it has been assumed that the action of CBH I possibly results in loosening and for unravelling of fiber structure (132). Interestingly, the strength properties, *i.e.* tensile index was even higher for the CBH I treated pulp than for the reference, obviously due to the more intensive fibrillation induced by the enzymatic treatment. At the pilot scale, using a two-stage secondary refining with a low-intensity refiner (wingdefibrator), an energy saving of 10-15 % with CBH I was obtained. Lately, the method has been tested at the industrial scale, and is under further development.

Enzymes can also be used for reducing the energy consumption in debarking or for removing final bark residues from the wood logs (133). The energy consumption in debarking was found to decrease by up to 80% by pectinases, acting specifically on the cambial layer between wood and bark. This concept, however, has not been further developed due to the growing tendency for developing dry debarking processes, where the solubilization of toxic compounds into debarking waters is prevented.

Papermakers are currently searching for options to fulfill the legislative and environmental demands to reduce the fresh water usage. One major problem in white water mill closure is the accumulation of dissolved and colloid substances (DCS) in the process waters. These substances consist mainly of hemicelluloses, pectins, dispersed wood resin, lignans, and dissolved lignin. The compositions of DCS have a direct impact on the paper machine runnability and paper quality, as well as on the need and efficiency of purification processes of white water. The composition and structure of extractives, glucomannan, pectin and lignans can be modified by suitable enzymes such as lipases, mannanases, pectinases and oxidative enzymes (134-139). These minor changes achieved by enzymes may result in significant modifications in the behaviour of DCS in white waters, and more significantly, may lead to potential improvements in the technical parameters (yield, strength and brightness) of pulp and decrease environmental loads.

Recycled fibres are increasingly used in paper manufacturing, although the environmental impacts are not self-evident. When aiming at more efficient and environmentally friendly deinking processes, enzyme-aided deinking is a potential alternative. The application of cellulase and hemicellulase mixtures in deinking has been studied in laboratory, pilot and mill scales (140, 141), and shown to result in savings of deinking chemicals. The use of enzymes in deinking of recovered paper is one of the most promising enzymatic applications in the pulp and paper industry and already used in mill scale. Brightness reversion, affecting especially the mechanical pulps, has also been the target of enzymatic improvement (108, 142). The mechanisms of natural enzymatic modification of lignin to prevent the browning reactions have been studied in biomimetic approaches (143, 144).

The enzymatic modification of papermaking properties; *i.e.* fibre engineering of chemical, mechanical and recycled pulps has been widely studied during the last decade. The main ideas have been to enhance the beatability, and thus the strength properties of fibres through increased fibrillation or to affect the amount of fines in pulp in order to increase the drainage or water retention of pulp. The effects of individual cellulases on the properties of unbleached or bleached kraft pulp have been studied in detail (145, 146). *T.reesei* cellobiohydrolases (CBH) have been found to have only a modest effect on pulp viscosity, whereas endoglucanases (EG), and especially EG II dramatically decrease pulp viscosity, and thus the strength properties after refining. In Douglas-fir chemical pulp, cellulases and cellulase-hemicellulase mixtures have been reported to enhance the beatability of coarse fibers and thus to improve the paper properties (147). Enzymes have thus significant potential for improving traditional pulp and paper manufacturing processes due to their specificity and environmental benefits.

THE KYOTO PROTOCOL IN 1997

The Conference on Climate Change (COP3) held in Kyoto in 1997, agreed on a protocol which includes each party's quantitative commitment to reduce its emissions of greenhouse gases, such as carbon dioxide, by 2010. The protocol specifies that the European Union will commit itself to reducing its greenhouse gas emissions by 8% of the 1990 level by 2010. As an essential element in achieving this goal, industry must reduce energy consumption in order to maintain development while helping to meet these targets. Obviously, the energy intensive mechanical pulp production is the continuous target for energy saving. Recently, the demand to increase the utilization of renewable raw materials for the production of chemicals and energy has again focused research towards total hydrolysis of lignocellulosic materials. In the transportation sector, biofuels are the only option which can reduce the CO₂ emissions by an order of magnitude. A recent proposal for a Directive of the European Parliament sets a target of substituting 20% of traditional fuels in the road transport sector by 2020. The objectives of this action plan are to reduce the European Union's growing dependency on external supply of oil, and to contribute to the achievement of the EU's greenhouse gas emission reductions as decided in Kyoto. Obviously, the improvement of enzymatic hydrolysis of lignocellulosics will become an increasingly important research goal. Lignocellulose enzymes will thus play key roles in the development of environmentally friendly products and processes also in the future, and will contribute to the prevention of the climate change.

CONCLUSIONS

Enzyme technology has already shown its potential for more sustainable processing of lignocellulosic raw materials. Advanced production technologies enable the production of monocomponent enzymes or tailored mixtures of enzymes which have potential applications in enzymatic modification of fibres. Protein engineering with high throughput screening systems allows the development of catalysts with higher specific activities or improved pH and temperature profiles. More efficient cellulolytic enzymes are the key for many processes, including partial hydrolysis for refining of mechanical fibres, fibre engineering or total hydrolysis of lignocellulosics. In fibre processing, enzymes carry out reactions which cannot be achieved by other means. High specificity, environment friendliness, and cost savings are currently the driving forces behind the development of new applications for lignocellulose processing.

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REFERENCES

1. Nevalainen, H.; Penttilä, M. 1995. In: The mycota II. Genetics and Biotechnology ; Kuck (Ed.) Springer-Verlag Berlin, Heidelberg.
2. Tomme, P.; Warren, R.A.J.; Gilkes, N.R. *Adv. Microb. Physiol.* **1995**, *37*, 1-81.
3. Kraulis, P.J., Clore, G.M., Nilges, M., Jones, T.A., Pettersson, G., Knowles, J.K.C. & Gronenborn, A.M. *Biochemistry* **1989**, *28*, 7241-7257.
4. Rouvinen, J.; Bergfors, T.; Teeri, T.; Knowles, J.K.C.; Jones, T.A. *Science* **1990**, *249*, 380-386.
5. Davies G.J.; Dodson, G.G.; Hubbard, R.E.; Tolley, S.P.; Dauter, Z.; Wilson, K.S.; Hjort, C.; Mikkelsen, J.M.; Rasmussen, G.; Schulein, M. *Nature* **1993**, *36*, :362-4.
6. Divne, C.; Ståhlberg, J.; Reinikainen, T.; Ruohonen, L.; Pettersson, G.; Knowles, J.K.C.; Teeri, T.T.; Jones, A. *Science* **1994**, *265*, 524-528.

7. Mattinen, M.-L.; Kontteli, M.; Kerovuo, J.; Linder, M.; Annila, A.; Lindeberg, G.; Reinikainen, T.; Drakenberg, T. *Prot. Sci.* **1997**, *6*, 294-303.
8. Sulzenbacher, G.; Driguez, H.; Henrissat, B.; Schulein, M.; Davies, G.J. *Biochemistry* **1996** *35*, 15280-7.
9. Kleywegt, G.J.; Zou, J.Y.; Divne, C.; Davies, G.J.; Sinning, I.; Stahlberg J.; Reinikainen, T.; Srisodsuk, M.; Teeri, T.T.; Jones, T.A. *J Mol Biol.* **1997**, *272*, 383-97.
10. MacKenzie, L.F.; Sulzenbacher, G.; Divne, C.; Jones, T.A.; Woldike, H.F.; Schulein, M.; Withers, S.G.; Davies, G.J. *Biochem J.* **1998**, *335*, 409-16.
11. Varrot, A.; Hastrup, S.; Schulein, M.; Davies, G.J. *Biochem J.* **1999** *337*, 297-304.
12. Davies, G.J.; Brzozowski, A.M.; Dauter, M.; Varrot, A.; Schulein, M. *Biochem J.* **2000**, *348*, 201-7.
13. Reinikainen, T.; Ruohonen, L.; Nevanen, T.; Laaksonen, L.; Kraulis, P.; Jones T.A.; Knowles, J.K.C.; Teeri, T.T. *Proteins: Structure, Function and Genetics* **1992** *14*, 475-482.
14. Srisodsuk, M.; Kleman-Leyer, K.; Keränen, S.; Kirk, T.K.; Teeri, T.T. *Eur. J. Biochem.* **1998**, *251*, 885-892.
15. Koivula, A.; Reinikainen, T.; Ruohonen, L.; Valkeajärvi, A.; Claeysens, M.; Teleman, O.; Kleywegt, G..J.; Szardenings, M.; Rouvinen, J.; Jones, T.A.; Teeri, T.T. *Prot. Eng.* **1996**, *9*, 691-699.
16. Linder, M.; Teeri, T.T. *Proc. Natl. Acad. Sci USA.* **1996**, *93*, 12251-12255.
17. Koivula, A.; Linder, M.; Teeri, T.T. 1998. In: *Trichoderma and Gliocladium*. Harman, G.E. & Kubicek, C.P. (Eds). London & Bristol, PA: Taylor & Francis Ltd., 1998. Vol. 2, 3-23.
18. Ståhlberg, J.; Divne, C.; Koivula, A.; Piens, K.; Claeysens, M.; Teeri, T.T.; Jones, T.A. *J. Mol. Biol.* **1996**, *264*, 337-349.
19. Linder, M.; Nevanen, T.; Teeri, T.T. *FEBS Lett.* **1999**, *447*, 13-16.
20. Carrard, G.; Linder, M. *Eur. J. Biochem.* **1999**, *262*, 637-643.
21. Carrard, G.; Koivula, A.; Söderlund, H.; Béguin, P. *Proc. Nat. Acad. Sci. USA* **2000**, *97*, 10342-10347.
22. Poutanen, K. *J. Biotechnol.* **1988**, *7*, 271-282.
23. Poutanen, K.; Sundberg, M. *Appl Microbiol. Biotechnol.* **1988**, *28*, 419-424.
24. Poutanen, K.; Puls, J. *Appl. Microbiol. Biotechnol.* **1988**, *28*,425-432.
25. Sundberg, M.; Poutanen, K. *Biotechnol. Appl. Biochem.* **1991**, *13*, 1-11.
26. Tenkanen, M.; Puls, J.; Poutanen, K. *Enzyme Microb. Technol.* **1992**, *14*, 566-574.
27. Ståhlbrand, H.; Siika-aho, M.; Tenkanen, M.; Viikari, L. *J. Biotechnol.* **1993**, *29*, 229-242.
28. Siika-aho, M.; Tenkanen, M.; Buchert, J.; Puls, J.; Viikari, L. *Enzyme Microb. Technol.* **1994**, *16*, 813-819.
29. Luonteri, E.; Siika-aho, M.; Tenkanen, M.; Viikari, L. *J. Biotechnol.* **1995**, *38*, 279-291.

30. Tenkanen, M.; Thornton, J.; Viikari, L. *J. Biotechnol.* **1995**, *42*, 197-206.
31. Luonteri, E.; Alatalo, E.; Siika-Aho, M.; Penttilä, M.; Tenkanen, M. *Biotechnol. Appl. Biochem.* **1998**, *28*, 179-188.
32. Luonteri, E.; Beldman, G.; Tenkanen, M. *Carbohydr. Polym.* **1998**, *37*, 131-141.
33. Luonteri, E.; Tenkanen, M.; Viikari, L. *Enzyme Microb. Technol.* **1998**, *22*, 192-198.
34. Tenkanen, M.; Siika-aho, M. *J. Biotechnol.* **2000**, *78*, 149-161.
35. Lappalainen, A.; Siika-aho, M.; Kalkkinen, N.; Fagerström, R.; Tenkanen, M. *Biotechnol. Appl. Biochem.* **2000**, *31*, 61-68.
36. Törrönen, A.; Harkki, A.; Rouvinen, J. *EMBO J.* **1994**, *13*, 2493.
37. Törrönen, A.; Rouvinen, J. *Biochemistry* **1995**, *34*, 847.
38. Sabini, E.; Schubert, H.; Murshudov, G.; Wilson, K.S.; Siika-aho, M.; Penttilä, M. *Acta Cryst.* **2000**, *56*, 3-13.
39. Hakulinen, N.; Tenkanen, M.; Rouvinen, J. *J. Struct. Biol.* **2000**, *132*, 180-190.
40. Biely, P.; Vršanská, M.; Tenkanen, M.; Kluepfel, D. *J. Biotechnol.* **1997**, *57*, 151-166.
41. Rättö, M.; Poutanen, K.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 470-473.
42. Perttula, M.; Rättö, M.; Konradsdottir, J.; Kristjansson, J.K.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1993**, *38*, 592-595.
43. Ritschkoff, A.-C.; Buchert, J.; Viikari, L. *J. Biotechnol.* **1994**, *32*, 67-74.
44. Rättö, M.; Mathrani, I.M.; Ahring, B.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1994**, *41*, 130-133.
45. Maijala, P.; Raudaskoski, M.; Viikari, L. *Microbiology* **1995**, *141*, 743-750.
46. Tenkanen, M.; Viikari, L.; Buchert, J. *Biotechnol. Techn.* **1997**, *11*, 935-938.
47. Kantelinen, A.; Hatakka, A.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1989**, *31*, 234-239.
48. Niku-Paavola, M.-L.; Karhunen, E.; Kantelinen, A.; Viikari, L.; Lundell, T.; Hatakka, A. *J. Biotechnol.* **1990**, *13*, 211-221.
49. Zurbriggen, B.; Penttilä, M.; Viikari, L.; Bailey, M. *J. Biotechnol.* **1991**, *17*, 133-146.
50. Bailey, M.J.; Viikari, L. *World J. Microbiol. Biotechnol.* **1993**, *9*, 80-84.
51. Bailey, M.; Buchert, J.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1993**, *40*, 224-229.
52. Puls, J.; Poutanen, K.; Körner, H.-U.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1985**, *22*, 416-423.
53. Poutanen, K.; Puls, J.; Linko, M. *Appl. Microbiol. Biotechnol.* **1986**, *23*, 487-490.
54. Poutanen, K.; Rättö, M.; Puls, J.; Viikari, L. *J. Biotechnol.* **1987**, *6*, 49-60.
55. Viikari, L.; Kantelinen, A.; Buchert, J.; Puls, J. *Appl. Microbiol. Biotechnol.* **1994**, *41*, 124-129.

56. Tenkanen, M.; Buchert, J.; Viikari, L. *Enzyme Microb. Technol.* **1995**, *17*, 499-505.
57. Tenkanen, M.; Luonteri, E.; Teleman, A. *FEBS Lett.* **1996**, *399*, 303-306.
58. Rahkamo, L.; Siika-aho, M.; Vehviläinen, M.; Dolk, M.; Viikari, L.; Nousiainen, P.; Buchert, J. *Cellulose* **1996**, *3*, 153-163.
59. Rättö, M.; Ritschkoff, A.-C.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 53-57.
60. Tenkanen, M.; Makkonen, M.; Perttula, M.; Viikari, L.; Teleman, A. *J. Biotechnol.* **1997**, *57*, 191-204.
61. Rahkamo, L.; Paakkari, T.; Suortti, T.; Viikari, L.; Buchert, J. *Cellulose* **1998**, *5*, 79-88.
62. Rahkamo, K.; Siika-aho, M.; Viikari, L.; Leppänen, T.; Buchert, J. *Holzforschung* **1998**, *52*, 630-634.
63. Itävaara, M.; Siika-aho, M.; Viikari, L. *J. Environ. Polym. Degrad.* **1999**, *7*, 67-73.
64. Palonen, H.; Tenkanen, M.; Linder, M. *Appl. Environ. Microbiol.* **1999**, *65*, 5229-5233.
65. Kotiranta, P.; Karlsson, J.; Siika-aho, M.; Medve, J.; Viikari, L.; Tjerneld, F.; Tenkanen, M. *Appl. Biochem. Biotechnol.* **1999**, *81*, 81-90.
66. Kantelinen, A.; Rantanen, T.; Buchert, J.; Viikari, L. *J. Biotechnol.* **1993**, *28*, 219-228.
67. Rättö, M.; Siika-aho, M.; Buchert, J.; Valkeajärvi, A.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1993**, *40*, 449-454.
68. Suurnäkki, A.; Kantelinen, A.; Hortling, B.; Buchert, J.; Viikari, L. *Holzforschung* **1994**, *48*, 291-296.
69. Tenkanen, M.; Puls, J.; Rättö, M.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1993**, *38*, 159-165.
70. Tenkanen, M. *Appl. Biochem.* **1998**, *27*, 19-24.
71. Viikari, L. *Appl. Microbiol. Biotechnol.* **1984**, *20*, 118-123.
72. Viikari, L. *CRC Crit. Rev. Biotechnol.* **1988**, *7*, 237-261.
73. Buchert, J.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1988**, *29*, 375-379.
74. Hallborn, J.; Walfridsson, M.; Airaksinen, U.; Ojamo, H.; Hahn-Hägerdal, B.; Penttilä, M.; Keränen, S. *Bio/Technology* **1991**, *9*, 1090-1095.
75. Hallborn, J.; Gorwa, M.-F.; Meinander, N.; Penttilä, M.; Keränen, S.; Hahn-Hägerdal, B. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 326-333.
76. Meinander, N.; Hahn-Hägerdal, B.; Linko, M.; Linko, P.; Ojamo, H. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 334-339.
77. Richard, P.; Toivari, M.H.; Penttilä, M. *FEBS Lett.* **1999**, *457*, 135-138.
78. Aristidou, A.; Penttilä, M. *Curr. Opin. Biotechnol.* **2000**, *11*, 187-198.
79. Toivari, M.H.; Aristidou, A.; Ruohonen, L.; Penttilä, M. *Metab. Eng.* **2001**, *3*, 236-249.
80. Tien, M.; Kirk, T.K. *Science* **1983**, *221*, 661-663.
81. Glenn, J.K.; Gold, M.H. *Arch. Biochem. Biophys.* **1985**, *242*, 329-341.
82. Niku-Paavola, M.-L.; Karhunen, E.; Salola, P.; Raunio, V. *Biochem. J.* **1988**, *254*, 877-884.

83. Saloheimo, M.; Barajas, V.; Niku-Paavola, M.-L.; Knowles, J. *Gene*, **1989**, *85*, 343-351.
84. Niku-Paavola, M.-L.; Karhunen, E.; Kantelinen, A.; Viikari, L.; Lundell, T.; Hatakka, A. *J. Biotechnol.* **1990**, *13*, 211-221.
85. Karhunen, E.; Niku-Paavola, M.-L.; Viikari, L.; Haltia, T.; Van der Meer, R.; Duine, J. *FEBS Lett.* **1990**, *267*, 6-8.
86. Kantelinen, A.; Hortling, B.; Ranua, M.; Viikari, L. *Holzforschung* **1993**, *47*, 29-35.
87. Saloheimo, M.; Niku-Paavola, M.-L. *Bio/Technology* **1991**, *9*, 987-990.
88. Jönsson, L.J.; Saloheimo, M.; Penttilä, M. *Curr. Genet.* **1997**, *32*, 425-430.
89. Saloheimo, M.; Niku-Paavola, M.-L.; Knowles, J.K.C. *J. Gen. Microbiol.* **1991**, *137*, 1537-1544.
90. Kiiskinen, L.-L.; Viikari, L.; Kruus, K. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 198-204.
91. Hakulinen, N.; Kiiskinen, L.-L.; Kruus, K.; Saloheimo, M.; Paananen, A.; Koivula, A.; Rouvinen, J. *Nature Struct. Biol.* **2002**, *9*, 601-605.
92. Viikari, L.; Ranua, M.; Kantelinen, A.; Sundquist, J.; Linko, M. 1986. 3rd Int. Conf. Biotechnology in the pulp and paper industry, Stockholm, pp. 67-69.
93. Viikari, L.; Ranua, M.; Kantelinen, A.; Linko, M.; Sundquist, J. 1987. 4th Int. Symp. Wood and pulping chemistry, Paris, Vol. 1, pp. 151-154.
94. Viikari, L.; Kantelinen, A.; Sundquist, J.; Linko, M. *FEMS Microbiol. Rev.* **1994**, *13*, 335-350.
95. Tolan, J.S.; Thibault, L. Proceedings TAPPI 2000 Pulping conference, Boston, Mass.
96. Viikari, L.; Tenkanen, M.; Suurnäkki, A. 2001, *In: Biotechnology* (Rehm, H.-J. & Reed, G., Eds) Vol. 10: Special Processes. Weinheim-New York: Wiley-VCH, 523-546.
97. Senior, D.J.; Mayers, P.R.; Saddler, J.N. *Biotech. Bioeng.* **1991**, *37*, 274-279.
98. Kantelinen, A.; Hortling, B.; Sundquist, J.; Linko, M.; Viikari, L. *Holzforschung* **1993**, *47*, 318-324.
99. Buchert, J.; Tenkanen, M.; Pitkänen, M.; Viikari, L. *Tappi J.* **1993**, *76*, 131-135.
100. Hortling, B.; Korhonen, M.; Buchert, J.; Sundquist, J.; Viikari, L. *Holzforschung* **1994**, *48*, 441-446.
101. Suurnäkki, A.; Kantelinen, A.; Buchert, J.; Viikari, L. *Tappi J.* **1994**, *77*, 111-116.
102. Suurnäkki, A.; Heijnesson, A.; Buchert, J.; Westermark, U.; Viikari, L. *J. Pulp Paper Sci.* **1996**, *22*, J91-J96.
103. Saake, B.; Clark, T.; Puls, J. *Holzforschung* **1995**, *49*, 60-68.
104. Buchert, J.; Carlsson, G.; Viikari, L.; Ström, G. *Holzforschung* **1996**, *50*, 69-74.

105. Teleman, A.; Harjunpää, V.; Tenkanen, M.; Buchert, J.; Hausalo, T.; Drakenberg, T.; Vuorinen, T. *Carbohydr. Res.* **1995**, *272*, 55-71.
106. Suurnäkki, A.; Li, T.-Q.; Buchert, J.; Tenkanen, M.; Viikari, L.; Vuorinen, T.; Ödberg, L. *Holzforschung* **1997**, *51*, 27-33.
107. Buchert, J.; Kantelinen, A.; Suurnäkki, A.; Viikari, L.; Janson, J. *Holzforschung* **1995**, *49*, 439-444.
108. Buchert, J.; Bergnor, E.; Lindblad, G.; Viikari, L.; Ek, M. *Tappi J.* **1997**, *80*, 165-171.
109. Suurnäkki, A.; Heijnesson, A.; Buchert, J.; Tenkanen, M.; Viikari, L.; Westermark, U. *J. Pulp Paper Sci.* **1996**, *22*, J78-J83.
110. Suurnäkki, A.; Heijnesson, A.; Buchert, J.; Viikari, L.; Westermark, U. *J. Pulp Paper Sci.* **1996**, *22*, J43-J47.
111. Buchert, J.; Teleman, A.; Harjunpää, V.; Tenkanen, M.; Viikari, L.; Vuorinen, T. *Tappi J.* **1995**, *78*, 125-130.
112. Vuorinen, T.; A. Teleman, A.; Fagerström, P.; Buchert, J.; Tenkanen M., 1996 International Pulp Bleaching Conference, Washington D.C., Proceedings, pp. 43-51.
113. Buchert, J.; Salminen, J.; Siika-aho, M.; Ranua, M.; Viikari, L. *Holzforschung* **1993**, *47*, 473-478.
114. Suurnäkki, A.; Clark, T.A.; Allison, R.W.; Viikari, L.; Buchert, J. *Tappi J.* **1996**, *79*, 111-117.
115. Buchert, J.; Ranua, M.; Kantelinen, A.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 825-829.
116. Clarke, J.; Rixon, J.; Ciruela, A.; Gilbert, H.; *Appl. Microbiol. Biotechnol.* **1997**, *48*, 177-183.
117. Rixon, J.; Clarke, J.; Hazlewood, G.; Hoylamd, R.; McCarthy, A.; Gilbert, H.; *Appl. Microbiol. Biotechnol.* **1996**, *46*, 514-520.
118. Buchert, J.; Ranua, M.; Siika-aho, M.; Pere, J.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1994**, *40*, 941-945.
119. Bourbonnais, R.; Paice, M. *Appl. Microbiol. Biotechnol.* **1992**, *36*, 823.
120. Call, H.P.; Mücke, I. *J. Biotechnol.* **1997**, *53*, 163-202.
121. Amann, M. 1997, Proc. 9th Int. Symp. on Wood and Pulp. Chem., Montreal, 1997, F4, pp.1-5.
122. Poppius-Levlin, K.; Wang, W.; Tamminen, T.; Hortling, B.; Viikari, L.; Niku-Paavola, M.-L. *J. Pulp Paper Sci.* **1999**, *25*, 90-94.
123. Nelson, P.J.; Chin, C.W.J.; Viikari, L.; Tenkanen, M. *Appita Journal* **1998**, *51*, 451-456.
124. Petit-Conil, M.; Semar, S.; Niku-Paavola, M.-L.; Sigoillot, J.C.; Asther, M.; Anke, H.; Viikari, L. 2002. In: Progress in Biotechnology, Vol. 21 (Viikari, L. & Lantto, R., Eds) Elsevier Science B.V., Amsterdam, pp. 193-201.
125. Hatakka, A. 2001. In: Biopolymers: Biology, Chemistry, Biotechnology, Applications. Vol. 1. Lignin, Humic Substances and Coal (Hofrichter, M. &

- Steinbuechel, A., eds), Wiley-VCH, Weinheim, Germany, Chapter 5, pp.129-180.
- 126.Grönqvist, S.; Suurnäkki, A.; Niku-Paavola, M-L.; Kruus, K.; Buchert, J.; Viikari, L. 2003, ACS Symposium series (chapter in this book).
 - 127.Niku-Paavola, M.-L.; Tamminen, T.; Hortling, B.; Viikari, L.; Poppius-Levlin, K., 2002, *In: Progress in Biotechnology, Vol 21* (Viikari, L. & Lantto, R., Eds) Elsevier Science B.V., Amsterdam, 121-130.
 - 128.Eggert, C.; Temp, U.; Dean, J.F.D.; Eriksson, K-E.L. *FEBS Lett.* **1996**, *391*, 144.
 - 129.Rättö, M.; Niku-Paavola, M.-L.; Raaska, L.; Mattila-Sandholm, T.; Viikari, L. *Material u. Organismen* **1996**, *30*, 279-292.
 - 130.Niku-Paavola, M-L.; Anke, H.; Poppius-Levlin, K.; Viikari, L. 2003, ACS Symposium series (chapter in this book).
 - 131.Oksanen, T.; Buchert, J.; Amann, M.; Candussio, A.; Viikari, L. 2002. *In: Progress in Biotechnology, Vol. 21* (Viikari, L. & Lantto, R., Eds), Elsevier Science B.V., Amsterdam, pp 255-262.
 - 132.Pere, J.; Siika-aho, M.; Viikari, L. *Tappi J.* **2000**, *83*, 8.
 - 133.Rättö, M.; Kantelinen, A.; Bailey, M.J.; Viikari, L. *Tappi J.* **1993**, *76*, 125-128.
 - 134.Fischer, K.; Messner, K. *TappiJ.* **1992**, *75*, 130-134.
 - 135.Mustranta, A.; Fagernäs, L.; Viikari, L. *Tappi J.* **1995**, *78*, 140-146.
 - 136.Kantelinen, A.; Jokinen, O.; Sarkki, M-L.; Pettersson, C.; Sundberg, K.; Eckerman, C.; Ekman R.; Holmbom, B. 1995, *Int. Symp. Wood Pulp Chem.* **1**, 605-612.
 - 137.Thornton, J.; Tenkanen, M.; Ekman, R.; Holmbom, B.; Viikari, L. *Holzforschung* **1994**, *48*, 436-440.
 - 138.Karlsson, S.; Holmbom, B.; Spetz, P.; Mustranta, A.; Buchert, J. *Appl. Microbiol. Biotechnol.*, **2001**, *55*, 317-320.
 - 139.Buchert, J.; Mustranta, A.; Tamminen, T.; Spetz, P.; Holmbom, B. *Holzforschung* **2002**, *56*, 579-584.
 - 140.Jeffries, T.W.; Klungness, J.H.; Stykes, M.S.; Rutledge-Cropey, K.; *Tappi J.* **1994**, *77*, 173-179.
 - 141.Welt, T.; Dinus, R.; *Prog. Pp. Recycl.* **1995**, *36*, 47.
 - 142.Hatakka, A.; Mettälä, A.; Rouhiainen, L.; Viikari, L. *Holzforschung* **1994**, *48*, 82-88.
 - 143.Hortling, B.; Rättö, M.; Forsskähl, I.; Viikari, L. *Holzforschung* **1993**, *47*, 155-162.
 - 144.Rättö, M.; Forsskähl, I.; Jansson, J.; Viikari, L. *Tappi J* **1993**, *76*, 67-70.
 - 145.Pere, J., Siika-aho, M., Buchert, J.; Viikari, L. *Tappi J.* **78** (1995) 71-78.
 - 146.Oksanen, T.; Pere, J.; Buchert, J.; Viikari, L. *Cellulose* **1997**, *4*, 329-339.
 - 147.Mansfield, S.D.; Saddler, J.N. *J. Pulp Paper Sci.* **1999**, *25*, 84-89.

Chapter 3

Lignocellulose Processing with Oxidative Enzymes

**S. Grönqvist, A. Suurnäkki, M-L. Niku-Paavola, K. Kruus,
J. Buchert, and L. Viikari**

VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Finland

Since the successful introduction of commercial hydrolytic enzymes to lignocellulose processing, the next generation of oxidative enzymes are now entering the markets. Significant progress in molecular biology have enabled us to better understand the electron transfer mechanisms in the lignocellulosic substrates and improve the production of these enzymes at a commercial scale. The most intensively studied application is enzyme catalysed delignification, for which several concepts have been introduced. Recently, other applications, such as oxidative fibre modification or activation of lignin to replace traditional adhesives have been actively studied. However, in spite of extensive research, the underlying mechanisms are still only partially understood. This paper reviews recent advances in the application of oxidative enzymes for lignocellulose processing.

Oxidative enzymes have potential in several applications in various industrial areas, such as the cosmetic, food, textile, chemical and pulp and paper sectors. In lignocellulose processing oxidative enzymes can be used for modification of lignin and extractives. The enzymology of lignin modification has been the focus of scientists for more than 30 years. Due to the mostly

promising results that have been obtained in bleaching and pulping, laccases and manganese-dependent peroxidases have been the most extensively studied groups of enzymes in this area. The first laccases have been on the market for some years, and laccase-based mediated bleaching systems have been developed and tested at a pilot-scale. The ability of laccases to oxidize lignin is currently being evaluated in the activation of fibre surfaces for bonding, grafting or glueing applications.

Enzymatic modification of fibre bound substrates represents a continuous challenge for scientists. Besides the diverse chemical compositions of the major components, the fibre cell wall matrix embeds these fractions to produce the rigidity and resistance typical of plants. The structures of carbohydrates; cellulose and hemicellulose, are chemically well understood, whereas lignin forms an undefined structure. In spite of extensive research, the mechanisms of enzymatic modification, especially degradation of lignin are not yet fully understood. This article reviews the latest achievements in oxidative modification of fibre components, while primarily focusing on lignin.

Oxidative Enzymes

Research related to lignin biodegradation has resulted in the identification of the essential enzymes in lignin degradation including oxidases, peroxidases, dehydrogenases and hydrogen peroxide generating enzymes. The only organisms capable of efficiently mineralising lignin are basidiomycetous white-rot fungi and related litter-decomposing fungi (1). Physiological conditions for lignin degradation, as well as secretion patterns of the lignolytic enzymes vary substantially among different fungal species (2). The most extensively studied lignolytic enzymes for various biotechnical applications include laccases and manganese-dependent peroxidases of white-rot fungi. Promising results with these enzymes have been obtained in pulp and textile dye bleaching as well as fibre modification (3).

Peroxidases

Fungal peroxidases participating in lignin biodegradation include lignin peroxidase (LiP, EC 1.11.1.14) (4), manganese-dependent peroxidase (MnP, EC 1.11.1.13) (5) and peroxidases having properties of both LiP and MnP and being either manganese-independent peroxidase (MIP) (6), LiP-like (7) or versatile peroxidase (8). There are also nonligninolytic fungal peroxidases, which do not have the characteristic substrate oxidation sites of either LiP or MnP (9). Purified ligninolytic enzymes have been shown to cause limited delignification

provided that additives are supplemented; veratryl alcohol and H_2O_2 for LiP (10) and manganese, H_2O_2 , organic acids and surfactants for MnP (11, 12).

Since the discovery of LiP and MnP in *Phanerochaete chrysosporium* (4, 5) these enzymes have been found to be secreted by many white-rot fungi, usually in multiple isoenzymes. LiP and MnP are heme-containing glycoproteins using hydrogen peroxide as an electron acceptor. LiP oxidizes nonphenolic subunits of lignin by a one-electron transfer mechanism resulting in formation of cation radicals, which are further decomposed chemically (13). It has been shown by various analysis with synthetic lignin and lignin model compounds that LiP is responsible for C_α - C_β bond cleavage, ring opening as well as many other reactions.

MnP oxidizes Mn(II) to Mn(III), which organic acids e.g. oxalic, malic, lactic, or malonic acid stabilize by chelating. Chelated Mn(III) oxidizes phenolic subunits in lignin and forms phenoxy radicals, which may further cleave bonds between aromatic rings and the C_α carbon atoms (1). Chelated Mn(III) is in general a powerful oxidant, which may also oxidize some nonphenolic aromatics such as dyes. Mn(III) also creates radicals from the co-oxidants present in the medium. Thiyl and peroxy radicals, formed from thiols and unsaturated fatty acids respectively, are highly reactive and mediate oxidation towards nonphenolic lignin structures (14, 15). However, Mn(III) is not capable of oxidizing recalcitrant nonphenolic units of lignin. The capacity of MnP to oxidize lignin is limited because the phenolic structures constitute only 10-15 % of all units in lignin (16). However, the research on various white-rot fungi has shown that MnP is more common than LiP (2, 17) and that it has an essential role in depolymerization of lignin (18).

In the past few years several crystal structures of peroxidases from different sources have been reported. The three-dimensional structures of both LiP (19, 20) and MnP have been solved (21). Interestingly, the overall folding and the secondary structure of peroxidases are highly conserved despite their low sequence homology. The structure information, as well as the vast sequence data have increased our knowledge of the action of peroxidases on aromatic substrates. However, the way these enzymes act towards polymeric lignin is still not fully understood. Significant progress in production of recombinant peroxidases (22) has recently been obtained. Further enhancement of production might also be possible by means of the genomic data of *Phanerochaete chrysosporium*, which has recently been made available (23).

Laccases

Laccases (EC 1.10.3.2) are probably the most commonly occurring oxidoreductases in white-rot fungi (24). Most of the isolated and characterised

laccases are from fungal origin. Well known laccase producers include *Trametes*, *Pleurotus*, *Coprinus*, *Myceliophthora*, *Phlebia*, *Pycnoporus*, *Rhizoctonia*, and *Schizophyllum* (25). Laccase or laccase-like activity has also been demonstrated by plants, some insects and a few bacteria (26). It is well recognised that laccases are involved in both polymerisation and depolymerisation processes of lignin. The plant origin laccases are reported to have an important role in wound response and lignin biosynthesis (27) whereas in fungi they are involved in lignin degradation, as well as in several other functions including pigmentation, fruiting body formation, sporulation, and pathogenesis (26, 28). A biological role of laccases in the oxidation of Mn^{2+} has also recently been proposed (29).

Laccases belong to the blue multi-copper oxidase family. The catalytic site of laccases contains four copper atoms per laccase molecule. The copper atoms can be classified into three types: one type 1 Cu, one type 2 Cu, and two type 3 Cu. The mononuclear site (type 1 Cu) functions as the primary electron acceptor, extracting electrons from the substrate. The copper is coordinated by two histidine nitrogens and a cysteine sulfur with a highly covalent Cu-S bond giving rise to the pronounced blue color of laccases. Type 2 and two type 3 Cu form the trinuclear center, where reduction of molecular oxygen takes place. It is not fully understood how the electrons are transferred from the mononuclear site to the trinuclear site. It has been proposed that the electrons are extracted through a conserved Cys-His pathway from the mononuclear site to the trinuclear site (30).

Laccases catalyze the four-electron reduction of dioxygen to water with four concomitant one-electron oxidation of the reducing substrate. The mononuclear site functions as a primary electron acceptor whereas the trinuclear center, the binding site of the dioxygen, accepts electrons from the mononuclear site. The exact nature of the reaction mechanism is still controversial and debated. The most widely accepted mechanism is that proposed by Messerschmidt *et al.* (30). Although laccases have been extensively studied, thus far only two crystal structures are available, namely the type 2 copper depleted laccase from *Coprinus cinereus* (31) and a recently published laccase from *Trametes versicolor* in four copper form (32). In addition, the crystallisation of laccases from *Trametes versicolor* and *Pycnoporus cinnabarinus* have been reported (33).

Laccases display a surprisingly broad specificity towards the reducing substrate. They catalyse oxidation of a wide variety of aromatics, especially phenolic, and inorganic substrates. Simple diphenols like hydroquinone and catechol, polyphenols, diamines, and aromatic amines are good substrates for most laccases. Since the description of the mediator concept in the early nineties (34-36), the range of potential mediator substrates has continued to increase. Mediators are small molecular weight compounds, which can be oxidised by laccase. The oxidised mediator then oxidises the actual substrate. A typical

example of a mediator is hydroxybenzotriazole (HBT), which has been studied intensively for delignification together with laccase (37). Laccase alone can only oxidise phenolic subunits in lignin. However, when combined with a mediator, non-phenolic groups can also be oxidised. Promising results with mediated oxidation (Figure 1) have been obtained in pulp delignification. In principle, different types of monomers, as well as polymers, can be oxidised by a suitable enzyme mediator combination. Because of the broad substrate specificity range of laccases, they possess great biotechnological potential. The most intensively studied applications for these enzymes include pulp delignification, textile dye bleaching, effluent detoxification as well as biopolymer modification (3).

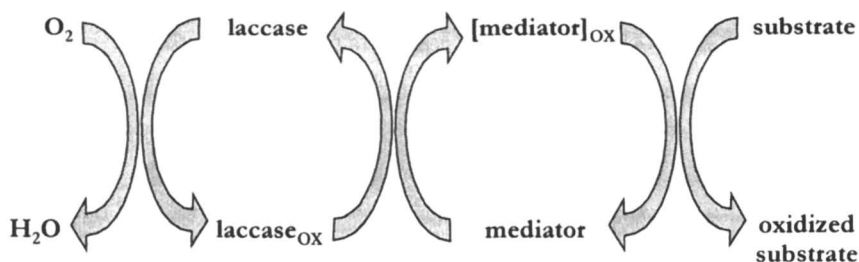


Figure 1. A schematic presentation of mediated oxidation by laccase.

Surface Chemistry Of Pulp Fibres

Wood fibres are mainly composed of cellulose, hemicellulose, *i.e.* xylan and glucomannan, lignin and extractives (38). As oxidative enzymes react with lignin both the chemistry and location of the lignin in the pulp fibres is of most importance for the enzyme activity. In mechanical pulping no major chemical changes in the fibre components occur, whereas during alkaline chemical pulping, *i.e.* kraft cooking, about 90 % of lignin is removed from the fibres. The larger average pore size of chemical fibres renders them more susceptible to the action of macromolecular enzymes. The enzymatic action even in chemical pulps is, however, limited to accessible surfaces, *i.e.* to fines and to the outermost surface and accessible pores of long fibres (39).

In addition to fibres process waters in paper manufacture also contain various wood derived compounds, *i.e.* dissolved and colloidal substances (DCS). These are also potential substrates for enzymatic oxidation. These wood components, such as extractives, carbohydrates and lignin are dissolved and

dispersed into the process waters (40, 41) during mechanical pulp production and bleaching. In chemical pulping and bleaching wood extractives are extensively modified and degraded (38).

The surface composition of different types of fibres has been analysed by ESCA (Electron Spectroscopy for Chemical Analysis). Unbleached softwood kraft pulps are reported to have a surface coverage of lignin of about 10-30% depending on the pulp kappa number (42). Surface coverage of lignin in unbleached hardwood kraft pulp is reported to be about 20 % (43). Partial enzymatic removal of xylan results in increased surface coverage of lignin in conventional unbleached pine kraft pulps (43). Concentrations of both lignin and xylan in primary and secondary fines of unbleached kraft pulps have been visualised by mechanical peeling techniques (44). In mechanical pulps about 33 % of the surfaces are covered by lignin and this surface coverage of lignin is not changed in the bleaching due to non-delignifying bleaching (45, 46). As oxidative enzymes are particularly active on the fibre surfaces, both mechanical and chemical pulps contain potential substrates for oxidation.

Bleaching Of Chemical Pulp

Today, bleaching of kraft pulps is mainly carried out with chlorine dioxide, hydrogen peroxide, oxygen and ozone in variable sequences. During the search for environmentally sound alternatives for chlorine based chemicals, enzymatic methods were also developed and commercialised. Enzyme-aided bleaching is used today in the pulp and paper industry to improve the bleachability of kraft pulps through the action of xylanases or other enzymes affecting the extractability of lignin. The effect of xylanase pretreatment on bleachability is, however, limited. The most promising direct enzymatic bleaching system is based on the use of oxidative enzyme, laccase, together with a mediator directly degrading lignin. In addition to laccase, the potential of MnP has been studied in chemical pulp bleaching. The effect of oxidative enzymes on the potential for lignin preserving bleaching of mechanical pulps has also been studied (47).

Laccase-Mediator Concept In Chemical Pulp Bleaching

In the laccase-mediator concept, the mediator oxidised by laccase enzyme acts directly on lignin and results in efficient delignification (Figure 1). In the initial study, the common substrate of laccases, ABTS was used as the mediator (35). The search for a more suitable mediator resulted in discovery of 1-hydroxybenzotriazole (HBT) (36). This delignification procedure is commonly referred to as the LMS (laccase-mediator-system) or Lignozyme process and it

has been demonstrated in pilot scale in totally chlorine free (TCF) bleaching sequence (37). A number of other mediators with great structural variety have been studied. The most effective mediators in delignification usually contain N-OH functional groups (48, 49), such as the most promising current mediators, violuric acid (VIO) and N-hydroxy-N-phenylacetamide (NHA). The latter mediator results in extremely fast delignification with no significant impact on cellulose structure (50). The performance of NHA was further improved by implementing a slow-release mediator system, based on a precursor of NHA (DiAc, N-acetoxy-N-phenylacetamide) (51). The delignification degree of laccase-HBT after an alkaline extraction has also been reported to be high, up to 40 % in low-kappa number softwood and hardwood kraft pulps (52). In high kappa number softwood kraft pulps, violuric acid has been reported to be as twice as efficient a mediator as both HBT and NHA in the LMS bleaching (53). In addition to nitrogen based mediators, inorganic mediators such as transition metal complexes or polyoxometalates containing *e.g.* molybdenum ion have recently been successfully tested for laccase-mediator bleaching (54, 55). Until now, only one fungal metabolite, 3-hydroxyanthranilate, has been introduced as a natural mediator (56). Other potential natural mediators are siderophores, which are strong iron chelating agents, have also been studied for lignin degradation (57). Several studies on the mechanisms of laccase-mediated delignification of pulps have been published (*e.g.* 58-66).

The efficiency of laccase-DiAc stage was recently demonstrated in pilot scale in an ECF bleaching sequence, LaEpD₀EoD₁ (67). The pilot scale bleaching trial with laccase-DiAc stage required 24 % less chlorine dioxide than the reference mill sequence (D₀EopD₁EpD₂) without strength loss, suggesting that laccase-DiAc stage could be an alternative for oxygen delignification stage in ECF bleaching. Optimisation of alkaline extraction stages and further development of enzyme suitable for alkaline conditions required in DiAc conversion to NHA and high shear forces were, however, found to be prerequisites for economical viability of the system. The LMS system has been shown to be able to replace either the oxygen delignification or ozone stage, (68-70). The development of the laccase-mediator concept is presented in Table I.

In addition to delignification, the effects of LMS on the physical properties of pulps have been determined. In high kappa number chemical pulps, both laccase-HBT treatment and HBT treatment alone enhanced the handsheet densification during PFI refining (71). The use of laccase with NHA and violuric acid resulted in similar bonding strength as compared to oxygen delignification, but without reduction in viscosity (50).

The combination of xylanase and laccase-mediator bleaching systems either sequentially or simultaneously has been reported to result in additive enhancement of bleachability (72-74). The application of LMS system employing HBT as mediator with xylanase treatment in one single stage was

found to be ineffective, apparently due to the inactivation of xylanase by the HBT (72). This inactivating effect of HBT has also been observed towards laccases (49) as HBT radicals undergo chemical reactions with the aromatic amino acid side chains of many laccases. Studies on new mediators have revealed that NHA caused less damage to enzymes (72, 75). In practice, it would be beneficial to combine the indirect xylanase treatment with the delignifying laccase-mediator treatment as the target substrates of these treatments are different and thus the maximal effect of both treatments could be exploited.

Table I. Steps in the development of laccase-mediator systems

<i>When</i>	<i>Description</i>
1986	Enzyme-mediator concept (ref. by 37)
1990, 1991	Redox cascade, often in the presence of chelating agents (ref. by 37)
1992	Laccase and ABTS as mediator (35)
1992	Laccase and HBT as mediator (ref. by 37)
1993, 1994	Laccase and mediators containing N-OH, N-oxide, oxime or hydroxamic acid-compounds (ref. by 37)
1994	Pilot plant trial with HBT in TCF sequence, degree of delignification > 50% (ref. by 37)
1997	Laccase and NHA: Lower costs, reduced laccase inhibition, biodegradable and higher selectivity (48)
2000	Slow-release mediator (NHA- DiAc) Better cost efficiency, less mediator carryover (51)
2000	Metal-complex mediator (54) Completely reversible mediation, only catalytic amounts of mediator needed
2001	Pilot plant trial with slow- release mediator (NHA- DiAc) in ECF sequence, high brightness (88 % ISO) without strength loss (67)

MnP In Pulp Bleaching

The use of manganese peroxidase (MnP) has also been studied for bleaching of chemical pulps (11, 12, 76-78). In small scale tests, demethoxylation, delignification and an increase of about 10 ISO units in brightness after alkaline stage has been reported (12, 76, 77). Unlike the laccase-mediator system, the MnP based system uses a natural mediator, Mn(II). MnP uses hydrogen peroxide as the electron acceptor and oxidises chelated Mn(II) to Mn(III). Stable chelated Mn(III) can then diffuse to the fibre matrix, which then leads to the formation of

phenoxy radicals on the phenolic structures within the lignin. However, the nonphenolic lignin structures are not attacked. It has, however, been suggested that other reactions, such as peroxidation of lipids (including certain extractives) initiated by MnP could also be involved in degradation of both phenolic and non-phenolic units in lignin (15). The applicability of MnP in pulp delignification on industrial scale is limited mainly due to the strictly controlled reaction conditions demanded by MnP, as well as to the obviously high price and limited availability of the enzyme. Calculations on the costs of the components needed (peroxide, additives, enzyme) have not been published.

Oxidative Enzymes In Fibre Modification

The properties of fibre products are determined both by the physical and chemical properties of the fibres and the chemical additives used in processing. Upgrading of fibre properties is in many cases of great interest. The availability of oxidative enzymes such as laccase, capable of radicalising papermaking fibres, has raised the idea of an alternative, environmentally sound approach to wood fibre upgrading by targeted modification of fibres by enzymatic or chemo-enzymatic methods (Table II).

The primary reaction of laccase and other phenoloxidases is the formation of phenolic or cationic radicals. The oxidative enzymes initiate radical formation in solubilised lignans and colloidal lignin, as well as in fibres that will react further without additional enzymatic action (79-82). Due to the high reactivity of these radicals (either with each other or with a secondary substrate), reactions such as polymerisation, depolymerisation, co-polymerisation and grafting can occur. The size of oxidases limits the range of the enzyme on the fibre surface (83). Hence, such enzymes can be used to carry out surface specific modification of fibres. When more extensive modification is needed, small molecular weight mediators can be used together with laccase.

The ability of laccases to oxidise fibre bound lignin is somewhat unclear. The most probable primary substrates are the colloidal and solubilised lignin fragments present in pulp suspensions or attached to the fibres. In addition, extractives have been proposed to act as mediators in the oxidation reaction (79-81). Indeed, it has been suggested that the presence of water-soluble extractives would be essential for radical formation in fibre bound lignin (81). The activity of laccases on lipophilic extractives and hydrophobic lignans has been reported in several papers (84-87).

It has been proposed that the state of lignin in wood fibres determines the dominating pathway of oxidation (81). Depending on the chemical and physical structure of the lignin polymer, different types of modifications of lignin can take place. Laccase treatments have been found to generate two oxidation species in

lignin, *i.e.*, via oxygen chemically transformed lignin products and initial oxidation radicals that have gained stabilisation (81, 88). The radicals formed are phenolic and they can be observed directly (80).

Functionalization Of Fbres By Oxidative Enzymes

The ability of oxidative enzymes to create long-living radicals to fibre surfaces can also be exploited as such or after further functionalisation of fibres with specific chemical components. The chemical changes caused by the enzymatic modification may include macro-scale modifications caused by radical-initiated polymerisation or depolymerisation reactions (Table II). Thus, it can be envisioned that the presence of surface lignin in mechanical and lignin-rich chemical pulp fibres offers possibilities for producing tailor-made or completely novel paper and board products.

Laccase catalysed radical coupling of compounds to lignin has been mostly carried out with defined substrates (89-96). Mai and Hütterman (91) suggest that organic peroxides are needed to start the copolymerisation of acrylamide with lignin oxidised by laccase. Success in grafting low molecular weight compounds to surface lignin activated by laccase has also been reported. According to Chandra and Ragauskas (97), laccase facilitates the coupling of phenolic acids to fibre surfaces. Lund *et al.* (95) reported attempts to graft phenolic monomers onto softwood kraft pulp. They (95) questioned, however, whether laccase is actually bound to the lignin in fibres. As consequence of laccase activation, increases in sheets strength have been reported (98). Improvement in wet strength of kraft pulp fibres with laccase in the presence of lignin rich extractives has also been reported (99).

Adhesion Of Fibres By Oxidative Enzymes

In conventional production of lignocellulose based composites, such as fibre or particle boards, synthetic adhesives are used in combination with hot pressing. Alternative enzymatic methods may allow the production of particle and fibreboards with less or even totally without hazardous adhesives, such as urea-formaldehyde, phenol-formaldehyde or isocyanide. Laccase catalysed bonding can be achieved by activation of additional lignin by the oxidative enzyme (two component system) or by the enzymatic activation of the lignin present in fibres (one component system). Besides laccases, peroxidases have been studied for activation of lignin (100, 101). In their first experiments, Haars and Hüttermann (102) used the two component system where laccase treated lignosulphonate was used as an additive to bond wood fibres. In later experiments Haars and

Table II. Fibre modification with oxidative enzymes

<i>Aim</i>	<i>Description</i>
Modification of pulp properties	<p>Generation of bonding strength on woody fibres by enzymatic phenol polymerisation with dehydrogenases (107)</p> <p>Modification of chemical and mechanical pulp fibres by laccase (108)</p> <p>Detection of laccase induced modification by luminescence spectroscopy (109)</p> <p>Studies on oxidative species generated in the lignin of wood fibres by a laccase catalysed treatment (81)</p> <p>Effect of cellulase, laccase and proteinase on papermaking properties of mechanical pulp fibres (110)</p> <p>Peroxidase treatment of pulp led to enhanced beating (111)</p> <p>Improved wet strength of paper material by the combined action of laccase and mediator (99, 112)</p> <p>Laccase treatment of mechanical pulp improves refining efficiency (113)</p>
Activation of wood fibres	<p>Enzymatic activation of middle lamella lignin of wood fibres as means for the production of binder-free fibre boards (114)</p> <p>Spectroscopic properties of oxidation species generated in lignin of wood fibres (81)</p> <p>Activation of lignin leads to copolymerisation with carbohydrates (115)</p> <p>Activity of laccase on TMP (116)</p>
Bonding of wood fibres	<p>The use of peroxidases and hydrogen peroxide in bonding of particle boards was suggested in 1972 (106)</p> <p>The use of laccase as radical donor in bonding of fibre boards was suggested in 1996 (106)</p> <p>Enzymatic activation of middle lamella lignin of wood fibres as means for the production of binder-free fibre boards (114)</p> <p>Enhanced autoadhesion of fibres by laccase (80, 105)</p> <p>Lignocellulose-derived adhesive for bonding of wood boards (117)</p> <p>Influence of extractives on enzymatic catalysed bonding (79)</p> <p>Properties of fibreboards obtained by peroxidase catalysed reaction (101)</p> <p>Phenol oxidising enzymes in production of fiberboards (118)</p> <p>Enzymatic activation of lignin leads to copolymerisation with carbohydrates (115)</p> <p>Bonding of MDF boards and lineboards (119)</p> <p>Method to manufacture fibreboards (120)</p>
Bonding of model compounds to lignin	<p>Copolymerisation of lignin with low-molecular weight compounds (96)</p> <p>Copolymerisation of phenolic compounds to lignin (95)</p>

Table II continues. Fibre modification with oxidative enzymes

Aim	Description
Bonding of model compounds to fibre bound lignin	Studies of the reactions of activated lignin and nucleophiles (90)
	Peroxides in enzymatic copolymerisation of lignin with acrylates (91)
	Effect of ions in the enzymatically induced synthesis of lignin graft copolymers (121)
	Copolymers from lignin and acryl compounds (92, 93)
	Oxidative coupling of water-soluble phenols with lignin (94)
	Precipitation of laccase polymerised vanillic acid, catechol, mimosa tannin and tannin acid dehydrogenatively to TMP (122)
	Grafting of N-containing phenolic monomers onto softwood pulp (95)
	Bonding of 4-hydroxyphenol acetic acid to fibres (97)

coworkers (spent sulphite liquor was used with laccase for particle board and wood laminate production (103). Kraft lignin, as well as concentrated process water from thermomechanical pulp (TMP) refining have also been studied for additives in glueing experiments using *Trametes hirsuta* laccase to prepare particle boards and MDF boards (104). Tensile strength measurements from the test fibre boards showed clearly that laccase treatment was comparable to a process where a synthetic reference adhesive, urea formaldehyde resins, was used. Fibreboards with better modulus of rupture and elasticity have generally been reported (105).

In the one component system, enzymatic activation of surface lignin has been exploited to enhance the adhesion between fibres through activation of surface lignin in production of binderless fibreboards (88, 89, 105, 106). The use of both laccases and peroxidases in the activation has been reported (101, 105). The improved bonding is thought to be due to physical changes on the fibre surface caused by the phenoxy radicals (105). Although the mechanism is not completely understood, it presumably involves direct oxidation of fibre surface lignin and the parallel radicalization of solubilized or colloidal lignin (80). These radicals will react further without enzymatic action. During hot pressing, fibre to fibre bondings are formed between radicals and other reactive groups situated on separate fibres (105).

Oxidative Enzymes In The Hydrolysis Of Lignocellulose

The interest in replacing fossil fuels with biofuels derived from lignocellulosic raw materials is increasing due to the worldwide concern about green house gases. The enzymatic hydrolysis of lignocellulosic materials has been studied in detail since the 1950's and significant advances in basic and applied enzymology have been achieved. The molecular structures, catalytic mechanisms and substrate specificities of major cellulases have been elucidated in detail. However, the heterogenous nature of the lignocellulosic matrice makes it difficult to understand the interactions of enzymes and their substrates, containing also lignin and hemicellulose. The accessibility of the substrate plays a key role in hydrolysis and is improved by using different pretreatment techniques (123). The role of residual hemicellulose and lignin as limiting factors in enzymatic hydrolysis has recently been reviewed in detail (124). The exact role of lignin in limiting hydrolysis, however, has been difficult to define. According to Mooney *et al.* (125), one of the most remarkable restrictions is the effect of lignin on fibre swelling and its resulting influence on the accessibility of cellulose. Obviously, the removal of both lignin and hemicellulose would leave the cellulose more accessible to contact with cellulases. Lignin is, however, thought to influence cellulase accessibility to cellulose in more ways than just acting as a barrier to prevent the enzymes from effectively binding to cellulose. Thus, it has been shown that the increase in pore volume observed after lignin removal corresponds to the increased accessibility of the substrate (126, 127). Lignin is also thought to negatively influence the hydrolysis reaction by irreversibly adsorbing the cellulase enzymes, thus preventing their action (128, 129). It has been observed that the extent to which lignin adsorbs cellulases, depends on the nature of the lignin (130). Therefore, lignin may be a rate limiting factor in the hydrolysis of cellulose.

In the present steam pretreatment technology, lignin is not dissolved from the fibrous material and may comprise up to 40% of the raw material. In comparative studies, lignin has been extracted to verify its role in hydrolysis (125). Although it may not be feasible to extract lignin during the pretreatment phase, the role of partial lignin removal during the hydrolysis of cellulose is interesting. The compatibility of enzymatic lignin degradation with cellulose hydrolysis has been studied using the laccase-mediator system on steam-pretreated softwood. Thus, it was observed that the degree of hydrolysis was improved significantly by combining the two enzymatic treatments (131). The inhibitory effects of the LMS system on cellulase activity decreased slightly the effect. Therefore, the slow-release mediator was also tested. The mechanism was expected to be based on removal of lignin fragments with sterical hindrance, modification of fibre surfaces improving cellulase desorption and eventually decreasing the inhibitory effect of aromatic compounds on cellulases.

References

1. Kirk, T.K. and Cullen, D In: Environmentally friendly technologies for the pulp and paper industry. Young, R.A. and Akhtar, M. Eds., New York, John Wiley & Sons, **1998**, pp. 273-307.
2. Hatakka, A. *FEMS Microbiol. Rev.*, **1994**, 13: 125-135.
3. Gianfreda, L., Xu, F., Bollag J.-M. *Biorem J*, **1999**, 3: 1-25
4. Tien M. and Kirk TK. *Proc Natl Acad Sci USA*, **1983**, 81, 2280-2284.
5. Glenn, J.K. and Gold, M.H. *Arch. Biochem. Biophys*, **1985**, 242, 329-341.
6. De Jong, E., Field, J.A. and de Bont, J.A.M. *FEBS Lett.*, **1992**, 299, 107-110.
7. Heinfling, A., Ruiz-Duenas, F., Martinez, M., Bergbauer, M., Szewzyk, U. and Martinez, A. *FEBS Lett.*, **1998**, 428, 141-146.
8. Camarero, S., Sarkar, S., Ruiz-Duenas, F.J., Martinez, M.J. and Martinez, A.T. *J Biol Chem.*, **1999**, 274 10324-10330.
9. Baunsgaard, L., Dalboge, H., Houen, G., Rasmussen, E.M. and Welinder, K.G. *Eur. J. Biochem.*, **1993**, 213, 605-611.
10. Arbeloa, M., de Leseleuc, J., Goma, G. and Pommier, J-C. *Tappi J.*, **1992**, 75, 215-221.
11. Paice, M.G., Reid, I.D., Bourbonnais, R., Archibald, F.S. and Jurasek, L. *Appl. Env. Microbiol.*, **1993**, 59, 260-265.
12. Kondo, R., Harazono, K. and Sakai, K. *Appl. Env. Microb.*, **1994**, 60: 4359-4363.
13. Kirk, T. and Farrell, R. *Ann. Rev. Microbiol.*, **1987**, 41, 465-505.
14. Warishii, H., Valli, K., Renganathan, V. and Gold, M.H. *J. Biol. Chem.*, **1989**, 264 14185-14191.
15. Bao, W., Fukushima, Y., Jensen Jr., K.A., Moen, M. and Hammel, K.E. *FEBS Lett.*, **1994**, 354, 297-300.
16. Lai, Y-Z. In: *Methods in Lignin Chemistry* (Lin, S.Y., Dence, C.W., Eds.) Berlin, Springer- Verlag, **1992**, pp. 423-434.
17. Vares, T. and Hatakka, A. *Can. J. Bot.* **1997** 75, 61-71.
18. Wariishi, H., Valli, K., and Gold, M.H. *Biochem. Biophys. Res. Comm.*, **1991**, 176, 269-275.
19. Edwards, S. L, Raag, R., Warishii, H., Gold, M.H., Poulos, T. L. *Proc. Natl. Acad. Sci USA*, **1993**, 90, 750-754.
20. Piontek, K., Glumoff, T. and Winterhalter, K. *FEBS Lett.* **1993**, 315, 119-124.
21. Sundaramoorthy, M., Kishi, K., Gold, M.H. and Poulos, T.L. *J. Biol. Chem.* **1994**, 269, 32759-32767.
22. Lokman, B.C., Joosten, V., Gouka, R.J., Verrips, C.T. and van den Hondel, C. *Proc. 6th Eur. Conf. on Fungal Genetics. Pisa, Italy, Abstract book*, 125., 2002

23. <http://www.jgi.doe.gov>
24. Käärik, A. *Stud. Forest. Suec.*, **1965**, No 31, pp. 1-80.
25. Xu, F. The encyclopedia of bioprocessing technology, fermentation, biocatalysis and bioseparation (Flinkinger, M.C and Drew, S. W. eds), **1999**, 1545-1554.
26. Thurston, C.F. *Microbiol.*, **1994**, 140, 19-26.
27. Bao, W., O'Malley, D.M., Whetten, R. and Sederoff, R.R. *Science*, **1993**, 260, 672-674.
28. Leatham, G.F. and Stahmann, M.A. *J. Bacteriol.*, **1981**, 144, 509-517.
29. Höfer, C. and Schlosser, D. *FEBS Lett.*, **1999**, 451, 186-190.
30. Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A. and Finazzi-Agro, A. *J. Mol. Biol.*, **1992**, 224, 179-205.
31. Ducros, V., Brzozowski, A.M., Wilson, K.S., Brown, S.H., Ostergaard, P., Schneider, P., Yaver, D.S., Pedersen, A.H. and Davies, G.J. *Nature Struct. Biol.*, **1998**, 5, 310-316.
32. Bertrand, T., Jolival, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C. and Mougou, C. *Acta Cryst.*, **2002**, D58, 319-321.
33. Antorini, M., Herpoel-Gimbert, I., Choinowski, T., Sigoillot, J-C., Asther, M., Winterhalter, K. and Piontek, K. *Biochim. Biophys. Acta*, **2001**, 36517, 1-6.
34. Bourbonnais, R., and Paice, M. *FEBS Lett.*, **1990**, 267, 99-102.
35. Bourbonnais, R. and Paice, M. *Appl. Microbiol. Biotechnol.*, **1992**, 36, 823.
36. Call, H-P. PCT world patent application WO 9429510, **1994**
37. Call, H.P. and Mücke, I. *J. Biotechnol.*, **1997**, 53: 163-202.
38. Sjöström, E. In *Wood Chemistry, Fundamentals and Applications*, 293 p., Academic Press., **1993**, San Diego, CA, USA.
39. Suurnäkki A., Heijnesson, A., Buchert, J., Tenkanen, M., Viikari, L. and Westermark, U. *J. Pulp Paper Sci.* **1996**, 22, J78-J83.
40. Ekman, R., Eckerman, C., Holmbom, B. *Nord. Pulp Pap. Res. J.*, **1990**, 5, 96-102.
41. Thornton, J., Ekman, R., Holmbom, B. and Örså, F. *J. Wood Chem. Technol.* **1994**, 14, 159-175.
42. Laine J. and Stenius P. *Cellulose I*, **1994**, 145-160.
43. Buchert, J., Carlsson, G., Viikari, L. and Ström G. *Holzforchung*, **1996**, 50, 69-74.
44. Suurnäkki, A., Heijnesson, A., Buchert, J., Viikari, L. and Westermark, U. *J Pulp Paper Sci.*, **1996**, 22, J 43-J47.
45. Mustranta, A., Koljonen, K., Holmbom, B., Stenius, P. and Buchert, J. **1998**. Proc. 5th Eur. Workshop Lignocellulosic and Pulp, Aveiro, Portugal, **1998**, pp. 11-14.

46. Mustranta, A., Koljonen, K., Lappalainen, A., Pere, J., Tenkanen, M., Stenius, P. and Buchert, J. Proc. 6th Eur. Workshop Lignocellulosic and Pulp, France, **2000**, pp. 15-18.
47. Petit-Conil, M., Semar, S., Niku-Paavola, M-L., Sigoillot, J.C., Asther, M., Anke, H. and Viikari, L. Progress in Biotechnology, Vol, 21, **2002**, in press.
48. Amann, M. Proc. 9th Int. Symp. on Wood and Pulp. Chem., Montreal, **1997**, F4, pp.1-5.
49. Freudenreich, I., Amann, M., Fritz-Langhals, E. and Stohrer, J. Proc. Int. Pulp Bleaching Conf., **1998**, Helsinki, Finland, pp. 71-76.
50. Hayness, K. and Ragauskas, A. Proc. Int. Pulp Bleaching Conf., Helsinki, Finland, **1998**, book 2, pp. 355-359.
51. Amann, M., Candussio, A., Müller, R. and Frey, V. Proc. TAPPI Pulping Conf., Boston, USA, **2000**.
52. Poppius-Levlin, K., Wang, W., Ranua, M., Niku-Paavola, M-L. and Viikari, L. Proceedings from the Biol. Sci. Symp., 327-333, **1997**.
53. Chakar, F.S., Allison, L., Kim, D.H. and Ragauskas, A.J. Proc. TAPPI Pulping Conf., Boston, USA, **2002**, Abstract book, 38.
54. Bourbonnais, R., Rochefort, D., Paice, M.G., Renaud, S. and Leech, D. Tappi J., **2000**, 83, 68.
55. Balakshin, M.Y., Evtuigin, D.V., Pascoal Neto, C. and Cavaco-Paulo, A. J. Molecular Catalysis B: Enzymatic, **2001**, 16, 131-140.
56. Eggert, C., Temp, U., Eriksson, K.-E.L. Appl Environ Microbiol, **1996**, 62: 1151-1158
57. Niku-Paavola, M-L., Anke, H., Poppius-Levlin, K. and Viikari, L. 223rd ACS National Meeting, Abstracts of Papers, Orlando, USA, **2002**, submitted.
58. Bourbonnais, R., Paice M., Reid, I., Lanthier, P. and Yaguchi, M. Appl. Environ. Microbiol., **1995**, 61, 1876-1880.
59. Sealey, J. and Ragauskas, A. Proc. 9th Int. Symp. on Wood and Pulp. Chem., Montreal, Canada, **1997**, pp. F1-1-F1-4.
60. Balakshin, M., Capanema, E., Chen, C-L., Gratzl, J., Kirkman, A. and Gracz, H. Proc. 10th Int. Symp. on Wood and Pulp. Chem., Yokohama, Japan, **1999**, Vol. 1, pp. 572-577.
61. Poppius-Levlin, K., Wang, W., Tamminen, T., Hortling, B., Viikari, L., Niku-Paavola, M.-L. J. Pulp Paper Sci., **1999**, 25, 90-94.
62. Chakar, F.S. and Ragauskas, A.J. J. Wood Chem. Technol., **2000**, 20, 169-184.
63. Chakar, F.S. and Ragauskas, A.J. Holzforschung, **2000**, 54, 647-653.
64. Balakshin, M.Y., Capanema, E., Chen, C-L., Gratzl, J., Kirkman, A. and Gracz, H. J. Molecular Catalysis. B: Enzymatic, **2001**, 13, 1-16.
65. Balakshin, M.Y., Chen, C-L., Gratzl, J., Kirkman, A. and Jakob, H. Holzforschung, **2002**, 54, 390-396.
66. Chandra, R.P., Chakar, F.S., Allison, L., Kim, D.H., Ragauskas, A.J. and Elder, T.J. Progress in Biotechnology, **2002**, Vol. 21, Elsevier, 151-164.

67. Amann, M. and Pfaller, R. GSF-Bericht, **2001**, 01/01, 73-81.
68. Poppius-Levlin, K., Wang, W. and Ranua, M. Proc. Int Pulp Bleaching Conf., Helsinki, **1998**, pp.77-85.
69. Chakar, F. and Ragauskas, A. Proc. 10 th Int. Symp. on Wood and Pulp Chem., Yokohama, Japan, **1999**, Vol. 1, pp. 566-570.
70. Paice, M., Bourbonnais, R., Renaud, S., Labonte, S., Sacciadis, G., Berry, R., Amann, M., Candussio, A. and Muller, R. Progress in Biotechnology, Vol. 21, **2002**, in press.
71. Wong, K.K.Y., Andersson, K.B. and Kibblewhite, R.P. Enzyme Microbiol. Technol., **1999**, 25, 125-131.
72. Viikari, L., Oksanen, T., Buchert, J., Amann, M. and Candussio, A. Proc. 10 th Int. Symp. on Wood and Pulp Chem. Yokohama, Japan, **1999**, Vol I, 504-507.
73. Surma-Slusarska, B. and Leks-Stepien, J. J. Wood Cham. Technol., **2001**, 21, 361-370.
74. Oksanen, T., Buchert, J., Amann, M., Candussio, A. and Viikari, L. Progress in Biotechnology, Vol. 21, **2002**, 255-262.
75. Pfaller, R., Amann, M. and Freudenreich, J. Proc. 7th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vancouver, **1998**; A99-A102.
76. Kaneko, R., Iimori, T., Miyawaki, S., Machida, M. and Murakami, K. Biosci. Biotech. Biochem., **1995**, 59, 1584-1585.
77. Paice, M.G., Bourbonnais, R. and Reid, I.D. Tappi J., **1995**, 78, 161-169.
78. Moreira, M. T., Sierra-Alvarez, R., Lema, J. M., Feijoo, G. and Field, J. A. Bioresource Technology, **2001**, 78, 71-79.
79. Hassingboe, J. M., Lawther, J. M. and Felby, C. Proc. 7th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vancouver, Canada, **1998**, Vol. A, A125-A128.
80. Felby, C., Nielsen B.R., Olesen, P.O. and Skibsted, L.H. Appl. Microbiol Biotechnol, **1997**, 48, 459-464.
81. Barsberg, S. and Thygesen, L. Biochemica et Biophysica Acta, **1999**, 1472, 625-642.
82. Aust, S., Barr, D., Grover, T., Shah, M., Namhyun, C. US patent 5 389 356, **1995**.
83. Paice, M. G., Bourbonnais, R., Reid, I.D., Archibald, F. S. and Jurasek, L. Journal of pulp and paper science **1995**, 21, 280-284.
84. Buchert, J., Mustranta, A., Tamminen, T., Spetz, P. and Holmbom, B. Holzforshung **2002**, 56, 579-584.
85. Buchert, J., Mustranta, A., Spetz, P., Ekman, R. and Luukko K. Proc. Pre-symp. of the 10 th Int. Symp. on Wood and Pulp. Chem.,Seoul, Korea, **1999**, P115-119.
86. Karlsson, S., Holmbom, B., Spetz, P., Mustranta, A. and Buchert, J. Appl. Microbiol. Biotechnol., **2001**, 55, 317-320.
87. Zhang, X. Pulp & Paper Canada, **2000**, 101, 59-61.

88. Barsberg, S., Felby, C. and Nielsen, K. Abstracts of Papers, 223rd ACS National Meeting, Orlando, **2002**, American Chemical Society: Washington, D.C, USA.
89. Hüttermann, A. VTT 163. VTT Symp., **1996**, pp. 143-148.
90. Hüttermann, A., Kharazipour, A., Schindler, K., Fastenrath, M., Noetzold, S., Schroeter, M., Hüttermann, J., Hüttermann A. H., von Kiedrowski, G., Baumberger, S., Lapierre, C. and Monties, B. Proc. 7th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vancouver, Canada, **1998**, Vol. A, 207-209.
91. Mai, C. and Hüttermann Proc. 7th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vancouver, Canada, **1998**; Vol B, 233-236.
92. Mai, C., Milstein, O. and Hüttermann, A. Appl. Microbiol. Biotechnol., **1999**, 51, 527-531.
93. Mai, C., Milstein, O. and Hüttermann, A. Journal of Biotechnology, **2000**, 79, 173-183.
94. Lund, M. and Ragauskas, A. Appl. Microbiol. Biotechnol., **2001**, 55, 699-703.
95. Lund, M., Felby, C. and Bjerrum, M. Proc. 7th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vancouver, Canada, **1998**; Vol. C, 139-142.
96. Milstein, O., Mai, C., Hüttermann, A., Srebotnik, E. and Messner, K. Proc. 6th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vienna, Austria, **1995**; 645-648.
97. Chandra, R. and Ragauskas, A. Proc. 11th Int. Symp. on Wood and Pulp Chem., Nice, France, **2001**, part II., pp. 39-42.
98. Jin, L., Nicholas, D. D. and Schultz T.P. Holzforschung, **1991**, 45, 467-468.
99. Lund, M. and Felby, C. Enzyme and Microbial Technology, **2001**, 28, 760-765
100. Nimz, H. H., Gurang, I. and Mogharab, I. Liebigs Ann Chem., **1976**, 1421-1434.
101. Kharazipour, A., Bergmann, K., Nonninger, K. and Hüttermann, A. J. Adhes. Sci. Technol., **1998**, 12, 1045-1053.
102. Haars, A. and Hüttermann, A. German patent DE 3037992, C2, **1983**
103. Haars, A., Kharazipour, A., Zanker, H. and Hüttermann, A. ACS Symp. ser. 385. American Chemical Society, Washington, USA, **1989**, pp 126-134.
104. Viikari, L., Hase, A., Qvintus-Leino, P., Kataja, K., Tuominen, S., and Gädda, L. PCT World Patent WO 9831762, **1998**.
105. Felby, C., Pedersen, L.S. and Nielsen, B.R. Holzforschung, **1997**, 51, 281-286.
106. Hüttermann, A., Mai, C and Kharazipour, A. Appl Microbiol Biotechnol, **2001**, 55:387-395.

107. Yamaguchi, H., Maeda, Y. and Sakata, I. *Mokuzai Gakkaishi*, **1994**, 40, 185-90
108. Viikari, L., Harkki, T., Niku-Paavola, M.-L., Buchert, J. and Poppius-Levlin, K. *Proc. 7th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vancouver, Canada, 1998*; Vol A, A121-A124.
109. Barsberg, S. Thygesen, L. and Felby, C. *Proc. 7th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vancouver, Canada, 1998*, Vol. C, pp. C123-C126.
110. Wong K. K. Y.; Richardson J. D. and Mansfield S. D. *Biotechnol. Prog.*, **2000**, 16, 1025-1029.
111. Sigoillot, J.C., Petit-Conil, M., Herpoel, I; Joseleau, J.P., Ruel, K., Kurek, B., de Choudens, C. and Asther, M. *Enzyme Microb. Technol.*, **2001**, 29, 160-165
112. Lund, M. and Felby, C. WO2000068500, **2000**.
113. Mansfield S. D. *Appita*, **2002**, 55, 49-53
114. Kharazipour, A. and Hüttermann, A. *Proc. 6th Int. Conf. Biotechnology in the Pulp and Paper Industry, Vienna, Austria, 1995*, 617-620.
115. Hüttermann, A., Majcherczyl, A., Braun-Lüllemann, A., Mai, C., Fastenrath, M., Kharazipour, A., Hüttermann, J. and Hüttermann A. H. *Naturwissenschaften*, **2000**, 87, 539-541.
116. Grönqvist, S., Buchert, J., Rantanen, K., Viikari, L. and Suurnäkki, A. *Enzyme Microb. Techn.* **2003**, 32, 439-445.
117. Viikari, L., Hase, A., Qvintus-Leino, P., Kataja, K., Tuominen, S. and Gädda, L. WO 9831761, **1998**.
118. Kharazipour, A., Schindel, K. and Hüttermann, A. *ACS Symp. Ser.*, 687 (*Enzyme Applications in Fiber Processing*), **1998**, 99-115.
119. Lund M; Hassingboe J; Felby C (2000) Sixth European workshop on lignocellulosics and pulp (EWLP 2000). Bordeaux, France, 3-6 Sept. 2000, 113-116
120. Viikari, L., Hase, A. Tuominen, S., Qvintus-Leino, P. and Laine, J., WO 2002002288, **2002**.
121. Mai, C., Schormann, W. and Hüttermann, A. *Enzyme Microb. Technol.*, **2001**, 28, 460-466.
122. Yamaguchi, H., Maeda, Y. and Sakata, I. *Jpn. Wood Res. Soc.*, **1992**, Vol. 38, no. 10, pp. 931-937.
123. Thompsen, D.N., Chen, H.C. and Grethlein, H.E. *Biores. Technol.*, **1992**, 39, 155-163.
124. Mansfield, S.D., Mooney, C.A. and Saddler, J.N. *Biotechnol. Progress*, **1999**, 15, 804-816.
125. Mooney, C.A., Mansfield, S.D., Touhy, M.G., Saddler, S.N. *Biores. Technol.*, **1998**, 64, 113-119.
126. Grethlein, H.E. *Bio. Technol.*, **1985**, 3, 155-160.

127. Wong, K.K.Y., Deverell, K.F., Mackie, K.L., Clark, T.A. and Donaldson, L.A. *Biotechnol. Bioeng.*, **1988**, 31, 447-456.
128. Converse, A.O., Ooshima, H. and Burns, D.S. *Appl. Biochem. Biotechnol.*, **1990**, 24/25, 67-73.
129. Lee, D., Yu, A.H.C, Wong, K.K.Y., Saddler, J.N. *Appl. Biochem. Biotechnol.*, **1994**, 45/46, 407-415.
130. Sutcliffe, R. and Saddler, J.N. *Biotechnol. Bioeng.* **1986**, 17, 749-762.
131. Palonen and Viikari, manuscript in preparation

Chapter 4

Bigrafting of Celestine Blue onto a High Kappa Kraft Pulp

Richard P. Chandra¹, Francois Wolfaardt²,
and Arthur J. Ragauskas¹

¹Institute of Paper Science and Technology, 500 10th Street NW,
Atlanta, GA 30318

²Sappi Forest Products Technology Centre, P.O. Box 3252,
Springs 1560, South Africa

Laccase was applied to high kappa (60) kraft pulps in combination with a phenolic cationic compound (celestine blue) before and after pulp refining. Nitrogen content and zeta potential indicated the attachment of celestine blue to the pulp fibers. Laccase and celestine blue treatment before refining and treatment with laccase alone on preredefined pulp resulted in tensile strength increases of 10-15%. The results here indicate the potential of laccase-facilitated grafting technology to modify high lignin content pulps to improve their performance in paper products.

Introduction

Researchers in industry are continually searching for methods and materials to improve the efficiency and cost effectiveness of their processes. In recent years, laccase has been investigated for diverse applications such as hair dyeing, textile dyeing, leather tanning, and detergent additives. Laccase (benzenediol:oxygen oxidoreductase) enzymes are useful in a wide range of applications because of their ability to oxidize many compounds, including phenols, aminophenols, polyphenols, polyamines, certain inorganic ions, and aryl diamine compounds (1). In the pulp and paper industry, laccase has been investigated mainly for its ability to delignify kraft pulps to facilitate subsequent bleaching processes. Bourbonnais and Paice (2) described the application of laccase to both phenolic and non-phenolic lignin compounds. These researchers revealed that laccase was unable to catalyze the oxidation of non-phenolic lignin model compounds in the absence of another compound (ABTS-2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)). The compound used to aid laccase was called a "mediator." The laccase-mediator combination was also shown to possess the ability to depolymerize lignin within kraft pulps (Paice 1989). The delignification of kraft pulps with laccase has been termed "biobleaching." Subsequent studies with the laccase-mediator system (LMS) focused on application to kraft pulps and lignin preparations (3,4, 5).

Overall, the many studies evaluating the effects of the LMS on kraft pulps have revealed that the LMS degrades lignin to facilitate subsequent chemical bleaching sequences. The role of the mediator compound was not completely understood; however, it was speculated that in addition to facilitating the oxidation of nonphenolic compounds, the mediator also enabled laccase to oxidize the lignin within the fiber wall (3). In the absence of mediators, laccase polymerizes lignin preparations (6). Second and third-generation mediators include HBT (1-hydroxybenzotriazole), NHAA (N-hydroxyacetanilide), and violuric acid (3,4,5,7,8). Chakar and Ragauskas (9) have thoroughly investigated the chemistry behind LMS delignification. Unfortunately, although much is now known regarding the mechanisms behind laccase-mediator delignification, this technology has not yet been applied on an industrial scale. Presently, the most significant stumbling blocks that have hindered the commercial application of this technology include the price and instability of mediators.

With the difficulties associated with implementing industrial-scale biobleaching technology, research efforts have shifted toward employing

laccase's robust oxidizing capabilities to improve other facets of pulp and paper processing. In alignment with these efforts, the polymerizing ability of laccase has been exploited to modify pulp to improve paper strength. Since laccase reacts with phenolic moieties contained primarily in the lignin fraction of pulp, highly lignified pulps such as mechanical and linerboard-grade kraft pulps have been targeted for laccase application to improve pulp properties. Felby et al. (10) have been forerunners in this area. With their work on the application of laccase to beech (*Fagus sylvatica*) fibers, Felby et al. were able to show that laccase was capable of oxidizing pulp fibers to improve strength properties of the resulting MDF (medium density fiberboard). These laccase-induced strength benefits were attributed to free-radical initiated bonds formed by laccase reacting with lignin at the fiber surface. Hassingboe et al. (11) later showed that laccase was able to oxidize colloidal lignin to "mediate" the oxidation of the lignin in beech fibers, resulting in improved pulp properties. Viikari (12) et al. and later Wong and Mansfield (13), also found that pretreatment of mechanical pulps with laccase resulted in increases in paper strength. Another avenue for improving pulp properties with laccases would be to employ the polymerizing ability of laccase to chemically attach a separate constituent to pulp.

Yamaguchi et al. (14,15) utilized laccase to polymerize various phenolic compounds to form dehydrogenative polymers (DHP). The DHP was subsequently coupled to thermomechanical pulp (TMP) with peroxidase for the formation of paperboard. After hot pressing for board formation, the ply-bond strength of the paperboard produced from DHP-coupled TMP increased twofold. Yamaguchi et al. also found an increase in paper tensile strength after laccase pretreatment of thermomechanical pulps (TMP) with subsequent combination with a vanillic acid DHP. The reaction was also performed with milled wood lignin (MWL); the molecular weight of the MWL increased after combination with the DHP. These authors rationalized their results as a chemical bonding between the lignin and the DHP facilitated by laccase during the laccase pretreatment of the TMP. Unfortunately, the nature of the chemical alteration of the surfaces of the treated fibers was not investigated.

Recently, Chandra and Ragauskas investigated the application of laccase in the polymerization of phenolic compounds to high kappa kraft pulps. This work was the first to explore laccase's ability to simultaneously react with monomeric 4-hydroxybenzoic acid and high kappa kraft pulps in an aqueous system, resulting in the attachment of these compounds to the pulp. It was shown that laccase in combination with 4-hydroxybenzoic acid could increase the carboxylic acid group content by 100% with a concomitant increase in kappa

number (16). This work was followed by studies elucidating the effects of the grafting treatment on paper physical properties (17) and the surface material isolated from the kraft pulps (18). Along the same lines as these previous studies, the present study describes the application of laccase and a cationic phenolic compound to high kappa kraft pulps. P ulps were treated before and after refining. The effects on both pulp properties and paper physical properties were evaluated.

Materials and Methods

Pulp. Softwood kraft pulp with high lignin content (kappa number 60) was obtained from a commercial mill located in the southeastern USA. The pulp was screened to remove fines and shives and then fluffed. Acetone-soluble compounds were removed from the pulp by extraction for 24 h. Each batch (~70 g dry weight) of extracted pulp was washed with 12 L of deionized water. The pulp was again fluffed, and samples (30.0 g dry basis) were weighed into heat-resistant pouches.

Laccase. Laccase was donated by Novozymes, Raleigh, NC. The laccase was Novo NS51002 isolated from *Trametes villosa*. The activity of laccase was measured by monitoring the rate of oxidation of syringaldazine (19). The change in $A_{530\text{nm}}$ of 0.001 per minute per mL of enzyme solution in a 100-mM potassium phosphate buffer (2.20 mL) and 0.216 mM syringaldazine in methanol (0.300 mL) was set to one unit (U) of activity. This test was done at 23°C and pH 4.5.

Pulp Treatment. The pH of the pulp was adjusted to 4.5 and the grafting treatment consisted of 200 mg celestine blue (Cel B.) (Aldrich Chemical Company, Milwaukee, Wisconsin) (Figure 1) with 5.00 mL of laccase (8.54×10^6 U/g pulp). The pulp consistency was adjusted to 15.0% and mixed thoroughly. All treatments were incubated in a water bath at 45.0°C for 120 min. Symbols used in the graphs are shown in Table 1. Treatments performed before pulp refining are denoted in figures as “before refining” and treatments applied on pre-refined pulps are denoted as “after refining.” The reactions were ceased by washing each sample with 12 L of deionized water and extracting residual dye with methanol for 24 h. Methanol was removed by washing with 8.0 L of water. The reactions with laccase + celestine blue (Lac+Cel B) were repeated three times for refined and unrefined pulp to calculate a least significant difference (LSD) value using the students t-value. For nitrogen content and zeta-

potential analysis, the results are the average of two determinations and the standard-deviations are expressed in the figures.

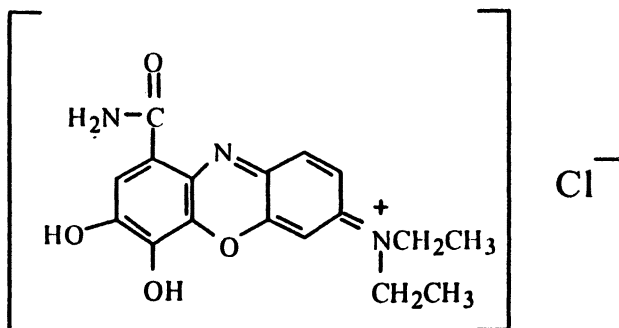


Figure 1: The structure of celestine blue used in grafting experiments

Table 1: Treatment Regime Applied to High Kappa Kraft Pulp Fibers

Treatment	Symbol
Control	Control
Laccase + Pulp	Lac
Celestine Blue + Pulp	Cel B
Laccase + Celestine Blue + Pulp	Lac + Cel B

Refining. All pulps were converted to the calcium (Ca^{2+}) form according to Scallan (20). In brief, samples were suspended at 1% csc and the pH was adjusted to 2.75 with 0.1 N H_2SO_4 . After stirring for 30 min, the samples were washed extensively with deionized water. Samples were resuspended in water at 1% csc, and the pH was adjusted to 10 with $\text{Ca}(\text{OH})_2$. After stirring for 30 min, the pulps were washed extensively. Treated pulps and controls were refined according to TAPPI Standard T 248 om-85. Handsheets were formed according to TAPPI Standard T 205 om-88.

Pulp Testing. After refining and grafting, 24.0 g of pulp was used to prepare 18 handsheets according to TAPPI Method T 305. One sheet of each replicated treatment was used to determine the influence of the treatment on kappa number according to TAPPI Method T 236. The sheets were conditioned for 24 h before measuring the weight and thickness of sheets to calculate the basis weight. Tensile strength, tearing resistance, bursting strength, and z-directional tensile strength (ZDT) were determined for five handsheets of each treatment according to TAPPI Methods T 494, T 414, T 403, and T 541, respectively. Handsheets were sent to Atlantic Microlab Inc. (Norcross, GA) for determination of nitrogen content by combustion followed by elemental analysis. The remaining 6.0 g of pulp was used to determine the surface charge of treated fibers by measuring the zeta potential of the pulp.

Zeta Potential. Zeta potential was measured on a Mutek SZP04. All pulp samples were pH-adjusted to 4.5 at a 1% csc prior to measurement. Samples were suspended in deionized water at 1% csc.

Results and Discussion

Enzymes such as cellulases, hemicellulases, oxidoreductases, and pectinases have been frequently studied for potential application in pulp fiber modification. Two examples include work by Oksanen et al. (21), who applied both hemicellulases and cellulases to kraft pulp fibers and found that cellulases increased density of paper sheets made from these fibers, and Mansfield et al. (22), who have shown that cellulase can be used to increase the tensile strength of paper sheets made from Douglas-fir (*Pseudotsuga menziesii*) kraft pulps. With the exception of a few studies (10,12,13), and our previous work (16), the use of oxidoreductase enzymes for fiber modification has not been as extensively explored. In the present study, an attempt was made to graft a water-soluble cationic phenolic compound (celestine blue) to high kappa kraft pulps with the aid of laccase. The first set of pulp samples were treated before refining, and the second set of pulp samples were treated after refining. After treatments, all samples were extracted with methanol to differentiate absorbed celestine blue from laccase-grafted celestine blue. Methanol was effective in removing the celestine blue from the pulp. It should be noted that celestine blue changed color from blue to a deep red upon reaction with laccase. The pulp and paper strength properties resulting from all treatments, including kappa number, nitrogen content, and zeta potential, was characterized.

In previous work, we have used the kappa number measurements to compare pulps that have undergone grafting treatment to control samples (16). This method was effective in demonstrating the increase in the aromatic fraction of the pulp with the grafting of phenolic compounds (23). The results of kappa number measurement are shown in Figure 2. The kappa number of the samples treated after refining was lower than those treated before refining. This is most likely due to the loss of fines material in the samples that were treated after refining. The fines material isolated after refining of kraft pulps has been shown to contain a high proportion of lignin (24). In both the samples treated before and after refining, there was no significant change in kappa number with the Lac+Cel B treatment. This was a surprising result; however, upon examining the kappa number test and the experimental conditions employed here, the most plausible explanation for the result would be the low dosage of celestine blue applied. One hundred milligrams (100 mg) of celestine blue was applied to facilitate complete dissolution in the reaction. According to the definition of kappa number, the percentage of lignin in a pulp sample can be approximated by kappa number \times 0.15. In the case of the pulp used here, a kappa number of 60 would have approximately 9% lignin content resulting in 2.7 g of lignin in a 30-g pulp sample (23). With a 100% grafting of celestine blue to the fibers, the total titratable material in the kappa test would increase to 2.7 g + 0.100 g = 2.8 g. A 2.8-g value of titratable material in the pulp would result in a kappa number of 62. Indeed, with such a small increase in kappa number, the test itself may not possess the sensitivity to demonstrate the grafting of celestine blue to the fibers. Therefore, nitrogen content was used to attempt to evaluate the ability of laccase to incorporate celestine blue into the pulp samples.

Since celestine blue contains a nitrogen group (Figure 1), nitrogen content measurement was a logical method for quantifying the amount of celestine blue incorporated into the pulp. As shown in Figure 3, nitrogen content of samples treated with the combination of laccase and celestine blue (Lac+Cel.B) before refining had the most prominent increase in nitrogen content. This was an encouraging result; however, the samples treated with Cel.B alone also showed slight increases in N content. This was most likely due to an inefficient removal of Cel. B during methanol extraction. Since refining pulps prior to treatment was expected to create a greater amount of surface area for reaction with laccase and celestine blue, it was surprising that the Lac+Cel B samples treated after refining did not show an increase in N content. Since the samples treated before refining showed the most pronounced change in nitrogen content, these samples were subjected to zeta-potential analysis.

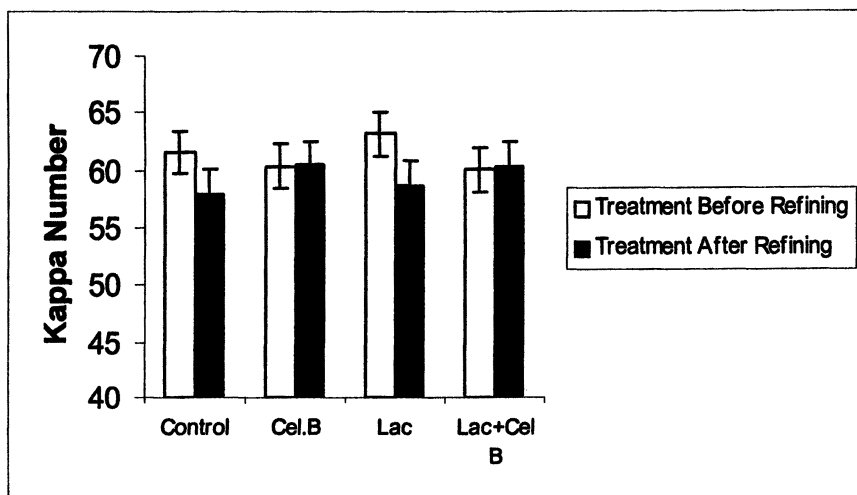


Figure 2: Kappa Number Results of Samples Treated with Laccase+Celestine Blue Before Refining and After Refining

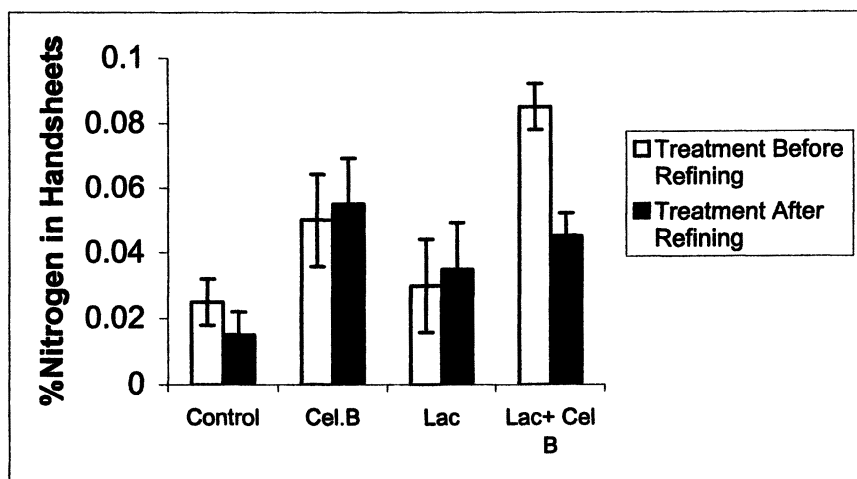


Figure 3: Nitrogen Results of Samples Treated with Laccase+Celestine Blue Before Refining and After Refining

Zeta-potential analysis has been frequently applied for the evaluation of the effects of wet-end additives that alter surface charge properties of pulps to alter their papermaking properties. Zeta potential involves measuring the charge balance at the boundary layer that surrounds a particle in an aqueous medium. Zeta-potential measurement results are shown in Figure 4. Samples possessed an overwhelming negative charge due to the natural negative charges imparted by

cellulose, hemicellulose, and lignin (20). Samples treated with Lac+Cel B displayed a tendency to be less negatively charged than the control samples. The results of zeta-potential testing for the Lac+Cel B samples is indicative of laccase-facilitated attachment of celestine blue to the fiber surface. In this study it was hoped that an increase in cationic charges at the fiber surface would result in an increase in fiber bonding and paper strength. This effect would be similar to cationic wet-end additives employed during the papermaking process (25).

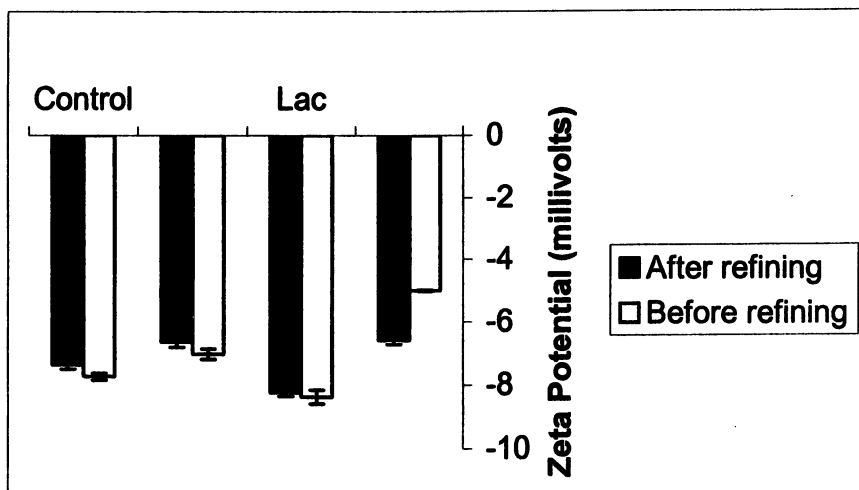


Figure 4: Zeta-potential Results of Samples Treated with Laccase+Celestine Blue Before Refining

Paper strength properties were measured on the pulps treated before and after refining. Properties tested include apparent density, burst, tensile, z-direction tensile, and tear. As shown in Figure 5, samples treated before refining had higher density values than samples treated after refining. This is most likely due to the loss of fines material with the samples treated after refining. These results demonstrate the importance of fines for filling in the open spaces between fibers during formation to increase the overall density of the paper sheet structure. Another effect of removing fines in the samples treated in this work is the removal of surface lignin (24). The high amount of surface lignin (24,26) present in kraft pulps would render them more amenable to grafting treatments with laccase. In the work described here, removal of lignin at the fiber surface via refining (24) may result in an ineffective grafting treatment with laccase and celestine blue. Accordingly, the treatment with laccase+Cel B applied after refining was ineffective while the treatment before refining was effective in

increasing density. Another interesting result was the increase in apparent density observed with laccase applied to the pulp after refining. The performance enhancements imparted by reaction with laccase and laccase celestine blue were evaluated further by burst, tensile, ZDT, and tear tests.

The results of both burst and tensile (Figures 6 and 7) were very similar to those obtained in apparent density measurements. Laccase treatment after

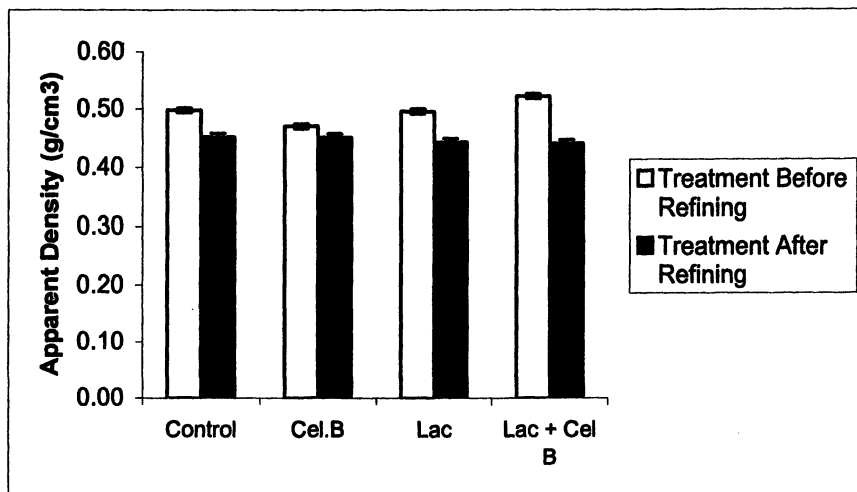


Figure 5: Apparent Density Results of Samples Treated with Laccase+Celestine Blue Before Refining and After Refining

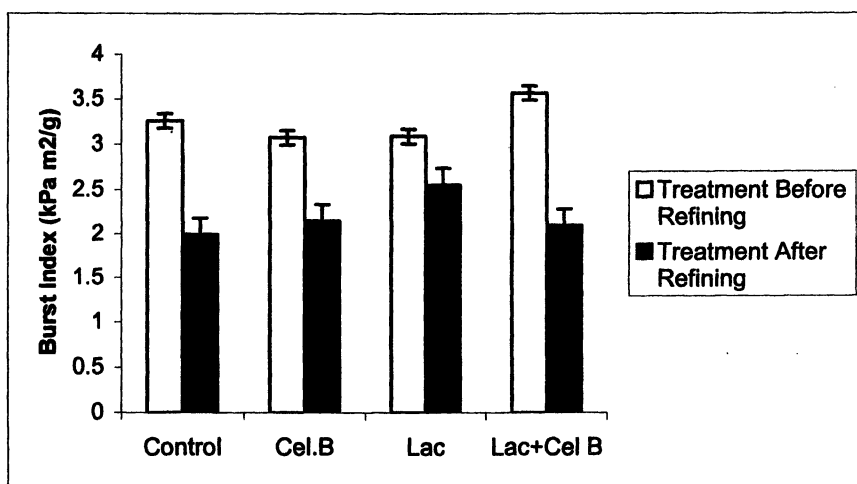


Figure 6: Burst Test Results of Samples Treated with Laccase+Celestine Blue Before Refining and After Refining

refining increased the tensile and burst strength of the pulp compared to the other postrefining treatments. This result may be due to the refining treatment creating an increase in surface area, thereby increasing the accessibility of the fiber to reaction with laccase. Laccase reaction with fibers is similar to the result obtained by Viikari et al. and others (12,27).

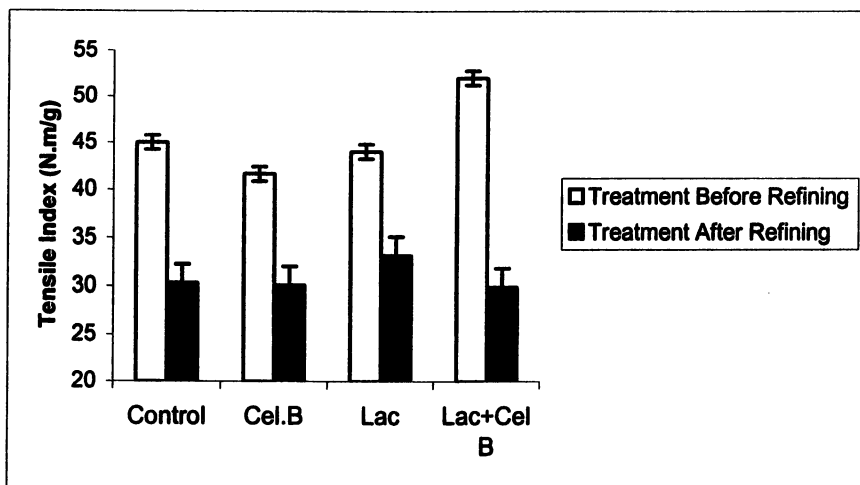


Figure 7: Tensile Testing Results of Samples Treated with Laccase+Celestine Blue Before Refining and After Refining

The z-direction tensile test has been shown to be an effective measure of internal bond strength for paper and paperboard (28). This test measures paper strength in the cross direction. The results of z-directional tensile strength are shown in Figure 8. Similar to the other tests, laccase treatment with celestine blue before refining resulted in increases in ZDT. Laccase treatment after refining also results in a sharp increase in ZDT close to the value of the samples treated with Lac+Cel.B before refining. Tear-testing results showed no significant change among the samples.

Yamaguchi et al. (14,15) utilized laccase to polymerize various phenolic compounds to form dehydrogenative polymers (DHP). The DHPs were subsequently coupled to thermomechanical pulp (TMP) in the presence of peroxidase for subsequent formation of paper by hot pressing. Hüttermann et al. (29) combined kraft lignin “activated” with laccase with spruce sulfite pulp with subsequent sheet formation and press drying. The resulting paper sheets in both the work of Yamaguchi et al. (14,15) and Hüttermann (29) had enhanced paper

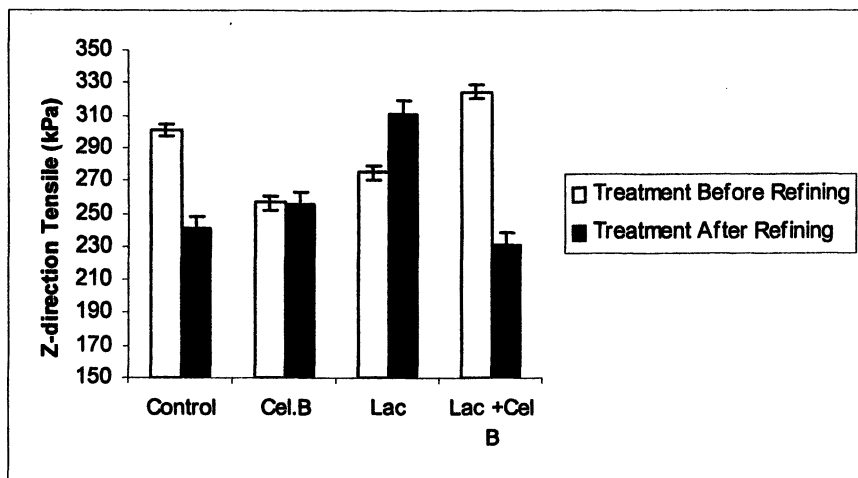


Figure 8: Z-direction Tensile Results of Samples Treated with Laccase+Celestine Blue Before Refining and After Refining

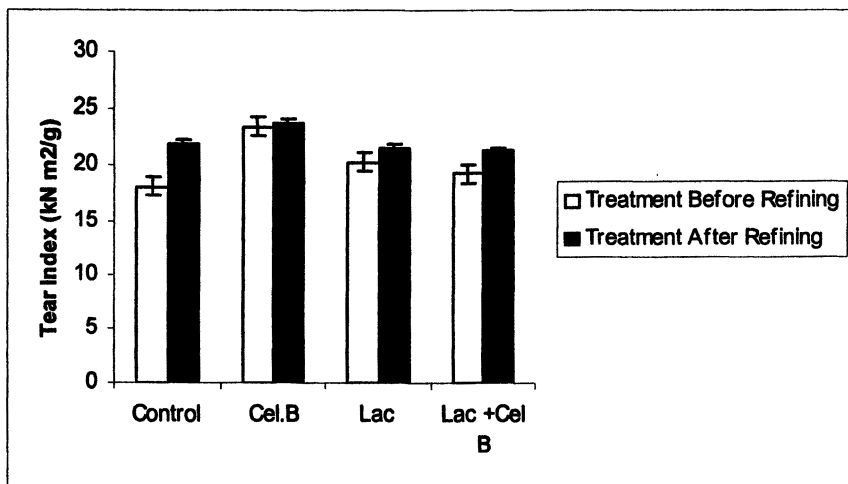


Figure 9: Tear-Test Results of Samples Treated with Laccase+Celestine Blue Before Refining and After Refining

strength properties. Unfortunately, it is difficult to compare the results obtained here to those of Yamaguchi et al. and Hüttermann, since the sheets in the present study were not formed by heating. Felby et al. (10) treated a ferulated arabinoxylan adduct with laccase in the presence of beech TMP fibers resulting in tensile strength increases. These authors rationalized their results by hypothesizing that laccase attached the ferulic acid portion of the adduct to the

lignin on the fiber while the arabinoxylan served to fill gaps in the fiber network to increase tensile strength. With all of these previous studies, it is apparent that laccase is capable of either coupling lignin or phenolic compounds to pulp fibers or reacting with the fibers themselves. The present study demonstrated both beneficial effects as laccase reacted with the preredefined pulp to increase paper strength, and laccase reacted with celestine blue to increase the strength of pulps treated before refining.

Conclusions

The work here displays the potential for laccase to be employed as a catalyst for grafting a variety of compounds to pulp fibers. With further development, laccase-facilitated grafting of compounds to pulp fibers can be used as a tool for tailoring the properties of high lignin content pulps to suit particular end-uses to expand the range of application for these products.

Acknowledgements

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References

1. Yaropolov, A.I., Skorobogatko, O.V., Vartanov, S.S., Varfolomeyev, S.D., *Appl. Biochem. Biotechnol.*, **1994**, *49*, 257-280.
2. Bourbonnais, R. and Paice, M.G. *FEBS Lett.* **1990**, *267*, 99-102.
3. Paice, M.G., Bourbonnais, R., Reid, I.D., Archibald, F.S., and Jurasek, L.. *J. Pulp Pap. Sci.* **1995**, *21*, 280-284.
4. Call, H. P.; Mucke, I. , *J. Biotechnol.*, **1997**, *53*, 163-202.
5. Poppius-Levlin, K., Wang, W., Tamminen, T., Hortling, B., Viikari, L., Niku-Paavola, M.-L. *J. Pulp Pap. Sci.* **1999**, *25*, 90-94.

6. Leonowicz, A., Szklarz, G., and Wojta-Wasilewska, M. *Phytochemistry*, **1985**, *24*, 393-396.
7. Chakar, F.S. and Ragauskas, A.J. TAPPI 1998 Pulping Conference, Montreal, Canada. **1998**, 109-117.
8. Chakar, F.S., Ph.D. thesis, Institute of Paper Science and Technology, Atlanta, GA, 1999.
9. Amann, M., Candussio, A., Muller, R., Frey, V., Paice, M. G., Bourbonnais, R., Labonte, S., Sacciadis, G., Renaud, S. *TAPPI Pulping/Process and Product Quality Conference*, Boston, MA, **2000**, 930-937.
10. Felby, C., Pedersen, L.S., and Nielsen, B.R., **1997**, *Holzforchung*, *51*, 281-286.
11. Hassingboe, J., Lawther, J.M., Felby, C., *7th International Conference on Biotechnology in the pulp and paper Industry*, Vancouver, B.C., **1998**, A125-128.
12. Viikari, L., Harkki, T., Niku-Paavlova, M-L., Buchert, J., and Popplus-Levlin, K.. *7th International Conference on Biotechnology in the Pulp and Paper Industry*, Vancouver, B.C., **1998**, A121-124.
13. Wong, K.K.Y, Richardson, J.D. and Mansfield, S.D., *Biotechnol. Progr.*, **2000**, *16*, 1025-1029.
14. Yamaguchi, H., Maeda, Y., Sakata, I., *Mokuzai Gakkaishi*, **1992**, *38*, 931-937.
15. Yamaguchi, H., Maeda, Y., Sakata, I., *Mokuzai Gakkaishi*, **1994**, *40*, 185-190.
16. Chandra, R.P. and Ragauskas, A.J. In *Biotechnology in the Pulp and Paper Industry*; Viikari, L. and Lantto, R.; Elsevier: Amsterdam, **2002**; Vol. 21, pp 165-172.
17. Chandra, R.P. and Ragauskas, A.J., *Enzyme Microb. Technol.* **2002**, *30*, 855-861.
18. Chandra, R.P. and Ragauskas, A.J. *International Symposium on Wood and Pulping Chemistry*, Nice, France., **2001**, *2*, 39-43.
19. Sealey, J., Ragauskas, A., *Enzyme Microb. Technol.*, **1998**, *23*, 422-426.
20. Scallan, A.M. and Grignon, *Svensk Papperstidning*, 1979, 41-45.
21. Oksanen, T., Pere, J., Buchert, J., and Viikari, L., *Cellulose* **1997**, *4*, 329-339.
22. Mansfield, S. and Saddler, J.N., *J. Pulp Pap. Sci.*, **1999**, *25*, 84-89.
23. Li, J. and Gellerstedt, G., *Nordic Pulp and Paper Research Journal*, **1998**, *3*, 147-158.
24. Heijnesson, A., Simonson, R., and Westermarck, U. *Holzforschung*, **1995**, *49*, 313-318.
25. Scott, W.E.; *Principles of Wet End Chemistry*; Tappi Press: Atlanta, GA, 1996.

26. Laine, J., Stenus, P., Carlsson, G., and Stroh, G. , *Cellulose*, **1994**, *1*,145-160.
27. Hansen, T.T., Holm, H.C., Franks, N.E., US Patent 5,603,804, 1997.
28. Koubaa, A. and Koran, Z. *Tappi*, **1995**,103-111.
29. Hüttermann, A., Kharazipour, A., Schindel, K., Fastenrath, M., Noetzold, S., Schroeter, M. *7th International Conference on Biotechnology in the Pulp and Paper Industry*, Vancouver, Canada, **1998**, A207-A209.

Chapter 5

Treatment of Pulp and Paper Industry Process Waters with Oxalate Oxidase: Compounds Interfering with the Activity

Simona Larsson¹, Pierre Cassland², Leif J. Jönsson³,
and Nils-Olof Nilvebrant^{1,*}

¹STFI Swedish Pulp and Paper Research Institute, Box 5604, SE-114 86
Stockholm, Sweden

²Applied Microbiology, Lund University/Lund Institute of Technology,
SE-221 00 Lund, Sweden

³Biochemistry, Division for Chemistry, Karlstad University, SE-651 88
Karlstad, Sweden

Removal of oxalic acid from bleaching filtrates using the enzyme oxalate oxidase is a possibility to prevent problems with scaling in the pulp and paper industry. Bleaching filtrates contain compounds that interfere with the action of oxalate oxidase and a selection of cations, anions and organic acids, as well as hydrogen peroxide was investigated with regard to potential inhibitory effect on oxalate oxidase. While the effects of chloride and sulfate were rather limited in the concentration range studied (up to 20 mM), chlorate severely decreased the oxalate oxidase activity at 1.5 mM and sulfite at 0.10 mM. Under the conditions investigated, oxalate oxidase could stand inclusion of hydrogen peroxide very well up to 1.0 mM, while only 30 % activity remained in the presence of 20 mM hydrogen peroxide. The effect of organic acids was studied at pH 3.8 and formic acid showed more inhibitory effect than glycolic acid, which in turn displayed more effect than acetic acid that had no inhibitory effect at a concentration of 20 mM. Among the cations studied, the negative effect on oxalate oxidase activity increased in the order: calcium, magnesium < manganese (II) < iron (III) < copper (II), iron (II).

Introduction

Precipitation of calcium oxalate, also known as scaling, is a current problem in the pulp and paper industry. Oxalic acid and calcium is present in bleaching filtrates due to occurrence in the raw material, but also due to formation of oxalic acid during bleaching (1-6). In order to decrease water usage, it is getting increasingly common to recirculate process water, which increases the problem with scaling. Selective removal of oxalic acid with the enzyme oxalate oxidase is a possibility to prevent scaling (7).

Oxalate oxidase from barley is a secreted glycoprotein that is also known as germin (8-9). It catalyses the conversion of oxalic acid and molecular oxygen to carbon dioxide and hydrogen peroxide. It is a manganese-containing homohexamer that also possesses superoxide dismutase activity. The molecular mass of the subunits has been estimated to 26 kDa (10-11).

A problem with the approach to eliminate oxalic acid by enzymatic treatment is the inhibition of oxalate oxidase that occurs in authentic process waters (7). In order to make the enzymatic treatment for removal of oxalic acid more efficient, it is therefore important to identify compounds in the bleaching filtrates that interfere with oxalate oxidase and with the help of this knowledge find the most appropriate method to remove or inactivate these interfering compounds or, alternatively, for enzymatic treatment select positions where the concentration of interfering compounds is low.

Coupled assays are commonly used for determination of oxalic acid in medical samples and in studies of the properties of oxalate oxidase. In the coupled assays, hydrogen peroxide is first formed in the reaction catalyzed by oxalate oxidase. The hydrogen peroxide is then utilized in a second reaction, in which a peroxidase generally is employed to oxidize an aromatic substrate to a colored compound that is quantified spectrophotometrically. When coupled assays are used to study the effect of various compounds on oxalate oxidase activity, it is not clear whether the added compounds affect the oxalate oxidase or the peroxidase. In assays that rely on quantification of molecular oxygen (12) or on reactions coupled with hydrogen peroxide (11, 13-14), the presence of substances that directly interact with molecular oxygen and hydrogen peroxide may also lead to erroneous conclusions regarding the effect on the catalytic action of oxalate oxidase.

Direct assays have also been employed to measure the effect of inhibitory compounds on barley oxalate oxidase activity (15-16). In these studies, measurements of substrate degradation were performed by using radiolabeled oxalic acid in combination with barley oxalate oxidase. However, due to that bleaching filtrates were not in the focus of attention in these investigations, a rather limited number of the studied compounds were the same as those that were examined in the current work.

Rather than relying on any of the coupled assays, we have instead used an assay in which oxalic acid is directly quantified with ion chromatography to identify compounds that potentially could act as inhibitors of oxalate oxidase. A selection of compounds relevant for the pulp and paper industry was investigated including cations, anions and organic acids, as well as hydrogen peroxide.

Materials and Methods

Selected compounds

The effect on the oxalate oxidase activity of selected inorganic cations, inorganic and organic anions, as well as hydrogen peroxide was tested. The pH of the stock solutions was adjusted to 3.8 using concentrated sodium hydroxide or hydrochloric acid.

For comparing the effect of the anions chloride and sulfate, the sodium and potassium salts were used at the concentrations 100 μM , as well as 1.0, 10 and 20 mM. Sulfite (with sodium as the counter-ion) was studied at the concentrations 1.0, 10 and 100 μM , as well as 1.0, 10 and 20 mM. Chlorate was tested at the concentrations 1.0, 1.5 and 2.0 mM.

The effect of acetic, formic and glycolic acid at pH 3.8 was tested at the concentrations 100 μM , as well as 1.0, 10 and 20 mM. Furthermore, the effect of formic acid at the concentrations 100 μM , as well as 1.0, 10 and 20 mM was tested at pH 2.2 and 4.5. The pH was adjusted with concentrated sodium hydroxide.

The effects of cations, namely calcium, cupric, ferrous, ferric, magnesium and manganous ions, were studied using both the chloride and the sulfate salts. The cations were applied at the concentrations 100 μM , as well as 1.0, 10 and 20 mM.

The effect of hydrogen peroxide was also studied. The concentrations 100 μM , as well as 1.0, 10 and 20 mM were used in the assays.

All compounds were obtained from Merck (Darmstadt, Germany), with the exception of chlorate and oxalic acid solutions, which were obtained from Acculon Reference Standard solutions (AccuStandard Inc., New Haven, CT, USA).

Assay of oxalate oxidase activity

The total volume of the assay was 2.00 mL. The assay mixture included the following components (in final concentrations): 250 mM succinate buffer (pH 3.8), 0.60 mM oxalic acid, and 100 $\mu\text{g/mL}$ oxalate oxidase from barley seedlings (Sigma-Aldrich, St. Louis, MO, USA). The tested compounds were then added

to the desired concentrations ranging from 1.0 μM to 20 mM. The assay mixture was incubated at room temperature (23°C). Samples for ion chromatography analysis (200 μl) were taken directly after the addition of oxalate oxidase (0 min) and at the end of the incubation (15 min). The samples were diluted 20 times and analysed immediately. All experiments were performed in duplicates and the mean values are presented.

Analyses

Oxalic acid was determined by ion-exchange chromatography (IC) with a Dionex 2020i-series ion chromatography system and using an IonPac AS4A-SC anion-exchange column (250 mm X 4 mm) and an AG4A-SC guard column (all from Dionex, Sunnyvale, CA, USA). The mobile phase consisted of 1.7 mM NaHCO_3 and 1.8 mM Na_2CO_3 and was applied at a flow rate of 2.0 mL/min. The concentration of oxalic acid was calculated by comparing the area of the oxalic acid peak with that of the external standard. The EZchrom software system version 2.31 (Scientific Software Inc., Pleasanton, CA, USA) was used for the quantification procedure.

Results

Inorganic anions

Chloride and sulfate were tested in concentrations up to 20 mM. Inclusion of chloride resulted in almost no inhibition even at the highest concentration

(Table I). Sulfate was more inhibitory than chloride. Slight inhibition (10 %) was observed for sulfate already at the concentration 1.0 mM (Table I). Sodium and potassium gave the same result when used as counter ions (data not shown).

Chlorate and, in particular, sulfite were much more inhibitory than chloride and sulfate (Table I). Chlorate and sulfite caused almost complete inhibition at the concentrations 1.5 and 0.10 mM, respectively.

Hydrogen peroxide

The potential inhibitory effect of hydrogen peroxide was also tested since it is commonly found in the process waters from the pulp and paper industry, where it is used as a bleaching agent. Hydrogen peroxide did not display any inhibition at the concentrations 0.10 and 1.0 mM (Table I). However, the activity of oxalate oxidase decreased with 15 % and 70 % at hydrogen peroxide concentrations of 10 mM and 20 mM, respectively.

Organic acids

Among different carboxylic acids that are present in bleaching filtrates, acetic, formic and glycolic acids are the most prominent (17-18). The potential inhibitory effect of these three acids on oxalate oxidase was therefore tested. Acetic acid was not inhibitory in the concentration range examined. Formic acid was the most inhibitory among the three organic acids. About one fourth of the activity was left when the concentration of formic acid was 20 mM at pH 3.8 (Table II).

Glycolic acid showed an intermediate effect. Oxalate oxidase displayed 45 % activity in the presence of 20 mM glycolic acid (Table II).

To test whether the difference between the three carboxylic acids was associated with the different pK_a values, additional experiments were conducted with formic acid. Reactions with formic acid were therefore also performed at pH 2.2 and 4.5. Formic acid should be mostly undissociated at pH 2.2 and mostly dissociated at pH 4.5. Performance of the reactions at either pH 2.2 or 4.5 led to a decrease in the rate of degradation of oxalic acid compared with the standard reaction at pH 3.8 (Table II). From the data in Table II, it can be calculated that the inclusion of 10 mM formic acid gave 7 % of the initial activity at pH 4.5, 42 % of the initial activity at pH 3.8, and 63 % of the initial

activity at pH 2.2. The inhibitory effect of formic acid was therefore more apparent at pH 4.5 than at pH 3.8 or 2.2.

Cations

Six different cations, including calcium, cupric, ferrous, ferric, magnesium and manganous ions, were tested. Both chloride and sulfate were employed as counter ions. For calcium, inclusion of the sulfate salt resulted in more degradation than when chloride was the counter ion (Table III). Addition of 20 mM calcium chloride gave a very slow reaction rate. The ferrous and the cupric ion followed by the ferric ion were the most inhibitory cations, displaying inhibition already at the lowest concentration tested (0.10 mM) (Table III). Calcium (chloride salt), magnesium and manganese displayed rather similar effect at the concentrations 10 and 20 mM (Table III). Calcium with sulfate as counter ion caused least interference (Table III). However, the low solubility of calcium sulfate should be considered when these results are interpreted.

Discussion

In an attempt to clarify what kind of compounds in bleaching filtrates that are interfering with the catalytic action of barley oxalate oxidase, the effect of different inorganic anions, organic acids, metal ions and hydrogen peroxide were investigated. These groups of compounds are relevant with regard to process waters in the pulp and paper industry. There are several previous reports

Table I. The effect of selected inorganic anions (the sodium forms of the salts were used) and hydrogen peroxide on the activity of oxalate oxidase at pH 3.8.*

<i>Compound</i>	<i>Concentration (mM)</i>	<i>Remaining activity (%)</i>
Chlorate	0.1	100
	1.0	62
	1.5	3.0
	2.0	0.0
Chloride	0.1	100
	1.0	100
	10	100
	20	96
Sulfate	0.1	100
	1.0	90
	10	59
	20	41
Sulfite	0.001	90
	0.01	61
	0.1	4.0
	1	0.0
Hydrogen peroxide	0.1	100
	1	100
	10	85
	20	30

*Reaction conditions: The reaction mix contained oxalic acid (0.6 mM), succinate buffer (250 mM), and barley oxalate oxidase (100 µg/mL). The reaction was allowed to proceed for 15 minutes, after which it was stopped. The remaining activity was calculated as the rate of consumption of oxalic acid compared to the rate in the absence of added compounds. The results are the mean values of two separate experiments. One hundred percent corresponds to a degradation rate of 2.87 ± 0.08 mg oxalic acid per liter per min.

Table II. The effect of organic acids on the activity of oxalate oxidase.

<i>Acid</i>	<i>pH</i>	<i>Concentration (mM)</i>	<i>Remaining activity (%)</i>
Acetic acid	3.8	0.1	100
		1.0	100
		10	100
		20	101
Formic acid	2.2	0	38
		0.1	39
		1	38
		10	24
		20	3
Formic acid	3.8	0.1	100
		1.0	100
		10	42
		20	24
Formic acid	4.5	0	14
		0.1	13
		1	6
		10	1
		20	0
Glycolic acid	3.8	0.1	100
		1.0	101
		10	60
		20	45

One hundred percent corresponds to the degradation rate at pH 3.8 with the reaction conditions indicated in Table I.

Table III. The effect of cations on the activity of oxalate oxidase at pH 3.8.

<i>Cation</i>	<i>Concentration (mM)</i>	<i>Remaining activity (%)</i>	
		<i>Chloride salt</i>	<i>Sulfate salt</i>
Calcium	0.1	100	100
	1.0	97	100
	10	30	62
	20	4.6	41
Copper (II)	0.1	89	89
	1.0	5.1	3.2
	10	2.7	0.0
	20	0.2	0.0
Iron (II)	0.1	45	18
	1.0	18	8.5
	10	0.3	0.4
	20	0.0	0.0
Iron (III)	0.1	87	82
	1.0	24	40
	10	2.6	17
	20	0.8	10
Magnesium	0.1	100	100
	1.0	100	100
	10	58	55
	20	1.1	9.3
Manganese (II)	0.1	100	100
	1.0	100	78
	10	26	19
	20	6.9	2.4

The reaction conditions are indicated in Table I.

concerning oxalate oxidase inhibitors (11-16, 19-21). The reported results do not cover the most important types of compounds encountered in bleaching filtrates from the pulp and paper industry. In addition, these studies are often contradictory, which probably could be explained by the fact that oxalate oxidase from different organisms show different properties as well as by the frequent usage of coupled or indirect assays, where it is not certain whether the observed inhibition is due to loss of oxalate oxidase or peroxidase activity.

Barley oxalate oxidase was rather insensitive to chloride (Table I). A similar result has been obtained for oxalate oxidase from grain sorghum (0.1 M NaCl), barley (0.01-1 mM NaCl), spiny amaranth (*Amaranthus spinosus*) (1 mM NaCl), and *Bougainvillea spectabilis* (0.1 M NaCl) (11, 13, 19, 21). However, some reports (14, 15) have indicated that relatively low levels of sodium chloride are inhibitory to oxalate oxidase.

Sulfate was more inhibitory than chloride, leading to 10 % loss in enzyme activity at a concentration of 1.0 mM (Table I). Previously, it has been reported that up to 1 mM sulfate has no effect on barley and *Amaranthus* oxalate oxidase (11, 14, 19). However, these studies were dependent on a coupled assay of oxalate oxidase involving a peroxidase, which may account for the difference in the obtained results.

We found that sulfite caused clear inhibition already at a concentration of 0.01 mM (Table I), which may be explained by its strong reducing strength. It is possible that sulfite under certain circumstances can be present in bleaching filtrates (22-23). The presence of chlorate also resulted in significant loss of oxalate oxidase activity at relatively low concentrations (1.0 mM) (Table I). Chlorate is very relevant in process waters from the pulp and paper industry, when chlorine dioxide is employed as a bleaching agent. To our knowledge, there are no other reports regarding the effect of chlorate and sulfite on oxalate oxidase activity.

Among the carboxylic acids, formic acid was most inhibitory, followed by glycolic acid whereas acetic acid did not display any inhibition under the conditions tested (Table II). It has previously been shown that even though other carboxylic acids display structural similarity with oxalic acid, they do not serve as substrates for barley and *Pseudomonas* oxalate oxidase (12, 14). At pH 3.8, acetic acid (pK_a 4.74) is mainly uncharged, whereas glycolic acid (pK_a 3.83) and formic acid (pK_a 3.75) appear in approximately equal amounts of charged and uncharged form. The experiments performed at pH 2.2 and 4.5 with formic acid indicate that the charged form is more inhibitory than the uncharged (Table II). The rate of the control reaction (with no formic acid present), set to 100 % at pH 3.8, decreased at pH 2.2 and, in particular, at pH 4.5. Since oxalic acid has pK_{a1} 1.23 and pK_{a2} 4.28, this may indicate that at least one of the carboxyl groups should be uncharged when it serves as substrate for oxalate oxidase. Thus, the low pH optimum of oxalate oxidase (pH 3.8) could possibly reflect that uncharged substrate is required for the catalysis. A recent report has also confirmed that oxalic acid that is negatively charged probably cannot enter the active site of barley oxalate oxidase at higher pH due to the negatively charged

surface caused by carboxylate groups of aspartate and glutamate residues (9). On the other hand, pH 2.2 is likely to be suboptimal for the enzyme and therefore the rate of the reaction decreased.

Metal ions can form salts with oxalic acid that have very low solubility constants. However, the pH used in the experiments with oxalate oxidase was below pK_{a2} for oxalic acid and thus precipitations did not occur when the different cations were added. Among the various metal ions tested, cupric and ferrous ions were the most inhibitory and they caused inhibition already at the lowest concentration tested (0.10 mM) (Table III). Regarding the inhibitory effect of copper, our findings are in accordance with previous reports concerning oxalate oxidase from barley and *Pseudomonas sp.* OX-53 (12, 16). In contrast, a stimulating effect on the catalytic activity of oxalate oxidase by cupric ions has been reported when coupled assays were used for barley and grain sorghum oxalate oxidase activity determination (11, 13-14, 20). On the other hand, 1 mM copper (II) showed no effect on the activity of oxalate oxidase from *Amaranthus* leaves (19). Regarding ferrous iron, our data are again in agreement with earlier results (16). In contrast, Koyama (12), Satayapal and Pundir (13), as well as Goyal *et al.* (19) have reported that ferrous ions in the concentration range 0.01-1 mM exhibit virtually no effect on *Pseudomonas* (0.1 mM $FeSO_4$), grain sorghum (0.5 mM $FeSO_4$) and *Amaranthus* (0.01-1 mM $FeSO_4$) oxalate oxidase. The mechanism behind the inhibition of oxalate oxidase by divalent iron has so far not been elucidated. It has been suggested that Fe^{2+}/Fe^{3+} interfere with the activity of barley oxalate oxidase by forming insoluble complexes with oxalic acid (11). It is, however, not entirely unlikely that the reason why strong inhibition of oxalate oxidase by ferrous ion was not observed in most of the studies in which a coupled assay was used is that the produced hydrogen peroxide is quickly consumed by horseradish peroxidase. Although Koyama did not use the coupled assay and employed measurement of oxygen consumption instead, catalase that was present in the assay consumed hydrogen peroxide (12). In the absence of peroxidase or catalase, the combination of ferrous ion and hydrogen peroxide would create Fenton's reagent, resulting in the formation of highly reactive hydroxyl radicals that may be very detrimental for the integrity of the enzyme.

At high concentrations, calcium sulfate caused less inhibition than calcium chloride. This is probably due to the low solubility of calcium sulfate (K_{sp} in water at 25 C°: $7.10 \cdot 10^{-5}$). Calcium and manganous ions were found to cause less interference than ferrous and cupric ion (Table III), which is in compliance with a previous study (16). In our study, low concentrations (up to 1 mM) of calcium and manganous ions did not affect oxalate oxidase from barley negatively (Table III), which is in good agreement with a previous report concerning oxalate oxidase from barley (14). Previous studies have also shown that 1 mM of manganese did not affect the activity of barley and *Bougainvillea spectabilis*

oxalate oxidase (20-21). The ferric ion showed more effect than calcium, manganese, and manganous ions (Table III). The presence of 1 mM ferric iron has even been reported to lead to an increase in the activity of barley oxalate oxidase (20). These results were, however, based on the coupled assay method in which peroxidase is employed.

No compound examined in this work was found to stimulate the enzymatic activity, which is a difference compared with some of the previous reports concerning oxalate oxidase. For example, the activity of barley oxalate oxidase has been reported to increase in the presence of calcium (0.01-1 mM) (11). In this case, one has to bear in mind that the reported stimulation may also possibly reflect the effect of the studied compound on horseradish peroxidase, which contains structural calcium ions (24).

Hydrogen peroxide, one of the reaction products, also showed a negative effect on oxalate oxidase. The inhibition of oxalate oxidase by hydrogen peroxide has not been reported previously. This result is also of importance since the concentrations that we tested must be considered relevant with regard to the pulp and paper industry.

In conclusion, our data provide relevant information regarding oxalate oxidase inhibitors both from fundamental and applied points of view since we have tested a number of compounds using a direct assay. Several of these compounds, such as chlorate, sulfite, formic acid, glycolic acid and hydrogen peroxide have not previously been tested with respect to the potential effect on the activity of oxalate oxidase. Furthermore, as these above mentioned compounds may be found in process waters from the pulp and paper industry, the results are of a practical importance and help to explain why the degradation of oxalic acid by oxalate oxidase may be inefficient when industrial samples are treated.

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References

1. Krasowski, J.A.; Marton, J. TAPPI 1982 Research and Development Division Conf. Asherville, NC, 29 Aug.-1 Sept., 1982, 129-138.

2. Nilvebrant, N.-O.; Reimann, A. 4th European workshop on lignocellulosics and pulp (EWLP), Advances in characterization and processing of wood, non-woody and secondary fibers, Stresa, Italy, 8-11, 1996, 485-491.
3. Ristolainen, M.; Alen, R. *Tappi J.* **1998**, *81*, 195-198.
4. Elsander, A.; Ek, M.; Gellerstedt, G. *Tappi J.* **2000**, *83*, 73-77.
5. Ulmgren, P.; Rådeström, R. *Nord. Pulp Pap. Res. J.* **2000**, *15*, 128-132.
6. Fiskari, J.; Gullichsen, J. *Pap. Asia* **2000**, *16*, 16-17.
7. Nilvebrant, N.-O.; Reimann, A.; de Sousa, F.; Cassland, P.; Larsson, S.; Hong, F.; Jönsson, L.J. *Prog. Biotechnol.* **2002**, *21*, 231-238.
8. Lane, B.G. *Biochem. J.* **2000**, *349*, 309-321.
9. Woo, E.J.; Dunwell, J.M.; Goodenough, P.W.; Marvier, A.C.; Pickersgill, R.W. *Nat. Struct. Biol.* **2000**, *7*, 1036-1040.
10. Dumas, B.; Saillard, A.; Cheviet, J.-P.; Freyssinet, G.; Pallett, K. C. R. *Acad. Sci. Paris* **1993**, *316*, 793-798.
11. Kotsira, V.P.; Clonis, Y.D. *Arch. Biochem. Biophys.* **1997**, *340*, 239-249.
12. Koyama, H. *Agric. Biol. Chem.* **1988**, *52*, 743-748.
13. Satyapal; Pundir, C.S. *Biochim. Biophys. Acta* **1993**, *1161*, 1-5.
14. Sugiura, M.; Yamamura, H.; Hirano, K.; Sasaki, M.; Morikawa, M.; Tsuboi, M. *Chem. Pharm. Bull.* **1979**, *27*, 2003-2007.
15. Chiriboga, J. *Biochem. Biophys. Res. Commun.* **1963**, *11*, 277-282.
16. Chiriboga, J. *Arch. Biochem. Biophys.* **1966**, *116*, 516-523.
17. Ristolainen, M.; Alen, R. *Tappi J.* **1998**, *81*, 195-198.
18. Topfer, A.; Stan, H.-J. *Vom Wasser* **2000**, *95*, 1-24.
19. Goyal, L.; Thakur, M.; Pundir, C.S. *Plant Sci.* **1999**, *142*, 21-28.
20. Kotsira, V.P.; Clonis Y.D. *Arch. Biochem. Biophys.* **1998**, *356*, 117-126.
21. Srivastava, S.K.; Krishnan, P.S. *Biochem. J.* **1962**, *85*, 33-38.
22. Jiang, Y.; Qian, Y.; Yu, T.; Zhan, H. *China Pulp Pap.* **2000**, *19*, 52-58.
23. Tredway, C.M. *Pulp. Pap.* **1979**, *53*, 73-75.
24. Howes, B.D.; Feis, A.; Raimondi, L.; Indiani, C.; Smulevich, G. *J. Biol. Chem.* **2001**, *276*, 40704-40711.

Chapter 6

Xylanase Treatment of D₀ Pulp to Enhance Bleaching

Jeffrey S. Tolan, and Corina Popovici

Iogen Corporation, 400 Hunt Club Road, Ottawa, Ontario
K1V 1C1A, Canada

Xylanase treatment is used to enhance the bleaching of kraft pulp. This technology is used in 15 mills in North America and enables the mills to bleach pulp while using 10% to 20% less chlorine dioxide or other oxidative chemicals. Xylanase treatment is carried out on brownstock prior to the bleach plant. This treatment location is satisfactory for most mills, but there is an interest in improving the benefits a mill can obtain from xylanase and in treating pulp in mills not amenable to brownstock treatments. This paper describes the xylanase treatment of pulp after the first chlorine dioxide (D₀) bleaching stage. Such treatments can take place in neutral wash stages or in the first alkaline extraction stages. There is significant opportunity using xylanase treatments in these stages to obtain a greater benefit than in conventional xylanase treatments of brownstock.

Introduction

Kraft pulping is the primary chemical pulping process used in the pulp and paper industry. In kraft pulping, wood chips (or other raw material) are cooked in alkali liquor consisting of sodium sulfide and sodium hydroxide at temperatures around 200 °C for 1 to 3 hours. The pulping process converts the stiff wood to flexible fibers suitable for paper. In addition, the

alkali dissolves about 90% of the lignin in the wood. At the point after pulping and washing the lignin away with water, the pulp is referred to as “brownstock”. Brown paper bags are an example of a product made from brownstock.

Complete removal of the lignin is essential in the production of most kraft pulp, which is used to make white paper. The lignin remaining in brownstock, which comprises 2% to 4% of the brownstock, is removed by bleaching with oxidative chemicals. For more than a century before 1990, the primary bleaching chemical was chlorine. Chlorine gas is an effective bleaching agent that produces a high-quality pulp inexpensively. Unfortunately, chlorine bleaching releases chlorinated lignin compounds into the mill effluent that have raised concerns over chronic toxicity (such as dioxins, furans and the large set of compounds lumped together as AOX).

To address the concerns associated with chlorine bleaching, the industry has adopted alternative bleaching technologies. These include the use of chlorine dioxide, oxygen, hydrogen peroxide and, to a lesser extent, ozone. These technologies are not without problems, including significant increases in capital and operating costs and decreases in pulp quality relative to that produced using chlorine.

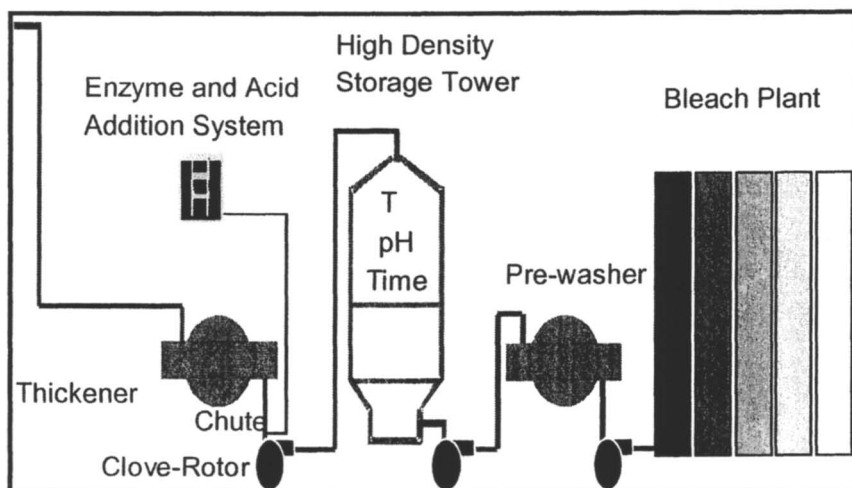


Figure 1. Brownstock treatment

The use of xylanase enzymes to treat pulp has been adopted by the industry to address these issues. The benefits of xylanase in bleaching kraft pulp were reported by researchers in Finland in 1986 (1). Numerous studies of the effects of xylanase, mechanism of action on pulp, and properties of xylanase enzymes have been published (2-5). Initial mill trials of the technology were carried out in 1991, and commercial use started in 1992. Today, 15 mills are using xylanase full-time in North America. This represents 4 million tonnes of pulp treated annually and corresponds to 16% of the pulp made in Canada and 12% in the US. This is also the largest penetration of biotechnology into the pulp and paper industry (6).

Xylanase is added to brownstock prior to the bleach plant (Figure 1). The enzyme is provided in an aqueous solution and is added to the pulp at or near the final brownstock washer. At this point, the pH and temperature of the pulp are adjusted to the optimum range for the enzyme. The pH is usually adjusted with sulfuric acid because brownstock is very alkaline. The temperature is controlled by the temperature of the brownstock wash water and can vary by a few degrees centigrade. Once added, the enzyme acts on the pulp in the high-density brownstock storage tower for 15 minutes to two hours, depending on the size of the tower and the degree of pulp channeling. The enzyme hydrolyzes and solubilizes 2% to 4% of the xylan in the pulp. This action does not directly bleach or delignify the pulp, but makes the pulp easier to bleach. The treated brownstock then proceeds to the bleach plant with a decreased requirement for bleaching chemicals.

Typical effects of xylanase are shown in Figure 2. The brightness of the pulp (after bleaching) increases with increasing total kappa factor (TKf), which is a measure of the amount of chlorine dioxide used. The TKf is defined as $TKf = (2.63 * C) / (10 * Kp)$, where C = Chlorine dioxide usage across the bleach plant, kg/t pulp, and Kp = pulp kappa number.

For a given TKf, the xylanase treated pulp reaches 0.5 to 1.0 points higher brightness than the untreated pulp. Therefore, if a mill follows xylanase treatment with the same usage of bleaching chemicals as an untreated pulp, the mill can achieve a higher pulp brightness.

Alternatively, the mill can maintain the same level of brightness using xylanase with a lower amount of bleaching chemicals than for an untreated pulp. From Figure 2, a bleached brightness of 90.7 is achieved with a TKf of 0.53 for an untreated pulp and 0.43 for an enzyme-treated pulp, thereby saving the mill 18.5% of its chlorine dioxide.

A third alternative is to combine the features of the first two and use xylanase treatment to achieve a higher brightness and a savings in chlorine dioxide. The increase in brightness and decrease in chemical usage will be somewhat less than that achieved if either parameter was decreased alone.

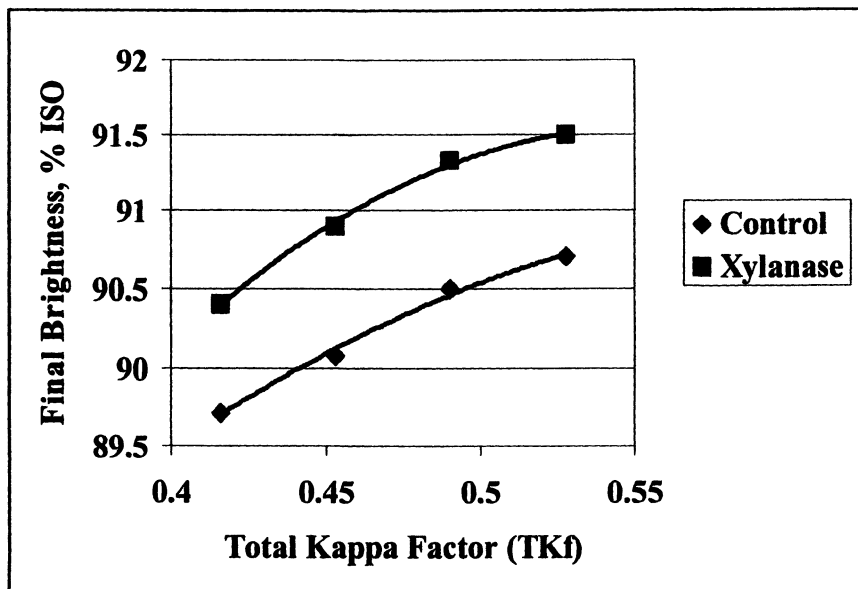


Figure 2. Effect of xylanase treatment on brightness and bleaching chemical charge (4).

The enzymatic treatment of brownstock has been successful in the industry. However, some aspects of the treatment are limitations. Some mills do not have brownstock pumps; in this case, it is difficult to disperse the enzyme uniformly into the pulp. In some mills, the brownstock high-density storage tower is so small or channels so badly as to not allow the required retention time to be achieved. Some brownstock pulps do not respond well to xylanase, for reasons that are not understood (2). Finally, there is always a desire to increase the benefits of xylanase in bleaching.

These reasons motivated a study of xylanase treatments of Do pulps, that is, pulps that have been through only the first chlorine dioxide (Do) bleaching stage. The Do stage itself contains the chlorine dioxide, a highly oxidative chemical, and is therefore not compatible with xylanase. After the Do stage, some mills have a neutral wash that is compatible with xylanase treatment. Most mills do not have a neutral wash and proceed directly to the first alkaline extraction (E₁) stage. This stage is at conditions that are too harsh for xylanase treatment, but these conditions were modified to accompany the enzyme in this study.

Materials and Methods

Pulp: Hardwood kraft brownstock from a mill in Quebec was used. The pulp was washed thoroughly with water and stored refrigerated.

Enzyme: The enzyme used was *Trichoderma xylanase II*, engineered for thermal and pH resistance (7). The aqueous solution of enzyme has a xylanase activity of 9800 Xu/ml based on the release of Remazol Brilliant Blue dye from xylan (8). The enzyme was stored refrigerated. The xylanase was made in submerged liquid culture by a strain of *Trichoderma reesei* and was highly pure, with only trace endoglucanase activity. No cellulose-oligomers were detected in the pulp filtrates.

Conventional xylanase treatments: 15 grams of pulp (dry basis) was diluted to 10% consistency in heat sealable plastic bags. The pH of the pulp was adjusted to 7.0 with dilute sulfuric acid. The enzyme was added to the pulp at dosages of 0, 0.25, 0.5, or 0.7 xu/g of pulp and manually kneaded into the pulp. The bags were placed in 57 or 60°C water bath for 1 hour, then filtered over a Buchner funnel. Filtrates were saved for xylose analysis.

Chlorine dioxide (Do) stages: After the enzyme treatment stage, the pulp was subjected to a standard Do stage. This immediately destroyed any remaining xylanase activity. A 10g/L solution of ClO₂ was made by bubbling chlorine into a column of sodium chlorate. The solution was standardized by titration with sodium thiosulfate and stored refrigerated. The ClO₂ was added to the pulp at kappa factors of 0.15, 0.17, 0.19 and 0.21. The bags were sealed and incubated in a water bath at 50°C for 1 hour. At this point, the pulp was filtered over a Buchner funnel and washed with 4 liters of water, then pressed to 20% consistency.

Neutral wash (Xw) stages: Some pulps were subjected to neutral washes, with (Xw) or without (W) xylanase present. The Do pulps were adjusted to pH 7 with dilute sodium hydroxide. Xylanase was added at dosages of 0, 0.25 or 0.5 xu/g of pulp. The enzyme was added as in a conventional xylanase treatment, by kneading the pulp. The bags were then placed in a water bath at 57°C for 1 hour, then filtered over a Buchner funnel. Filtrates were saved for xylose analysis. The pulps were then washed with 4 liters of water at 25°C, then pressed to 20% solids consistency.

Conventional alkali extraction (E₁ stages): Following Do or Xw, most pulps were subjected to conventional alkaline extraction (E₁) stages. The pulp was adjusted to pH 11 at 10% consistency with a measured amount of dilute sodium hydroxide. The pulps were incubated in a water bath at 75°C in the presence of a small amount of hydrogen peroxide to simulate a mill extraction stage. After 1 hour, the pulp was filtered over a Buchner funnel and then washed with 4 liters of water.

Mild extraction (X_E) stages: Some pulps were subjected to mild extraction (X_E) stages rather than E_1 stages. For mild extraction stages, the pulp was adjusted to pH 7 at 10% consistency with a measured amount of dilute sodium hydroxide. Xylanase was added at dosages of 0, 0.7 or 1.0 xu/g and kneaded into the pulp, as described in the other xylanase stages. The pulps were incubated in a water bath at 60°C in the presence of a small amount of oxygen and hydrogen peroxide to simulate a mill extraction stage. After 1 hour, the pulp was filtered over a Buchner funnel and the filtrate saved for xylose analysis. The pulp was then washed with 4 liters of water.

Second chlorine dioxide (D_1) stages: All pulps were subjected to standard D_1 stages following E_1 or X_E stages. The chlorine dioxide dosages were $\frac{1}{2}$ that of the D_0 stage. The stages were run at 10% consistency in a water bath at 75°C for 3 hours. The pulp was then filtered over a Buchner funnel and washed with 4 liters of water.

Second extraction (E_2) stages: All pulps were subjected to standard E_2 stages following D_1 stages. The pulp was adjusted to pH 11 with dilute sodium hydroxide and incubated in a water bath at 75°C at 10% consistency for 1 hour. The pulps were then filtered over a Buchner funnel and washed with 4 liters of water.

Third chlorine dioxide (D_2) stages: All pulps were subjected to standard D_2 stages following E_2 stages. The chlorine dioxide dosages were 2 kg/t pulp. The stages were run at 10% consistency in a water bath at 75°C for 3 hours. The pulp was then filtered over a Buchner funnel and washed with 4 liters of water.

Post-bleaching: Bleached pulps were soured with SO_2 , pressed into handsheets, and dried overnight at 25°C. The ISO brightness of the dry handsheets was measured.

Analysis

Brownstock kappa number was measured by TAPPI method T 236 cm-85. Bleached brightness was measured by the PAPTAC method E.1. The concentration of chlorine dioxide solutions was measured by titration with sodium thiosulfate. The concentration of xylose in pulp filtrates was measured by adding sulfuric acid to the filtrate to a concentration of 2% (w/v) and incubating in a 121°C steam autoclave for 1 hour. This acid hydrolysis converts xylose oligomers to monomers. The xylose concentration was then measured by HPLC. The xylose concentration and the quantity of filtrate were used to calculate the amount of xylose released from the pulp.

Results

Table I and Figure 3 show that xylanase treatment in a neutral wash can be beneficial to pulp bleaching. The untreated pulp is represented by the curve of lowest brightness as a function of TKf. This pulp requires a TKf of 0.43 to reach 89.5 ISO brightness.

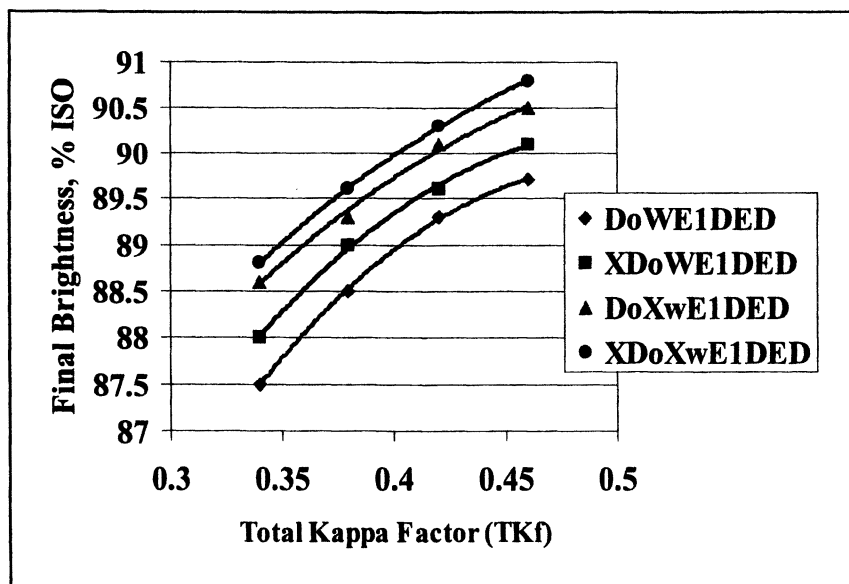


Figure 3. Xylanase in neutral wash

The pulp with a conventional 0.5 xu/g xylanase treatment of the brownstock requires a TKf of 0.41 to reach 89.5 brightness. This is a savings of 4.7% relative to the untreated pulp. This is a lower benefit than the typical range of 15% to 20% for a hardwood pulp. Partly this was due to the use of a lower xylanase dosage than the typical 1-2 xu/g dosage used in a mill. In addition, the low response of this pulp to xylanase was the reason this pulp was chosen deliberately as a candidate for an alternative xylanase treatment.

The 0.5 xu/g xylanase treatment in the neutral wash resulted in a better response than the conventional xylanase treatment. This pulp was bleached to 89.5 ISO brightness with 8% less ClO₂ than the untreated pulp.

Finally, the best response was with 0.25 xu/g in the conventional brownstock treatment and then an additional 0.25 xu/g in a neutral wash treatment. This pulp was bleached to 89.5 ISO with 12.7% less ClO₂ than the untreated pulp. This equals the sum of the benefits from the separate conventional and the neutral wash treatments.

Table I: Xylanase treatments in neutral wash stage (Pulp: Hardwood, Kappa # 15.1)

PARAMETER	SEQUENCE			
	D_oWE_1DED (Untreated)	XD_oWE_1DED (Conventional) X	$D_oX_wE_1DED$ (Neutral Wash) X_w	$XD_oX_wE_1DED$ (Two-stage) X and X_w
<i>X Dosage</i> (xu/g)	0	0.5	0	0.25
<i>X_w Dosage</i> (xu/g)	0	0	0.5	0.25
<i>Total Dosage</i> (xu/g)	0	0.5	0.5	0.5
<i>ClO₂ (kg/t) to 89.5 ISO</i>	22.7	21.6	20.6	19.8
<i>Savings</i>	0	4.7%	8%	12.7%

Table II and Figure 4 show that xylanase treatment in a mild extraction stage can be beneficial to pulp bleaching. The untreated pulp is represented by the curve of lowest brightness as a function of TKf. This pulp requires a TKf of 0.47 to reach 87 ISO brightness.

The pulp with a conventional 0.7xu/g xylanase treatment of the brownstock requires a TKf of 0.43 to reach 87 ISO brightness. This is a savings of 10.3% relative to the untreated pulp. This is a lower benefit than the typical range of 15% to 20% for a hardwood pulp. This was another pulp that was chosen deliberately as a candidate for an alternative xylanase treatment because of its low response.

The 1 xu/g xylanase treatment in the mild (pH 7, 60°C) extraction stage resulted in a similar response as the conventional xylanase treatment. This pulp was bleached to 87 ISO brightness with 9% less ClO₂ than the untreated pulp.

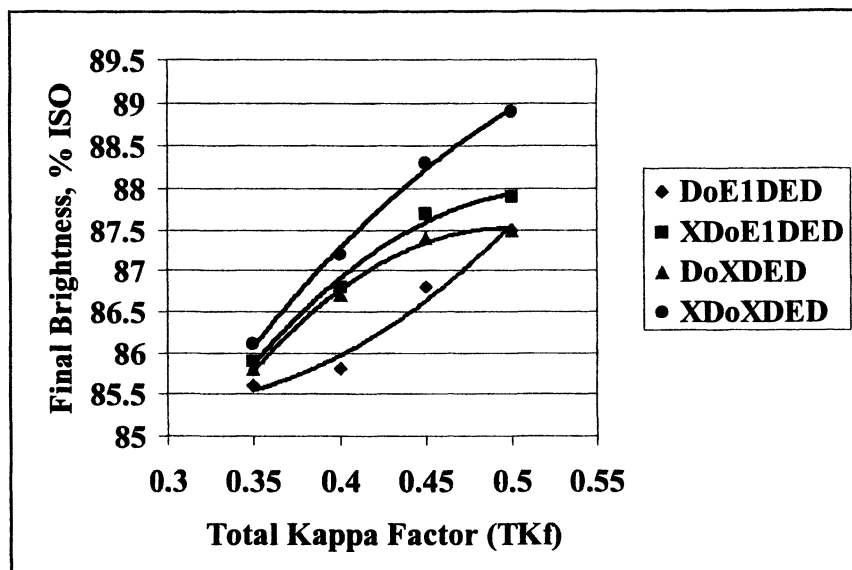


Figure 4. Xylanase in mild extraction stage.

By using the mild extraction stage, the sodium hydroxide use in that stage decreased by 57%.

Finally, the best response was with 0.7 xu/g in the conventional brownstock treatment and then an additional 0.7 xu/g in a mild extraction stage. This pulp was bleached to 87 ISO with 18.9% less ClO_2 than the untreated pulp. This is close to the sum of the chemical savings from the separate xylanase treatments of brownstock and during mild extraction. This two-stage treatment also saved 57% of the caustic used in the extraction stage.

Figure 5 shows the amount of xylose released by xylanase treatments of brownstock, Do pulp, and split evenly between brownstock and Do pulp. Two trends are interesting. The shape of the dosage response curve changes with Do bleaching. Also, the xylose released by the split treatments is almost the independent sum of the two separate treatments.

Discussion

Xylanase treatment of Do pulp is at least as effective as conventional brownstock treatment. When carried out in a neutral wash stage, the xylanase treatment offers several potential advantages over conventional xylanase

treatments. In the neutral wash, there is always a stock pump present. The towers are designed to minimize pulp channeling. The towers have a level of instrumentation that is not present in brownstock storage towers. These aspects of neutral wash treatments address several of the shortcomings of conventional xylanase treatments. One limitation of neutral wash xylanase treatments is their availability is limited to a minority of mills. Mills with extra towers or mills with Papricycle stages (9) are the primary candidates for these xylanase treatments.

Table II: Xylanase treatments in mild extraction stage (Pulp: Hardwood, Kappa # 14.9)

PARAMETER	SEQUENCE			
	D_oE_1DED (Untreated)	XD_oE_1DED (Conventional) X	D_oX_EDED (Mild Extraction) X_E	XD_oX_EDED (Two-stage) X and X_E
X Dosage (xu/g)	0	0.7	0	0.7
X_E Dosage (xu/g)	0	0	1	0.7
Total(xu/g)	0	0.7	1	1.4
Extraction T , pH	75°C, pH 11	75°C, pH 11	60°C, pH 7	60°C, pH 7
NaOH (kg/t)	10	10	4.3	4.3
Savings	---	---	57%	57%
ClO ₂ (kg/t) to 87 ISO	23.3	20.9	21.2	18.9
Savings	---	10.3%	9%	18.9%

When carried out in a mild extraction stage, the xylanase treatments carry some additional advantages over neutral wash treatments. Xylanase in mild extraction stages not only decreases the requirement for chlorine dioxide, but also allows this to happen with a reduced level of caustic and at a reduced temperature. This mild xylanase extraction stage potentially allows a mill to save on energy costs as well as caustic and ClO₂. Unlike neutral washes, almost every mill is equipped with alkaline extraction stages.

The use of a two-stage xylanase treatment, that is, treatment of brownstock and of Do pulp, offers benefits roughly equal to the sum of the separate benefits of conventional and Do-treatments. This offers an opportunity for mills to obtain an increased benefit from xylanase treatment. The difficulty in implementing this operation is the need for and complexity of two enzyme addition systems in the bleach plant.

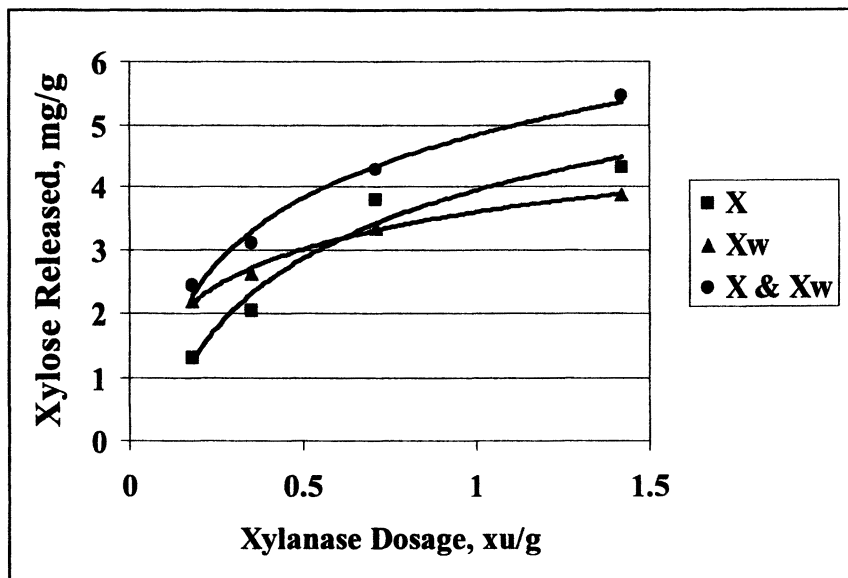


Figure 5. Xylose release for xylanase treatments on Brownstock and Do pulp.

The observation that the bleaching benefits obtained on Do treated pulp are additive to those from conventional pulp implies that the treatment of the Do pulp is independent of the conventional xylanase treatment. This implies further that, from the point of view of xylanase, the character of the pulp is changed substantially by the Do stage. This stage removes 80% of the lignin remaining in the brownstock. How this might change the response of the pulp to xylanase is a matter for speculation.

References

1. Viikari, L.; Ranua, A.; Kantelinen, A.; Linko, M.; Sundquist, J. *Bleaching with enzymes*, Biotechnology in the Pulp and Paper Industry, Proc. 3rd International Conference, Stockholm, 67, 1986.
2. Tolan, J.S.; Olson, D.; Dines, R.E. *Enzymes for Pulp and Paper Processing*, Jeffries, T.W.; Viikari, L., Eds.; ACS Symposium Series 655, American Chemical Society: Washington DC, 1996; pp 25-35.
3. Suurnakki, A.; Tenkanen, M.; Buchert, J.; Viikari, L. *Hemicellulases in the Bleaching of Chemical Pulps*, *Advances in Biochemical Engineering Biotechnology*; Eriksson, K.E.L., Ed; Springer: Berlin, Germany, 1997; Vol. 57, pp 261-287.

4. Tolan, J.; Guenette, M. *Using Enzymes in Pulp Bleaching: Mill Applications, Advances in Biochemical Engineering Biotechnology*; Eriksson, K.E.L., Ed; Springer: Berlin, Germany, 1997; Vol. 57, pp 289-310.
5. *Xylans and Xylanases*; Visser, J.; Beldman, G; Kusters-van Someren, M.A.; Voragen, A.G.J., Eds.; *Progress in Biotechnology 7, Proceedings of an International Symposium, Wageningen, The Netherlands, Dec. 8-11, 1991*; Elsevier: Amsterdam, The Netherlands, 1992; Vol. 7.
6. Tolan, J.S.; Popovici, C. *Mill usage and mechanistic studies of xylanase to enhance bleaching, Biotechnology in the Pulp and Paper Industry: 8th ICBPPI Meeting*; Viikari, L.; Lantto, R., Eds.; *Progress in Biotechnology 21*; Elsevier: New York, 2002; Vol. 21, pp. 263-270.
7. White, T.; Watkinson, J.; Sung, W.; Yaguchi, M.; Wood, M.; Huang, F. *Protein engineering of xylanase for pulp bleaching applications; 7th International Conference on Biotechnology in the Pulp and Paper Industry*; Vancouver, Canada, June 16-19, 1998.
8. Bailey, M.J.; Poutanen, K. *Collaborative testing of a Xylanase Assay Method, Xylans and Xylanases*; Visser, J.; Beldman, G; Kusters-van Someren, M.A.; Voragen, A.G.J., Eds.; *Progress in Biotechnology 7*; Elsevier: Amsterdam, The Netherlands, 1992; Vol. 7, pp 155-161.
9. *Pulp Bleaching: Principles and Practice*; Dence, C.W.; Reeve, D.W., Eds.; TAPPI Press: Atlanta, GA, 1996; pp 315-318.

Chapter 7

Cellulose-Binding Domains as a Tool for Paper Recycling

H. Pala¹, R. Pinto¹, M. Mota, A. P. Duarte^{1,2}, and F. M. Gama¹

¹Centro de Engenharia Biológica-IBQF, Universidade do Minho, Largo do Paco, 4719 Braga codex, Portugal (telephone: 351-253-604400; fax: 351 253)

²Current address: Departamento de Ciência e Tecnologia do Papel, Universidade da Beira Interior, 6201-001 Covilhã, Portugal

Treatment of secondary paper fibres with cellulose-binding domains allows for improvements of pulp drainability and of paper mechanical properties. The interfacial system fibre-water-fibre, and after drying, fibre-air-fibre, may be affected by the CBD treatment, influencing the pulp and paper technical properties. Inverse Gas Chromatography provides experimental evidence that support this hypothesis.

Introduction

Cellulose-binding domains (CBD) are functionally independent protein modules present in many cellulases. These modules are essential for the effectiveness of the enzymatic action, namely for the hydrolysis of insoluble cellulose and especially of the crystalline regions. In fact, whenever the CBD is removed, the hydrolysis of insoluble substrates by the catalytic domain alone proceeds at a much lower rate (1, 2, 3). The enzyme affinity and adsorption on cellulose is controlled by the binding core structure, topology and charge (4). The enzymatic modification of paper pulps depends on the CBD used. Indeed, a Kraft pulp treated with proteins from *Trichoderma reesei* - native cellobiohydrolase I and a recombinant cellobiohydrolase I with an endoglucanase I-binding domain - have different technical properties (3).

The binding domains retain their functionality when separated from the rest of the protein. It has been suggested that CBDs have the ability to disrupt the cellulose fibres surface (5, 6). This ability would explain the striking importance of CBDs in the hydrolysis of insoluble substrates: not only they allow for a concentration of the enzymes in the surface of the fibres, but also the swelling and fibre surface disruption favours the formation of new binding sites, facilitating the hydrolytic process. The CBD action on the fibre surface is certainly a topic that requires additional experimental studies.

To this point, research on the CBD properties has focused mainly on the CBD/cellulose interaction and on the binding/catalytic domains interdependence. In the present paper, the ability of family I CBD (obtained by proteolytic digestion of *Trichoderma reesei* cellulases) to modify the technical properties of a secondary pulp for paperboard production is demonstrated. Paper fibres upgrading with cellulases has been attempted since over the last 20 years. As shown in previous work (7, 8), enzymatic modification of fibres is not simply a hydrolytic process. In fact, the modification of the interfacial properties may be a rather important aspect. To characterise the modification of interfacial properties by CBDs, Inverse Gas Chromatography was used in the present work.

Methods and Materials

Cellulose-binding domains

The cellulose-binding domains (CBD) preparation was obtained by ultrafiltration after proteolytic digestion of Celluclast 1.5L, according to the protocol described in Lemos *et al.* (9). The final CBD solution did not show cellulolytic activity (9); protein quantification was done by the Bradford method (10).

Cellulose powder: Whatman CF-11 was used in the Inverse Gas Chromatography experiments.

Paper pulp: The pulp used in this study was obtained after disintegration of old paperboard containers, representing a mixture of 60% Kraft paper, 20% fluting and 20% test liner. It was kindly supplied by the company *Portucel Viana*.

CBD treatment of paper pulps

Paper pulps (a mass of wet pulp equivalent to 70 g of oven dried (o.d.) pulp) were disintegrated with a blender in sodium citrate buffer 0.05 M, pH 5.0, for 10 minutes. The CBD solution (representing 10% of the total reaction volume to assure a good dispersion) was then added to the mixture and allowed to react for 30 min, with continuous slow mixing (3% consistency, at 50°C). Then, the fibres were recovered by filtration. The filtrate was forced through the fibre cake, in order to avoid the loss of shorter fibres. Reducing sugars in the filtrate were measured using the DNS method (11). The pulp was treated with 1.1 mg protein/g o.d. pulp. Control assays were executed in the absence of CBD. Each assay, with and without CBDs, was performed twice with good reproducibility.

Preparation of paper sheets

Paper sheets (60 g/m²) were prepared using an Auto Dinamic Sheet Former (DSF), manufactured by Noram – Lorentzen & Wettre.

Pulp and Paper Testing

Determination of the pulp and paper properties followed the usual standard procedures: drainage rate (ISO 5267/1), burst (ISO 2758), tensile strength (ISO 1924/2), tear (ISO 1974), sheet density (ISO 534) and permeability to air flow (ISO 5636/3): Test pieces for paper characterisation were obtained from the middle region of the DSF sheets; a four-sheet set (0.198 m² each) was available for each experimental condition. The coefficients of variation (drainage, tensile, burst and tear) were less than 1%.

Inverse Gas Chromatography

In gas chromatography, the retention volume of a volatile substance depends on its interaction with the stationary phase. In Inverse Gas Chromatography (IGC), the solid adsorbent to be characterised is the stationary phase, while known probe molecules are used as volatiles. When the measurements are carried out at infinite dilution of the solutes or at zero surface coverage of the adsorbent, then the net retention volume V exclusively derive from the interaction between adsorbent and adsorbate. The net retention volume is given as: $V = F(t_r - t_0)$, where F is the corrected flow rate of carrier gas, t_r is the retention time of the respective solute and t_0 is the retention time of the marker (methane). The complete thermodynamic characterisation of the adsorbent solid phase is

possible, by using the appropriate probes and measuring the retention volume at different temperatures, as described elsewhere (12, 13). When aiming at comparing the properties of different solids, the direct comparison of the retention volumes, at a defined temperature, provides an accurate measure of the relative affinity of the materials for each probe.

In this work, the effect of CBDs on the surface properties of a model cellulose powder (Whatman CF-11) was studied. A suspension of cellulose (total cellulose weight of 10 g) was prepared in sodium acetate buffer 0.05 M, pH 5.0. The CBD solution (representing a minimum of 10% of the total reaction volume, to assure a good dispersion) was then added to the mixture (final concentration: $3\% w_{\text{cellulose}}/v_{\text{buffer}}$; $0.2\% w_{\text{protein}}/w_{\text{cellulose}}$) and allowed to react for 30 min, with continuous slow mixing, at 50°C. The mixture was then centrifuged at 4000 rpm. The supernatant was removed, the fibres were washed with distilled water (300 ml) and again centrifuged (method 1). In another experiment, the same procedure in the preparation of the samples was carried out, with the exception that the fibres were washed twice with 400 ml of distilled water (method 2). For both methods, the respective control was prepared, using a buffer solution without CBDs. The obtained material was packed in a Chrompack stainless steel column of 1 m length and 4 mm internal diameter. About 5 grams were packed in each column. The exact weight was recorded and used to correct the values of retention volume. The non-processed Whatman CF-11 fibres were also analysed.

Chromatographic measurements at infinite dilution were carried out with a Chrompack CP9001 gas chromatograph equipped with a flame ionisation detector. The carrier gas was helium. The sample was injected with a Hamilton Gastight 1750SL syringe, and its concentration was adjusted such that the minimum attenuation was used in the determination of the retention time, ensuring practically infinite dilution. The retention volume of each probe, V , was calculated as the average of at least 5 values.

Results and Discussion

Table I summarises the properties of the paper sheets and the effect of the cellulose-binding domains (CBD) treatment. A positive effect on both the pulp and paper properties is detected. In fact, both drainage and strength (specially tensile and burst) are improved by the CBD treatment. The paper sheets do not show any major change in the density or permeability parameters. This trend contrasts with the effect typically obtained when enzymes are applied for fibre modification (7); although improving drainage to a large extent, the enzymatic hydrolysis normally worsens the paper mechanical properties. Furthermore, as expected, no solubilisation was detected after the pulp treatment. Indeed, the possibility of excessive (hydrolytic) modification of the fibres, with reductions both in paper yield and fibre quality, is not expected to be an issue when using CBDs.

Table I. Effect of the CBD Application on the Properties of Pulp and Paper

	Drainage (°SR)	Tensile (Nm/g)*		Burst (KPam ² /g)	Tear (mNm ² /g)*		Permeability to air (ml/min)
		MD	TD		MD	TD	
Control	43	30.3	11.6	1.0	3.6	6.8	2714
CBD assay	35	36.5	12.6	1.1	3.7	5.8	2831

* MD, machine direction; TD, transversal direction.

The possible application of CBD in recycling has been previously proposed (7). However, the present work provides further evidence that the non-hydrolytic peptides may be a valuable additive for the paper industry. In the previous work, paper strength was measured in handsheets where, as a consequence of random deposition, fibres do not present a determined orientation. By contrast, the dynamic sheet former (DSF) used in this work allows for an optimal fibre orientation during sheet formation. As in the paper-machine, the obtained paper sheets can be characterised under two directions (Figure 1): along with, and transversally to the fibres orientation in the paper sheet (machine-direction, MD; and transversal-direction, TD). The results show that the magnitude of the modification achieved with the CBDs is greater in the “stronger” direction of the paper sheet. The effectiveness of CBD may be associated to a better fibre alignment (and increased interaction sites between fibres), which would explain the increased paper resistance to tensile forces in the machine-direction. Resistance improvement along the transversal direction is inferior (20% MD versus 9% TD) because although fibre alignment provides an increased number of connections, the inter-fibre length connection along this direction is lower.

A relevant question remains unanswered: why do the non-hydrolytic CBDs allow for both drainage and strength improvements? In our opinion, CBD modify the fibres interfacial properties and thereby the pulp and paper properties. The high fibre-affinity of the peptide and its surface activity may be responsible for the fibre surface modification. In fact, an increased strength of the fibre-water interaction has been observed in paper fibres and powdered celluloses treated with cellulases (8, 14). The referred results show that the adsorbed enzymes increase the fibre water-affinity, leading to a stronger water adhesion. This effect may be responsible for the fibre stabilisation in aqueous suspension, thus avoiding the formation of preferential draining channels and leading to a more homogeneous paper sheet. Upon drying, the inter-fibre interaction is again possible (Figure 2). CBD and enzymes should modify the interfacial properties in a similar way. However, the hydrolytic activity is often detrimental to the paper strength, owing to the reduction in the intrinsic fibre strength, while a positive effect is obtained with CBD. The catalytic activity, and the much higher

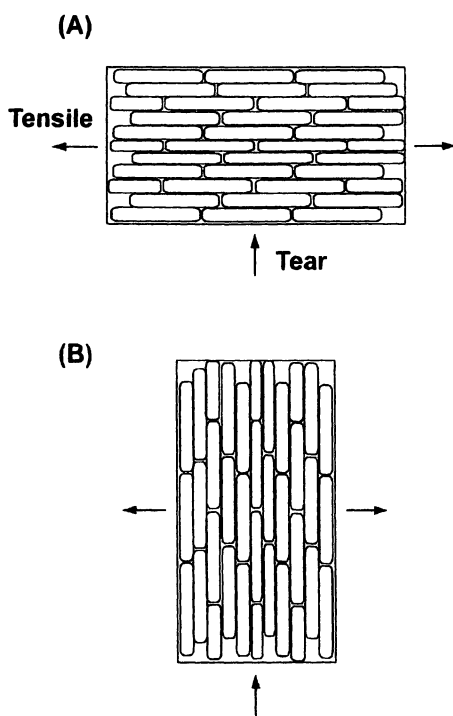


Figure 1. Paper direction influencing paper strength

Fibre alignment in paper sheets increases the number of interfiber contact sites thus improving paper strength. When tensile is measured along the paper MD (A), the number of interfibre bounds opposing the applied strength is higher than when it is determined along the paper TD (B), thus explaining the increased paper resistance to tensile in the former situation. As in respect to tear, paper TD (A) offers a higher resistance to the applied force because in this direction fibres themselves have to be broken.

molecular weight of glycosyl hydrolases (*versus* CBD), may contribute for the different effects in the paper properties. More data on the use of CBD for pulp and paper modifications is necessary in order to support these hypotheses.

It must be remarked, with regard to Figure 2, that the interaction between adjacent fibres is critically affected by the presence of water. The Hamacker constant, a measure of van der Waals forces between surfaces (15), is much higher for condensed phases interacting across air, as opposed to the interaction across water. In the case of cellulose, the Hamacker constants, determined according to the Lifshitz theory are 5.8×10^{-20} J (interaction across air) and 0.8×10^{-20} J (interaction across water) (16). Therefore, the fibre stabilising effect in aqueous suspension (a consequence of steric and hydration phenomena) no longer applies upon fibre dehydration, when the attracting van der Waals forces between the fibres are higher and hydration effects are not present. The CBD activity may be interpreted as a refining-like process, according to Milichovsky molecular interpretation of the process (17). Indeed, it has been recently shown (6) that CBD may lead to fibre disruption and crystallinity reduction, directly affecting the fibres hydration layers.

The characterisation of a model cellulose using IGC shows that washing the fibres modifies significantly their surface properties, possibly due to the removal of ions or other adsorbed chemicals (Table II, CF-11 *versus* Control 1 and 2).

The differences between the two controls suggest that washing the fibre, as carried out by method 1 (washed once with 300 ml of distilled water), is not sufficient. Indeed, the retention volumes obtained for Control 1 lie between those of the non-treated fibres CF-11 and Control 2, prepared by more thoroughly washing the fibres with distilled water (2x400ml). Comparing the Controls with the respective CBD assays suggest the same general trend. Interestingly, the treatment with CBDs does not affect the dispersive properties of the surface, since the retention volume of neutral probes is not affected (Table II, Control *versus* CBD). The interaction with an acidic probe, chloroform, is also not affected. It seems that the cellulose surface has essentially an acidic character, since it interacts preferentially with basic and amphoteric probes, which have larger retention volumes. The presence of CBDs contributes to a reduction of this acidic character, as can be concluded from the significant reduction in the retention volume detected for tetrahydrofuran, diethyl ether, acetone and ethyl acetate. The major modification CBDs introduce in the surface properties seems to be a reduction in the concentration of acidic groups, presumably because a part of the cellulosic surface is not accessible following the CBD treatment. Apparently, the stabilisation of the fibres in aqueous suspension is therefore a consequence of steric and hydration effects, as suggested elsewhere (8), and not

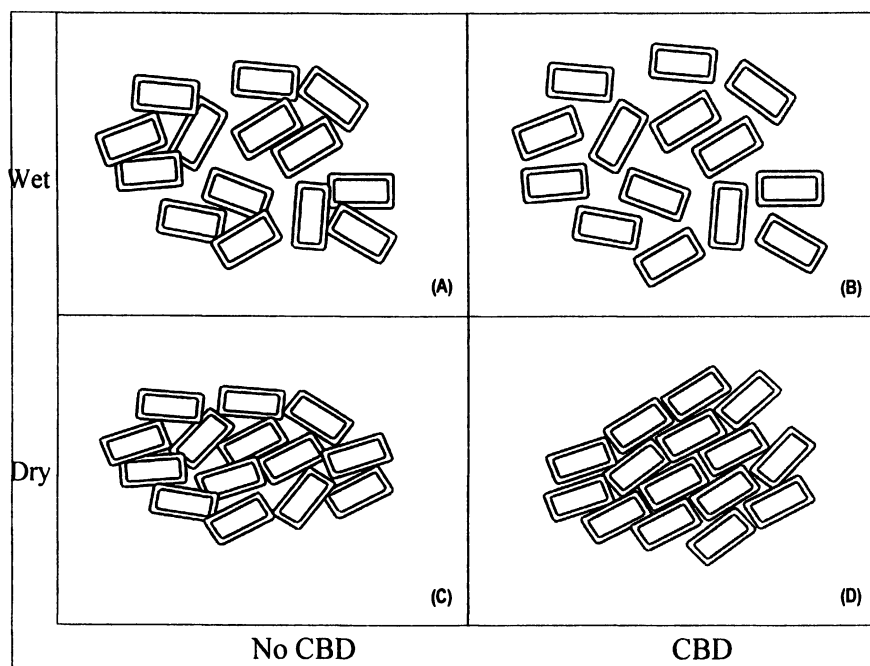


Figure 2. Interfacial fibre modification by enzyme adsorption

(A) Fibres in suspension, with no CBD. The fibres hydrophobic character causes aggregation. The presence of the aggregates affects sheet structure, due to the occurrence of preferential dewatering areas during sheet formation. The fibres arrangement in the paper sheet (C) does not follow an optimal orientation pattern, thus affecting the product final characteristics. (B) Fibres in suspension, with CBD. The stabilised fibres allow water to flow freely, leading to a better-formed sheet: the fibres orient themselves towards the same direction, increasing the surfaces contact for bonding (D).

Table II: Retention Volume of the Probes Used in the Inverse Gas Chromatography Experiments

<i>Probe</i>	<i>V_n (ml)</i>				
	<i>CFII</i>	<i>Control 2*</i>	<i>CBD 2*</i>	<i>Control 1*</i>	<i>CBD 1*</i>
Hexan - n	0.231±0.009	0.604±0.014	0.471±0.019	0.465±0.017	0.509±0.006
Heptan - n	0.76±0.012	1.745±0.015	1.439±0.015	1.309±0.007	1.438±0.007
Octan - n	2.061±0.025	5.030±0.021	4.123±0.028	3.737±0.016	4.127±0.038
Decan - n	16.277±0.025	42.652±0.105	35.205±0.119	31.391±0.045	34.070±0.706
Chloroform - a	0.453±0.022	1.343±0.016	1.051±0.025	0.974±0.012	0.986±0.019
THF - b	2.894±0.041	10.121±0.113	7.502±0.026	6.556±0.102	5.324±0.201
Diethyl eter - b	0.713±0.015	2.520±0.031	1.982±0.021	1.674±0.021	1.297±0.012
Acetone - p	3.635±0.068	11.836±0.297	7.800±0.178	7.253±0.190	4.255±0.166
Ethyl acetate - p	5.296±0.266	21.364±0.476	14.722±0.211	13.218±0.538	8.313±0.424

* Samples prepared according to method 1 or 2 (material and methods section);
n-neutral; a-acidic; b-basic; p-amphoteric

of the increase of surface polarity. A comprehensive thermodynamic characterisation of the fibres interface will provide a better understanding of the complex phenomena associated to the CBDs modification of cellulosic fibres.

Conclusions

Cellulose-binding domains seem to be a performant additive for paper recycling. They probably share some of the good enzyme properties: hydration and slight surface disruption of the fibres (in a way similar to refining), without its drawbacks: fibre solubilization, intrinsic fibre strength reduction. The hydration and stabilisation of the fibres may lead to better paper sheet formation, resulting in improved paper resistance. Enzymes may have a similar effect, in some cases, but hydrolysis of the fibres surface is, probably, mainly detrimental (7). Inverse Gas Chromatography confirms that CBDs modify the interfacial properties of a model cellulose. The major effect of CBDs adsorption on the surface properties of Whatman fibres is the reduction of the acidic character, with maintenance of the dispersive energy.

Acknowledgements

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References

1. Kilburn, D.G., Gilkes, N.R., Ong, E., Greenwood, J.M., Miller Jr., R.C. and Warren, R.A.J. *Biotechnology in Pulp and Paper Manufacture*. Kirk, K. and Chang, H.-M., Eds, **1990**, Chapter 55, 551-557.
2. Suurnäkki, A., Oksanen, T., Linder, M., Niku-Paavola, M.-L., Tenkanen, M., Siika-aho, M., Viikari, L. and Buchert, J. *7th Int. Conf. on Biotechnol. in the Pulp and Paper Ind.* **1998**, C107-C110.
3. Suurnäkki, A., Tenkanen, M., Siika-aho, M., Niku-Paavola, M.-L., Viikari, L. and Buchert, J. *Cellulose* **2000**, 7, 189-209.
4. Reinikainen, T., Takkinen, K. and Teeri, T.T. *Enzyme and Microbial Technol.* **1997**, 20, 143-149.
5. Din, N., Gilkes, N.R., Tekant, B., Miller Jr., R.C., Warren, R.A.J. and Kilburn, D.G. *Bio/Technol.* **1991**, 9: 1096-1099.
6. Xiao, Z., Gao, P. and Wang, T. *Biotechnol. Lett.* **2001**, 23, 711-715.

7. Pala, H., Lemos, M.A., Mota, M. and Gama, F.M. *Enzyme and Microbial Technol.* **2001**, *29*, 274-279.
8. Pala, H., Mota, M. and Gama, F.M. *Biocatalysis and Biotransformation* **2002**, *20*:5, 353-361
9. Lemos, M.A., Teixeira, J.A., Mota, M. and Gama, F.M. *Biotechnol. Lett.*, **2000**, *22*, 703-707.
10. Bradford, M.M. *Anal. Biochem.* **1976**, *72*, 248-254.
11. Bernfeld, P. *Methods in Enzymology*, Vol. 1. Collowick, S.P. and Kaplan, N.D., Eds, Academic Press, N.Y., **1995**, 149-152.
12. Panzer, U. and Schreiber, H.P. *Macromolecules*, **1992**, *25*, 3633-3637.
13. Belgacem, M.N., Blayo, A. and Gandini, A. *J. Colloid Interfacial Sci.*, **1986**, *182*, 431-436
14. Dourado, F., Mota, M., Pala, H. and Gama, F.M. *Cellulose* **1999**, *6*, 265-282.
15. Israelachvili, J. *Intermolecular and surface forces*. 2nd edition, Academic Press, **1992**, p.399
16. Bergström, L., Stemme, S., Dahlfors, T., Arwin, H. and Ödberg, L. *Cellulose* **1999**, *6*, 1-13.
17. Milichovsky, M. *Tappi J.* **1990**, *73* (10), 221-232.

Chapter 8

Modulation of Wood Fibers and Paper by Cellulose-Binding Domains

Oded Shoseyov¹, Ilan Levy¹, Ziv Shani², and Shawn D. Mansfield³

¹The Institute of Plant Sciences and Genetics in Agriculture, the Faculty of Agricultural, Food and Environmental Quality Sciences. The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100 Israel

²CBD–Technologies Ltd., Tamar Science Park, P.O. Box 199, Rehovot 76100, Israel

³CRC Chair in Wood and Fibre Quality, Department of Wood Science, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada

Recombinant cellulose-binding domains (CBD) have previously been shown to modulate the elongation of different plant cells *in vitro*. Using *Acetobacter xylinum* as a model system, CBD was found to increase the activity of the cellulose synthase, up to fivefold, in a dose-dependent manner. In *Populus*, the introduction of a *cbd* gene under the control of the elongation specific *cel1* promoter led to significant increases in biomass production in selected clones compared with wild-type plants. An analysis of the ensuing wood characteristics from the transgenic trees demonstrated significant increases in both fiber cell length and the average degree of cellulose polymerization. Additionally, a significant decrease in microfibril angle was observed. These results coincided with increased burst, tear and tensile indexes of paper prepared from these wood fibers. The mechanism by which CBD affects cell wall metabolism remains unknown. A physio-mechanical mechanism was postulated whereby CBD

localizes between adjacent cellulose microfibrils and separates them in a wedge-like action. *In vitro* experiments with petunia cell suspensions supports this hypothesis, as increasing concentrations of CBD displayed an abnormal shedding of cell wall layers, indicating that CBD has the potential to cause non-hydrolytic cell wall disruption activity in-vivo. Additionally, CBD fusion proteins may also be used to cross-link and introduce functional molecules into and onto lignocellulosic-based fibre networks. Consequently, these CBD fused molecules can be used to improve the physio-mechanical performance of paper sheets, and to alter its surface properties.

Early studies of CBD-cellulose interactions clearly suggest that the presence of CBD increases the effective concentration of enzyme on insoluble cellulosic substrates, and thereby assists the enzyme through the phase transfer from soluble fraction (the enzyme) to insoluble fraction (the substrate) (1-7). It was later reported that CBDs were present in both hydrolytic and non-hydrolytic proteins. In proteins that possess hydrolytic activity (cellulases, xylanases), the CBD has been shown to be a discrete domain that concentrates the catalytic domains on the surface of the insoluble substrate (5, 8-12). In contrast, the CBDs in proteins that do not exhibit hydrolytic activity compose part of a scaffolding subunit that organizes the catalytic subunits into a cohesive multi-enzyme complex known as a cellulosome. This enzymatic complex was found to function more efficiently in the degradation of cellulosic substrates (1, 13-17). Removal of the CBD from the cellulase molecule or from the scaffolding in cellulosomes has been shown to dramatically decrease enzymatic activity (18-23).

Today, more than 200 putative sequences, in over 40 different species, have been identified. The binding domains are classified into 16 different families based on amino acid sequence, binding specificity and structure (5, 8-11). CBDs can contain anywhere from 30 to 180 amino acids, and exist as a single, double or triple domain in one protein. Their location within the parental protein can be either from the C- or N-terminus, and occasionally centrally positioned in the polypeptide chain. The affinity and specificity towards different cellulose allomorphs can also vary significantly (for an extended review on CBDs, see 8, 9, 11, 16, 17, 24). The three-dimensional structure of representative members of different CBD families has been resolved by crystallography and NMR (25-31), and indicate that CBDs from different families are structurally similar and that their cellulose binding capacity can be attributed, at least in part, to several aromatic amino acids that compose their hydrophobic surface. CBDs from the same organism have also been shown to differ in their binding specificity (23)

and occasionally two CBDs located on the same enzyme, can also exhibit this distinction (32).

Biochemical studies have shown that CBD binding to cellulosic substrates is characterized in decrease entropy. For example, in the case of CBD_{Cex} from *C. fimi*, which binds to crystalline cellulose, the decrease in entropy can be attributed to a net loss in conformational freedom of the polysaccharide and protein side chains. Water hydration upon binding may be another factor leading to lower entropy (33). On the other hand, binding of CBD_{Ni} from *Cellulomonas fimi* to amorphous cellulose is characterized by decreased enthalpy. This phenomenon can be ascribed to heat release, which occurs upon complex formation that transpires through hydrogen and van der Waals bonding between the equatorial hydroxyl of the glucopyranosyl ring and the polar amino acids (32). Although the interaction of CBDs with their corresponding cellulosic substrates is occasionally irreversible, contact with the cellulose surface is dynamic. Using fluorescence recovery techniques Jervis *et al.* (34) demonstrated that CBD_{Cex} is mobile on the surface of crystalline cellulose when it appears in isolated form or as a module of a xylanase protein. Furthermore, it was hypothesized that the binding of family IIa CBDs from *C. fimi* to cellulose occurs either along or across the chain (35).

This review will describe the potential applications of CBDs in fiber modification, both *in vivo* and *in vitro*.

Wood Fiber Modification Using CBD

The gram-negative bacterium *Acetobacter xylinum* has long been regarded as a model of cellulose biosynthesis primarily because cellulose microfibril synthesis is set apart from cell wall formation (36). Consequently, cellulose is produced as separate ribbons composed of microfibrils and their interactions with other polysaccharides do not exist as in the plant cell wall. Since polymerization and crystallization of cellulose is a coupled process in *A. xylinum* cellulose biosynthesis, any interference during the crystallization phase results in accelerated polymerization (37). It has also been shown that some organic substances with affinity for cellulose can also alter cell growth and cellulose-microfibril assembly *in vivo*. For example, carboxymethylcellulose (CMC) and fluorescent brightening agents (calcofluor white ST) prevent microfibril crystallization, thereby enhancing polymerization. These molecules bind to the polysaccharide chains immediately following extrusion from the cell surface, thus preventing normal assembly of the microfibrils and cell walls (38). Shpigel *et al.* (39) have shown that like other organic cellulose-binding substances, family III CBD derived from *Clostridium cellulovorans* could modulate

cellulose biosynthesis. It was apparent that CBD increased the rate of cellulose synthesis activity in *A. xylinum* up to fivefold compared to a control. Electron microscopy of the cellulose synthesized in the presence of CBDs revealed that the newly formed fibrils are spread out into a splayed ribbon instead of the uniform, thin, packed ribbon as was evident in the control fibers. The mechanism by which CBD affects cell wall metabolism remains unknown. A physio-mechanical mechanism has been proposed whereby; CBDs adhere to, and slides between adjacent cellulose fibers and separate them in a wedge-like action (40). This hypothesis is supported by *in vitro* experiments that show that *Petunia* cell suspensions treated with increasing concentrations of CBD displayed abnormal shedding of cell wall layers, suggesting that CBDs can cause non-hydrolytic cell wall disruption *in vivo* (40).

Several protocols were tested to analyze the effect of CBDs on living plant cells. In these studies it was found that Family III CBDs from *C. cellulovorans* could modulate cell elongation. At low concentrations, this CBD enhanced elongation of *Prunus persica* L. pollen tubes and *A. thaliana* root seedlings, whereas at high concentrations, CBD inhibited root elongation in a dose-dependent manner. It was demonstrated that cellulose-xyloglucan networks, similar to plant cell walls, could be formed when employing the *A. xylinum* model system in a medium containing xyloglucan (41-44). NMR analysis indicated that 80 to 85% of the xyloglucan adopts a rigid conformation; in all probability aligned with the cellulose chain, whereas, the remainder is more mobile. The xyloglucan, when present during cellulose synthesis in the *A. xylinum* model system, causes the cellulose to become more amorphous and increases its tensile strength (45). When CBDs were present, it was shown that the CBDs could compete with xyloglucan for the binding of cellulose (39). These findings support the hypothesis that, at least part of the effect CBD exerts on the plant cell wall, is via cellulose-xyloglucan interactions.

Table I. Fibre properties of wild type and transgenic *Populus* trees

Clone	LWL ^a (mm)	Coarseness (mg/m)	Microfibril angle (degrees)	Crystallinity (CI)
WT	0.829 ± 0.005 ^b	0.914 ± 0.011 ^b	30.59 ± 0.517 ^c	39.1 ± 3.12
CBD1 ^d	0.920 ± 0.004 ^b	0.941 ± 0.014 ^b	28.48 ± 0.554 ^c	43.0 ± 3.12
CBD2 ^d	0.966 ± 0.006 ^b	0.962 ± 0.010 ^b	27.38 ± 0.397 ^c	45.3 ± 3.12

^aLength weighted fiber length.

^bNumber of fibers analyzed: 13500.

^cNumber of fibers analyzed: 100.

^dTwo different clones

Shoseyov *et al.* (46) have also shown that CBDs can also modulate plant growth in transgenic plants. The introduction of the Family III *cbd* gene from *C. cellulovorans* under the control of the elongation-specific *cel1* promoter (47-48) into *Populus* resulted in significant alteration in fiber properties. The transgenic wood fibers were significantly longer they displayed higher coarseness and smaller microfibril angles (Table I). An analysis of fiber crystallinity by FT-IR (Table I) also suggests that there was a trend of greater crystallinity within the modified fibers.

Additional changes were detected in the nature of the cellulose polymers themselves. Figure 1 demonstrates that the transgenic trees contain a larger degree of cellulose polymers ranging from 2000 to 6000 MW, while exhibiting a lower concentration of cellulose molecules ranging from 200 to 900 MW compared with wild type trees. Therefore, CBD expression in the transgenic trees resulted in a significant shift in the cellulose polymers to a higher molecular weight. Although many morphological and ultrastructural changes were detected in the transgenic fibers, an extensive comparison with the wild type (Table II) revealed that the chemical composition had not changed.

Table II. Chemical composition of wild type and transgenic *Populus* trees

Clone	Natural sugars (%)					Lignin (%)		Ash (%)	Ext. (%)	Total
	Gal	Ara	Glu	Xyl	Man	ISL	SL			
WT	0.8	0.6	43.6	19.8	2.5	17.3	4.1	0.6	10.1	99.4
CBD1	0.8	0.6	42.5	19.8	2.4	17.2	4.0	0.6	10.9	98.8
CBD2	0.8	0.6	43.3	20.4	2.4	17.1	3.9	0.6	10.0	99.1

NOTE: Gal- Galactos; Ara- Arabinose; Glu- Glucose; Xyl- Xylose; Man- Mannose
ISL- Acid Insoluble Lignin; SL- Acid Soluble Lignin

Pulp produced from the modified and wild type trees were used to make standard laboratory handsheets, and these sheets were tested for standard paper strength properties. It was apparent that no differences in the pulp yield were observed between the transgenic and wild type trees (data is not shown). The results (Figure 2) clearly show that paper made from fibers originating from the transgenic trees, have improved strength properties as measured by tensile, burst, and tear indices when compared with sheets produced from the wild type trees.

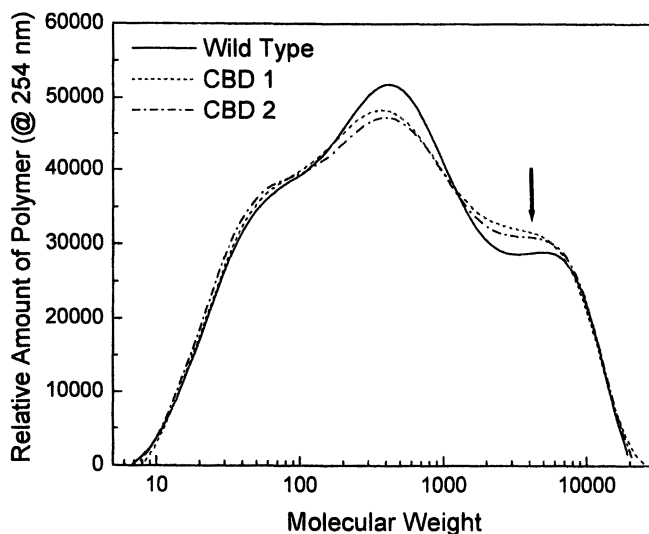


Figure 1. Molecular weight distribution of isolated cellulose polymers in wild type and transgenic trees expressing CBD.

Modulating Cellulose Containing Materials with CBD

Din *et al.* (49) reported that CBD_{CenA} from *C. fimi* endoglucanase A is capable of non-hydrolytic disruption of cellulosic fibers, which results in the release of small particles. In addition, it was shown that CBD_{CenA} could prevent the flocculation of microcrystalline bacterial cellulose (50). Similar phenomena were observed for other CBDs (40, 51-54), including CBD_{CloS} (Figure 3). However, this observation is not common to all CBDs (6). The first direct evidence for the involvement of CBDs in fiber surface alteration was reported by Lee *et al.* (55), who demonstrated using atomic force microscopy that the Cel7A (formally CBH I) generated distinct tracks along the longitudinal axis of cellulosic fibers, while treatment with EG II caused the peeling and smoothing of the fiber surfaces. When cellulases that lacked CBDs were subsequently used, no effect on the surface of the cotton fiber was detected. Additional information to support this observation came from the study carried out by Suurnäkki *et al.* (7). In this application the actions of endoglucanases, cellobiohydrolases and the catalytic domains from *T. reesei* on bleached chemical pulp were compared. According to

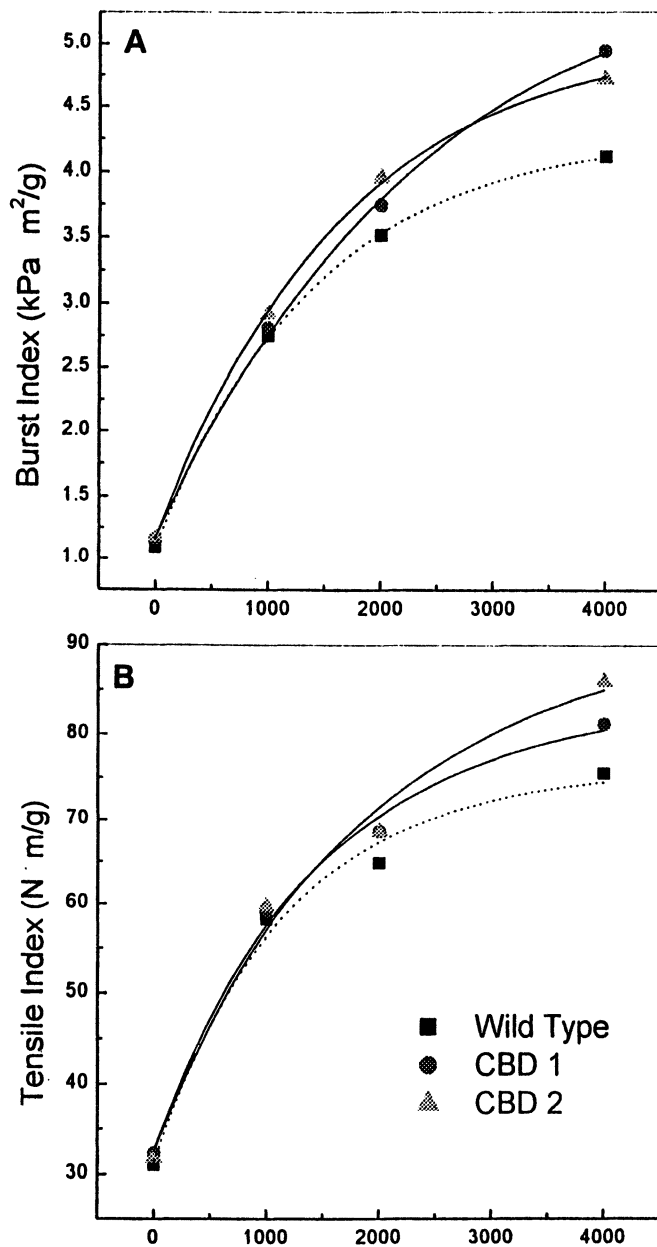


Figure 2. Mechanical properties of papers made of fibers from both wild type and transgenic plants expressing CBD (A) Burst Index, (B) Tensile Index and (C) Tear Index.

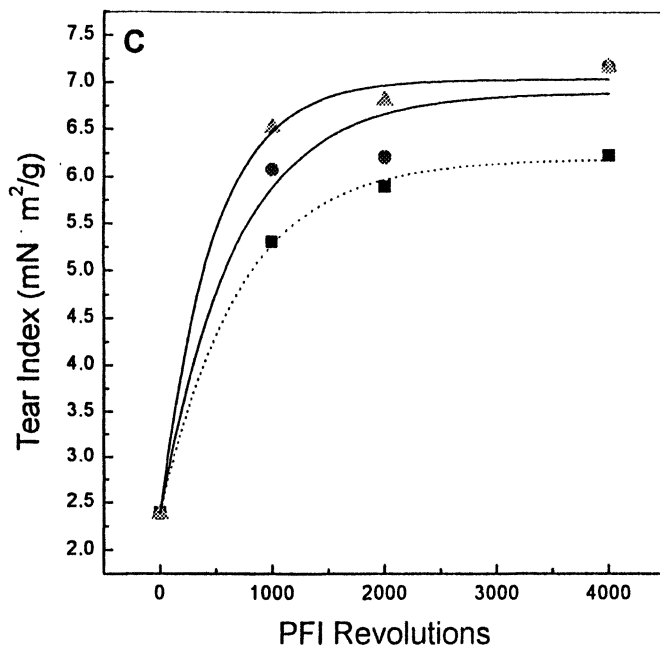


Figure 2. *Continued.*

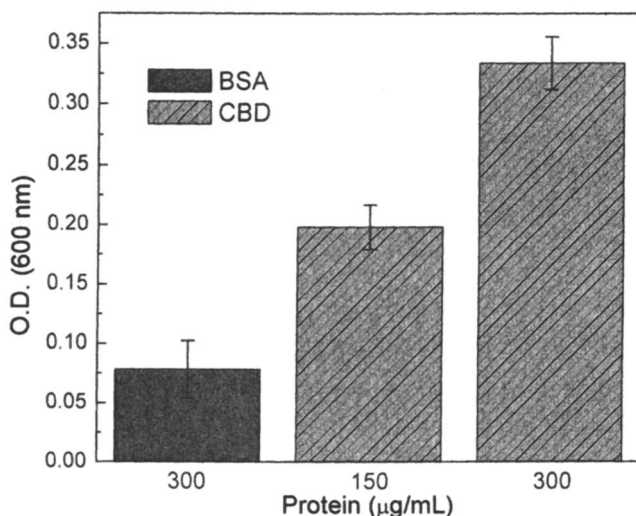


Figure 3: Non-hydrolytic fiber disruption of cellulose filter paper at different concentrations of cellulose-binding domain (CBD) originated from *Clostridium cellulovorans*.

this research, the presence of CBD in the intact enzyme had a beneficial effect on pulp properties such as viscosity and strength after PFI refining.

The tensile strength of paper is imparted by both the inherent intrinsic strength of the fibres, as well as by the amount and strength of the fiber-to-fiber bonds (56). Intra-fiber interaction has been shown to improve stress transfer between the adjoining fibers and is considered to be one of the most important factors affecting overall stress development in the fiber network under tensile deformation (57, 58). Earlier studies have shown that the low strength of dry-formed structures can be improved by adding binder materials or bicomponent fibers (59). Recently, we demonstrated that CBDs could modify paper properties. Two CBDs belonging to family III (from *C. cellulovorans*) that had been fused together to form a cellulose cross-linking protein (CCP) were applied onto filter paper. These treatments significantly improved the tensile strength (Figure 4A and B) (60). A potential explanation to the effect of CBD and CCP is that these molecules may change the interfacial properties of the fibers and thereby alter the fiber-to-fiber interaction. Additionally, applying CBDs to cellulosic fibers has the potential to improve paper recycling. It has been demonstrated that the application of CBD on secondary fibers, such as old paperboard containers, results in increased tensile and burst indexes as well as improvement in pulp drainage (61).

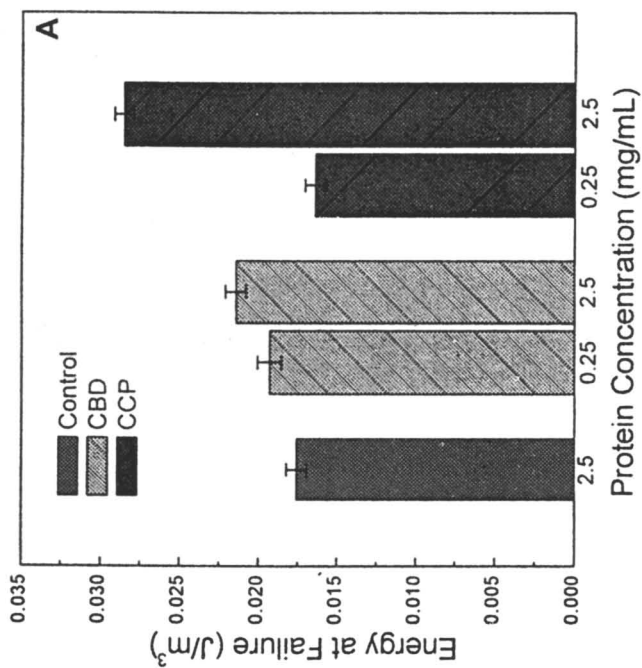
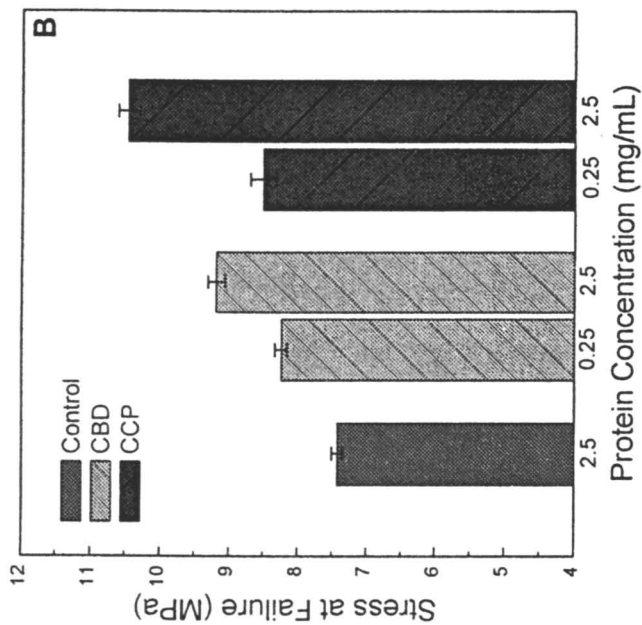
Although CCP application has been shown to improve mechanical properties of paper sheets (Figure 4C and D), further investigation on the effect(s) of CCP demonstrated that CCP did not interfere with the effect of cationic starch on the dry strength of the paper but rather indicated a synergistic effect (unpublished data). Recently, Kitaoka and Tanaka (62) reported on the production of a novel papermaking reagent by covalently binding activated anionic polyacrylamide (A-PAM) to CBD originating from *Trichoderma viride* 1,4- β -glucanase (CBD-A-PAM). In this manner they were able to produce a molecule, which contained more than one CBD that is capable of cellulose fiber cross-linking. It was subsequently shown that the dry and wet tensile strength of the ensuing paper to which the CBD-A-PAM was added internally increased. We propose that the increase in stress to failure caused by CBD and CCP is related to the nature of the CBD's binding site, which is a large hydrophobic planar surface with several attachment sites (3, 25, 26, 63, 64). CCP is an efficient cross-linker due to its larger size and to the number of attachment sites it contains that enable it to cross-link cellulosic materials. Applying a single CBD molecule to the paper also improved its mechanical properties, but to a lesser extent than CCP.

The CCP construct can also be employed as a sizing agent. The application of CCP to fibre networks using different methods (imbibing, spraying or blade coating) results in a hydrophobic paper surface (Figure 5). It is hypothesized that at relatively high CCP concentrations most of the binding sites on the cellulose are occupied by a single CBD moiety, and therefore, the second CBD moiety (the non-bound moiety) of CCP exposes its hydrophobic amino acids and consequently increases the surface hydrophobicity (40, 60, 65).

Another study demonstrated that polysaccharide structure modification could be achieved using isolated CBDs. It has been suggested that the surface of cellulosic polysaccharide (ramie cotton fibers) was roughened after treatment with CBD (CBD_{CenA} from *C. fimi*). It was proposed that these treatments could be used in order to alter dyeing characteristics of cellulose fibers (66). Cavaco-Paulo *et al.* (67) demonstrated the effect of CBD on the dye affinity to cotton fibers, and showed increased levels of dye affinity following treatments with family II CBD from *C. fimi*.

Conclusions

Clearly there is strong indications that cell wall modification, mediated by CBD, could provide an effective mean for fiber improvement *in vivo* and the consequent improved fiber properties can significantly enhance the quality of ensuing paper products. In addition, the binding of cellulose-binding domains to cellulosic polymers, under a wide range of environmental conditions, without the



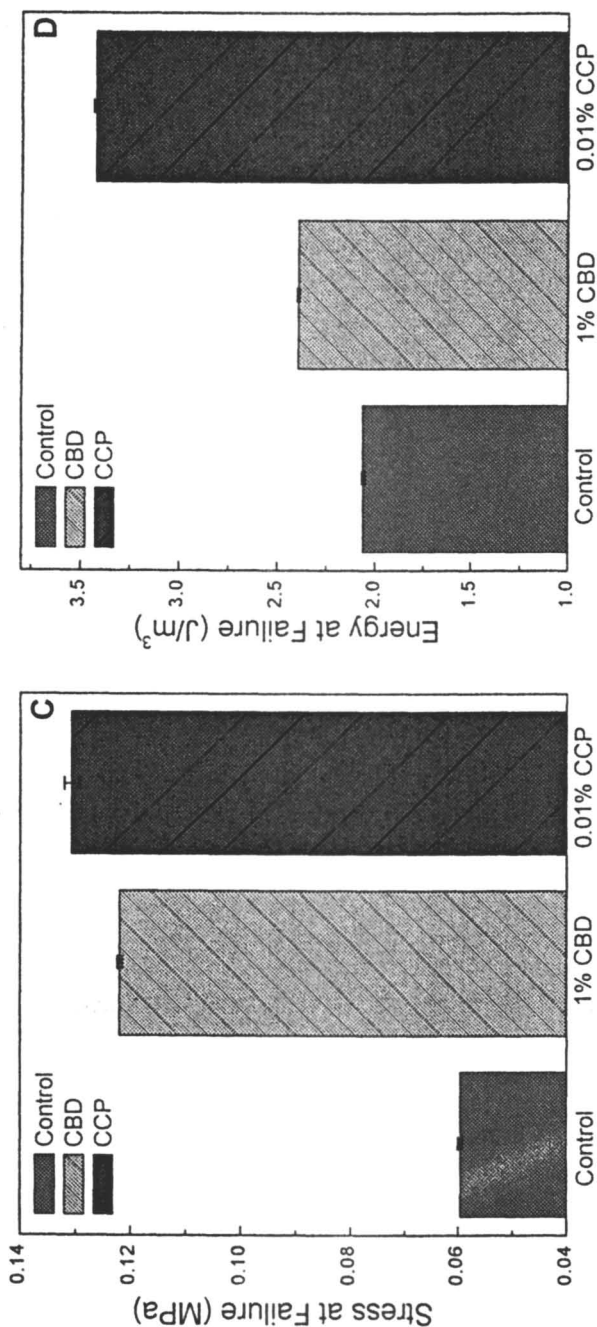


Figure 4. The effect of protein treatment on the mechanical properties of papers treated with cellulose-binding domain (CBD) or cellulose cross-linking protein (CCP). Stress at failure (A) and energy adsorption (B) of Whatman filter paper No. 1 coated with CBD or CCP, and papers hand sheets prepared with CBD or CCP in the furnish (C and D). Protein concentration relates to the coating solution (in A and B), or to the furnish (in C and D) as percent protein relative to fibers (w/w).

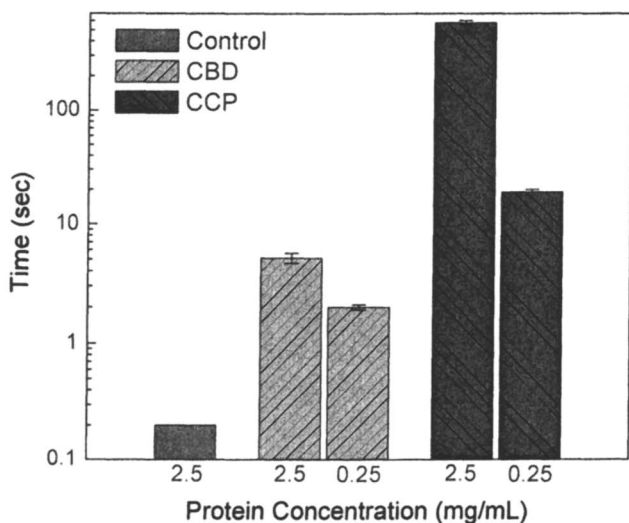


Figure 5. Water-absorption time of papers treated with cellulose-binding domain (CBD) or cellulose cross-linking protein (CCP) at different concentrations. Whatman filter paper No. 1 was immersed in protein solution and then left to dry at room temperature. Protein concentration relates to the amount of protein in the coating solution.

need for chemical reactions, makes them attractive moieties for the design of a new class of paper modifying agents that are environmentally friendly.

References

1. Shoseyov, O.; Doi, R. H. *Proc. Nat. Acad. Sci. USA* **1990**, *87*, 2192-2195.
2. Beguin, P.; Aubert, J. P. *FEMS Microbiol. Rev.* **1994**, *13*, 25-58.
3. Din, N.; Forsythe, I. J.; Burtnick, L. D.; Gilkes, N. R.; Miller, R. C. Jr.; Kilburn, D. G. *Mol. Microbiol.* **1994**, *11*, 747-755.
4. Linder, M.; Lindeberg, G.; Reinikainen, T.; Teeri, T. T.; Pettersson, G. *FEBS Lett.* **1995**, *372*, 96-98.
5. Tomme, P.; Warren, R. A.; Gilkes, N. R. *Adv. Microb. Physiol.* **1995**, *37*, 1-81.

6. Bolam, D. N.; Ciruela, A.; McQueen-Mason, S.; Simpson, P.; Williamson, M. P.; Rixon, J. E.; Boraston, A.; Hazlewood, G. P.; Gilbert, H. J. *Biochem. J.* **1998**, *331*, 775-81.
7. Suurnakki, A.; Tenkanen, M.; Siika-aho, M.; Niku-Paavola, M. L.; Viikari, L.; Buchert, J. *Cellulose* **2000**, *7*, 189-209.
8. Gilkes, N. R.; Henrissat, B.; Kilburn, D. G.; Miller, R. J.; Warren, R. A. J. *Microbiol. Rev.* **1991**, *55*, 303-315.
9. Tomme, P.; Driver, D. P.; Amandoron, E. A.; Miller, R. C. Jr.; Warren, R. A. J.; Kilburn, D. G. *J. Bacteriol.* **1995**, *177*, 4356-4363.
10. Linder, M.; Margolles-Clark, E.; Reinikainen, T.; Teeri, T. T. *J. Biotechnol.* **1997**, *57*, 49-57.
11. Tomme, P.; Boraston, A.; McLean, B.; Kormos, J.; Creagh, A. L.; Sturch, K.; Gilkes, N. R.; Haynes, C. A.; Warren, R. A. J.; Kilburn, D. G. *J. Chromatogr. B. Biomed. Sci. Appl.* **1998**, *715*, 283-296.
12. Teeri, T. T.; Koivula, A.; Linder, M.; Wohlfahrt, G.; Divne, C.; Jones, T. A. *Biochem. Soc. Trans.* **1998**, *26*, 173-178.
13. Woodward, J.; Hayes, M. K.; Lee, N. E. *Bio/Technology* **1988**, *6*, 301-304.
14. Doi, R. H.; Goldstein, M.; Hashida, S.; Park, J. S.; Takagi, M. *Crit. Rev. Microbiol.* **1994**, *20*, 87-93.
15. Beguin, P.; Alzari, P. M. *Biochem. Soc. Trans.* **1998**, *26*, 178-185.
16. Bayer, E. A.; Shimon, L. J.; Shoham, Y.; Lamed, R. *J. Struct. Biol.* **1998**, *124*, 221-234.
17. Bayer, E. A.; Chanzy, H.; Lamed, R.; Shoham, Y. *Curr. Opin. Struct. Biol.* **1998**, *8*, 548-557.
18. Van Tilbeurgh, H.; Tomme, P.; Claeysens, M.; Bhikhabhai, R. *FEBS Lett.* **1986**, *204*, 223-227.
19. Tomme, P.; Van Tilbeurgh, H.; Pettersson, G.; Van Damme, J.; Vandekerckhove, J.; Knowles, J.; Teeri, T. T.; Claeysens, M. *Eur. J. Biochem.* **1988**, *170*, 575-581.
20. Hefford, M. A.; Laderoute, K.; Willick, G. E.; Yaguchi, M.; Seligy, V. L. *Protein Eng.* **1992**, *5*, 433-439.
21. Goldstein, M. A.; Takagi, M.; Hashida, S.; Shoseyov, O.; Doi, R. H.; Segel, I. H. *J. Bacteriol.* **1993**, *175*, 5762-5768.
22. Coutinho, J. B.; Gilkes, N. R.; Kilburn, D. G.; Warren, R. A. J.; Miller, R. C. *FEMS Microbiol. Lett.* **1993**, *113*, 211-218.
23. Carrard, G.; Linder, M. *Eur. J. Biochem.* **1999**, *262*, 637-643.
24. Henrissat, B. *Cellulose* **1994**, *1*, 169-196.
25. Xu, G. Y.; Ong, E.; Gilkes, N. R.; Kilburn, D. G.; Muhandiram, D. R.; Harris-Brandts, M.; Carver, J. P.; Kay, L. E.; Harvey, T. S. *Biochemistry* **1995**, *34*, 6993-7009.

26. Tormo, J.; Lamed, R.; Chirino, A. J.; Morag, E.; Bayer, E. A.; Shoham, Y.; Steitz, T. A. *EMBO J.* **1996**, *15*, 5739-5751.
27. Johnson, P. E.; Joshi, M. D.; Tomme, P.; Kilburn, D. G.; McIntosh, L. P. *Biochemistry* **1996**, *35*, 14381-14394.
28. Brun, E.; Moriaud, F.; Gans, P.; Blackledge, M. J.; Barras, F.; Marion, D. *Biochemistry* **1997**, *36*, 16074-16086.
29. Sakon, J.; Irwin, D.; Wilson, D. B.; Karplus, P. A. *Nat. Struct. Biol.* **1997**, *4*, 810-818.
30. Mattinen, M. L.; Linder, M.; Drakenberg, T.; Annala, A. *Eur. J. Biochem.* **1998**, *256*, 279-286.
31. Notenboom, V.; Boraston, A. B.; Kilburn, D. G.; Rose, D. R. *Biochemistry* **2001**, *40*, 6248-6256.
32. Brun, E.; Johnson, P. E.; Creagh, A. L.; Tomme, P.; Webster, P.; Haynes, C. A.; McIntosh, L. P. *Biochemistry* **2000**, *39*, 2445-2458.
33. Creagh, A. L.; Ong, E.; Jervis, E.; Kilburn, D. G.; Haynes, C. A. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12229-12234.
34. Jervis, E. J.; Haynes, C. A.; Kilburn, D. G. *J. Biol. Chem.* **1997**, *272*, 24016-24023.
35. McLean, B. W.; Bray, M. R.; Boraston, A. B.; Gilkes, N. R.; Haynes, C. A.; Kilburn, D. G. *Protein Eng.* **2000**, *13*, 801-809.
36. Ross, P.; Mayer, R.; Benziman, M. *Microbiol. Rev.* **1991**, *55*, 35-58.
37. Benziman, M.; Haigler, C. H.; Brown, R. M. J.; White, A. R.; Cooper, K. M. *Proc Natl. Acad. Sci. USA* **1980**, *77*, 6678-6682.
38. Haigler, C. H. In: *Biosynthesis and biodegradation of cellulose*; Haigler, C. H.; Weimer, P. J., Ed. Marcel Dekker Inc: New York, 1991; pp 99-124.
39. Shpigel, E.; Roiz, L.; Goren, R.; Shoseyov, O. *Plant Physiol.* **1998**, *117*, 1185-1194.
40. Levy, I.; Shani, Z.; Shoseyov, O. *Biomol. Eng.* **2002**, *19*, 17-30.
41. Atalla, R. H.; Hackney, J. M.; Uhlin, I.; Thompson, N. S. *Int. J. Biol. Macromol.* **1993**, *15*, 109-112.
42. Hayashi, T.; Ohsumi, C. *Plant Cell Physiol.* **1994**, *35*, 419-424.
43. Hackney, J. M.; Atalla, R. H.; VanderHart, D. L. *Int. J. Biol. Macromol.* **1994**, *16*, 215-218.
44. Whitney, S. E. C.; Brigham, J. E.; Darke, A. H.; Reid, J. S. G.; Gidley, M. J. *Plant J.* **1995**, *8*, 491-504.
45. Brett, C. T. *Int. Rev. Cytol.* **2000**, *199*, 161-199.
46. Shoseyov, O.; Shani, Z.; Shpigel, E. US patent 6,184,440, 2001.
47. Shani, Z.; Dekel, M.; Tsabary, G.; Shoseyov, O. *Plant Mol. Biol.* **1997**, *34*, 837-842.
48. Shani, Z.; Dekel, M.; Sig Jensen, C.; Tzfira, T.; Goren, R.; Altman, A.; Shoseyov, O. *J. Plant Physiol.* **2000**, *156*, 118-20.

49. Din, N.; Gilkes, R. N.; Tekant, B.; Miler, R. C. Jr.; Warren, R. A. J.; Kilburn, D. G. *Bio/Technology* **1991**, *9*, 1096-1099.
50. Gilkes, N. R.; Kilburn, D. G.; Miller, R. C. Jr.; Warren, R. A.; Sugiyama, J.; Chanzy, H.; Henrissat, B. *Int. J. Biol. Macromol.* **1993**, *15*, 347-351.
51. Krull, L. H.; Dintzis, F. R.; Griffin, H. L.; Baker, F. L. *Biotech. Bioeng.* **1988**, *31*, 321-327.
52. Boraston, A.; Bray, M.; Burn, E.; Creagh, A. L.; Gilkes, N.; Guarna, M.; Jervis, E.; Johnson, P.; Kormos, J.; McIntosh, L.; McLean, B.; Sandercock, L.; Tomme, P.; Haynes, C.; Warren, A.; Kilburn, D. G. In *Carbohydrate from Trichoderma reesei and other microorganisms*. Claeyssen, M.; Nerinx, W.; Piens, K., Ed. The Royal Society of Chemistry: Cambridge, UK, 1998; pp 139-146.
53. Banka, R. R.; Mishra, S.; Ghose, T. K. *World J. Microb. Biotech.* **1998**, *14*, 551-558.
54. Gao, P. J.; Chen, G. J.; Wang, T. H.; Zhang, Y. S.; Liu, J. *Acta Biochimica et Biophysica Sinica* **2001**, *33*, 13-18.
55. Lee, I.; Evans, B. R.; Woodward, J. *Ultramicroscopy* **2000**, *82*, 213-221.
56. Roberts, J. C. *The Chemistry of Paper*; The Royal Society of Chemistry: Cambridge, UK, 1996.
57. Askling, C.; Wagberg, L.; Rigdahl, M. *J. Mater. Sci.* **1998**, *33*, 1997-2003.
58. Gassan, J.; Bledzki, A. K. *J. Appl. Polym. Sci.* **1999**, *71*, 623-629.
59. Villalobos, J. A. *Tappi J.* **1981**, *64*, 129.
60. Levy, I.; Nussinovitch, A.; Shpigel, E.; Shoseyov, O. *Cellulose* **2002**, *9*, 91-98.
61. Pala, H.; Lemos, M. A.; Mota, M.; Gama, F. M.; *Enzyme Microb. Tech.* **2001**, *29*, 274-279.
62. Kitaoka, T.; Tanaka, H. *J. Wood Sci.* **2001**, *47*, 322-324.
63. Gilkes, N. R.; Jervis, E.; Henrissat, B.; Tekant, B.; Miller, R. C. Jr.; Warren, R. A.; Kilburn, D. G. *J. Biol. Chem.* **1992**, *267*, 6743-6749.
64. White, A.; Withers, S. G.; Gilkes, N. R.; Rose, D. R. *Biochemistry* **1994**, *33*, 12546-12552.
65. Levy, I.; Shoseyov, O. *Biotech. Adva.* **2002**, *In press*.
66. Gilkes, N. R.; Kilburn, D. G.; Miller, R. C. Jr.; Warren, A. US patent 5,821,358, 1998.
67. Cavaco-Paulo, A.; Morgado, J.; Andraus, J.; Kilburn, D. G. *Enzyme microb. Tech.* **1999**, *25*, 639-643.

Chapter 9

Cellulose-Binding Domains: Tools for Innovation in Cellulosic Fiber Production and Modification

**Michaël Quentin, Henry van der Valk, Jan van Dam,
and Ed de Jong**

**Department of Fibre and Paper Technology, ATO BV, P.O. Box 17,
6700 AA Wageningen, The Netherlands**

Plant cell walls are composed of cellulose, nature's most abundant macromolecule, and therefore represent a renewable resource of special technical importance. Cellulose degrading enzymes involved in plant cell wall loosening (expansins), or produced by plant pathogenic microorganisms (cellulases), share similarities favouring the degradation of this highly crystalline substrate. Most of the cellulases and cell wall loosening expansins share a multi-domain structure, which includes a cellulose-binding domain (CBD). CBDs possess the intrinsic ability to strongly and specifically bind to cellulose. CBDs may be applied to engineer hybrid enzymes able to bind to cellulose on one end, and to display enzymatic or chemical reactivity on the other, providing innovative solutions to modify cellulosic surfaces or to immobilise biocatalysts on it. In transgenic plants, CBDs influence polysaccharide synthesis and their assembly in the cell wall. Therefore, CBDs represent biotechnological tools to modify cellulosic fibres either during their growth or during post harvest processing.

Introduction

Plant cell walls serve multiple purposes. Besides their primary function in preventing protoplast rupture, they define cell size and shape and they are instrumental in controlling rate and direction of cell growth. In addition, the wall provides a physical barrier against biotic and abiotic stresses. It should be born in mind that the wall is part of the plant metabolism and may be involved in signal transduction as well. Plant cell walls contain a number of polymeric compounds that together can bear the inner stress of the cell, or turgor pressure. Actually, the rigidity of most plant cells and tissues depends on the tensile strength of the wall answering the turgor pressure. The main stress-bearing compound of the wall is the crystalline cellulose microfibril (CMF) that consist of at least 36 β -1,4-D-glucose polymer chains. Though the CMFs constitute the major part of the wall, a number of other polysaccharides occur, such as xyloglucans and pectin. Other regular components of the wall are structural proteins that add to wall strength and enzymes that are involved in wall modification. The cell walls, mainly polysaccharides, represent a huge resource for carbohydrates; they constitute a major part of the world's biomass and provide a rich source of food and energy. The mechanical properties of the composite wall and its diversity have allowed a variety of technical applications. One of the main applications that will be discussed here is the use of plant fibres as raw material for textile and paper industries. To improve the quality of both raw material and the resulting final products, targeted manipulation of the wall could be of great importance. The applications in textile and paper industries fully depend on the quality of the plant fibres. Their quality in turn depends on the amount and crystallinity of the cellulose together with their interconnecting compounds, mainly hemicellulose and lignin.

Chemical and physical properties of fibres, and their further technical applications vary depending on both their species and tissue origin (*1*). Sources of plant fibres used in textile industries are derived from the epidermal hairs of seeds and fruits, *i.e.* cotton and kapok, which are almost entirely composed of cellulose. Other sources of fibres for textiles are sclerenchyma cells of various origins. They include the phloem fibres of primary and secondary phloem, the peri-vascular and cortex fibres, such as those from flax, hemp, ramie, jute and kenaf. Those bast fibres are bundles of elongated cells with thick cell walls, rich in highly crystalline cellulose. They derive their special properties, great strength, elasticity and indigestibility from their generally lignified secondary wall. The conducting fibres of gymnosperms are solely tracheids and the wood is known as softwood. The wood of angiosperms is called hardwood: it usually contains xylem vessels as well as tracheids and non-conducting sclerenchyma. Wood fibres are the main source for paper production; they are highly lignified

and inflexible and therefore need extensive processing. Material derived from parenchyma with non-thickened cell walls includes the paper made from pitch of papyrus and raffia.

Cell wall modification may occur already *in planta* using genetic engineering (2,3). Fibre modification may occur also as part of the technological processes that use specifically designed enzymes. Cellulose-binding domains (CBDs) are discrete domains present in many wall-degrading enzymes. Their strong and specific binding to cellulose makes them the ideal tool to target fibre cellulose, not only *in-planta*, but also in the biotechnological design of cell wall modifying enzymes. The role and function of microbial CBDs have been described in detail and their mode of action in both natural and heterologous enzymes is well understood (4,5). Recently, a bacterial CBD isolated from *Cellulomonas fimi* has been shown to modify cell wall structure *in planta* (6-8). Plants expressing a CBD of different, fungal, origin have become available as well. Following, we will present a general description of wall composition and architecture and consider first the use of individual CBDs of different origin to directionally modify the wall *in planta* and, secondly their use in targeting any specific enzyme or otherwise functional group to cellulose fibres during industrial processing.

Plant cell-wall composition and architecture

Higher plant cell walls constitute a contiguous extra-cellular matrix throughout the plant body. Though the wall is persistent once deposited, during cell growth it is yet highly dynamic. This wall is called the primary wall and consists predominantly of crystalline cellulose microfibrils (CMFs) that are embedded in a matrix of hemicellulose, pectin and a variety of proteins. The proteins are both structural proteins and enzymes that provide the highly dynamic character to the primary wall. The wall deposited after cessation of growth is called the secondary wall, which has approximately the same constituents as the primary wall however at different quantities. Callose, a non-linear β -1,3-D-glucose polymer may be transiently present in many cell walls (9). Variations in type, proportion and distribution of the various components relate to size, shape, strength and function of the various plant tissues (10). Moreover, at cell differentiation the wall may become incrustated or covered with a number of other -non-glucan- substances with peculiar properties needed for specialised cell function. Examples are the waxes and cutin of the epidermis, suberin in cork and endodermis, and lignin in xylem and sclerenchyma fibres.

From the non-glucan substances only lignin occurs in sclerenchyma fibres, the specialised stress bearing cells that are of interest here.

Because comprehension of wall composition and architecture is a prerequisite to manipulate and next to improve fibre quality, processing and utilisation, we will briefly review those wall components, *i.e.* cellulose, hemicellulose, structural proteins, enzymes and lignin that occur in sclerenchyma fibres of dicotyledons.

2.1 Cellulose

The major stress-bearing constituent of plant cell walls is cellulose, a normally homogenous and linear chain of β -1,4-D-glucose residues. In crystalline form, each glucose unit in the polymer chain is rotated 180° relative to its neighbours, so that in fact the basic repeating unit is cellobiose (Figure 1). Cellulose is synthesised at the surface of the plasma membrane by a complex of 6 cellulose synthases, seen as 'terminal complexes' or 'rosettes' in freeze-fractured cell membrane surfaces. At least 36 individual cellulose chains form crystalline CMF by hydrogen bonding between the free hydroxyl functions and oxygen residues of neighbouring cellulose chains (11,12). In addition, the CMF are interrupted, and covered all over, by less crystalline cellulose (12,13) (Figure 1), which may confer them a relatively heterogeneous structure. Besides wall thickness, the spatial organisation of the CMF in the wall, or wall texture, will determine its mechanical properties (14-16). Wall texture depends on the pattern of CMF deposition, but its control is far from understood. At least in primary cell walls, CMF orientation relates to cell growth and cell polarity as the majority of the CMF are deposited transverse to the axis of elongation. The deposition of the CMF has been discussed to be controlled by the cortical cytoskeleton (17), but models presuming some sort of self-organisation have been proposed as well (16).

2.2 Hemicellulose

Hemicelluloses are a diverse group of polysaccharides in the plant cell wall that contain xylose as a main component and whose function is to cross-link CMFs. Thus hemicelluloses provide rigidity to the CMF and strength to the wall. In addition, they space CMF and thus add to control of wall porosity and the deposition of new material during wall assembly. Modification of wall plasticity,

required for cell elongation, involves remodelling of the complex cellulose-hemicellulose network (18). In the primary wall of dicotyledons and non-graminaceous monocotyledons, the most common hemicellulose is xyloglucan (XG). In angiosperms (hardwoods), the major component is xylan, whereas in gymnosperms (softwoods) and grasses, glucuronoarabino-xylans and mannans are the most abundant types.

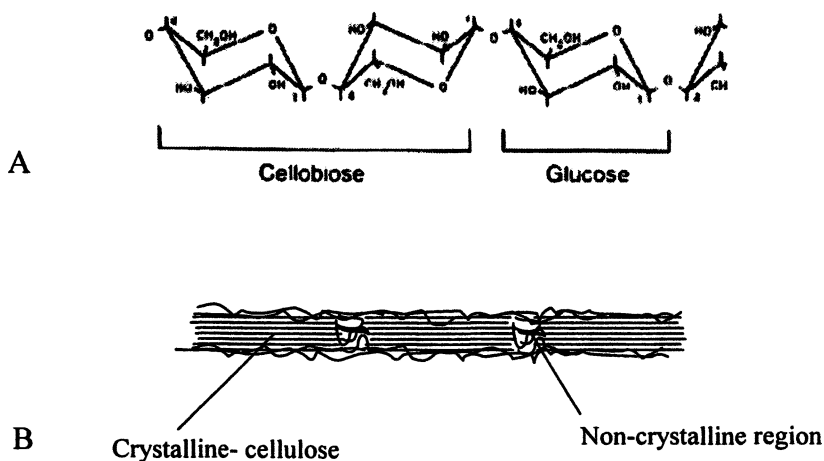


Figure 1. Structure of cellulose. (A) β -glucosidic bonds. (B) Schematic diagrams of a longitudinal section through cellulose microfibrils illustrating the crystalline (micellar) and non-crystalline regions.

The XGs are linear chains of β -1,4-D-glucose (Figure 2), but unlike the unbranched cellulose, 75% of the glucosyl residues are substituted at O-6 position by α -D-xylose. Additional sugars, β -D-galactose and α -L-arabinose are added at the O-2 position of some xylosyl units. α -L-fucose can be added at the O-2 of a subtending galactosyl unit, providing a trisaccharide side chain (19-21). Xylans are composed of β -1,4-D-xylosyl chains, which are substituted with glucuronic acid residues or acetyl residues at the O-2 position, or with arabinose or arabinofuranose residues at the O-3 position (Figure 2). The frequency and composition of the branches are dependent on the source of the xylan (22).

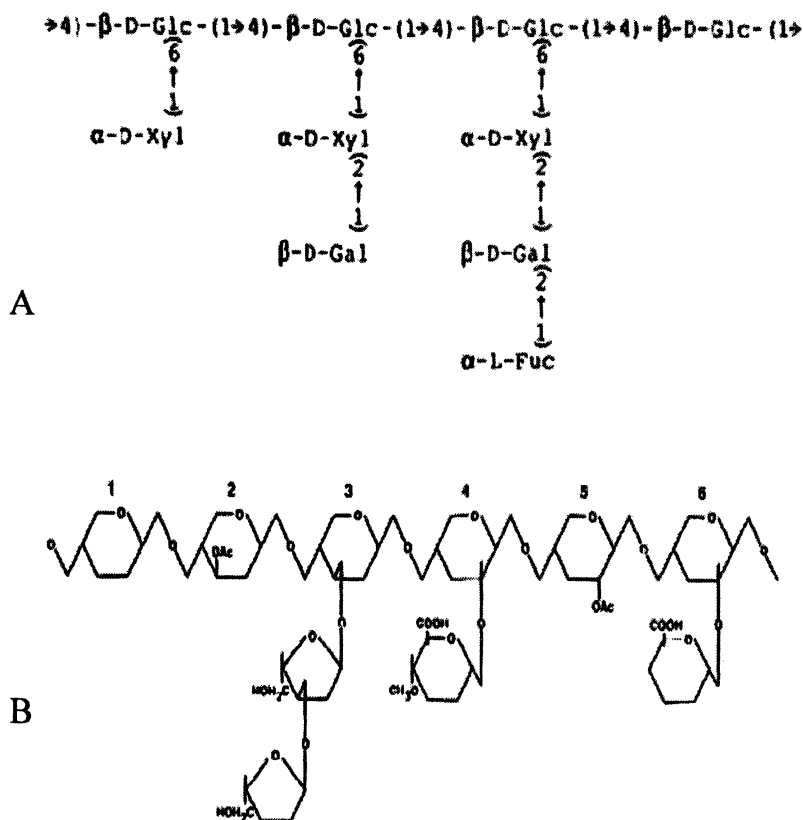


Figure 2. Structure of principle hemicelluloses. (A) Average structural repeat of cell wall xyloglucan. (B) Part of a hypothetical xylan molecule. 1, xylose; 2, acetyl xylose; 3, α -L-arabinofuranose side chain; 4, 4-O-methyl-D-glucuronic acid side chain; 5, acetyl xylose; 6, D-glucuronic acid.

Hemicelluloses do not mutually interact. The XGs coat cellulose after its deposition and cross-link two CMF by hydrogen bonds between the glucan backbone of the XG and the cellulose chain. The number of bonds between CMF and XG, and thus wall strength depends to the degree of substitution of the xyloglucan backbone, and on the nature of the side chain. The presence of fucosylated trisaccharide side chains confers a flat conformation of the XGs

required for its association with cellulose (23-25). The binding involves at least 4 hydrogen bonds, with O-O distances between 2.6 and 3.0 Å (25).

The linear xylans associate to cellulose by hydrogen bonds also, but unlike for XGs, the strength of the binding, i.e. the number of hydrogen bonds, decreases proportionally with substitution.

Less abundant non-cellulose polysaccharides, such as glucomannans, galactoglucomannans, and galactomannans, supposedly cross-link the CMF in some primary gymnosperm walls. In some algae, pure mannans can hydrogen bond into paracrystalline arrays, which are similar in structure to cellulose.

2.3 Pectin

In the primary wall, the cellulose/hemicellulose network is embedded in a pectic matrix (19). The pectic polymers contain linear homogalacturonan blocks or 'smooth regions', and in the same molecule, branched blocks of rhamnogalacturonans or 'hairy region' with neutral sugar side-chains (26). Homogalacturonans are homopolymers of α -1,4-D-galactosyluronic acids. Two types of 'hairy regions' occur, rhamnogalacturonan I and II. In rhamnogalacturonans I, the homogalacturonan chain is interrupted by the presence of α -1,2-L-rhamnosyl residues, which may be glycosylated at the O-4 position. Glycosyl chains are rich in arabinosyl and galactosyl residues. Rhamnogalacturonan II show by far the richest diversity in sugars and linkages of all known polysaccharides.

Pectin is synthesised as methylesters, which once deposited in the cell wall are modified by the enzyme pectin methylesterase. De-esterified pectin components can cross-link with Ca^{2+} . Those structures called 'egg-box' give rise to a gel-like structure participating to the cell wall cohesion (27,28). In addition to Ca^{2+} bridges, ferulic acids, which are esterified to arabinose and galactose, can participate in the cross-linking of pectins (19,27). Pectin is a main component of the middle lamellae, it adheres cells together and in the primary wall it determines cell porosity.

2.4 Lignin

In differentiated cells, walls are impregnated with the aromatic polymer lignin. Lignins are polymers composed of phenylpropanoid alcohol units, which are derived from coniferyl-, sinapyl- and p-coumaryl-alcohol, depending on the

plant source. Linking occurs by a variety of chemical bonds, including ester and ether bonds and carbon-carbon-linkages. The result is a three dimensional network of highly hydrophobic polymers. In addition, lignin may also be covalently linked to hemicellulose and cellulose (29,30). Thus lignins provide an extreme rigidity to cell walls that are capable to resist mechanical stress, like in tracheids and hardwood fibres. Lignin is highly disadvantageous for processing and use of fibres as raw material for textiles and wood-free paper.

2.5 Proteins

Protein constituents of the cell walls are structural proteins and enzymes (31). Extensine, a hydroxyprolin-rich glycoprotein is the main structural protein in the cell wall and adds to wall rigidity and strength by interlocking the CMF. Side-chains from pectin or other polysaccharide containing 1 to 4 arabinofuranosyl residues and single galactopyranosyl residues may be attached covalently to the hydroxyproline and serine residues respectively.

Cell-wall dynamics largely depend on wall-modifying enzymes that may be secreted or activated by acidification of the wall (32). They will be discussed in connection to the CBDs in paragraph 3.2.

Cellulose-binding domains: a common feature in many cell-wall modifying enzymes

The variety of polysaccharides-degrading enzymes, produced by phytopathogens and involved in the plant cell wall dynamics, typically reflects the diversity of structure among cell-wall constituents, and the variety of linkages between each polysaccharide. Cellulases, mannanases, xylanases, pectinases and other enzymes involved in the hydrolysis of polysaccharides are modular proteins comprising multiple structural and functional domains. Typically, these enzymes contain, in addition to the catalytic domain, one or more carbohydrate-binding modules (33). Specific CBDs were first demonstrated in the micro-organisms *Trichoderma reesei* (34) and *Cellulomonas fimi* (35). Since then, CBDs have been extensively characterised from many cellulases and xylanases, and have recently also been identified in plant cell-wall modifying enzymes (36).

3.1 Microbial cellulose-binding domains

Most plant cell wall degrading bacteria and fungi produce enzymatic complexes (cellulosome) and individual enzymes acting synergistically on (hemi-)cellulosic polysaccharides. Individual enzymes share a multi-domain structure composed of a catalytic domain and a CBD interconnected by a flexible and often highly glycosylated linker sequence (33). In cellulosomal complexes, enzymes are non-covalently associated to a scaffolding unit carrying a CBD (37,38). CBDs are present in many investigated cellulases (33,39-41), hemicellulases (33,39,40,42), one fungal (43) and two bacterial (44,45) pectinases.

CBDs are structurally well-defined domains, internal or linked to the N- or C-terminus of the catalytic domain (33,39,46). To date, 240 CBDs are registered in the NCBI protein databank, and most of them are classified into 13 families according to their amino acid sequence homologies (39). Most reported CBDs belong to families I, II and III. Family I contains small CBDs (33-40 amino acid residues), all of fungal origin (Table 1). The other families contain bacterial CBDs (Table 1) much larger in size (90-240 amino acids). To date, the tertiary structures of 8 CBDs from families I to V have been solved (47-54). Families I, II, III and V differ by the structure of their members, but similarities responsible for the same substrate binding mechanism were observed between those families. A planar linear strip including aromatic residues, with one of their faces exposed to the matrix, may be involved in hydrogen bonding to cellulose. Those aromatic amino acids are mainly tyrosine in fungal CBDs and tryptophane in bacterial CBDs. In the same planar strip, polar amino acids are responsible for stabilising the orientation of the cellulose binding residues. Conserved cysteine causes proper folding of the domain through disulfide bridges. CBDN1 from the family IV differs by its unique selectivity for amorphous cellulose and soluble oligosaccharides (48). The binding site of CBDN1 is not flat, but is a cleft containing a central strip of hydrophobic residues that is flanked on both sides by polar hydrogen-bonding group. This cleft can accommodate single strand carbohydrate chains, which explains the selectivity of CBDN1 for amorphous cellulose.

The structure-function relationship of CBDs was extensively studied by exchanging catalytic and binding domains in cellulases (55-58), by using site directed mutagenesis of aromatic amino acids of the flat face of the CBD (59-63), or by chemical modification of those residues (64). This illustrates why even in the same family, specific CBD properties are variable. Modification of the amino acids involved in binding results in a change of the substrate affinity (60). While bacterial CBDs have an affinity restricted to crystalline cellulose, some fungal CBDs are able to bind both crystalline and amorphous forms of the substrate (65,66). Different CBDs may direct the enzymatic activity to different

sites of the heterogeneous surface of the cellulose (58,63). Reversibility of the binding also depends on the CBD structure. It is now thought that binding of most CBDs to cellulose is reversible, and that cellulases are able to slip along the cellulosic fibres. The exchange rates, or ability of the CBD to move along the fiber also vary (67-69). For different CBDs, different environmental conditions (e.g. pH, temperature) are required for optimal binding (62). Each parameter, which varies from one CBD to another, can affect the specificity of an enzyme.

CBDs participate in the required close association of the catalytic domain with the insoluble substrate (41,70). It appears that the presence of CBDs increases the effective concentration of the cellulases on cellulose (5,41). Removal of the CBD always results in a decreased activity of the enzyme on insoluble substrates (35,71). CBDs allow cellulases to act on crystalline cellulose by destabilising the hydrogen bond structure of the cellulose, making the polysaccharide chains more accessible for endo-glucanases, only being able to hydrolyse amorphous substrate. Bacterial CBDs contribute to the non-hydrolytic disruption of cotton fibre (41,72). The same effect for fungal CBDs was reported on cotton fiber and CF-11 cellulose (73,74). The physical modifications on cellulose fibre due to this disrupting effect may also differ from one CBD to another. Lee *et al.* (75) observed EG II from *Trichoderma reesei* was having a peeling and smoothing effect on cotton fibres, while CBHI was able to penetrate cellulose surface and to pry apart microfibrils. How CBDs participate in breaking down hydrogen bonds of the substrate is not yet understood, but appears to be independent from the catalytic domain. CBDs with different specificities may also play a role in targeting the catalytic domains to distinct regions of the substrate, but the mechanism of specific substrate-site recognition by each CBD remains unknown (58).

3.2 Plant cellulose-binding domains

The structural rigidity and strength of the plant cell wall is thought to depend on the integrity of the cellulose/hemicellulose network. Wall loosening proteins associated to cell expansion, may act at the interface of the cellulose and hemicelluloses, and affect particularly cell walls structure and plasticity (76). The acid growth hypothesis proposes that auxine activates a plasma-membrane proton pump. The acidification of the wall activates growth-specific hydrolases, which cleave the bonds that join microfibrils to other polysaccharides. Relaxation of the wall by loosening enzymes, together with an uptake of water leads to an increase in cell size. Those proteins involved in the loosening of the plant cell-wall are endo-1,4- β -glucanases (77), xyloglucan endo-

Table 1. Amino acid sequences of cellulose-binding domains

enzyme	sequence	reference
<i>CBHI</i>	TQSHY <u>GQCGGIG</u> YSGPTV CASGTT CQYLNP <u>YY</u> SQCL	(39)
<i>CBHII</i>	CSSVWGQCGGQNW SGPTCC ASGSTCVYSND <u>YY</u> SQCL	
<i>EGI</i>	TQTHWGQCGGIG YSGCKT CTSGTT CQYSND <u>YY</u> SQCL	
<i>EGIII</i>	QQTVWGQCGGIG WSGPTN <u>C</u> APGSA <u>CS</u> TLNP <u>YY</u> AQCI	

In bold: aromatic amino-acids involved in the binding. Underlined: conserved cystein involved in disulfide bridging.
 In *italic*: conserved domains.

Table 1b. Amino acid sequence of *Cellulomonas fimi* CBDs (family II)

enzyme	Sequence	reference
<i>CenA</i>	APGCRVDYAVTNQ WPGFGANVTITNLGDPVSS WKLDWYTAGORIQQ LWNGTASTNGGQVSTSLP WNGSIPTGGTASFNGSWGAGSNPTPASFSLN GTTCTGT	(39)
<i>CenB</i>	TPSC ^T VVYSTNS WNVGFTGSKIINTGTIPLT WTLGFAPSGQQVTQG WS ATWSQIGTIVTATGLS WNATLQPGGSTDIGFNGSHPGTNPASFIVNGE VCG	

CenD TGSCAVVITYTANGWSGGFTAAVTLTNTGTTALSG WTLGFAPSPGQTLTQ
 GWSARWAQSGSSVTATNEA WNAVLPAGASVEIGFSGHTGTNTAPATFT
 VGGATCITTR

In **bold** : conserved tryptophane residues. *Underlined and italic* : conserved cysteine involved in disulfide bridges.

Table 1c. Amino acid sequence of the faEG3 CBD (strawberry endoglucanase)

<i>enzyme</i>	<i>Sequence</i>	<i>reference</i>
<i>FaEG3</i>	QSSYNQLLPVVTSPKQTPVPKLTPAAPASTSGPIAIAQKVTSS WVSKGVT YYRYSTTVTNKSGKTLNKLTLISKLYGPL WGLTSKTGDSYVFPS WLNSL PAGKSLEGFVYIH	(36)

In **bold** : conserved tryptophane residues.

Table 1d. Amino acid sequence of the CsEXP1 CBD (cucumber expansin)

<i>enzyme</i>	<i>Sequence</i>	<i>reference</i>
<i>CsEXP1</i>	GSRTGWQSMRNWGQN WQSNNYLNGQGLSFQVTLSDGRTLTA YNLVPS NWQFGQTYEGPQF	(88)

In **bold** : conserved tryptophane residues.

transglycosylases (XETs) (78,79) and expansins (80). Such loosening proteins may also be associated to fruit ripening and abscission.

The substrate of XET is restricted to XGs. This enzyme cleaves and then re-joins xyloglucan chain, participating to both loosening and assembly of the cell wall (78,79); and it has never been shown to carry any CBD.

Endo-1,4- β -glucanases from plants usually don't possess a CBD and are not able to degrade crystalline cellulose, neither have they been shown to hydrolyse native cellulose (77). Their participation to wall loosening involves cleavage of the xyloglucan chains inter-linking cellulose (18,77,81). Nevertheless, two unique endo-1,4- β -glucanases were shown to possess a putative cellulose-binding domain (36,82). Trainotti *et al.* (36) suggested the presence of homologous CBD sequences in an endoglucanase from cotton and in two genes from *Arabidopsis thaliana*. The endo-1,4- β -glucanases faEG3, expressed during strawberry fruit ripening, exhibits a typical linker peptide and a CBD sharing high homology with a CBD sequence from the bacteria *Dictyostelium discoideum* (Table 1). The unusual faEG3 was shown to loosen and disorganise cellulose microfibrils during fruit ripening (36,83).

Apart from the endo-glucanase Cell2A from *Trichoderma reesei* (84), none of the known XETs or endo-glucanases has been shown to induce extension of isolated plant tissues. However, another class of proteins, called expansins, enhances extension of boiled cucumber hypocotyls and wheat coleoptiles. Action of expansins on cell walls seems not to be related to any endo- or exo-cellulase activity (85,86). According to substrate specificity and sequence similarities, expansins have been classified in two subgroups, α and β , sharing 25% amino acid identity. At their C-terminus, α - and β -expansins all exhibit a serie of conserved tryptophanes (Table 1), sharing homology with the CBD of bacterial cellulases (76,80,87). Expansins have been shown to bind reversibly to crystalline cellulosic substrates (76,86,88). Comparative studies have shown that expansins, unlike bacterial CBDs, don't compete with xyloglucan for binding to cellulose, suggesting they may interfere with different sites of the substrate (76). Like bacterial CBDs, expansins were shown to weaken cellulose paper without apparent hydrolytic activity (89), and to enhance hydrolysis of cellulose by cellulases. The participation of expansin to cell expansion may be mediated by the loosening of non-covalent association within the cellulose/hemicellulose network. The occurring separation of cellulose from their hemicellulose coating would allow the microfibrils to slide passed each other. The mechanism by which hydrogen bonds are disrupted, and the participation of the CBD in this mechanism, is however not yet understood.

CBD as a biotechnological tool

4.1 Plant cell wall modification

The intriguing role of CBDs in microbial hydrolases and in cell-wall loosening enzymes may be of interest also in plant cell wall modification. *In-planta*, by interacting with cellulose, individual CBDs might directly, or indirectly, affect some structural aspects of the cellulose-xyloglucan network.

In-vitro, a family III CBD from the bacterium *Clostridium cellulovorans*, showing affinity for crystalline cellulose, promotes peach pollen tube growth and *Arabidopsis thaliana* seedlings elongation at low concentration, which effect is reverted at high concentration (6). When expressed in transgenic poplar trees and tobacco plants under the control of the *Cell* (endo- β -1,4-glucanase) promotor, specific for elongating tissues (90,91), the CBD from *Clostridium cellulovorans* enhances growth rate and increased plant biomass.

A CBD cloned from *Aspergillus japonicus*, having affinity for both bacterial microcrystalline cellulose and carboxymethyl cellulose (66) was expressed in *Arabidopsis thaliana* under the control of a constitutive promotor. Transgenic plants exhibit shorter roots and flowering stems (Figure 3). A cytological analysis reveals a defect in cell elongation, and suggests the fungal CBD may affect cell-wall structure potentially through an alteration of cellulose crystallisation (unpublished data).

The bacterial CBD from *Clostridium cellulovorans*, which disrupt crystalline cellulose, has been shown not to have any expansin activity on cucumber hypocotyls or wheat coleoptiles (76). Neither the CBD from *Aspergillus japonicus* enhanced stress relaxation of wheat coleoptiles exposed to 50 μ g purified peptide per ml of acetate buffer at pH 5.0 for 10 hours (unpublished data). Bacterial CBD increased the rate of cellulose synthesis in *Acetobacter xylinum*, and appeared to interfere in the crystallisation of the bacterial cellulose ribbon (6). The CBD from *Clostridium* also appeared to compete with xyloglucan for coating the cellulose surface (6). Thus, it may be hypothesised that bacterial CBD act on cellulose-hemicellulose interaction, and facilitate the cell wall loosening. Over-expression of a fungal CBD, much smaller in size and suspected to bind to different forms of cellulose *in-planta* leads to a different effect on the cell wall. Small dyes, such as calcofluor-white, Congo red or coumarine, associating with native cellulose during its secretion (92), or herbicides perturbing cortical microtubule polymerisation and thus cellulose orientation (17), have been shown to affect cellulose crystallisation and deposition. It is assumed that a CBD, which may coat native cellulose or intercalate with microfibrils, would have a similar effect. Antibodies annihilating the activity of endo-1,4- β -glucanases involved in cell wall loosening are known to affect cell wall structure (93,94). Covering the substrate sites of cell-wall loosening enzymes, CBDs could mimic this effect of antibodies, resulting in a defect in cell elongation.

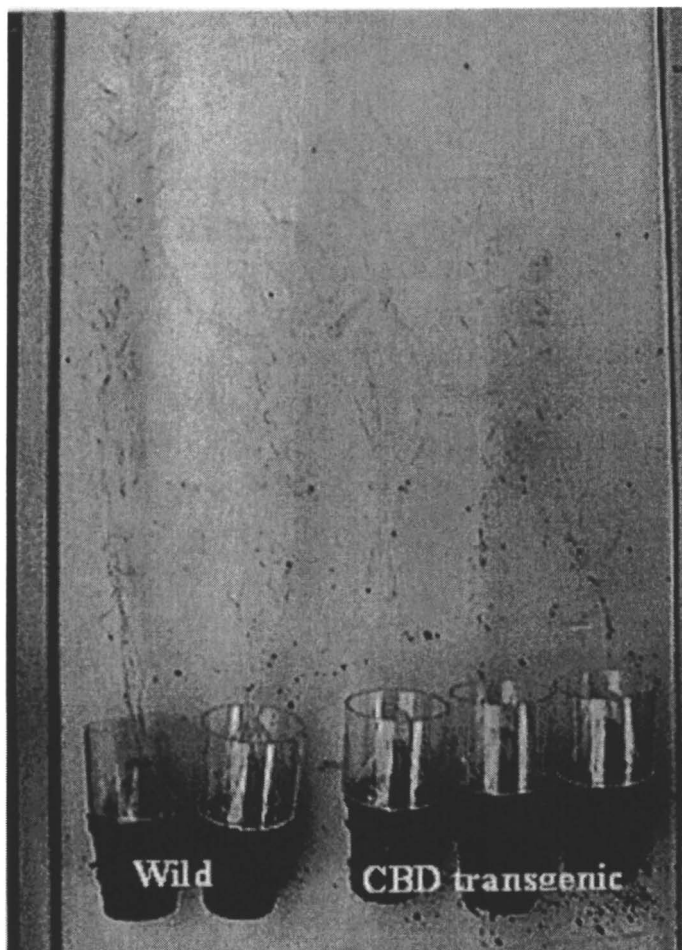


Figure 3. *Transgenic Arabidopsis thaliana* expressing a fungal CBD. *Arabidopsis thaliana* was transformed with the CBD from *Aspergillus japonicus* (Quentin et al. 2002), fused to Cell signal peptide (Shani et al. 2000) via a linker peptide, under the control of an enhanced 35S constitutive promoter. Transgenic plants are smaller and thinner than the wild type.

It is difficult to specify a unique and clear mode of action of CBDs on cell walls. It is predicted that variations in the substrate specificity, in the size and the disrupting ability of the cellulose-binding proteins, as well as variations in the promoter controlling their level of expression and tissue localisation, would differently alter cell-wall structure and plant development. Variation in biomass yield, fibre length, the degree of polymerisation or crystallinity of the cellulose, and polysaccharide composition would provide relevant possibilities of exploitation of the modified plant cell walls. Transgenic poplar trees, expressing the CBD from *Clostridium*, exhibit longer fibre cells and a higher degree of polymerisation of cellulose (7). Paper prepared from those fibres has been shown to present increased burst, tear and tensile indices, reflecting stronger fibre-to fibre interactions and thus paper strength (8).

4.2 Enzyme technology

4.2.1 Fiber processing.

Enzymes produced by cell-wall degrading micro-organisms are increasingly applied during textile and paper processing, since they can be produced at large scale, and due to their high substrate specificity for plant polysaccharides.

The production of wood-free paper requires removal of lignin, while causing minimal degradation of cellulose, since lignin's presence avoids the formation of hydrogen bonds between fibres, limits the swelling of fibres and makes them stiff. Cellulases together with hemicellulases are used in pulping and fibre modification participating in the enhancement of the fibrillation of fibres, increasing the inter-fibre bonds responsible for the strength of the product. They also influence the drainage rate, which is limiting the papermaking speed (95,96). They are of great interest in modification and deinking of recycled paper. Treatment of pulps with xylanases and mannanases, dissolving hemicelluloses closely associated to fibres, renders the fibres more accessible to bleaching chemicals (97) and lead to a more efficient delignification when using lignin-degrading enzymes like laccases (42). Peroxydases, being used in lignin oxidation, could also have a benefit in the bleaching of pulps (96). Lipases and esterases are used to remove hydrophobic content (pitch) of the woods. Non-wood fibre-yielding plants such as flax and jute require pectinase treatment to degrade the middle lamella separating fibre bundles. The use of purified

polygalacturonases result in better processing than dew-retting achieved by microorganisms which often lead to cellulose degradation (98).

4.2.2. Targetting of enzymatic activity

CBDs are able to maintain their own properties when isolated from different types of cellulases (34,71,99). Structurally and functionally independent from the catalytic domain, CBDs could be used to construct fusion proteins, using recombinant technology, without affecting the biological activity of the fusion partner. The utilisation of CBDs as affinity tags for protein affinity-purification and immobilisation on cellulosic material is now in hand (65,100-108).

Recently, studies focused on the key-role of CBDs in association with a catalytic activity, during enzyme treatments of pulps (109-112), cotton fibres (113,114), or during paper recycling (115). The presence of a CBD in cellulases and xylanases appear to enhance their activity on pulp, and to improve their bleaching and beating effects on fibres. Paper made of those treated fibres also possesses better properties of drainability.

The behaviour of enzymes in conditions encountered during processing such as high temperature (116), and high agitation conditions (113) has also been investigated. Due to the high agitation levels required during textile processing, the presence of a CBD didn't appear to have any benefit on xylanase activity (113).

Increasing the availability of CBDs with different specificity, would be a way to enhance enzyme efficiency, or to multiply the potential uses in production of cellulosic material. Targeting the activity of enzymes normally deprived of a CBD such as pectinases, laccases, lipases, would provide innovative solutions to processes associated to fibre extraction and modification. The production of CBD-hybrids with affinity for specific substrate surfaces would lead to an increased value of the final product. This technology is in development in different laboratories (117).

Recent reports show that the benefit of individual CBD peptides in pulp treatment has to be considered. For unknown reasons, fungal CBD peptides modify fibre surface characteristics and improve pulp drainability and paper strength (115,118). A fusion protein including two CBDs from *Clostridium cellulovorans* increased strength and water-repellence of Whatman filter paper (119). Cellulases immobilised on cotton fabric were shown to increase dyeability (116). Immobilisation of protein on the cellulose surface affects the charge, hydrophobicity and reactivity of the fibres. It gives interesting perspectives for production of new kinds of paper, cellulosic composites or packaging material with added functionality.

4.2.3. Enzyme stability

Knowledge on the modular organisation of cellulolytic enzymes or of recombinant enzymes would also provide new solutions for enzyme engineering. Combinations of different catalytic domains with varying CBDs, modified nature of the linker peptides, and the interactions between those domains, affect properties of the enzymes such as binding capacity, protease sensitivity or thermostability.

Removal of the linker region of cellulase sequences was shown to have (66,120), or not to have (121,122) an effect on the substrate binding capacity of the enzyme. Linker peptides seem to avoid misfolding of cellulases, and contribute to their stability. Conferring flexibility and separation, they allow domains to act independently. Most linker peptides from cellulases are protease sensitive. But susceptibility of linker sequences to such enzymes seems to depend on the size of those regions (120) and on the nature of their amino acid sequence, which determines their level of glycosylation (66,120,122,123). We have shown that an extremely large linker region cloned from *Aspergillus japonicus*, particularly rich in potentially O-glycosylated serine and threonine, could protect a CBD hybrid from protease activity in *E. coli* (66).

Recent studies showed that modification in the combination of domains of cellulases from *Clostridium thermocellum* might affect their thermostability (124). Riedel *et al.* (125) characterised one CBD from family III without affinity towards cellulose, and apparently only present in the cellulase to maintain the catalytic activity and the thermostability of the enzyme. Description of multiple combinations of domains and their interactions, and characterisation of natural linker sequences, may solve the problem of protease sensitivity of recombinant enzymes during their production. Furthermore, it would allow the utilisation of those enzymes in the high temperature conditions, required during fibre transformation processes.

Concluding remarks.

CBD-technology represents an innovative approach to modify cell-wall synthesis and composition, and produce fibre-yielding plants with new intrinsic properties.

CBDs have earlier been considered beneficial for enzyme activity in bio-processing. Used as affinity tag, they are commonly used to purify or immobilise recombinant proteins on a cellulosic matrix. They could be used to engineer

hybrid protein able to specifically modify cellulosic surfaces. Being part of the modular structure of cellulases, CBDs together with catalytic domains and linker sequences participate in the enzyme stability. Production of protease-resistant and thermostable CBD-hybrids would benefit from a better understanding of enzyme structure and domain interactions.

The mechanisms of substrate recognition and binding of CBDs on cellulose surfaces is not yet understood. Neither the effect of the binding on the chemical and physical structure of cellulosic surfaces is known. Characterisation of CBDs with specificity restricted to different sites present on the heterologous surface of the substrate, and increased knowledge's on the optimal conditions for binding, would favour a fine targeting of recombinant enzymes and allow restricted modification of the cell walls and fibre surfaces.

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References

1. McDougall, G.J.; Morrison, I.M.; Steward, D.; Weyers, J.D.B.; Hillman, J.R. *J. Sci. Food Agr.* 1993, 62, pp 1-20.
2. Yuan, L.; Knauf, V. *Curr. Opin Biotech.* 1997, 8, pp 227-233.
3. Chapple, C.; Carpita, N. *Curr. Opin. Plant Biol.* 1998, 1, pp 179-185.
4. Levy, I.; Shoseyov, O. *Biotechnol. Adv.* In Press.
5. Linder, M.; Teeri, T.T. *J. Biotechnol.* 1997, 57, pp 15-28.
6. Shpigel, E.; Roiz, L.; Goren, R.; Shoseyov, O. *Plant Physiol.* 1998, 117, pp 1185-1194.
7. Shoseyov, O.; Shani, Z. *US Patent* 6,005,092, 1999.
8. Levy, I.; Shani, Z.; Shoseyov, O. *Biomol. Eng.* In Press.
9. Stone, B.A.; Clarke, A.E. In *Chemistry and Physiology of higher Plant (1-3)-B-glucans*, I.T.U. press Ed., 1992.
10. Carpita, N.; McCann, M. In *Biochemistry and Molecular Biology of Plants*; Buchanan, B.G.W., Gruissem, W., Jones, R. Eds; American Society of Plant Physiologist: Rockville, USA; 2000, pp 52-108.
11. Delmer, D.P.; Amor, Y. *Plant cell.* 1995, 7, pp 987-1000.

12. Delmer, D.P. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1999, 50, pp 245-276.
13. Hon, D.N.S. *Cellulose*. 1994, 1, pp 1-25.
14. Kersten, S.; Decraemer, W.F.; Verbelen, J.P. *Plant physiol.* 2001, 127, pp 381-385.
15. Emons, A.M.C.; Mulder, B.M. *Trends Plant Sci.* 2000, 5, pp 35-40.
16. Emons, A.M.C.; Schel, J.H.N.; Mulder, B.M. *Plant Biol.* 2002, 4, pp 22-26.
17. Satiat-Jeuennaitre, B. *Biol. Cell.* 1987, 59, pp 89-96.
18. Rose, J.K.C.; Bennett, A.B. *Trends Plant Sci.* 1999, 4, pp 176-183.
19. Carpita, N.C.; Gibeaut, D.M. *Plant J.* 1993, 3, pp 1-30.
20. Fry, S.C. *J. Exp. Bot.* 1989, 40, pp 1-11.
21. Hayashi, T. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1989, 40, pp 139-168.
22. Thompson, J.A. *FEMS Microbiol. Rev.* 1993, 104, pp 65-82.
23. Whitney, S.E.C.; Brigham, J.E.; Darke, A.H.; Grant Reid, J.S.; Gidley, M.J. *Plant J.* 1995, 8, pp 491-504.
24. Pauly, M.; Abersheim, P.; Darvill, A.; York, W.S. *Plant J.* 1999, 20, pp 629-639.
25. Finkenstadt, V.L.; Hendrixson, T.L.; Millane, R.P. *J. Carbohydr. Chem.* 1995, 14, pp 601-611.
26. Schols, H.A.; Voragen, A.G.J., In *Pectins and Pectinases*; Visser, J., Voragen, A.G.J. Eds; Progress in biotechnology: 14; Elsevier: Amsterdam, The Netherlands; 1996, pp 3-19.
27. Fry, S.C. *Annu. Rev. Plant. physiol.* 1986, 37, pp 165-186.
28. Jarvis, M.C. *Plant Cell Environ.* 1984, 7, pp 153-164.
29. Lewis, N.G.; Yamamoto, E. *Annu. Rev. Plant physiol. Plant Mol. Biol.* 1990, 41, pp 455-496.
30. Whetten, R.W.; MacKay, J.J.; Sederoff, R.R. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1998, 49, pp 585-609.
31. Cassab, G.I. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1998, 49, pp 281-309.
32. Cosgrove, D.J. *Annu. Rev. Cell Dev. Bi.* 1997, 13, pp 171-201.
33. Gilkes, N.R.; Henrissat, B.; Kilburn, D.G.; Miller Jr, R.C.; Warren, W.A.J. *Microbiol. Rev.* 1991, 55, pp 303-315.
34. Van Tilbeurgh, H.; Tomme, P.; Claeysens, M.; Bhikhabhai, R.; Pettersson, G. *FEBS Lett.* 1986, 204, pp 223-227.
35. Gilkes, N.R.; Warren, R.A.J.; Miller Jr, R.C.; Kilburn, D.G. *J. Biol. Chem.* 1988, 263, pp 10401-10407.
36. Trainotti, L.; Spolaore, S.; Pavanello, A.; Baldan, B.; Casadoro, G. *Plant Mol. Biol.* 1999, 40, pp 323-332.
37. Bayer, E.A.; Shimon, L.J.W.; Shoham, Y.; Lamed, R. *J. Struct. Biol.* 1998, 124, pp 221-234.

38. Bayer, E.A.; Chanzy, H.; Lamed, R.; Shoman, Y. *Curr. Opin. Struc. Biol.* 1998, 8, pp 548-557.
39. Tomme, P.; Warren, R.A.J.; Miller Jr, R.C.; Kilburn, D.G.; Gilkes, N.R. In *Enzymatic degradation of insoluble carbohydrates*; Saddler, J.N., Penner, M.H. Eds; ACS Symp. Ser. 618; American Chemical Society: San Diego, USA; 1995, pp 143-163.
40. Warren, R.A.J. *Annu. Rev. Microbil.* 1996, 50, pp 183-212.
41. Henrissat, B. *Cellulose.* 1994, 1, pp 169-196.
42. Kulkarni, N.; Shendye, A.; Rao, M. *FEMS Microbiol. Rev.* 1999, 23, pp 411-456.
43. Wei, Y.D.; Goodwin, P.H. *Accession number AF158256.* 2000.
44. Brown, I.E.; M.H., M.; Charnock, S.J.; Davies, G.J.; Black, G.W. *Biochem. J.* 2001, 355, pp 155-165.
45. McKie, V.A.; Vinken, J.P.; Voragen, A.G.J.; van den Broek, L.A.M.; Stimson, E.; Gilbert, H.J. *Biochem. J.* 2001, 355, pp 167-177.
46. Henrissat, B.; Claeysens, M.; Tomme, P.; Lemesle, L.; Mornon, J.P. *Gene.* 1989, 81, pp 83-95.
47. Brun, E.; Moriaud, F.; Gans, P.; Blackledge, M.J.; Barras, F.; Marion, D. *Biochemistry.* 1997, 36, pp 16074-16086.
48. Johnson, P.E.; Joshi, M.D.; Tomme, P.; Kilburn, D.G.; McIntosh, L.P. *Biochemistry.* 1996, 35, pp 14382-14394.
49. Kraulis, P.J.; Clore, G.M.; Nilges, M.; Jones, T.A.; Petterson, G.; Knowles, J.; Gronenborn, A.M. *Biochemistry.* 1989, 28, pp 7241-7257.
50. Tormo, J.; Lamed, R.; Chirino, A.J.; Morag, E.; Bayer, E.A.; Shoham, Y.; Steitz, T.A. *EMBO J.* 1996, 15, pp 5739-5751.
51. Xu, G.Y.; Ong, E.; Gilkes, N.R.; Kilburn, D.G.; Muhandiram, D.R.; Harris-Brandts, M.; Carver, J.P.; Kay, L.E.; Harvey, T.S. *Biochemistry.* 1995, 34, pp 6993-7009.
52. Mattinen, M.L.; Linder, M.; Drakenberg, T.; Annila, A. *Eur. J. Biochem.* 1998, 256, pp 279-286.
53. Hoffren, A.M.; Teeri, T.T.; Teleman, O. *Protein Eng.* 1995, 8, pp 443-450.
54. Raghobhama, S.; Simpson, P.J.; Szabó, L.; Nagy, T.; Gilbert, H.J.; Williamson, M.P. *Biochemistry.* 2000, 39, pp 978-984.
55. Srisodsuk, M.; Lehtio, J.; Linder, M.; Margolles-Clark, E.; Reinikainen, T.; Teeri, T.T. *J. Biotechnol.* 1997, 57, pp 49-57.
56. Kim, H.; Masatoshi, G.; Heo-Jin, J.; Kyung Hwa, J.; Il, K.; Kensuke, F. *Appl. Biochem. Biotech.* 1999, 75, pp 193-204.
57. Tomme, P.; Driver, D.P.; Amandoro, E.A.; Miller Jr, R.C.; Warren, R.A.J. *J. Bacteriol.* 1995, 117, pp 4356-4363.
58. Carrard, G.; Koivula, A.; Söderlund, H.; Béguin, P. *Proc. Natl. acad. Sci.* 2000, 97, pp 10342-10347.

59. Linder, M.; Mattinen, M.L.; Kontelli, M.; Lindeberg, G.; Stahlberg, J.; Drakenberg, T.; Reinikainen, T.; Petterson, G.; Annila, A. *Protein Sci.* 1995, 4, pp 1056-1064.
60. Linder, M.; Lindeberg, G.; Reinikainen, T.; Teeri, T.T.; Pettersson, G. *FEBS Lett.* 1995, 372, pp 96-98.
61. Reinikainen, T.; Ruohonen, L.; Nevanen, T.; Laaksonen, L.; Kraulis, P.; Jones, T.A.; Knowles, J.K.; Teeri, T.T. *Protein Sci.* 1992, 14, pp 475-482.
62. Linder, M.; Nevanen, T.; Teeri, T.T. *FEBS Lett.* 1999, 447, pp 13-16.
63. Wang, H.; Jones, R.W. *Appl. Microbiol. Biot.* 1997, 48, pp 225-231.
64. Bray, M.R.; Johnson, P.E.; Gilkes, N.R.; McIntosh, L.P.; Kilburn, D.G.; Warren, R.A.J. *Protein Sci.* 1996, 5, pp 2311-2318.
65. Reinikainen, T.; Takkinen, K.; Teeri, T.T. *Enzyme Microb. Tech.* 1997, 20, pp 143-149.
66. Quentin, M.; Ebbelaar, M.; Derksen, J.; Mariani, C.; van der Valk, H. *Appl. Microbiol. Biot.* 2002, 58, pp 658-662.
67. Jervis, E.J.; Haynes, C.A.; Kilburn, D.G. *J. Biol. Chem.* 1997, 272, pp 24016-24023.
68. Carrard, G.; Linder, M. *Eur. J. Biochem.* 1999, 262, pp 637-643.
69. Linder, M.; Teeri, T.T. *Proc. Natl. acad. Sci.* 1996, 93, pp 12251-12255.
70. Klyosov, A.A. *Biochemistry.* 1990, 29, pp 10577-10585.
71. Gilkes, N.R.; Jervis, E.; Henrissat, B.; Tekant, B.; Miller Jr, R.C.; Warren, R.A.J.; Kilburn, D.G. *J. Biol. Chem.* 1992, 267, pp 6743-6749.
72. Din, N.; Gilkes, N.R.; Tekant, B.; Miller Jr, R.C.; Warren, R.A.J.; Kilburn, D. *Bio/Technology.* 1991, 9, pp 1096-1099.
73. Gao, P.J.; Chen, G.J.; Wang, T.H.; Zhang, Y.S.; Liu, J. *Acta Bioch. Bioph. Sin.* 2001, 33, pp 13-18.
74. Xiao, Z.; Gao, P.; Qu, Y.; Wang, T. *Biotechnol. Lett.* 2001, 23, pp 711-715.
75. Lee, I.; Evans, B.R.; Woodward, J. *Ultramicroscopy.* 2000, 82, pp 213-221.
76. Cosgrove, D.J. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1999, 50, pp 391-417.
77. Brummel, D.A.; Lashbrook, C.C.; Bennett, A.B., in *Enzymatic conversion of Biomass for Fuels Production*; Himmel, M.E., Baker, J.O., Overend, R.P. Eds; ACS Symp. Ser. 566; American Chemical society: Washington, USA; 1994, pp 100-29.
78. Campbell, P.; Braam, J. *Tren. Plant Sci.* 1999, 4, pp 3611-366.
79. Thompson, J.E.; Fry, S.C. *Plant J.* 2001, 26, pp 23-34.
80. Cosgrove, D.J. *Plant physiol. biochem.* 2000, 38, pp 109-124.
81. Hayashi, T. *The cutoskeletal basis of plant growth and form.* 1991. 131-144.
82. Catala, C.; Bennett, A.B. *Plant physiol.* 1998, 118, pp 1535.
83. Trainotti, L.; Ferrarese, L.; Dalla Vecchia, F.; Rascio, N.; Casadoro, G. *J. Plant Physiol.* 1999, 154, pp 355-362.
84. Yuan, S.; Wu, Y.; Cosgrove, D.J. *Plant Physiol.* 2001, 127, pp 324-333.

85. McQueen-Mason, S.; Durachko, D.M.; Cosgrove, D.J. *Plant Cell*. 1992, 4, pp 1425-1433.
86. McQueen-Mason, S.; Cosgrove, D.J. *Plant Physiol*. 1995, 107, pp 87-100.
87. Darley, C.P.; Forrester, A.M.; McQueen-Mason, S.J. *Plant Mol. Biol*. 2001, 47, pp 179-195.
88. Cosgrove, D.J. *Plant Physiol*. 1998, 118, pp 333-339.
89. McQueen-Mason, S.; Cosgrove, D.J. *Proc. Natl. Acad. Sci*. 1994, 91, pp 6574-6578.
90. Shani, Z.; Dekel, M.; Tsabary, G.; Shoseyov, O. *Plant Mol. Biol*. 1997, 34, pp 837-842.
91. Shani, Z.; Dekel, M.; Sig Jensen, C.; Tzfira, T.; Goren, R.; Altman, A.; Shoseyov, O. *J. Plant. Physiol*. 2000, 156, pp 118-120.
92. Herth, W. *J. cell Biol*. 1980, 87, pp 442-450.
93. Inouhe, M.; Nevins, D.J. *Plant Physiol*. 1991, 96, pp 426-431.
94. Hoson, T.; Nevins, D.J. *Physiol. Plant*. 1989, 77, pp 208-215.
95. Bhat, M.K. *Biotechnol. Adv*. 2000, 18, pp 355-383.
96. Kirk, T.K.; Jeffries, T.W. In *Enzyme for Pulp and Paper Processing*; Jeffries, T.W., Viikari, L. Eds; ACS Symp. Ser. 655; American Chemical Society: Washington; 1996, pp 2-14.
97. de Jong, E.; Wong, K.K.Y.; Martin, L.A.; Mansfield, S.D.; Gama, F.M.; Saddler, J.N. In *Enzyme for Pulp and Paper Processing*; Jeffries, T.W., Viikari, L. Eds; ACS Symp. Ser. 655; American Chemical Society: Washington; 1996, pp 44-62.
98. Sharma, H.S.S.; Van Sumere, C.F.V. *The Genetic Engineer and Biotechnologist*. 1992, 12, pp 19-23.
99. Lemos, M.A.; Teixeira, J.A.; Mota, M.; Gama, F.M. *Biotechnol. Lett*. 2000, 22, pp 703-707.
100. Greenwood, J.M.; Gilkes, N.R.; Miller Jr, R.C.; Warren, R.A.J. *Biotechnol. Bioeng*. 1994, 44, pp 1295-1305.
101. Lehtio, J.; Wernerus, H.; Samuelson, P.; Teeri, T.T.; Stahl, S. *FEMS Microbiol. Lett*. 2001, 195, pp 197-204.
102. Levy, I.; Shoseyov, O. *J. Pept. Sci*. 2001, 7, pp 50-57.
103. Linder, M.; Nevanen, T.; Soderholm, L.; Bengs, O.; Teeri, T.T. *Biotechnol. Bioeng*. 1998, 60, pp 642-647.
104. Ong, E.; Gilkes, N.R.; Miller Jr, R.C.; Warren, R.A.J.; Kilburn, D.G. *Enzyme Microb. Tech*. 1991, 13, pp 59-65.
105. Richins, R.D.; Mulchandani, A.; Chen, W. *Biotechnol. Bioeng*. 2000, 69, pp 591-596.
106. Rotticci-Mulder, J.C.; Gustavsson, M.; Holmquist, M.; Hult, K.; Martinelle, M. *Protein Expres. Purif*. 2001, 21, pp 386-392.
107. Shpigel, E.; G oldlust, A.; Efroni, G.; Avraham, A.; Eshel, A.; Dekel, M.; Shoseyov, O. *Biotechnol. Bioeng*. 1999, 65, pp 17-23.

108. Tomme, P.; Boraston, A.; McLean, B.; Kormos, J.; Creagh, A.L.; Sturch, K.; Gilkes, N.R.; Haunes, C.A.; Warren, R.A.J.; Kilburn, D.G. *J. Chromatogr. B*. 1998, 715, pp 283-296.
109. Suurnäkki, A.; Tenkanen, M.; Siika-Aho, M.; Niku-Paavola, M.L.; Viikari, L.; Buchert, J. *Cellulose*. 2000, 7, pp 189-209.
110. Gerber, P.J.; Heitmann, J.A.; Joyce, T.W.; Buchert, J.; Siika-Aho, M. *J. Biotechnol.* 1999, 67, pp 67-65.
111. Gerber, P.J.; Joyce, T.W.; Heitmann, J.A.; Siika-Aho, M.; Buchert, J. *Cellulose*. 1997, 4, pp 255-268.
112. Rixon, J.E.; Clarke, J.H.; Hazlewood, G.P.; Hoyland, R.W.; McCarthy, A.J.; Gilbert, H.J. *Appl. Microbiol. Biot.* 1996, 46, pp 514-520.
113. Azevedo, H.; Bishop, D.; Cavaco-Paulo, A. *Enzyme Microb. Tech.* 2000, 27, pp 325-329.
114. Boisset, C.; Chanzy, H.; Schülein, M.; Henrissat, B. *Cellulose*. 1997, 4, pp 7-20.
115. Pala, H.; Lemos, M.A.; Mota, M.; Gama, F.M. *Enzyme Microb. Tech.* 2001, 29, pp 274-279.
116. Andreas, J.; Azevedo, H.; Cavaco-Paulo, A. *J. Mol. Catal. B-Enzym.* 1999, 7, pp 233-239.
117. Shoseyov, O.; van der Valk, H.C.P.M.; Gama, M.; Fagerstrom, R.; Lovitt, R.W.; Vind, J.; Tenenbaum, A.; Sampaio, L. *CBD hybrids, a new EC consortium. Engineering of hybrid proteins containing cellulose binding domains. Novel biotechnological tools for pulp, paper and textile industries.* In *8th international conference on biotechnology in the pulp and paper industry*; 2001, Helsinki, Finland: VTT Biotechnology.
118. Kitaoka, T.; Tanaka, H. *J. Wood Sci.* 2001, 47, pp 322-324.
119. Levy, I.; Nussinovitch, A.; Shpigel, E.; Shoseyov, O. *Cellulose*. In Press.
120. Srisodsuk, M.; Reinikainen, T.; Pentillä, M.; Teeri, T.T. *J. Biol. Chem.* 1993, 268, pp 20756-20761.
121. Black, G.W.; Rixon, J.E.; Clarke, J.H.; Hazlewood, G.P.; Ferreira, L.M.A.; Bolam, D.N.; Gilbert, H.J. *J. Biotechnol.* 1997, 57, pp 59-69.
122. Shen, H.; Schmuck, M.; Pilz, I.; Gilkes, N.R.; Kilburn, D.G.; Miller Jr, R.C.; Warren, R.A.J. *J. Biol. Chem.* 1991, 266, pp 11335-11340.
123. Langsford, M.L.; Gilkes, N.R.; Singh, B.; Moser, B.; Miller Jr, R.C.; Warren, R.A.J.; Kilburn, D.G. *FEBS Lett.* 1987, 225, pp 163-167.
124. Kataeva, I.A.; Blum, D.L.; Li, X.L.; Ljungdahl, L.G. *Protein Eng.* 2001, 14, pp 167-172.
125. Riedel, K. *FEMS Microbiol. Lett.* 1998, 164, pp 261-267.

Chapter 10

Ethanol from Wheat Straw Cellulose by Wet Oxidation Pretreatment and Simultaneous Saccharification and Fermentation

C. Felby¹, H. B. Klinke², H. S. Olsen³, and A. B. Thomsen^{2,*}

¹Plant Fiber Laboratory, Royal Veterinary and Agricultural University, Højbakkegaard Alle 1, Taastrup, Denmark
²Risøe National Laboratory, Frederiksborgvej 399, Roskilde, Denmark
³Novozymes A/S, Krogshøjvej 36, Bagsvaerd, Denmark

Alkaline wet oxidation (WO) was used as a pretreatment method for fractionation wheat straw to a solubilised hemicellulose and a solid cellulose fraction. The WO was performed at initially 6% dry (solid) matter content. In the process the hemicellulose was solubilised and some of the lignin and pectin substances oxidized, resulting in a final solid matter content of 3% in the hydrolysate. By filtration, the solid cellulose product was increased to 10% dry matter that was effectively hydrolyzed and fermented to ethanol by simultaneous saccharification and fermentation (SSF) process using the filtrate as process water. The compact fermentation bulk was liquefied by enzyme treatment prior to the SSF in order to decrease the viscosity and facilitate good mixing conditions during the fermentation. The lipophilic compounds associated to the surface of wheat straw remained in a more concentrated form in the solid fractions after WO and SSF.

Introduction

The use of biomass as a source for liquid fuels has the potential to grow from a niche market to a major industry of environmental and strategic importance (1,2). The main product will be ethanol derived from fermentation of C6 sugars. Present day technology is mainly based on corn starch and sugar cane juice as carbohydrate sources for the fermentation. The projected growth for ethanol as liquid fuel within the next decades is so large, that the demand can only be met if cellulose can be exploited for this application (3). Cellulose is available in large quantities as agricultural and forest residues, of which wheat, corn, rice and softwood are dominant. The use of cellulose as C6 source is a challenging task as contrary to starch or sucrose, the polymer is embedded in a lignin/hemicellulose matrix and has a crystalline structure that requires very harsh conditions to open.

State of the art conversion of cellulose containing plant biomass to ethanol can roughly be described as a pre-treatment followed by enzymatic hydrolysis of cellulose to glucose which subsequently is fermented to produce ethanol (2). A number of cellulase enzymes catalyze the hydrolysis of crystalline cellulose, but when applied on native cellulose the rate of hydrolysis is very slow due to the low accessibility of the cellulose in the lignin/hemicellulose matrix (4). In this aspect the pre-treatment is a key process as it removes or loosens the lignin/hemicellulose matrix from the cellulose, making fast and efficient hydrolysis of the cellulose possible, as is needed for industrial exploitation.

Several pre-treatment technologies are known including steam explosion (5,6), organosolv extraction (7), ammonia fiber explosion (8), supercritical extraction (9), and dilute acid pre-treatment (10). All of these techniques are based on acidic hydrolysis or solubilization of the lignin and hemicellulose. Another approach is to use an oxidative technique, known as wet oxidation, which utilize the low redox potential of lignin compared to cellulose and hemicellulose. Wet oxidation is an exothermic reaction where lignocellulosic biomass, oxygen, water and in some cases carbonate is added to a high- pressure reactor. Typical operating conditions are 150-195 °C at 6-12 bar pressure of oxygen or ambient air at alkaline or weak acidic conditions. During the alkaline treatment the main reactions taking place are alkaline hydrolysis of lignin and hemicellulose combined with oxidative breakdown of mainly the lignin. The initial alkaline pH plays an important role for the solubilization and oxidation of lignin as well as preventing the sugar components from degrading to furfurals (11). The cellulose and hemicellulose yields are high >96 % and 68 % respectively. Only low amounts of fermentation inhibitors are formed during the process (12, 13). The

main reaction products are heat, CO₂, H₂O and carboxylic acids. The heat can be recovered and used in other production steps, and the carboxylic acids are good substrates for methane production (14). This also offers an opportunity for coupling ethanol production with generation of heat and electricity in already existing power plants.

It is well known, that enzymatic hydrolysis and fermentation of the cellulosic fraction from pre-treated lignocellulosics is improved by removing the liquid hemicellulose fraction. Washing pre-treated lignocellulosic substrates with water improves enzymatic hydrolysis of cellulose (15, 16) and fermentation to ethanol (17, 18). Total cellulose conversion to ethanol by simultaneous saccharification and fermentation (SSF) has been reported on washed pre-treated solids from acidic pre-treated wheat straw (19), herbaceous crops (20) and wood (21). However, to avoid excessive water use in the manufacture of bioethanol, the liquid fraction from pre-treatment should be included in the process stream together with the cellulosic fibres, i.e. the whole slurry. High solid ratio of pre-treated wheat straw (12.5 %) in the SSF was used in the studies of Mohagheghi et al. (22), however, this investigation was made on washed solid material and did not include the hydrolysate derived from the dilute acid pre-treatment. In addition the high solid ratio required special laboratory equipment to achieve efficient mixing.

Identification of possible by-products is an important issue in bioethanol production as ethanol itself is a low-value product. Wheat straw has a significant content of lipophilic extractives (23) associated to the surphase of the plant fibres. Lipophilic compounds are less reactive than phenol components (e.g. lignin), besides they are not water soluble, thus, they might still be present on the solid material after the pretreatment. Furthermore, as lipophilic compounds do not serve as substrate in an ethanol fermentation they could be isolated from the solid residue from the SSF-process.

This paper describes the use of wet oxidation as pre-treatment for fermentation of wheat straw cellulose to ethanol by simultaneous fermentation and saccharification (SSF) at high substrate concentration using *Saccharomyces cerevisiae*. A liquefaction stage is introduced prior to SSF by means of enzymatic hydrolysis to ensure a good mixing in the simple lab-shaker set-up. The effect of hydrolysate from alkaline wet oxidised wheat straw on enzymatic hydrolysis and SSF at different enzyme loadings is investigated. Emphasis is given to the enzymatic hydrolysis of the pretreated wheat straw cellulose and the chemical composition of the process streams. The isolation and characterisation of lipophilic compounds in wheat straw is investigated during the different steps of the ethanol process.

Methods and materials

Wheat Straw

Straw from wheat (*Triticum aestivum* L.) variety Husar was obtained from Risoe National Laboratory.

Chemicals

Multicomponent cellulases Celluclast 1.5L (endo- and exo-cellulase) and Novozyme 188 (β -glucosidase) was obtained from Novozymes A/S, Bagsvaerd, Denmark. Enzyme activity of the cellulases was measured as filter paper units (FPU) according to (24).

Yeast (*Saccharomyces cerevisiae*) sold as bakers yeast was obtained from De Danske Sprit Fabrikker, Aalborg, Denmark (Danisco A/S). Sodium carbonate, urea and sulphuric acid were purchased from Sigma, St. Louis, Mo, USA.

Wet oxidation pre-treatment

Wet oxidation (WO) was carried out in a 2 litre loop reactor with continuous circulation and stirring, allowing heating and cooling times of 1-2 min (11). Pre-treatment conditions were 10 min at 195 °C, 6.5 g/l Na_2CO_3 and 12 bar O_2 (12). Ground wheat straw (65 g, 5 mm, 92 % dry matter), was mixed with 1 L water and Na_2CO_3 , before adding oxygen pressure and heating the suspension. Initial pH was 10 and after the reaction pH dropped to 5.9. After cooling, the pre-treated wheat straw was separated by filtration into a solid fraction and a liquid fraction for further analysis.

Chemical and physical characterization following wet oxidation and fermentation

Solid fraction: Determination of cellulose hemicellulose, lignin and non-cell wall material (NCWM) was done by the sequential gravimetric detergent fiber procedure according to Goering and van Soest (1970). The NCWM includes pectins and lipophilic components.

Liquid fraction: Total organic carbon (TOC) was analyzed on a Shimadzu TOC-500 with IR detection after combustion at 680 C (Pt-catalyst). Compensation for inorganic carbon was made by acidification.

Aromatics: The phenols and 2-furoic acid were isolated from the liquid fraction by solid phase extraction on polystyrene divinylbenzene polymer columns: IST Isolute ENV+ 100 mg/ml (International Sorbent Technology Ltd., Mid-Glamorgan, UK). The aromatic compounds were identified and quantified by gas chromatography (GC) with mass selective detection (MS) (12). Lignin was precipitated by HCl at pH 1.5 (12) and dissolved in eluent (5 mg/ml) for size exclusion chromatography. Column for polyphenol separation was TSKgel G2500PW (Tosoh Biosep, Tokyo, Japan), 7.5 mm i.d. x 30 cm. Eluent was 70% MeOH in 50 mM NaCl at a flow of 1 ml/min. The HPLC (Shimadzu Corp., Kyoto, Japan) was fitted with an UV detector and a RI detector. Lignin was determined by UV-detection at 254 nm and polystyrene standards were used for calibration of molecular weight.

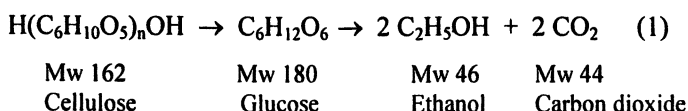
Lipophilic extractives: Untreated, wet oxidized, and SSF treated wheat straw were soxhlet extracted by a mixture of toluene: acetone: ethanol (4:1:1 v/v) for 5 hours. The extracts were carefully evaporated at 50 °C until dryness, and the weight residue was considered the total lipophilic extractives. Analysis of lipophilic extractives from untreated wheat straw and following wet oxidation and SSF was done directly on the solids by combined methylation and pyrolysis GC/MS essentially as described by Hardell and Nilvebrant (26). Methylation reagent was 10 % aqueous tetramethylammonium hydroxide (TMAH). Pyrolysis was performed at 400 °C in a filament pulse pyrolyzer (Pyrrola, Pyrol AB, Sweden) fitted on a HP-5890 gas chromatograph with a VG TRIO-1 quadrupole mass spectrometer. The pyrolysate was separated on a DB-1 column (J&W Scientific, USA, 30m x 0.25 mm, film thickness 0.25 µm) with He as carrier gas at 1mL/min. The column temperature program was 5 min hold at 50 °C and then increased by 10 °C/min to 320 °C.

Sugars and ethanol: Glucose, xylose, arabinose, acetic acid and ethanol were quantified by HPLC with RI-detection (Shimadzu Corp., Kyoto, Japan) after separation on an Aminex HPX-87H column (Bio-Rad Laboratories Inc., Hercules, CA, USA), using 4 mM H₂SO₄ as eluent and 0.6 ml/min flow at 63 °C. Conversion factors for dehydration on polymerization was 162/180 for glucose and was 132/150 for xylose and arabinose.

Liquefaction and Simultaneous Saccharification and Fermentation (SSF)

Liquefaction (presaccharification) and SSF fermentation were made in 250 ml blue cap flasks with yeast locks filled with concentrated H₂SO₄. A 10% (w/w) suspension was prepared by mixing 25 g filter cake (solid fraction) with 225 mL filtrate (hydrolysate) in each flask. In order to reduce the viscosity a liquefaction stage was introduced prior to the SSF. The liquefaction of the suspension was done by treating the suspension for a total of 24 hours with a cellulase mixture of 83/17 v/v of Celluclast 1.5L and Novozyme 188. Enzyme loading was 5 FPU/g DM. pH and temperature was 4.7 and 50°C, respectively.

Following liquefaction the flasks were cooled to 32 °C and incubated with 800 mg *Saccharomyces cerevisiae* (Baker's yeast). Additional enzyme mixture was added giving a total enzyme loading of 5, 10 or 20 FPU/g DM. For yeast nutrition 16 mM of urea was added to each flask. The fermentation was monitored by CO₂ loss, as determined by weighing the flasks at regular intervals. The ethanol yield during fermentation was calculated from the CO₂ loss by multiplication of the conversion factor (i.e. the molar ratio of EtOH/CO₂) according to reaction scheme (1): EtOH (g) = CO_{2, loss}(g)/1.045. The final ethanol concentration was determined by the HPLC procedure described earlier. All experiments were carried out in duplicate.



Degree of cellulose hydrolysis

The enzymatic hydrolysis of cellulose to glucose was determined by the method of Bjerre *et al.* (11) with Celluclast 1.5L and Novozym 188 (83/17 mixture). Approx. 100 mg solid fraction was suspended in 5 ml 0.04 M acetate buffer corresponding to a 2 % dry matter, and incubated with the enzymes at pH 4.8 and 50 °C for 24 hours. The liquid fraction from the wet oxidized wheat straw was adjusted to pH 4.8 by addition of 2 M H₂SO₄ prior to the addition of solid fraction and enzymes. Cellulose hydrolysis, as determined by glucose concentration, was measured by HPLC as described above.

Results and discussion

Pre-treatment by wet oxidation

WO pre-treatment resulted in an efficient separation of the wheat straw in to a cellulose-rich solid fraction and a hemicellulose-rich liquid fraction (hydrolysate), see Figure 1. The chemical analysis and mass balance show that approximately 96 % of the cellulose was recovered during wet oxidation treatment (Figure 2). Due to oxidation, 49 % of the hemicellulose and 65% of the lignin were converted, and thus removed as CO₂, H₂O, carboxylic acids and other components during the wet oxidation. The solid fraction had a high cellulose content (72%), but some lignin and hemicellulose were still present (Table 1). Analysis of the liquid fraction showed that much of the lignin and hemicellulose were oxidized to organic acids (Table 1) of which formic and acetic acid were the main acids (12). Total organic carbon (TOC) of the liquid fraction was 11g C/ L.

Enzymatic hydrolysis of products

An important evaluation parameter of pre-treatment is the total glucose yield, defined as the glucose formed by enzymatic saccharification corrected for the glucose lost during pre-treatment. The total glucose yield of the water-washed pre-treated solid fractions of wheat straw has previously been reported to be 57 % by alkaline hydrolysis (27), 32 % by liquid ammonia pre-treatment (18) and 85 % by steam pre-treatment (28). By alkaline wet oxidation of wheat straw and enzymatic hydrolysis at 30 FPU/g DM, the total glucose yield was 66% (Figure 3).

To the best of our knowledge it has not previously been investigated how the liquid fraction affect the total glucose yield by enzymatic hydrolysis of the cellulosic fraction of pre-treated wheat straw. Cellulose hydrolysis was found to be about 70 % for both the washed cellulose fraction in a buffer as the solid fraction directly suspended in hydrolysate (Figure 3). At low enzyme loadings (5-10 FPU/g), lower yields were obtained in hydrolysate (WO liquid fraction adjusted to pH 4.8) when compared to hydrolysis in a acetate buffer. Thus, the enzymatic hydrolysis was inhibited by components in the liquid fraction. This is most likely caused by the adsorption of some of the enzymes to lignins or inhibition by phenols or other degradation products (29). Analysis of water-soluble lignins isolated by acid precipitation was found to have a M_w of 1 kDa, consisting of phenol oligomers of an average of 5 phenol units (data not shown). These polyphenols accounted for an additional 18% of the TOC of the liquid fraction (Table 1). At the high enzyme loadings (20-30 FPU/g) only slight inhibition was observed when comparing the cellulose conversion in liquid fraction with that obtained in buffer solution.

The fractionation of wheat straw by alkaline wet oxidation resulted in an efficient solubilisation of the hemicellulose with a relatively uniform molecular weight distribution. The soluble hemicellulose sugars were present as oligomers and polymers consisting mainly of xylose and arabinose (measured after acidic hydrolysis, Table 1). Thomsen et al. (30) found that hemicellulose isolated from alkaline wet oxidised wheat straw consisted of a high and low molecular weight fraction. The high molecular weight fraction contributed with 27.5% having a molecular weight (M_w) of 13.000 corresponding to an average degree of polymerisation of 72 and the low molecular weight fraction contributed with 73% having a M_w of 302 corresponding to an average degree of polymerisation of 1.7. At 30 FPU/g DM enzyme loading, 55 % of these hemicellulose components were converted to xylose and arabinose monomers. Celluclast was shown to have significant β -xylosidase activity and thus has the ability to hydrolyze the hemicellulose present as xylan (31). The hydrolysed hemicellulose can be fermented by a C5 yeast or bacterium to ethanol or by lactobacillus to lactic acid (32, 30). Alternatively, the polymeric hemicellulose may be recovered and used as filler in plastics (33).

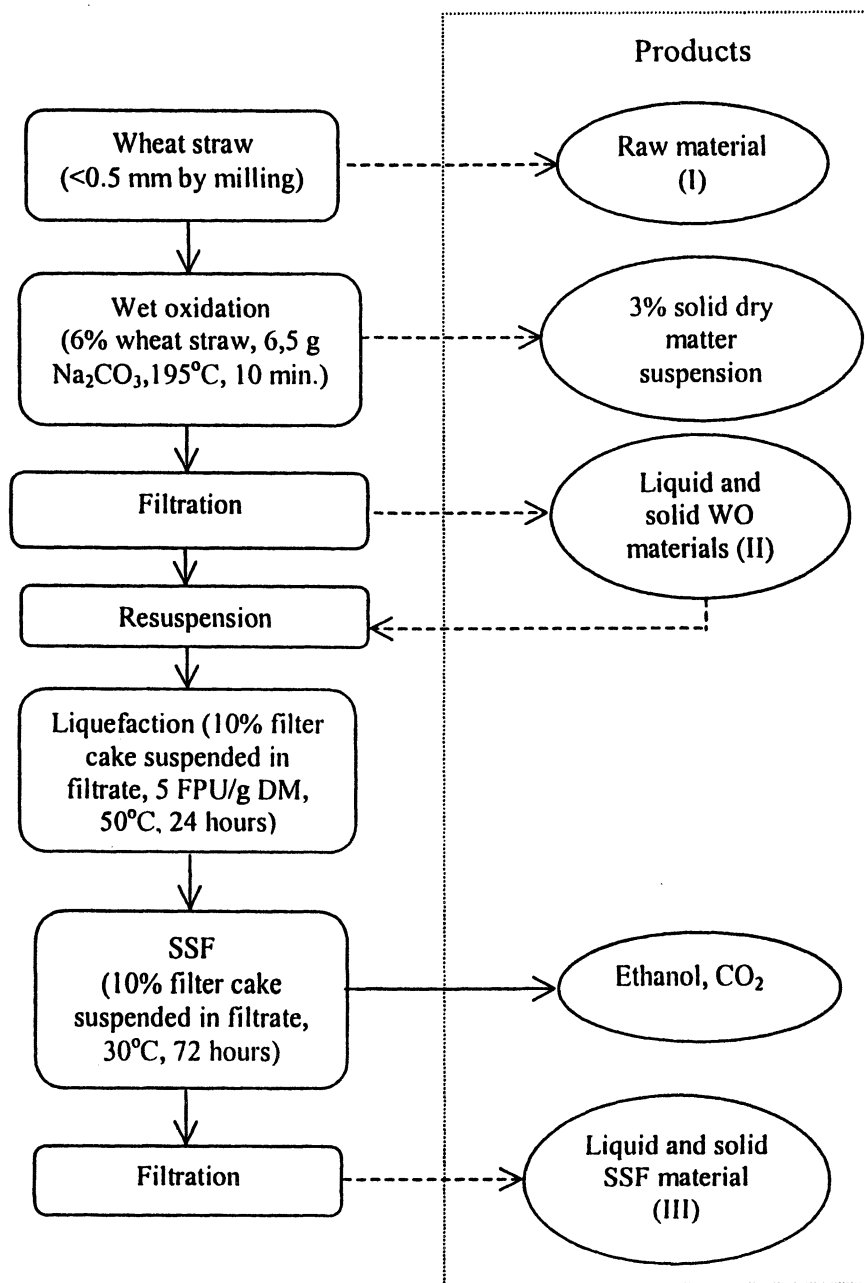


Figure 1. Process diagram for the treatment of wheat straw by wet oxidation followed by SSF (simultaneous saccharification and fermentation) with indication of samples drawn analyses (I, II, III).

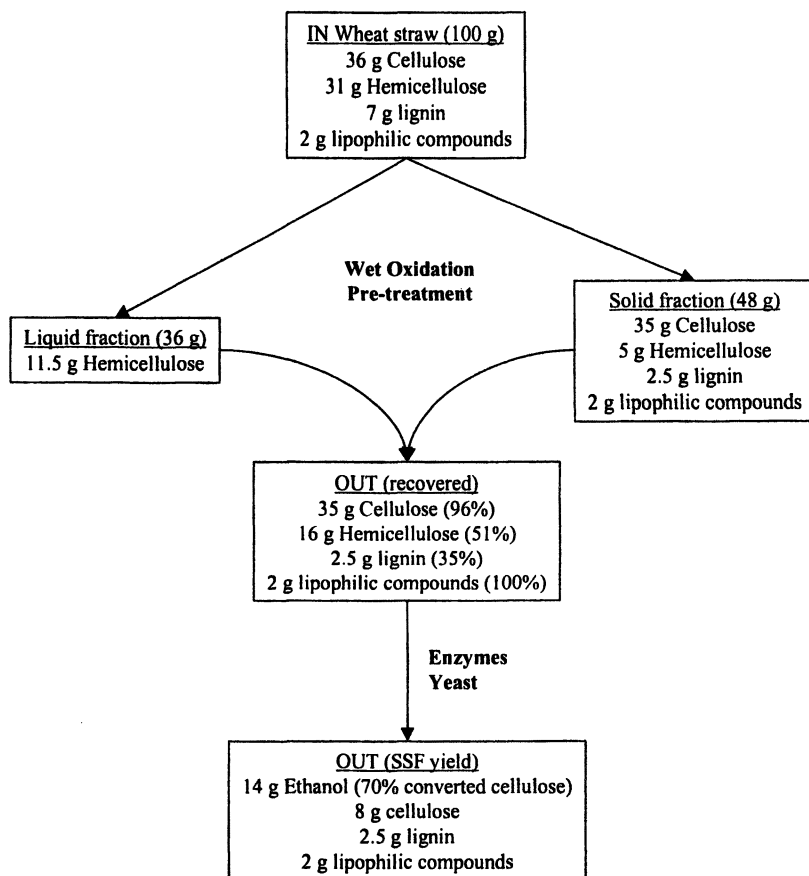


Figure 2. Mass balance for wet oxidation and simultaneous saccharification and fermentation (SSF) of cellulose, hemicellulose, lignin and lipophilic components.

Table 1. Chemical composition of wheat straw, wet oxidized (WO) solid and liquid fractions, and SSF solid material.

	Wheat straw (%)	WO Solid fraction (%)	WO Liquid fraction (%)*	SSF solid residue (%**)
Cellulose	36	72	-	NA
Hemicellulose	31	10	33	NA
Lignin	7	5	-	NA
Non cell wall material	16	2	-	NA
Organic acids	-	-	17	-
Polyphenols	-	-	18	-
Phenols	-	-	2	-
Wax	2	4	-	10
Other	-	-	30	-
Ash	8	6	1	-

NA = not analysed. *Determined on total organic carbon (TOC) basis, ash not included (13). A gravimetric composition of the liquid fraction is not possible because of volatile and semi-volatile components like carboxylic acids.

Liquefaction and SSF

After wet oxidation, the solid material in hydrolysate contributed with only 3 % dry matter due to hydrolysis and oxidation of hemicellulose and lignin. In order to investigate SSF at a high solid ratio the wet oxidized hydrolysate was filtered before resuspending the filter cake in the wet oxidized filtrate (Figure 1). Thereby a solid fraction of 10 % pretreated wheat straw was obtained for the SSF. Pretreated wheat straw at dry matter contents at 10 % or higher introduces a high viscosity with only limited mixing when treated in a simple lab shaker without forced agitation. Therefore a liquefaction/ presaccharification stage with cellulases was introduced prior to the fermentation, in analogy to the liquefaction of starch used for fermentation at dry matter levels of 30 % or higher (Figure 1). The liquefaction was achieved by adding 5 FPU of Celluclast/g DM at 50°C for 24 hours. This gave a good solubilisation of solids and resulted in a suspension with low viscosity and rich in fermentable carbohydrates whereupon the SSF process was initiated.

The fermentation curve is shown in Figure 4. At the highest enzyme loading, 70 % of the cellulose was converted to ethanol corresponding to a final ethanol concentration of 56 g/L. This is significantly higher than the ethanol yield of 38 g/L using 12.5 % pretreated wheat straw as obtained in work of Mohagheghi et al (22). More importantly, the experiments of Mohagheghi et al (22) were made using washed filter cakes with no hydrolysate added. Our results indicate that the cellulose from wet oxidized wheat straw is available to enzymatic hydrolysis.

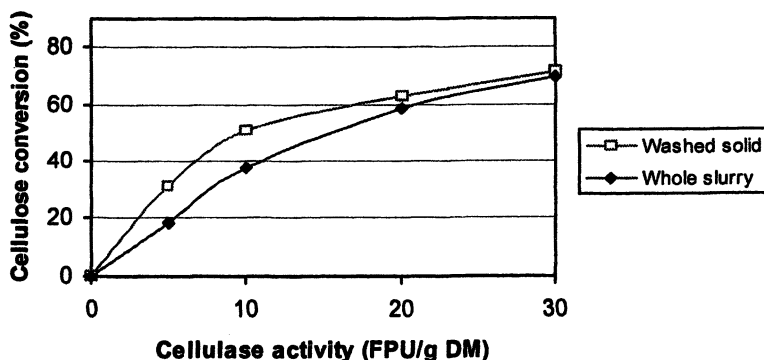


Figure 3. Enzymatic hydrolysis of the cellulosic fraction of wet oxidized (WO) wheat straw in buffer (washed solid) and in liquid fraction of WO wheat straw. Hydrolysis was done at 2% consistency, pH 4.8 and 50°C.

Furthermore, at the applied concentrations the hydrolysate has no negative influence on the fermentation rate in accordance with the enzyme hydrolysis tests at the highest enzyme loadings (Figure 2). At the lowest enzyme loading (5 FPU/g DM) a lower rate of SSF was observed probably due to enzyme inhibition in accordance with the enzymatic tests. At 10 and 20 FPU/g DM loadings the rates of fermentation were similar for the first 24 hours and more than 85 % of the total fermentation was completed after 48 hours. These results are in accordance with the testing of the enzymatic convertibility of cellulose, where 72 % cellulose was converted in 24 hours at 50 °C at 30 FPU/g (Figure 4).

The SSF process was divided into two phases. The first phase was characterized by a fast fermentation rate during the first 48 hours and the second phase by a slow fermentation rate during the next 100 hours after which the SSF was stopped (Figure 3). The first rapid fermentation phase illustrated by CO₂ loss is similar to that of the first 24 hours in the enzyme tests at 10 and 20 FPU/g DM measuring the glucose liberation. The rate of fermentation in the first 24 hours is close to what can be achieved in a similar lab-scale set-up using starch as a substrate (34). Thus, there was no limiting factor of cellulose accessibility to the enzymatic hydrolysis during the first period of hydrolysis. The decrease in hydrolysis rate after 48 hours indicate that the cellulose can be divided into two pools: High accessible cellulose (approximately 70%) and low accessible cellulose (approximately 30%). The composition of these two pools from the wet oxidation pre-treatment is yet to be described, but some of the factors to be considered are the cellulose crystallinity and the remaining lignin and hemicellulose in the solid fraction (Table 1). These fractions are most likely closely associated with the cellulose and as such they may be responsible for the

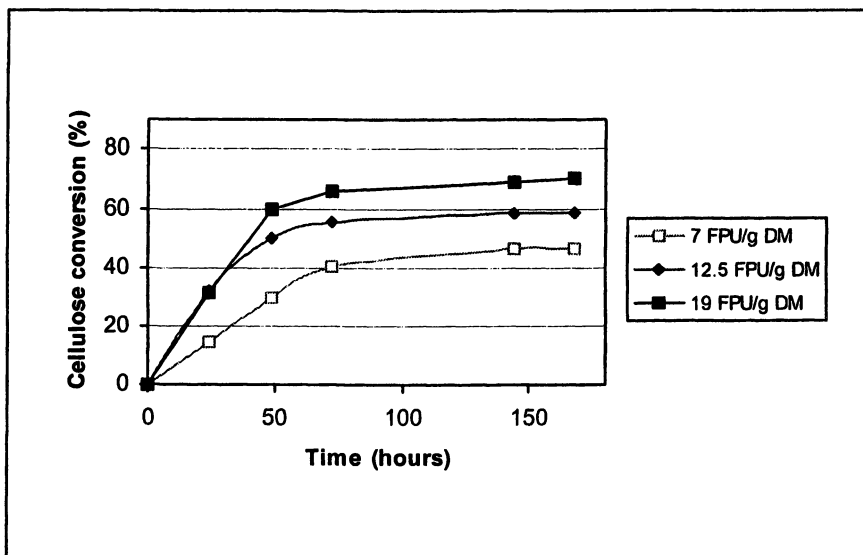


Figure 4. Cellulose conversion during hydrolysis and fermentation of solid fraction.

low accessible cellulose fraction. As the enzyme preparations used (Celluclast and Novozyme 188) also contained hemicellulases, as previously discussed, these may have improved the accessibility of cellulose to enzymatic hydrolysis.

The fermentations were done at 10 % dry matter of the solid fraction, however, the concentration of soluble compounds in the added liquid fraction corresponded to only 6% dry matter. The liquid fraction from alkaline wet oxidized wet straw was previously found not to inhibit the ethanol productivity of *Saccharomyces cerevisiae* up to a level corresponding to 17% dry matter content of wheat straw (13). Hence, the soluble compounds found in the liquid fraction are considered negligible with respect to yeast inhibition in a set-up using more concentrated hydrolysate.

Any evaluation of cellulose-based processes for ethanol production should be compared to the present technology used for hydrolysis and fermentation of starch, as this is the most cost-effective process used at present. The technical specifications for the starch based process show fermentation efficiencies higher than 90 % and dry matter contents in the hydrolysis and fermentation of 30 % or more (35). If cellulose is to be an economically attractive substrate for ethanol production, fermentation efficiency and dry matter content must approach the starch process. This means that the dry matter content must be increased by a factor of 2-3 and that cellulose accessibility must be improved by further 10-20 %. Such a development requires improved technologies and processes e.g. more efficient enzymes. Another aspects to be considered for high dry matter fermentation is the yeast viability. Fermentation at 25-35% dry matter introduces several new stress factors to the yeast, of which one of them is osmotic pressure (36). Compared to starch raw materials, cellulose is a nutrient and protein deficient substrate, hence, the yeast should be supplied with the nitrogen and minerals necessary for its optimal growth.

Lipophilic extractives retained in the solid products

The residue from SSF has a high lignin content and could be burnt for energy production because of its high heat of combustion. However, it was observed that the lipophilic extractives found in the raw material were retained in the solid fraction during wet oxidation pre-treatment, enzymatic hydrolysis and fermentation (Figure 2, Table 1). Lipophilic extractives analyzed in the untreated and wet oxidized wheat straw consisted of mainly triglycerides and free fatty acids primarily C16, C18, C18:1, C18:2. Palmitic acid (C16) was the main fatty acid of the wax in both the untreated and wet oxidized wheat straw. Thus, the

reaction conditions used for wet oxidation pre-treatment of wheat straw did not oxidize the lipophilic components. It should be noted that the wet oxidation process was originally developed for decomposing polluted materials in wastewater, sludge, and polluted soil (37), therefore it was surprising to find all lipophilic compounds intact after the WO pre-treatment. However, the reaction temperature for the pre-treatment of biomass by wet oxidation is considerably lower (150-195°C) than that originally used for the treatment of pollutants (260-300°C).

After SSF fermentation, the lipophilic components were still present in the fiber residues. Thus, following wet oxidation and SSF, the content of lipophilic components in the dry matter was increased from 2 % in the raw material to 4 % and 10 %, respectively, in the treated fibers (Table 1). It is well known that plant waxes and fatty acids are used for cosmetics e.g. in soap and shampoo, thus this process offers the possibility to produce these potentially value-added products in a more concentrated form.

Conclusion

Cellulose from wheat straw can be effectively used to produce ethanol in a SSF process using wet oxidation as pre-treatment. The ethanol yield was 56 g/L using 10% DM of wet oxidized solids as substrate in SSF. Enzyme tests showed that components (probably polymeric lignin) in hydrolysates decreased the cellulose hydrolysis rate at low enzyme addition. Additionally, enzymes were used for hemicellulose hydrolysis. At high enzyme leading no inhibition was observed. A liquefaction stage was introduced prior to SSF using low enzyme addition (5 FPU/g DM) facilitating good mixing conditions in the SSF. Lipophilic extractives in wheat straw were unaffected by both wet oxidation and SSF and was present in the solid residues in a concentrated form.

Acknowledgement

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References

1. Wyman, C.E. Ethanol from lignocellulosic biomass – Technology economics and opportunities. *Bioresource Technology*. **1994**, 50: 3-16.
2. Wyman, C.E. Twenty years of trials, tribulations, and research progress in bioethanol technology - Selected key events along the way. *Applied Biochemistry and biotechnology*. **2001**, 91: 5-21.
3. Wheals, A.E.; Basso, L.C; Alves, D.M.G.; Amorim, H.V. Fuel ethanol after 25 years. *Trends in Biotechnology*. **1999**, 17: 482-487
4. Boisset, C.; Petrequin, C.; Chanzy, H.; Henrissat, B.; Schulein M. Optimized mixtures of recombinant *Humicola insolens* cellulases for the biodegradation of crystalline cellulose. *Biotechnology and Bioengineering*. **2000**, 72: 339-345
5. Clark, T.A.; Mackie, K.L. Steam explosion of the softwood *Pinus Radiata* with sulfur dioxide addition, I. Process optimization. *Journal of wood Chemistry and Technology*, **1987**, 7: 373-381.
6. Wu, M.M.; Chang, K.; Gregg, D.J.; Boussaid, A.; Beatson, R.P.; Sessler, J.N. Optimization of steam explosion to enhance hemicellulose recovery and enzymatic hydrolysis of cellulose in softwoods. *Applied Biochemistry and Biotechnology*. **1999**, 77-79:47-54.
7. Chum, H.L.; Douglas, L.J.; Feinberg, D.A.; Schroeder, H.A. Evaluation of steam explosion to enhance hemicellulose recovery and enzymatic hydrolysis of cellulose in softwoods. pretreatments of biomass for enzymatic hydrolysis of cellulose. National Renewable Energy Laboratory. Golden CO. 1985.
8. Holtzapfle, M.T.; Jun, J.H.; Ashok, G.; Patibandla, S.L.; Dale, B.E. The ammonia freeze explosion (AFEX) process: A practical lignocellulosic pretreatment. *Applied Biochemistry and Biotechnology*. **1990**, 28/29: 59-64
9. Reyes T, Bandyopadhyay SS and McCoy PJ. Extracton of lignin from wood with supercritical alcohol. *Journal of Supercritical Fluids*. **1989**, 22: 80.

10. Himmel, M.E.; Adney, W.S.; Baker, J.O.; Elander, R.; McMillan, J.D.; Nieves, R.A.; Sheehan, J.J.; Thomas, S.R.; Vinzant, T.B.; Zhang, M. Advanced bioethanol production technologies: A perspective. *Fuels and Chemicals from Biomass. ACS symposium Series 666*: 2-45. 1997.
11. Bjerre, A.B.; Olesen, A.B.; Fernqvist, T.; Plöger, A.; Schmidt, A.S. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. *Biotechnology and Bioengineering*. 1996 49: 568-577.
12. Klinke, H.B.; Ahring, B.K.; Schmidt, A.S.; Thomsen, A.B. Characterization of degradation products from alkaline wet oxidation of wheat straw. *Bioresource Technology*. 2002 82: 15-26.
13. Klinke, H.B.; Olsson, L.; Thomsen, A.B.; Ahring, B.K. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: Wet oxidation and fermentation by yeast. *Biotechnology and Bioengineering*. In press.
14. Ahring, B.K.; Thomsen, A.B.; Continuous conversion of solid lignocellulosic biomass to ethanol by subjecting biomass slurry to high temperature and/or oxygen enriched atmosphere, hydrolyzing, fermenting, separating ethanol and treating wastewater. Patent WO200160752-A1. 2001.
15. Mes-Hartree, M.; Saddler, J.N. The nature of inhibitory materials present in pretreated lignocellulosic substrates which inhibit the enzymatic hydrolysis of cellulose. *Biotechnology Letters*. 1983, 5:531-536.
16. Sinitsyn, A.P.; Clesceri, L.S.; Bungay, H.R. Inhibition of cellulases by impurities in steam-exploded wood. *Applied Biochemistry and Biotechnology*. 1982, 7:455-458.
17. Fox, D.J.; Gray, P.P.; Dunn, N.W.; Marsden, W.L. Comparison of alkali and steam (acid) pretreatments of lignocellulosic materials to increase enzymic susceptibility: Evaluation under optimised pretreatment conditions. *Journal of Chemical Technology and Biotechnology*. 1989, 44:135-146.
18. Mes-Hartree, M.; Dale, B.E.; Craig, W.K. Comparison of steam and ammonia pretreatment for enzymatic hydrolysis of cellulose. *Applied Microbiology and Biotechnology*. 1988, 29:462-468.

19. Spindler, D.D.; Wyman, C.E.; Grohmann, K.; Mohagheghi A. Simultaneous saccharification and fermentation of pre-treated wheat straw to ethanol with selected yeast strains and β -glycosidase supplementation. *Applied Biochemistry and Biotechnology*. **1989**, 20/21:529-54
20. Spindler, D.D.; Wyman, C.E.; Grohmann K. Evaluation of pretreated herbaceous crops for the simultaneous saccharification and fermentation process. *Applied Biochemistry and Biotechnology*. **1990**, 24/25:275-286.
21. Spindler, D.D.; Wyman, C.E.; Grohmann, K. The simultaneous saccharification and fermentation of pretreated woody crops to ethanol. *Applied Biochemistry and Biotechnology*. **1991**, 28/29:773-786.
22. Mohagheghi A, Tucker M, Grohmann K, Wyman C. High solids simultaneous saccharification and fermentation of pretreated wheat straw to ethanol. *Applied Biochemistry and Biotechnology*. **1992**, 33:67-81.
23. Sun, R.C.; Sun, X.F. Identification and Quantitation of Lipophilic Extractives from Wheat Straw. *Industrial Crops and Products*. **2001**, 14: 51-64.
24. Ghose, T.K. Measurement of cellulase activities. *Pure and Applied Chemistry*. **1987**, 59: 257-268
25. Goering, H.K.; van Soest, P.J. Forage fiber analyses (apparatus, reagent, procedures and some applications). Agricultural handbook Agricultural research service, USDA, Washington, DC, 379: 1-20. 1970.
26. Hardell, H.L.; Nilvebrant, N.O. Analytical pyrolysis of spruce milled wood lignins in the presence of tetramethylammonium hydroxide. *Nordic Pulp and Paper Research Journal*, **1996**, 2: 121-126.
27. Detroy, R.W.; Cunningham, R.L.; Bothast, R.J.; Bagby, M.O.; Herman, A. Bioconversion of wheat straw cellulose/hemicellulose to ethanol by *Saccharomyces uvarum* and *Pachysolen tannophilus*. *Biotechnology and Bioengineering*. **1982**, 24: 1105-1113.
28. Nidetzky, B.; Steiner, W.; Hayn, M.; Esterbauer, H. Enzymatic hydrolysis of wheat straw after steam pretreatment: Experimental data and kinetic modelling. *Bioresource Technology*. **1993**, 44: 25-32.

29. Converse, A.O. Substrate factors limiting enzymatic hydrolysis. In: *Bioconversion of Forest and Agricultural Plant Residues*, eds. JN Saddler. CAB International, Oxon, UK. 1993
30. Thomsen, A.B.; Klinke, H.B.; Hoffmeyer, P.; Lilholt, H. Materials derived from biomass concepts and examples. *Proceedings of the 23th Risø International Symposium on Material Science: Sustainable Natural and polymeric composites – Science and Technology*. Editors: H. Lilholt, B. Madsen, H.L. Toftegaard, E. Cendre, M. Megris, L.P. Mikkkelsen, B.F. Sørensen Risø National Laboratory, Roskilde, Denmark, 2002. Pp 77-91.
31. Thygesen, A.; Thomsen, A.B.; Schmidt, A.S.; Jørgensen, H.; Ahring, B.K.; Olsson, L. Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. *Enzyme and Microbial Technology*. In press.
32. Zaldivar, J.; Nielsen, J.; Olsson, L. *Applied Microbiology and Biotechnology*. 2001, 56: 17-34.
33. Amash, A.; Zugenmaier, P. Study on cellulose and xylan filled polypropylene composites. *Polymer Bulletin*. 1998, 40: 215-258.
34. Wang, S.; Ingledew, W.M.; Thomas, K.C.; Sosulski, K.; and Soulski, F. W. Optimization of fermentation temperature and mash specific gravity for fuel alcohol production. *Cereal Chemistry*. 1999, 76:82-86.
35. Olsen, H.S.; Andersen, E. Utilization of enzymes in agricultural product processing. *Proceedings of the International Conference on Agricultural Science and Technology*, Nov 7-9 2001, China; 52-67.
36. Thomas, K.C.; Hynes, S.H.; Ingledew, W.M. Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. *Process Biochemistry*. 1996, 31: 321-331
37. Thomsen, A.B. Degradation of quinoline by wet oxidation: Kinetics and reaction mechanisms. *Water Resources*. 1996, 136-146.

Chapter 11

Siderophores as Natural Mediators in Laccase-Aided Degradation of Lignin

M-L. Niku-Paavola¹, H. Anke², K. Poppius-Levlin³,
and L. Viikari¹

¹VTT Biotechnology P.O. Box 1500, FIN-02044 VTT, Finland

²Institut fuer Biotechnologie und Wirkstoff-Forschung c/o University of
Kaiserslautern, D-67663 Kaiserslautern, Germany

³KCL, Science and Consulting, P.O. Box 70, FIN-02151 Espoo, Finland

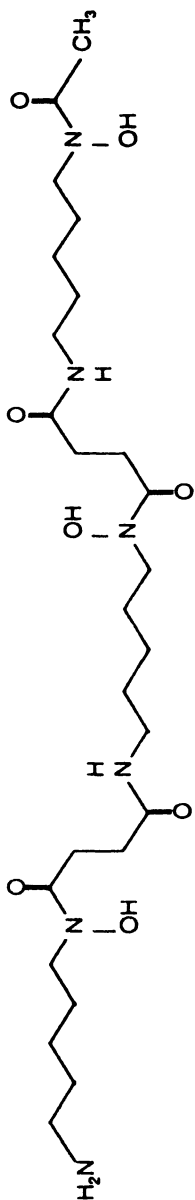
Hydroxamate siderophores, microbial metal chelating agents have structural similarities with synthetic mediators successfully used in laccase-aided delignification processes. The iron-free forms of fungal hydroxamate siderophores; desferrioxamine B, desferritriacetylfusigen and nocardamin, were shown to have mediator characteristics in laccase catalyzed oxidative reactions. Ferricrosin, an iron-containing form of siderophore, did not react with laccase. It was concluded that the iron-binding structures, free hydroxyl groups in the hydroxamate siderophores, are the targets for laccase. GPC, ¹H NMR and ¹³C NMR analysis indicated degradation and modification of siderophores during oxidation by laccase. The reaction mechanism differs from the corresponding reactions of synthetic mediators. When compared on molar basis, the ability of the oxidized siderophore to degrade lignin was better than that obtained by the synthetic mediator 1-hydroxybenzotriazole (HBT), due to the higher number of N-OH-groups in the siderophore molecule. The role of siderophores as natural mediators in delignification and as models for mediators is thus worth of further evaluation.

Introduction

In nature white-rot fungi are the most efficient lignin degrading organisms. During growth on lignin containing substrates they secrete several enzymes capable to modify lignin substructures [reviewed in (1)]. The role of individual enzymes in lignin degradation has been studied intensively in order to develop environmentally friendly delignification processes. Along with peroxidases, laccases (benzenediol: oxygen oxidoreductase EC 1.10.3.2) are the most important enzymes participating in delignification by white rot fungi. When used without additives, purified laccases mainly polymerize lignin *in vitro*. However, when suitable, electron transferring additives are provided for laccases in the reaction, true delignification takes place (2, 3). In this laccase-mediator concept, the primary substrate of laccase mediates the oxidation towards the secondary substrate, *i.e.* lignin.

Mediated oxidation by laccase has been shown to be a specific and efficient delignification method. Over 55% delignification of pine kraft pulp was achieved (4) and ozone and oxygen stages in TCF bleaching could successfully be replaced by the laccase-mediator system when using HBT (5). The mediated oxidation concept has been shown to be effective also for elimination of toxic environmental wastes, for chemical synthesis and textile applications (6, 7, 8, 9). The first mediators introduced were 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and 1-hydroxybenzotriazole (HBT). During laccase action both form radicals with high redox potential and good stability, the former dication (10) and the latter nitroxide radicals (11). Several mediators are able to delignify efficiently, but still suffer from drawbacks, such as high costs, for larger scale applications. New, more effective mediators have been screened to decrease production costs and to minimize eventual environmental risks.

Natural compounds present or secreted in the environment of white rot fungi could be potential candidates as mediators. Indeed, several low molecular weight compounds secreted by white rot fungi have been shown necessary for lignin degradation, such as veratryl alcohol (12) and aliphatic carboxylic acids (13). However, until now only one metabolite of a white rot fungus, 3-hydroxyanthranilate, has been introduced as a natural mediator (14). It has a hydroxyl and an amino group as targets for laccase oxidation. Siderophores, fungal and bacterial metabolites responsible for microbial iron chelation are classified as hydroxamates, phenolates and catecholates (15). Iron chelating compounds are commonly produced by wood-decaying fungi (16). Close structural similarities exist between the synthetic mediators and natural hydroxamates which contain several N-OH, C=O and NH groups in one molecule. Two of the siderophores studied in this work are shown in Figure 1. Both siderophores consist of the same basic three-subunit structure.

**DESFERRIOXAMINE B**

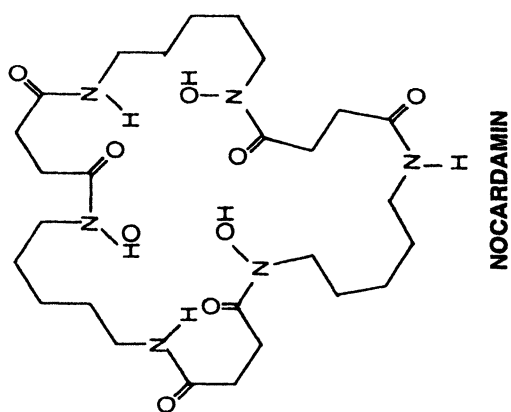


Figure 1. Structure of the siderophore desferrioxamine B and nocardamin.

In this work, the potential of hydroxamate siderophores acting as mediators for delignification was studied. The reactivity of siderophores as laccase substrates was analyzed and the reaction products were monitored by GPC, ^1H NMR and ^{13}C NMR. The reactions with laccase, siderophores and pulp were followed by reactivity measurements based on oxygen consumption and by efficiency in one-step alkaline delignification. The known synthetic mediators HBT and ABTS were used as references.

Materials and Methods

Enzyme

The laccase was partially purified from *Trametes hirsuta* strain VTT-D-443 as described previously (17). The enzyme was dosed using ABTS as substrate (18).

Substrates

The commercial hydroxamates studied were desferrioxamine B mesylate (DFA) (Sigma), glycine hydroxamate (GHA) (Sigma), tyrosine hydroxamate (THA) (Sigma) and hydroxylamine (HA) (Baker). Desferriacetylfulsigen (DAF), nocardamin (Noc) and ferricrosin (Frc) were produced and purified as described (19, 20). Reference mediators were HBT (Sigma) and ABTS (Boehringer).

Pine kraft pulp (kappa 25, brightness 27 (ISO), viscosity 1060 ml/g) was produced in laboratory scale.

The reactivity of substrates

The reactivity of substrates was measured as oxygen consumption during the reaction with laccase in closed erlenmeyer flasks using a Clark type oxygen electrode (Orion 97-08) (17). The reactivities were expressed as initial reaction rates (O_2 $\mu\text{mol}/\text{l}/\text{min}$) calculated from the first reaction minutes and as relative reactivity (O_2 moles consumed/mole of substrate oxidized after 15 minutes reaction). The measurements were performed in reaction conditions where neither laccase dose or O_2 concentration limited the reaction: the mediator concentration was low (0.1 mM) and laccase dose was high (20 $\mu\text{kat}/\text{l}$). The pulp consistency in reactivity measurement was 0.5% and laccase dose 4000 nkat/ g pulp (d.w). Reactions were performed in 25mM succinate buffer, pH 4.5 at 20°C.

Pulp treatments

Pulp was chelated before laccase treatment to eliminate the ferric ions disturbing the reactions of siderophores. Chelation conditions were: pulp consistency 10%, sodium salt of EDTA 0.4% on pulp, pH 2-3, 80°C 1h.

Delignification efficiency of siderophores was assayed using the previously optimized conditions of laccase-mediated oxidation (21). Laccase dose was 100 nkat per g pulp (d.w.), mediator concentrations were HBT 3%, DFA 4% and nocardamin 2.5% of pulp d.w. Delignification conditions were: pulp consistency 10%, pH adjusted to 4.5 with sulfuric acid, oxygen 3 bar, 45°C 2h, alkaline extraction with 1% NaOH, 60°C 1h.

Analytical methods

For the gel permeation chromatography (GPC) analysis, 1.5 mM siderophore (DFA) in 25 mM succinate buffer pH 4.5 was treated with laccase at a dose of 5 μ kat/l at 20° C for 2h. The reaction mixture was mechanically stirred and infused with oxygen. The relative molar mass distributions were analysed by using the method originally developed for lignin with Fractogel TSK HW-55 in 0.5 M NaOH (21).

For NMR spectroscopy, 10 mg DFA (15 μ mol) was dissolved in 1 ml water, pH was adjusted to 4.5, and siderophore was treated with laccase dose of 2.5 μ kat/l. The reaction mixture was mechanically stirred and infused with oxygen at 20° C for 20h. The preparation was finally freeze-dried and thereafter dissolved in D₂O. The siderophore concentration in reference sample was 10 mg per ml D₂O.

¹H NMR and ¹³C NMR analyses were performed at VTT Chemical Technology by Dr. E. Ämmälähti. Qualitative ¹H NMR and COSY spectra were recorded at 35° C at 600 MHz and ¹³C NMR spectra at 150 MHz on a Varian Unity instrument.

Viscosity, brightness and kappa number of the pulps were analysed according to SCAN-C15:1988, ISO 2470 and SCAN -C1-1977, respectively.

Results

Oxidation of siderophores by laccase

The reactivities of siderophores with laccase were demonstrated by oxygen consumption measurements (Figure 2). Laccases catalyze the four-electron reduction of oxygen to water with four concomitant one-electron oxidations of the reducing substrate molecule(s).

The iron-free forms of siderophores; DFA, DAF and Noc were shown to be substrates of laccase. Frc, the iron-containing form of the siderophore, as well as the siderophore analogues GHA, THA and HA did not react with laccase.

The initial reaction rate in laccase catalyzed oxidation of the siderophore Noc was as high as that of the reference mediator ABTS in spite of the higher molecular weight and cyclic structure of the former. The reaction curve shows that the oxidation of ABTS was completed already within the first minute. During the oxidation of 0.1 mM ABTS 0.9 mg oxygen/l, corresponding to 0.028 mmol oxygen/l, was reduced to water. Each ABTS molecule was thus the donor of one electron. Based on the structure of Noc, it can be anticipated that this siderophore is able to donate at least three electrons. The reaction was completed after 35 min, and altogether 0.18 mmol oxygen/l was consumed (result not shown). Thus, it seems that at least six electrons per molecule of Noc are transferred to oxygen, either in the primary enzymatic reaction or in the secondary non-enzymatic reactions. It is interesting to observe that DFA, having the same structural groups as Noc but aliphatic backbone, was a much worse substrate for the laccase. The ability of the laccase to oxidize HBT was surprisingly poor compared to the very high efficiency of HBT in delignification.

Because the iron-containing form of siderophore was not oxidized by laccase, it was expected that the iron-binding structures, the free hydroxyl groups in the hydroxamate siderophores, would be the target structures for laccase. This was confirmed when the initial reaction rate and the reactivity of the iron-free siderophore DFA was shown to decrease gradually by increased amounts of iron added to the laccase catalyzed reaction (Table I). The reaction was over 90% inhibited when the concentration of iron was equal to that of the siderophore.

Analysis of reaction mechanism of laccase oxidation

The oxidation mechanism of DFA by laccase was more closely followed by analyzing the reaction products of DFA during the oxidation by gel permeation chromatography (Figure 3). The molecular mass of DFA is 657 Da, and it consists of three subunits. In the chromatogram, DFA is seen as a single peak at the start of the reaction. After oxidation by laccase, DFA was partially degraded into components, with lower molecular masses than the parent molecule. No polymerisation as with the synthetic mediators (21) was observed during the oxidative reaction by laccase.

The ^{13}C NMR analysis of DFA after laccase oxidation (Figure 4) clearly indicated differences in the area of carbonyl groups (180-190 ppm). Also the signals on the CH/CH₂-area (30-40 ppm) were increased, as compared to the spectrum of the original DFA. This is in accordance with the ^1H NMR spectrum of the corresponding samples (Figure 5). The effect of laccase oxidation can be seen in displacement of the H₁' signal from 2.59 to 2.31 ppm and the H₂' signal from 2.29 to 2.23 ppm. This indicates modification in the area of the (CH₂)₂-repeating unit of DFA. A new signal appeared at 1.71 ppm, very close to the methyl signal of DFA. The identification of this signal would demand additional studies. The N-OH groups of the synthetic mediators were shown to be converted to NH-groups in the laccase catalyzed oxidation (22). Surprisingly, no new NH-groups could be detected in COSY-spectra of DFA (results not shown).

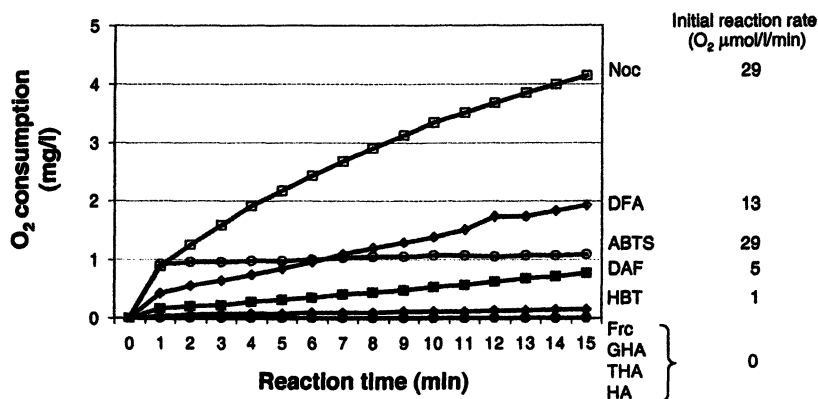


Figure 2. Oxidation of siderophores, siderophore analogues and reference mediators by laccase.

Table I. Inhibition of laccase catalyzed oxidation of siderophore by iron*.

Concentration of FeCl ₃ in reaction mixture mM	Initial reaction rate O ₂ consumption μmol/l/min	Relative reactivity oxygen/DFA mol/mol (after 15 min reaction)	Inhibition %
0	13	0.44	0
0.01	5	0.43	2
0.05	5	0.24	45
0.10	1	0.02	95

*Siderophore (DFA) concentration 0.1mM

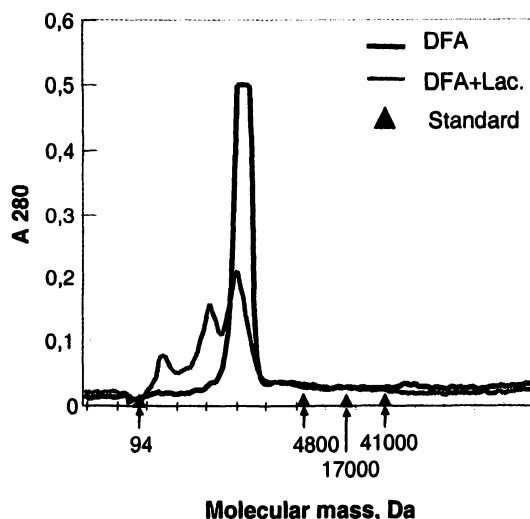


Figure 3. Molecular mass distribution of DFA in laccase reaction.

Oxidation and delignification of fibres

The oxidation of siderophore (DFA), kraft fibres and their combinations by laccase was followed by using the oxygen consumption measurement method (Figure 6). The relative reactivity of kraft fibres appeared to be quite low, only roughly 0.1 mg oxygen/l was consumed corresponding to 0.003 mmol oxygen/l. The amount of lignin calculated from the kappa number of the fibres was 0.19 g/l. Previous analyses had stated 2mM phenolic units /g of this kraft lignin (21). Thus, the concentration of phenolic hydroxyl groups was 0.38 mM and only 3% of them were oxidized by the enzyme alone. Taking into account the limited accessibility and reactivity of fibre bound lignin by laccase alone, this is not an unexpected result. In the presence of both DFA and fibres, the oxygen consumption by laccase was significantly increased. The calculated sum of the individual oxygen consumption of fibres and DFA was, however, only about half of the measured oxygen consumption of the combined system. This gives evidence that oxidation of the siderophore by laccase in the presence of a secondary substrate (fibres) may undergo redox recycling. The increased oxygen consumption in the presence of the secondary substrate implies that oxygen may be consumed to reoxidation of the siderophore mediator, and/or to secondary enzymatic or non-enzymatic oxidative reactions of the partially oxidized lignin.

Due to the competitive reactions between iron chelation and oxidation of siderophores by laccase, the metals from the pulp were removed before the laccase-siderophore treatments. Table II shows the results of the delignification

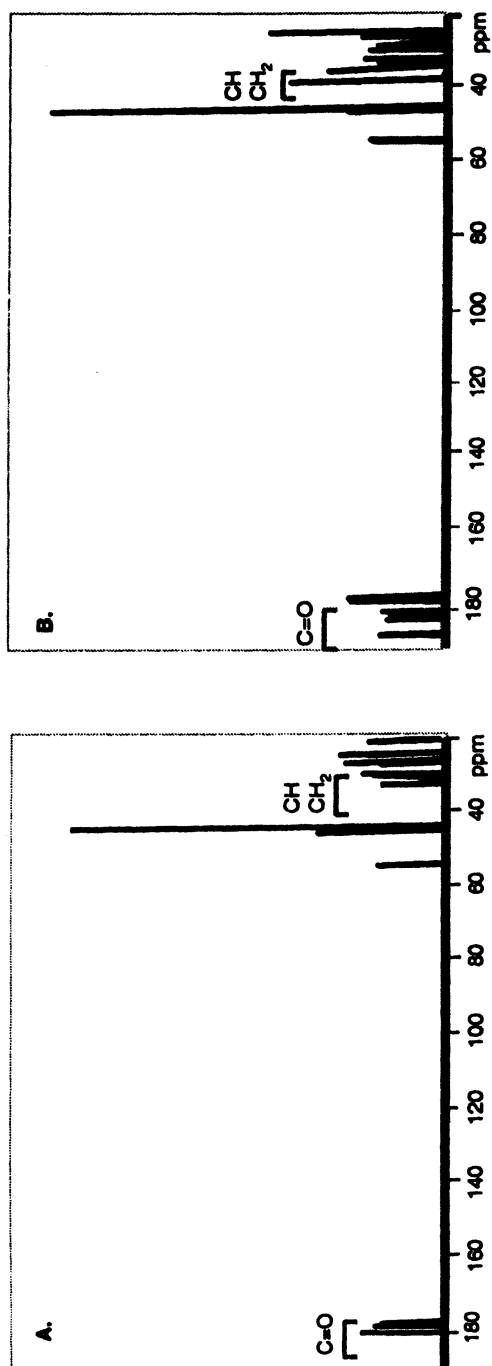


Figure 4. ^{13}C NMR spectra of DFA in laccase reaction.
A = DFA, B = DFA + Laccase.

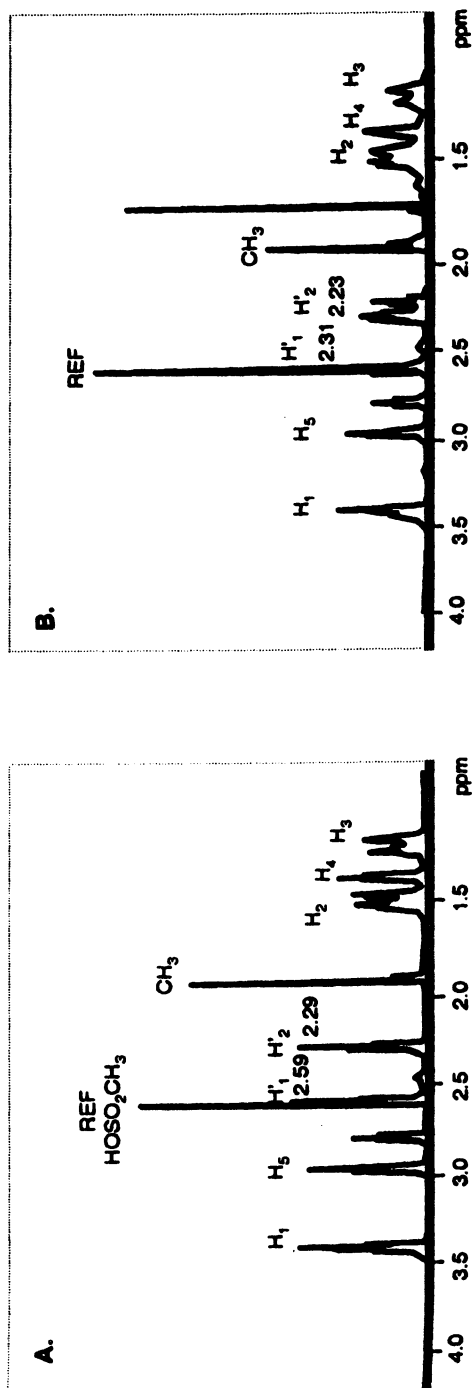


Figure 5. ¹H NMR spectra of DFA in laccase reaction. A = DFA, B = DFA + Laccase. Proton series in DFA are indicated in the upper part of the figure.

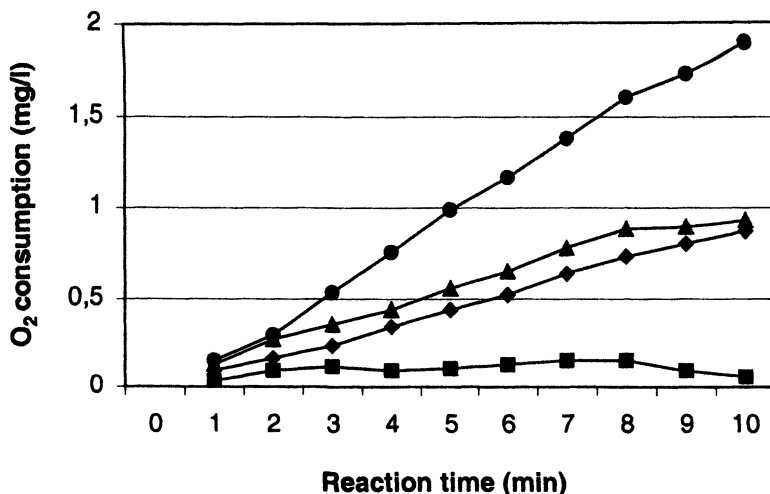


Figure 6. Oxidation of kraft fibres (■-■), siderophore DFA (◆-◆) and their combination (●-●) by laccase. The calculated sum of the separate oxidation of fibres and DFA (▲-▲).

efficiency of siderophores, as compared with HBT. After the enzyme-mediator treatment, only a mild alkaline extraction was performed. Thus, the kappa numbers may give more evidence to the effects of the treatments than the brightness values which were not essentially increased during the mild treatment conditions. The reduction in kappa number was 32% with HBT and 12-20% with siderophores. While the brightness values were essentially not improved under the test conditions, the viscosity values implied that the siderophore mediators were specific towards lignin degradation. When compared on molar basis the siderophores seemed to be more effective which can be explained by their higher molar electron transferring capacity.

Table II. Delignification of kraft pulp by HBT or siderophores oxidized by laccase.

Mediator, % of d.w.	Mediator, mmol per g d.w.	Kappa no	Brightness %	Viscosity ml/g
None		25	27	1060
HBT 3%	0.2	17	27	1010
DFA 4%	0.06	20	29	1070
Noc 2.5%	0.04	22	29	1070

Discussion

Iron-free forms of the natural mediators, hydroxamate siderophores were shown to share some of the common characteristics of synthetic, delignifying mediators. The efficiency of siderophores as delignifying mediators was not as good as that of the reference mediator, HBT. However, when compared on molar basis, the efficiency of siderophores was more pronounced which can be explained by their higher molar electron transferring capacity. As indicated previously, the efficiency of any mediator molecule in the delignification is a sum of several characteristics (22). The laccase used in this work was from *Trametes hirsuta* which belongs to the high redox potential group of laccases. Previously, it has been shown that laccases show differences in their substrate specificities for various mediators (23).

When compared with the oxidation of the common substrate for laccases, ABTS, the siderophore nocardamin, consisting of a trimeric structure, showed equally good characteristics. Obviously, due to its structure, this natural mediator had a higher molar capacity to transfer electrons which was highlighted in the oxygen transfer rates. During the oxidation of siderophores with trimeric structures by laccase, the molecule was partially split obviously into dimeric and monomeric subunits. This is different from the synthetic mediators which are polymerised by the laccase action (21).

It is obvious that the iron-binding structures, free hydroxyl groups in the hydroxamate siderophores, are the targets for laccase. Only iron-free forms of siderophores reacted with laccase and the initial reaction rate as well as the reactivity of the siderophore decreased gradually when increased amounts of iron were added to the reaction mixture. The inhibition was nearly complete when the concentration of iron was equal to that of the siderophore.

Previously, hydroxamate siderophore was shown to degrade lignin slightly also in the iron containing form, however, without the presence of laccase (24). The degradation of lignin in black liquor in the presence of siderophore was claimed to be due to reduction of chelated ferric iron to the ferrous stage with concomitant liberation of hydroxyl radicals. Also phenolate and catecholate siderophores are proposed to be mediators for fungal Fenton system in order to produce hydroxyl radicals and to cause non-enzymatic wood degradation (25).

The microbial delignification processes in nature may well proceed by exploitation of different components present in the decaying wood material or excreted by the fungus. All potential components for mediated oxidations are present in the biological environment; the different fungi secreting enzymes, organic acids, siderophores or other natural mediators as well as degradation products of lignocellulosics, eventually also participating in the degradative process.

Although it may not be feasible to use siderophores as industrial mediators they definitely play an important role in microbial degradative and metabolic processes in nature. They may also be useful in attempts to understand the role of chelated transition metals in the natural degradation processes and may serve

as biomimetic models for developing mediated oxidation systems for various purposes.

Abbreviations

ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate), DAF (desferritriacetylfusigen), DFA (desferrioxamine B mesylate), Frc (ferricrosin), GHA (glycine hydroxamate), HA (hydroxylamine), HBT (1-hydroxybenzotriazole), Noc (nocardamin), THA (tyrosine hydroxamate).

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References

1. Hatakka, A. *FEMS Microbiol. Rev.* **1994**, *13*, 125-135.
2. Bourbonnais, R.; Paice, M.G. *Appl. Microbiol. Biotechnol.* **1992**, *36*, 823-.
3. Call, H-P. WO 94/29510, 1994.
4. Bourbonnais, R.; Paice, M.G. *Tappi J.* **1996**, *79*, 199-204.
5. Poppius-Levlin, K.; Wang, W.; Ranua, M. *International Pulp Bleaching Conference*, Helsinki, Finland, June 1-5, 1998, pp 77-85.
6. Johannes, C.; Majcherczyk A.; Huttermann, A. *Appl. Microbiol Biotechnol.* **1996**, *46*, 313-317.
7. Potthast, A.; Rosenau, T.; Chen, C.L.; Gratzl, J.S. *J. Mol Catal. A: Chem.* **1996**, *108*, 5-9.
8. Niku-Paavola, M-L.; Viikari, L. *J. Mol Catal. B: Enzymatic.* **2000**, *10*, 435-444.
9. Schneider, P.; Pedersen, A.H. PCT World Patent Application WO95/01426, 1995.
10. Bourbonnais, R.; Paice, M.G.; Freiermuth, B.; Bodie E.; Borneman, S. *Appl. Environ. Microbiol.* **1997**, *63*, 4627-4632.
11. Potthast, A.; Koch, H.; Fischer, K. *Proc. of the 9th International Symposium on Wood and Pulping Chemistry*, Canada, Montreal, June 9-12, 1997, pp F2-1-F2-4.
12. Koduri, R.S.; Tien, M. *J. Biol. Chem.* **1995**, *270*, 22254-22258.

13. Warishii, H.; Valli K.; Gold, M.H. *Biochem. Biophys. Res. Comm.* **1991**, *176*, 269-275.
14. Eggert, C.; Temp, U.; Dean, J.F.D.; Eriksson, K-E.L. *FEBS Lett.* **1996**, *391*, 144-148.
15. Neilands, J.B. *Microbiol. Sci.* **1984**, *1*, 9-14.
16. Goodell, B.; Jellison, J.; Liu, J.; Daniel, G.; Paszczynski, A.; Fekete, F.; Krishnamurthy, S.; Jun, L.; Xu, G. *J. Biotechnol.* **1997**, *53*, 133-162.
17. Niku-Paavola, M-L.; Wang, W.; Poppius-Levlin, K.; Anke, H.; Viikari, L. *Proceedings of the 9th International Symposium on Wood and Pulping Chemistry*, Canada, Montreal, June 9 - 12, 1997, pp 80-1-80-3.
18. Niku-Paavola, M-L., Karhunen, E., Salola, P. and Raunio, V. *Biochem. J.* **1988**, *254*, 877-883.
19. Anke, H. *J. Antibiotics* **1977**, *30*, 125-128.
20. Anke, H.; Kinn, J.; Bergquist, K-E.; Sterner, O. *Biol. Metals* **1991**, *4*, 176-180.
21. Poppius-Levlin, K.; Wang, W.; Tamminen, T.; Hortling, B.; Viikari, L.; Niku-Paavola, M-L. *J. Pulp Pap. Sci.* **1999**, *25*, 90-94.
22. Freudenreich, J.; Amann, M.; Fritz-Langhals, E.; Stohrer, J. *International Pulp Bleaching Conference*, Finland, Helsinki, June 1-5, 1998, pp 71-76.
23. Pfaller, R.; Amann, M.; Freudenreich, J. *7th Int. Conf. Biotechnology in the Pulp and Paper Industry*, Canada, Vancouver, June 16-19, 1998, pp A99-A102.
24. Santiago, M.F.; Peralta-Zamora, P.; Duran, N. In *Proc. Braz. Symp. Chem. Lignins Other Wood Compon.*; Ramos, L.P., Ed.; Universidade Federal do Parana, Departamento de Quimica: Curitiba, Brazil, 1997, Vol. 6, pp 567-575.
25. Goodell, B.; Qian, Y.; Jellison, J.; Richard, M.; Qi, W. In *Biotechnology in the Pulp and Paper Industry: 8th ICBPPI Meeting*; Viikari, L.; Lantto, R., Eds.; Progress in Biotechnology; Elsevier Science B.V.: Amsterdam, The Netherlands, 2002; Vol. 21, pp 37-47.

Chapter 12

Microbial Strategies for the Depolymerization of Glucuronoxylan: Leads to Biotechnological Applications of Endoxylanases

James F. Preston¹, Jason C. Hurlbert², John D. Rice¹,
Anuradha Ragunathan¹, and Franz J. St. John¹

¹Department of Microbiology and Cell Science, P.O. Box 110700,
University of Florida, Gainesville, FL 32611-0700

²Department of Structural Biology, St. Jude Children's Hospital
Memphis, TN 38105

The hemicellulose fractions of crop residues and hardwoods are underutilized resources for conversion to alternative fuels and bio-based products. The predominant hemicellulosic polymer in these resources is 4-O-methylglucuronoxylan in which 4-O-methyl-D-glucuronopyranosyl (MeGA) residues are linked α -1,2- to 5 to 15% of the xylose residues comprising a nearly linear β -1,4-xylan backbone. Enzymatic processing of methylglucuronoxylan to fermentable xylose utilizes microbial endoxylanases, β -xylosidases and α -glucuronidases. Endoxylanases have been assigned to glycohydrolase families, GH5, GH10 and GH11, each with unique sequences and structural motifs. Based upon the detailed characterization of selected members, the GH10 and GH11 xylanases respectively generate aldotetrauronic (MeGAXXX) and aldopentaauronic (XMeGAXXX) acids, along with xylooligosaccharides, as predominant limit products (Biely, P.; Vrsanka, M; Tenkanen, M; Klupful, D.; J. Biotechnol. 1997, 57, 151-166). The GH5 xylanase from *Erwinia chrysanthemi* cleaves β -1,4-xylosidic linkages at a residue adjacent to a xylose residue that is substituted with a MeGA residue and catalyzes the formation of products, all of which retain one or more MeGA substitutions, with no release of xylose or xylobiose (Hurlbert, J.C.; Preston, J.F.; J. Bacteriol. 2001, 183, 2093-2100). This xylanase apparently recognizes the MeGA substitution site and generates

limit products that are the result of a single cleavage between substitution sites. Other GH5 xylanases identified on the basis of amino acid sequence alignments await further definition. The aldotetrauronic acid product generated by GH10 enzymes can be taken up and processed by intracellular α -glucuronidase, exoxylanase and /or β -xylosidase for conversion to fermentable substrate (Shulami, S.; Gat, G.; Sonenshein, A.L.; Shoham, Y.; *J. Bacteriol.* **1999**, *181*, 3695-3704). The products generated by the GH5 and GH11 xylanases may require secretion of β -xylosidase and/or other xylanases to form products that can be assimilated. The GH5 and GH11 enzymes may complement other enzymes secreted by the host and neighboring microorganisms, and their retention during evolution supports important roles in the strategies employed by their hosts to develop niches for growth. Secreted GH11 enzymes are likely the best candidates for engineering enzymes for biobleaching applications. Secreted GH10 xylanases, along with intracellular α -glucuronidase and β -xylosidase, are presently the best targets for engineering bacterial biocatalysts to convert hemicellulose to useful products.

Introduction

With a range of mechanisms and applications, microbial xylanases have become a subject of intensive and revealing research. The successful applications of these endoxylanases to the areas of biobleaching of Kraft pulp and food processing are important factors for expanding research into mechanisms and enzyme production (1,2,3), with a goal of developing the most efficient catalysts for different processes. The increasing interest in the utilization of renewable resources for the production of alternative fuels and biobased or "green" chemicals has brought further attention to the different enzymes with respect to their structure, function and catalytic activities for the depolymerization of complex polysaccharides comprising lignocellulosic biomass.

The targets of these enzymes are the β -1,4-linked xylans in the hemicellulose fraction, which constitutes as much as 20% of the lignocellulosic biomass of crop residues and hardwoods. The predominant carbohydrate polymer in this fraction is 4-O-methylglucuronoxylan (MeGAX_n) in which 4-O-methyl-D-glucuronopyranosyl (MeGA) residues are linked α -1,2- to xylose residues comprising a linear β -1,4-xylan backbone. Depending upon the source, 5 to 20% of the xylose residues may be substituted with MeGA. Additional substitutions with L-arabinofuranosyl and acetyl, feruloyl and p-coumaroyl moieties may occur to variable extents in MeGAX_n polymers (4,5). The complexities and variabilities of the structures of glucuronoxylans from different sources may account in part for the evolution of different families of xylanases

that have different substrate specificities. Of equal importance may be the processing of substrate to product that may be taken up and metabolized by the xylanase-producing organism, or secretion of an enzyme that is able to complement other glycoside hydrolases and lyases to digest and solubilize the polymers within the lignocellulosic fraction.

The strategies employed by different microorganisms to digest xylans and utilize the released carbohydrate differ significantly, not only with respect to the xylanolytic catalysts, but also with respect to the location of the catalytic domain relative to cellulose and other carbohydrate binding domains (6,7). Such domains affect the interaction of an enzyme with the complex substrates that comprise the structural lignocellulosic biomass. In addition to the xylanases, arabinofuranosidases (8,9), acetyl esterases (10,11), and feruloyl esterases (11-13) participate in the formation of an optimal substrate for the xylanases. α -Glucuronidases (12,14) and β -xylosidases (12,14) are needed as intracellular and/or secreted enzymes to allow complete conversion to fermentable substrate. A representative structure for hardwood MeGAXn is presented below, along with sites of substitution on the xylan backbone and sites for hydrolytic cleavages catalyzed by different enzymes.

The options are further expanded with the complex relationships of endoxylanases, endoglucanases, arabinofuranosidases, O-acetyl esterases, and feruloyl esterases in the context of structures like the cellulosomes that are common to anaerobic bacteria. In such cases, cohesion domains on scaffoldin proteins and dockerin domains on the enzymes contribute to the assembly and structural integrity of functional glycoside hydrolase systems. The correct assembly of these is presumably required for the efficient digestion of extracellular polymers and vectorial uptake and metabolism of the fermentable carbohydrates (15). Similar multienzyme complexes have been identified in the anaerobic fungi as well (16-18). The aerobic saprophytic yeast and fungi on the other hand tend to secrete individual enzymes that digest the lignocellulosic polysaccharides to monosaccharides that are then taken up and metabolized.

A common process for the conversion of lignocellulosic biomass involves the pretreatment of woody biomass with dilute acid, e.g. 0.5% w/w H₂SO₄, at high temperatures (120 to 160°C) to hydrolyze the hemicellulose fraction to release pentoses, and at the same time release the cellulose fraction for subsequent digestion with microbial cellulases (19,20). The dilute acid pretreatment protocol has been commercially adopted for the production of ethanol from hardwood and crop residues. The cellulose fraction may be efficiently digested to fermentable carbohydrates through the action of fungal cellulases. Yeast and bacteria may then serve as biocatalysts for the conversion of hexoses (mainly glucose) and pentoses (mainly xylose) to ethanol and other biobased products. The conditions for acid hydrolysis are chosen to maximize the release of pentoses, and minimize their subsequent dehydration to furfural. The formation of furfural not only results in a loss of fermentable substrate, but also in the formation of an inhibitor of the fermentation process (21,22). The 4-O-methyl-D-glucuronic acid residues that are irregularly linked α -1,2 to xylose

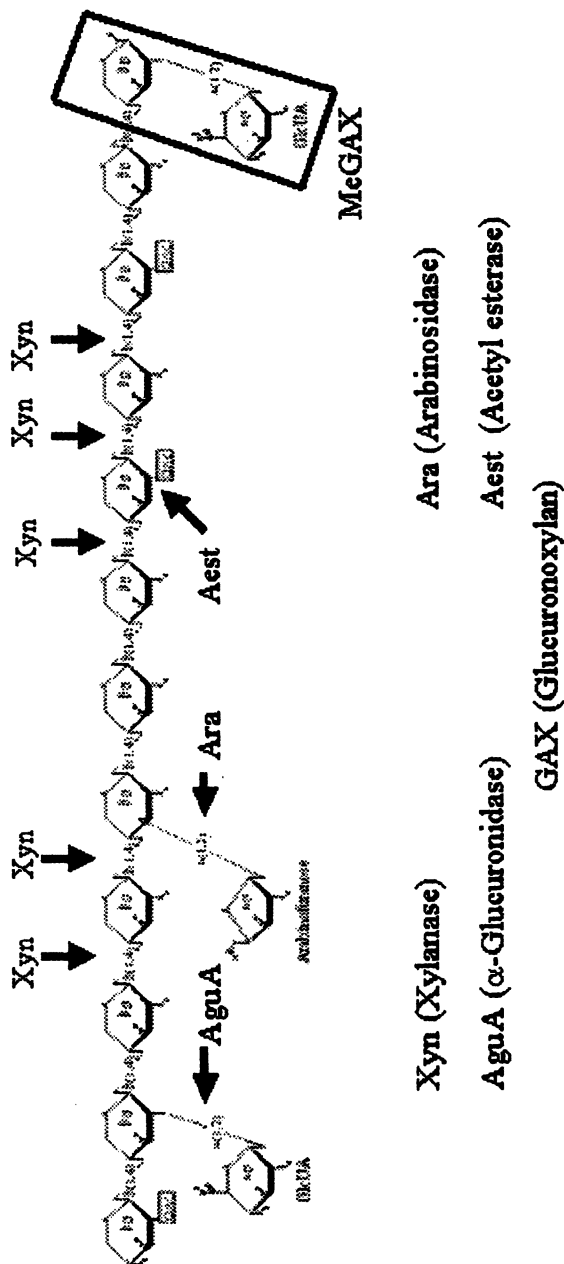


Figure 1. A hypothetical structure of methylglucuronoxylan obtained from hardwood biomass showing potential substitutions on the xylan backbone. The arrows denote sites that may be cleaved by endo- β -1,4-xylanases (Xyn), α -glucuronidases (AguA), arabinofuranosidases (Ara), and xylan-O-acetyl esterases (Aest). The blocked disaccharide, 2'-O-(4-O-methyl- α -D-glucopyranosyluronic acid)-xylose, MeGAX, is a product generated in high yield during dilute acid pretreatment protocols for digestion and saccharification of hardwood and crop residues.

residues in the xylan backbone are not readily released during the dilute acid pretreatment, and are present in the hydrolysate predominantly as the aldobiuronic acid, 2'-O-(4-O-methyl- α -D-glucuronopyranosyl) xylose (MeGAX) (23). Lesser amounts of the aldotriuronic acid, 2'-O-(4-O-methyl- α -D-glucuronopyranosyl) xylobiose (MeGAX₂), also are found in these hydrolysates. Xylose residues that are substituted with methylglucuronic acid residues are not fermentable by ethanologenic bacterial biocatalysts currently in use and represent a carbohydrate resource that is currently unavailable for fermentation (24).

The limitations of dilute acid pretreatment protocols to maximize the release of fermentable substrate from the hemicellulose fraction have prompted development of enzyme based protocols to lessen the dependence on the concentration of acid and high temperature. It is anticipated that relatively mild acidic conditions may be chosen that result in solubilization and hydrolysis of L-arabinofuranosyl and O-acetyl residues with only partial acid hydrolysis of the glucuronoxylan. Such conditions should result in negligible loss of xylose to furfural, but require the presence of endoxylanases, β -xylosidase and α -glucuronidase activities to completely convert the polymer into fermentable sugars. For the development of ethanologenic bacterial catalysts to efficiently utilize hemicellulose, an immediate objective is to identify endoxylanases that can generate aldouronic acids that can be taken up and fermented.

Relationships of structural properties, catalytic mechanisms and family assignments of endoxylanases

Enzymes with endo-1,4- β -xylanase activity belong to the EC 3.2.1.8 of the glycohydrolase family. On the basis of primary sequence comparisons and the established relationships between certain sequences and solved structures, as well as mechanisms of glycosidic bond cleavage, they have been assigned to one of three families, GH5, GH10, or GH11, of glycoside hydrolases within the CAZy data base (25). The xylanase members of each of these families catalyze the cleavage of β -1,4-linked xylose residues through an acid/base catalyzed double displacement reaction that results in the retention of the configuration at the anomeric carbon (26-28). The primary structures of the xylanase members of each family allow the maturation of a properly folded and active protein configuration in which two glutamic acid residues are juxtaposed such that one glutamate acts as a proton donor and accepts a glycosyl moiety released during the cleavage, and the second glutamate serves as a nucleophile, attacking the hydrogen atom of a water molecule and allowing the water derived hydroxyl group to displace the glycosyl linkage to the glutamate residue that served as the proton donor. The process can be mechanistically viewed as a glycosylation of the proton donor residue that occurs during the cleavage of a glycosidic bond in the polymer, followed by a deglycosylation of the proton donor that is mediated by the action of the nucleophile and a water molecule (29).

The folded structures of the different families provide different environments that affect substrate interaction and the processing of the polymer to give different products. To date, the structures of several bacterial and fungal GH10 and GH11 endoxylanases have been solved. The catalytic domains of GH10 enzymes have an 8-fold α/β barrel structure typified by that produced by *Cellulomonas fimi* (30) shown in Fig. 2a; the catalytic domain for the GH11 enzymes is comprised of two β -sheets separated by a single short α -helix, designated a β -jelly roll fold (25) and typical of the GH11 xylanase from *Bacillus circulans* (31) shown in Fig. 2b. The structure of a GH5 endoxylanase has not been published. The solved structure of the catalytic domain of the GH5 endoglucanase from *Bacillus agaradhaerans* is that of the 8-fold α/β barrel motif, and typical of other GH5 endoglucanases (32). However, the fraction of α -helix content of the GH5 *Erwinia chrysanthemi* endoxylanase is 15% at conditions of optimal activity (33), compared to 34% for the GH5 endoglucanase, and approximate values of 40% and 5% for the respective catalytic domains of GH10 and GH11 xylanases. A solved GH5 xylanase will be needed before a structural category can be definitively assigned to these enzymes.

General biochemical and catalytic properties of representatives of xylanase families

The catalytic domains of representatives of each family are similar in size, and at least for the GH10 and GH11, appear to have similar substrate preferences, and generate similar products from polymeric glucuronoxylans. Comparisons of some of these, selected on the basis of complete sequences and, where available, kinetic properties using either oat spelt or birch wood xylan as substrate, are listed in Table 1.

The definitive studies with the unsequenced xylanases of *Streptomyces* sp. B-12-2 (35) are included and show little difference in the activities of GH10 and GH11 enzymes toward oat spelt and birch wood xylan. The absence of studies with different enzymes from different sources, using identical substrate preparations, limits the interpretation of comparative kinetic properties. Nevertheless, we may conclude that both the mesophilic bacteria and fungi contain GH10 and GH11 enzymes with k_{cat} values from 100 to 1000 sec^{-1} , which are much greater values than the 0.47 sec^{-1} estimated for the GH5 xylanase from *Erwinia chrysanthemi*. The lower value seen for the *Erwinia* GH5 xylanase should reflect in part the fact that this enzyme cleaves only one time between MeGA substitutions. In contrast, the GH10 and GH11 enzymes introduce cleavages between substitution sites, and then process the products further to release xylobiose and xylotriose. In the absence of studies on initial cleavage rates, product inhibition, and substrate binding, it is too early to dismiss the members of the GH5 xylanases with respect to biotechnological applications.

The markedly different structures of the GH10 and GH11 xylanases provide catalytic properties that have further justified the classification of these

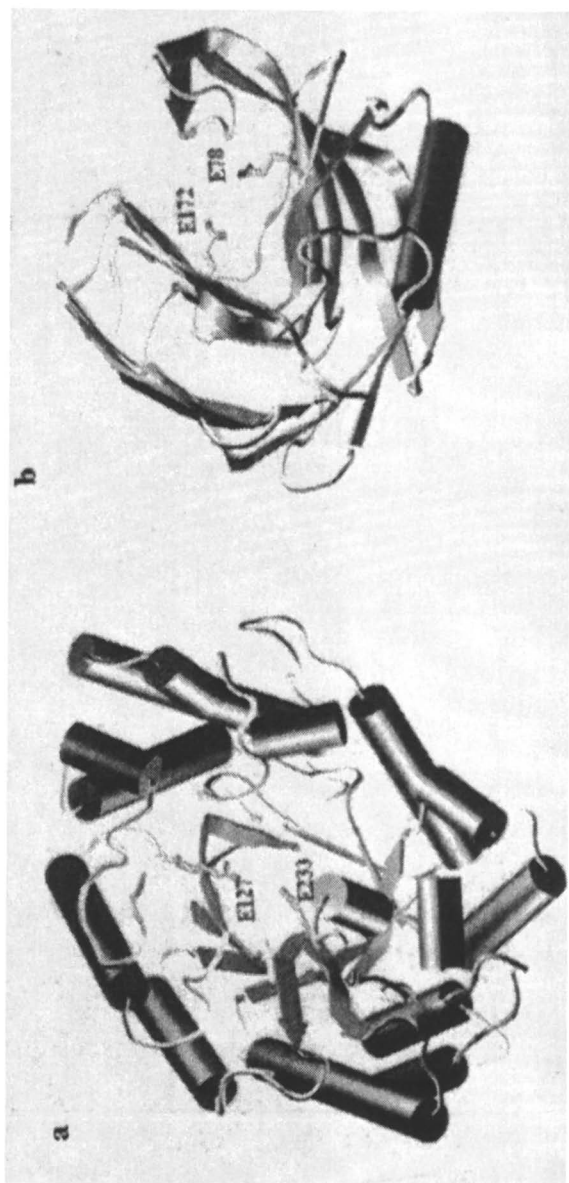


Figure 2. Structures of GH10 and GH11 endoxylanases. a) Catalytic domain of *Cellulomonas fimi* GH10 cellulase/xylanase, PDB 2EXO (30). The structure displays the 8-fold α/β barrel. E127, the proton donor, and E233, the nucleophile, are positioned to catalyze the double displacement reaction with retention of the anomeric configuration in the product. b) Molecule of *Bacillus circulans* GH11 endoxylanase, PDB 1BVV (31). The structure displays two β -folds separated by a short α -helix. E78, the proton donor, and E172, the nucleophile, are positioned to catalyze the double displacement reaction with retention of the anomeric configuration in the product. Figures were drawn with VMD 1.7.1 and rendered with POV-Ray(34).

Table I. Comparative properties of representatives of different families of endoxylanases.

<i>Family/Species</i>	<i>MW^a</i>	<i>k_{cat}(sec⁻¹)^{b,c}</i>	<i>limit product^d r eference</i>
<i>GH5/E. chrysanthemi</i> Q46961 ^e	41.2	0.47 ^c	XGAX(X) _n X (33)
<i>GH10/S. lividans</i> P26514 ^e	32.7	134 ^c	GAX ₃ , X ₂ , X ₃ (36, 38)
<i>GH10/C. albidus</i> P07529 ^e	31.5	nd ^f	GAX ₃ , X ₂ , X ₃ (36)
<i>GH10/B. stearotherm.</i> P40493 ^e	43.8	206 ^b	nd ^f (37)
<i>GH10/P. fluorescens</i> P14768 ^e	38.3	1002 ^b	nd ^f (63)
<i>GH10/Streptomyces</i> sp B-12-2 xyl3	36.2	204 ^b	X ₃ and others (35)
<i>GH10/Streptomyces</i> sp B-12-2 xyl3	36.2	222 ^c	X ₂ and others (35)
<i>GH11/T. reesei</i> P36217 ^e	20.0	nd ^f	XGAX ₃ , X ₂ , X ₃ (36)
<i>GH11/S. lividans</i> P26515 ^e	22.8	nd ^f	XGAX ₃ , X ₂ , X ₃ (36)
<i>GH11/B. pumulis</i> P00694 ^e	22.5	166	nd ^f (39)
<i>GH11/Streptomyces</i> sp B-12-2 xyl1b	23.2	163 ^b	X ₃ and others (35)
<i>GH11/Streptomyces</i> sp B-12-2 xyl1b	23.2	187 ^c	X ₂ and others (35)

a- Values in kDa represent catalytic domains from deduced sequences, defined in Pfam; for the xylanases from *Streptomyces* sp B-12-2, molecular weights were determined by SDS-PAGE

b-oat spelt xylan

c-birch wood xylan

d-aldouronic acid limit products deduced from ¹³C-NMR

e-accession numbers for SwissProt or TrEMBL

f-nd, not determined

enzymes into distinct families (40,41), and the processing of glucuronoxylans leads to limit products that further support this classification (36). The GH10 xylanases from *Streptomyces lividans* generate, as the smallest aldouronic acid, aldotetrauronic acid in which the MeGA residue is linked to the reducing terminus of β -1,4-xylotriose. The GH11 xylanases from *Trichoderma reesei* and *Streptomyces lividans* generate aldopentauronic acid in which the MeGA is linked to a xylose residue penultimate to the reducing terminus of β -1,4-xylotetraose (36). All members of both GH10 and GH11 xylanases generate xylobiose and xylotriose, and in some cases free xylose. In contrast, the GH5 xylanase from *Erwinia chrysanthemi* generates a product the size of which is determined by the distance between the xylose residues that are substituted by the 4-O-methylglucuronic acid residues (33). In this case, the cleavage occurs preferentially at glycosidic linkages adjacent to xylose that is substituted with MeGA, and neither xylose nor xylobiose is formed. Along with the preference for substrates most extensively substituted with MeGA residues, the GH5 xylanase from *Erwinia chrysanthemi* has an apparent requirement for MeGA to allow binding and cleavage. It should be noted that different members of the GH10 families have been shown to preferentially cleave xylooligosaccharides at different glycosidic linkages (42), and generalizations on the preferred formation of limit products from complex substrates as signatures for members of a family may prove untenable. This point may be further supported with the discovery of a GH5 xylanase from *Trichoderma reesei* that has limited endo-action on glucuronoxylan and then processes released products by exo-action with the formation of xylose. (43).

Family relationships of catalytic domains deduced from amino acid sequence

In the absence of detailed structural information, amino acid sequence alignments provide a way in which to compare the xylanases and consider the relationships of the different families. This approach, emphasizing hydrophobic cluster analysis to predict secondary and tertiary structures (44,45), has been particularly well developed in the accessible WEB sites of CAZyModO (46). The extensive and expanding DNA sequence databases have provided a useful resource in which to explore the distribution and sequence of genes encoding the GH5, GH10, and GH11 endoxylanase families, and recent comparisons of the GH11 xylanases have established the strong relationships among the members of this family (47). Using the MACAW program (48,49) to align and identify shared sequence blocks of the members of each family, as they are currently assigned in CAZy, organizations are seen within their primary structures that support their distinctive assignments to their respective families, as shown in Figure 3. The program has not found blocks of more than 20 amino acids in a member of one family that can be aligned with another, and the members of the

GH5 xylanases cannot be aligned with members of the GH5 endoglucanases. It should be noted that the alignments and comparisons that we are currently applying consider only the regions that comprise the catalytic module or domain. Several of the GH10 and GH11 endoxylanases contain cellulose binding domains or other carbohydrate binding domains that, in addition to the catalytic domains, affect the overall catalytic efficiencies of the xylanases with respect to the depolymerization of polymeric substrates.

As noted in the alignment, each xylanase has two glutamate residues that are separated by approximately 100 amino acids. The specific glutamates that function as proton donors and nucleophiles have been identified in the solved structures for the GH10 xylanases from *Penicillium simplicissimum* (50), *Streptomyces lividans* (40), and *Cellulomonas fimi* (30), and the GH11 xylanases from *Bacillus agaradhaerans* (51), *Bacillus circulans* (31), *Streptomyces lividans*, and *Trichoderma reesei* (52). The assignments for other members of these families may be logically inferred from the alignment of specific sequences and the block distributions within the catalytic domains. For members of the GH5 family, the absence of solved structures and site-directed mutation studies make the designations of these glutamate residues as catalytic residues tentative, although logical. When the homologous regions containing these glutamates are compared (Table II), a signature is obtained for each enzyme that further supports the assignment of each to its respective family. An additional relationship appears between the number of amino acids separating the two active site glutamates in a catalytic domain and the assigned family. In this case the GH5 and GH11 xylanases, with 90 amino acids separating the catalytic sites, share a difference with the GH10 xylanases that have 105 residues between active site glutamates. The putative active site regions in the GH5 endoglucanase (Cel5A) from *Bacillus agaradhaerans* has a proton donor region, ¹⁵⁷NVIYEIANEPN, with E-165 as the proton donor, and ²⁴⁶QGAAIFVSEWG, with E-254 as the nucleophile, and 89 amino acids separating the two sites. Thus, while the GH5 endoglucanases and endoxylanases are similar in the length of the peptide separating the two sites, they bear no resemblance with respect to the amino acid sequences surrounding the glutamate residues in the active sites. The emphasis on hydrophobic cluster analysis to define sequence motifs may not be justified when classifying xylanases along with glucanases to family 5 of the glycoside hydrolases.

Microbial diversity and phylogenetic distribution of endoxylanases

The phylogenetic relationships of the amino acid sequences for the different families provide insight into the origins and evolution of genes encoding these enzymes, and also gives evidence for the significant divergence of the genes encoding the different endoxylanase families. As shown in Figure 4, the deduced translated sequences of genes encoding regions including the active

Table II. Positions of active site residues of family GH5, GH 10 and GH11 endoxylanases. Species are listed with SWISS-PROT or TrEMBL accession numbers. *Aeromonas caviae* and *Erwinia chrysanthemi* have been respectively designated as *Aeromonas punctata* and *Pectobacterium chrysanthemi*.

Glycoside Hydrolase Family	Species	Sequence		Residues Between Active Sites
		Proton donor	Nucleophile	
GH5	<i>A.punctata</i> (P70733)	¹⁶⁴ LYAI SVQNEPD	²⁵⁵ GKELWMTEVYYP	90
	<i>B.subtilis</i> (Q45070)	¹⁶³ LYAI SVQNEPD	²⁵³ GKDLWMTEVYYP	91
	<i>P.chrysanthemi</i> (Q938A4)	¹⁵⁷ LYAI S I QNEPD	²⁴⁶ GKQVWMTEHLVD	88
	<i>C.acetobutylicum</i> (Q97TI2)	¹⁶³ LYALSL QNEPD	²⁵³ GKD IWMTEHYLE	89
	<i>M.incognita</i> (Q9NHE0)	¹⁵⁰ LYALSL QSEPD	not present	
GH10	<i>P.simplicissimum</i> (P56588)	¹²⁷ WDVLNE I FNE	²³² KE I AI TELDI	106
	<i>S.lividans</i> (P26514)	¹⁶⁴ WDVVNEAFAD	²⁷¹ VDVAI TELDI	109
	<i>C.fimi</i> (Q59278)	¹⁶³ WDVVNEAFAD	²⁶⁸ VDVRI TELDI	107
	<i>A.punctata</i> (O83007)	¹²⁹ WDVVNEAVED	²³⁵ VQLHVTELDI	107
	<i>B.stearothermophilus</i> (P45703)	¹²⁸ WDV INEAVAD	²³⁴ V I LHI TELDI	107
GH11	<i>A.punctata</i> (Q43993)	⁹⁸ WTRNALIEYYV	¹⁹² YQVMATEGYQS	93
	<i>B.stearothermophilus</i> (P45705)	⁹⁷ WTRNALIEYYV	¹⁹¹ YQVLATEGYQS	93
	<i>B.circulans</i> (P09850)	⁹⁹ WTRSPLI EYYV	¹⁹⁴ YQVMATEGYQS	94
	<i>S.lividans</i> (P26220)	¹²⁷ WTSNPLVEYYV	²²⁰ YMI MATEGYQS	92
	<i>T.reesei</i> (P36217)	¹¹¹ WSRN PLIEYY I	²⁰³ YQ I VAVEGYFS	88

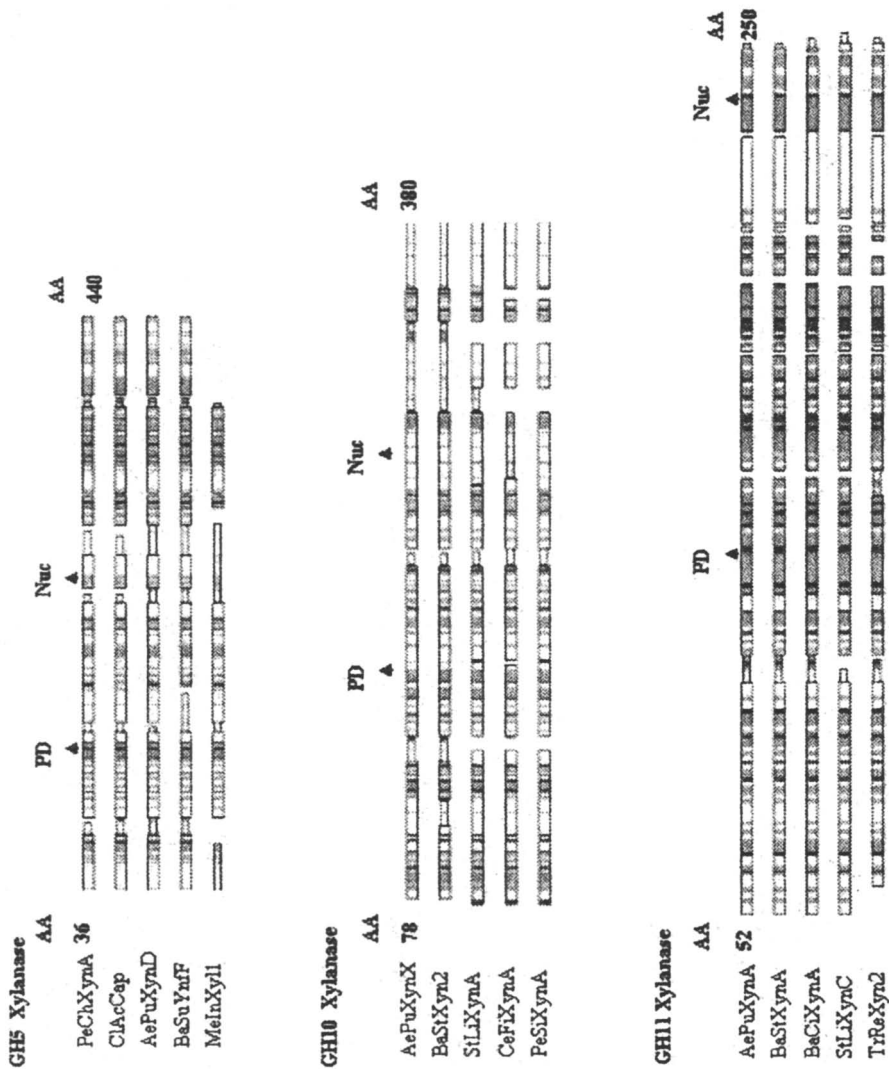


Figure 3. Alignments of selected GH5, GH10, and GH11 endoxylanases. Alignments were made with the MACAW program (48, 49) to identify shared sequence blocks of amino acids for representative members of each family. Sequences were identified from the CAZyModO site and retrieved from SWISS-PROT and TrEMBL databases with the following relationships to accession numbers: PeChXynA, Q93844; ClAcCap, Q97T12; AePuXynX, O83007; BaSiXyn2, BaSynjF, Q45070; MeInXyl, Q9NHE0; AePuXynX, O83007; BaSiXyn2, P45703; StLiXynA, P26514; CeFlXynA, Q59278; PeSiXynA; P56588; ApunXynA, Q43993; BaSiXynA, P45705; BaCiXynA, P09850; StLiXynC, P26220; TrReXyn2, P36217. Selected sequences are the same as those listed and identified in Table 2. Alignments were made using the segment pair overlap search method with a pairwise cutoff score of 42, and mean score shading. The blocks represent those sequences that met the criteria for alignment set by the cutoff. There were no block alignments seen between members of different xylanase families, or between GH5 xylanases and GH5 glucanases, with a cutoff score greater than 20 residues. The dark colored regions indicate identities in alignment, with the darkest reflecting amino acids, e.g. tryptophan, histidine, proline, that are expected to exert relatively strong effect on structure. The light colored regions may include similarities in amino acid properties as well as identities. The positions of active site glutamate residues that serve as proton donors (PD) and nucleophiles (Nuc) are aligned, along with the blocks, for the respective members of each family.

site glutamates and all amino acids between them indicate the members of each family may be viewed as distantly related orthologs. Since the residues surrounding the active sites and the number of residues separating these are nearly identical for the members of given family (Table 2), the differences within a family reflect the evolutionary changes that have still allowed the positioning of these active sites in the folded proteins to provide, at least for some of the GH10 and GH11 xylanases, a characteristic signature with respect to product formation. The determination of product formation in other GH5 xylanases in addition to the *Erwinia chrysanthemi* xylanase is needed to further test this hypothesis. The identification of a gene in the root-knot nematode, *Meloidogyne incognita*, with significant homology to bacterial GH5 endoxylanase of *Erwinia chrysanthemi* was surprising. As both the metazoan eukaryote and the bacterium are plant pathogens, it is possible that the GH5 gene in the nematode was obtained by horizontal gene transfer from the bacterium, or vice versa. A gene (*ynfF*) with significant homology to the gene encoding the GH5 xylanase in *Aeromonas caviae* (*punctata*) was found as an ORF during the sequencing of the *Bacillus subtilis* genome, pointing out the probability that genomic databases will reveal important information on the xylanolytic potential of other microorganisms. Of further interest here is the relatively close relationship between the GH5, GH10, and GH11 endoxylanases from the Gram negative *Aeromonas caviae* and Gram positive *Bacillus* spp. (*Bacillus subtilis* or *Bacillus* (*Geobacillus*) *stearothermophilus*), suggesting the possibility of genetically mobile regions that may reflect an organization of these genes in the genome that allows collective transfer. The clustering of the genes involved in the metabolism of glucuronoxylan in *Bacillus stearothermophilus* T6 (14) provides supporting evidence for this suggestion.

The presence of both GH10 and GH11 xylanases in *Trichoderma reesei* attest to a repertoire of endoxylanases in one organism with different mechanisms, and presumably different functions. Enzymes representing both of these families are also found in the bacteria, *Cellulomonas fimi*, *Aeromonas caviae*, *Bacillus stearothermophilus*, and *Streptomyces lividans*. Of some biotechnological interest is the presence and expression of genes encoding GH5 as well as GH10 and GH11 endoxylanases in different strains of the Gram negative *Aeromonas caviae* (55-58). This is the only species known to express genes encoding enzymes of all three families. As Gram negative bacteria, they may provide genes for construction of synthetic operons to allow maximal expression, translation, and secretion of functional enzymes in the ethanologenic Gram negative bacterial biocatalysts that are currently used in the industrial production of ethanol from lignocellulosic biomass.

Relationship of xylanase mechanism to applications in processing lignocellulosics

The solubilization of lignocellulosic biomass is necessary to render the structural polymers of the cellulose and hemicellulose fractions accessible to

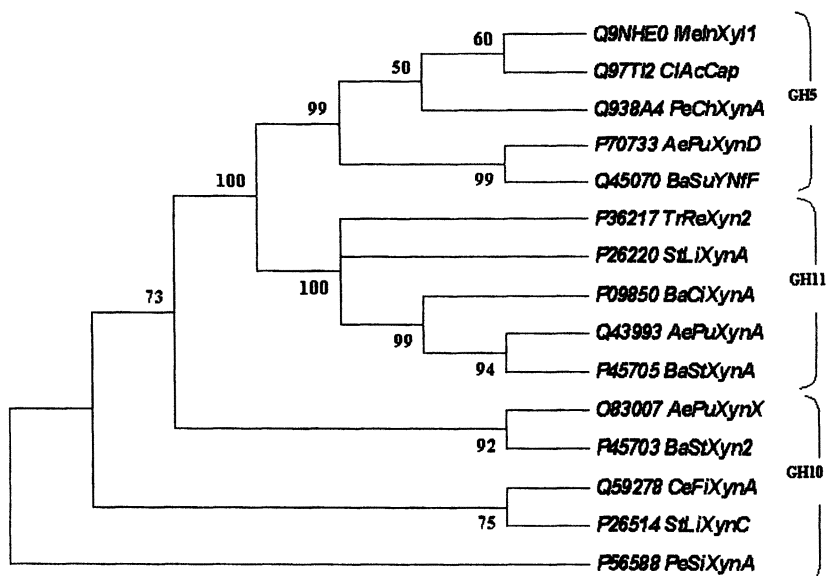


Figure 4. Phylogenetic tree representing the evolutionary relationship of regions between the conserved catalytic proton donor and the nucleophilic residues in the GH5, GH10 and GH11 xylanases. Amino acid sequences archived with SWISS-PROT or TrEMBL are the same as those compared in Figure 3 and Table 2. The sequences compared were those between and inclusive of sequences listed in Table 2. The phylogenetic tree was constructed using MEGA2 (53) with the Neighbor-Joining method (54). The scale represents a relative evolutionary distance, and the whole numbers are bootstrap values for 1,000 replicates.

enzymes that convert them to products that can be taken up and metabolized by microbial biocatalysts. For the use of hardwood and crop residues, a dilute acid pretreatment step will likely serve this need. Conditions milder than those currently used to maximize the release of xylose will have the advantage of decreased furfural formation, but will depend upon xylanases, α -glucuronidases, and β -xylosidase to allow the conversion of partial hydrolysates to fermentable substrates. Many xylanolytic fungi secrete enzymes with these activities, and are able to solubilize the glucuronoxylans to free sugars. Bacteria, on the other hand, secrete the endoxylanases to allow the partial depolymerization of the polymer and the formation of aldouronic acids that are taken up and further metabolized by periplasmic or intracellular α -glucuronidase, β -xylosidase, and exoxylanase activities. This has perhaps been best documented in *Bacillus stearothermophilus*, in which the genes encoding these enzymes are clustered in

one particular region of the genome (14). Using this as a model, the most effective endoxylanase for a bacterial biocatalyst will be of the GH10 family, and may be anchored to the surface of the cell. The formation of aldotetrauronic acid should be followed by uptake and further metabolism to release free xylose and methylglucuronate that may then be fermented. This type of system has a naturally occurring analogy in the compartmentalization of endoglucanases and endoxylanases in the cellulosomes of many species of *Clostridium* and other cellulolytic anaerobic bacteria, a system that has presumably evolved to allow the efficient vectorial digestion of lignocellulosics for subsequent fermentation. The GH11 xylanases that generate aldopentauronate are secreted, and the generated products will likely require processing with an extracellular β -xylosidase to allow them to be taken up by the cells for further metabolism. The GH5 xylanase secreted by *Erwinia chrysanthemi* generates a product that will require the combined actions of other extracellular endoxylanases and β -xylosidase to provide products that can be taken up and fermented. The diagram in Figure 5 depicts the different options available for coupling the depolymerization of glucuronoxylans to fermentation and formation of biobased products.

The GH11 endoxylanases have already found important applications in the biobleaching of Kraft pulp as well as the processing of foods (1-3). Their relatively small size, efficient secretion, broad substrate preference, and relatively high k_{cat} will continue to make this type of xylanase a focus of research and application development. For the application of extracellular enzymes to the formation of free xylose, the complementation of a GH11 xylanase with extracellular β -xylosidase may be sufficient to allow uptake for further processing and fermentation. The engineering of bacterial GH11 enzymes for increased catalytic activity and high-level secretion in *Trichoderma reesei* (59) would seem to provide an opportunity for the commercial applications comparable to those of the fungal endoglucanases.

The initial interest in the GH5 xylanase from *Erwinia chrysanthemi* was related to its potential role as a virulence factor in a phytopathogenic bacterium (60). Our continued interest in this xylanase is related to its constitutive expression and secretion in a bacterium that is closely related to the strains of *Escherichia coli* and *Klebsiella oxytoca* that have been engineered as efficient ethanologenic bacterial biocatalysts (61,62). As in the case of the endoglucanase *celZ* from this same bacterium, it should be quite feasible to engineer ethanologenic strains of *E. coli* for its secretion. Future investigations will evaluate the substrate preferences of this in comparison with members of the GH10 and GH11 families. The retention of the GH5 xylanase as the sole activity expressed in two different isolates of *E. chrysanthemi* suggests a property of selective advantage in the maceration and colonization of a plant tissues that requires the digestion of native plant polymers. The presence and expression of genes encoding GH5 as well as GH10 and GH11 enzymes from *Aeromonas caviae* suggests a function that is different for each family. The most recent description of a GH5 xylanase from *Trichoderma reesei* (43) indicates an enzyme with greater versatility than the GH5 xylanase from *E. chrysanthemi*,

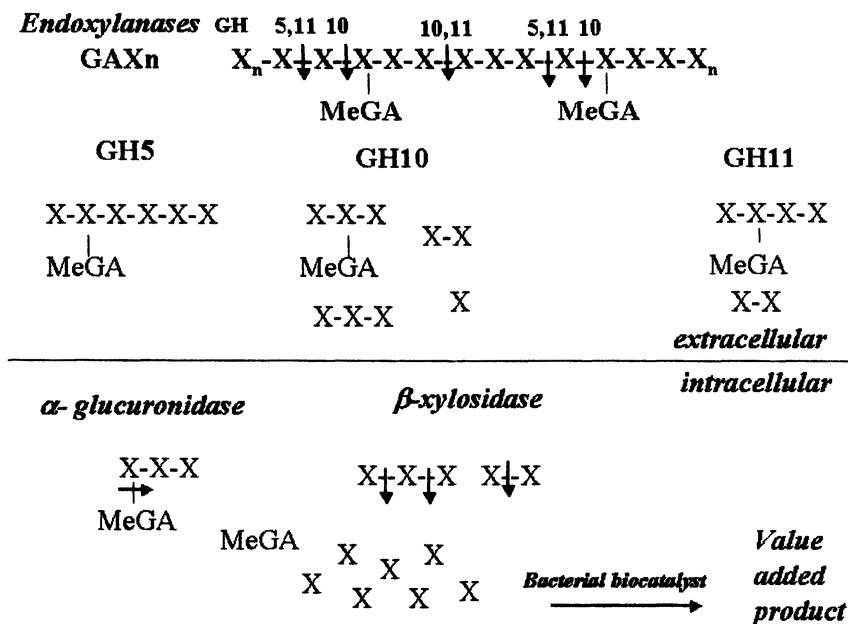


Figure 5. Comparison of products generated by GH5, GH10, and GH11 endoxylanases and requirements for processing and conversion by bacterial biocatalysts to value added products. The intracellular compartmentalization of β -xylosidase and α -glucuronidase in bacteria argues for the most efficient depolymerization process to be catalyzed by a GH10 endoxylanase bound to the cell surface, either by itself or as part of a complex analogous to a cellulosome.

and an ability to digest acetylated glucuronoxylans. The further investigation of the GH5 endoxylanases, particularly with respect to substrate preferences, will assist in their adoption in biotechnological applications.

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References

1. Viikari, L.; Kantelinen, A.; Sandquist, J.; Linko, M. *FEMS Microbiol. Rev.* **1994**, *13*, 338-350.
2. Jeffries, T. *Curr. Opin. Biotechnol.* **1996**, *7*, 337-342.
3. Beg, Q.K.; Kapoor, M.; Mahajan, L.; Hoondal, G.S. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 326-338.
4. Timell, T.E. *Adv. Carbohydrate Chem.* **1964**, *19*, 247-302.
5. Puls, J.; Schusell, J. *Chemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis*. In: *Hemicellulose and Hemicellulases*, Coughlin, M.P.; Hazlewood, G.P., Eds. Portland Press, London 1992, pp 1-26.
6. Linder, M.; Teeri, T.T.; *J. Biotechnol.* **1997**, *57*, 15-28.
7. Black, G.W.; Rixon, J.E.; Clarke, J.H.; Hazelwood, G.P.; Ferreira, L.M.A.; Bolam, D.N.; Gilbert, H.J. *J. Biotechnol.* **1997**, *57*, 59-69.
8. de Vries, R.P.; Visser, J. *Microbiol. Molec. Biol. Rev.* **2001**, *65*, 497-552.
9. Beylot, M.H.; Emani, K.; McKie, V.A.; Gilbert, H.J.; Pell, G. *Biochem. J.* **2001**, *358*, 599-605.
10. Christov, L.; Prior, B.A. *Enzyme Microb. Technol.* **1993**, *15*, 460-475.
11. Tenkanen, M., *Biotechnol and Appl. Biochem.* **1998**, *27*, 19-24.
12. de Vries, P.; Kester, H.C.M.; Poulsen, C.H.; Benen, J.A.E.; Visser, J. *Carbohydrate Res.* **2000**, *327*, 401-410.
13. Blum, D.L.; Kataeva, I.A.; Li, X.L.; Ljungdahl, L.G. *J. Bacteriol.* **2000**, *184*, 1346-1351.
14. Shulami, S.; Gat, G.; Sonenshein, A.L.; Shoham, Y. *J. Bacteriol.* **1999**, *181*, 3695-3704.
15. Bayer, E.A.; Shimon, L.J.W.; Shoham, Y.; Lamed, R. *J. Struct. Biol.* **1998**, *124*, 221-234.
16. Raghothama, S.; Eberhardt, R.Y.; Simpson, P.; Wigelsworth, D.; White, P.; Hazlewood, G.P.; Nagy, T.; Gilbert, H.J.; Williamson, M.P. *Nature Struct. Biol.* **2001**, *8*, 775-778.
17. Blum, D.L.; Li, X.L.; Chen, H.Z.; Lungdahl, L.G. *Appl. Environ. Microbiol.* **1999**, *65*, 3990-3995.
18. Aylward, J.H.; Gobius, K.S.; Xue, G.P.; Simpson, G.D.; Dalrymple, B.P. *Enzyme Microb. Technol.* **1999**, *24*, 609-614.
19. Grohmann, K.; Torget, R.; Himmel, M. *Biotechnol. Bioengin. Symposium No. 15* **1986**, *15*, 59-80.
20. Sheehan, J.; Himmel, M. *Biotechnol. Progr.* **1999**, *15*, 817-827.
21. Zaldivar, J.; Martinez, A.; Ingram, L.O. *Biotechnol. Bioengin.* **1999**, *65*, 25-33.
22. Gutierrez, T.; Buszko, M.L.; Ingram, L.O.; Preston, J.F. *Appl. Biochem. Biotechnol.* **2002**, *98-100*, 337-340.

23. Lai, Y.-Z. *Chemical degradation*. In: *Wood and Cellulosic Chemistry*. Hon, D.N.-S.; Shiraishi, N., Eds. Marcel Dekker, Inc., New York. 1991. pp 455-523.
24. Rodriguez, M.E.; Martinez, A.; York, S.W.; Zoubi-Hasona, K.; Ingram, L.O.; Shanmugam, K.T.; Preston, J.F. American Society for Microbiology, 101st General Meeting, 2001, Abstracts p 535.
25. Coutino, P.M.; Henrissat, B. *Carbohydrate-active enzymes: an integrated database approach*. In: *Recent Advances in Carbohydrate Engineering*, H.J. Gilbert, G. Davies, B. Henrissat, and B. Svensson, eds., The Royal Society of Chemistry, Cambridge, 1999, pp 1-12.
26. Withers, S.G. In: *Carbohydrate Bioengineering*. Petersen, S.B.; Svenson, S.; and Pederson, S., Eds. Elsevier Science 1995, pp 97-113.
27. Davies, G.J.; Mackensie, L.; Varrot, A.; Dauter, M.; Brosowski, A.M.; Schulein, M.; Withers, S.G. *Biochem.* **1998**, *37*, 11707-11713.
28. Joshi, M.D.; Sidhu, G.; Pot, I.; Brayer, G.D.; Withers, S.G., McIntosh, L.P. *J. Mol. Biol.* **2000**, *299*, 255-279.
29. Lawson, S.L.; Wakarchuck, W.W.; Withers, S.G.. *Biochem.* **1997**, *36*, 2257-2265.
30. White, A.; Withers, S.G.; Gilkes, N.R.; Rose, D.R.. *Biochem.* **1994**, *33*,12546-12552.
31. Sidhu, G.; Withers, S. G.; Nguyen, N. T.; McIntosh, L. P.; Ziser, L.; Brayer, G. D. *Biochem.* **1999**, *38*, 5346-5354.
32. Davies, G.J.; Dauter, M.; Brzozowski, A.M.; Bjornvad, M.E.; Andersen, K.V.; Schlulein, M. *Biochem.* **1998**, *37*, 1926-1932.
33. Hurlbert, J.C.; Preston, J.F. *J. Bacteriol.* **2001**, *183*, 2093-2100.
34. Humphrey, W.; Dalke, A. ; Schulten, K. *J. Molec. Graphics.* **1996**,*14.1*, 33-38.
35. Elegir, G; Szakacs, G; Jeffries, T.W. *Appl. Environ. Microbiol.* **1994**, *60*, 2609-2615.
36. Biely, P.; Vrsanska, M; Tenkanen, M.; Kluepfel, D. *J. Biotechnol.* **1997**, *57*, 151-166.
37. Khasin, A.; Iris, A.; Shoham, Y. *Appl. Environ. Microbiol.* **1993**, *59*, 1725-1730.
38. Roberge, M; Shareck, F.; Morosoli, R.; Kluepfel, D.; Dupont, C. *Biochem.* **1997**, *36*, 7769-7775.
39. Ko, E.P.; Akatsuka, H.; Moriyama, H.; Shinmyo, A.; Hata, Y.; Katsube, Y.; Urabe, I.; Okada, H. *Biochem. J.* **1992**, *288*, 117-121.
40. Ducros, V.; Charnock, S.J.; Derewenda, U.; Derewenda, Z.S.; Dauter, Z.; Dupont, C.; Shareck, F.; Morosoli, R.; Kluepfel, D.; Davies, G. *J. Biol. Chem.* **2000**, *275*, 23020-23026.
41. Harris, G.W.; Jenkins, J.A.; Connerton, I.; Cummings, N., Loleggio, L.; Scott, M.; Hazlewood, G.P.; Laurie, J.I.; Gilbert, H.J.; Pickersgill, R.W. *Structure.* **1994**, *2*, 1107-1116.

42. Charnock, S.J.; Sprurway, T.D.; Xie, H.; Beylot, M.-H.; Virden, R.; Warren, R.A.; Hazlewood, G.P.; Gilbert, H.J. *J. Biol. Chem.* **1998**, *273*, 32187-32199.
43. Tenkanen, M.; Salcheimo, M.; Biely, P.; Siika-aho, M.; Vrasanska, M. Abstracts of the 223rd National Meeting of the American Chemical Society. ACS Symposium: Application of Enzymes to Lignocellulosics. 2002.
44. Henrissat, B.; Bairoch, A. *Biochem. J.* **1993**, *293*, 781-788.
45. Henrissat, B.; Callebaut, I.; Fabrega, S.; Lehn, P.; Mornaan, J.-P.; Davies, G. *Proc. Natl. Acad. Sci.* **1995**, *92*, 7090-7094.
46. Coutino, P.M.; Henrissat, B. 1999. Carbohydrate-active enzymes server at URL <http://afmb.cnrs-nrs.fr/~cazy/CAZY/index.html>.
47. Sapag, A.; Wouters, J.; Lambert, C.; de Ioannes, P.; Eyzaguirre, J.; Depiereux, E. *J. Biotechnol.* **2002**, *95*, 109-131.
48. Karlin, S.; Altschul, S.F. *Proc. Natl. Acad. Sci.* **1990**, *87*, 2264-2268.
49. Schuler, G.D.; Altschul, S.F.; Lipman, D. *Proteins: Structure, Function, and Genetics.* **1991**, *9*, 180-190.
50. Schmidt, A.; Gubitz, G.M.; Kratky, C. *Biochem.* **1999**, *38*, 2403-2412.
51. Sabini, E.; Wilson, K.S.; Danielson, S.; Schulein, M.; Davies, G.J. *Acta Crystallog. D.* **2001**, *57*, 2344-2347.
52. Torronen, A.; Rouvinen, J. *Biochem.* **1995**, *34*, 847-856.
53. Kumar, S.; Tamura, K.; Jakobsen, I.B.; Nei, M. *Bioinformatics* **2001**, *17*, 1244-1245.
54. Saitou, N.; Nei, M. *Molec. Biol. and Evol.* **1987**, *4*, 406-425.
55. Kubata K.B.; Suzuki T.; Horitsu H.; Kawai K.; Takamizawa K. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1463-1464.
56. Okai, N.; Fukasaku, M.; Kaneko, J.; Tomita, T.; Muramoto, K.; Kamio, Y. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1560-1567.
57. Suzuki, T.; Ibata, K.; Hatsu, M.; Takamizawa, K.; Kawai, K. *J. Ferment. Bioeng.* **1997**, *84*, 86-89.
58. Usui, K.; Ibata, K.; Suzuki, T.; Kawai, K. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1346-1352.
59. Bergquist, P.L.; Te'o, V.S.; Gibbs, M.D.; Cziferszky, A.C.E.; De Faria, F.P.; Azevedo, M.O.; Nevalainen, K.M.H. *Appl. Biochem. Biotechnol.* **2002**, *98*, 165-176.
60. Keen, N.T.; Boyd, C.; Henrissat, B. *Mol. Plant-Microbe Interactions* **1996**, *9*, 651-657.
61. Burchhardt, G.; Ingram, L.O. *Appl. Environ. Microbiol.* **1992**, *58*, 1128-1133.
62. Asghari, K.; Bothast, R.J.; Doran, J.B.; Ingram, L.O. *J. Industrial Microbiol.* **1996**, *16*, 42-47.
63. Charnock, S.J.; Lakey, J.H.; Virden, R.; Hughes, N.; Sinnott, M.L.; Hazlewood, G.P.; Pickersgill, R.; Gilbert, H.J. *J. Biol. Chem.* **1997**, *272*, 2942-2951.

Chapter 13

Comparison of Catalytic Properties of Acetyl Xylan Esterases from Three Carbohydrate Esterase Families

Maija Tenkanen^{1,5}, Jaime Eyzaguirre², Riitta Isoniemi¹,
Craig B. Faulds³, and Peter Biely⁴

¹VTT Biotechnology, Espoo, Finland

²Pontificia Universidad Católica de Chile and Universidad Nacional Andrés Bello, Santiago, Chile

³Institute of Food Research, Norwich, United Kingdom

⁴Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia

⁵Current address: University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki FIN-00016, Finland

Most natural hemicelluloses are acetylated. Some xylans carry also esterified phenolic acid side groups. Carbohydrate-acting esterases (CE) have been recently classified according to structural similarities into several CE families. However, this classification does not necessarily predict the substrate specificity of these enzymes. In this work eight different hemicellulose-acting esterases from three different esterase families were studied. The substrate specificities and the modes of action of esterases from different families varied significantly. Some esterases acted on a wide range of substrates, i.e. on acetyl and on feruloyl groups in xylan and on acetyl groups in glucomannan, while others were strictly specific for one substrate. The CE family 1 and 5 esterases deacetylated first at the position 3 and 2, respectively, whereas the CE family 4 esterase performed a fast double deacetylation at both positions in methyl per-*O*-acetyl- β -D-xylopyranoside.

Introduction

Esterases are a versatile group of hydrolytic enzymes. One sub-group of esterases includes those acting on ester linkages in carbohydrates. Many polysaccharides are esterified in nature. One of the most common types is acetylation. For example hemicelluloses, pectins and chitin carry acetyl groups. Other common esterified substituents in polysaccharides are methanol and hydroxycinnamic acid groups.

The main hemicelluloses are xylans, which are composed of 1,4-linked β -D-xylopyranosyl units (1). The backbone of xylan is substituted at carbon 2 and 3 positions, with type and degree depending on the origin. Hardwood xylans carry 4-O-methylglucuronic acid side groups whereas softwood xylans are substituted with L-arabinofuranosyl units. Xylans from annual plants may contain only the latter side groups, or both types of substitutions. Furthermore, hardwood xylans are acetylated whereas softwood xylans are not (2). Xylans from annual plants may, in addition to acetyl groups, carry esterified phenolic hydroxycinnamic acids such as ferulic acid, which are mainly linked to carbon 5 of arabinose side groups (3).

The other main hemicelluloses are glucomannans consisting of randomly alternating 1,4-linked β -D-glucopyranosyl and β -D-mannopyranosyl units. The glucomannan backbone is often also substituted at the carbon 6 position with D-galactopyranosyl residues and at carbon 2 and 3 with acetyl groups (1,2). Glucomannans are the main hemicelluloses in softwoods whereas xylans are main hemicelluloses in hardwoods and annual plants. Glucomannans and galactomannans also occur in annual plants, especially in seeds, tubers and bulbs.

Acetyl xylan esterases and acetyl glucomannan esterases are enzymes capable of releasing acetyl groups from polymeric hemicelluloses (4,5). Esterases liberating hydroxycinnamic acids, such as ferulic acid, are generally named and classified based to their respective substrate specificities (6). The traditional classification of esterases is according to their substrate specificities (IUB). However, this classification is not very clear due to the rather wide substrate specificities of many esterases. Currently acetyl xylan esterases (EC 3.1.1.72) and feruloyl esterases (EC 3.1.1.73) are classified in a separate group from acetyl esterases (EC 3.1.1.6). However, acetyl esterases acting on glucomannans are not yet classified under an own EC number.

More recently several carboxyl esterases, especially those acting on carbohydrates, have been grouped on the basis of hydrophobic cluster analysis and amino acid sequence similarities (<http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>). Currently the classification consists of 13 different carbohydrate esterase families. Acetyl xylan esterases are found in the CE families 1-7 indicating the structural versatility of these esterases. All classified feruloyl

esterases belong to the CE family 1. No acetyl glucomannan esterases have yet been classified. Glycosyl hydrolases have been classified similarly into several families on the basis of hydrophobic cluster analysis. This structural grouping has also been shown to follow differences in substrate specificities and catalytic properties, for example in the case of xylanases (7).

In order to find out if the acetyl xylan esterases belonging to the different CE families possess distinctive catalytic properties we collected eight different esterases studied previously in four laboratories, and compared their action on various acetylated substrates. It has been noticed previously that different acetyl xylan esterases differ in their action on per-*O*-acetylated methylglycopyranosides (8-10). Thus acetylated sugars were tested in this work, in addition to polymeric acetylated xylan and glucomannan, to determine if these substrate could be used for the classification of carbohydrate-acting esterases.

Experimental

Enzymes

Eight purified esterases were included in the study (Table I). The AXE from *S. lividans* was a generous gift from Dr. Claude Dupont (University of Quebec, Canada). Other enzymes used were: endo- β -xylanase II (XYL), α -glucuronidase (GLUR), β -xylosidase (β X) and endo- β -mannanase (MAN) from *Trichoderma reesei* (11-14), β -mannosidase (β M) from *Aspergillus niger* (15), and commercial α -galactosidase (α G) and β -galactosidase (β G) (Megazyme, Ireland).

Activity assays

Acetyl xylan esterase and acetyl glucomannan esterase activities were measured at pH 5 using 2.5% steam-extracted birch wood xylooligomers and water-extracted spruce wood galactoglucomannan, respectively, as substrates (16,17). Activities on α -naphthyl acetate (Sigma N-8505) and α -naphthyl propionate (Sigma N-0376) were assayed with 1 mM substrate at pH 5 according to Poutanen and Sundberg (16). All enzyme activities are expressed in SI units, katal (kat = mol/s).

The effect of phenylmethylsulfonyl fluoride (PMSF, Sigma P-7626) on esterase activity was tested by incubating enzymes for 60 min with 1 mM PMSF after which the residual activity was determined with α -naphthyl acetate.

Table I. Carbohydrate-acting esterases examined in this study

<i>Enzyme</i>	<i>Organism</i>	<i>CE family^a</i>	<i>Mw (kDa)</i>	<i>pI</i>	<i>pH optimum</i>	<i>Ref.</i>
FAE	<i>Aspergillus oryzae</i>	1	30	3.6	4.5-6.0	18
AXE I	<i>Penicillium purpurogenum</i>	1	48	7.5	5.3	19,20
AXE	<i>Schizophyllum commune</i>	1	31	3.4	7.7	8, 21
AXE	<i>Streptomyces lividans</i>	4	34	9.0	6.0-7.5	22,23
AXE II	<i>Penicillium purpurogenum</i>	5	23	7.8	5.5-6.0	19,24
AXE	<i>Trichoderma reesei</i>	5	34	6.8, 7.0	5.0-6.0	25,26
FAE A	<i>Aspergillus niger</i>	UC	30	3.3	5.0	27,28
AGME	<i>Aspergillus oryzae</i>	UC	36	4.6	5.0-5.5	15

^a UC = unclassified

Hydrolysis experiments

Acetyl-4-*O*-methylglucuronoxylan (Ac-xylan) was obtained from birch wood holocellulose by DMSO extraction (29) and acetyl-galactoglucomannan (Ac-mannan) from spruce thermomechanical pulp by aqueous extraction (15). Arabinoxylan with esterified ferulic acid (Fe-xylan) was from steam-extracted wheat straw (18).

The hydrolysis experiments were carried out for 24 h at 40°C and pH 5 (0.2 M Na-citrate buffer) or pH 6 (0.2 M Na-phosphate buffer) as indicated later in the text. The concentration of Ac-xylan and Fe-xylan used was 10 g/l; Ac-mannan, 7.5 g/l; enzymes, 4 mg of enzyme protein/g of substrate. The amounts of other enzymes used in the synergistic studies were: XYL (20 000 nkat/g), β X (5 000 nkat/g), GLUR (1 000 nkat/g), MAN (5 000 nkat/g), α G (1 000 nkat/g), β M (1000 nkat/g) and β G (1 000 nkat/g). After incubation the reactions were terminated by keeping the samples in boiling water bath for 5 minutes, after which they were centrifuged and the amount of free acetic acid was measured using an enzyme-based kit (Boehringer Test Combination 148 261).

Methyl 2,3,4-tri-*O*-acetyl- β -D-xylopyranoside (Ac-Me-Xylp), methyl 2,3,4,6-tetra-*O*-acetyl- β -D-mannopyranoside (Ac-Me-Manp) and methyl 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranoside (Ac-Me-Glcp) were obtained by acetylation

of commercially available methyl β -glycosides (Sigma) with acetic anhydride in pyridine (30). Methyl 2,4-di-*O*-acetyl- β -D-xylopyranoside, which was used as a standard, was a kind gift from Dr. Jan Hirsch (Slovak Academy of Sciences, Slovakia), and methyl 3,4-di-*O*-acetyl- β -D-xylopyranoside and methyl 5-(*trans*)-feruloyl- α -L-arabinofuranoside (Me-5-Fe-Araf) were kindly provided by Dr. Maria Mastihubova (Slovak Academy of Sciences, Slovakia).

Reactions with esterified monosaccharides were conducted at 40°C and pH 6 (0.1 M Na-phosphate buffer). The substrate concentration was 10 mM except for Ac-Me-Manp, which was used at 6.4 mM concentration (a saturated solution at 25°C). At time intervals the products formed were analysed by thin layer chromatography (TLC) or by gas liquid chromatography (GLC). TLC was carried out on Silica gel 60 (Merck) in the solvent system ethylacetate-benzene-2-propanol (2:1:0.1 v/v). Compounds were detected with orcinol/H₂SO₄ reagent. For GLC analysis aliquots of the reaction mixtures were immediately frozen on dry ice and lyophilized. Residues were trimethylsilylated and analysed by GLC as described earlier (8). The products of the first and the second deacetylation were identified on the basis of chromatographic mobility (TLC) and retention times of known derivatives established previously (8).

Results

Classification of the esterases

Currently the classification of carbohydrate active esterases consists of 13 distinct CE families (<http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>). Acetyl xylan esterases are found in the first seven families. All hydroxycinnamoyl esterases so far classified belong to the CE family 1. The first five families contain both eukaryotic and prokaryotic esterases whereas the CE family 6 has only fungal and the CE family 7 only bacterial members. CE family 8 contains mainly pectin methyl esterases and the CE families 12 and 13 pectin acetyl esterases. The CE family 4 is interesting in that it includes enzymes having both acetyl xylan and acetyl chitin esterases. Many of the members within all these families are grouped based only on open reading frames and thus no information on the activities of the corresponding proteins is available.

Recently three-dimensional structures of two acetyl xylan esterases from the CE family 5 (31-34) and two feruloyl esterases from the CE family 1 (35,36) have been solved. All four of these esterases display an ($\alpha/\beta/\alpha$)-sandwich fold with the catalytic triad Ser-His-Asp in the active site. Two of these enzymes, AXE II from *P. purpurogenum* and AXE from *T. reesei*, were included in the present study.

Eight acetyl xylan esterases, of which four have previously been classified to belong in the CE families 1, 4 and 5, were used in this work (Table I). Two of the esterases, FE from *A. oryzae* and AXE from *S. commune*, having the N-terminal amino acid sequences SGSLQ QVTDF GDNPT (Tenkanen, unpublished) and ASLQQ VSNFG TNPTG VQMFI YVPDQ LAANP PIIVA MHYYT GTAQA YFQGT (P. Biely, M. Hrmova, G. B. Fincher, unpublished), respectively, were both assigned into CE family 1 based on the amino acid sequence homology. The N-terminal amino acid sequence of AGME from *A. oryzae* was XEXTTTNPTYFFTDG (X unknown) (Tenkanen, unpublished), which is not sufficiently homologous with existing sequences for a proper classification of AGME. The deduced amino acid sequence of FAE A from *A. niger* shows no homology with esterases in the existing CE families (28). However, it is highly homologous to three other FAEs from *A. tubingensis*, *A. awamori* and *P. funiculosum* (Swiss-Prot, www.expasy.ch/sprot), neither of which has yet been classified in any of the CE families.

Action on acetylated xylan and glucomannan

All esterases showed acetyl xylan esterase activity regardless whether they were assigned as acetyl or feruloyl esterases (Table II). However, the specific acetyl xylan esterase activity was rather low for FAE A from *A. niger*. On the other hand, FE from *A. oryzae* possessed a significant acetyl xylan esterase activity as has been already reported earlier (18). The highest specific acetyl xylan esterase activities were detected for AXEs from *T. reesei* and *S. lividans*.

Table II. Specific activities on acetylated xylan (AXE) and glucomannan (AGME) measured at pH 5

Enzyme	Organism	CE family	AXE (nkat/mg)	AGME (nakt/mg)	AXE / AGME
FAE	<i>A. oryzae</i>	1	170	57	3.0
AXE I	<i>P. purpurogenum</i>	1	96	6	16
AXE	<i>S. commune</i>	1	310	80	3.8
AXE	<i>S. lividans</i>	4	760	-	
AXE II	<i>P. purpurogenum</i>	5	130	-	
AXE	<i>T. reesei</i>	5	1300	-	
FAE A	<i>A. niger</i>	UC	3	1	3.0
AGME	<i>A. oryzae</i>	UC	43	450	0.1

UC = unclassified, - no activity

Five of the eight esterases tested showed activity on acetylated glucomannan (Table II). All three CE family 1 enzymes were able to act on glucomannan whereas members of the CE families 4 and 5 were inactive against glucomannan. Both unclassified *Aspergillus* esterases were also able to liberate acetic acid from glucomannan. The highest specific acetyl glucomannan esterase activity was detected for AGME from *A. oryzae*, which was also the only enzyme with higher specific activity on acetylated glucomannan than on acetylated xylan.

Six of the esterases could liberate most of the esterified acetic acid from xylan (Figure 1). The degree of deacetylation was not enhanced by the presence of other xylanolytic enzymes (results not shown). The two unclassified *Aspergillus* esterases possessed more limited action on Ac-xylan. They needed the assistance from other xylanolytic enzymes as the degree of deacetylation increased in the presence of xylanase, and even further by AGME after supplementation with α -glucuronidase and β -xylosidase (Figure 2).

Efficient deacetylation of glucomannan was obtained with AXE from *S. commune* and the two esterases FE and AGME from *A. oryzae*. AXE I from *P. purpurogenum* and FAE A from *A. niger* showed limited action (Figure 1). This is in accordance to the activity assay measurements as these two esterases also showed very low Ac-glucomannan esterase activity. The action of any of the esterases on glucomannan was not significantly improved by the presence of other glucomannan-degrading enzymes (results not shown).

Two of the studied esterases, which have already been named feruloyl esterases, were able to hydrolyse ferulic acid from polymeric xylan (results not shown). They also were the only enzymes active on methyl 5-feruloyl-arabinofuranoside (Table III) showing that phenolic acid, which is larger than the acetyl moiety, can be accommodated in the active site of only a few of the carbohydrate-active esterases.

Deacetylation of methyl glycopyranosides

All of the esterases studied, except AXE from *S. lividans*, showed activity on all acetylated glycosides tested (Table III). AXE of *S. lividans* was able to act on Ac-Me-Xylp and Ac-Me-Glcp but not on Ac-Me-Manp, indicating that configuration of the hydroxyl group at position 2 is crucial for the enzyme activity. Interestingly both tested AXEs from CE family 5 did show activity on Ac-Me-Manp even though they were not able to liberate acetic acid from polymeric glucomannan. Thus the substrate binding sites of the CE family 5 esterases seem to afford more freedom to low molecular mass substrates for productive binding than the substrate binding site of AXE from *S. lividans*, which did not show any activity on fully acetylated mannopyranoside. However, xylan lacking the C-6 hydroxymethyl group seems the only polymeric substrate, which fits in the rather tight active site of the CE family 5 esterases (33,34).

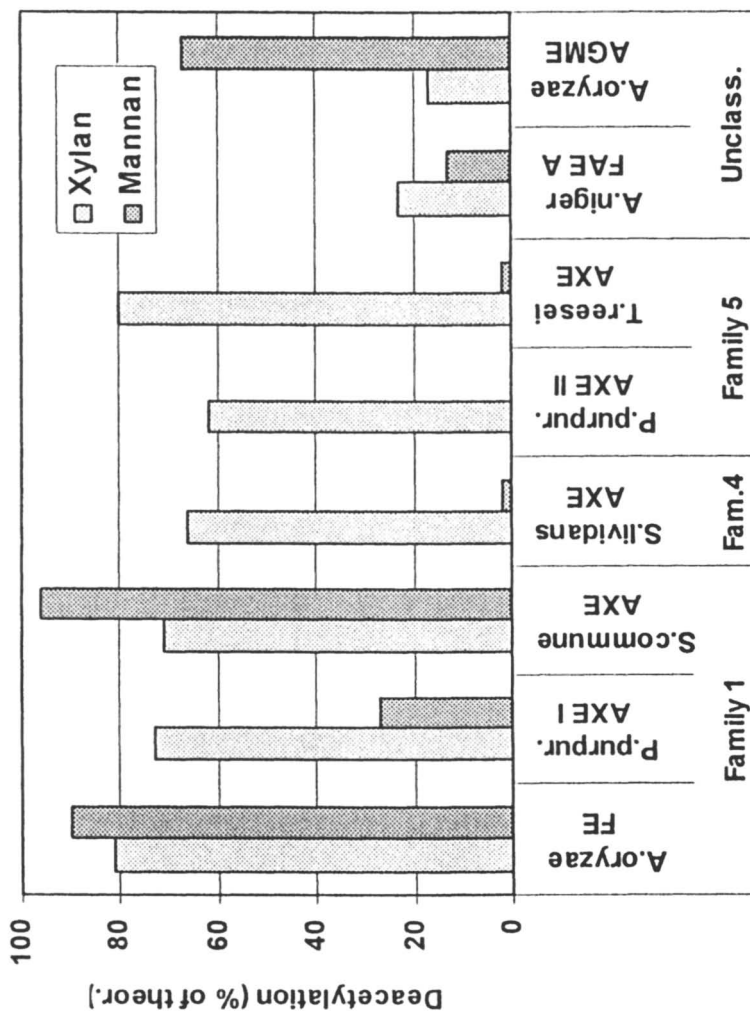


Figure 1. Deacetylation of Ac-xylan and Ac-glucosmannan with different carbohydrate esterases (4 mg/g). Hydrolysis experiments were conducted for 24 hours at 40°C and pH 5, with exceptions of AXE from *S. commune*, AXE from *S. lividans* and AXE II from *P. purpurogenum* where pH 6 was used.

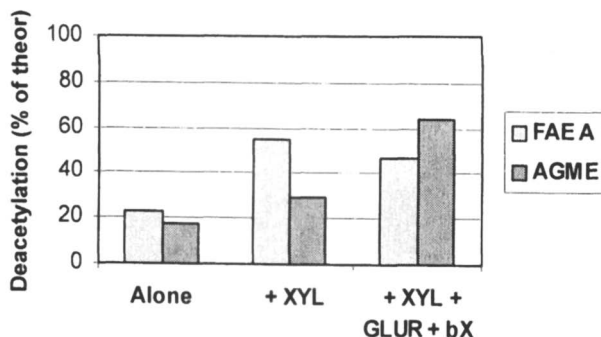


Figure 2. Action of FAE A from *A. niger* and AGME from *A. oryzae* on Ac-xylan alone and in combination with endo- β -xylanase (XYL), α -glucuronidase (GLUR) and β -xylosidase (bX) from *T. reesei*. Experiments were carried out for 24 h at 40°C and pH 5.

Table III. Action of esterases on methyl per-*O*-acetyl-glycopyranosides and on methyl-5-feruloyl-arabinofuranoside

Enzyme	Organism	CE family	Ac-Me-Xylp	Ac-Me-Manp	Ac-Me-Glcp	Me-5-Fe-Araf
FAE	<i>A. oryzae</i>	1	+	+	+	+
AXE I	<i>P. purpurogenum</i>	1	+	+	+	-
AXE	<i>S. commune</i>	1	+	+	+	-
AXE	<i>S. lividans</i>	4	+	-	+	-
AXE II	<i>P. purpurogenum</i>	5	+	+	+	-
AXE	<i>T. reesei</i>	5	+	+	+	-
FAE A	<i>A. niger</i>	UC	+	+	+	+
AGME	<i>A. oryzae</i>	UC	+	+	+	-

UC = unclassified, + deacetylation or deferuloylation, - no activity

According to the pattern of deacetylation of Ac-Me-Xylp, the six classified esterases investigated can be clearly divided into three separate groups, which coincide with their classification into the CE families (Table IV). The esterases belonging to the CE family 1 deacetylate first position 3 leading to 2,4-di-*O*-Me-Xylp. The second deacetylation of 2,4-di-*O*-Me-Xylp by these enzymes took place at either position, since 2-*O*-Ac- and 4-*O*-Ac-Me-Xylp were generated in similar amounts. This observation suggests that once the acetyl group from position 3 had been removed, the enzymes do not recognize the 2-position as the only second target in the molecule. AXE from *S. commune* and FE from *A.*

oryzae show a strikingly similar pattern of deacetylation of 2,3,4-tri-*O*-Ac-Me-Xylp, as identified by GLC analysis of the products formed (results not shown). Only the CE family 1 esterases completely deacetylated Me-Xylp.

AXE II from *P. purpurogenum*, which belongs to the CE family 5, differed clearly in the regioselectivity of the first deacetylation of 2,3,4-tri-*O*-Ac-Me-Xylp from the CE family 1 member AXE I from the same organism. AXE II, similarly as AXE from *T. reesei* (10), attacked position 2 faster than position 3 (Table IV). Surprisingly the specificity on position 2 did not restrict the action on Ac-Me-Manp. Nevertheless, neither of these CE family 5 enzymes showed activity on acetylated glucomannan. In contrast to AXE from *T. reesei*, AXE II from *P. purpurogenum* did not show such a fast second deacetylation of position 3 in 3,4-di-*O*-Ac-Me-Xylp.

AXE from *S. lividans* has already been previously reported to catalyse the second deacetylation of 2,3,4-tri-*O*-Ac-Me-Xylp immediately after the first deacetylation, so that the product of the first deacetylation is never observed (9). The double deacetylation affords only one product, 4-*O*-Ac-Me-Xylp. However,

Table IV. Main products of the first and the second deacetylation of methyl 2,3,4-tri-*O*-Ac- β -D-xylopyranoside by the esterases

Enzyme	Organism	CE family	Main diacetate(s) Formed	Main monoacetate(s) formed
FAE	<i>A. oryzae</i>	1	2,4-di- <i>O</i> -Ac	2- <i>O</i> -Ac + 4- <i>O</i> -Ac
AXE I	<i>P. purpurogenum</i>	1	2,4-di- <i>O</i> -Ac (+ 3,4-di- <i>O</i> -Ac)	2- <i>O</i> -Ac + 4- <i>O</i> -Ac
AXE	<i>S. commune</i>	1	2,4-di- <i>O</i> -Ac	2- <i>O</i> -Ac + 4- <i>O</i> -Ac
AXE	<i>S. lividans</i>	4	Traces of 2,3-di- <i>O</i> -Ac not as intermediate ^a	4- <i>O</i> -Ac
AXE II	<i>P. purpurogenum</i>	5	3,4-di- <i>O</i> -Ac	4- <i>O</i> -Ac (+ 2- <i>O</i> -Ac)
AXE	<i>T. reesei</i>	5	Traces of 2,4-di- <i>O</i> -Ac + 2,3-di- <i>O</i> -Ac ^a	4- <i>O</i> -Ac (+ 2- <i>O</i> -Ac)
FAE A	<i>A. niger</i>	UC	2,4-di- <i>O</i> -Ac	2- <i>O</i> -Ac + 4- <i>O</i> -Ac
AGME	<i>A. oryzae</i>	UC	2,4-di- <i>O</i> -Ac	4- <i>O</i> -Ac (+ 2- <i>O</i> -Ac)

UC = unclassified, ^a The second deacetylation at position 2 and 3 is much faster than the first one thus no diacetate intermediate is accumulated.

the reaction mixture contained a small amount of 2,3-di-*O*-Ac-Me-Xylp, which persisted in the reaction mixture even when the starting triacetate was no longer present.

Analogous differences in deacetylation as seen with 2,3,4-tri-*O*-Ac-Me-Xylp were exhibited by the investigated esterases on 2,3,4,6-tetra-*O*-Ac-Me-Glcp. This substrate has the same configuration of acetyl substituents as 2,3,4-tri-*O*-Ac-Me-Xylp, but is larger, due to the C-6 acetylated hydroxymethyl group. AXE from *S. commune* and FE from *A. oryzae*, both members of CE family 1, attacked the glucopyranoside in an identical manner, deacetylating first the position 3. The second deacetylation took place quite selectively at position 2 leading to 4,6-di-*O*-Ac-Me-Glcp in high yields. Thus those enzymes preferred the secondary acetyl groups and did not instantly attack the primary acetyl group at position 6 as more nonspecific lipases do (8,37). Deacetylation of 2,3,4,6-tetra-*O*-Ac-Me-Glcp by AXE from *S. lividans* and AXE from *T. reesei* at positions 2 and 3 proceeded almost simultaneously to also give 4,6-*O*-Ac-Me-Glcp as the main product. AXE from *T. reesei* did not stop its action on the level of diacetylated glucopyranoside, but converts it further to 4- and 6-monoacetylated derivatives of Me-Glcp.

Interesting results were obtained with 2,3,4,6-tetra-*O*-Ac-Me-Manp, which differs from the xylopyranoside and glucopyranoside in an axial orientation of the hydroxyl group at the position 2. This change makes the substrate resistant to hydrolysis by AXE from *S. lividans* and also changes the regioselectivity of the first and the second deacetylation by other esterases capable of attacking acetylated mannopyranoside. The esterases of the CE family 1 deacetylate Ac-Me-Manp at positions 2 and 3, leading to high yields of 4,6-di-*O*-Ac-Me-Manp. The esterases of the CE family 5 behave as if completely disoriented with respect to regioselectivity of 2,3,4,6-tetra-*O*-Ac-Me-Manp. Early reaction mixtures contained all four possible triacetates, some of them predominating over the others.

Activity on α -naphthyl esters

All investigated esterases, except AXE from *S. lividans*, also possessed activity on artificial α -naphthyl acetate (Table V). Thus the alcohol moiety of the substrate does not need to be a sugar molecule for these esterases. The AXE from *S. lividans* has already been reported to show negligible activity on p-nitrophenyl acetate (22). The enzymes were not strictly specific acetyl esterases as they were able to hydrolyse α -naphthyl propionate (Table V). The specific activity on α -naphthyl acetate was, however, higher than that on α -naphthyl propionate, showing the preference on acetyl esters. The CE family 1 esterases seemed slightly more specific for the acetyl moiety than the CE family 5 esterases.

Table V. Specific activities on aryl esters

Enzyme	Organism	CE family	A-Naphthyl acetate (nkat/mg)	α -Naphthyl propionate (nkat/mg)	Ratio Ac / Pr
FAE	<i>A. oryzae</i>	1	562	115	4.9
AXE I	<i>P. purpurogenum</i>	1	120	24	5.0
AXE	<i>S. commune</i>	1	987	354	2.8
AXE	<i>S. lividans</i>	4	-	-	
AXE II	<i>P. purpurogenum</i>	5	82	59	1.4
AXE	<i>T. reesei</i>	5	106	80	1.3
FAE A	<i>A. niger</i>	UC	539	447	1.2
AGME	<i>A. oryzae</i>	UC	690	nd	

UC = unclassified, - no activity, nd = not determined

Inhibition by PMSF

Serine esterases, such as AXE from *T. reesei*, are inhibited by phenylmethylsulfonyl fluoride (PMSF) (26). The effect of PMSF on the other esterases in this study was also tested. Surprisingly the CE family 5 esterases were the only ones that were almost completely inhibited by PMSF (Table VI). Esterases in the CE family 1 were only partially inhibited even though they also contain the characteristic catalytic triad Ser-His-Asp in the heart of active site (36,37). The effect of PMSF on AXE from *S. lividans* was not tested, as this enzyme did not have activity on α -naphthyl acetate. Furthermore the unclassified FAE A from *A. niger* was not inhibited at all by PMSF, but sequence data showed the presence of the classical catalytic triad. Thus it seems that esterases in different CE families have clearly distinguished active sites and catalytic amino acids. Some of them may contain catalytic cysteine or histidine residues instead of serine in the active site in analogy to proteases. Tests with specific inhibitors for sulfhydryl and imidazole groups and effects of chelating agents would be of interest in future studies. It may well be that different inhibitors may also be used in classification of carbohydrate-acting esterases.

Discussion

The present work studied the catalytic differences of eight acetyl xylan esterases from three carbohydrate esterase (CE) families 1, 4 and 5 (<http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>). Esterases from the CE family 1 form a versatile group of enzymes containing both fungal and bacterial

Table VI. Inhibition of the esterases with 1 mM PMSF. Activity was measured with α -naphthyl acetate

<i>Enzyme</i>	<i>Organism</i>	<i>CE family</i>	<i>Inhibition</i>
FAE	<i>A. oryzae</i>	1	49 %
AXE I	<i>P. purpurogenum</i>	1	17 %
AXE	<i>S. commune</i>	1	10 %
AXE	<i>S. lividans</i>	4	no activity on the substrate used
AXE II	<i>P. purpurogenum</i>	5	88 %
AXE	<i>T. reesei</i>	5	85 %
FAE A	<i>A. niger</i>	UC	ND
AGME	<i>A. oryzae</i>	UC	nd

UC = unclassified, ND = no detectable inhibition, nd = not determined

esterases, which possess activity on various substrates. All three esterases from the CE family 1 studied here could liberate acetyl groups from xylan and glucomannan. Furthermore one of them was able to act on hydroxycinnamoyl (feruloyl) esters. They all deacetylated methyl per-*O*-acetyl- β -D-xylopyranoside, -glucopyranoside and -mannopyranoside, from which the first acetyl group was removed from position 3. AXE I from *P. purpurogenum* differed somewhat from FE from *A. oryzae* and AXE from *S. commune* by showing limited action on acetylated glucomannan as well as in its pattern of deacetylation of methyl per-*O*-acetyl-glycopyranosides.

The two esterases from the CE family 5 were clearly distinguishable from the CE family 1 esterases. They were specific for acetylated xylan and did not act on acetylated glucomannan. However, they were able to liberate acetyl groups from methyl per-*O*-acetyl- β -D-mannopyranoside in addition to -xylopyranoside and -glucopyranoside. The CE family 5 esterases showed preference for acetyl groups at position 2 in methyl 2,3,4-tri-*O*-acetyl- β -D-xylopyranoside. The axial substituent at position 2 in mannopyranoside caused these enzymes to lose the regioselectivity of the first deacetylation, indicating the importance of a right configuration of the acetyl substituent at this position. AXE II from *P. purpurogenum* and AXE from *T. reesei* had comparable properties. This could be expected based on the three dimensional structural similarities of these enzymes (33,34). Some differences were, however, observed in their action on methyl 2,3,4-tri-*O*-acetyl- β -D-xylopyranoside. AXE II from *P. purpurogenum* did not show as a comparable rapid second deacetylation of position 3 as did AXE from *T. reesei*.

The acetyl xylan esterase from the CE family 4 possessed the strictest substrate specificity. AXE from *S. lividans* did not have activity on acetylated glucomannan, methyl per-*O*-acetyl- β -D-mannopyranoside or artificial α -

naphthyl acetate. It quickly deacetylated both positions 2 and 3 of the sugar acetates. Unfortunately there was only one member of this CE family available for this study and thus the generality of the catalytic properties of this family could not be established. Furthermore, it was the only bacterial esterase included in this study. The CE family 4 differs from the CE families 1 and 5 in that it mainly contains several chitin and chitooligosaccharide deacetylating esterases.

The two unclassified enzymes showed properties incomparable to those of the other investigated esterases. They appear, therefore, to be members of other CE families. Based on the amino acid sequence homologies, neither of these enzymes could be classified into the existing 13 CE families, thus they likely belong to new CE families. Furthermore, FAE A from *A. niger* is highly homologous to other *Aspergillus* cinnamoyl/feruloyl esterases. Thus these enzymes seem definitely to belong to a new CE family which includes esterases with high activity on esters of hydroxycinnamic acids and low or even negligible activity on acetyl xylan and acetyl glucomannan.

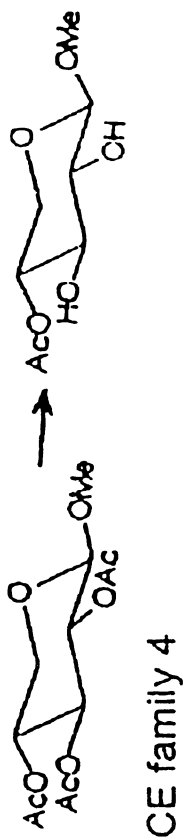
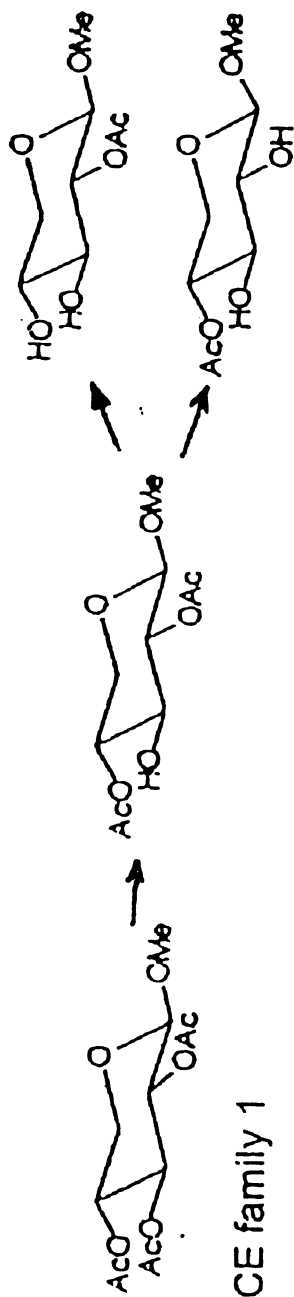
The differences in substrate specificities and catalytic properties seem to follow the structural classification of acetyl xylan esterases based on the amino acid sequences and the hydrophobic cluster analysis (Table VII). According to the regioselectivity of the first and the second deacetylation of methyl glycosides, the investigated esterases fit surprisingly well into three groups corresponding their classification into CE families (Figure 3). However, proper classification of acetyl xylan esterases can be based only on activities on both natural acetylated xylan and glucomannan and on action pattern on acetylated xylose and mannose. Lack of action on α -naphthyl acetate or on p-nitrophenyl acetate as well as inhibition studies give further information on the catalytic action. It will be of great interest to analyze in the future the properties of more acetyl xylan esterases, particularly from the CE families 2,3,6 and 7, in order to get more complete picture on differences in the mode of action of these enzymes.

The targeted deacetylation of natural acetylated polysaccharides as well as chemically acetylated polysaccharides or mono- and oligosaccharides, is of interest as specific chemical acetylation and deacetylation is difficult and requires several synthetic steps. Controlled enzymatic removal of certain acetyl groups opens up possibilities to produce partially acetylated structurally defined carbohydrates. For example some of the esterases studied could be useful in manufacturing at position 2 and/or 3 deacetylated cellulose acetate as they exhibited clear activity as well as regioselectivity on per-*O*-acetylated methyl β -D-glucopyranoside. Enzyme assisted regioselective deacetylation of cellulose acetates has also recently been reported (38,39). Another potential application of carbohydrate esterases is in the synthesis of sugar esters, particularly due to their activity on secondary alcohols.

Table VII. Summary of the acetyl xylan esterase classification

<i>CE Family</i>	<i>Activity on</i>					<i>1st deacetylation</i>
	<i>Ac-xylan</i>	<i>Ac-mannan</i>	<i>Ac-xyl</i>	<i>Ac-man</i>	<i>Ac-glc</i>	<i>α-Naphthyl acetate</i>
1	+	+	+	+	+	C-3
4	+	-	+	-	+	C-2/3
5	+	-	+	+	+	C-2

+ action, - no action



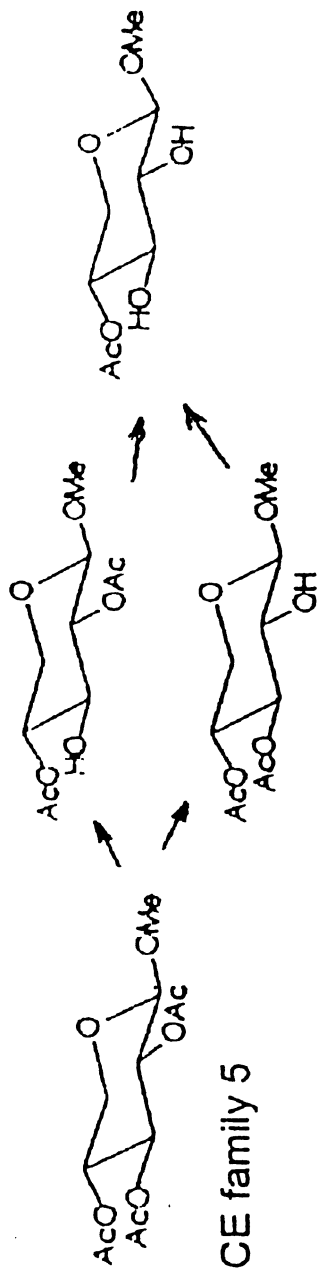


Figure 3. Action of the esterases from the CE families 1, 4 and 5 on methyl 2,3,4-tri-O-acetyl- β -D-xylopyranoside.

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References

1. Aspinall, G.O. *Adv. Carbohydr. Chem.* **1959**, *14*, 429-468.
2. Timell, T.E. *Wood Sci Technol.* **1967**, *1*, 45-70.
3. Mueller-Harvey, I.; Hartley, R.D.; Harris, P.J.; Curzon, E.H. *Carbohydr. Res.* **1986**, *148*, 71-85.
4. Biely, P. *Trends Biotechnol.* **1985**, *3*, 286-290.
5. Christov, L.P.; Prior, B.A. *Enzyme Microb. Technol.* **1993**, *15*, 460-475.
6. Williamson, G.; Kroon, P.; Faulds, C.B. *Microbiology* **1988**, *144*, 2011-2023.
7. Biely, P.; Vršanská, M.; Tenkanen, M.; Kluepfel, D. *J. Biotechnol.* **1997**, *57*, 151-166.
8. Biely, P.; Cote, G.L.; Kremnicky, L.; Weisleder, D.; Greene, R.V.; *Biochim. Biophys. Acta* **1996**, *1298*, 209-222.
9. Biely, P.; Cote, G.L.; Kremnicky, L.; Greene, R.V.; Dupont, C.; Kluepfel, D. *FEBS Lett.* **1996**, *396*, 257-260.
10. Biely, P.; Cote, G.L.; Kremnicky, L.; Greene, R.V.; Tenkanen, M. *FEBS Lett.* **1997**, *420*, 121-124.
11. Tenkanen, M.; Puls, J.; Poutanen, K. *Enzyme Microb. Technol.* **1992**, *14*, 566-574.
12. Siika-aho, M.; Tenkanen, M.; Buchert, J.; Puls, J.; Viikari, L. *Enzyme Microb. Technol.* **1994**, *16*, 813-819.
13. Poutanen, K.; Puls, J. *Appl. Microb. Biotechnol.* **1988**, *28*, 425-432.
14. Stålbrand, H.; Siika-aho, M.; Tenkanen, M.; Viikari, L. *J. Biotechnol.* **1993**, *29*, 229-242.
15. Tenkanen, M.; Thornton, J.; Viikari, L. *J. Biotechnol.* **1995**, *42*, 197-206.
16. Poutanen, K.; Sundberg, M. *Appl. Microbiol. Biotechnol.* **1988**, *28*, 419-424.
17. Tenkanen, M. *Biotechnol. Appl. Biochem.* **1998**, *27*, 19-24.
18. Tenkanen, M.; Schuseil, J.; Puls, J.; Poutanen, K. *J. Biotechnol.* **1991**, *18*, 69-84.
19. Egana, L.; Gutierrez, R.; Caputo, V.; Peirano, A.; Steiner, J.; Eyzaguirre, J.; *Biotechnol. Appl. Biochem.* **1996**, *24*, 133-139.
20. Gordillo, F.; Eyzaguirre, J. 2002, GeneBank accession number AF529173.
21. Halgasova, N.; Kutejova, E.; Timko, J. *Biochem. J.* **1994**, *298*, 751-755.
22. Shareck, F.; Biely, P.; Morosoli, R.; Kluepfel, D. *Gene* **1995**, *153*, 105-109.

23. Dupont, C.; Roberge, M.; Shareck, F.; Morosoli, R.; Kluepfel, D. *Biochem. J.* **1996**, *319*, 881-886.
24. Gutierrez, R.; Cederlund, E.; Hjelmquist, L.; Peirano, A.; Herrera, F.; Ghosh, D.; Duax, W.; Jörnval, H.; Eyzaguirre, J. *FEBS Lett.* **1998**, *423*, 35-38.
25. Sundberg, M.; Poutanen, K. *Biotechnol. Appl. Biochem.* **1991**, *13*, 1-11.
26. Margolles-Clark, E.; Tenkanen, M.; Söderlund, H.; Penttilä, M. *Eur. J. Biochem.* **1996**, *237*, 553-560.
27. Faulds, C.; Williamson, G. *Microbiology* **1994**, *140*, 779-787.
28. de Vries, R.P.; Michelsen, B.; Poulsen, C.H.; Kroon, P.A.; van den Heuvel, R.H.H.; Faulds, C.B.; Williamson, G.; van den Hombergh, J.P.T.W.; Visser, J. *Appl. Environ Microbiol* **1997**, *63*, 4638-4644.
29. Häggglund, E.; Lindberg, B.; McPherson, J. *Acta Chem. Scand.* **1956**, *10*, 1160-1164.
30. Horton, D. *Methods Carbohydr. Chem.* **1963**, *2*, 368-373.
31. Pangborn, W.; Erman, M.; Li, N.; Burkhart, B.M.; Pletnev, V.Z.; Duax, W.L.; Gutierrez, R.; Peirano, A.; Eyzaguirre, J.; Thiel, D.J.; Ghosh, D. *Protein Struct. Funct. Genet.* **1996**, *24*, 523-524.
32. Ghosh, D.; Erman, M.; Sawicki, M.; Lala, P.; Weeks, D.R.; Li, N.; Pangborn, W.; Thiel, D.J.; Jörnval, H.; Gutierrez, R.; Eyzaguirre, J. *Acta Crystallogr. Sect. D. Biol. Crystallogr.* **1999**, *55*, 779-784.
33. Hakulinen, N.; Tenkanen, M.; Rouvinen, J.; *J. Structural. Biol.* **2000**, *132*, 186-190.
34. Ghosh, D.; Sawicki, M.; Lala, P.; Erman, M.; Pangborn, W.; Eyzaguirre, J.; Gutierrez, R.; Jörnval, H.; Thiel, D.J. *J. Biol. Chem.* **2001**, *276*, 11159-11166.
35. Schubot, F.D.; Kataeva, I.A.; Blum, D.L.; Shah, A.K.; Ljungdahl, L.G.; Rose, J.P.; Wang, B.-C. *Biochemistry* **2001**, *40*, 12524-12532.
36. Prates, J.A.M.; Turbauriech, N.; Charnock, S.J.; Fontes, C.M.G.A.; Ferreira, M.A.; Davies, G.J. *Structure* **2001**, *9*, 1183-1190.
37. Shaw, J.-F.; Klivanov, A.M. *Biotechnol. Bioeng.* **1987**, *29*, 648-651.
38. Moriyoshi, K.; Ohmoto, T.; Ohe, T.; Sakai, K. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1708-1713.
39. Altaner, C.; Saake, B.; Puls, J. *Cellulose* **2002**, *8*, 259-265

Chapter 14

Manganese Peroxidase and Its Role in the Degradation of Wood Lignin

Annele Hatakka¹, Taina Lundell¹, Martin Hofrichter²,
and Pekka Maijala¹

¹Department of Applied Chemistry and Microbiology, P.O. Box 56 (Viikki
Biocenter), 00014 University of Helsinki, Helsinki, Finland

²Unit of Environmental Biotechnology, International Graduate School
at Zittau, 02763 Zittau, Germany

White-rot wood-rotting and litter-decomposing fungi produce lignin-degrading enzymes the most common of which are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. According to literature and our own results, MnP and laccase are common ligninolytic enzymes whereas LiP is less common. Molecular properties of MnPs produced by several fungi are well known. MnP oxidizes Mn(II) to Mn(III), which in chelated form is a powerful oxidizing system, and when amended with unsaturated lipids is also able to mineralize up to 16 % of ¹⁴C-labelled synthetic lignin to ¹⁴CO₂. The results obtained until now indicate that the mineralization of lignin may occur outside the fungal cell wall. This points to the key role of MnP in lignin degradation. MnP in a mixture of Tween 80, Mn²⁺, Mn-chelating organic acid and hydrogen peroxide generating system resulted both in depolymerization of milled pine wood, and polymerization of the insoluble part of pine wood, but depolymerization was the most prominent reaction. Many suitable fungi for biopulping produce MnP whereas the production of LiP does not seem to be necessary in this application. The role of laccase is unclear. However, selective lignin degradation and efficiency in biopulping require a proper balance between lignin and cellulose degradation, and therefore also thorough studies to clarify the significance of cellulose and hemicellulose degradation in lignin-selective fungi are needed.

Wood-rotting fungi that cause white rot in wood are the most efficient lignin degraders in nature. They are a taxonomically heterogeneous group of higher basidiomycetous fungi, which are characterized by their ability to depolymerize and mineralize lignin using extracellular oxidative enzymes. Physiological conditions for lignin degradation and secretion patterns of the ligninolytic enzymes vary between different fungi (1, 2). In the past, researchers have tried to find correlations between the expression of different ligninolytic enzymes and lignin degradation. This has been difficult since even now we do not know all the enzymes and factors necessary for lignin degradation, and very little is known about the expression of different enzymes and production of small molecular weight compounds when fungi grow on wood. Most studies have been carried out in liquid cultures or under otherwise artificial conditions.

The most important lignin degrading enzymes were discovered and characterized in the beginning of the 1980's, while before that only laccase had been known. Since the detection of lignin peroxidases (LiPs) in 1983 and manganese peroxidases (MnPs) in 1984 (review: 3), these enzymes have been isolated from many different fungi and characterized in detail. The enzymology and molecular biology of lignin degradation have mainly been studied in *Phanerochaete chrysosporium* (4, 5, 6), but many other white-rot fungi efficiently degrade lignin (1, 2), and the idea how lignin is degraded has become more versatile. There are fungi, e.g. *Ceriporiopsis subvermispora* that show better selectivity for lignin than *P. chrysosporium*, that is, they degrade and remove relatively more lignin than polysaccharides already in the early stages of wood decomposition (7, 8, 9). These fungi could also have direct applications such as biopulping. How these fungi degrade lignin is a very interesting question. It is important to know which enzymes are expressed and functional when fungi grow under natural or nearly natural conditions, i.e. on wood or straw.

Discovery and Characteristics of Lignin Degrading Peroxidases

Lignin peroxidase (LiP, ligninase) was discovered from the most studied white-rot fungus *Phanerochaete chrysosporium* and published almost simultaneously by two research groups (10, 11). The finding of manganese peroxidase (MnP) was published about a year later (12). LiPs and MnPs are heme-containing glycoproteins that require hydrogen peroxide as an oxidant. Most fungi secrete several isoenzymes into their cultivation medium. The progress in the clarification of the mechanisms and properties of these enzymes has been rapid. LiP oxidizes non-phenolic lignin substructures by abstracting one electron and generating cation radicals that are then decomposed chemically (3, 13). Reactions of LiP using a variety of lignin model compounds and synthetic lignin have thoroughly been studied, and its capability for C_α - C_β bond cleavage, ring opening and other reactions have been demonstrated (3).

Kirk and Farrell (3) launched the concept of "enzymatic combustion" in their review. Progress in understanding the enzyme functions in the molecular level has been remarkable (14). However, heterologous expression of these heme peroxidases has been difficult. Instead, homologous expression of peroxidases was developed (15). Recently some progress has been achieved in heterologous expression of peroxidases, but the amounts of recombinant products have been so low that economically feasible production of these enzymes in large scale is not yet possible, hampering the use of ligninolytic peroxidases in different applications. 3-D structure of LiP (16, 17) and MnP (18) were solved in the early 1990's.

Properties and Occurrence of Manganese Peroxidase

Manganese peroxidases (MnPs) are somewhat larger heme proteins than LiPs with molecular masses of 47-60 kDa, are glycosylated and have usually acidic pI's and low pH optima (4, 19) (Table 1). The heme iron is in the ferric, high spin, pentacoordinate state and is ligated to the proximal histidine. Its crystal structure (18), and DNA sequence alignments show that the heme environment of MnP is similar to that of other plant and fungal peroxidases, especially to LiP. Crystal structures of substrate binding site mutants of MnP indicated that there is only one major Mn binding site (20).

MnP oxidizes Mn^{2+} to Mn^{3+} that is stabilized by organic acids such as oxalate, malate, lactate and malonate chelating the oxidized manganese ions (6, 21). Chelated Mn^{3+} then oxidizes phenolic rings in lignin and lignin model compounds to phenoxyl radicals, which leads to the decomposition of these structures. It is in general a powerful oxidant, and may also oxidize certain non-phenolic aromatics, e.g., methoxybenzenes, carboxylic acids, thiols, and unsaturated aliphatic compounds (lipids). Figure 1 shows examples of reactions catalyzed by MnP. Chelated Mn(III) is stable and slow enough to diffuse to some distance in the lignocellulosic complex, and oxidation by Mn(III) could therefore be the first reaction to start lignin depolymerization. However, it is not strong enough to attack recalcitrant non-phenolic units of lignin. MnP activity results in the formation of phenoxyl radicals that may subsequently cause the cleavage of some bonds between aromatic rings and the C_{α} carbon atoms (6).

A little less than 60 basidiomycetous white-rot and litter decomposing fungi have been demonstrated to produce MnP either in liquid or solid state cultivations (22). So far, MnP has not been found in bacteria or in fungi other than basidiomycetes, i.e. not in molds or yeasts. MnP is more common than LiP (1, 2, 23, 24). Table 1 shows examples of MnPs of wood-rotting and litter-decomposing fungi.

A comparison of MnPs from *P. chrysosporium* and *Bjerkandera* sp. BOS55 indicates that white-rot fungi produce two different types of MnPs, those strictly requiring Mn^{2+} and those showing high Mn^{2+} affinity but also having a Mn^{2+} independent activity (25). One of the regulators of the production of LiPs, MnP

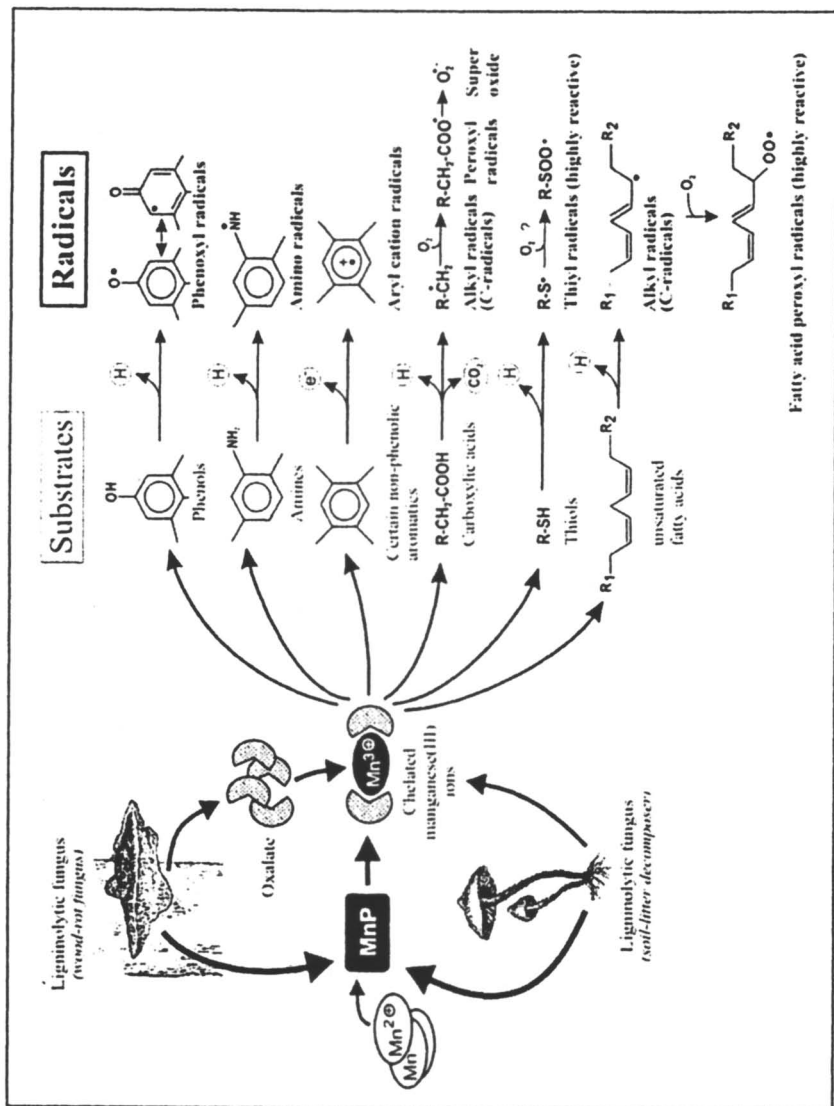


Figure 1. Radicals formed by manganese peroxidase (references, see 2 and 22)

Table 1. Molecular Masses and Isoelectric Points of Manganese Peroxidase by Some White-Rot Wood-Rotting and Litter-Decomposing Fungi

<i>Fungus</i>	<i>Molecular masses M_r (kDa)</i>	<i>Isoelectric points (pIs)</i>	<i>References</i>
<i>Abortiporus biennis</i>	38-45	3.9-6.5	24
<i>Agaricus bisporus</i>	ND	3.35, 3.25, 3.3	45
<i>Agrocybe praecox</i>	42	6.3-7.0	46
<i>Bjerkandera sp. BOS55</i>	44, 45	3.45, 3.40	25, 47
<i>Bjerkandera adusta</i>	ND	3.45, 3.35	48
<i>Ceriporiopsis subvermispora</i>	53-63	4.1-4.6 (liquid), 3.2, 3.3, 3.4, 3.5 (wood)	49, 50
<i>Collybia dryophila</i>	43		51
<i>Dichomitus squalens</i>	48, 48.9	3.9, 4.15	52
<i>Flavodon (Irpex) flavus</i>	43-99		53
<i>Heterobasidion annosum</i>	ND	3.3, 3.5, 3.7	54
<i>Lentinula edodes</i>	49	3.2	55
<i>Nematoloma frowardii</i>	42-44 (liquid) 50 (straw)	3.1-4.0 (liquid); 3.2 (straw)	30, 56
<i>Panus tigrinus</i>	43	2.95, 3.2	57
<i>Phanerochaete chrysosporium</i>	46	4.2- 4.9; 4.9 (aspen pulp)	19, 58, 59, 60
<i>P. flavido-alba</i>	45	3.55-5.85	61
<i>Phanerochaete sordida</i>	45	3.3, 4.2, 5.3	62
<i>Phlebia radiata</i> 79 (ATCC 64658)	44-48	3.6, 3.85, 4.85 (liquid culture), 3.3- 5.9 (straw)	27, 63, 64
<i>Phlebia tremellosa</i> 2845 (ATCC 48754)	MnPs:	ND	65
<i>Pleurotus eryngii</i>	43 (2 isoenzymes)	3.65, 3.75 (2 isoenzymes)	66
<i>Pleurotus ostreatus</i>	42-45	3.5, 3.7, 4.3 (liquid) 3.5, 3.7 (wood)	67, 68
<i>Rigidoporus lignosus</i>	42	3.5, 3.7	69
<i>Stropharia coronilla</i>	41, 41, 43	6.3-7.1, 3.5-3.7, 5.1	70
<i>Trametes gibbosa</i>	40-43	ND	24
<i>Trametes hirsuta</i>	40-45	ND	24
<i>Trametes versicolor</i>	49	2.9-3.2	71

ND = not determined or reported; LiP, lignin peroxidase; MnP, manganese peroxidase

and “hybrid” MnPs may be the concentration of Mn in the medium. Fungi can respond differently to supplemented Mn^{2+} (24, 26, 27). Manganese content of the natural substrate, wood, may thus also regulate the expression of the MnPs vs. LiPs.

Role of Manganese Peroxidase in the Degradation of Lignin and Lignin-Related Compounds

Depolymerization of lignin by MnPs from *Phanerochaete chrysosporium*, *Phlebia radiata* and *Nematoloma frowardii* has been demonstrated (26, 28, 29, 30, 31, 32). MnP also causes depolymerization of chlorolignin (33), demethylation of lignin (34) and bleaching of pulp (35). The enzyme mediates initial steps in the degradation of high-molecular weight lignin (26). MnP in the presence of suitable organic acids is even able to mineralize lignin and lignin model compounds to considerable amounts (29). Table 2 summarizes some experiments where MnP, LiP or different mixtures of enzymes have been used to degrade polymeric lignin model compounds.

Purified MnP from *P. radiata* converted pine milled wood in the presence of the surfactant Tween 80 as a source of unsaturated fatty acids, Mn(II), malonate and H_2O_2 -producing system (glucose – glucose oxidase), and as depolymerization products, produced fragments having predominant molecular mass of ca. 0.5 kDa. MnP oxidized an aromatic non-phenolic β -O-4 dimer (lignin substructure), which is not normally attacked by MnP, in the presence of pine wood meal (36). Thus, certain wood components may actually enhance the degradative ability of MnP in the way similar to that caused by Tween 80, unsaturated fatty acids, or thiols. These results further point to the importance to study lignin degradation under natural conditions, not only the expression of enzymes or enzyme isoforms or isoenzymes, but also to study the degradation processes by these enzymes.

The exact mechanism that MnP uses to oxidize non-phenolic residues is not completely understood. *Ceriporiopsis subvermispota* has been reported to degrade non-phenolic lignin structures by one-electron oxidation of the aromatic ring (37) or by hydrogen abstraction at the benzyl position (38). This fungus does not produce LiP (39), although *lip*-like genes have been found (40). Lipid peroxidation may be involved in the degradation of lignin and other recalcitrant molecules by this fungus (38, 41). The MnP-lipid system is indeed strong enough to degrade C_α - C_β and β -aryl ether bonds of non-phenolic β -O-4 lignin models (42). In the presence of Mn(II), MnP promotes the peroxidation of unsaturated lipids generating transient lipoxyradical intermediates. These lipoxyradicals have been shown to oxidize non-phenolic lignin model compounds, and it was found that the MnP-lipid peroxidation system is able to depolymerize both phenolic and non-phenolic (methylated) synthetic lignins

Table 2. Examples of *In Vitro* Studies on the Degradation of Lignin by Manganese Peroxidase or by Enzyme Mixtures

<i>Enzyme(s)</i>	<i>Lignin model compound^a</i>	<i>Results of degradation</i>	<i>References</i>
LiP (crude)	¹⁴ C-methylated spruce lignin	Depolymerization	10
MnP	Four ¹⁴ C-labelled DHPs, 0.5 – 7 hrs	No ¹⁴ CO ₂ , repolymerization, syringyl-DHP depolymerized, guaiacyl-DHP polymerized and depolymerized	28
MnP	Chlorolignin	Depolymerization of lignin	33
MnP, laccase	¹⁴ C-(lignin)-labelled lignocellulose	6.5% soluble products, synergistic action of laccase and MnP	69
LiP, MnP LiP + MnP	¹⁴ C-(methoxyl)-DHP	3-6% demethoxylation	34
LiP, MnP, laccase	Alkali lignin	LiP degraded guaiacyl and syringyl structures in non-phenolic methylated lignin, MnP and laccase required phenolic OH-groups	72
LiP, MnP, LiP + MnP	Hardwood lignin	LiP + MnP: 5.1% reduction in lignin content, solubilization of lignin	73
MnP	¹⁴ C-labelled milled straw lignin	4.2-9.7% as ¹⁴ CO ₂ , low molecular wt products	29
MnP	¹⁴ C-(ring)-DHP	¹⁴ CO ₂ evolution, 4-6%, water-soluble products (30-50%)	31
MnP	¹⁴ C-(ring)-DHP, 16 d	¹⁴ CO ₂ evolution, 7% in 16 d	30
MnP	¹⁴ C-(ring)-DHP	¹⁴ CO ₂ evolution 12% in 36 hrs, 16% in 166 hrs	32

^a DHP = synthetic lignin, dehydrogenation polymerizate

(38). This finding supports the general involvement of lipid peroxidation in fungal polymer degradation.

MnP from *P. radiata* catalyzes peroxidation of linoleic acid and other unsaturated lipids. The reaction system cleaves the non-phenolic dimeric β -O-4 lignin model compound, and also promotes the MnP-catalyzed mineralization of ^{14}C -labeled synthetic lignin and ^{14}C -labeled wheat straw in a cell-free system (32). Kapich et al. (43) used a non-phenolic β -O-4 dimeric lignin model compound to show that different peroxy radical (ROO^{\bullet}) generating systems, e.g. MnP-Mn(II)-linoleic acid, oxidized the model to products which indicated hydrogen abstraction and electron transfer. Some (about 20 %) of the ^{14}C -labelled products were missing and indicated the formation of volatile products (e.g. CO_2). They further presented a hypothesis that biogenic peroxy radicals may be the actual agents of lignin biodegradation. Evidence was given that *C. subvermispora* produces unsaturated fatty acids, e.g. 9,12-octadecadienoic acid, at the early stages of cultivation on wood meal. These and other compounds could form peroxides suggesting their involvement in free radical generation *in situ*, i.e. in wood cell walls (44).

Crude and purified MnPs from *N. frowardii* and *P. radiata* catalyze partial depolymerization of ^{14}C -(ring)-labelled synthetic lignin (dehydrogenation polymerizate, DHP) into water-soluble fragments (30-50 %) and simultaneously, a release of $^{14}\text{CO}_2$ ranging from 4-6 % is detected (31). Both linoleic acid and Tween 80 promotes the MnP-catalyzed direct mineralization of lignin (31). In the case of ^{14}C -DHP, the stimulating effect of Tween 80 and linoleic acid is even greater than that of glutathione, which also enhances the mineralization of lignin by MnP (29). Wood rotting fungi contain high amounts of lipids (74), but more studies are needed to verify the role of lipids in the degradation of lignin in wood.

Role of Manganese Peroxidase in Fungi Causing Preferential Degradation of Lignin in Wood

Preferential degradation of lignin compared to the degradation of wood polysaccharides, above all cellulose, is called selective degradation of lignin. Several fungi that are efficient lignin degraders in nature and especially suitable for selective lignin degradation, apparently do not produce LiP. Many of these fungi produce substantial amounts of MnP on lignocellulose medium. Table 3 shows some representative results from a screening of about 90 white-rot fungi for their selectivity on spruce wood block test (75), and preliminary indications of the enzymes they produce when growing on wood chips.

C. subvermispora represents a particularly efficient lignin-degrading fungus that does not produce LiP but high levels of MnP. Because it is one of the most promising fungi for biopulping, i.e. fungal pretreatment of wood chips for

mechanical pulping (76), its lignin degrading system has been intensively studied (Table 1). *C. subvermispora* secretes multiple MnP and laccase isoforms on wheat straw medium (77). Molecular masses and isoelectric points of MnP and laccase isoforms were close to those reported when wood chips have been used as a solid substrate (49, 78).

Pleurotus eryngii secretes high amounts of MnP but only low amounts of laccase into a straw medium (79). Production of multiple forms of MnP has been found during growth on different solid lignocelluloses (wood, pulp, straw) by several white-rot fungi, e.g. *P. chrysosporium* (60, 77), *Rigidoporus lignosus* (69), *Phlebia radiata* (64), *C. subvermispora* (49, 77) and *N. frowardii* (30). MnP was the main ligninolytic enzyme during the treatment of kraft pulp with *Trametes versicolor*, although laccase activity was also present (80). *P. radiata* MnP isoforms purified from wheat straw cultures had a molecular mass of 50 kDa and *pI*s of 3.4-3.9 and 4.9-5.3 (64). Unlike *P. radiata*, MnP from *N. frowardii* apparently forms only one MnP isozyme with a molecular mass of 54 kDa and a relatively low *pI* of 3.0-3.2 (30).

Selective lignin degradation and efficiency in biopulping require a proper balance between lignin and cellulose degradation. Hence, in addition to studies on lignin-degrading enzymes, also thorough studies to clarify the significance of cellulose and hemicellulose degradation in lignin-selective fungi are needed.

The Possible Role of Manganese Peroxidase in Lignin Degradation: Cell Free Mineralization of Lignin

Kirk and Farrell (3) summarized the physiological conditions that support lignin degradation by *P. chrysosporium* (measured as $^{14}\text{CO}_2$ evolution from ^{14}C -labelled DHP), and proposed the concept "enzymatic combustion". Similar "enzymatic combustion" could be applicable to some other white-rot fungi, e.g. *P. radiata* (81, 82). Lignin degradation is usually thought to proceed so that at first fungi degrade the lignin polymer outside of the hyphae to smaller lignin fragments, and subsequently these compounds are taken up by the growing fungus and intracellularly converted to CO_2 , water and new fungal biomass (6). However, if a significant part of lignin is mineralized outside the fungal cell wall, many unusual features in lignin biodegradation by white-rot fungi could be better understood. For example, enzymatic reactions are usually specific, but unspecificity is characteristic to fungal lignin degradation. If the reactions were catalyzed by MnP-Mn-chelator-system, they would be chemical, not enzymatic, specific reactions. The degradation of lignin to $^{14}\text{CO}_2$ is optimal under high oxygen tension, even in oxygen concentrations that are toxic to fungal growth. If part of the ^{14}C -labelled lignin is not taken into the fungal cell but is processed and decomposed outside the fungal cell, the positive effect of high oxygen tension on lignin degradation could be possible. It is known that fungi cannot

grow using lignin as their sole carbon and energy source, and the rationale of this could follow from extracellular mineralization of lignin. If we assume that lignin degradation products are not taken inside the fungal hyphae, they cannot be used as a carbon source and thus support fungal growth. Many studies indicate that lignin degradation is secondary metabolism, not directly connected to growth supporting metabolism. This could also be in agreement with extracellular lignin mineralization.

MnP has been found in almost all white-rot fungi so far, but it is possible that some fungi express the enzyme only on lignocellulose medium. It was earlier assumed that the function of MnP would be nearly the same as that of laccase, that is to oxidize phenolic substructures of lignin, but it is not understood why fungi produce two enzymes of similar function. However, there are still many open questions: MnP is not a strong oxidant, and the system MnP-Mn-chelator requires free phenolic groups, and there are only 10-15 % free phenolic groups in lignin (83). How fungi lacking LiP degrade or attack non-phenolic structures is still an open question. The involvement of lipid peroxidation and the presence of cryptic *lip* genes have been suggested, and probably small molecular weight compounds have an important role in lignin degradation.

White-rot fungi do have aromatic metabolism, which indicates that part of the lignin degradation products can be taken inside the fungal cell wall and metabolized there. Usually dimeric lignin models, or monomeric aromatic compounds such as veratric acid and vanillic acid have been used as substrates. Unlike bacteria, basidiomycetous fungi cannot grow on aromatic compounds, and actually many of these compounds, e.g. vanillin, are toxic to them in higher concentrations (84). The relationship between extracellular vs. intracellular mineralization occurring under natural conditions is not clear and needs further studies.

References

1. Hatakka, A. *FEMS Microbiol. Rev.* **1994**, *13*, 125-135.
2. Hatakka, A. *Biopolymers. Biology, Chemistry, Biotechnology, Applications. Vol 1. Lignin, Humic Substances and Coal*; Hofrichter, M.; Steinbüchel, A., Eds.; Wiley-VCH: Weinheim, 2001, pp 129-180.
3. Kirk, T.K.; Farrell, R. L. *Annu. Rev. Microbiol.* **1987**, *41*, 465-505.
4. Gold, M.H.; Alic, M. *Microbiol. Rev.* **1993**, *57*, 605-622.
5. Cullen, D. *J. Biotechnol.* **1997**, *53*, 273-289.
6. Kirk, T.K.; Cullen, D. *Environmentally Friendly Technologies for the Pulp and Paper Industry*; Young, R.A.; Akhtar, M., Eds.; John Wiley & Sons, Inc.: New York, 1998, pp 273-307.
7. Eriksson, K.-E.L.; Blanchette, R.A.; Ander, P. *Microbial and Enzymatic Degradation of Wood and Wood Components*; Springer-Verlag: Berlin Heidelberg, 1990.

Table 3. Examples of the Production of Ligninolytic Enzymes by Some White-Rot Fungi and the Lignin Loss vs. Cellulose Loss of Spruce Wood Blocks During 10 Weeks Fungal Growth

<i>Fungus</i>	<i>Ligninolytic enzymes produced^a</i>	<i>Lignin loss/ cellulose loss on spruce</i>
<i>Phellinus viticola</i> T255 ^b	MnP ++; laccase +	3.3
<i>Climacocystis borealis</i> T261i ^b	MnP +++; Laccase +	2.9
<i>Fibricium rude</i> PO141i ^b	MnP +; Laccase +++	2.6
<i>Phlebia tremellosa</i> 76-24 ^c	MnP +; laccase ++	1.5
<i>Phanerochaete chrysosporium</i> ME446 ^b	ND	1.3
<i>Ceriporiopsis subvermispota</i> CZ-3 ^d	MnP +++; laccase +	1.2

^a observed qualitatively on spruce wood chip cultivations; high production, +++; medium, ++; low, +; no production, -; ND = not determined or reported; LiP, lignin peroxidase; MnP, manganese peroxidase.

^b Majjala, P., Jussila, J., Laine, S., Lankinen, P., Hatakka, A., 2002, unpublished results

^c The strain 76-24 did not produce MnP in liquid cultures (65).

^d references (49) and (50).

8. Blanchette, R. A.; Burnes, T. A.; Eerdmans, M. M.; Akhtar, M. *Holzforchung* **1992**, *46*, 109-115.
9. Messner, K.; E. Srebotnik, E., *FEMS Microbiol. Rev.* **1994**, *13*, 351-364.
10. Tien, M.; Kirk, T.K. *Science* **1983**, *221*, 661-663.
11. Glenn, J. K.; Morgan, M.A.; Mayfield, M.B.; Kuwahara, M.; Gold, M.H. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 1077-1083.
12. Kuwahara, M.; Glenn, J.K.; Morgan, M.A.; Gold, M.H. *FEBS Lett.* **1984**, *169*, 247-250.
13. Kirk, T.K.; Tien, M.; Kersten, P.J.; Mozuch, M.D.; Kalyanaraman, B. *Biochem. J.* **1985**, *236*, 279-287.
14. Martinez, A. *Enzyme Microb. Technol.* **2002**, *30*, 425-444.
15. Mayfield, M.B.; Kishi, K.; Alic, M.; Gold, M.H. *Appl. Environ. Microbiol.* **1994**, *60*, 4303-4309.
16. Piontek, K.; Glumoff, T.; Winterhalter, K. *FEBS Lett.* **1993**, *315*, 119-124.
17. Poulos, T.L.; Edwards, S.L.; Wariishi, H.; Gold, M.H. *J. Biol. Chem.* **1993**, *268*, 4429-4440.
18. Sundaramoorthy, M.; Kishi, K.; Gold, M.H.; Poulos, T.L. *J. Biol. Chem.* **1994**, *269*, 32759-32767.

19. Gold, M.H.; Wariishi, H.; Valli, K. *Biocatalysis in Agricultural Biotechnology*, Whitaker, J.R.; Sonnet, P.E., Eds.; ACS Symp. Ser. No. 389: Washington, DC, 1989, pp 128-140.
20. Sundaramoorthy, M.; Kishi, K.; Gold, M.H.; Poulos, T.L., *J. Biol. Chem.* **1997**, *272*, 17574-17580.
21. Kishi, K.; Wariishi, H.; Marquez, L.; Dunford, B.H.; Gold, M.H.; *Biochemistry* **1994**, *33*, 8694-8701.
22. Hofrichter, M. *Enzyme Microb. Technol.* **2002**, *30*, 454-466.
23. Orth, A. B.; Royse, D. J.; Tien, M. *Appl. Environ. Microbiol.* **1993**, *59*, 4017-4023.
24. Vares, T.; Hatakka, A. *Can. J. Bot.* **1997**, *75*, 61-71.
25. Palma, C.; Martinez, A.T.; Lema, J.M.; Martinez, M.J. *J. Biotechnol.* **2000**, *77*, 235-245.
26. Perez, J.; Jeffries, T. W. *Appl. Environ. Microbiol.* **1992**, *58*, 2402-2409.
27. Moilanen, A.-M.; Lundell, T.; Vares, T.; Hatakka, A. *Appl. Microbiol. Biotechnol.* **1996**, *45*, 792-799.
28. Wariishi, H.; Valli, K.; Gold, M.H. *Biochem. Biophys. Res. Comm.* **1991**, *176*, 269-275.
29. Hofrichter, M.; Scheibner, K.; Bublitz, F.; Schneegaß, I.; Ziegenhagen, D.; Martens, R.; Fritsche, W., *Holzforschung* **1999**, *53*, 161-166.
30. Hofrichter, M.; Vares, T.; Kalsi, M.; Galkin, S.; Scheibner, K.; Fritsche, W.; Hatakka, A. *Appl. Environ. Microbiol.* **1999**, *65*, 1864-1870.
31. Hofrichter, M.; Vares, T.; Scheibner, K.; Galkin, S.; Sipilä, J.; Hatakka, A. *J. Biotechnol.* **1999**, *67*, 217-228.
32. Kapich, A.; Hofrichter, M.; Vares, T.; Hatakka, A. *Biochem. Biophys. Res. Commun.* **1999**, *259*, 212-219.
33. Lackner, R.; Srebotnik, E.; Messner, K. *Biochem. Biophys. Res. Comm.* **1991**, *178*, 1092-1098.
34. Ander, P.; Hatakka, A.I.; Lundell, T.K.; Pettersson, B.; Stalmasek, M.; Volc, J. *Lignocellulosics: Science, Technology, Development and Use*, Kennedy, J.F.; Phillips, G.O.; Williams, P.A., Eds.; Ellis Horwood Ltd.: Chichester, 1992, pp 109-119.
35. Paice, M.G.; Reid, I.D.; Bourbonnais, T.; Archibald, F.S.; Jurasek, L. *Appl. Environ. Microbiol.* **1993**, *59*, 260-265.
36. Hofrichter, M.; Lundell, T.; Hatakka, A. *Appl. Environ. Microbiol.* **2001**, *67*, 4588-4593.
37. Srebotnik, E.; Jensen Jr.; K.A.; Kawai, S.; Hammel, K.E. *Appl. Environ. Microbiol.* **1997**, *63*, 4435-4440.
38. Bao, W.; Fukushima, Y.; Jensen Jr.; K.A.; Moen, M.; Hammel, K.E., *FEBS Lett.* **1994**, *354*, 297-300.
39. Srebotnik, E.; Jensen Jr.; K.A.; Hammel, K.E. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 12794-12797.
40. Rajakumar, S.; Gaskell, J.; Cullen, D.; Lobos, S.; Karahanian, E.; Vicuña, R. *Appl. Environ. Microbiol.* **1996**, *62*, 2660-2663.
41. Moen, M.A.; Hammel, K.E. *Appl. Environ. Microbiol.* **1994**, *60*, 1956-1961.

42. Jensen, K.A. Jr.; Bao, W.; Kawai, S.; Srebotnik, E.; Hammel, K.E. *Appl. Environ. Microbiol.* **1996**, *62*, 3679-3686.
43. Kapich, A.N.; Jensen, K.A.; Hammel, K.E. *FEBS Lett.* **1999**, *461*, 115-119.
44. Enoki, M.; Watanabe, T.; Nakagame, S.; Koller, K.; Messner, K.; Honda, Y.; Kuwahara, M. *FEMS Microbiol. Lett.* **1999**, *180*, 205-211.
45. Lankinen, V. P.; Bonnen, A.M.; Anton, L. H.; Wood, D.A.; Kalkkinen, N.; Hatakka, A.; Thurston, C.F. *Appl. Microbiol. Biotechnol.* **2001**, *55*, 170-176.
46. Steffen, K.; Hofrichter, M.; Hatakka, A. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 819-825.
47. ten Have, R.; Hartmans, S.; Teunissen, P.J.M.; Field, J.A. *FEBS Lett.* **1998**, *422*, 391-394.
48. Heinfling, A.; Martinez, M.J.; Martinez, A.T.; Bergbauer, M.; Szewzyk, U.; *FEMS Microbiol. Lett.* **1998**, *165*, 43-50.
49. Lobos, S.; Larrain, J.; Salas, L.; Cullen, D.; Vicuña, R. *Microbiology* **1994**, *140*, 2691-2698.
50. Rüttimann, C.; Schwember, E.; Salas, L.; Cullen, D.; Vicuña, R. *Biotechnol. Appl. Biochem.* **1995**, *16*, 64-76.
51. Steffen, T.K.; Hatakka, A.; Hofrichter, M. *Appl. Environ. Microbiol.* **2002**, *68*, 3442-3448.
52. Périé, F.H.; Sheng, D.; Gold, M.H. *Biochem. Biophys. Acta* **1996**, *1297*, 139-148.
53. Raghukumar, C.; D'Souza, T.M.; Thorn, R.G.; Reddy, C.A. *Appl. Environ. Microbiol.* **1999**, *65*, 2103-2111.
54. Majjala, P. *Heterobasidion annosum and wood decay: enzymology of cellulose, hemicellulose, and lignin degradation*, Diss. Biocentri Viikki Universitatis Helsingiensis 6/2000
(URL: <http://thesis.helsinki.fi/julkaisut/mat/bioti/vk/majjala>)
55. Forrester, I.T.; Grabski, A.C.; Mishra, C.; Kelley, B.D.; Strickland, W.N.; Leatham, G.F.; Burgess, R.R. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 359-365.
56. Schneegaß, I.; Hofrichter, M.; Scheibner, K.; Fritsche, W. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 602-605.
57. Maltseva, O.V.; Niku-Paavola, M.-L.; Leontievsky, A.A.; Myasoedova, N.M.; Golovleva, L.A. *Biotechnol. Appl. Biochem.* **1991**, *13*, 291-302.
58. Farrell, R.L.; Murtagh, K.E.; Tien, M.; Mozuch, M.D.; Kirk, T.K. *Enzyme Microb. Technol.* **1989**, *11*, 322-328.
59. Pease, E.; Tien, M. *J. Bact.* **1992**, *174*, 3532-3540.
60. Datta, A.; Bettermann, A.; Kirk, T.K. *Appl. Environ. Microbiol.* **1991**, *57*, 1453-1460.
61. Ben Hamman, O.; de la Rubia, T.; Martinez, J. *FEMS Microbiol. Lett.* **1999**, *177*, 137-142.
62. Rüttimann-Johnson, C.; Cullen, D.; Lamar, R.T. *Appl. Environ. Microbiol.* **1994**, *60*, 599-605.
63. Lundell, T.; Hatakka, A. *FEBS Lett.* **1994**, *348*, 291-296.

64. Vares, T.; Kalsi, M.; Hatakka, A. *Appl. Environ. Microbiol.* **1995**, *61*, 3515-3520.
65. Vares, T.; Niemenmaa, O.; Hatakka, A. *Appl. Environ. Microbiol.* **1994**, *60*, 569-575.
66. Martinez, M.J.; Ruiz-Dueñas, F.J.; Guillén, F.; Martinez, A.T. *Eur. J. Biochem.* **1996**, *237*, 424-432.
67. Becker, H.G.; Sinitsyn, A.P. *Biotechnol. Lett.* **1993**, *15*, 289-294.
68. Sarkar, S.; Martinez, M.J.; Martinez, A.T. *Biochim. Biophys. Acta* **1997**, *1339*, 23-30.
69. Galliano, H.; Gas, G.; Seris, J.L.; Boudet, A.M. *Enzyme Microb. Technol.* **1991**, *13*, 478-482.
70. Steffen, K.; Hofrichter, M.; Hatakka, A. *Enzyme Microb. Technol.* **2002**, *30*, 550-555.
71. Johansson, T.; Nyman, P.O. *Arch. Biochem. Biophys.* **1993**, *300*, 49-56.
72. Martinez-Inigo, M.J.; Kurek, B. *Holzforschung* **1997**, *51*, 543-548.
73. Thompson, D.N.; Hames, B.R.; Reddy, C.A.; Grethlein, H.E. *Biotechnol. Bioengin.* **1998**, *57*, 704-717.
74. Kapich, A.N.; Shishkina, L.N. *Microbiology* (transl. from Russian) **1995**, *64*, 366-371.
75. Hatakka, A.; Maijala, P.; Mettälä, A.; Hakala, T.; Hauhio, L.; Ellmen, J. *Biotechnology in the Pulp and Paper Industry*; Viikari, L.; Lantto, R., Eds.; Elsevier Science B.V., 2002, pp 81-88.
76. Akhtar, M.; Blanchette, R.A.; Myers, G.C.; Kirk, T.K. *Environmentally Friendly Technologies for the Pulp and Paper Industry*, Young, R.A.; Akhtar, M., Eds.; John Wiley & Sons, Inc.: New York, 1998, pp 309-419.
77. Hatakka, A.; Vares, T.; Bocchini, P.; Galletti, G. C.; Hatakka, A. *TAPPI Biological Sciences Symposium*, TAPPI: San Francisco, 1997, pp 405-412.
78. Fukushima, Y.; Kirk, T.K. *Appl. Environ. Microbiol.* **1995**, *61*, 872-876.
79. Camarero, S.; Sarkar, S.; Ruiz-Dueñas, F.J.; Martinez, M.J.; Martinez, A.T. *J. Biol. Chem.* **1999**, *274*, 10324-10330.
80. Reid, I.D. *Appl. Environ. Microbiol.* **1998**, *64*, 2117-2125.
81. Hatakka, A.; Uusi-Rauva, A.K. *Eur. J. Appl. Microbiol. Biotechnol.* **1983**, *17*, 235-242.
82. Hatakka, A.I.; Lundell, T.K.; Tervilä-Wilo, A.L.M.; Brunow, G. *Appl. Microbiol. Biotechnol.* **1991**, *36*, 270-277.
83. Lai, Y.-Z. *Methods in Lignin Chemistry*, Lin, S.Y.; Dence, C.W., Eds.; Springer-Verlag: Berlin, 1992, pp 423-434.
84. Ander, P.; Hatakka, A.; Eriksson, K.-E. *Arch. Microbiol.* **1980**, *125*, 189-202.

Chapter 15

Influence of Growth Substrate and Free Ferulic Acid on the Production of Feruloyl Esterase by *Aureobasidium pullulans*

Karl Rumbold¹, Georg Gübitz², Karl-Heinz Robra²,
and Bernard Prior¹

¹Department of Microbiology, University of Stellenbosch, Stellenbosch,
South Africa

²Department of Environmental Biotechnology, Graz University
of Technology, Graz, Austria

The effect of the carbon source and supplemented free ferulic acid on the production of extracellular feruloyl esterase, β -xylanase and α -arabinofuranosidase by *Aureobasidium pullulans* was examined. *Aureobasidium pullulans* was cultivated on birchwood xylan, oat spelt xylan glucose, xylose, sugarcane bagasse and dissolving pulp as carbon source without and with the addition of ferulic acid. Production of feruloyl esterase activity was highest on birchwood xylan (16.8 U/mg protein). Ferulic acid (0.01% w/v) supplementation led to growth inhibition on all the carbon sources. Feruloyl esterase is also produced with glucose as carbon source, which is a repressor for xylanase production. It is therefore suggested that feruloyl esterase is regulated independently from xylanase. *Aureobasidium pullulans* feruloyl esterase was found to possess activity against α - and β -naphthylacetate and naphthol-AS-D-chloroacetate but not against longer chain naphthyl substrates and others as determined in a screening with 14 esterase substrates.

INTRODUCTION

Hydroxycinnamic acids like ferulic acid and *p*-coumaric acid are bound as substituents on hemicellulose polymers in plant cell walls (1). Esterified to α -arabinofuranosyl sidechains in arabinoxylans (2), ferulic acid substituents can occur as single residues, or as dehydrodimers coupling two polysaccharide chains (3-4) or can be etherified to lignin (5-6).

Feruloyl esterases catalyze the release of ferulic acid from plant material (7-9). They play a key role in the cooperative decomposition of plant cell wall material together with other major hemicellulolytic enzymes. Feruloyl esterases are therefore of importance to lignocellulose utilizing industries (10) and have potential medical applications due to their ability to release antioxidant hydroxycinnamates from dietary fiber (11).

The production of feruloyl esterases is apparently regulated independently from other hemicellulolytic enzymes. *Aspergillus niger* feruloyl esterase production is stimulated by supplementing free ferulic acid to oat spelt xylan as the only carbon source, while xylanase levels are repressed (12). In contrast, *Streptomyces avermitilis* feruloyl esterase production is independent from the amount of ferulic acid present in the carbon source, but is also induced under conditions different to those of xylanase (13).

The black yeast *Aureobasidium pullulans* produces a whole range of hemicellulolytic enzymes, with xylanase being produced at high level with high specific activity (14) but is repressed when glucose is present in the growth medium (15). Previous work indicated, that the synthesis of β -xylosidase and α -arabinofuranosidase is dependent on arabinoxylan as inducer, whereas xylanase and acetyl esterase are also produced when xylose is used as the carbon source (16).

Recently, we discovered that *A. pullulans* also produces extracellular feruloyl esterase. The purpose of this study was to investigate the composition of the growth medium on the production of feruloyl esterase in comparison to the production of endo-xylanase and α -arabinofuranosidase. The specificity of the crude feruloyl esterase to a range of esterase substrates was also evaluated.

MATERIALS AND METHODS

Strain and culture conditions

Aureobasidium pullulans NRRL Y2311-1 was cultivated in 50 ml yeast nitrogen base (0.7% w/v), L-Asparagine (0.2% w/v) and KH_2PO_4 (0.5% w/v) with 1% (w/v) of the respective carbon source, birchwood xylan (BWX, Sigma, Steinheim, Germany), oat spelt xylan (OSX, Sigma, Steinheim, Germany), dissolving pulp (DP) from *Eucalyptus grandis* (SAPPI Saiccor, Umkomaas, South Africa), sugarcane bagasse (SCB, Tongaat-Hulett, Mt Edgecombe, South Africa), glucose (GLC) and xylose (XYL). In a second set the respective cultures were supplemented with 0.01 % (w/v) ferulic acid (FA, Sigma, Steinheim,

Germany). The cultures were incubated on a rotary shaker (120 rpm) at 30°C for 84 hours and one ml samples were taken every 12 h for assay. Growth was followed by microscopic enumeration of yeast cells.

Enzyme assays

Culture supernatant recovered after centrifugation (10 min, 20000g) was assayed for feruloyl esterase activity using 4-nitrophenyl-5-*O*-*trans*-feruloyl- α -L-arabinofuranoside (Sigma) as substrate (17), for α -arabinofuranosidase activity using 4-nitrophenyl- α -L-arabinofuranoside as substrate (16). Endo-xylanase activity was determined using birchwood xylan as substrate (18). One unit (U) of activity was defined as the amount of enzyme releasing 1 μ mol product/min. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) based on the Coomassie Blue method (18) with bovine serum albumin as standard.

Screening of esterase substrates

α -naphthyl acetate, β -naphthyl acetate, β -naphthyl propionate, α -naphthyl butyrate, β -naphthyl valerate, β -naphthyl laurate, α -naphthyl myristate, α -naphthyl palmitate, naphthol AS β -Cl-propionate, naphthol AS benzoate, naphthol AS phenylacetate, naphthol AS acetate, naphthol AS-D acetate and naphthol AS-D chloroacetate as well as Fast Blue BB salt and carboxyl esterase from porcine liver were purchased from Sigma (Steinheim, Germany). Esterase activity was determined qualitatively by adding 50 μ l culture supernatant of *A. pullulans* cultivated on BWX to 1 ml of 0.1 M sodium phosphate buffer (pH 7) containing 25 μ l Fast Blue solution (100 mg in 5 ml double distilled H₂O). The reaction was started by adding 50 μ l of the respective substrate solution (60 mg in 5 ml acetone) and the formation of a purple color pigment could be observed if an enzymatic reaction occurred.

RESULTS AND DISCUSSION

A. pullulans was grown on six different carbon sources at a concentration of 1% w/v, without and with the addition of 0.01 % w/v FA, over a 84 hours period until late stationary phase. The activity-time-course profiles of feruloyl esterase activity are presented in Fig. 1. On BWX, the highest activity of 16.8 U/mg protein was reached after 60 hours. On OSX, activity also showed a maximum after 60 hours (9.1 U/mg protein). Both substrates are equally well suited for the growth of *A. pullulans* and production of feruloyl esterase, although the activity is 1.8 fold better in the case of BWX.

No activity was produced on DP as a result of poor growth, which is probably because DP consists to 95% from cellulose and *A. pullulans* lacks cellulase activity (14). On SCB, feruloyl esterase activity reached 7 U/mg protein after 60 hours. This is noteworthy, because SCB is more difficult to

Table I. Specific activities of *A. pullulans* enzymes on various carbon sources

Carbon source	<i>Feruloyl</i>	<i>Xylanase</i>	<i>Arabinofuranosidase</i>
	<i>esterase</i>		
<i>Specific activity U/mg protein</i>			
Birchwood xylan	16.8 ± 0.1	1310 ± 5	1.6 ± 0.02
Oat spelt xylan	9.1 ± 0.08	1250 ± 3	1.4 ± 0.06
Glucose	4.2 ± 0.1	0	0.2 ± 0.05
Xylose	2 ± 0.09	420 ± 5	0.2 ± 0.06
Dissolving pulp	0	0	0
Sugarcane bagasse	7 ± 0.09	390 ± 5	1.2 ± 0.05

degrade and to metabolize as carbon source than BWX or OSX, which impinged on growth and protein production.

Surprisingly, feruloyl esterase activity was also present when *A. pullulans* was grown on monosaccharides as carbon source, reaching maxima of 4.2 U/mg protein after 60 hours on GLC and 2 U/mg protein after 36 hours on XYL. This is particularly interesting, since the production of other hemicellulases like xylanase is suppressed in presence of GLC, as shown in Table I. These findings also indicate that the production of feruloyl esterase from *A. pullulans* may also be regulated independently to xylanase production. This conclusion is further supported by the observation that XYL induces xylanase and xylosidase synthesis (Table I) but that feruloyl esterase activity of *A. pullulans* grown on XYL was 50% lower than with cells grown on GLC (Fig. 1).

No feruloyl esterase activity was detected in the cultures when supplemented with 0.01% FA (Fig. 1). This is as a result of almost no growth of *A. pullulans*. At a 10 times lower concentration of free FA, the growth and the production of feruloyl esterase was still only half of the amount of that observed without the addition of FA (data not shown), suggesting that free FA does not only negatively affect the production of feruloyl esterase, but moreover inhibits growth of *A. pullulans*. These results are in contrast to data reported previously for *A. niger*, where FA at an amount of 0.03% (w/v) was found to stimulate feruloyl esterase production (12, 20).

Maximum levels of feruloyl esterase, xylanase and arabinofuranosidase produced by *A. pullulans* are shown in Table I. When *A. pullulans* was cultivated on BWX or OSX, high levels of all three enzymes could be found in the culture supernatant.

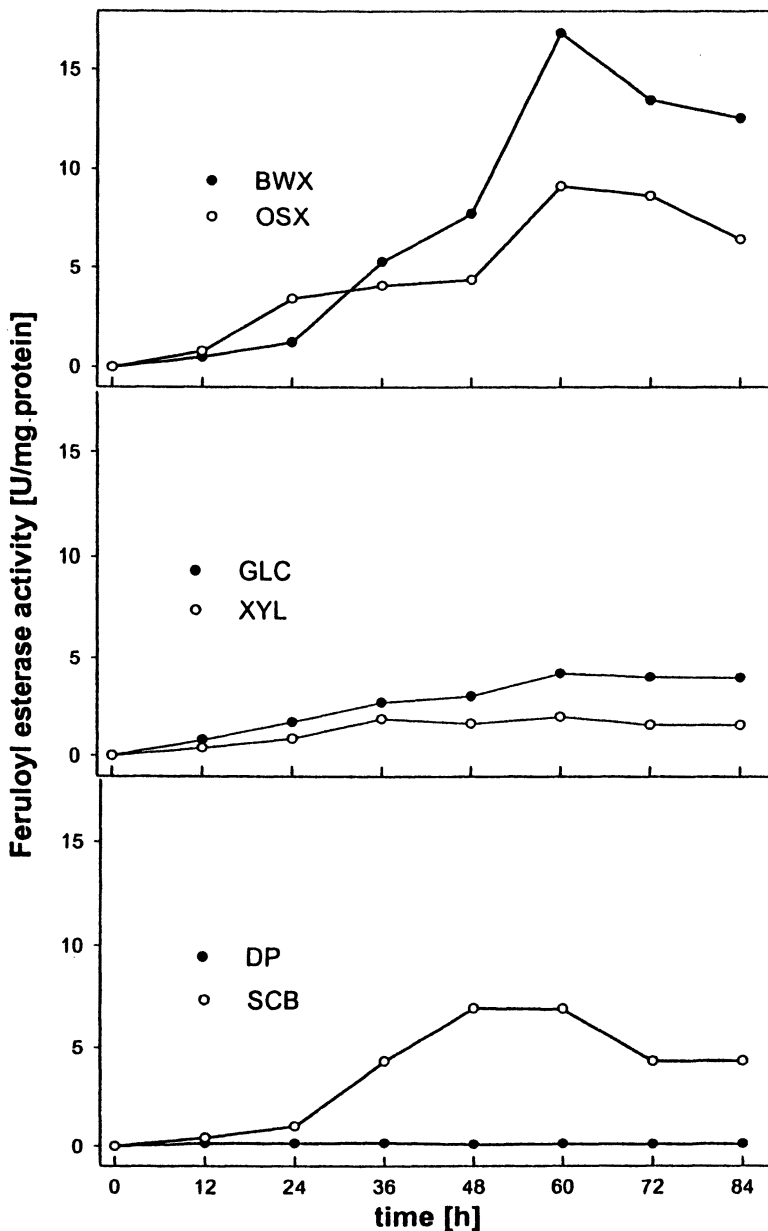


Figure 1. Effect of various carbon sources and free ferulic acid on the production of feruloyl esterase from *Aureobasidium pullulans*. The upper graph shows feruloyl esterase activity produced on birchwood xylan (BWX) and oat spelt xylan (OSX), the middle graph shows activity on glucose (GLC) and xylose (XYL) and the lower graph shows activity on dissolving pulp (DP) and sugarcane bagasse (SCB).

Xylanase production by *A. pullulans* was induced when grown on XYL and repressed when grown on GLC due to *cre*-mediated glucose repression, previously reported in promoter studies with *xylA* from *A. pullulans* by Wymelenberg et al. (21). Arabinofuranosidase was produced at very low levels on both XYL and GLC, whereas feruloyl esterase levels are higher when grown on GLC than on XYL. Feruloyl esterase could possibly be involved in the exopolysaccharide metabolism of *A. pullulans*, which produces pullulan preferably when grown on GLC (22). This would be an indication for the relative high feruloyl esterase levels on GLC in comparison to growth on XYL. Cultivation on SCB resulted in relative lower levels of feruloyl esterase and xylanase, partly due to low fermentability resulting in weaker growth caused by the fibrous nature of the material.

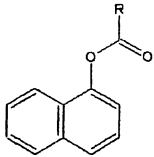
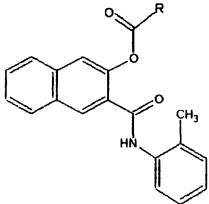
The substrate range of *A. pullulans* feruloyl esterase was determined by screening the culture supernatant produced on BWX with 14 commercially available α -naphthyl and naphthol-AS esters as described in Materials and Methods. Carboxyl esterase from porcine liver was used as a reference. *A. pullulans* culture supernatant showed activity on α - and β -naphthyl acetate, but no activity on aliphatic esters of a chain length from C 3 to C 16 (Table II). Activity was also shown on naphthol AS-D acetate and naphthol AS-D chloroacetate. Analogous to feruloyl esterases from other organisms (23), *A. pullulans* feruloyl esterase activity is limited to acetyl esters. However, the enzyme can also catalyze acetyl esters with an additional phenolic moiety such as in naphthol-AS-D acetate and naphthol AS-D chloroacetate (Table II). The phenolic moiety is reported to also contribute to feruloyl esterase catalytic specificity in general (23-24), although naphthol AS-D chloroacetate has not been previously reported as a substrate for feruloyl esterases. In contrast, the carboxyl esterase failed to catalyze the substrates with a phenolic moiety.

In summary, production of feruloyl esterase is stimulated when *A. pullulans* is grown on BWX, OSX and SCB, but also on GLC and XYL. However, FA supplemented to the medium affected fungal growth and therefore did not increase feruloyl esterase activity. It is also suggested, that the synthesis of feruloyl esterase is independently regulated from xylanase synthesis. Feruloyl esterase from *A. pullulans* acts on α - and β -naphthyl acetate, as well as naphthol AS-D chloroacetate as substrates.

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Table II. Comparison of substrate range

Substrate	Chain length	AP	PLE	Structure
α -naphthyl acetate	C2	+	+	 naphthyl substrate
β -naphthyl acetate	C2	+	+	
β -naphthyl propionate	C3	-	+	
α -naphthyl butyrate	C4	-	+	
β -naphthyl valerate	C5	-	+	
β -naphthyl laurate	C12	-	-	
α -naphthyl myristate	C14	-	-	 naphthol AS-D substrate
α -naphthyl palmitate	C16	-	-	
naphthol AS β -Cl-propionate		-	-	
naphthol AS benzoate		-	-	
naphthol AS phenylacetate		-	-	
naphthol AS acetate		-	-	
naphthol AS-D acetate		+	-	
naphthol AS-D chloroacetate		+	-	

AP = *A. pullulans* feruloyl esterase + activity
 PLE = Porcine liver carboxyl esterase - no activity

References

1. Carnachan, S. M.; Harris, P. J. Ferulic acid is bound to the primary cell walls of all gymnosperm families *Biochem Syst Ecol* **2000**, *28*, 865-879.
2. Saulnier, L.; Thibault, J. F. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans *J Sci Food Agric* **1999**, *79*, 396-402.
3. Hatfield, R. D.; Ralph, J.; Grabber, J. H. Cell wall cross-linking by ferulates and diferulates in grasses *J Sci Food Agric* **1999**, *79*, 403-407.
4. Hernanz, D.; Nunez, V.; Sancho, A. I.; Faulds, C. B.; Williamson, G.; Bartolome, B.; Gomez-Cordoves, C. Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley *J Agric Food Chem* **2001**, *49*, 4884-4888.
5. Iiyama, K.; Lam, T. B. T.; Stone, B. A. Phenolic bridges between polysaccharides and lignin in wheat internodes *Phytochemistry* **1990**, *29*, 733-737.

6. Lam, T. B. T.; Kadoya, K.; Iiyama, K. Bonding of hydroxycinnamic acids to lignin: ferulic and *p*-coumaric acids are predominantly linked at the benzyl position of lignin, not the [beta]-position, in grass cell walls *Phytochemistry* **2001**, *57*, 987-992.
7. Faulds, C. B. and Williamson, G. Release of ferulic acid from wheat bran by a ferulic acid esterase (FAE-III) from *Aspergillus niger* *Appl Microbiol Biotechnol* **1995**, *43*, 1082-1087
8. Bartolome, B.; Garcia-Conesa, M. T.; Williamson, G. Release of the bioactive compound, ferulic acid, from malt extracts *Biochem Soc Trans* **1996**, *24*, 379-380.
9. Kroon, P. A. and Williamson, G. Release of ferulic acid from sugar-beet pulp by using arabinanase, arabinofuranosidase and an esterase from *Aspergillus niger* *Biotechnol Appl Biochem* **1996**, *23*, 263-267.
10. Williamson, G.; Kroon, P. A.; Faulds, C. B. Hairy plant polysaccharides: a close shave with microbial esterases *Microbiology* **1998**, *144*, 2011-2023.
11. Andreasen, M. F.; Kroon, P. A.; Williamson, G.; Garcia-Conesa, M. T. Esterase activity able to hydrolyze dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals *J Agric Food Chem* **2001**, *49*, 5679-5684
12. Faulds, C. B.; de Vries, R. P.; Kroon, P. A.; Visser, J.; Williamson, G. Influence of ferulic acid on the production of feruloyl esterases by *Aspergillus niger* *FEMS Microbiol. Lett.* **1997**, *157*, 239-244.
13. Garcia, B. L.; Ball, A. S.; Rodriguez, J.; Perez-Leblic, M. I.; Arias, M. E.; Copa-Patino, J. L. Induction of ferulic acid esterase and xylanase activities in *Streptomyces avermitilis* UAH30 *FEMS Microbiol Lett* **1998**, *158*, 95-99.
14. Leathers, T. D. Color variants of *Aureobasidium pullulans* overproduce xylanase with extremely high specific activity *Appl Environ Microbiol* **1986**, *52*, 1026-1030.
15. Leathers, T. D.; Kurtzman, C. P.; Detroy, R. W. Overproduction and regulation of xylanase in *Aureobasidium pullulans* and *Cryptococcus albidus* *Biotechnol Bioeng* **1984**, *14*, 225-240.
16. Myburgh, J.; Prior, B. A.; Kilian, S. G. Production of xylan-hydrolyzing enzymes by *Aureobasidium pullulans* *J Ferment Bioeng* **1991**, *72*, 135-137.
17. Biely, P.; Mastihubova, M.; van Zyl, W. H.; Prior, B. A. Differentiation of feruloyl esterases on synthetic substrates in α -arabinofuranosidase-coupled and UV-spectrophotometric assays *Anal Biochem* **2002**, accepted.
18. Bailey, M. J.; Biely, P.; Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity *J Biotechnol* **1992**, *23*, 257-270.
19. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Anal Biochem* **1976**, *72*, 248-254.
20. Faulds, C. B.; Williamson, G. Effect of hydroxycinnamates and benzoates on the production of feruloyl esterase by *Aspergillus niger* *J Sci Food Agric* **1999**, *79*, 450-452.

21. van den Wymelenberg, A.; Cullen, D.; Spear, R.; Andrews, J. Regulated expression of green fluorescent protein under the control of *Aureobasidium pullulans* xylanase gene *xynA* *FEMS Microbiol Lett* **1999**, *181*, 205-209.
22. Vijayendra, S. V. N.; Bansal, D.; Prasad, M. S.; Nand, K. Jaggery: A novel substrate for pullulan production by *Aureobasidium pullulans* CFR-77 *Process Biochem* **2001**, *37*, 359-364.
23. Williamson, G.; Faulds, C. B.; Kroon, P. A. Specificity of ferulic acid (feruloyl) esterases *Biochem Soc Trans* **1998**, *26*, 205-209.
24. Prates, J. A. M.; Tarbouriech, N.; Charnock, S. J.; Fontes, C. M. G. A.; Ferreira, L. M. A.; Davies, G. J. The structure of the feruloyl esterase module of xylanase 10B from *Clostridium thermocellum* provides insights into substrate recognition *Structure* **2001**, *9*, 1183-1190.

Chapter 16

In Situ Solid-State Fermentation and Utilization of Xylanase in Pulp Bleaching

Judit Szendefy¹, George Szakács¹, Sándor Kemény²,
and Lew Christov³

¹Department of Agricultural Chemical Technology, Technical University of Budapest, 1111 Budapest, Gellért tér 4, Hungary

²Department of Chemical Unit Operations, Technical University of Budapest, 1111 Budapest, Műegyetem rkpt3, Hungary

³Sappi Biotechnolgy Laboratory, Department of Microbiology, Biochemistry and Food Science, University of the Free State, P.O. Box 330, 9300 Bloemfontein, South Africa and Sappi Forest Products Technology Centre, Sappi Management Services, P.O. Box 3252, 1560 Springs, South Africa

Solid substrate fermentation (SSF) was evaluated for microbial production of xylanase using oxygen delignified soda-aq pulp from *Eucalyptus grandis* as a carbon source and an enzyme inducer. Screening of a number of *Thermomyces lanuginosus* isolates yielded xylanase activities of over 5,000 IU/g dry material (DM) with very low levels (<0.4 FPU/g DM) of cellulase being concurrently produced. The *in situ* produced enzymes (whole SSF culture) were used without a prior downstream processing of xylanase in pulp bleaching at various mixing ratios of SSF material to raw pulp. Optimum conditions for SSF of xylanase by *T. lanuginosus* TUB F-980 were determined in terms of moisture content (83%), final pH (7.0) and time course (≥5 d).

A fractional factorial experimental design was employed to examine the impact of various nitrogen sources on xylanase production and determine the optimum nitrogen concentration (2g/l) and composition (56% corn steep liquor and 44% ammonium sulfate). Under these optimized conditions, a xylanase yield of 13,757 IU/g DM was attained, which represented a 2.3-fold increase in enzyme activity over the non-optimized SSF process. Biobleaching of pulp with the *in situ* crude SSF xylanase of *T. lanuginosus* TUB F-980 induced a brightness gain of up to 2.1 points over control and up to 35% savings of chlorine dioxide. It was demonstrated that the cost-effectiveness of biobleaching with SSF enzymes could be improved over the use of commercial liquid enzyme products due to the significantly lower enzyme production costs associated with the SSF process. The SSF enzymes outperformed the commercial enzymes by 43% and 62.5% when compared at equal enzyme costs and equal bleaching efficiency, respectively.

Introduction

The industrial road of pulp and paper manufacture has gone over the years along routes that were energy intensive and environmentally unfriendly. In the last decade, however, with the advent of biotechnology, the pulp and paper technology is becoming increasingly more environmentally cautious and cost effective. The observation of Viikari *et al.* in 1986 (1) that enzymes can enhance pulp brightness has initiated a new era in pulp bleaching. Pretreatment of pulp with xylanases was shown to reduce the amount of chlorine used in the bleaching process, affording a positive environmental impact.

Xylanases can attack and hydrolyze pulp hemicelluloses thereby xylan redeposited on the fiber surface, xylan-derived chromophores or lignin-xylan complexes are removed from pulp rendering residual lignin more accessible to bleach chemicals (2). Xylanase preparations free of cellulase activity are especially useful as the presence of cellulase may decrease both pulp strength and yield during biobleaching (3).

Currently, the commercial xylanases are mainly produced by conventional submerged fermentation (SF) (4), which is an inherently expensive operation best suited for high value antibiotics and other pharmaceutical products. An economically viable alternative for enzyme production and application would be solid substrate fermentation (SSF). It offers numerous advantages over the submerged fermentation systems, including high volumetric productivity, high concentration of the products, less effluent generation and simple fermentation

equipment (5,6). It has been proven that many enzymes and other biochemicals can be produced by SSF at a fraction of the cost of SF production (7,8). Fungal xylanases are frequently produced by SSF (4,5,9,10) and the conditions of the solid fermentation were optimized from different aspects in numerous studies (11-17).

Eucalyptus wood is a major raw material for the pulp and paper industry in countries such as South Africa, Brazil, India and Australia. In this work, the production and application of xylanase using thermophilic fungi on oxygen delignified soda-*aq* pulp from *Eucalyptus grandis* by SSF is described. The pulp, which is initially used as a carbon source and inducer of xylanase production, subsequently becomes the target substrate of enzyme application in the biobleaching process.

A new approach of enzyme production and utilization was introduced by evaluating the efficiency of the whole SSF material (mixture of fungal biomass, residual substrate and enzymes), without extracting and purifying the xylanases. The whole SSF material (*in situ* crude enzyme) was successfully used for enzymatic bleaching of pulp. The premise of the present work is that SSF is employed for enzyme production on pulp only if the *in situ* and *on-site* produced enzyme-enriched pulp substrate is used directly in pulp bleaching, without a prior downstream processing of enzyme. *Thermomyces lanuginosus* TUB F-980, which was the best-performing isolate from the bleaching trials, was further evaluated in SSF experiments to optimize the moisture content of pulp, medium composition and time course of xylanase production by SSF.

Materials and Methods

Cultures

Thermophilic fungi were purchased from the following culture collections: ATCC (American Type Culture Collection, Manassas, Virginia); CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands); NRRL (Northern Regional Research Center, USDA, Peoria, Illinois); TUB (Technical University of Budapest, Hungary); WFPL (Western Forest Products Laboratory, Vancouver, Canada). The strains were maintained on potato dextrose agar (PDA) slants and Petri plates by incubating the cultures at 45 °C and then storing them at room temperature.

Substrate

Oxygen delignified pulp from *Eucalyptus grandis* was obtained from a soda-*aq* pulp producing mill in South Africa. Prior to use, pulp was thoroughly

washed with distilled water until neutral pH of wash waters was attained, air-dried and then stored at 4 °C until use.

Solid Substrate Fermentation (SSF)

Non-optimized SSF was carried out in 500-ml cotton plugged Erlenmeyer flasks. Five gram pulp substrate (dry weight equivalent) was wetted with 25 ml salt solution, setting the moisture content to 83%. The composition of the salt solution was as follows (in g/l): NH_4NO_3 , 5; KH_2PO_4 , 5; NaCl, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1; and (in mg/l): $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2; MnSO_4 , 1.6; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.45; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5. The pH of the medium was adjusted to 6.8 before sterilization.

When the optimum moisture content of SSF was determined, different moisture levels (50-86%) were adjusted with the above-described salt solution and water, keeping the salt concentration per 5 g eucalyptus pulp constant.

Ammonium sulfate, ammonium nitrate, potassium nitrate, corn steep liquor (50 % dry weight) and defatted soybean meal were used as the nitrogen sources. Their concentrations were calculated as to provide 0.009 g nitrogen per gram carbon source (0.9%). The pH of the medium was adjusted to pH 6.8 before sterilization.

In the time course experiment, 5 g pulp was supplemented to 83 % moisture content with the following optimized medium solution (g/l): KH_2PO_4 , 11.39; corn steep liquor (50 % dry weight), 28.18; $(\text{NH}_4)_2\text{SO}_4$, 4.11; and (in mg/l): $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2; MnSO_4 , 1.6; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.45; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5. The pH of the medium was adjusted to pH 6.8 before sterilization. The substrate was sterilized at 121°C for 30 min. After cooling, the medium was inoculated with a spore suspension of the fungus to a final concentration of 2.5×10^6 spores g^{-1} dry matter (DM) substrate. The spore suspension was prepared by washing spores from the surface of 6 day-old sporulating PDA plates with water containing 0.1 % Tween-80. The inoculated flasks were incubated at 45°C for 5 days. All SSF experiments were carried out in duplicate and results were shown as average values.

Enzyme Extraction

Enzyme activity was determined from the culture extract of SSF samples: 5 g (dry weight) fermented substrate was extracted with 100 ml water containing 0.1 % Tween-80 by shaking for 1 h at room temperature (25°C). At the end of the extraction, suspension was centrifuged (3000 rpm, 10 min) and the supernatants were stored at 4°C until the assays were performed.

Enzyme Assay

Xylanase activity of the supernatant was determined by the method of Bailey *et al.* (18). The substrate solution contained 1% birchwood xylan (Fluka, No. 95588), solubilized in 0.05 M citrate-phosphate buffer (pH 6). All necessary dilutions of supernatants were also carried out using 0.05 M citrate-phosphate buffer (pH 6). The reaction mixture consisted of 1.8 ml substrate solution and 0.2 ml properly diluted enzyme. After 5 min incubation at 50°C, the liberated reducing sugars (xylose equivalent) were estimated by the dinitrosalicylic acid method (19). One unit (IU) of xylanase was defined as the amount of enzyme releasing 1 μmol xylose equivalent per minute under the assay conditions. Filter paper activity (FPA) was determined as described by Ghose (20).

Experimental Design

A 2^{5-1} fractional factorial design leading to sixteen sets of experiments, performed in duplicate, was used to verify the most significant factor affecting the xylanase activity. The variables were coded according to Eq. 1:

$$x_i = (X_i - X_0) / \Delta X_i \quad (1)$$

where x_i is the coded value of a variable, X_i is the real value of the variable, X_0 is the real value of a variable at the center point, and ΔX_i is the step change value. The significance level of each variable effect and interactions is determined by Student's test:

$$t(16) = \frac{b_j}{s_{b_j}}$$

where b_j is the regression coefficient calculated as follows:

$$b_j = \frac{\sum_i y_i x_{ji}}{\sum_i x_{ji}^2}, \quad b_0 = \frac{\sum_i y_i}{\sum_i x_{ji}^2}, \quad \sum_i x_{ij}^2 = N$$

y_i - xylanase activity, x_{ji} - transformed factors, b_0 - intercept, N - number of trials

$s_{b_j}^2$ is the variance of regression coefficient: $s_{b_j}^2 = \frac{s_y^2}{N}$

The effect of a factor is considered to be significant, if $t_{\alpha/2} < t(16)$. $t_{\alpha/2} = 2.12$ when $\alpha = 0.05$ and degrees of freedom is 16.

The range and the levels of the variables investigated in this study are shown in Table I. The xylanase activity was taken as the dependent variable or response, y_i . In order to fit an empirical second-order polynomial model, a 3^3 factorial design was performed. The range and the levels of the variables investigated in this study are shown in Table II. The quadratic model for predicting the optimal point was expressed according to Eq. 2:

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad (2)$$

where Y is the response variable, b the regression coefficients, and x_i the coded levels of the variable. The significance level of each variable effect, and interactions is determined by Student's t test. $t_{\alpha/2} = 2.052$ when $\alpha = 0.05$ and degrees of freedom is 27. Statsoft, Inc. (2000) was used for the regression analysis of the experimental data obtained.

Table I. Range of values for the 2^{5-1} fractional factorial design

Nitrogen content of supplements (%)	Variables X_i	Levels	
		-1 (0.25 g N/l)	+1 (1.3 g N/l)
7.5	X_1 : Defatted soybean meal	3.32 (g/l)	17.29 (g/l)
4	X_2 : Corn steep liquor (50 % dry weight)	6.25 (g/l)	32.5 (g/l)
35	X_3 : NH_4NO_3	0.71 (g/l)	3.71 (g/l)
21	X_4 : $(\text{NH}_4)_2\text{SO}_4$	1.18 (g/l)	6.13 (g/l)
0	X_5 : KH_2PO_4	0 g N/l	0 g N/l
		1.10 g KH_2PO_4 /l	7.68 g KH_2PO_4 /l

Table II. Range of values for the 3^3 factorial design

Variables X_i	Levels		
	-1	0	+1
X_1 : KH_2PO_4	4.39 (g/l)	8.78 (g/l)	13.16 (g/l)
X_2 : Total N content	1 (g/l)	2 (g/l)	3 (g/l)
X_3 :	0	0.5	1
CSL/ $(\text{NH}_4)_2\text{SO}_4$	(0% CSL/100% $(\text{NH}_4)_2\text{SO}_4$)	(50% CSL/50% $(\text{NH}_4)_2\text{SO}_4$)	(100% CSL/0% $(\text{NH}_4)_2\text{SO}_4$)

Enzyme Treatment of Pulp

Biotreatment of eucalyptus oxygen-delignified soda-aq pulp with the intact SSF material was carried out in different SSF material to raw pulp ratios (1:50, 1:100, 1:200, 1:400, w/w) under standardized conditions (60°C, pH 6, 10% pulp consistency, 3 h). For reference, enzyme pretreatments with commercial enzymes, Xylanase P (Iogen Corp., Canada), Pulpzyme HC (Novozymes, Denmark) and Cartazyme NS 10 (Clariant, UK) were carried at their pH optima (pH 5.0, 7.0 and 7.0, respectively) as described above using a charge of 5 IU xylanase/g pulp.

Chemical Bleaching of Pulp

The enzyme pretreated pulp was subsequently bleached in a DED sequence. Controls were prepared under the same conditions, but omitting the enzyme. To evaluate the bleaching efficiency of the *in situ* SSF xylanase, pulp was bleached with reduced chlorine dioxide charges, as indicated. Following chemical bleaching, pulp samples were washed, air-dried and analyzed for brightness using a ColorTouch 2 brightness machine (Technidyne Corp., New Albany, IN, USA) according to T452 om-98 of the Standard Methods of the Technical Association of the Pulp and Paper Industry (Tappi, Atlanta, GA, USA).

Results and Discussion

Screening of Thermophilic Fungal Isolates

Initially thermophilic fungi, representing different genera and species, were screened in the SSF process on eucalyptus post-oxygen soda-aq pulp for xylanase production (Fig.1). Some species such as *Acremonium thermophilum*, *Humicola grisea* var. *thermoidea* and *Humicola insolens* showed very weak growth on the substrate and did not practically produce xylanase. Other isolates (*Malbranchea cinnamomea*, *Myceliophthora thermophila*, *Sporotrichum thermophile* and *Talaromyces emersonii*) grew very well on pulp, but yielded low xylanase concentration. Two species (*Thermoascus aurantiacus* and *Thermomyces lanuginosus*) appeared as the best xylanase producers. However, *T. lanuginosus* was selected for further evaluation studies because of its significantly lower levels of cellulase activity produced simultaneously (<0.5 FPU/g), compared to the two *T. aurantiacus* strains (>8 FPU/g; Fig. 1).

Nine *Thermomyces lanuginosus* isolates were evaluated in a second screening step for xylanase and cellulase SSF production on pulp (Fig. 2).

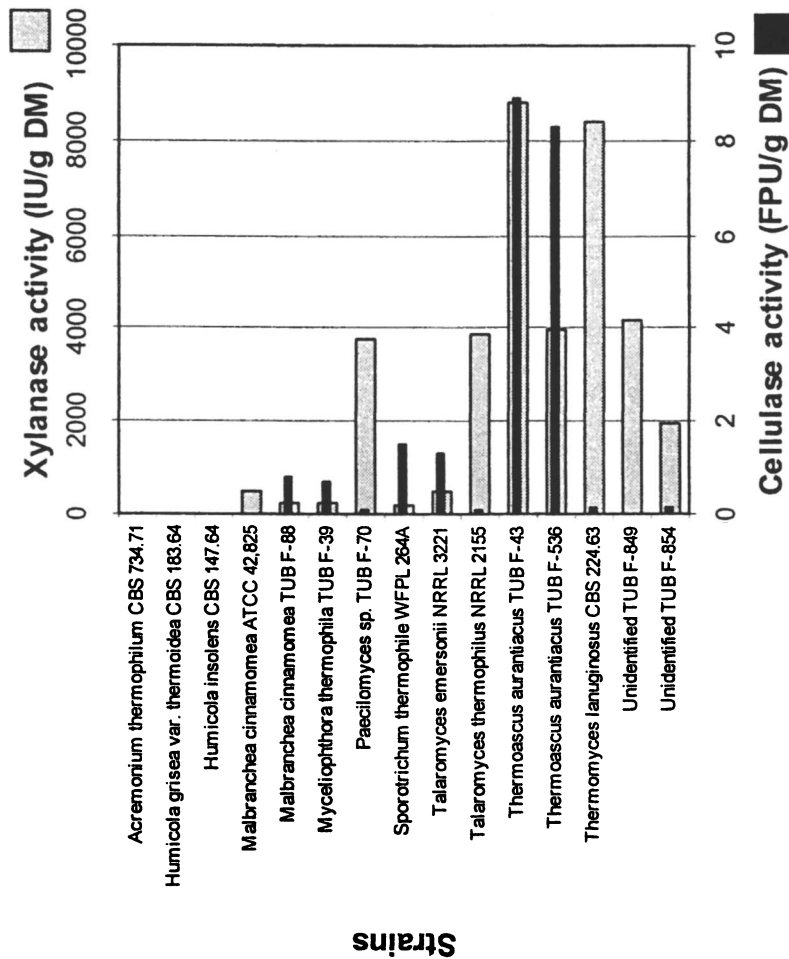


Figure 1. Xylanase production by thermophilic fungi representing different species in SSF on eucalyptus pulp (45 °C; initial pH 6.8; moisture content 83 %; fermentation time 5 d)

Interestingly, the difference among the isolates in xylanase production was not considerable. One strain (ATCC 38905) produced much higher cellulase activity (about 1.1 FPU/g) over the average level of 0.2 FPU/g.

Six thermophilic fungi, isolated from South African soils, were also screened for xylanase production in SSF (Fig. 3). Two isolates (TUB F-980 and TUB F-1026) showed good xylanase production (>6000 U/g) accompanied by a relatively low cellulase activity (<0.15 FPU/g). These two isolates were later identified as *Thermomyces lanuginosus*.

Biobleaching Efficiency of *in situ* Crude SSF Xylanase

Thermophilic fungi that produced 2000 IU/g or more xylanase on eucalyptus oxygen delignified soda-aq pulp were evaluated for the biobleaching efficiency of their SSF produced enzymes on the same pulp (Fig. 4). The SSF sample (*in situ* crude enzyme) produced at the end of the SSF fermentation of pulp was a mixture of substrate pulp residue, fungal biomass and different enzymes. This material, obtained without a downstream processing (enzyme extraction and purification), represents a relatively inexpensive source of crude enzymes for biotechnological purposes (6,8).

In most cases, the brightness increase over control reached or exceeded 1 brightness point at the 1:50 ratio (SSF material/raw pulp, w/w; Fig. 4). The solid culture of *T. lanuginosus* TUB F-980 xylanase appeared as most efficient in the bleaching trials (brightness gain of 1.3 to 1.8 points over control). This strain was selected for further optimization of xylanase production on pulp by SSF.

Optimization of Moisture Content in SSF

The moisture content of the medium in SSF is of importance to the growth of microorganisms, production and expression of enzymes. The optimum moisture levels have to be determined for each system and microorganism (21,22). To examine the effect of moisture content on xylanase production of *T. lanuginosus* F-980 in SSF, moisture levels of 50, 67, 75, 80, 83 and 86 % were tested (Fig. 5). At 50 % moisture content, a very weak growth and low xylanase yields were observed. Maximum xylanase activity of over 7000 U/g was measured at 83% initial moisture content.

Optimization of Nitrogen Content in SSF Using Experimental Design

The Kjeldahl nitrogen content of eucalyptus pulp (0.0064%) was extremely low and likely not enough to support growth and enzyme production. Therefore, the influence of different organic and inorganic nitrogen additives on xylanase production at 83 % substrate moisture level was studied (Fig. 6). The

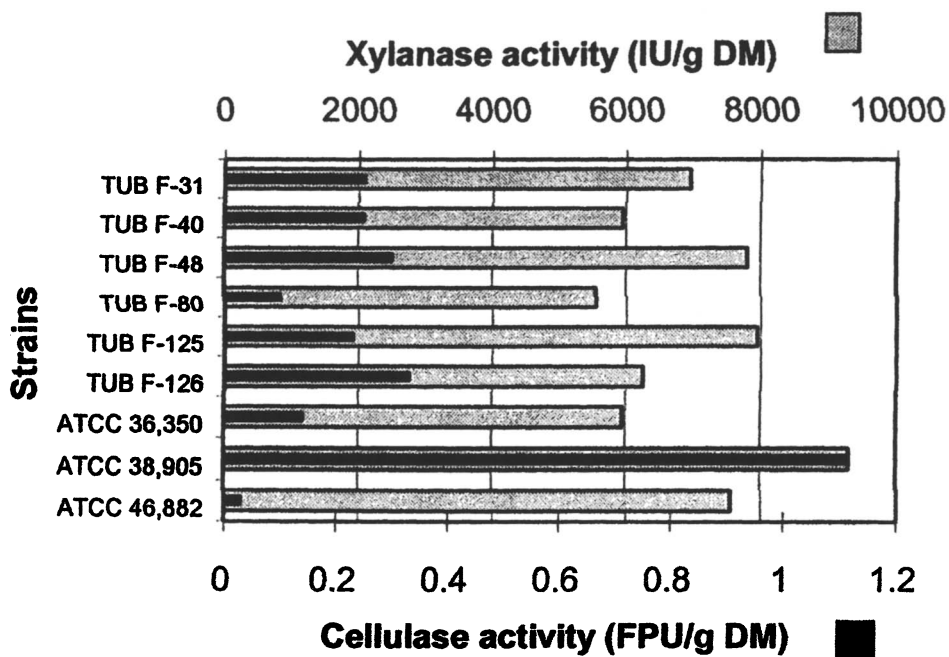


Figure 2. Xylanase production by *Thermomyces lanuginosus* strains in SSF on eucalyptus pulp (45 °C; initial pH 6.8; moisture content 83 %; fermentation time 5 d)

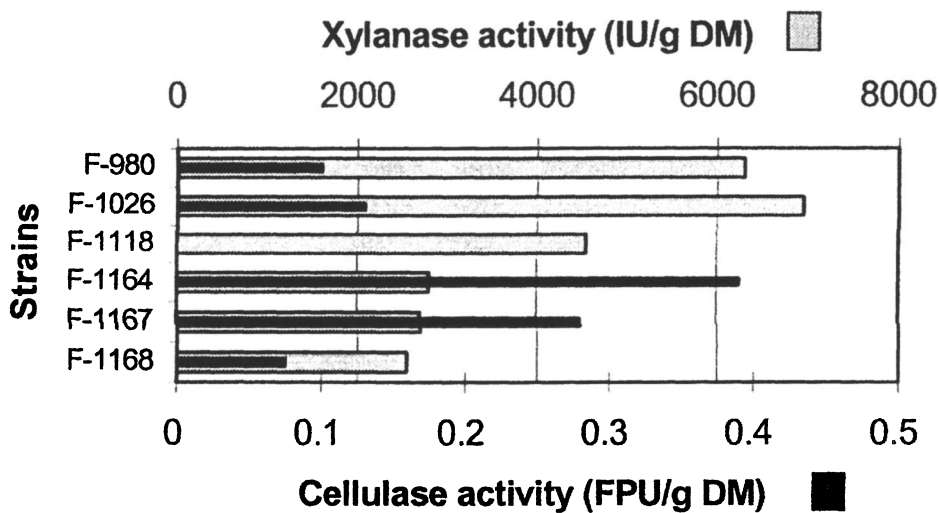


Figure 3. Xylanase production by South African thermophilic isolates in SSF on eucalyptus pulp (45 °C; initial pH 6.8; moisture content 83 %; fermentation time 5 d)

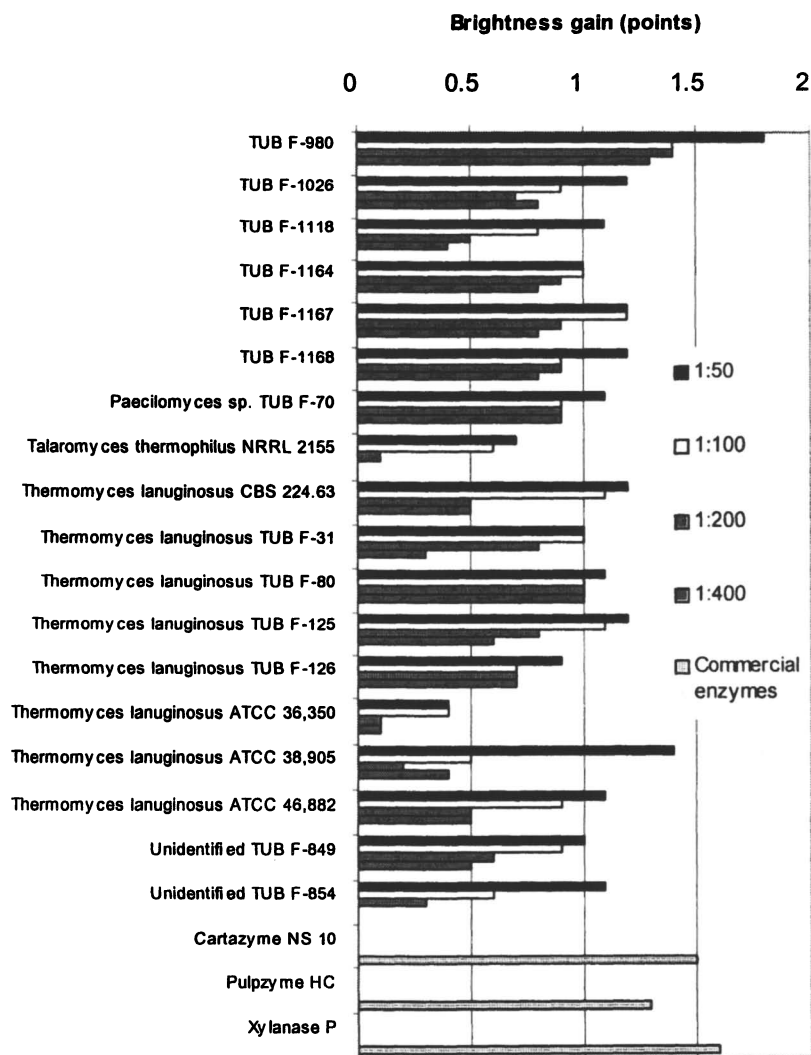


Figure 4. Brightness gains induced by *in situ* SSF crude xylanase at different SSF/raw pulp ratios during biobleaching of eucalyptus pulp

ammonium nitrate in the basal medium (see Materials and methods) was replaced by various inorganic and organic ingredients with a total nitrogen concentration fixed at 1.75 g/l. Strain F-980 produced more xylanase in presence of organic nitrogen as compared to inorganic nitrogen salts. Fig. 6 also shows that *T. lanuginosus* TUB F-980 was a nitrate-negative strain. It should be noticed that although the initial pH for all five media tested was the same (pH 6.8), the final pH values at the end of SSF differed. For example, final pH values of 8.0, 7.6, 6.1 and 6.1 were measured for soybean meal, corn steep liquor (CSL), ammonium sulfate and ammonium nitrate containing media, respectively. As shown in this study, the final pH of the SSF fermentation seemed to have a major impact on xylanase yield.

A 2⁵⁻¹ fractional factorial design was chosen to further evaluate the influence of nitrogen and phosphorous on xylanase yield. Four nitrogen sources (soybean meal, CSL, ammonium nitrate, ammonium sulfate) were selected at two concentration levels for the experimental design. Phosphorus is one of the most important nutrients, therefore, supplementation with potassium dihydrogen phosphate was selected as the fifth factor. The design and the results of this experiment are given in Table III. The effects are shown in a Pareto chart (Fig. 7), the regression coefficients, *t*- and *p* values are presented in Table IV. It can be concluded from the Pareto chart and Table IV. that KH₂PO₄, ammonium sulfate and CSL had a positive effect on xylanase production. Although soybean meal was the best nitrogen source when it was used as single supplement (Fig.6), it showed a significant negative effect on the xylanase production as compared to other nitrogen sources (Fig. 7 and Table III). Soybean meal also exhibited a negative interaction with the other nitrogen sources when they were used in pairs (combinations) (Fig. 7 and Table III). The best combination of nitrogen sources was CSL as organic nitrogen with ammonium sulfate as inorganic nitrogen.

Based on these results, CSL and ammonium sulfate were chosen to determine the optimum content and composition of nitrogen in the SSF medium. Potassium dihydrogen phosphate was also selected for quantitative analysis. The three selected factors were examined on three levels in a 3³ full factorial design. The design of this experiment is given in Table V. together with the results of the experiment. Regression analysis was performed to fit the response function with the experimental data. The regression coefficients, *t*- and *p* are presented in Table VI. The fit of the polynomial model (R²) was calculated to be 0.97, indicating that 97% of the variability in the response could be explained by the model. The response equation was obtained as follows and represented a suitable model for xylanase production:

$$Y = 13264.74 + 949.72 x_1 - 795.11 x_1^2 - 2415.11 x_2^2 + 1352.78 x_3 - 5577.11 x_3^2 + 270.38 x_1 x_2 - 323.71 x_1 x_3 - 673.75 x_2 x_3 - 534.42 x_2^2 x_3 + 2000.92 x_2^2 x_3^2$$

The three-dimensional graphs obtained from the calculated response surface are presented in Figs. 8 and 9. For calculation purposes, the normalized coded variables x_1 , x_2 and x_3 were defined as: $x_1 = (X_1 - 8.78)/4.39$, $x_2 = (X_2 - 2)/1$ and $x_3 = (X_3 - 0.5)/0.5$. The fitted surface had a maximum point at 11.39 g/l KH₂PO₄ ($x_1 = 0.597$), 2.01 g/l total nitrogen ($x_2 = 0.014$) with a share of 56% and 44% from the CSL and ammonium sulfate, respectively ($x_3 = 0.12$). The model predicts a maximum response of 13,607 IU xylanase/g with a variation of 517 IU/g for this point.

Impact of Final pH on Xylanase Production in SSF

Fig. 10 depicts the response xylanase activities of the 3^3 design in terms of final fermentation pH as measured in the SSF cultivations after 5 days. The points were distributed according to the amount of total nitrogen and its $\text{CSL}/(\text{NH}_4)_2\text{SO}_4$ ratio in the fermentation medium. There seems to be a correlation between the nitrogen content of the wetting solution and the evolved final fermentation pH. The highest final pH (8.0) was measured when the total nitrogen (3 g/l) consisted of only CSL. When the same amount of nitrogen was provided by ammonium sulfate alone, the final pH was 6.2. The highest xylanase yield was achieved by addition in the wetting solution of 2 g/l total nitrogen at a ratio of $\text{CSL}/(\text{NH}_4)_2\text{SO}_4$ of 0.5, resulting in final pH values around 7. Therefore, it appeared that the maximum xylanase production was dependent on the type and concentration of nitrogen in the SSF wetting solution, as well as the final fermentation pH. Apparently, the final pH is influenced by the choice of nitrogen source.

Time-course of Xylanase Production in SSF

The time-course of xylanase fermentation was studied on both non-optimized and optimized media. As a result of the medium optimization, the xylanase yield was significantly increased (Fig. 11). For instance, after 5 days, a xylanase activity of 13,757 IU/g was achieved on the optimized medium which was more than double the activity produced on the non-optimized medium. This is in agreement with the values that the model predicted, and verifies both the validity of the response model and the existence of the optimal point.

Pulp Bleaching with *in situ* Crude SSF Xylanase of *T. lanuginosus*

Biobleaching of soda-*aq* pulp with the *T. lanuginosus* TUB F-980 crude SSF xylanase produced *in situ* on the optimized medium using the same mixing ratios of SSF material to raw pulp demonstrated the advantages of the media optimization studies. As a result of the 2-fold increase in the xylanase yield using the optimized medium, the enzyme charge on pulp increased proportionally which resulted in enhancement in the bleaching performance of the crude SSF xylanase. Thus, the brightness of the enzyme pretreated bleached pulp increased by an additional 0.3-0.4 points (Table VII) which was translated in additional 5% reduction of the chlorine dioxide consumption (Table VIII) during biobleaching of eucalyptus soda-*aq* pulp with the crude SSF xylanase of *T. lanuginosus* TUB F-980 (23).

Table III. Experimental design and results of the 2^{5-1} fractional factorial design

No	Soybean meal	CSL	NH_4NO_3	$(NH_4)_2SO_4$	KH_2PO_4	Xylanase activity (IU/g)	
						Flask 1	Flask 2
1	-	-	-	-	+	5789	5538
2	+	-	-	-	-	6210	6009
3	-	+	-	-	-	6153	6153
4	+	+	-	-	+	7487	7881
5	-	-	+	-	-	6736	6009
6	+	-	+	-	+	8210	8177
7	-	+	+	-	+	8842	9359
8	+	+	+	-	-	6421	6896
9	-	-	-	+	-	7052	7389
10	+	-	-	+	+	8526	7586
11	-	+	-	+	+	9894	9753
12	+	+	-	+	-	6947	7093
13	-	-	+	+	+	7368	8472
14	+	-	+	+	-	6526	6758
15	-	+	+	+	-	7684	7980
16	+	+	+	+	+	6358	5743

Table IV. Regression coefficients, t - and p values of xylanase production in 2^{5-1} fractional factorial design

<i>Factor</i>	<i>Effect</i>	<i>t(16)-Value</i>	<i>p-Value</i>
Mean/Interc.	7281.22	67.21945	0.000000
(1)Soybean	-751.81	-3.47033	0.003155
(2)CSL	225.19	1.03945	0.314048
(3)NH ₄ NO ₃	129.94	0.59978	0.557049
(4)(NH ₄) ₂ SO ₄	578.69	2.67119	0.016729
(5)KH ₂ PO ₄	1060.44	4.89492	0.000162
1 by 2	-915.06	-4.22388	0.000645
1 by 3	-168.31	-0.77692	0.448540
1 by 4	-505.06	-2.33134	0.033135
1 by 5	-464.81	-2.14555	0.047598
2 by 3	-96.81	-0.44688	0.660950
2 by 4	-3.31	-0.01529	0.987990
2 by 5	-104.56	-0.48266	0.635878
3 by 4	-1048.81	-4.84126	0.000181
3 by 5	-120.56	-0.55651	0.585562
4 by 5	-276.56	-1.27660	0.219962

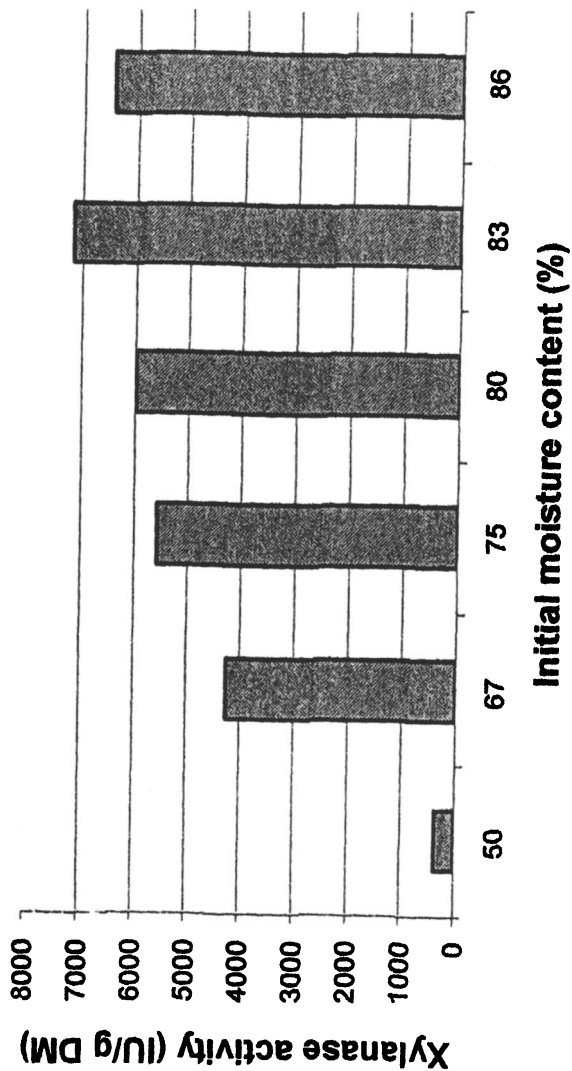


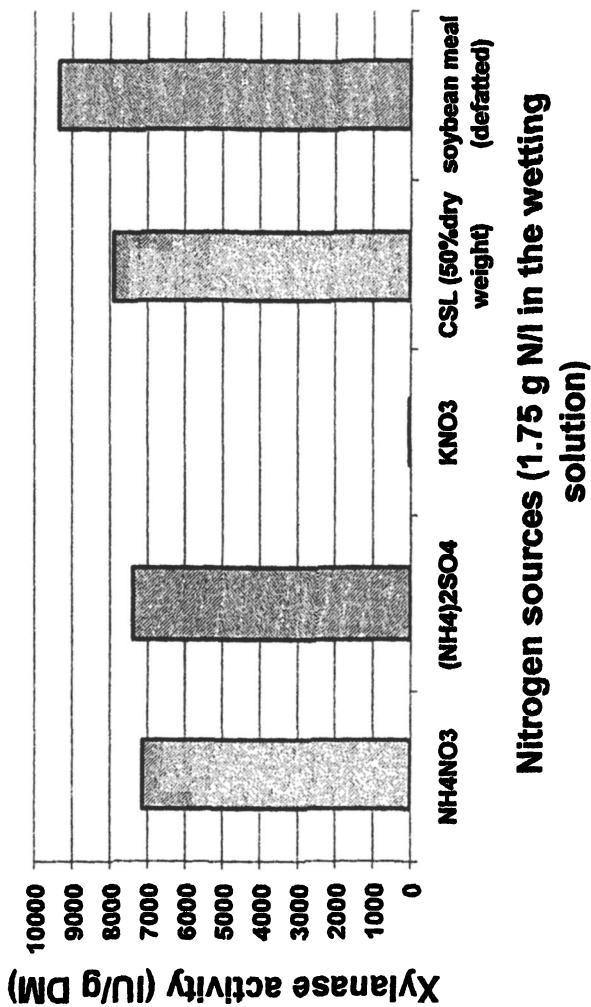
Figure 5. Impact of initial moisture content on xylanase production by *Thermomyces lamuginosus* TUB F-980 in SSF on eucalyptus pulp (45 °C; initial pH 6.8; fermentation time 5d)

Table V. Experimental design and results of the 3³ factorial design

No	KH_2PO_4	Nitrogen	CSL/(NH ₄) ₂ SO ₄	Xylanase activity (IU/g)	
				Flask 1	Flask 2
1	-1	-1	-1	5087	4186
2	-1	-1	0	9711	8837
3	-1	-1	+1	7191	7191
4	-1	0	-1	5202	4070
5	-1	0	0	11329	11860
6	-1	0	+1	7528	8315
7	-1	+1	-1	5087	4116
8	-1	+1	0	8786	8256
9	-1	+1	+1	6629	6517
10	0	-1	-1	5665	5233
11	0	-1	0	10337	11011
12	0	-1	+1	9101	8764
13	0	0	-1	6936	6279
14	0	0	0	12832	13256
15	0	0	+1	9101	8876
16	0	+1	-1	7168	6279
17	0	+1	0	11329	13256

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18	0	+1	+1	6966	8876
19	+1	-1	-1	6705	7209
20	+1	-1	0	11329	11163
21	+1	-1	1	8989	9213
22	+1	0	-1	6936	6512
23	+1	0	0	13873	13258
24	+1	0	+1	9213	8989
25	+1	+1	-1	9480	8721
26	+1	+1	0	11792	11279
27	+1	+1	+1	7528	8090



*Figure 6. Effect of different nitrogen sources (supplements) on xylanase production by *Thermomyces lanuginosus* TUB F-980 in SSF on eucalyptus pulp (45 °C; initial pH 6.8; moisture content 83 %; fermentation time 5 d)*

Table VI. The regression coefficients, *t*- and *p* values of xylanase production of the 3³ full factorial design

Factor	Regression coefficient	<i>t</i> (35)-Value	<i>p</i> -Value
Mean/Interc.	13264.74	43.2263	0.000000
(1)KH ₂ PO ₄ (L)	949.72	4.9260	0.000020
KH ₂ PO ₄ (Q)	-795.11	-2.3810	0.022838
(2)Nitrogen (L)	69.83	0.3622	0.719375
Nitrogen(Q)	-2415.11	-7.2322	0.000000
(3) CSL/(NH ₄) ₂ SO ₄ (L)	1352.78	7.0165	0.000000
CSL/(NH ₄) ₂ SO ₄ (Q)	-5577.11	-16.7010	0.000000
1L by 2L	270.38	2.5604	0.014929
1L by 2Q	314.79	1.7211	0.094069
1Q by 2L	-77.63	-0.4244	0.673872
1Q by 2Q	144.79	0.4570	0.650465
1L by 3L	-323.71	-3.0654	0.004171
1L by 3Q	-114.96	-0.6285	0.533748
1Q by 3L	-18.29	-0.1000	0.920910
1Q by 3Q	258.29	0.8153	0.420408
2L by 3L	-673.75	-6.3802	0.000000
2L by 3Q	65.75	0.3595	0.721399
2Q by 3L	-534.42	-2.9218	0.006058
2Q by 3Q	2000.92	6.3160	0.000000

R²=0.9716; Adj.:.95699

MS Residual=267634.6

Table VII. Brightness gains (points) over control induced during bleaching of eucalyptus oxygen delignified soda-aq pulp with *in situ* SSF crude xylanase of *Thermomyces lanuginosus* F-980

SSF medium	Activity (IU/g DM)	Ratio SSF material/raw pulp (w/w)			
		1:400	1:200	1:100	1:50
Non-optimized	6037	1.3	1.4	1.4	1.8
Optimized	13757	1.6	1.8	1.8	2.1

Table VIII. Reduction of chlorine dioxide charges (%) during bleaching of eucalyptus oxygen delignified soda-aq pulp with *in situ* SSF crude xylanase of *Thermomyces lanuginosus* F-980

SSF medium	Activity (IU/g DM)	Ratio SSF material/raw pulp (w/w)			
		1:400	1:200	1:100	1:50
Non-optimized	6037	20	25	25	30
Optimized	13757	25	30	30	35

Comparison of enzyme bleaching efficiency on equal units base

In this work, commercial enzymes were used in biobleaching at equal enzyme charges of 5 IU/g pulp. In comparison, the SSF enzymes were used at various SSF pulp to raw pulp ratios which, if translated into xylanase enzyme dosages on pulp, ranged (under non-optimized conditions) from as low as 15 IU/g (at a ratio of 1:400) to as high as 118 IU/g (1:50). These enzyme charges increased nearly 2.3-fold following optimization of xylanase production. An enzyme dose equivalent to 5 IU/g could be obtained with the SSF enzymes at a ratio of about 1:1200 under non-optimized conditions. As shown in Table VII, the brightness gain produced with the SSF enzymes at 15 IU/g pulp (1:400) under non-optimized conditions was 1.3 points, i.e. already lower than the average gain of 1.4 points produced with the commercial enzymes (Fig. 4). Therefore, it can be expected that at a charge of 5 IU/g, the bleaching efficiency of the SSF enzymes would be even lower than at 15 IU/g. This suggests that at equal enzyme charges on pulp, the commercial enzymes would be much more efficient in pulp bleaching than the SSF enzymes.

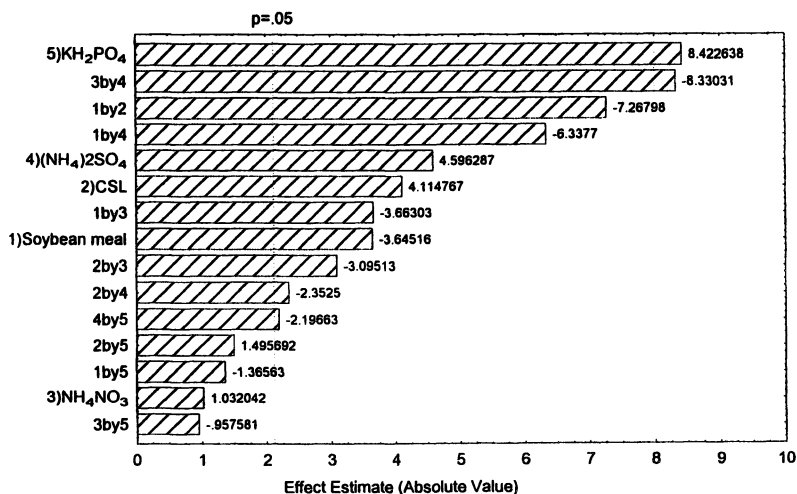


Figure 7. Pareto chart of effects of the 2^{5-1} fractional factorial design.

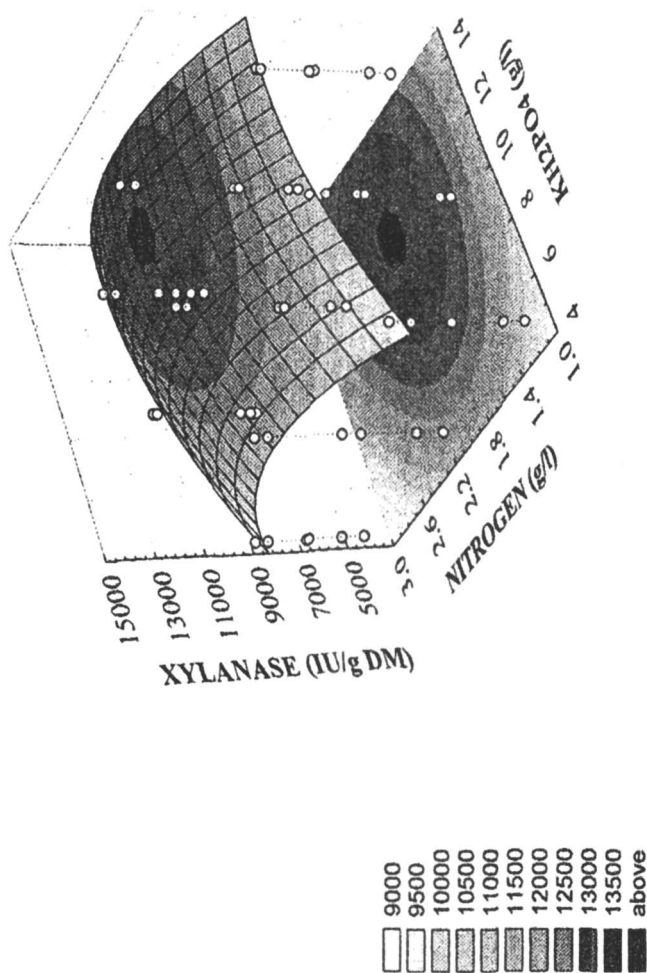


Figure 8. Fitted surface of xylanase production by *Thermomyces lanuginosus* TUB F-980 in SSF on eucalyptus pulp in terms of KH_2PO_4 and nitrogen content in the wetting salt solution (45 °C; initial pH 6.8; moisture content 83 %; fermentation time 5 d)

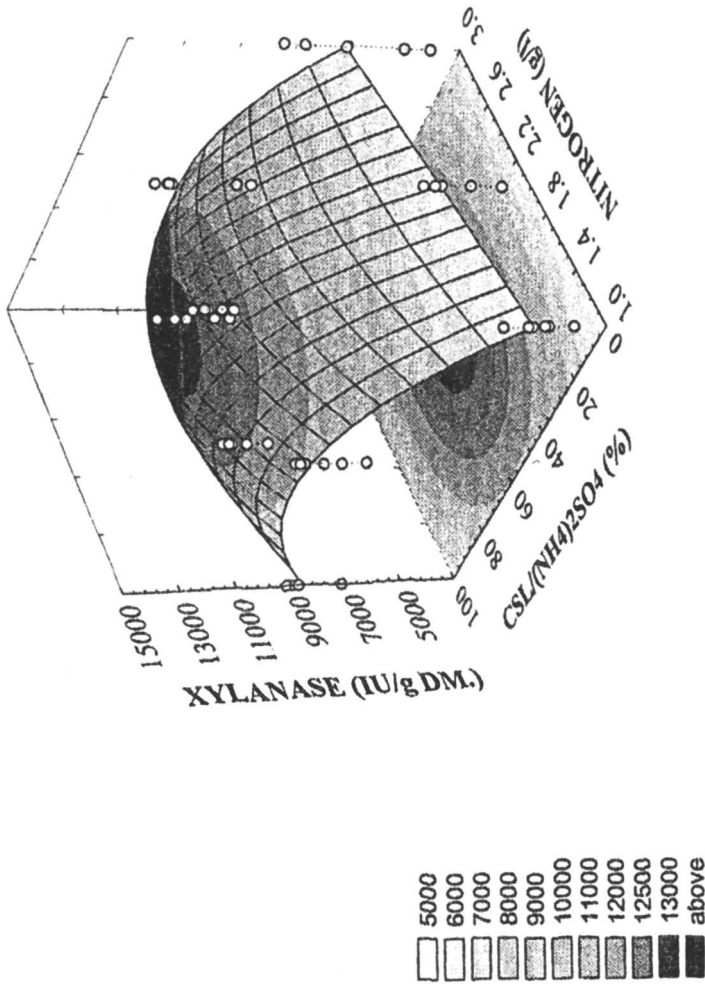


Figure 9. Fitted surface of xylanase production by *Thermomyces lanuginosus* TUB F-980 in SSF on eucalyptus pulp in terms of nitrogen content and composition (corn steep liquor and $(\text{NH}_4)_2\text{SO}_4$) in the wetting salt solution (45 °C; initial pH 6.8; moisture content 83 %; fermentation time 5 d)

Comparison of enzyme bleaching efficiency on equal cost base

Based on the enzyme activities assayed and prices of products supplied by the enzyme manufacturers, the average costs of the three commercial enzymes to treat a ton of pulp when used at a charge of 5 IU/g pulp are approximately USD 2.0. The brightness gain induced by these commercial products averaged to 1.4 brightness points (Fig. 4).

There are different technological routes and equipments for scaling up of the SSF process, e.g. tray (koji) cultivation, composting bed (pile), rotating drum bioreactor, packed bed reactor, etc. The tray method was first introduced in the Oriental countries (Japan, China, etc.) and is largely used these days to prepare fermented food products such as soy sauce, tempeh, sake, etc. on a large scale (21,24). Substrates such as steamed rice, soybean, etc. are fermented in automated tray (koji) fermentors under controlled conditions of aeration, pH, temperature, humidity, etc. The koji method would also be a suitable cultivation strategy to ferment relatively soft substrates such as cellulose pulps (unpublished data). The estimated cost for enzyme production by the SSF tray method is USD 150 per ton or USD 0.15 per kg of fermented material (7). Thus, at a ratio of 1:75 (13.3 kg SSF pulp/t untreated pulp, w/w), the production costs of SSF enzymes would equal to USD 2.0. At this ratio (1:75), the bleaching effect induced under optimized conditions would amount between 1.8 points (produced at a ratio of 1:100) and 2.1 points (at 1:50), with an average of about 2.0 points (Table VII). Therefore, at equal costs of USD 2.0/t pulp, the SSF enzymes would outperform the commercial enzymes by 0.6 points or 43% on average.

Cost comparison at equal enzyme bleaching efficiency

A direct cost comparison between the SSF and commercial enzymes can be made at the same bleaching efficiency as measured by the brightness gain induced. Using the SSF enzymes, a brightness increase of 1.4 points is achievable under non-optimized conditions at a ratio of 1:200 (Table VII), i.e. using 5 kg SSF material per ton of untreated pulp. At USD 150/t SSF pulp according to the koji method (7), the costs to produce 5 kg SSF pulp would be USD 0.75. Compared to the cost of USD 2.0/t incurred for the use of commercial enzymes, this represents a cost reduction of USD 1.25/t pulp. Therefore, the costs of the SSF enzymes needed to attain the same bleaching efficiency as commercial enzymes are 62.5% lower.

Conclusions

Solid-state fermentation of xylanase has been found advantageous over the classical submerged fermentation method of enzyme production as it can offer the possibility of a direct use of *in-situ* xylanase for pulp pretreatment and

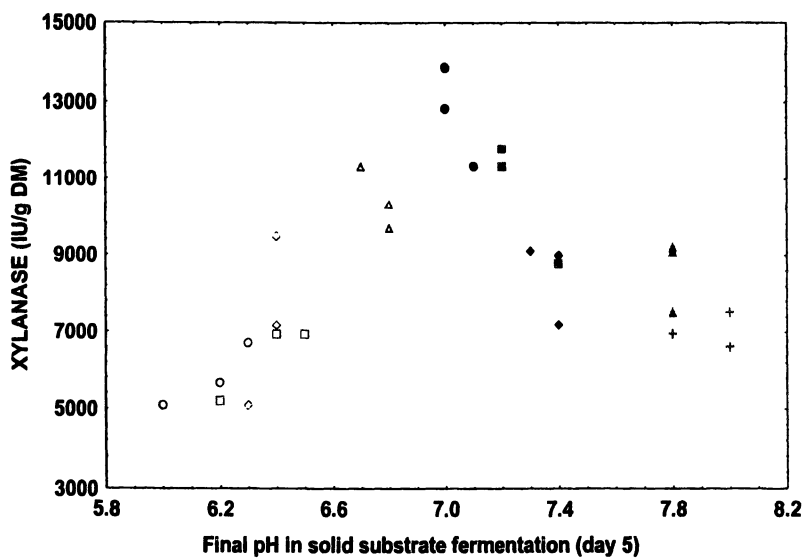


Figure 10. Effect of nitrogen supplements used in combinations on xylanase yield and fermentation final pH.

1 g/l N, CSL/(NH₄)₂SO₄ = 0 (○) ; 2 g/l N, CSL/(NH₄)₂SO₄ = 0 (□) ; 3 g/l N, CSL/(NH₄)₂SO₄ = 0 (◇) ;
 1 g/l N, CSL/(NH₄)₂SO₄ = 0.5 (Δ) ; 2 g/l N, CSL/(NH₄)₂SO₄ = 0.5 (●) ; 3 g/l N, CSL/(NH₄)₂SO₄ = 0.5 (■) ;
 1 g/l N, CSL/(NH₄)₂SO₄ = 1 (◆) ; 2 g/l N, CSL/(NH₄)₂SO₄ = 1 (▲) ; 3 g/l N, CSL/(NH₄)₂SO₄ = 1 (+)

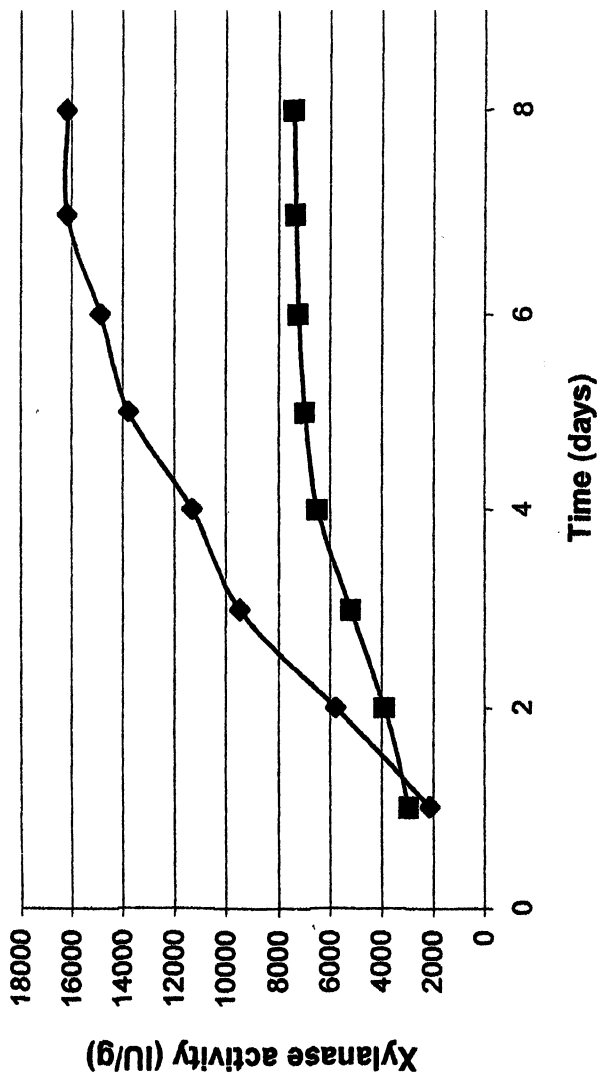


Figure 11. Xylanase production by *Thermomyces lanuginosus* TUB F-980 on optimized (◆) and non-optimized (■) media in SSF on eucalyptus pulp (45 °C; initial pH 6.8; moisture content 83 %)

bleaching without a prior downstream processing of enzyme. The pulp, which is initially used as a carbon source for enzyme production, subsequently becomes the target substrate of enzyme application. This approach could certainly improve the economics and enhance the efficiency of the biobleaching technology due to the operational simplicity of SSF, high volumetric productivity and concentration of enzyme and production of substrate-specific enzymes in a water-restricted environment.

In this work, it was shown that optimization of moisture content of the pulp substrate, medium composition, pH and time-course of SSF process can significantly improve the xylanase yields. Employment of fractional factorial experimental design was found especially useful in the selection of the optimum composition of nitrogen source in the SSF medium. The xylanase production levels correlated well with the final pH of fermentation which in turn was dependent on the type, and concentration of organic and inorganic nitrogen in SSF. The xylanase activity produced experimentally by SSF was in good agreement with the values that the model predicted and verified both the validity of the response model and the existence of the optimal point. Since the biobleaching effect is enzyme dose-dependent, the use of increased enzyme charges on the pulp enhanced the bleaching performance of the *in situ* produced crude SSF enzymes, resulting in improved brightness of bleached pulp or reduced consumption of chlorine-containing bleach chemicals.

The *in situ* crude SSF xylanases could induce bleaching effects comparable to those of commercially available enzyme products when applied in bleaching of eucalyptus soda-alkali pulp under the specified conditions of this study. In an attempt to evaluate the cost-effectiveness of the biobleaching process, assessment of the bleaching performance of SSF and commercial enzymes was made. The commercial xylanases, however, were used at their pH and temperature optima whereas the crude SSF enzymes were applied on pulp at standardized (non-optimized) bleaching conditions. It was demonstrated that the cost-effectiveness of biobleaching with SSF enzymes could be improved over the use of commercial liquid enzyme products due to the significantly lower enzyme production costs associated with the SSF process. Thus, the SSF enzymes outperformed the commercial enzymes by 43% and 62.5% when compared at equal enzyme costs and equal bleaching efficiency, respectively. However, when used at equal enzyme charges, the commercial enzymes were more efficient than the SSF enzymes. Apparently, mixing of the enzymes into the raw pulp, which is facilitated in the case of use of liquid commercial products, would be a crucial factor for the bleaching performance of the crude SSF enzymes.

Since the costs of enzyme bleaching depend on the production costs and bleaching efficiency of xylanase, both fermentation technologies (liquid and SSF) will have to compete for a cutting edge in lowering the enzyme production costs. This study certainly demonstrates the great potential of the SSF process to

enhance the competitiveness of the enzymatic bleaching technology in the pulp and paper industry.

Acknowledgements

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References

1. Viikari, L.; Rauna, M.; Kantelinen, A.; Linko M.; Sunquist, J. In *Proceedings of the 3rd International Conference on Biotechnology in the Pulp and Paper Industry*, 1986, Stockholm, Sweden, pp. 67-69.
2. Wong, K.Y.W., De Jong, E., Saddler, J.N. and Allison, R.W. Mechanism of xylanase aided bleaching of kraft pulp. *Appita J.*, **1997**, *50*, 509-518.
3. Viikari, L.; Suurnakki, A.; Buchert, J. In *Enzymes for Pulp and Paper Processing*; Jeffries, T.W. and Viikari, L.; Eds.; ACS Symposium Series 655, American Chemical Society, Washington, D.C., 1996, pp. 15-24.
4. Haltrich, D.; Nidetzky, B.; Kulbe K.; Steiner, W.; Zupancic, S. *Bioresource Technol.* **1996**, *58*, 137-161.
5. Pandey, A.; Selvakumar, P.; Soccol, C.R.; Nigam, P. *Curr. Sci.* **1999**, *77*, 149-162.
6. Tengerdy, R.P. *J. Sci. Ind. Res.* **1996**, *55*, 313-316.
7. Szakacs, G.; Tengerdy, R.P. In *Enzymes for Pulp and Paper Processing*; Jeffries, T.W. and Viikari, L.; Eds.; ACS Symposium Series 655, American Chemical Society, Washington, D.C., 1996, pp.175-182.
8. Szakacs G.; Urbanszki, K.; Tengerdy, RP. In *Glycosyl Hydrolases for Biomass Conversion*; Himmel, M. E.; Baker, J. O.; Saddler, J. N.; Eds., ACS Symposium Series 769, American Chemical Society, Washington, D.C., 2001, pp.190-203.

9. Archana A.; Sharma A.; Satyanarayana, T. In *Thermophilic Moulds in Biotechnology*, Johri, B. N.; Satyanarayana, T; Olsen, J.; eds., Kluwer Academic Publ., Boston, 1999, pp.169-190.
10. Pandey, A.; Soccol, C. R.; Mitchell, D. *Process Biochem.* **2000**, *35*, 1153-1169.
11. Purkarthofer H.; Sinner M.; Steiner, W. *Enzyme Microb. Technol.* **1993**, *15*, 677-682.
12. Alam, M.; Gomes, I.; Mohiuddin, G.; Hoq, M.M. *Enzyme Microb. Technol.* **1994**, *16*, 298-302.
13. Kalogeries, E.; Christakopoulos, P.; Kekos, D.; Macris, B.J. *J. Biotechnol.* **1998**, *60*, 155-163.
14. Jain, A. *Process Biochem.* **1995**, *30*, 705-709.
15. De Souza, M.C.O.; Roberto, I.C.; Milagres, A.M.F. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 768-772.
16. Ghanem, N.B.; Yusef, H.H.; Mahrouse, H. K. *Bioresource Technol.* **2000**, *73*, 113-121.
17. Beg, Q.K.; Bhushan, B.; Kapoor, M.; Hoondal, G.S. *Enzyme Microb. Technol.* **2000**, *27*, 459-466.
18. Bailey, J.M.; Biely, P.; Poutanen, K. *J. Biotechnol.* **1992**, *23*, 257-270.
19. Miller, G.L. *Anal. Chem.* **1959**, *31*, 426-428.
20. Ghose, T.K. *Pure & Appl. Chem.* **1987**, *59*, 257-268.
21. Doelle, H.W.; Mitchell, D.A.; Rolz, C.E. *Solid Substrate Cultivation*; Elsevier Appl. Sci.: London, 1992.
22. Pandey, A.; Soccol, C.R.; Rodriguez-Leon, J.A.; Nigam, P. *Solid-State Fermentation in Biotechnology: Fundamentals and Applications*; Asiatech Publishers, Inc.: New Delhi, 2001.
23. Christov, L.; Szakacs, G.; Tengerdy, R. Biobleaching of ligno-cellulose pulp. South African Patent 2002/3513, 2002.
24. Steinkraus, K.H. *Handbook of Indigenous Fermented Foods*. Marcel Dekker, Inc.: New York, 1983.

Chapter 17

Characterization of the Activity Profile in Cellulases Derived from Recombinant Strains of *Trichoderma reesei*

F. A. Zandoná¹, P. D. Cancelier², M. Siika-Aho³, and L. P. Ramos^{2,*}

¹Tuiuti of Paraná University, Curitiba, PR, Brazil

²Research Center in Applied Chemistry (CEPESQ), Universidade Federal do Paraná (UFPR) P.O. Box 19081, Curitiba, PR, Brazil 81531-990

³VTT Biotechnology, P.O. Box 1500, FIN-02044, Espoo, Finland

Enzyme preparations from eight deletion strains of *Trichoderma reesei* were characterized with regard to their activity profile against several model substrates, and subsequently compared to the complete cellulase system of the industrial preparation Celluclast 1.5L (Novozymes, Denmark). The recombinant enzymes (Röhm Enzyme Finland Oy, Finland) comprised of 4 single (A, CBH I-; B, CBH II-; C, EG I-; D, EG II-), 2 double- (E, CBH I/II-, F, EG I/II-) and 2 tri-deletion (G, EG II-/CBH I/II-; H, CBH II-/EG I/II-) enzymes. Compared to Celluclast 1.5L, deletion of both genes encoding for EG I and EG II (enzyme F) resulted in very little endoglucanase activity in the enzyme preparation, whereas the absence of both CBH I and CBH II (enzyme E) reduced filter paper activity by more than 75%. In general, for saccharification experiments in which less than 2% of the substrate was hydrolyzed to soluble sugars, EG-rich preparations (enzyme E at 200 mg/g) caused a considerable decrease in the degree of depolymerization (DP) of substrates with high amorphous character (*e.g.*, filter paper fibers), whereas cellulose DP was not affected when substrates having a higher supramolecular organization were used (*e.g.*, Avicel, Sigmacell or cotton fibers). By contrast, when CBH-rich preparations (enzyme F, 200 mg/g) were used, greater cellulose saccharification yields were observed (14%), and changes in cellulose DP were primarily due to the progressive removal of cellobiose residues from the *termini* of cellulose molecules.

Introduction

The extracellular enzyme system produced by *Trichoderma reesei* is composed of three major enzyme components: endoglucanases (EC 3.2.1.4), exocellobiohydrolases (EC 3.2.1.91) and β -glucosidases (EC 3.2.1.21) (1,2). These enzymes are usually glycosylated, occur in multiple forms, have distinct specific activities, and except for EG III (see below), possess the ability to adsorb onto cellulose through a cellulose binding domain (3). In general, the random action of endoglucanases gradually increases the availability of cellulose chain ends, therefore increasing the specific surface area of the substrate for exoglucanase catalysis. At least five endoglucanases (EG I to V) have been identified in the cellulase system secreted by the soft-rot fungus *T. reesei* (2,4,5). On the other hand, two unrelated cellobiohydrolases (CBH I and II) have been described in the culture filtrate of this fungus (6), and these enzymes have been shown to act synergistically during the enzymatic hydrolysis of cellulose (2,7). In fact, it has been shown that CBH II exhibits some endoglucanase properties, as it was able to access substrate sites that are normally accessed by typical endoglucanases such as EG I and II (8,9). For this reason, Henrissat *et al.* (10) suggested a new nomenclature for cellulases that is based solely on structural features, i.e., the glycoside hydrolase families.

One important issue about designing an ideal cellulase system is the full characterization of those enzyme components which are considered essential for hydrolysis. Hence, several authors have tried to study the kinetic behaviour of purified enzyme components using a wide variety of substrates ranging from chromogenic materials (11) to bacterial (12) and microcrystalline cellulose (13). As a result, the strategic combination of key specific activities would provide an effective way to reproduce or further improve the effectiveness by which cellulosic substrates are hydrolyzed (7). Through this working hypothesis, it is possible to establish a degree of synergism greater than those found in natural systems because highly complementary enzymes would be mixed at an optimal ratio (14,15). However, this ideal ratio may vary from one substrate to another, depending on its chemical composition and supramolecular structure.

Enzyme purification is not only tedious but sometimes ineffective at isolating key components of the cellulase system without any cross-contamination with endoglucanases. To circumvent this problem, several groups are actively working on the utilization of genetic engineering to enhance properties, specific activities and productivity of selected enzymes (7,15). Hence, the combination of these cloned enzymes may generate what once has been referred to as cellulases of fourth generation (16), and the best possible preparations may eventually arise from the combination of enzymes from heterologous systems. However, until these specialty enzymes are made available, other methods must be assessed to address the relative importance of each of the individual cellulase categories in the saccharification of cellulose.

During the last few decades, genes for both cellobiohydrolases and endoglucanases have been cloned and characterized. With the further elucidation of the biological function of these genes through targeted transformation, many

different recombinant strains have been produced, and have been characterized by having genes either replaced or deleted from the genome of the parental strain.

While transformations like these are usually of low frequency, Karhunen *et al.* (17) described for the first time the high frequency gene replacement technique in the *T. reesei* mutant VTT-D-79125. The expression of the EG I cDNA (*egl1*) at the *cbh1* locus, under the control of the powerful cellobiohydrolase I (*cbh1*) promoter, produced a 2-fold increased in the endoglucanase activity of the transformant, and the one step gene replacement of CBH I cDNA reached a frequency as high as 63%.

Based on the favorable results of this pioneering work, Suominen *et al.* (18) applied this one-step gene replacement technique to evaluate the effects of deleting individual cellulase genes. These experiments were aimed at targeting and replacing the genes that encode for EG I, EG II, CBH I and CBH II, producing four monodeletion transformants whose properties looked promising for several industrial applications such as biorefining or biopolishing of delignified pulp fibers. Based on their common genetic background, the authors stated that the only difference among the strains was the lack of one gene and its promoter. Nevertheless, CBH II was reported as the only enzyme whose production was affected by the deletion of other cellulase genes.

Deletion of the gene encoding CBH I decreased protein secretion in the *T. reesei* culture filtrate by 40%, whereas monodeletion of CBH II, EG I or EG II did not change the total amount of protein produced by the parental strain (18). However, a 70% decrease in filter paper activity was observed as a result of CBH I deletion, and this effect was much more pronounced than that observed when the CBH II gene was deleted using the same technique. Likewise, by knocking out the gene encoding for both EG I or EG II, there was a decrease in the endoglucanase activity of the resulting enzyme preparation. However, the lack of EG II was more detrimental to the hydrolysis of both filter paper and hydroxyethylcellulose (HEC), suggesting that this endoglucanase component played a much more important role than EG I in the total hydrolysis of cellulose.

Since the monodeletion strains from *T. reesei* mutant VTT-D-79125 were available, double- and trideletion strains were also constructed from this same parental strain and the properties of these recombinant enzymes have been the subject of both academic (19) and applied investigations (20). For instance, EG-rich preparations were produced when both genes encoding for CBH I and II were deleted. Preparations like this have important applications in the hydrolysis of amorphous cellulose derivatives, and have been proposed for several industrial processes including biopolishing of textile fibers (21), biobleaching of kraft pulps (22) and fiber modification in pulp and paper manufacturing (23-26).

The main focus of this work is the characterization of the activity profile of several recombinant enzymes with the aim of establishing a direct evidence to the relative importance of each enzyme component on the hydrolysis efficiency of model substrates.

Material and Methods

Enzymes

The commercial enzyme preparation used in this study, Celluclast 1.5L (C 1.5L), was kindly supplied by Novozymes (Denmark), whereas the recombinant enzyme preparations were gifts from Röhme Enzyme Finland Oy (Finland). These enzymes were formerly classified as mono, di and tri-deletion enzymes, namely 4 single (A, CBH I-; B, CBH II-; C, EG I-; D, EG II-), 2 double- (E, CBH I/II-; F, EG I/II-) and 2 tri-deletion enzymes (G, EG II-/CBH I/II-; H, CBH II-/EG I/II-).

Protein content was determined using the Bradford assay employing Coomassie staining, and the Lowry assay after protein precipitation with trichloroacetic acid (TCA) (27).

Determination of enzyme activities against model substrates

The activity profile found in each enzyme preparation was determined according to I.U.P.A.C. recommendations (27) using the following substrates: filter paper Whatman No. 1 (FP, Sigma), carboxymethylcellulose of medium viscosity (CMC, Sigma), hydroxyethylcellulose (HEC, Fluka), oat spelt xylan (OSX, Sigma), larchwood xylan (LWX, Sigma), cellobiose of analytical grade (Sigma) and *p*-nitrophenyl- β -D-glucoside (PNPG, Sigma). Activity against *p*-nitrophenyl- β -D-lactoside (PNPL, Sigma) was carried out in the presence of β -glucosidase (D-glucono-1,5- β -lactone) and/or CBH (cellobiose) inhibitors (11), whereas Azocasein (AZC, Sigma) was used as substrate for protease activity according to Leighton *et al.* (28).

Reducing sugar release was monitored in several of these assays by dinitrosalicylic acid (DNS) (27), whereas filter paper and cellobiose hydrolysates were analyzed by high performance liquid chromatography (HPLC). By contrast, UV spectroscopy at 405 nm (Varian, model Cary 100) was used to determine activity against chromogenic substrates such as PNPG and PNPL (11).

HPLC analyses of enzyme hydrolysates were performed using a Shimadzu HPLC (Kyoto, Japan) workstation, model LC10AD, provided with a SIL10A autosampler and two detectors arranged in series, model SPD10A for UV spectroscopy and model RID10A for refractive index. The HPLC analysis was performed at 65°C in an Aminex HPX-87H column (Bio-Rad) at 65°C and elution was carried out isocratically with 8 mM H₂SO₄ at 0.6 mL min⁻¹.

Enzymatic hydrolysis of cellulosic materials

Enzymatic hydrolysis of cellulosic substrates was performed at 2% (m/m) substrate consistency in 2 mL of a 50 mM sodium acetate buffer, pH 4.8, for 2 h at 50°C, using an enzyme loading of 200 mg protein/g substrate and agitation at every 15 min. Due to their differences in origin, accessibility and degree of polymerisation, four cellulosic substrates were chosen for this experiment: Avicel PH101 (Fluka), Sigmacell Type 50 (Sigma), freeze-dried Whatman #1 filter

paper fibers (Whatman) and alkali-washed cotton fibers (Johnsson). The reaction was stopped by transferring the reaction mixture to an ice bath. HPLC analyses of enzyme hydrolysates were performed using a Shimadzu HPLC (Kyoto, Japan) workstation as described previously. The hydrolysis residue was isolated by centrifugation, washed thoroughly with water and freeze-dried prior to carbonylation. A minimum of two replicates were carried out for each of the enzyme treatments, as well as for the analytical procedures used to determine hydrolysis yields and changes in the degree of polymerization of cellulose.

Determination of the degree of polymerization of cellulose

The molecular mass distribution of cellulose was obtained by gel permeation chromatography (GPC) of their tricarbonyl derivatives. The procedure used for carbonylation (29) was an adaptation of the method previously described by Schroeder and Haigh (30) and Miller *et al.* (31). Approximately 50 mg of the freeze-dried fibers were placed in a vial and dried over phosphorus pentoxide for 24 h. To the dry residue, 0.5 mL of phenyl isocyanate and 3 mL of dry pyridine were added and the vials were tightly sealed. The reaction was carried out in a heating block at 80°C for 48 h. The reaction mixture was constantly homogenized in a Vortex during the reaction to allow better contact between sample and reagents. After cooling, the reaction was stopped by adding 1 mL of methanol. The carbonylated residue was precipitated in a mixture of methanol:water (4:1, v/v) and washed thoroughly to remove any residual pyridine. The resulting derivative was then freeze-dried from a water suspension and kept in a desiccator against phosphorus pentoxide.

The GPC analysis of the tricarbonyl derivatives was carried out using a Shimadzu LC10AD liquid chromatograph. The tricarbonylated samples were solubilized in tetrahydrofuran (THF, 0.5-1.0 mg·mL⁻¹) and filtered through a Teflon membrane with a pore size of 0.45 μm. Analyses were carried out by auto-injection (Shimadzu SIL10A) on a series of one guard column and four Tosoh TSK-GEL (type H8) columns at 45°C, with exclusion limits ranging from 4x10⁷ to 1x10³ units of molecular mass. THF was the elution solvent at a flow rate of 1 mL·min⁻¹, and detection was performed by UV spectroscopy (Shimadzu SPD10A) at 240 nm. The large peak eluting after a retention volume of 38 mL was attributed to the reagents and reaction by-products (32).

The GPC calibration curve was generated from the elution profile of twenty monodisperse polystyrene standards. Universal calibration was carried out according to Valtasaari and Saarela (33) based on the Mark-Houwink coefficients for cellulose tricarbonylate in THF ($K_c = 2.01 \times 10^{-5}$, $\alpha_c = 0.92$) and polystyrene in THF ($K_p = 1.18 \times 10^{-4}$, $\alpha_p = 0.74$). A typical dispersion of 2-3% was observed in our bimodal calibration curve as a result of quadratic fitting. Both the number average (MM_N) and the mass average (MM_M) molecular mass

of cellulose tricarbonylate were determined as described previously (34). The degree of polymerisation (DP) of cellulose was obtained by dividing the molecular mass of the tricarbonylated polymer by the corresponding molecular mass of the tricarbonyl derivative of anhydroglucose ($DP = MM/519$) (29).

Results and Discussion

The catalytic properties of eight enzyme preparations derived from genetically modified *T. reesei* strains (Röhm Enzyme Finland Oy) have been investigated and compared. We attempted to ascertain whether these properties could be correlated with the relative importance of both major endoglucanases (EG I and II) and cellobiohydrolases (CBH I and II) on the efficiency of hydrolysis of model substrates and other cellulosic materials.

Initially, the protein content of each enzyme preparation was determined. Both Lowry and Bradford assays (27) resulted in similar ranges of protein contents, but the former assay, carried out after protein precipitation with TCA 10%, provided better accuracy and lower standard deviations in experiments carried out in triplicates (Table I). For this reason, the Lowry assay was chosen as the method of choice for the determination of protein in this study.

Protein content varied considerably among the recombinant enzymes because these preparations have been concentrated to varying degrees after cultivation. Both preparations void of CBH I (enzyme A) and CBH II (enzyme B) were characterized by having low protein content (22.1 and 31.3 mg/mL, respectively), whereas higher protein contents were detected in those preparations in which at least one of the two major EG components of *T. reesei* (EG I and II) was missing (Table I). On the other hand, the protein content in double and trideletion enzymes varied between 37 mg/mL for enzyme G (lacking CBH I, CBH II and EG II) and 67 mg/mL for enzyme E (lacking CBH I and CBH II).

Because the enzyme system produced by the parental strain VTT-D-79125 was not available for this study, comparisons had to be made with another *T. reesei* complete cellulase system. For this purpose, Celluclast 1.5L (C 1.5L) was shown to have relatively high filter paper (FPase) and carboxymethylcellulase (CMCase) activities, while its β -glucosidase activity was very low (Table I).

Deletion of the gene encoding for CBH I (enzyme A) resulted in a proportional increase in the specific activities of the remaining cellulases, but the FPase activity decreased considerably in the enzyme system (Table II).

Likewise, deletion of CBH II (enzyme B) had a similar effect on FPase activity and this was in contradiction to the preliminary work of Suominen *et al.* (18), who demonstrated that, when activities were expressed in relation to the amount of protein secreted, the FPase activity in enzyme A corresponded to only 70% of the FPase activity found in enzyme B.

Table I. Protein content of the enzyme preparations

<i>Enzyme</i>	<i>Deletion</i>	<i>Lowry (mg/ml)</i>	<i>Bradford (mg/ml)</i>
A	CBH I	21.00 ± 0.28	20.60 ± 1.55
B	CBH II	27.00 ± 0.38	27.40 ± 1.46
C	EG I	127.00 ± 1.22	126.30 ± 8.34
D	EG II	41.00 ± 0.23	45.10 ± 1.61
E	CBH I & II	67.00 ± 0.97	67.60 ± 3.09
F	EG I & II	47.00 ± 0.48	49.00 ± 3.15
G	EG II, CBH I & II	37.00 ± 0.36	36.60 ± 2.12
H	CBH II, EG I & II	41.00 ± 0.26	42.30 ± 1.06
C 1.5L	--	168.50 ± 0.57	167.30 ± 4.92

Table II. Activity against model substrates

Enzyme	FPase (IU/mg)	CMCase (IU/mg)	HECase (nkat/mg)	OSXase (IU/mg)	BWXase (IU/mg)
A	0.47	28.61	149.97	11.31	3.20
B	0.45	11.72	124.87	3.82	1.22
C	0.57	5.91	165.97	1.92	0.06
D	0.56	8.17	107.08	5.20	1.67
E	0.19	32.40	336.15	19.15	2.11
F	0.58	0.67	61.17	1.06	5.29
G	0.015	13.51	138.91	31.50	4.88
H	0.19	0.67	32.01	17.31	5.91
C 1.5L	0.86	19.25	247.75	2.42	1.57

FP – filter paper; CMC – carboxymethylcellulose; HEC – carboxymethylcellulose; OSX – oat spelt xylan; BWX – birchwood xylan.

The FPase activities of enzymes A (CBH I-) and B (CBH II-) were lower than those determined in enzymes C (EG I-) and D (EG II-), suggesting that the effective saccharification of filter paper depends upon the availability of both CBHs for hydrolysis. However, the filter paper assay measures the release of reducing sugars in aqueous media, and do not account for the catalytic action of endoglucanases that act in the middle of the cellulose chains and generate oligosaccharides that remain adhered to the cellulose surface after partial hydrolysis. On the other hand, double deletions of both EG I and II (enzyme F) had the same effect on FP activity than any of the two monodeletions on EG I and II. Possibly, the decrease of endo-exo synergism was compensated by the increased dosage of CBHs as their relative portion in the preparation increased.

The lowest FPase activity (0.015 IU/mg) among all recombinant enzymes was detected in enzyme G, which corresponded to a trideletion of CBH I, CBH II and EG II. Therefore, this enzyme contains only EG I plus other minor endoglucanase components found in *T. reesei*. Interestingly, the other trideletion enzyme involved in this study, which was composed almost exclusively of CBH I, had an FPase activity of only 0.19 IU/mg. This low FPase activity probably accounts for the absence of synergism when CBH I is acting alone, without the cooperation of the other main cellulases of the complex (CBH II, EG I and II).

The endoglucanase activity of each enzyme preparation was determined against two model substrates, CMC and HEC. The former is the classical substrate for determining endoglucanase activity but the use of the latter offers several advantages because HEC solutions have lower viscosity than equivalent CMC solutions and the assay is carried out for shorter incubations times (10 min). These two advantages have the following benefits: (a) the assay is faster and easier to handle; (b) due to the lower viscosity of the HEC solution, an increased substrate availability is attained at equivalent substrate concentrations; (c) lower viscosities also result in better mass transfer during the assay; and (d) shorter residence times provide activity measurements during the initial rate of hydrolysis, avoiding undesired effects such as end-product inhibition.

According to the IUPAC recommendations (27), CMCase activities in cellulase preparations are reported in international units (IU/mL or IU/mg), whereas HECCase activities are expressed in nanokatal (nkat/mL or nkat/mg). However, differences in the procedures used to carry out these assays may be influential enough to compromise a direct comparison between the susceptibility of these substrates to endoglucanases (see above). In general, CMCCase activities expressed in IU/mg were lower than HECCase activities expressed in nkat/mg (Table II). However, when both substrates were assayed according to the same experimental procedure, little difference was observed in the ability of independent enzyme preparations to hydrolyze CMC or HEC, suggesting that endoglucanases such as EG I and II present similar activities against these cellulose derivatives, regardless of differences in their chemical structures (data not shown).

Specific endoglucanase activity when EG II was the only EG present in the enzyme preparation (enzyme C, 165.97 nkat/mg on HEC) was about 1.6 times higher than that observed when EG I was the predominant EG (enzyme D,

107.08 nkat/mg on HEC) (Table II). Considering that the specific activity of EG II on HEC is nearly two times as high as that of EG I (35), this suggests that the amounts of EG I and II (as protein) were more or less the same in these recombinant enzymes.

The enzyme lacking both CBH I and II that contained both major endoglucanases (enzyme E) displayed the highest activity against CMC and HEC (Table II). The main reason for this was likely an increase in the relative amount of endoglucanases when the major enzyme component CBH I was omitted from the mixture. On the other hand, the complete absence of both EG I and II (enzyme F) resulted in a dramatic decrease in the endoglucanase activity of the preparation, and the remaining activity was probably due to other *T. reesei* endoglucanase components such as EG III.

Enzyme A (CBH I-) was shown to have an activity against CMC of 28.61 IU/mg (149.97 nkat/mL against HEC), whereas only 11.72 IU/mg (124.87 nkat/mL against HEC) could be detected in the preparation which lacked CBH II (enzyme B). Suominen *et al.* (18) observed that the deletion of CBH I increased both endoglucanase and β -glucanase activities in relation to the parental strain, and this was attributed to a possible increase in EG II secretion as a result of the CBH I knock-out. Therefore, the occurrence of higher EG II levels in enzyme A may have been responsible for its higher endoglucanase activity. Likewise, enzyme E is derived from a *T. reesei* transformant deficient in both CBH I and II and its endoglucanase activity should have been equivalent or even greater than that observed for enzyme A. Indeed, both HECase and CMCcase activities in enzyme E were nearly 2.24 and 1.13 times greater than those detected in enzyme A, characterizing a probable summation of the effects derived from the deletion of both CBH I and II.

The best enzyme preparations against CMC had also the highest levels of xylanase activity, and this appeared to be related to the lack of CBH I and/or to the occurrence of the xylan-degrading EG I in the enzyme mixtures (Table II). When enzyme A (CBH I-) was assayed against oat spelt xylan (OSX), an activity of 11.31 IU/mg was found, whereas an even higher xylanase activity of 19.15 IU/mg was detected in the enzyme missing of CBH I and II (enzyme E). In fact, these correspond to a xylanase activity 5- to 6-fold higher than that of Celluclast 1.5L. Both EG I- enzymes (C and F) showed the lowest xylanase activity against OSX (1.92 and 1.06 IU/mg, respectively), with both CBH II- (enzyme B) and EG II- (enzyme D) preparations falling at an intermediate activity range of 3.82 to 5.20 IU/mg. On the other hand, the highest OSXase activity (31.50 IU/mg) was found in the trideletion enzyme G (EG II-, CBH I/II-). In general, replacement of OSX by birch wood xylan (BWX) decreased the xylanase activity of all enzymes, except the double deletion enzyme F. Since these substrates differ in both origin and chemical composition (BWX is almost exclusively composed of xylose, whereas OSX contains other carbohydrates such as arabinose), differences in enzyme activities were somewhat expected.

Compared to Celluclast 1.5L, all of the recombinant enzymes used in this study had an enhanced ability to release *p*-nitrophenol (PNP) from *p*-nitrophenyl-

β -D-glucoside (PNPG) (Table III). As a result of this higher β -glucosidase activity, glucose was always the main reaction product obtained during hydrolysis of substrates such as filter paper. Likewise, Suominen *et al.* (18) demonstrated that the monodeletion of CBH I or CBH II (enzymes A and B, respectively) resulted in the secretion of β -glucosidase activities higher than that of the parental strain. This suggests that the β -glucosidase activity in the culture broths of the *T. reesei* VTT-D-79125 mutant strain (4.8 nkat/mg of PNPase activity according to Suominen *et al.*) is higher than that of Celluclast 1.5L.

The PNPase activity found in most of the recombinant enzymes fell within 0.70 and 0.85 U/mg (Table III). Only one enzyme had a PNPase activity lower than this (0.41 IU/mg for enzyme C). By contrast, monodeletion of either CBH I (enzyme A) or CBH II (enzyme B) resulted in greater PNPase activities (1.33 and 1.25 IU/mg, respectively). Activity of the recombinant enzymes against cellobiose was similar to that observed against PNPG, except for those systems in which CBH I was absent. Enzymes A (CBH I-less), E (CBH I/II-less) and G (EG II, CBH I/II-less) had cellobiase activities as high as 2.03, 2.75 and 3.15 IU/mg. These results were in fair agreement with Suominen *et al.* (18), who demonstrated that replacement and/or deletion of both CBH I and II caused an increase in β -glucosidase secretion, an effect that was greater when CBH I was the target enzyme for transformation.

CBH activity was determined in the recombinant enzymes by measuring the difference in PNP release from PNPL in the presence and absence of cellobiose, with D-glucono-1,5- β -lactone added as a β -glucosidase inhibitor (Table III). All of the CBH-rich enzymes (Celluclast 1.5L, F and H) displayed differential activities beyond 0.4 IU/mg (0.6, 0.47 and 0.40 IU/mg, respectively). However, no suitable explanation has yet been found to explain the relatively high differential activity found in enzyme E (0.29 IU/mg).

Since *T. reesei* produces proteases under certain fermentation conditions, and the hinge domains of CBH I and other cellulases are susceptible to protease cleavage, protease activity was measured in all of the recombinant enzymes used in this study (Table III). Interestingly, a residual protease activity was found in most of the recombinant enzyme preparations. Removal of CBD from CBH I (36) or from other *T. reesei* cellulases does not change their mode of action on soluble substrates or pulp (35), but the efficiency by which cellulose is hydrolyzed is partially lost together with their ability to adsorb onto the substrate. Efforts are now being made to investigate this issue because the occurrence of partial proteolysis in these samples could explain why some of the recombinant enzymes behaved differently from what one would generally expect.

Hydrolysis of cellulosic materials by the recombinant enzymes

To investigate the actual performance of the recombinant enzymes in hydrolyzing cellulose, four substrates with distinct chemical properties and supramolecular organization were chosen: Avicel PH101, Sigmacell type 50,

Table III. Activity against cellobiose and several chromogenic substrates

Enzyme	Activity (U/mg) against					
	Cellobiose	PNPG	PNPL	PNPLc	AZC	
A	2.03	1.33	1.22	0.94	0.38	
B	1.13	1.25	0.78	0.61	0.39	
C	0.60	0.41	0.37	0.14	0.08	
D	0.80	0.79	0.75	0.74	0.22	
E	2.75	0.79	1.24	0.95	0.18	
F	0.69	0.70	0.84	0.37	0.21	
G	3.15	0.83	0.92	0.77	0.37	
H	0.51	0.85	0.78	0.38	0.20	
C 1.5L	0.02	nd	1.39	0.79	bdl	

nd – not determined; bdl – below detection limits; PNPL – assay performed in the presence of D-glucono-1,5- β -lactone; PNPLc – assay performed in the presence of D-glucono-1,5- β -lactone and cellobiose; AZC – protease activity against Azocasein

freeze-dried Whatman #1 filter paper fibers and fully bleached cotton fibers. Hydrolyses were carried out for 2 h at high protein loadings (200 mg/g), and release of soluble sugars was monitored in each reaction hydrolysate by HPLC.

The effect of the recombinant enzymes against cellulosic materials of high supramolecular organization (Avicel, Sigmacell and cotton fibers) was shown to be lower than that obtained when filter paper was used as substrate for hydrolysis (Table IV). This was in agreement with previous findings that highly crystalline cellulosic materials have a tighter supramolecular organization that restricts swelling and limits the yield by which cellulose is hydrolyzed. Enzyme E (depleted of CBH I and II) had the lowest activity against these recalcitrant substrates, whereas all of the CBH-containing enzyme preparations were capable of hydrolyzing Avicel, Sigmacell and cotton fibers to some extent.

Hydrolysis with the recombinant enzymes also resulted in different ratios of glucose-to-cellobiose (Glc:Glc₂) in the enzyme hydrolysates. When Sigmacell was used as a substrate, the highest Glc:Glc₂ ratios were produced by enzymes deficient in either CBH I or II. Glucose was the only hydrolysis product produced by enzyme E because the cellobiose concentration was below the lowest detection limit of the method, whereas enzyme A produced a Glc:Glc₂ ratio of 17.6:1 (m/m) and enzyme B, 21.3:1 (m/m). These increased Glc:Glc₂ ratios were attributed to the lack of exoglucanase activity in these enzyme preparations, as well as to their high cellobiase activity (Table III). Likewise, EG deficient enzymes (enzymes C, D and F) produced lower Glc:Glc₂ ratios in Sigmacell hydrolysates (within 8.8:1 to 10.7:1) because of their relatively low cellobiase activity and high CBH content. By contrast, Celluclast 1.5L resulted in the lowest Glc:Glc₂ ratio (2.9:1) because (a) it is primarily composed of cellobiohydrolases and (b) exhibits little cellobiase activity.

Despite their rather large differences in cellulose DP (Table V), Sigmacell, Avicel and cotton fibers provided similar hydrolysis yields for all of the enzyme preparations tested herein (Table IV), and this seemed to be related to the high degree of crystallinity of these substrates. Nevertheless, powdered cellulosic materials were always hydrolyzed to a greater extent than cotton fibers and we have attributed this to their higher availability of chain ends. On the other hand, freeze-dried filter paper fibers yielded the highest levels of enzymatic hydrolysis because of their higher degree of fiber porosity and supramolecular disorder.

The mode of action of the recombinant enzymes was further investigated by measuring the degree of polymerization (DP) of cellulose before and after hydrolysis with the double-deletion enzymes E and F. In general, enzyme E could not hydrolyze more than 2% of the above mentioned substrates in relation to their dry mass (Table IV), and the hydrolysis of Avicel (average DP_w of 277 AnGlc, Figures 1A), Sigmacell (average DP_w of 319 AnGlc, Figures 1B) and bleached cotton fibers (average DP_w of 2310 AnGlc, Figures 2A) resulted in very small variations in cellulose DP (that is, little hydrolysis had occurred in all cases) (Table V). However, a considerable shift in the cellulose DP distribution was observed when freeze-dried filter paper fibers (average DP_w of 1829 AnGlc, Figure 2A) were used as substrates for hydrolysis (Table V, Figures 2B).

Table IV. Enzymatic hydrolysis of cellulosic materials (2 h at 45°C) under equivalent protein loadings of 200 mg/g of cellulose.

Enzyme	Glucose yield (% m/m) upon hydrolysis of:			
	Sigmacell	Avicel	Cotton fibers	Filter paper
A	3.18	4.27	0.98	7.89
B	2.56	3.02	1.95	9.19
C	3.06	3.59	1.66	9.15
D	3.96	4.4	2.98	11.73
E	0.83	1.82	0.39	1.89
F	5.84	6.27	2.1	14.01
G	1.25	0.93	0.36	1.91
H	1.2	2.18	1.25	5.79
C 1.5L	8.12	5.82	4.19	13.86

Table V. Effect of the enzyme treatment on the degree of polymerization and polydispersity (P) of cellulosic substrates

Substrate	Untreated			Treated with E			Treated with F		
	DP _w	DP _n	P	DP _w	DP _n	P	DP _w	DP _n	P
Avicel	277	74	3.76	273	75	3.64	259	72	3.60
Sigmacell	319	90	3.52	334	98	3.38	346	100	3.46
Filter paper	182	270	6.78	106	163	6.54	113	190	5.97
	9			8			6		
Cotton	231	313	7.37	232	278	8.38	239	301	7.94
	0			7			0		

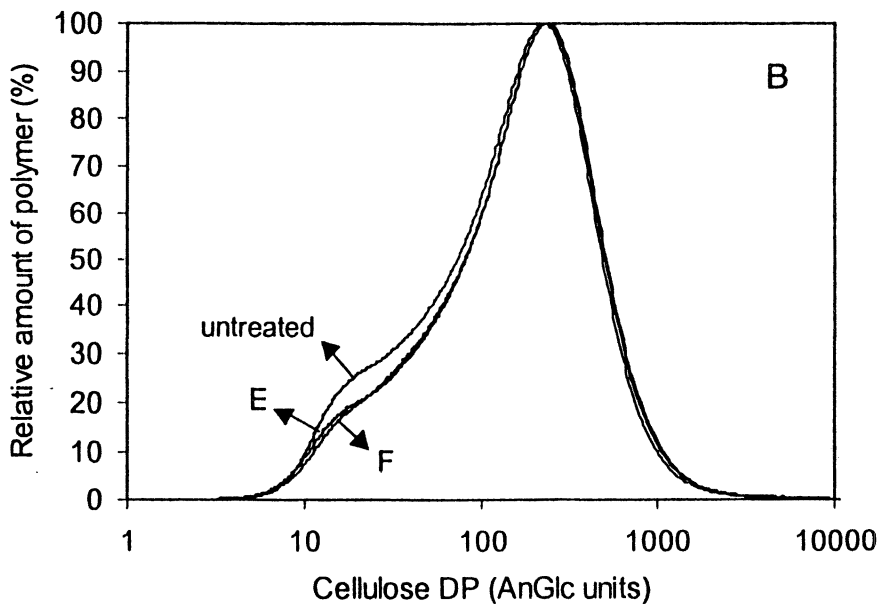
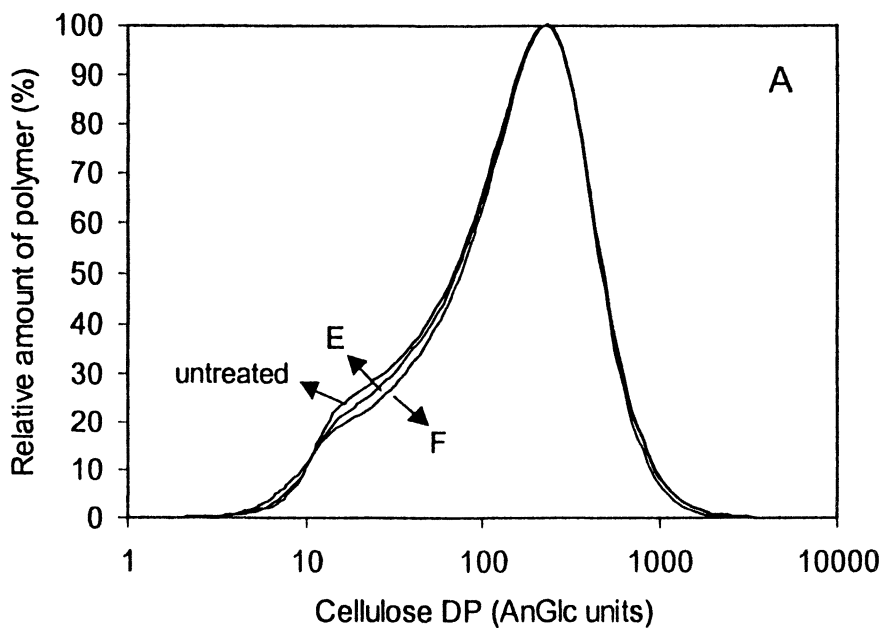


Figure 1. Effect of the enzyme treatment on the degree of polymerization (DP) of Avicel (A) and Sigmacell (B).

This observation was readily attributed to the high endoglucanase activity of enzyme E, because both EG I and II have very limited action against substrates with high molecular order and crystallinity indexes (*e.g.*, Avicel, Sigmacell and cotton fibers). By contrast, even though the release of soluble sugars would not account for more than a 2% mass loss, enzyme E caused a significant shift in the cellulose DP of Whatman #1 filter paper fibers because this substrate is not as well organized as microcrystalline cellulose and cotton fibers. This kind of rapid depolymerization of cellulose during the early stages of hydrolysis using *Trichoderma* cellulases has been attributed to the random attack of endoglucanases at the more exposed, accessible regions of the substrate (37,38). Interestingly, a similar DP profile was also obtained from partially hydrolyzed substrates derived from enzyme F, a CBH I-rich preparation. In this case, changes in cellulose DP were due primarily to the progressive removal of cellobiose residues from the *termini* of cellulose molecules, probably causing a considerable reduction of the length of the cellulose crystallite (37,39,40).

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References

1. Coughlan, M. P. *Biotechnol. Gen. Eng. Rev.* **1985**, 3, 39-109.
2. Wood, T. In: *Enzyme Systems for Lignocellulose Degradation*; Coughlan, M. P., Ed.; Elsevier: New York, NY, 1989; pp. 19-35.
3. Tomme, P.; Warren, A. J.; Miller, R. C. Jr.; Kilburn, D.; Gilkes, N. In: *Enzymatic Degradation of Insoluble Carbohydrates*; Saddler, J. N., Penner, M. H., Eds.; ACS Symposium Series 618; American Chemical Society: Washington, DC, 1995; pp. 142-163.
4. Saloheimo, M.; Nakari-Setälä, T.; Tenkanen, M.; Penttilä, M. *Eur. J. Biochem.* **1997**, 249, 584-591.
5. Saloheimo, A.; Henriessat, B.; Hoffrén, A.-M.; Teleman, O.; Penttilä, M. *Molecular Microbiology* **1994**, 13, 219-228.
6. Fägerstam, L.G.; Pettersson, L.G. *FEBS Lett.* **1980**, 119, 97-100.
7. Nidetzki, B.; Steiner, W.; Clayessens, M. In: *Enzymatic Degradation of Insoluble Carbohydrates*; Saddler, J. N., Penner, M. H., Eds.; ACS

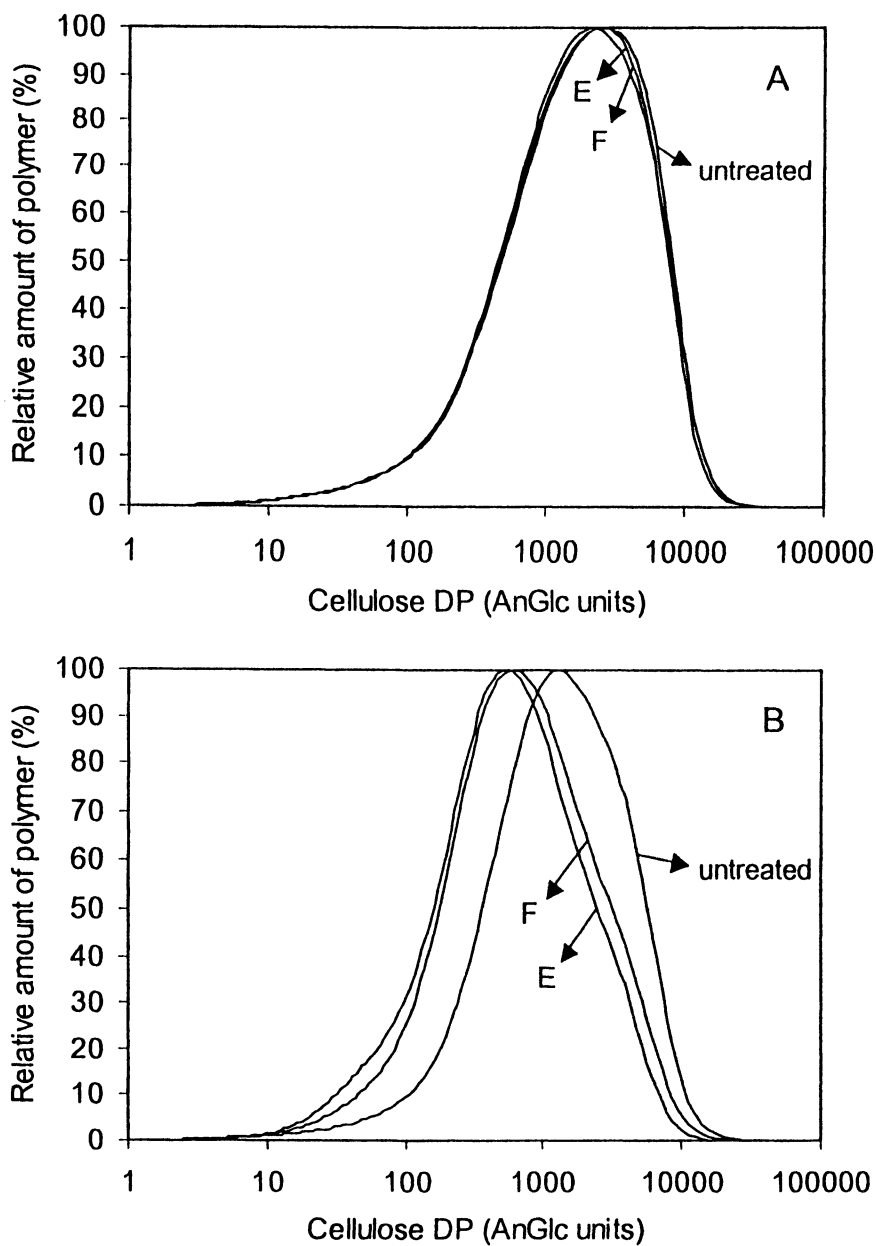


Figure 2. Effect of the enzyme treatment on the degree of polymerization (DP) of cotton (A) fibers and freeze-dried filter paper (B).

- Symposium Series 618; American Chemical Society: Washington, DC, 1995; pp. 90-112.
8. Claeysens, M.; Tomme, P.; Breher, C. F.; Hehre, E. J. *FEBS Lett.* **1990**, *263*, 89-92.
 9. Henrissat, B.; Driguez, H.; Viet, C.; Schulein, M. *Bio/Technology* **1985**, *3*, 722-726.
 10. Henrissat, B.; Teeri, T.; Warren, A. *FEBS Lett.* **1998**, *425*, 352-354.
 11. Claeysens, M.; Aerts G. *Biores. Technol.* **1992**, *39*, 143-146.
 12. Sridosuk, M.; Kleman-Leyer, K.; Keränen, S.; Kirk, T. K.; Teeri, T. *Eur. J. Biochem.* **1998**, *251*, 885-992.
 13. Fan, L. T.; Gharpuray, M. M.; Lee, Y. H. *Cellulose Hydrolysis*; Springer-Verlag: New York, NY, 1987.
 14. Baker, J. O.; Adney, W. S.; Thomas, S. R.; Nieves, R. A.; Chou, Y.-C.; Vinzant, T. B.; Tucker, M. P.; Laymon, R. A.; Himmel, M. E. In: *Enzymatic Degradation of Insoluble Carbohydrates*; Saddler, J. N., Penner, M. H., Eds.; ACS Symposium Series 618; American Chemical Society: Washington, DC, 1995; pp. 113-141.
 15. Thomas, S. R.; Laymon, R. A.; Chou, Y.-C.; Tucker, M. P.; Vinzant, T. B.; Adney, W. S.; Baker, J. O.; Nieves, R. A.; Mielenz, J. R.; Himmel, M. E. In: *Enzymatic Degradation of Insoluble Carbohydrates*; Saddler, J. N., Penner, M. H., Eds.; ACS Symposium Series 618; American Chemical Society: Washington, DC, 1995; pp. 208-236
 16. Klyosov, A. A. *Biochemistry* **1990**, *29*, 10577-10585.
 17. Karhunen, T.; Mäntylä, A.; Nevalainen, H.; Suominen, P. L. *Mol. Gen. Genet.* **1993**, *241*, 515-522.
 18. Suominen, P. L.; Mäntylä, A.; Karhunen, T.; Hakola, S.; Nevalainen, H. *Mol. Gen. Genet.* **1993**, *241*, 523-530.
 19. Ramos, L. P.; Rodrigues, C.; Zandoná F^o. A.; Mooney, C.; Saddler, J. N. In: *Proceedings of the Fifth Brazilian Symposium on the Chemistry of Lignins and Other Wood Component*; Ramos, L. P., Ed.; UFPR: Curitiba, PR, 1997; pp. 413-421.
 20. Cavaco-Paulo, A. In: *Proceedings of the Fifth Brazilian Symposium on the Chemistry of Lignins and Other Wood Component*; Ramos, L. P., Ed.; UFPR: Curitiba, PR, 1997; pp. 404-412.
 21. Cavaco-Paulo, A.; Almeida, L. *Biocatalysis* **1994**, *10*, 353-360.
 22. Buchert, J.; Ranua, M.; Siika-aho, M.; Pere, J.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1994**, *40*, 941-945.
 23. Pere, J.; Siika-aho, M.; Buchert, J.; Viikari, L. *Tappi J.* **1995**, *6*, 71-78.
 24. Pommier, J.-C.; Fuentes, J.-L.; Goma, G. *Tappi J.* **1989**, *72*, 187-191.
 25. Pommier, J.-C.; Goma, G.; Fuentes, J.-L.; Rousset, C.; Jokinen, O. *Tappi J.* **1990**, *73*, 197-202.
 26. Mansfield, S.; Wong, K. K. Y.; Jong, E.; Saddler, J. N. *Tappi J.* **1996**, *79*, 125-132.
 27. Ghose, T.K. *Pure and Appl. Chem.* **1986**, *59*, 257-268.
 28. Leighton, T. J.; Doi, R. H.; Warren, R. A. J.; Kelln, R. A. *J. Mol. Biol.* **1973**, *76*, 103-122.

29. Ramos, L. P. In: *Proceedings of the Seventh Brazilian Symposium on the Chemistry of Lignins and Other Wood Components - Poster Presentations*; UFV: Belo Horizonte, MG, 2001; pp.17-24.
30. Schroeder, L. R.; Haigh, F. C. *Tappi* **1979**, *62*, 103-105.
31. Miller, D.; Senior, D.; Sutcliffe, R. *J. Wood Chem. Technol.* **1991**, *11*, 23-32.
32. Wood, B. F.; Conner, A. H.; Hill Jr., C. G. *J. Appl. Polym. Sci.* **1986**, *32*, 3703-3712.
33. Valtasaari, L.; Saarela, K. *Paperi ja Puu* **1975**, *57*, 5-10.
34. Yau, W. W.; Kirkland, J. J.; Bly, D. D. *Modern Size-exclusion Liquid Chromatography*; John Wiley & Sons: New York, NY, 1979. pp. 7-12.
35. Suurnäkki, A.; Tenkanen M.; Siika-aho, M.; Niku-Paavola, M.-L.; Viikari, L.; Buchert, J. *Cellulose* **2000**, *7*, 189-209.
36. van Tilbeurgh H.; Tomme P.; Claeysens M.; Bhikhabhai, R.; Pettersson G. *FEBS Lett.* **1986**, *204*, 223-227.
37. Ramos, L. P.; Nazhad, M. M.; Saddler, J. N. *Enzyme Microb. Technol.* **1993**, *15*, 821-831.
38. Ramos, L. P.; Zandoná F^o, A.; Deschamps, F. C.; Saddler, J. N. *Enzyme Microb. Technol.* **1999**, *24*, 371-380.
39. Betrabet, S. M.; Paralikar, K. M. *Cellulose Chem. Technol.* **1977**, *11*, 615-625.
40. Betrabet, S. M.; Paralikar, K. M. *Cellulose Chem. Technol.* **1978**, *12*, 241-252.

Chapter 18

Amplification of Cellulase Genes and Cellulase Hyperproducers in *Trichoderma*: Minireview

H. Toyama, T. Hotta, N. Yamagishi, and N. Toyama

**Department of Food Science and Technology, Faculty of Horticulture,
Minamikyushu University, Takanabe, Miyazaki 884-0003, Japan**

Nuclear diameter in conidia and mycelia of *Trichoderma reesei* could be enlarged by a mitotic arrester, colchicine. This result means that chromosomes, including cellulase genes can be amplified by such reagent. Using this reaction, we constructed cellulase hyperproducers of this fungus. A haploidizing reagent, Benomyl, was used in order to carry out chromosomal (genetical) recombination. As the primary selection, double layer selection medium including selection substrates, Avicel, wood powder, or absorbent cotton contributed to selecting hyperproducers. As the secondary selection, Avicel liquid medium test could be used. In this report, we demonstrate the nuclear changes by colchicine treatment and the consequent pathway of selection of cellulase hyperproducers in *Trichoderma*.

Trichoderma is known producer of stable cellulase in high yields, and is widely utilized for industrial cellulase production (1, 2). The cellulase from this fungus is also widely used for processing of foods such as baby foods (3). This enzyme also contributes to recycling of cellulosic wastes (4). However, the cellulase productivity of this fungus is inadequate for many purposes and productivity needs to be increased (5). Colchicine is a well-known mitotic arrester and is widely used for plant breeding (6). In the breeding of

Trichoderma, chemical mutation techniques and / or genetic engineering techniques are usually applied (7, 8). Therefore, we attempted to construct an alternative breeding system for this fungus using autopolyploidization and haploidizing techniques.

Autopolyploidization

Colchicine treatment of normal conidia

Dried green mature conidia (mononucleate) of *Trichoderma* were incubated statically in Mandels' medium containing 1.0% (w/v) glucose (Wako), 0.5% (w/v) peptone (Difco), and 0.1% (w/v) colchicine (Wako) (pH 6.0) at 28°C. Nuclear changes were observed by nuclear staining with Giemsa and DAPI solutions. During colchicine treatment, the diameter of the nuclei continued to increase (9). After about 7 days of incubation, multiple smaller nuclei were generated from the single larger nucleus (autopolyploid nucleus) in a conidium. It is suspected that one larger nucleus was collapsed in a narrow conidium followed by the generation of multiple smaller nuclei. As a result, a multinucleate conidium was produced. This phenomenon is called multinucleation. When the colchicine treatment was prolonged, multiple minute nuclei were generated from nuclei in a conidium. We call this micronucleation.

Colchicine treatment of swollen conidia

Dried green mature conidia were incubated in Mandels' medium containing 1.0% glucose and 0.5% peptone (pH 6.0) for 10 h at 28°C using a rotary shaker to prepare swollen conidia (10). These swollen conidia were incubated without shaking in Mandels' medium containing 1.0% glucose, 0.5% peptone, and 0.1% colchicine (pH 6.0) at 28°C. As the inner volume of these swollen conidia was larger than that of the original conidia, larger autopolyploid nuclei could be produced in the swollen conidia. The nuclear changes in a swollen conidium were the same as those in the original conidium during colchicine treatment. Multinucleation and micronucleation also occurred.

Colchicine treatment of the mycelial mat

A mycelial mat (10 mm x 10 mm) of this fungus was incubated without shaking in Mandels' medium containing 1.0% glucose, 0.5% peptone, and 0.1% colchicine at 28°C, and nuclear changes were observed by nuclear staining with Giemsa solution and DAPI solution. Autopolyploidization, multinucleation, and micronucleation were also observed in the mycelia during colchicine treatment. Many nuclei appeared when multinucleation occurred in the mycelia (11).

Therefore, we called this structure a polykaryon. The nuclear diameter of autopolyploid nuclei was not uniform because these nuclei were not synchronized before the experiments.

Multinucleation

Multinucleation occurred when conidia or mycelia containing autopolyploid nuclei were treated with chemical reagents, e.g., higher concentrations of colchicine (2%), coumarin (Wako), or trypan blue (Wako), and when such conidia or mycelia were incubated at higher temperatures (12). Micronucleation also occurred on prolongation of this treatment. However, multinucleation was distinguished from haploidization because it was unknown whether genetic recombination occurred.

Haploidization

Benomyl treatment of swollen conidia in the liquid medium

When the swollen conidia containing autopolyploid nuclei were incubated without shaking in Mandels' medium containing 1.0% glucose, 0.5% peptone, and 0.6 μ g/ml benomyl (Sigma) (pH 6.0), multiple smaller nuclei were generated from the single larger nucleus (autopolyploid nucleus) in a swollen conidium. The diameter of such smaller nuclei decreased when the treatment time was prolonged. White colonies appeared with a high frequency on the medium when such conidia were spread on a potato dextrose agar (PDA) medium containing 0.1% (v/v) Triton X-100 (Wako) followed by incubation at 28°C.

Benomyl treatment of swollen conidia on the solid medium

There were few white colonies when the swollen conidia containing autopolyploid nuclei were incubated on a PDA medium containing 0.1% Triton X-100 and 0.6 μ g / ml benomyl (pH 6.0) at 28°C. Almost all of the colonies generated green conidia.

Benomyl treatment of mycelial mat on the solid medium

Fan-shaped sectors were produced around the colony when a mycelial mat containing autopolyploid nuclei was incubated on the PDA medium containing 0.6 μ g / ml benomyl (pH 6.0) at 28°C (13). Some sectors generated white

conidia. The diameter of nuclei in these sectors was almost the same as that of the original strain.

Successive autopolyploidization and haploidization

When a haploidized mycelial mat (a fan-shaped sector) was incubated in Mandels' medium containing 1.0% glucose, 0.5% peptone, and 0.1% colchicine again at 28°C, autopolyploidization occurred again, and the nuclear diameter also increased. Haploidization could be also carried out on such mycelial mats (14).

Nuclear changes and cellulase productivity

Changes in cellulase productivity of colchicine-treated conidia

Green mature conidia were incubated in 0.001% or 0.01% (w/v) colchicine solution for 24 h at 28°C using a reciprocal shaker (125 strokes / m). These treated conidia were collected and spread on PDA medium containing 0.1% Triton X-100 followed by incubation for 4 days at 28°C. Two colonies that produced a deeper yellow pigment were isolated. One yielded mononucleate conidia and the other binucleate conidia. The colony with mononucleate conidia was named strain A and the binucleate conidia colony was named strain B. The conidia derived from A and B were re-treated with 2.0% colchicine for up to 10 days. Colonies were isolated by plating treated conidia on PDA plates containing 0.1% Triton X-100 followed by incubation for 4 days at 28°C. Two colonies that generated multinucleate conidia were selected from among 26 (strain A) and 32 (strain B) colonies, respectively, by nuclear staining. The colony derived from strain A was named strain C and the other derived from strain B was called strain D. One loopful of conidia was grown in 50 ml of Mandels' medium containing 1.0% (w/v) Avicel and 0.5% (w/v) peptone (pH 5.0) in a 100-ml Erlenmeyer flask for 5 days using a rotary shaker (160 rpm) at 30°C. Mycelia were then removed on a 3G-3 glass filter and the filtrate was used as the source of cellulase. Avicel-hydrolyzing activity, CMC-hydrolyzing activity, and Salicin-hydrolyzing activity were measured using 1.0% Avicel, 1.0% CMC-Na (carboxymethylcellulose sodium-salt) (D.S. 0.7), and 1.0% Salicin (Wako), respectively, suspended in 0.1 M acetate buffer (pH 5.0) as substrates. Enzymes (2ml) and substrates (4ml) were mixed and incubated for 60 min at 40°C. The enzyme reaction was then stopped by addition of 0.1 N HCl. The amount of glucose generated was measured using the Glucose-test Wako (Wako). Enzyme activity was defined as the amount of enzyme that produces 1 μ mol of glucose per min. Avicel hydrolyzing activity, CMC hydrolyzing activity, and Salicin hydrolyzing activity were compared among the conidial variants (strain A, B, C,

and D). The cellulase productivity of the conidial strains A increased more than that of B and the productivity increased in both C and D as shown in Table 1 (15).

Changes in cellulase productivity of colchicine-treated mycelia

We attempted to select the strains with higher Avicel degrading activity from among the conidia of the haploidized colony derived from colchicine-treated mycelial mat using the double-layer selection medium (for primary selection) and Avicel liquid medium (for secondary selection) (16). For the primary selection, 96 ml of the Mandels' medium containing 1.0% Avicel, 0.5% peptone, 1.0% (w/v) substrate, 0.1% Triton X-100, 1.5% agar, and conidia (the bottom layer medium) was added to a deep glass plate (150 mm in diameter and 60 mm in depth) and left at 4°C in order to harden the agar. After hardening agar, 196 ml of Mandels' medium containing 1.0% Avicel, 0.5% peptone, 1.0% substrate, 0.1% Triton X-100, and 1.5% agar (the upper layer medium) was added on the bottom layer medium followed by hardening of the agar at 4°C. Avicel, wood powder (*Fagus crenata*), and absorbent cotton (for medical use) were used as the substrates for selection. Ten loopfuls of the conidia generated on the haploidized colony were incubated in the medium for primary selection for 6 days at 28°C. The colonies which could break thorough the selection layer were used for the secondary selection. Ten colonies were selected on the selection medium containing Avicel, four colonies were selected on the selection medium containing absorbent cotton, and four colonies were selected on the selection medium containing wood powder.

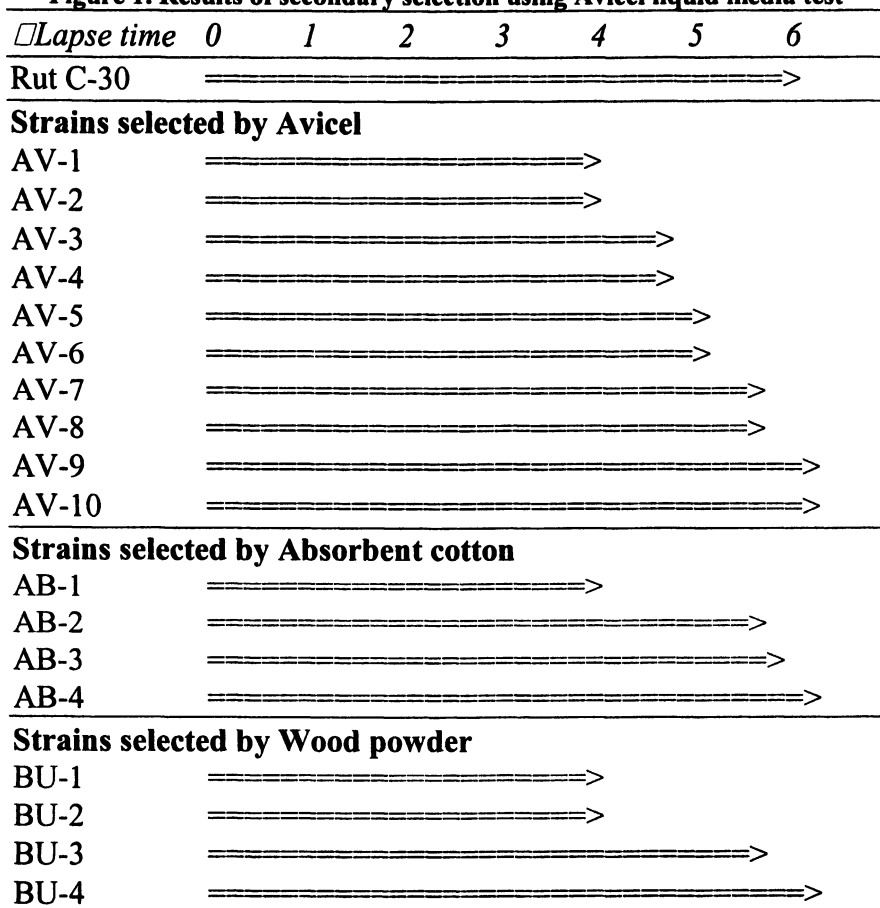
The Mandels' medium containing 1.0% Avicel and 0.5% peptone was used (pH 5.0) as the Avicel liquid medium for the secondary selection. A mycelial mat (2 mm x 2 mm) of the colonies selected by the primary selection was added to the medium for secondary selection and incubated by a rotary shaker (TAITEC NR-30, Koshigaya, Japan) (80 rpm) at room temperature (16 - 23°C). After the Avicel liquid medium became transparent, the amount of Avicel sedimentation was observed by leaving it for 1 h. *T. reesei* Rut C-30 took 6 days to make the Avicel liquid medium transparent whereas strains selected by Avicel, AV-1 and AV-2, took 4 days to make the medium perfectly transparent as shown in Fig. 1. The strain selected by absorbent cotton, AB-1, and those selected by wood powder, BU-1 and BU-2 could also make the medium transparent after 4 days of incubation. All of the selected strains were compared with *T. reesei* Rut C-30 in cellulose hydrolyzing activity by wheat bran culture. A mycelial mat (2 mm x 2 mm) of the selected strains was added to flasks of the wheat bran medium and incubated at 28°C for 6 days and these flasks were shaken once a day. After incubation, 15 ml of 0.1 M acetate buffer (pH 5.0) was added, stirred using a glass rod, and left to stand for 1 h. The enzyme solution was then extracted from the wheat bran culture using a nylon cloth. The extracts were

Table 1. Cellulose hydrolyzing activity in conidial strains

<i>Conidia</i>	<i>Avicel</i> <i>hydrolyzing activity</i> (IU / ml)	<i>CMC</i> <i>hydrolyzing activity</i> (IU / ml)	<i>Salicin</i> <i>hydrolyzing activity</i> (IU / ml)
Original strain	5.9	4.4	2.2
Conidia A	7.4	10.4	10.0
Conidia B	4.4	2.2	9.6
Conidia C	14.6	12.3	11.6
Conidia D	18.9	18.8	12.2

NOTE: One loopful of conidia was added to cellulose medium and the activity was measured after 5 days at 30°C. A was derived from the conidia treated with 0.001% colchicine for 24 h at 28°C (mononucleate). B was derived from the conidia treated with 0.01% colchicine for 24 h at 28°C (binucleate). C was derived from the conidia A after treatment with 2.0% colchicine for 10 days at 28°C (multinucleate). D was derived from the conidia B after treatment with 2.0% colchicine for 10 days at 28°C (multinucleate).

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Figure 1. Results of secondary selection using Avicel liquid media test

A mycelia mat (2mm \times 2 mm) was added to the liquid Mandels' medium containing 1.0% (w/v) Avicel and 0.5% (w/v) peptone (pH 5.0) and incubated for 6 days at room temperature (16-23°C) using a rotary shaker (80 rpm). The degree of transparency of the medium and the amount of Avicel sedimentation were compared everyday. The arrows show the time necessary for making the medium transparent

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centrifuged at $\times 5510$ g, and the top clear portion was used as the enzyme solution. As substrates for the enzyme reactions, 1.0 g of Avicel, CM-cellulose (Wako), or Salicin (Wako) were added to 99 ml of 0.1 M acetate buffer (pH 5.0) followed by 0.2 ml of enzyme solution and 4.0 ml of substrate mixed, and incubated for 60 min at 40°C using a reciprocal shaker (THOMASTAT T-22S, Tokyo, Japan). The agitation speed was 125 strokes / min. The reaction mixture was filtered with filter paper (no. 2, Whatman). The amount of reducing sugar in the reaction mixture was measured using the Glucose CII test Wako (Wako). IU was based on the amount of enzyme-producing reducing sugar equivalent to 1 μ mol of glucose per minute. Although only Avicel-hydrolyzing activity increased in AV-1 selected by Avicel, all activity, Avicel, CMC, and Salicin hydrolyzing activity, increased in AV-2. In the strain AB-1 selected by absorbent cotton, Avicel hydrolyzing activity increased especially as shown in Table 2. In the strains BU-1 and BU-2 selected by wood powder, all Avicel, CMC, and Salicin hydrolyzing activity increased.

Conclusion and consideration for future

This report demonstrates that colchicine is effective even in a cellulolytic fungus, *Trichoderma*. As colchicine has been widely utilized for breeding plants for a long time, this reagent had assumed to be effective only in plants. But, we show here that colchicine is also effective on microorganisms. We have already reported that colchicine can modify nuclei in fungi; *Aspergillus kawachii* (17), *Basydiomycetes*; *Pleurotus ostreatus* (18), *Flammulina velutipes* (19), *Lentinus edodes* (20), and yeast; *Saccharomyces cerevisiae* (21). As colchicine is also active on *Basydiomycetes*, which can digest lignin, further investigation should be also carried out on such organisms. Moreover, one of the merits of this autopolyploidization technique is that many and various genes can be amplified simultaneously. So, this advantage should be also utilized effectively in the production of *Trichoderma* strains for specific applications. From the results described here, we conclude that these autopolyploidization and haploidization techniques are effective on breeding of the fungus, *Trichoderma*.

References

1. Hayward, T.K.; Hamilton, J.; Tholudur, A.; Mcmillan, J.D. *Appl. Biochem. Biotechnol.* **2000**, 84/86, 859.
2. Heikinheimo, L.; Buchert, J.; Miettinen-Oinonen, A.; Suominen, P. *Text Res. J.* **2000**, 70, 969.

Table 2. Cellulose hydrolyzing activity of the strains selected by Avicel, absorbent cotton, and wood powder

<i>Strains</i>	<i>Avicel</i>	<i>CMC</i>	<i>Salicin</i>	<i>(IU / ml)</i>
Rut C-30	35	28	20	
<i>Strains selected by Avicel</i>				
AV-1	75	30	22	
AV-2	49	47	57	
AV-3	47	39	35	
AV-4	37	41	39	
AV-5	32	55	22	
AV-6	30	12	45	
AV-7	30	24	49	
AV-8	28	22	16	
AV-9	24	45	20	
AV-10	16	32	26	
<i>Strains selected by absorbent cotton</i>				
AB-1	53	28	30	
AB-2	39	26	26	
AB-3	36	22	22	

AB-4	34	16	20
<i>Strains selected by wood powder</i>			
BU-1	75	57	34
BU-2	67	61	67
BU-3	40	22	30
BU-4	28	24	23

NOTE: AV lines were selected by Avicel, AB lines were selected by absorbent cotton, and BU lines were selected by wood powder.

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3. Ado, Y. *Biosci. Bioind.* **1989**, 47, 840.
4. Ju, L.-K.; Afolabi, O.A. *Biotechnol. Prog.* **1999**, 15, 91.
5. Sun, T.; Li, Z.; Liu, D. *Biotechnol. Tech.* **1996**, 10, 889.
6. Takayanagi, R.; Kitaura, T.; Mako, M.; Miura, Y. *Breed. Sci.* **1996**, 46, 313.
7. Morikawa, Y.; Kawamori, M.; Shinsha, Y.; Oda, F.; Takasawa, S.; Ado, Y. *Agric. Biol. Chem.* **1985**, 49, 1869.
8. Durand, H.; Clanet, M.; Tiraby, G. *Bioenergy* **1985**, 84, 246.
9. Toyama, H.; Toyama, N. *J. Ferment. Bioeng.* **1990**, 69, 51.
10. Rosen, D.; Edelman, M.; Galun, E.; Danon, D. *J. Gen. Microbiol.* **1974**, 83, 31.
11. Toyama, H.; Toyama, N. *W. J. Microbiol. Biotechnol.* **1995**, 11, 326.
12. Toyama, H.; Toyama, N. The bulletin of the faculty of horticulture, Minamikyushu univ. **1995**; No.25(A), pp 31-36.
13. Toyama, H.; Toyama, N. *Appl. Biochem. Biotech.* **2000**, 84/86, 419.
14. Toyama, H.; Toyama, N. *Appl. Biochem. Biotech.* **2001**, 91/93, 787.
15. Toyama, H.; Toyama, N. *J. Indust. Microbiol.* **1995**, 15, 121.
16. Toyama, H.; Yamagishi, N.; Toyama, N. *Appl. Biochem. Biotech.* **2002**, 98/100, 257.
17. Toyama, H.; Toyama, N. *Microbios* **1997**, 90, 23.
18. Toyama, H.; Toyama, N. *J. Biotechnol.* **1995**, 35, 97.
19. Toyama, H.; Toyama, N. *Microbios* **1995**, 81, 155.
20. Toyama, H.; Toyama, N. *Microbios* **2000**, 101, 73.
21. Toyama, H.; Toyama, N. *Acta Biotechnologica* **1998**, 18, 305.

Chapter 19

A Novel Laccase from the Ascomycete *Melanocarpus albomyces*

Kristiina Kruus¹, Laura-Leena Kiiskinen¹, Markku Saloheimo¹,
Nina Hakulinen², Juha Rouvinen², Arja Paananen¹,
Markus Linder¹, and Liisa Viikari¹

¹VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Espoo, Finland

²Department of Chemistry, University of Joensuu, P.O. Box 111,
FIN-80101 Joensuu, Finland

A novel laccase from the ascomycete *Melanocarpus albomyces* was isolated, purified and characterized. The ultraviolet-visible absorption and electron paramagnetic resonance spectra indicated that the three types of coppers were present. Redox potential of the T1 copper of *M. albomyces* laccase was determined to be 0.46 ± 0.01 V. The enzyme had very interesting pH and temperature behaviour. It had a pH optimum measured with guaiacol at neutral and slightly alkaline pH and substantial activity still at pH 8. The laccase showed very good thermostability, retaining full activity for two hours at 60°C. The gene encoding the *M. albomyces* laccase was isolated and sequenced. The length of the open reading frame of the *M. albomyces* laccase was 623 amino acid residues. The copper binding residues were well conserved and the amino acid sequence had high homology to other ascomycete laccases. Interestingly the secreted laccase was processed both from the amino and carboxy terminus. The laccase was also crystallized with all four coppers present.

Introduction

Laccases (EC 1.10.3.2) were first discovered from the extract of the Japanese laquer tree *Rhus vernicifera* as early as in 1883. Laccase or laccase-like activity has been demonstrated by other higher plants, including apricots, mango, rosemary, mung beans, poplar, maple, and tobacco. Some insects and a few bacteria have also been reported to produce laccase activity. However, the best known laccases are of fungal origin, especially those belonging to the class of white-rot fungi (1).

Laccases are multi-copper containing enzymes, which catalyze the oxidation of a variety of organic and inorganic substrates coupled to the reduction of molecular oxygen to water with the one-electron oxidation mechanism. Laccases have a surprisingly wide substrate specificity range catalyzing a variety of aromatic, especially phenolic, as well as inorganic substrates (1). Laccases of plant origin are reported to have an important role in wound response and lignin synthesis (2) whereas in fungi they are involved in lignin degradation, as well as in several other functions including pigmentation, fruiting body formation, sporulation, and pathogenesis (1, 3).

Due to their catalytic properties laccases have gained considerable interest in various industrial areas. The most intensively studied applications include pulp delignification, textile dye bleaching, effluent detoxification, detergent components, as well as biopolymer modification (4). Laccases are currently used in large scale in finishing of denim fabrics. It is essential for many applications to obtain an enzyme, which works at a relatively high pH-range and elevated temperatures. Therefore we screened for laccase activity from our culture collection of thermophilic fungi. An interesting laccase produced by a thermophilic ascomycete *Melanocarpus albomyces* was discovered. *M. albomyces* has previously been reported to produce xylanases and cellulases with pronounced thermal stability and activity at alkaline pH range (5, 6).

Material and Methods

Fungal Strains and Enzymes

Melanocarpus albomyces (VTT D-96490) was maintained on oatmeal agar (Difco Laboratories, Detroit, Mich.). The strains was originally isolated from a soil sample in Saudi Arabia

Trametes hirsuta (VTT D-443) laccase was produced and purified as reported in Rittstieg *et al.* (7).

Media and Culture Conditions

M. albomyces was cultivated on liquid medium containing (per liter) 25 g glucose, 27.5 g yeast extract, 0.5 mg Indulin AT (Sigma, St. Louis, Mo.) and 0.04 liter mineral stock solution containing (per liter) 1.0 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.0 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.16 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 1.0 g Na_2EDTA . First, 0.1 liter of medium was inoculated with agar pieces cut off from well grown mycelium on oatmeal agar. After two days cultivation at 37 °C (160 rpm), the culture was homogenized and used to inoculate one liter of culture medium, which was again cultivated at 37 °C (160 rpm) for two days and then used to inoculate 15 liters culture medium in lab-scale fermenter (Chemap LF 20SG, Switzerland). Fermentation was carried out at 37 °C with agitation speed of 400 rpm and aeration 0.5 vvm. The lower pH limit was 5.0 (adjusted with NaOH); no upper pH control was used. Laccase activity in the culture filtrate was measured daily and the enzyme was collected when laccase activity reached its maximum. The mycelium was removed by filtration (Whatman no 1).

Protein and Enzyme Activity Determination

Laccase activity was measured according to Niku-Paavola *et al.* (8) using ABTS (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate), Boehringer Mannheim; Mannheim, Germany) as a substrate. The laccase activity was also measured with syringaldazine (Sigma) according to Leonowicz and Grzywnowicz (9), guaiacol (Sigma) according to Paszczynski *et al.* (10), and 2,6-dimethoxyphenol (Sigma) according to Wariishi *et al.* (11). All these activity assays were carried out in 25 mM succinate buffer (pH 4.5) at 25 °C using a double-beam spectrophotometer (Lambda 20, Perkin-Elmer, Überlingen, Germany). Activities were expressed as nanokatals. The protein concentration was determined using the BioRad DC Protein Assay Kit (BioRad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard.

Protein purification

The clear culture supernatant was concentrated and the buffer changed to 10 mM acetate buffer, pH 5, with Amicon 8400 ultrafiltration unit using a PM30 membrane (Millipore Corp., Bedford, Mass.). The subsequent purification steps: anion exchange chromatography (DEAE Sepharose), hydrophobic interaction chromatography (Phenyl Sepharose), and finally gel filtration (Sephacryl S-100 HR) were carried out as described in Kiiskinen *et al.* (12).

SDS-PAGE (12 % Tris-HCl Ready Gel, Bio-Rad) was performed according to Laemmli (13). Protein bands were visualized by staining with Coomassie Brilliant Blue (R 350; Pharmacia) and compared with molecular weight markers (Prestained Protein Marker Broad Range #7708S; New England BioLabs, Beverly, Mass.).

Determination of isoelectric point

The isoelectric point of *M. albomyces* laccase was determined by isoelectric focusing within the pH range of 3.5 – 9.5 (Ampholine™ PAGplate, Pharmacia, Uppsala, Sweden) on a LKB 2117 Multiphor II Electrophoresis System (LKB Pharmacia, Bromma, Sweden) according to the manufacturer's instructions. Laccase active bands were visualized by staining the gel with 2 mM ABTS in 25 mM succinate buffer (pH 4.5).

Enzyme activity and stability with respect to pH and temperature

The pH optimum for purified *M. albomyces* laccase was determined in McIlvaine buffer within a pH range of 2.2–8.0 using guaiacol as substrate. The temperature stability was determined by incubating the enzyme solution (200 nkat/ml) at 60 mM citrate buffer (pH 6) at different temperatures and following the residual enzyme activity.

Spectra

The optical absorption spectrum of purified *M. albomyces* laccase was measured with a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan). EPR spectra were recorded with a Bruker ESP 300 X-band spectrometer (Bruker, Karlsruhe, Germany) at 9.44 GHz and 12 K (cryostat from Oxford Instruments, Oxford, UK). Modulation frequency was 100 kHz, modulation amplitude 0.99 mT, sweep time/scan 168 s, sweep width 0.10 T, microwave power 1.00 mW, and protein concentration 50 μM.

Redox potential of the T1 copper

The redox potential of the T1 type copper of the laccase was determined according to Xu *et al.* (14) in anaerobic titration using $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$

($E_0 = 0.433$ V) redox couple in 100 mM Tris buffer, pH 7. The spectra were recorded by using a Cary100 spectrophotometer (Varian, Inc., Australia).

Isolation of the *M. albomyces* laccase gene

Genomic DNA was isolated from *M. albomyces* according to Raeder and Broda (15). The DNA was digested partially with Sau3A, separated in 15-30% sucrose gradient (22 000 RPM, 20 h) and used in construction of a cosmid library into the Supercos I (Stratagene) vector. The library was screened by colony hybridisation with the *Podospora anserina* laccase gene *lcc2* as a probe in a hybridisation mix without formamide (16) at 57⁰ C. The filters were washed in 2xSSC, 0.1% SDS, 57⁰ C. The laccase gene was subcloned as a 4.5 kb EcoRI fragment into the plasmid pBluescript SK- (Stratagene). *M. albomyces* total RNA was isolated with the Trizol reagent kit (Life Sciences). Race PCR of the laccase cDNA was performed with the RLM-RACE kit (Ambion) according to manufacturer's instructions. The RACE products were cloned into the pCR2.1TOPO vector (Invitrogen). DNA sequencing reactions were performed with the Big Dye kit (Applied Biosystems) and run with the ABI Prism 3100 Genetic Analyser (Applied Biosystems). Other nucleic acid manipulations were done with standard methodology (16).

Crystallization of the purified *M. albomyces* laccase

The purified *M. albomyces* laccase was crystallized in hanging drops at 22°C from a solution containing 2 μ l of protein solution (6 mg protein/ml in 100 mM sodium phosphate buffer, pH 7) and 2 μ l of reservoir solution (25% PMME2000, 0.2 M lithium sulphate, and 0.1 M sodium acetate, pH 4.2). The X-ray data was collected by using R-Axis IIC imaging plate aread detector and Ru200HB rotating anode (Rigaku, Japan) equipped with confocal optics (Osmic, USA).

Results and Discussion

Production and purification of the *M. albomyces* laccase

Laccase production in lab-scale fermentation took place during secondary metabolism at the late fermentation stage. The production started after 96 hrs

fermentation, when the cells had reached the stationary phase. The maximum activity was reached within 200 hrs, after which the protein was collected. It is not known why *M. albomyces* produces a laccase. The fungus has recently been reported to produce several thermostable and alkaline xylanases, as well as cellulases (5, 6, 17). Therefore, it is tempting to speculate that laccase production is involved in degradation of lignocellulose material. Interestingly, the isoelectric focusing PAGE and subsequent active staining with ABTS indicated the presence of only one isoform of laccase in the culture supernatant (Figure 2B), whereas many laccases have been reported to be produced as multiple isoforms.

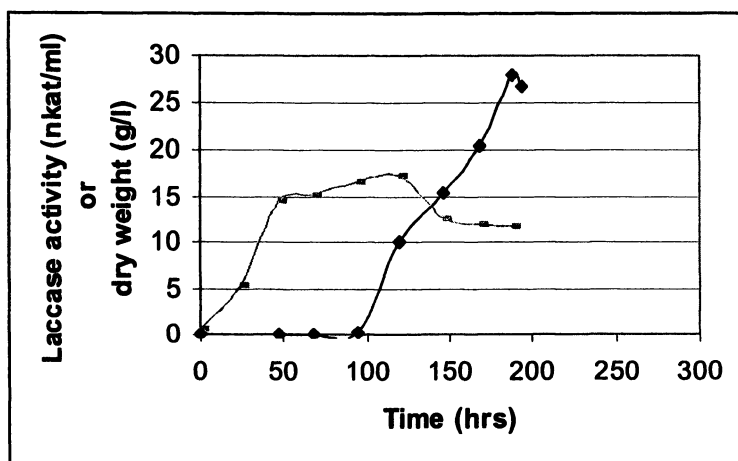


Figure 1. Production of *M. albomyces* laccase a in the 15 liter lab-scale fermenter. Laccase activity (◆) and cell mass (—).

Laccase was purified to electrophoretic homogeneity as shown in Figure 2A. It had a molecular weight of about 80 kDa, as determined on SDS-PAGE, and the pI was 4.0. The purified laccase had a pronounced blue color typical for laccases, which is caused by the coordination of the type 1 copper atom. This copper is coordinated by two histidine nitrogens and a cysteine sulfur with a highly covalent Cu-S bond giving rise to the typical blue color (18).

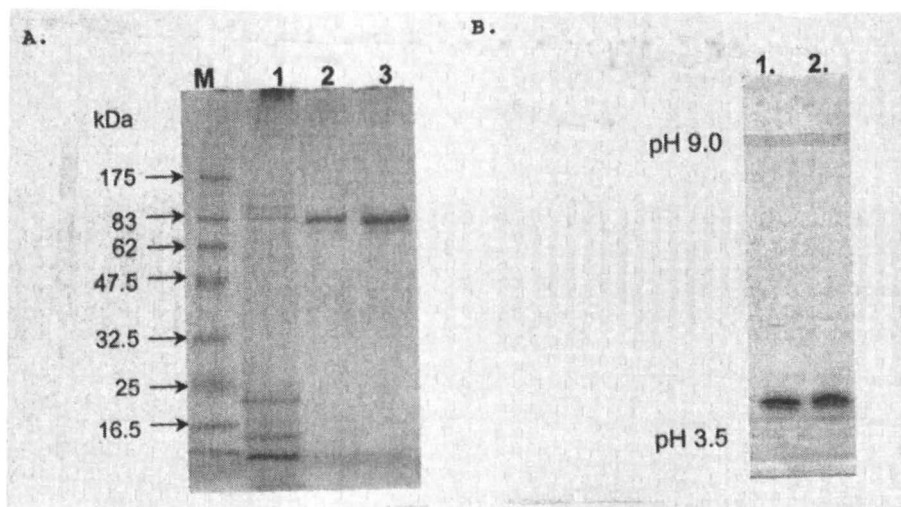


Figure 2. SDS-PAGE showing the purification of the *M. albomyces* laccase (A). Lanes: M Molecular weight marker, 1. culture supernatant (120 μg protein) 2. and 3. purified laccase (2 and 4 μg protein, respectively). IEF/zymogram (B) from the culture supernatant (lane 1) and from the purified laccase (lane 2).

Characterization of the purified laccase

The UV-visible and EPR spectra from the purified *M. albomyces* laccase were determined. According to the spectral data, all three types of coppers are present in the laccase. The ultraviolet-visible absorption spectrum of the laccase showed two peaks at 280 and 600 nm and a shoulder at 330 nm as shown in Figure 3A. The peak at 600 nm is typical for the type 1 Cu (T1), and the shoulder at 330 nm suggests the presence of the type 3 binuclear Cu pair (T3). The EPR spectrum (Figure 3B) is characteristic of fungal laccases and it reveals the presence of type 2 (T2, hyperfine line above 2700 G) and type 1 (region 2900-3200 G) Cu ions. The estimated parameters (g_{II} and A_{II}) are 2.18 and 0.010 cm^{-1} for the narrowly spaced signal and 2.25 and $\approx 0.019 \text{ cm}^{-1}$ for the widely spaced signal.

The redox potential of the T1 type copper of the *M. albomyces* laccase was determined according to Xu *et al.* (14) using the ferri- and ferrocyanide redox pair. Figure 4 A shows the anaerobic titration of *M. albomyces* laccase, where the enzyme is gradually reduced *i.e.* absorbance decreased by addition of ferrocyanide. The absorbance values were plotted as a function of redox couple concentrations (Figure 4). The results show a slope close to unity, indicating that the T1 copper redox reaction is a one-electron transfer process. The line applies

Nernst equation, and the redox potential of the enzyme can be calculated using the intersection of the y axis from the graph. The redox potential of the *M. albomyces* laccase was 0.46 ± 0.01 V. The enzyme belongs thus to the medium redox potential class of laccases, as classified by Eggert *et al.* (19). The highest redox potentials typically 0.7-0.8 V are reported to fungal laccases whereas plant origin laccases have lower redoxes.

The purified *M. albomyces* laccase was able to oxidize typical substrates for laccases: a variety of phenolic compounds and non-phenolic ABTS. The highest activity was observed towards ABTS (840 nkat/mg) followed by syringaldazine (380 nkat/mg), 2,6-dimethoxyphenol (290 nkat/mg), and guaiacol (90 nkat/mg). However, tyrosine was clearly not a substrate for this laccase, thus it does not belong to the group of tyrosinase type of polyphenol oxidases (EC 1.10.3.1; EC 1.14.18.1).

The purified *M. albomyces* laccase showed a very interesting pH optimum with quaiacol as substrate (Figure 5). The optimum is surprisingly broad ranging from 5.0 to 7.5. The enzyme has still 80 % of its maximum activity at pH 8.0. Compared to the pH optimum of the well-known laccase from the white-rot fungus *Trametes hirsuta*, the *M. albomyces* laccase is clearly different. It has much wider pH optimum and the highest activity at a neutral and slightly alkaline pH, where the *T. hirsuta* laccase has totally lost its activity. The purified laccase remained stable within the pH range of 4-8 for 22 hours. However, at pH-values lower than 4, the enzyme quickly lost its activity (12).

Besides an interesting pH optimum, the *M. albomyces* laccase showed also good thermostability (12). The activity remained unaltered after prolonged incubation, over 100 h, at 40 °C, whereas it showed a half-life ($t_{1/2}$) of about 50 h, 5 h, and 12 min at 50, 60, and 70 °C, respectively (Figure 6). The combination of thermal stability and optimum activity at neutral pH, is not very common among fungal laccases. Many other laccases that have a pH optimum at a neutral pH range are not as thermostable as *M. albomyces* laccase. For example, laccases from *Coprinus friesii*, *Panaeolus papilionaceus* and *Panaeolus sphinctrinus* have unusually high pH optima (7-8) with dimethoxyphenol, but do not remain active at 60 °C (20).

Similarly, the neutral laccases from *Myceliophthora thermophila* (21), *Chaetomium thermophilum* (22), and *Coprinus cinereus* (23) retain less than 75 % of maximum activity at 60 °C after 1 h. The unique combination of good thermal stability and activity at a neutral to alkaline pH range makes the *M. albomyces* laccase very promising for applications taking place at elevated temperatures and pHs.

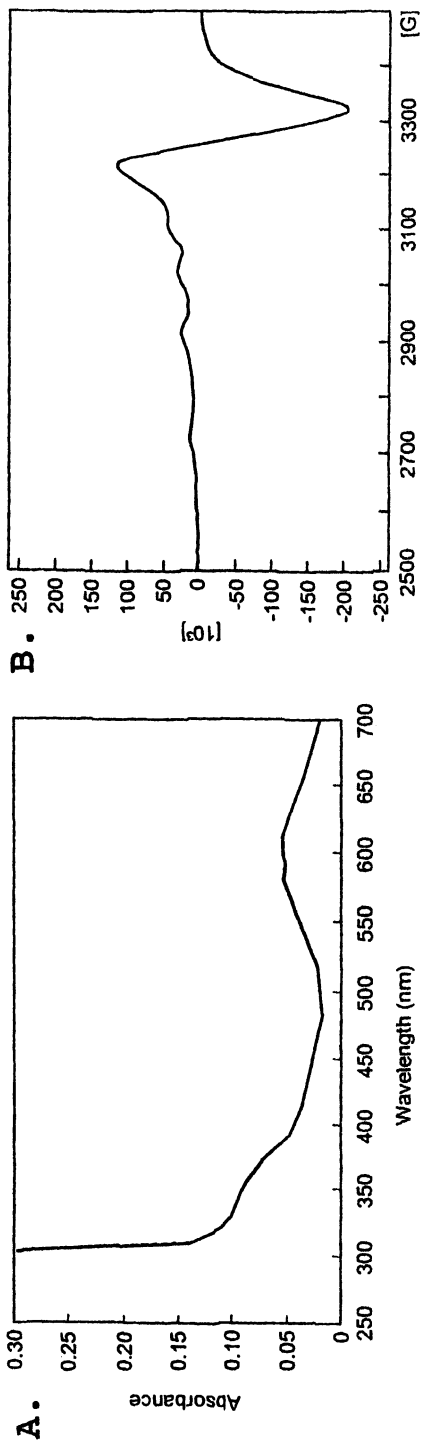


Figure 3. The ultraviolet-visible absorption spectrum of *M. albomyces* laccase (A) and the EPR spectrum of *M. albomyces* laccase (B).

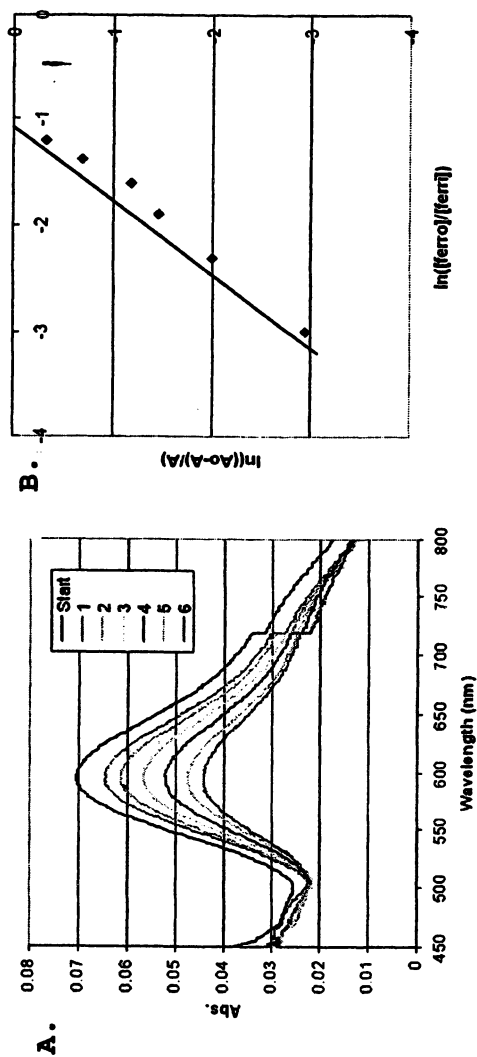


Figure 4. A) Determination of the redox potential of the T1 type copper of the *M. alabomyces* laccase by anaerobic titration. Starting point and number of ferrocyanide additions are shown in the inset. B) The maximum absorbance values at 600 nm plotted as a function of redox couple concentrations.

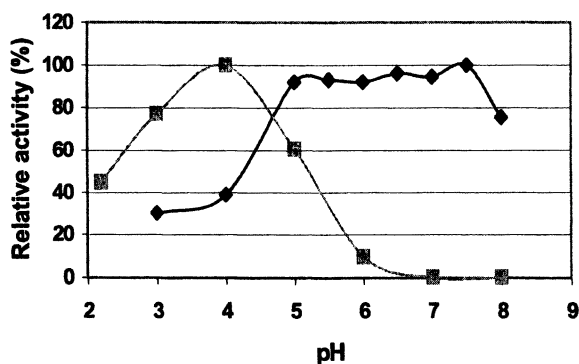


Figure 5. pH optima of *M. albomyces* (♦) and *T. hirsuta* (■) laccases; guaiacol was used as substrate.

Isolation of the laccase gene

The gene encoding the *M. albomyces* laccase was isolated from the *M. albomyces* genomic library using heterologous probes based on the gene fragments of *Podospira anserina lac 2*. The *M. albomyces* laccase gene contained five introns. The length of the open reading frame of the gene is 623 amino acids, which is typical for fungal laccases. The deduced amino acid sequence is shown in Figure 7. The copper binding residues are well conserved. The sequences analysed from three tryptic peptides of the purified *M. albomyces* laccase could be identified from the deduced amino acid sequence, confirming that the right gene was cloned. Direct sequencing of the amino terminus of *M. albomyces* laccase indicated that the first residue of the mature enzyme was glutamic acid. However, the deduced amino acid sequence suggested the presence of a propeptide (28 residues) between the signal sequence and the mature protein. The carboxyl terminal sequencing also revealed C-terminal processing; the last 14 amino acid residues were cleaved at some point. Interestingly, similar C-terminal processing has been reported on other ascomycete laccases, namely *Myceliophthora thermophila*, and *Neurospora crassa* (21). The cleavage site in *M. albomyces* laccase is Asp-Ser-Gly-Leu↓Lys, and the site is conserved. The function of the C-terminal processing is not known.

Comparison of the amino acid sequence of the *M. albomyces* laccase to the other known laccases revealed highest identity to the ascomycete laccases. The highest degree of sequence identity was observed with *Myceliophthora thermophila* (72%), *Podospira anserina* (68%), *Scytalidium thermophilum* (65%), and *Neurospora crassa* (63%). Laccases from basidiomycetes showed more limited sequence identity (24-28 %) to the *M. albomyces* laccase. The comparatively low level of sequence identity between the laccases of ascomycete

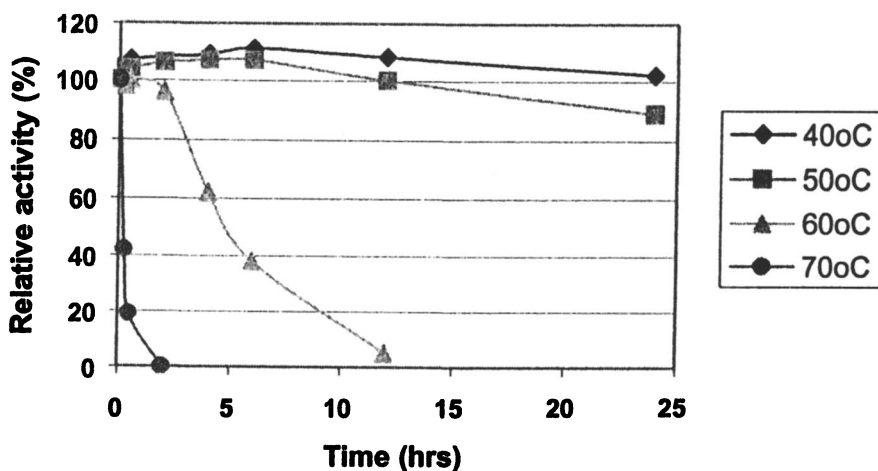


Figure 6. Thermal stability of the purified *M. albomyces* laccase determined at different temperatures.

and basidiomycete origin probably reflects the large phylogenetic distance between these classes. The lowest identity was discovered to *Aspergillus nidulans* conidial laccases and plant-originated laccases.

Crystallization of the laccase

Although extensive research aiming at crystallization of laccases has been carried out, thus far only two crystal structures are available, namely the type 2 copper depleted laccase from *C. cinereus* (24) and a recently published laccase from *Trametes versicolor* in four copper form (25). In addition, the crystallization of laccases from *Trametes versicolor* and *Pycnoporus cinnabarinus* has been reported (26). It has been speculated that one reason for difficulties in crystallizing laccases is related to their high degree of glycosylation (18). Interestingly, high quality laccase crystals were obtained without enzymatic deglycosylation of the *M. albomyces* laccase, even though this laccase is heavily glycosylated, having nine putative N-glycosylation sites.

Initially, crystals of *M. albomyces* laccase grew as thin and disordered plates by the hanging-drop vapour diffusion method. Later, larger single crystals (about 0.6 x 0.2 x <0.05 mm) were obtained by seeding technique, where original crystals were used as seeds. The diffraction data were collected at 2.4 Å resolution.

The structure was solved with the molecular replacement method by using the coordinates of *C. cinereus* laccase (26 % identity). The molecular structure includes all four copper atoms. The structure will be described elsewhere (27).

1-60 AKTFTSALALVVGMLAPGAVVWAPPSTPAQRDLVELREARQEGGKDLRPREPTCNTPSNR
 61-118 ACWSDGEDINIDYEVSTEDIGVTQS ↓ YVFNLTEVDNWMGPPDGVVKEKVMLIN ↓ GNIM GP ↓
 119-179 NIVANWGDTVEVTVINNLVTNGTSL EHWIIG HIHKDINLIIDGANGVTECPIPPKGGQRTYRW
 180-240 KARQYTTSS MYHSH FSAQYGNIGVVTIQINGPASH LYDLDLQVFLIDYYYYKAADDL VHF^T
 241-310 QNNAP^TFSDNVIIN^TAVN^TTE^TGE^TQYANV^TL^TTGCKRHRI^TKILN^TST^TENHF^TQVSI^TVNHT^M
 311-370 TVIAADMVIVNAMTVDSI^TFLAVGQRYDVYIDASRAI^TDN^TYWFNV^TFOUQAACUUSI^TN^TPI^A
 371-440 ALFHYAQA^TCGULL^TDEG^TTP^TVVDHQCLD^TLDYK^TLV^TRSV^TVNS^TFVKR^TDN^TL^TLV^TALDL^TIG
 441-509 TPLFVWKVNGSDINVDWGRPIIDYILLTGNTSYPVSDNIVOVD^TAVDO ↓ WTYWLIENDPEGP
 510-569 FSLP HPMHLH ↓ GHDFLVLGRSPDVPAA^TSQORFVDFDPAVDLARLNGDNPPRRD^TTMLPAGG
 570-630 WLLLAFR^TDNPGAWL^TF HCHIAW IVSGGLSVD^TFLERPADLRORISOEDDED^TDFNRV^TCD^TEWRA
 631-659 YWPTNPYPKIDSGLKRRRWVEESELVLR

Figure 7. Deduced amino acid sequence of the *M. albomyces* laccase. The signal peptide is highlighted in italics and the N-terminal pro-peptide and the cleaved C-terminus of the laccase are in red. The copper binding sites are in boxes and the underlined sequences were confirmed by amino acid sequencing. The green solid arrows indicate the position of introns and the arrow (↓) the C-terminal cleavage site.

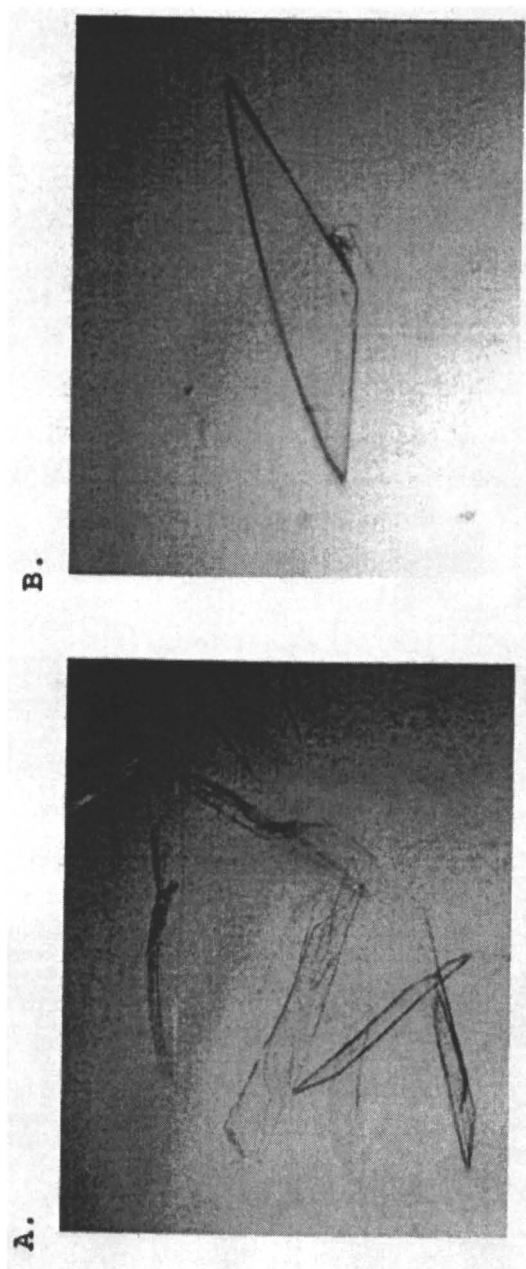


Figure 8. Crystals of *M. albomyces* laccase. A) Original crystals, B) a seeded crystal.

This new ascomycete laccase structure opens up new possibilities to understand and analyze the structures as well as the reaction mechanisms of laccases.

Acknowledgments

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References

1. Thurston C. (1994) The structure and function of fungal laccases. *Microbiology* 140: 19-26.
2. Bao W, Fukushima Y, Jensen KA Jr, Moen MA and Hammel KE. (1994) *FEBS Lett.* (354) 297-300.
3. Leatham, G.F., Stahmann, M.A. (1981) Studies on the laccase of *Lentinus edodes*: specificity, localization, and association with the development of fruiting bodies. *J. Gen. Microbiol.* 125, 147-157.
4. Gianfreda L, Xu F, Bollag J-M (1999) Laccases: a useful group of oxidoreductive enzymes. *Biorem J* 3: 1-25.
5. Jain A, Garg SK, Johri BN (1998) Properties of a thermostable xylanase produced by *Melanocarpus albomyces* IIS-68 in solid state fermentation. *Biores. Technol.* 64: 225-228.
6. Prabhu KA, Maheshwari R (1999) Biochemical properties of xylanases from a thermophilic fungus, *Melanocarpus albomyces*, and their action on plant cell walls. *J. Biosci.* 24: 461-470.
7. Rittstieg, K., Suurnäkki, A., Suortti, T., Kruus, K., Gübitz, G. and Buchert, J. (2002) Investigations on the laccase-catalyzed polymerization of lignin model compounds by means of high performance liquid chromatography, *Enzyme Microbial Technology*, in press.
8. Niku-Paavola M-L, Karhunen E, Salola P, Raunio V (1988) Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochem J* 254: 877-884.
9. Leonowicz A, Grzywnowicz K (1981) Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. *Enzyme Microb. Technol.* 3: 55-58.

10. Paszczynski A, Huynh V-B, Crawford R (1985) Enzymatic activities of an extracellular Mn-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol Lett. 29: 37-41.
11. Wariishi H, Valli K, Gold MH (1992) Oxidation of Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. J. Biol. Chem. 267: 23688-23695.
12. Kiiskinen, L.-L., Viikari, L. & Kruus, K. (2002) Purification and characterization of a novel laccase from the ascomycete *Melanocarpus albomyces*. Appl. Microbiol. Biotechnol. 59: 198-204.
13. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
14. Xu F, Shin W, Brown SH, Wahleitner JA, Sundaram UM, and Solomon EI (1996) A study of a series of fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. Biochim. Biophys. Acta 1292 303-311.
15. Raeder, U. and Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1, 17-20.
16. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA.
17. Vehmaanperä J, Elovainio M, Haakana H, Joutsjoki V, Lantto R, Mäntylä A, Paloheimo M, Suominen P, Londesborough J, Miettinen-Oinonen A (April 1997) Cellulases, the genes encoding them and uses thereof. International patent application WO97/14804.
18. Davies G.J., Ducros, V. (2001) Laccase. In Metalloproteins, Messerschmidt A., Huber, R., Poulos T., Wieghardt, K. (Eds.) John Wiley&Sons, Ltd, New York, pp 1359-1368.
19. Eggert C, LaFayette PR, Temp U, Eriksson K-EL, Dean JFD (1998) Molecular analysis of a laccase gene from the white rot fungus *Pycnoporus cinnabarinus*, Appl. Environ. Microbiol. 64, 1766-1772.
20. Heinzkill M, Bech L, Halkier T, Schneider P, Anke T (1998) Characterization of laccases from wood-rotting fungi (family *Coprinaceae*). Appl. Environ. Microbiol. 64: 1601-1606.
21. Berka R.M., Schneider P., Golightly E.J., Brown S.H., Madden M., Brown K.M., Halkier T., Mondorf K., Xu F. (1997) Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. Appl. Environ. Microbiol. 63: 3151-3157.
22. Chefetz B, Chen Y, Hadar Y (1998) Purification and characterization of laccase from *Chaetomium thermophilum* and its role in humification. Appl. Environ. Microbiol. 64: 3175-3179.
23. Schneider P, Caspersen MB, Mondorf K, Halkier T, Skov LK, Østergaard PR, Brown KM, Brown SH, Xu F (1999) Characterization of a *Coprinus cinereus* laccase, Enzyme Microb. Technol. 25: 502 – 508.

24. Ducros, V., Brzozowski, A.M., Wilson, K.S., Brown, S.H., Ostergaard, P., Schneider, P., Yaver, D.S., Pedersen, A.H. and Davies, G.J. (1998) Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. *Nature Struct. Biol.* **5**, 310-316.
25. Bertrand, T., Jolival, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., Mougin C. (2002) Crystal structure of a four copper laccase complex with arylamine: insight into substrate recognition and correlation with kinetics. *Acta Cryst. D* **58**, 319-321.
26. Antorini, M., Herpoel-Gimbert, I., Choinowski, T., Sigoillot, J-C., Asther, M., Winterhalter, K. and Piontek, K. (2001) *Biochim. Biophys. Acta* **365**: 1-6.
27. Hakulinen N, Kiiskinen L-L, Kruus K, Saloheimo M, Paananen, A., Koivula A and Rouvinen J, Crystal structure of laccase from *Melanocarpus albomyces* in four copper form . *Nature Struct. Biol.* **9**: 601-605.

Chapter 20

Glycoside Hydrolase Gene Cluster of *Acidothermus cellulolyticus*

**Shi-You Ding, William S. Adney, Todd B. Vinzant,
Stephen R. Decker, John O. Baker, Steven R. Thomas,
and Michael E. Himmel***

**Biotechnology Division for Fuels and Chemicals, National Bioenergy
Center, National Renewable Energy Laboratory, 1617 Cole Boulevard,
Golden, CO 80401**

We previously reported a highly thermostable endoglucanase EI from culture filtrates of *Acidothermus cellulolyticus* (U.S. Patent 5,536,655). In this study, additional 13-kb *A. cellulolyticus* genomic DNA downstream of EI was sequenced by using genomic library screening, primer walking and two-step inverse PCR techniques. Sequence analysis has revealed a glycoside hydrolase gene cluster, which contains at least five open reading frames (ORFs), encoding EI (Cel5A), Cel6A-Cel12A, Cel5B, Cel48A and Cel74A. Based on the sequence homology of deduced amino acid sequence, Cel6A-Cel12A contains two catalytic domains, glycoside hydrolase family 6 and 12 (GH6 and GH12), and two Carbohydrate-Binding Modules (CBM) belonging to families 2 and family 3, respectively. Cel5B contains a GH5 catalytic domain at its N-terminal and family 2 and 3 CBM at its C-terminal. Cel48A contains a family 3 CBM, a GH48 and a family 2 CBM domains. Cel74A contains a GH74 and a family 3 CBM. The catalytic domains of all these enzymes have been cloned and expressed in *E. coli*. The characterization of expressed enzymes is ongoing. These novel potential thermostable enzymes found in this study may prove useful in biomass conversion and other industrial applications.

Introduction

More than 50 million acres in the United States are currently available for energy crop production. Alternative domestic biomass resources include agricultural and forestry wastes, municipal solid wastes, industrial wastes, and terrestrial and aquatic crops grown solely for energy production (1). Biofuels are produced from biomass and include ethanol, methanol, biodiesel, and additives for reformulated gasoline. These fuels are desirable because they add little, if any, net carbon dioxide to the atmosphere and because they can greatly reduce ozone formation and carbon monoxide emissions (2). Biomass can be converted to sugars by the action of either acid or enzymes, or both. For the case of enzyme-mediated hydrolysis of cellulose, biomass must first be processed to reduce size and facilitate subsequent handling. Mild acid treatment is then used to hydrolyze part or all of the hemicellulose content of the feedstock. Finally, the liberated sugars are fermented to ethanol by yeasts, bacteria, or other suitable organisms, and the ethanol is recovered by distillation or other separation methods and used as fuel.

Conversion of Plant Biomass to Fuels and Sugars

An alternative to ethanol production from starch is the conversion of cellulose and hemicelluloses from plant fibers. Conversion of cellulose to ethanol through the concerted action of cellulases and saccharolytic fermentation is the most common method of converting plant fiber to ethanol. This process, SSF using the yeast *Saccharomyces cerevisiae*, is incomplete, as it does not utilize the entire sugar content of the plant biomass, namely the hemicellulose fraction.

The cost of producing ethanol from biomass can be divided into three areas of expenditure: pretreatment costs, fermentation costs, and other costs. Pretreatment costs include biomass milling, pretreatment reagents, equipment maintenance, power and water, and waste neutralization and disposal. The fermentation costs include enzymes, nutrient supplements, yeast maintenance and scale-up, and waste disposal. Other costs include biomass purchase, transportation and storage, plant labor, plant utilities, ethanol distillation, and administration (which may include technology-use licenses). One of the major expenses incurred in SSF is the cost of the enzymes (2).

Cellulase Enzymes

Efficient conversion of plant biomass to glucose and other fermentable sugars is an essential step in the production of ethanol or other biofuels from biomass. Cellulases are an important component of this process, where approximately one

kilogram of cellulase can digest fifty kilograms of cellulose. Within this process, thermal tolerant enzymes have taken precedent, due to their ability to function at elevated temperatures and under other conditions including pH extremes, solvent presence, detergent presence, proteolysis, etc. (3).

Glycoside hydrolases are a large and diverse family of enzymes that hydrolyse the glycosidic bond between two carbohydrate moieties or between a carbohydrate and a non-carbohydrate moiety. Glycoside hydrolase enzymes are classified into glycoside hydrolase (GH) families based on significant amino acid similarities within their catalytic domains. Enzymes having related catalytic domains are grouped together within a family (4, 5), where the underlying classification provides a direct relationship between the GH domain amino acid sequence and how a GH domain will fold. This information ultimately provides a common mechanism for how the enzyme will hydrolyse the glycosidic bond within a substrate, *i.e.*, either by a retaining mechanism or inverting mechanism.

The enzymatic degradation of cellulose requires the coordinate action of at least three different types of β -1,4-glucanases. Endo- β -1,4-glucanases (EC 3.2.1.4) cleave the cellulose strand randomly along its length, thus generating new chain ends. Exo- β -1,4-glucanases (EC 3.2.1.91) are processive enzymes and cleave cellobiosyl units (β -1,4-glucose dimers) from free ends of cellulose strands. Most fungal, and some bacterial cellulase systems display two types of exoglucanases: those that cleave from the reducing end and those that cleave from the nonreducing end of the cellulose strand. Lastly, β -D-glucosidases (cellobiases: EC 3.2.1.21) hydrolyze cellobiose to glucose. All three of these general activities are required for efficient and complete hydrolysis of cellulose to glucose.

Cellulases and xylanases are produced by a variety of bacteria and fungi to degrade the β -1,4 glycosidic bonds of cellulose and xylan and to so produce successively smaller fragments of these polysaccharides in the process of dismantling the plant cell wall. At present, cellulases and xylanases are found within at least 11 and 2 different GH families, respectively. Many cellulases and xylanases are characterized by having a multiple domain unit within their overall structure, a GH or catalytic domain is joined to a carbohydrate binding module (CBM) by a glycosylated linker peptide (7). The CBM can provide cellulose specificity to the enzymes, ensuring that the GH domain come in contact with the cellulose, while the linker peptides can provide the GH domain with the flexibility to recognize and act upon a target substrate.

Acidothermus cellulolyticus

Highly thermostable enzymes have been isolated from the cellulolytic thermophile *Acidothermus cellulolyticus* *gen. nov., sp. nov.*, a bacterium originally isolated from decaying wood in an acidic, hot springs at Yellowstone

National Park and deposited with the American Type Culture Collection (ATCC 43068) (8). This microorganism was shown to be a gram variable actinomycete able to grow on plant biomass under a wide range of pHs and temperatures as high as 55°C. We report here the preliminary assignment of 5 new glycoside hydrolases from *A. cellulolyticus* based on peptide sequences deduced from newly discovered genes.

Material and Methods

Organism and Growth Condition

A. cellulolyticus ATCC was grown by described method (8).

Molecular Cloning of Cel5B, Cel6A-Cel12A, Cel48A, and Cel74A

Genomic Library Screening and Primer Walking

Acidothermus cellulolyticus genomic DNA was isolated from *A. cellulolyticus* cultures grown on cellulose and purified by cesium chloride gradients. Genomic DNA was partially digested with Sau3AI and separated on agarose gels. DNA fragments in the range of 9-20 kilobase pairs were isolated from the gels. This purified Sau3AI digested genomic DNA was ligated into the BamHI predigested EMBL3 lambda vector (Clontech, San Diego, Calif.). Phage DNA was packaged according to the manufacturer's specification and plated with *E. coli* LE392 in top agar, which contained the soluble cellulose analog, carboxymethylcellulose (CMC). The plates were incubated overnight (12-24 hours) to allow transfection, bacterial growth, and plaque formation. Plates were stained with Congo Red followed by destaining with 1 M NaCl. Lambda plaques harboring endoglucanase clones showed up as unstained plaques on a red background.

A positive clone (SL-3) containing approximately 14-kb *A. cellulolyticus* genomic DNA was isolated by Congo Red staining method. SL-3 was then digested with *Bam*HI and subcloned into pDR540 (Pharmacia Biotech, St Albans, U.K), after second round Congo Red staining, a plasmid (NREL501) containing 9-kb BamHI fragment was isolated and sequenced by primer walking according to the instruction (<http://dna.biotech.iastate.edu/>, Iowa state University, DNA Sequencing & Synthesis Facility).

Because of the repeat sequences, later known as almost identical CBM sequences in different genes, primer-walking method could not finish the sequencing of entire plasmid. NREL501 was then subcloned into pUC19 using restriction enzymes *Pst*I and *Eco*RI for further sequencing. Each subclone was sequenced from both the forward and reverse directions, and primer walking method also employed for the plasmids which insert DNA fragment are more than 1-kb. Sequences obtained from above approaches were then assembled together to ensure the both strands are well overlap.

Two-step Inverse PCR (TSiPCR)

The downstream of NREL501 sequencing was performed by using the two-step inverse PCR method (9). The plasmid named NREL501 contains a 9-kb *Bam*HI fragment and encodes CMC activity by Congo Red screening. Five μ g *A. cellulolyticus* genomic DNA was digested extensively by *Pst*I, *Eco*RI, and *Bam*HI and self-ligated by T4 DNA ligase at 4°C overnight, respectively. One μ L ligation was used as template for the first PCR, the second PCR was then applied to specify the PCR product by using a nested primer pair and template diluted from the first PCR. The PCR product was then sequenced directly by PCR primer and by primer walking method if necessary. PCR was performed by Peqin-Elmer GeneAmp® PCR System 2400 and the enzyme (*Pfu Turbo*) was obtained from Stratagene.

Results and Discussion

Sequencing Strategy

Approximately 13-kb *A. cellulolyticus* genomic DNA was sequenced (Figure 1). The sequencing strategy comprised three major steps. The first step is direct sequencing by primer walking from both strands of NREL501. Because of the similarity of CBM sequences among different genes, the primer-walking method could not go through the multi-CBM region. All four new enzymes contained CBMs from either families 2 or 3 (one enzyme had multiple CBMs). CBMs from these families were almost identical in sequence (see Figure 3). The second step was subcloning NREL501. The 9-kb *Bam*HI fragment was digested by *Pst*I and *Eco*RI, all *Pst*I and/or *Eco*RI fragments were subcloned into pUC19 and sequenced to ensure no sequencing mistake. The primer walking method was also used to sequence the subclone, which had more than 1-kb insert. The third step was to continue sequencing downstream of 9 kb *Bam*HI fragment by TSiPCR approach. Primers were designed starting from the 3'-terminal

Table 1. Primers Used in This Study

<i>Name</i>	<i>Nucleotide sequence</i>
GUXNT-Seq1	GTCACTCTTATTCACCCGG
GUXCT-Seq1	AGGCTGCGTACTGGGCC
GH12 F1	GTCAGCAGACCTCGTGGAAGA
GH12 R1	AGATTACTGATCGACGTCGCAC
GH12 R2	TGGTCACACCATTCTGGTTAG
GUXA Cat F1	CAGTTTGACCAGCTTGTCGCGAA
GUX1-NT	GATATACATATGGGGTGTGGGAATATCCGCGCC
GUX1-CT	AGAGAGGGATCCTCAAAGAGCGAGTACCGC
GUX1 F1	ACTGGTGACGGCCTCTACAT
GUX1 F2	ACCGACTACTCTCGGTTCA
GUX1 R1	GAAGTTGTCTCGTGCCCGTAGT
E2 F1	GGCTTTGTGTCGACGGGATCGT
E2 F2	TCGTCTTCAATGAAGGTGCA
E2 R1	GAAGCGATCGACGGACCGCA
E2-F3	CTCTGGAGAAGCACAGATT
E2-F4	AATCACCCCGGTACCTTCGA
E2-F5	TCAACTCTGATCGGATGCTCTA
E2-F6	GCTGCTTCGCAAGGTGTTC
Gux1-F3	ACCGTGACCGTGACAACCAC
ManA-R1	TGGTGAGGACGACAATCA
E2-F7	ATGACCAGCACCAATACGGA
E2-R2	CGGCATCCCATCGATACATC
E2-R3	TCTGCCGCAATGCTGACGA
Gux1-F4	CACGTCGTACACGTACACAGT
E2-R4	CGCCGTCTCTGCTCCAGAAGA
E2-R5	CTTGTGCGGGATGAAGCCGGT
GUX1-N	TCCAACGACCCGTACATCCA
GUX1-C	CGACCCGGATGGGAAGAGCAT

Table 2. Calculated Parameters for *A. cellulolyticus* Glycoside Hydrolases

Enzyme Name	MW Mature peptide	# AA	Provisional Activity Assignment	**pI Calculated	*GOR Estimated Percentage Secondary Structure Hh Ee Cc	**Predicted N-linked glycosylation sites	Catalytic Acceptor/ Donor Residue
Cel5A	56477	521	endoglucanase	5.20	13% 27% 61%	3	Glu/Glu
Cel5B	76937	726	mannanase	4.68	11% 27% 63%	6	Glu/Glu
Cel6A	45269	423	endoglucanase	4.40	20% 20% 59%	6	Asp/Asp
Cel12A	37926	368	endoglucanase	4.94	6% 38% 56%	8	Glu/Glu
Cel48A	115435	1087	exoglucanase	4.57	12% 29% 59%	11	unknown
Cel74A	94971	910	xyloglucanase	4.88	1.5% 33% 65%	3	unknown

*GOR secondary structure prediction method version IV (26). Hh = alpha helix, Ee = strand, and Cc = (random) coil.

**PROSITE Release 17.11, of 17-May-2002

Table 3. Signal Peptide Found for *A. cellulolyticus* Glycoside Hydrolases (using SignalSeq*)

Enzyme	Calculated Signal Peptide
Cel5A	MPRALRRVPG SRVMLRVGVV VAVLALVAAL ANLAVPRPAR A
Cel5B	MGLVRRPAPA FVATAAGTAV AAAATLGSIT MPSATA
Cel6A-Cel12A	MERTQOSGRN CRYQRGTTRM PAISKRLRAG VLAGAVSIAA SIVPLAMQHP AIA
Cel48A	MPGLRRRLRA GIVSAAALGS LVSGLVAVAP VAHA
Cel74A	MDRSENIRLT MRSRRRLVSLL AATASFVAAA ALGVLPIAIT ASPAHA

* from Nielsen and coworkers (27).

sequence of *Bam*HI fragment in NREL501, *Eco*RI, *Bam*HI and *Pst*I were used to digest the genomic DNA, the digested DNA was self-ligated separately (Table I). TSiPCR was employed to amplify specific DNA fragment for sequencing. A few rounds TSiPCR resulted another 4-kb DNA sequence downstream of 9-kb *Bam*HI fragment (NREL501).

Sequences obtained from above methods were then assembled together. There is 0.7-kb overlapping between NREL501 and the previously reported 3-kb *A. cellulolyticus* genomic DNA sequence (GeneBank Acc# U33212), which encoding endoglucanase EI. The NCBI Blast program (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to analyze the assembled 13-kb *A. cellulolyticus* genomic DNA segment. Sequence analysis revealed four open reading frames (ORFs). According to the deduced amino acid sequence homology, all are multi-domain putative glycosides hydrolases and are named Cel6A-Cel12A, Cel5B, Cel48A and Cel74A(partial) (Figure 1), containing one or two CBMs and a catalytic domain (Cel6A-Cel12A has two catalytic domains). The catalytic domains belong to family 6, 12, 5, 48 and 74, respectively (CAZY-Carbohydrate-Active enzymes (<http://afmb.cnrs-mrs.fr/CAZY/index.html>)).

Sequence alignments and comparisons of the deduced amino acid sequences of the *A. cellulolyticus* enzymes were conducted using the ClustalW program. An examination of the amino acid sequence alignments of these new enzymes is shown in Figures 2 and 3. In these figures, the notations are as follows: an asterisk "*" indicates identical or conserved residues in all sequences in the alignment; a colon ":" indicates conserved substitutions; a period "." indicates semi-conserved substitutions; and a hyphen "-" indicates a gap in the sequence.

A. cellulolyticus Glycoside Hydrolases

The cellulolytic, thermophilic bacterium *A. cellulolyticus* was first isolated from biomass containing water and mud samples collected in Yellowstone National Park hot springs in 1985 (8). Studies of *A. cellulolyticus* culture filtrates have shown that it produces thermal stable endoglucanases (10-12) and beta-glucosidases (13), and probably xylanases (8). Table 2 shows the calculated molecular weights and pIs of the mature peptides, secondary structure estimation, and predicted number of N-linked glycans for the 6 glycosyl hydrolases discovered from *A. cellulolyticus* todate. Note that the number of N-linked glycan sites are only rough predictions because the occurrence of these

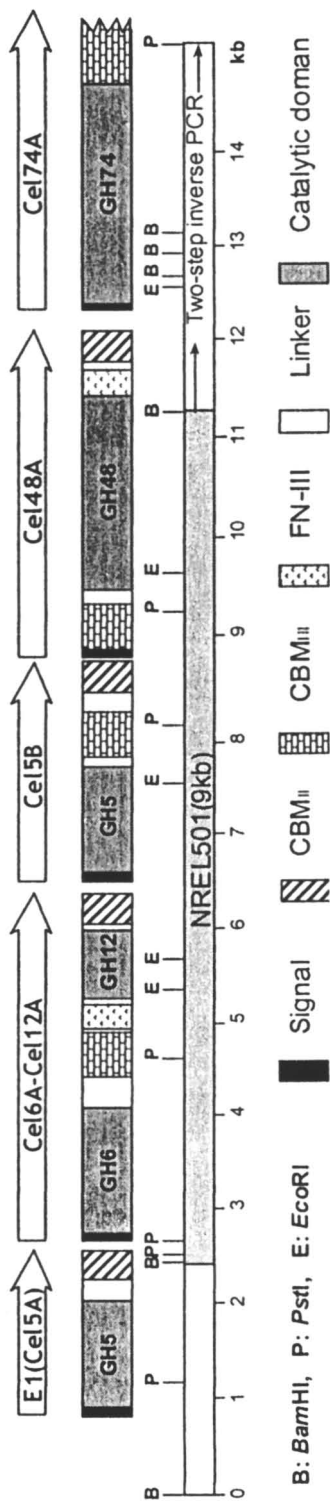


Figure 1. DNA sequencing and gene structure of the *A. cellulolyticus* glycoside hydrolase gene cluster.

Plasmid (NREL501) contains 9-kb *A. cellulolyticus* genomic DNA was sequenced by primer walking and subcloning. Restriction enzymes *Pst*I, *Eco*RI and *Bam*HI were used to digest NREL501, fragments were subcloned into pUC19 and sequenced. The downstream sequence of NREL501 was obtained by two-step inverse PCR (9) and sequenced by the primer walking method (Table 1). CBM^{II}: Family 2 Carbohydrate-Binding Module. CBM^{III}: Family 3 Carbohydrate-Binding Module. FN-III: Family III fibronectin-like sequence.

motifs on the surface of these proteins has not been confirmed. Table 3 shows the calculated signal sequence and protease site for each enzyme. Figure 2 shows the alignments of new *A. cellulolyticus* enzymes (catalytic domain only) and their most similar enzyme sequences published in GeneBank, respectively. Based on the sequence similarity between the putative *A. cellulolyticus* enzymes and the enzymes which have experimental data confirmed activity, we have identified five novel glycoside hydrolase sequences deduced from the nucleotide sequence. Figure 4 illustrates the detailed secondary structure prediction based on GOR for the 6 new *A. cellulolyticus* enzymes. Note the considerable dissimilarity between even the two family 5 enzymes.

Endoglucanase EI (Cel5A)

The first cellulase enzyme isolated from *A. cellulolyticus*, the endoglucanase EI, was shown to display maximal activity on carboxymethylcellulose at 83°C (14). This novel cellulase enzyme and the gene coding it are described in detail in U.S. Patents #5,275,944 and #5,536,655, respectively. The *A. cellulolyticus* EI endoglucanase is one of the most active cellulases known. In combination with the exocellulase CBH I from *Trichoderma reesei*, EI gives the highest saccharification and degree of synergism of all cellulases tested by Baker and coworkers (15). Endoglucanase EI (Cel5A) was previously isolated, sequenced, and characterized kinetically (15). In 1996, the x-ray crystallographic structure of the EI catalytic domain was reported by Sakon and coworkers (16). EI is an imperfect α/β barrel protein with an unusually planar substrate-interactive surface and an extended cellodextrin binding platform comprising the -2 to -6 glycosyl subsites.

We demonstrated that active EI catalytic domain could be expressed in *E. coli* using a variety of expression vectors and this host is currently used for routine protein engineering work. Full length EI is best expressed from *Streptomyces lividans* TK24, presumably because this host produces fewer serine proteases. We have also tested a strain of *Pichia pastoris* designed for expression and secretion of the *A. cellulolyticus* EI endoglucanase (data not shown). In this construction, the mature EI coding sequence was joined in the same translational reading frame to the yeast alpha factor signal sequence present in pPIC9. Transformants have not yet been analyzed for gene copy number, but unoptimized fermentations have already yielded 1.5 g/L of EI. EI has also been expressed in an active and thermal stable enzyme from a variety of plants (17).

Figure 2. Alignments of selected glycoside hydrolase enzymes with deduced amino acid sequences obtained from this study using ClustalW.

2A. Alignment of Selected Glycoside Hydrolase Family 5 Sequences

Cel5B_Ace: *A. cellulolyticus* Cel5B catalytic domain (AA 37-411)E1_Ace: *A. cellulolyticus* E1 catalytic domain (AA 42-400, GeneBank Acc. # U33212)Man_Tre: *T. reesei* beta-mannanase catalytic domain (AA 20-372, GeneBank Acc. # 11514387)Eng_Bpo: *Bacillus polymyxa* endo-beta-1,4-gucanase catalytic domain (AA 33-397, GeneBank Acc. # M33791)Catalytic Nucleophile/Base: Glu, Catalytic Proton Donor: Glu (shown in bold)

```

E1_Ace      -----AGGGYHTSGREILDANNVVR IAGIN - -WFGFETCNVYVHGLWSRDRYSMLDQ 52
Eng_Bpo     -----ASVKGYHTQGNKI VDESGKEAAFNGLN - -WFGLETPNYTLHGLWSRSMDDMLDQ 53
Cel5B_Ace   -----APAGFVTASGGQFVNLNGLPYRYGGTNNYLSYQS -HADVDDVLAKAQAMNLSV 52
Man_Tre     AVLQPVRASSFVTISGTQFNIDGKVGYFAGTNCYWCSEFLTNHADVDSTFHSISSGLKV 60
            . : * . . . . * * : . : : : : : : : : : * .

E1_Ace      IKSLGYNITRLPYSDDI LKPGTMPNSINFYQMNQDLQG -----LTSLQVMDKIVAYAGQ 106
Eng_Bpo     VKKEGYNLIRLPYSNQLFDSRRPDSIDYHK -NPDLVG-----LNPIQIMDKLIEKAGQ 106
Cel5B_Ace   IRTWGFIDIG-SLDGSVPTIDGNKNGFYFQYWDPTGAPAYNDGPTGLQGLDYAIASAAA 111
Man_Tre     VRVWGFNDVN -TQP-----SPGQIWFQKLS -ATGS -TINTGADGLQTLTDYVVVQSAEQ 109
            : : * : . . . . : : : : . . . . : * : * : *

E1_Ace      IGLRI ILDRHR--PDCSGQ-----SALWYTSVSEATWISDLQALAQR----- 147
Eng_Bpo     RGIQI ILDRHR--PGSGGQ-----SELWYTSQYPESRWISDWKMLADR----- 147
Cel5B_Ace   HGLRVI VVLTNDWKEFGGMDQDKWYGLPYHDNFYTDPTQQAYKNWVNHLLNRVNSITG 171
Man_Tre     HNLKLI I PFVNNWSDYGGINAYVNAFGG-NATTWYTNATAQTQYRKYVQAVVSR----- 162
            . : : * : . . . . * . . . . : * : . : : : : : : : : :

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E1_Ace      --YKGNPTVVGFDLHNEPHDPACWG-----CGDPSIDWRLAAERAGNAVLSVNPILLI 198
Eng_Bpo     --YKNNPTVIGADLHNEPHGQASWG-----TGNASTDWRLLAAORAGNAVLSVNPWLI 198
Cel15B_Ace VTYKNDPTIFAWELANERPCVSGTLPSTSGTCTQATIVNW--VDQMSAYVKSIDPNHMV 228
Man_Tre     --YANSTAI FAWELGNEPRCNG-----CSTDVIVQW---ATSVSQYVVKSLDSNHLV 208
:* . . . . . : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
E1_Ace      FVEGVQS---YNGDSYWWGGNLQAGQYVVVLNVPNRNLVYSADHYAT-SVYPQTFWSDPT 254
Eng_Bpo     LVEGVVDHNVQGNNSQYWWGGNLTGVANYPVVLDVPNRVVYSPHDYGP-GVSSQPWFNDPA 257
Cel15B_Ace SVGDEGFYIGSTQSGWPYNDPSDGVDDNALLRVKN-----IDFGTYHLYPNYWGQAD 282
Man_Tre     TLGDEGLGLSTGDG-AYPYTYG-EGTDFAKNVQIKS-----LDFGTFHLYPDSWGTNYT 260
:* . . . . . : : : : : : : : : : * : * : * : * : * : * : * :
E1_Ace      FPNMMPGIWNKNWGYLFNQNIAPVWLGEFG--TTLQSTTDQTLWKLTVQYLRPTAQYGAD 312
Eng_Bpo     FPSNLPAIWDQITWGYISKQNIAPVLVGEFGRNVDLSCPEGKQNALVHYI-----GAN 311
Cel15B_Ace WGT---QWIKDHIANAAAIGKPTILEEFGWQTPDRDSVYQITWTQVTRTNG----- 329
Man_Tre     WGN---GWIQTHAAACLAAGKPCVFEFYGAQQNPCTNEAPWQTTSLTTRG----- 307
:* . . . . . * : * : * : * : * : * : * :
E1_Ace      SFQWTFWSWNPDSGDTGGILKDDWQTVDTVKDGYLAPIKSSIFDPVG----- 359
Eng_Bpo     NLYFTYWSLNPNSGDTGGLLLDDWTTWNRPKQDMLGRIMKPVVSVAAQAEAAAE 365
Cel15B_Ace EAGNFWMLAGNVNGQYPNYDGFNVYFSSST--ATVLAASEALAI STG----- 375
Man_Tre     MGGDMFWQWGDITFANGAQNSNDPYTVWYNSSN--WQCLVKXHVDAING----- 353
:* . . . . . * : * : * : * : * : * : * :

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2B. Alignment of Selected Glycoside Hydrolase Family 6 Sequences.

GH6_Ace: *A. cellulolyticus* Cel6A-Cel12A catalytic domain GH6 (AA 54-477), CBHA_Cfi: *Cellulomonas fimi* cellobiohydrolase A catalytic domain (AA 41-471, GeneBank Acc. # L25809), E3_Tfu: *Thermomonospora fusca* cellobiohydrolase E3 catalytic domain (AA 176-596, GeneBank Acc. # U18978) Catalytic Nucleophile/Base: Asp, Catalytic Proton Donor: Asp (shown in bold)

GH6_Ace	-ATHVDN	NPYAGATFF	VPYWAQ	EVQSEAA	NQTN -	ATLAAK	MRVV	STYSTAV	WMDRIA	AIN	58
CBHA_Cfi	APVHVD	NPYAGAV	QYVNP	TWAASV	NAAAG	RQSDP	PALAAK	MRTVAG	QPTAV	WMDRISA	IT 60
E3_Tfu	-GEKVD	NPFEGAK	LYVNP	VWSAKAA	AEFG-----	GS	AVANES	TAVW	LDRI	GAIE	48
	:	*****	**	***	*
	:	*****	**	***	*
	:	*****	**	***	*
GH6_Ace	GVN---	GGPLT	TYLDA	ALSQQ	QGT-	TPE	VEI	EIV	YDLP	GRDCA	ALAS
CBHA_Cfi	GNA---	DGNGL	KFLHD	NAVAQ	KAA	GVPL	VFN	LV	YDLP	GRDC	FALAS
E3_Tfu	GNDSP	TTGSM	GLRDH	LEEA	VRQ	SGD-	-	PLT	IQV	VI	NLPGR
	*	..	**	**	*	*	*	*	*	*	*
	*	..	**	**	*	*	*	*	*	*	*
	*	..	**	**	*	*	*	*	*	*	*
GH6_Ace	QTYET	QYID	PIAS	ILSN -	PKYSS	LRI	VTI	IEP	DSL	PNV	NMSI
CBHA_Cfi	ARYKSE	YIDP	IA	DLDN -	PEYES	IRI	AAT	IEP	DSL	PNL	TNIS
E3_Tfu	DRYKSE	YIDP	IA	IMW	FADY	ENLR	I	V	AI	IEI	DSL
	*
	*
	*

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GH6_Ace
CBHA_Cfi
E3_Tfu
--GIEYALTKLHAI PNVIYIYMDAAHSGWLGWPNNASGYVQEVQKVLN-ASIGVNGIDGFV 225
--GVKYALDKLHAI PNVIYIYIDIGHSGWLGWDSNAGPSATLFAEVAKSTTAGFASIDGFV 229
VNGVGYALRKLGEI PNVIYIYIDAHHGWIGWDSNFGPSVDIFYEAAANASGSTVDYVHGFI 224
* : *** ** ***** * : * * * * * . . . . . : : * * * * *

GH6_Ace
CBHA_Cfi
E3_Tfu
TNTANYTPLKEPFMT-ATQQVGGQPVESANFYQWNPDIIDEADYAVDLYSRLVLAAGFPSSI 284
SDVANTTPLEEPLLDSSLTINNTPIRSSKIFYEWNDFDEIDYTAHMRLLVAAGFPSSI 289
SNTANYSATVEPYLD-VNGTVNGQLIRQSKWVDWNOYVDELSFVQDLRQALIAKGFERSDI 283
: : * * * * * . . . . . : : * * * * * . . . . . : : * * * * *

GH6_Ace
CBHA_Cfi
E3_Tfu
GMLIDTLRNGWGGPNEPTGPSTATDVNTFVNQSKIDLROHRLWCNQNAGLQPPQASP 344
GMLVDTSRNGWGGPNRPTSI TASTDVNAYVDANRVDRRVHRGAWCNPLGAGIGRFPPEATP 349
GMLIDTSRNGWGGPNRPTGPSSTDLNTYVDESRIIDRRIHPGNWCNQAAGLGERPTVNP 343
***: ** ***** . . . . . : : * * * * * . . . . . : : * * * * *

GH6_Ace
CBHA_Cfi
E3_Tfu
TDFPNAHLDAYVVIKPPGESDGTSAASDPTTGKKSDPMCPTYTTS -- YGVLTNALPNS 401
SGYAASHLDAFVVIKPPGESDGASTDI PNDQKKRFRDMCDPTFVSPKL-NNQLTGATPNA 408
--- --APGVDAYVVIKPPGESDGASEEI PNDEGKGFDRMCDPTYQGNARNGNPNPSGALPNA 399
. . . . . : : * * * * * : : * * * * * : : * * * * *

GH6_Ace
CBHA_Cfi
E3_Tfu
PIAGQWFPAQFDQLVANARPVP 424
PLAGQWFEEQFVTLVKNAYPVIG 431
PISGHWFSAQFREL LANAYPPL- 421
* : * * * * * * * * * * * : * * * * *

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2C. Alignment of Selected Glycoside Hydrolase Family 12 Catalytic Domain Sequences.

GH12_Ace: *A. cellulolyticus* Cel6A-Cell12A catalytic domain GH12 (AA 861-1090).
 EGIII_Tre: *T. reesei* EGIII catalytic domain (AA 17-234, GeneBank Acc.# AAE59774).
 CelB_Sli: *Streptomyces lividans* endoglucanase catalytic domain (AA 41-261, GeneBank Acc. # U04629).
 Catalytic Nucleophile/Base: Glu, Catalytic Proton Donor: Glu (shown in bold)

```

GH12_Ace      CTPGPNQGVTSVQDEYRVQTNENSSAQOCLTINTATGAWTVSTANFSGGTGGAPATY 60
Eng_Sli       DTTICEPFGTTIQG-RYVQNNRWGSTAPQCVTA-TDTG-FRVTQADGSAPTNGAPKSY 57
EGIII_Tre    -QTSCDQWATFTGNG--YTVSNNLWASAGSGFCVTAVLSLGGASWHADWQWSGGQNNV 57
               . : . . : : * . * . * . * . * . * . * . * . * . * . * . * . * .
GH12_Ace      PSYKKGCHWGNCTTKNVGMPIQISQIGSAVTSWSTTQVSSGAYD-VAYDIWTNSTPTTTG 119
Eng_Sli       PSVFNKGCHYTNCSPG-TDLPVRLDTVSAAAPSSISYGFVDGAVN-ASYDIWLDPTARTDG 115
EGIII_Tre    KSYQN-----SQIAIPQKRTVNSISSMPTTASWSYSGSNIRANVAYDLFTAANPNHVT 110
               * : . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GH12_Ace      QPNGTEIMWLNSRGGVQPFSGQATGVTVAGHTWNVWQQQTSWKIISYVLTGATSIS 179
Eng_Sli       -VNQTEIMWFNVRVGIQPIGSPVGT-ASVGGRTWEVWGGGNSNDVLSFVA-PSAISGW 172
EGIII_Tre    YSGDYELMIWLKYGDI GPIGSSQGT-VNVGGQSWILYGYNGAMQVYFVAQTNTN-Y 168
               . * : * * * * . * . . . . . * : : * : : * : : * : : * : : * : : .
GH12_Ace      NLLDKAIFADAAAAR-GSLNTSDYLLDVEAGFEIWQGGQGLGNSNFSVSVTSIG 230
Eng_Sli       SFDVMDFVRATVAR-GLAENDWYLTSVQAGFEPWQNGAGLAVNSFSSTVE-- 221
EGIII_Tre    SGDVKVFFNYLRDNKGYNAAGQYVLSYQFGTEPFTGSGTLNVAASWTASIN-- 218
               . * : : . . . * . * : . . * * : * * : * * : * * : * * : * * : : .

```


2E. Alignment of Selected Glycoside Hydrolase Family 74 Sequences.

GH74_Ace: *A. cellulolyticus* Cel74A catalytic domain (AA 47-786).
 Cel74A_Aac: *Aspergillus aculeatus* endoglucanase (Avicelase III) catalytic
 domain (AA 21-746, GeneBank Acc. # BAA29031). Cel74A_Tfu: *T. fusca*
 Cel74A catalytic domain (AA 47-781, [http://bahama.igi-psf.org/prod/bin/
 microbes/tfus/home.tfus.cgi](http://bahama.igi-psf.org/prod/bin/microbes/tfus/home.tfus.cgi))

```

Cel174A_Ace      ATTQPYTWSNVAIGGGG-FVDGIVFNEGAPGILYVRTDIGMYRWDAAANGRWIPLLDWVG 59
Cel174A_Tfu      SATTGTYTRNVEIVGGG-FVPGIVFNQSEPDLIYARTDIGGAYRWDPATERWIPLLDHVG 59
Cel174A_Aac      AASQAYTWKNVVTGGGGFTPGIVFNPSAKGVAYARTDIGGAYRLN-SDDTWTPLMDWVG 59
                :::   *** **   ***** . . : * .***** **   : :   *   ** : * **
Cel174A_Ace      WNNWGYNGVVSIAADPINTNKVAAVGYMYSWDPNDGAILRSSDQGATWQITPLPFKLG 119
Cel174A_Tfu      WDDWGHSGVVSIAATDVPDPRVYAAVGYTNDWDPNNGAIKRSTDRGEITWETTELPFKLG 119
Cel174A_Aac      NDTWHDWGDALATDPVDTRVYVAVGYMYSWDPNVGSILRSTDQGDITWETKLPFKVG 119
                : *   * : : : : : : : : : : : : : : : : : : : : : * * : * * : * : * : * : *
Cel174A_Ace      GNMPGRGMGERLAVDPNNDNILYFGAPSGKGLWRSTDSGATWSQMTNFPDVGTYIANPTD 179
Cel174A_Tfu      GNMPGRGMGERLAIDPNDNSVLYLGPAGSHGLMKS TDYGKTWOKVTSFPNPGNYVADPSD 179
Cel174A_Aac      GNMPGRGMGERLAVDPNKNSILYFGARSGLMKS TDYGATWSNVTSTFTWTGTIYFQDSSS 179
                ***** : : : : : : : : : : : : : : : * * * : : : * * : * * : * : * : * : *
Cel174A_Ace      TTGYQSDIQGVVWVAFDKSSSSLGQASKTI FVGVDADPNPWFWSRGGATWQAVPGAP-T 238
Cel174A_Tfu      VGGYLDGNQGVVWVDFPTSSSPGHVTKDI YVGVADKQNTVYRSTDDGGQITWERIPGQP-T 238
Cel174A_Aac      T--YTSDPVGIAWVTFDSTSGSSGSATPRIFVGVADAGKSVFKSE DAGATWAWVSERFQY 237
                . . * . * : : : : : * * : * * : * * : * : * : * * : * * : * * : * : *

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Continued on next page.

```

Cel174A_Ace      GFIPHKGVDFPVNHVLYIATNTGGPYDGSSEDVWKFVTSWTWTRISPVSPSTDTANDYF 298
Cel174A_Tfu      GFLAQKGVDFHVNGLLYIATSDTGGPYDGSDEWVRYDTTGTWDTITPA---DPDGFY 295
Cel174A_Aac      GFLPHKGVLSPEKTLIYSYANGAGPYDGTNGTVHKYKINITSGVWTDISPT---SLASTYY 294
**:::**: : ***: :: .*****: * * :. :. *:*:* *:*:*: . . :
Cel174A_Ace      GYGLTIDRQHPNTIMVATQISWWPDTIIFRSTDGGATWTRIMDWTSYPNRSLRYVLDIS 358
Cel174A_Tfu      GFSGLTIDRQNPDTIMVVSQILWNPDIQIWRSTDRGETWSRIWEFSGYDPDRLRYNHDIS 355
Cel174A_Aac      GYGLSVDLQVPGTILMVAALNCWPPDELIFRSTDSGATWSPIWENNGYPSINYYYSYDIS 354
*:*:*: * * *:*:*: ***** *:*:*:* * ** : *:*:*:* * * . * ***
Cel174A_Ace      AEPWLTFGVQPNPVPSPKLGWMDAEMAIIDPFNSDRMLYGTGATLYATNDLTKWDSGGQI 418
Cel174A_Tfu      AAPWLDFNRQDNPPPEVSPKLGWMTQAFEDPFNSDRMLYGTGATLYGSDNLTNWDEGKKI 415
Cel174A_Aac      NAPWIQDITSTD--QFPVVRVGMVVEALAIIDPFNSHMLYGTGLIVYGGHDLINWDSKHNV 412
**: . :. . . :*:*:* : * : *:*:*:*: ***** *:*:* *:*:*:* * * . :
Cel174A_Ace      HIAPMVKLEETAVNDLISPPSGAPLISALGDLGCFTHADVAVPSTIFTSPVFTTTSV 478
Cel174A_Tfu      DIKVRAQGIEETAVQDLIAPPDTELVSALGDIIGFVHDDITVVPDAMFDSPPFHGNTRSI 475
Cel174A_Aac      TVKSLAVGIEEMAVLGLITPPGGPALLSAVGGDDGGFYHSDLLDAAPNQAYHTPTYGTNGI 472
: . *:*:* * * *:*:*: *:*:*:* *:*:* * * * : *:*:* * * . . . :
Cel174A_Ace      DYAELNPSIIVRAGSDFPSSQPNDRHVAFSTDGCKNWFQSGPEGGVTGGTVAASADCSR 538
Cel174A_Tfu      DFAELNPSVMARVG--EAVDGEVDSHIGISTCGSHHWAGQEPGVTGACITVAVNADCSR 533
Cel174A_Aac      DYAGNKPSNIVRSG----ASDDYPTLALSSNFSGSTWYADYAAASTGTGVALSADGDT 527
** : ** : * * * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :

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```
Cel174A_Ace          FVWAPGDPGQPVVYAVGFGNSWAASQGVPANAQIRSDRVNPKTFYALSNGTFYRSTDGGV 598
Cel174A_Tfu          IVWSPDGTG--VHYSITLGSWTTPSQGVPAGARVEADRVNPKFYAFANGTFYTSIDGGA 591
Cel174A_Aac          VLLMSSTSG--ALVSKSQG-TLTAVSSLPSGAVIASDKSDNTVYGGSAAGAIYVSKNTAT 584
..*... : *.: :. .:*. * : *.: *.: *.: *.: *.: *.: *.: *.: *.: *.:
TFQP-VAAGLPSGAVGVMFHAVPGKEGDLWLA-----ASSGLYHSTNGGSSWSAI-TGV 651
TFTKSSAAGLPTKG--NIRFAAVPGHEGDIWLAGGETNSTYGMWRSTDSGATFTRI-TAV 648
SFTKIVSLGSSTTVN--AIRAHPSIAGDVWAS-----TDKGLWHSTDYGSTFTQIGSGV 636
.+ + . . * * * * * : *.: *.: *.: *.: *.: *.: *.: *.: *
```


Figure 3. Alignments of Family 2 and 3 CBM and type-III fibronectrin-like peptides amino acid sequences obtained from this study.

3A. Sequence alignment of Family III CBMs found in this study

Cel16A - Cel112A	VSGGLKVQYKNNDSAPGDNQIKPGLQLVNTGSSSVDLSTVTVRYWFTRDGSSSTLVNCD
Cel174A	VSGGVKVQYKNNDSAPGDNQIKPGLQVVNTGSSSVDLSTVTVRYWFTRDGSSSTLVNCD
Cel15B	VSGGVKVQYKNNDSAPGDNQIKPGLQLVNTGSSSVDLSTVTVRYWFTRDGSSSTLVNCD
Cel148A	AAVTLKAQYKNNDSAPSDNQIKPGLQLVNTGSSSVDLSTVTVRYWFTRDGSSSTLVNCD
Cel16A - Cel112A	WAAAMGCGNIRASFGSVNPATPTADTYLQLSFTGGTLAAGGSTGEIQNRVNKSDWSNFTET
Cel174A	WAAIGCGNIRASFGSVNPATPTADTYLQXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Cel15B	WAAAMGCGNIRASFGSVNPATPTADTYLQLSFTGGTLAAGGSTGEIQNRVNKSDWSNFTET
Cel148A	WAAAMGCGNIRASFGSVNPATPTADTYLQLSFTGGTLAAGGSTGEIQNRVNKSDWSNFTET
Cel16A - Cel112A	NDYSYGTNTTFQDWTKVTVYVNGVLVWGTEPSGT
Cel174A	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Cel15B	NDYSYGTNTAFQDWTKVTVYVNGRLVWGTEPSGT
Cel148A	NDYSYGTNTTFQDWTKVTVYVNGVLVWGTEPSGA

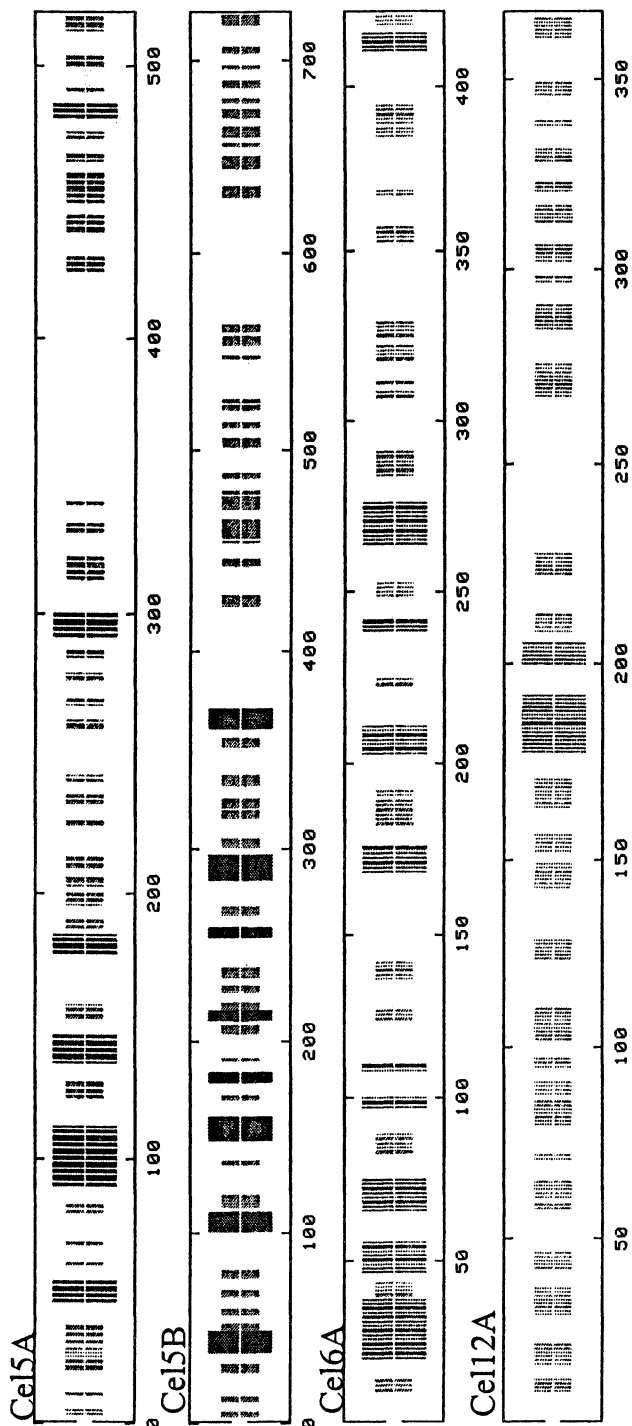
3B. Sequence alignment of Family II CBMs found in this study

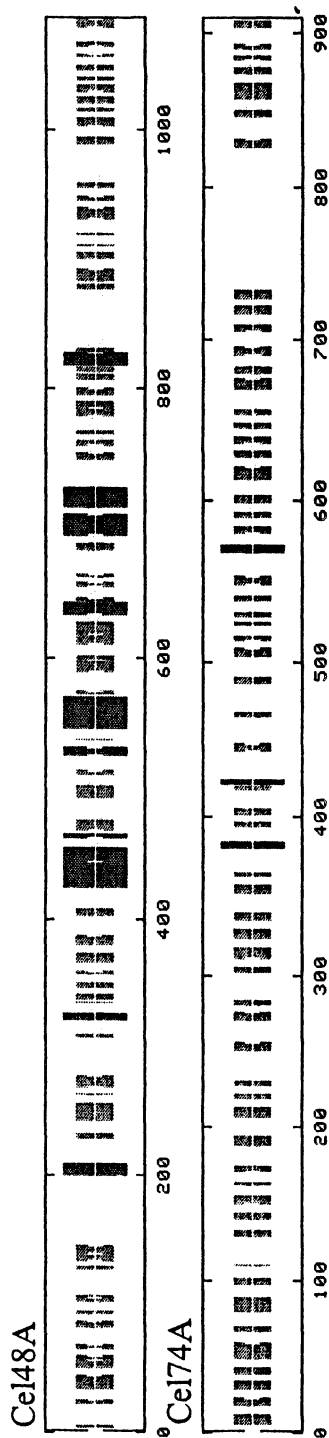
Cel16A - Cel112A	GVACRATYYVNSDWGSGGFTATVTVTNTGSRATNGTVAWSFGGNQTVTNYWNTALITQSGA
Cel15B	GVGCRATYYVNSDWGSGGFTATVTVTNTGSRATSGTVAWSFGGNQTVTNYWNTALITQSGA
E1 (Cel15A)	GARCTASYQVNSDWGNGFTVTVAVTNSGVAKTWTVSWTFGGNQTITNSWNAAVTQNGQ
Cel148A	GASCTATYYVNSDWGSGGFTTIVTVTNTGTRATSGTWTWSFAGNQTVTNYWNTALITQSGK
Cel16A - Cel112A	GSVTATNLSYNNV IQPGQSTTFGFNGSYSGTNAAPTLSCTAS
Cel15B	GSVTATNLSYNNV IQPGQSTTFGFNGSYSGTNAAPTLSCTAS
E1 (Cel15A)	GSVTARNMSYNNV IQPGQNTTFGFQASYTGSNAAPTVAACAAS
Cel148A	GSVTAKNLSYNNV IQPGQSTTFGFNGSYSGTNAAPTLSCTAS

3C. Sequence alignment of Fibronectin type III found in this study

Cel16A - Cel112A	DVTPPSVPTGLVVTGVSGSSVSLAWNASTDNDVGVAHYNNVYRNGVLVGQPTVTSFTDTGLAA
Cel148A	DTTTPPSVPTGLQVLTGTTSSVLSWTASTDNDVGVAHYNNVYRNGTLVGQPTATSFTDTGLAA
Cel16A - Cel112A	GTAYTYTVAAVDAAGNTSAPSTPV
Cel148A	GTSYTYTVAAVDAAGNTSAQSFAG

Figure 4. Secondary structure plots of *A. cellulolyticus* enzymes (Blue = α -helix; Red = strand; Yellow = random coil) determined by GOR secondary structure prediction method version IV (26).





Putative Mannanase Cel5B

The *A. cellulolyticus* mannanase gene consists of a coding sequence for a GH family 5 catalytic domain, a type III CBM, and a type II CBM. This gene can be summarized as (GH5-CBM_{III}-CBM_{II}). The molecular weight of this novel enzyme deduced from the nucleotide sequence is 76,937 Daltons. Importantly, only one of the two known family 5 mannanases is thermal tolerant (ie, *Caldocellum saccharolyticum* (*Caldicellulosiruptor saccharolyticus*; P22533).

Multi-Domain Enzyme Cel6A-Cel12A.

The multi-domain cellulase gene consists of a coding sequence for a GH family 6 catalytic domain, a type III CBM, an FN peptide, a GH family 12 catalytic domain, and a type II CBM. This gene can be summarized as (GH6-CBM_{III}-FN_{III}-GH12-CBM_{II}). The molecular weight of this novel enzyme deduced from the nucleotide sequence is 124,597 Daltons. This enzyme is unique because it contains two catalytic domains, two CBM, and two linker peptide regions. Each domain is unique. Enzymatic degradation of β -glucan is accomplished through glycosyl hydrolase family 12 enzymes (18). Although these endo-acting enzyme are active on β -(1 \rightarrow 4) glycosidic linkages, they are differentiated from other β -(1 \rightarrow 4)-acting enzymes by the distinction of being able to hydrolyze the β -(1 \rightarrow 4) linkages in mixed β -(1 \rightarrow 3, 1 \rightarrow 4)-linked polysaccharides. New cellulases classified as belonging to GH families 6 and 12 are of special interest to bioethanol applications, because relatively few examples are known (i.e., 9 members of family 6 and 3 members of family 12 are currently known). Furthermore, none of the known family 6 and 12 cellulases is thermal tolerant and development of biomass conversion processes compatible with elevated temperatures. Gibbs and coworkers (19) have recently described the multi-catalytic, multi-domain structure for the majority of the glycosyl hydrolases produced the extreme thermophile *Caldicellulosiruptor saccharolyticus*.

Putative Exoglucanase Cel48A.

The exoglucanase gene consists of a coding sequence for a type III CBM, a GH family 48 catalytic domain, an FN3 peptide, and a type II CBM. This gene can be summarized as (CBM_{III}-GH48-FN3-CBM_{II}). The molecular weight of this novel enzyme deduced from the nucleotide sequence is 115,435 Daltons. Relatively few bacterial exo-type cellulases are currently known, and only 3 have been placed in family 48. More importantly, none of these family 48 exoglucanases is thermal tolerant.

Putative Exoglucanase Cel74A.

The *A. cellulolyticus* Cel74A gene consists of a coding sequence for a GH family 74 catalytic domain and a type III CBM. This gene can be summarized as (GH74- CBM_{III}). The molecular weight of this novel enzyme deduced from the nucleotide sequence is >94,971 Daltons. Family 74 enzymes have been shown to demonstrate activity on plant xyloglucan. Plant produced endotransglycosidases or xyloglucan hydrolases specific for xyloglucans are believed to mediate plant cell wall structural changes induced by growth, seed germination, flowering, senescence, environmental conditions, or physical stresses (20). The proposed mechanism for this interaction involves cleavage of the xyloglucan by specific xyloglucanases to allow the cell wall network to loosen. Specific xyloglucan glycosyl transferases insert preformed xyloglucan "blocks" into these cleavage sites to expand the wall. Additionally, data from Grabber and coworkers (21) indicates that in alfalfa, xyloglucan in the primary cell wall is intimately involved with lignin, to the point of decreased xylose release relative to other sugars when treated with fungal polysaccharidases. We presume that bacterial xyloglucanases act in concert with other GHs to degrade immature plant cell walls in a similar way. New enzymes belonging to GH family 74 are of special interest to industry, because relatively few examples are known (i.e., only one member of family 74 is currently confirmed). More importantly, production of a thermal tolerant family 74 glycoside hydrolase has not previously been demonstrated.

Conclusion

The goal of our research program focuses on the expression of highly active endoglucanases and exoglucanases, which act synergistically to degrade the crystalline cellulose in biomass. The first enzyme we identified from *A. cellulolyticus* was endoglucanase EI. This enzyme has been shown to be highly active and thermal stable. These data imply that *A. cellulolyticus* may produce a high performance and diverse glycosyl hydrolase system, a prediction based in part on the rich biomass content of the hot spring from which it was originally isolated. The enzymes coded by the new genes we report in this study have the potential to be equally active and stable.

An interesting secondary finding is that the five glycoside hydrolase genes found in this study are closely linked. Clustering of glycosyl hydrolase genes in anaerobic bacteria known for production of cellulosomes has been well established. The genetic organization of the gene cluster of *Clostridium cellulolyticum* was recently discussed by Belaich and coworkers (22). Furthermore, a five-gene cluster centered on the gene in *Clostridium cellulovorans* that encodes endoglucanase EngL has been cloned and sequenced (23). As is the case with the *A. cellulolyticus* gene cluster, a family 5 mannanase gene, *manA*, was found downstream of an endoglucanase, *engL*. There is also evidence that lignocellulose catabolism genes may be clustered on the *Streptomyces viridosporus* chromosome (24). In contrast, Gibbs (19) reported the sequencing of three gene clusters of glycosyl hydrolases from *Caldicellulosiruptor* sp. strain Tok7B.1. These clusters are not closely linked, and each is different in its organization from those described for *C. saccharolyticus*. The catalytic domains of the enzymes belong to glycosyl hydrolase families 5, 9, 10, 43, 44, and 48 and the cellulose binding domains of the enzymes from *Caldicellulosiruptor* sp. are types IIIb, IIIc, or VI.

It appears most likely that new, aggressive cellulase systems will be comprised of key enzymatic activities obtained from different microbial sources (25). Furthermore, depending on the treatment history of the biomass sample, augmentation with cell wall degrading enzymes including phenolic esterases and hemicellulose debranching enzymes may further reduce the requirement for high loadings of expensive cellulases.

Acknowledgments

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References

1. Wiselogle, A.; Tyson, S.; Johnson, D. In *Handbook on Bioethanol: Production and Utilization*; Wyman, C.E., Ed., Taylor & Francis, Washington, DC. **1996** 105-118
2. Bergeron, P. In *Handbook on Bioethanol: Production and Utilization*; Wyman, C.E., Ed., Taylor & Francis, Washington, DC. **1996** 90-103
3. Cowan, D.A. In *The Archaeobacteria, Biochemistry and Biotechnology*; Danson, M.J., Eds., University Press, Cambridge, UK. **1992**, 149-159.
4. Henrissat, B.; Bairoch, A. *Biochem. J.* **1996** *316*, 695-696.
5. Henrissat, B.; Davies, G.; *Curr. Opin. Struct. Biol.* **1997** *7*, 637-644.

6. Himmel, M.E.; Adney, W.S.; Baker, J.O.; Nieves, R.A.; Thomas, S.R. In *Handbook on Bioethanol: Production and Utilization*; Wyman, C.E., Ed., Taylor & Francis, Washington, DC. **1996** 143-161.
7. Koivula et al., *Protein Expression and Purification* **1996** 8, 391-400
8. Mohagheghi, A.; Grohmann, K.; Himmel, M.; Leighton, L.; Updegraff, D. *Int. J. Systematic Bacteriology* **1986** 36, 435-443.
9. Ding, S.Y.; Bayer, E.A.; Steiner, D.; Shoham, Y.; Lamed, R. A scaffoldin of the *Bacteroides cellulosolvens* cellulosome that contains 11 type II cohesions. *J. Bacteriology* **2000** 182 (17): 4915-4925.
10. Himmel, M.E.; Adney, W.S.; Grohmann, K.; Tucker, M.P. United States patent No. 5,275,944; *Thermostable Purified Endoglucanase from Acidothermus cellulolyticus*, January 4, **1994**.
11. Adney, W.S.; Thomas, S.R.; Nieves, R.A.; Himmel, M.E. United States patent No. 5,366,884; *Thermostable Purified Endoglucanase II from Acidothermus cellulolyticus*, November 22, **1994**.
12. Himmel, M.E.; Tucker, M.P.; Adney, W.S.; Nieves, R.A. United States patent No. 5,432,075; *A Low-Molecular Weight, Thermostable β -D-glucosidase from Acidothermus cellulolyticus*, July 11, **1995**.
13. Adney, W.S.; Tucker, M.P.; Nieves, R.A.; Thomas, S.R.; Himmel, M.E. *Biotech. Lett.* **1995** 17, 49-54.
14. Tucker, M.P.; Mohagheghi, A.; Grohmann, K.; Himmel, M.E. *Bio/Technology* **1989** 7, 817-820.
15. Baker, J.O.; Adney, W.S.; Nieves, R.A.; Thomas, S.R.; Wilson, D.B.; Himmel, M.E. *Appl. Biochem. Biotechnol.* **1994** 45/46, 245-256.
16. Sakon, J.; Adney, W.; Himmel, M.; Thomas, S.; Karplus, P.A. *Biochemistry* **1996** 35, 10648-10660.
17. Hooker, B.S.; Dai, Z.; Anderson, D.B.; Quesenberry, R.D.; Ruth, M.F.; Thomas, S.R. In *Handbook on Bioethanol: Production and Utilization*; Wyman, C.E., Ed., Taylor & Francis, Washington, DC. **1996** 55-90.
18. Keitel, T.; Thomsen, K.K.; Heinemann, U. *J. Mol. Biol.* **1993** 232, 1003-1004.
19. Gibbs, M.D.; Reeves, R.A.; Farrington, G.K.; Anderson, P.; Williams, D.P.; Bergquist, P.L. *Curr. Microbiol.* **2000** 40, 333-40.
20. Kaku, T. *Plant Cell. Physiol.* **2002** 43, 21-26.
21. Grabber, J.H.; Panciera, M.T.; Hatfield, R.D. *J. Agric. Food Chem.* **2002** 50, 2595-2600.
22. Belaich J.P.; Tardif, C.; Belaich, A.; Gaudin, C. *J. Biotechnol.* **1997** 57, 3-14.
23. Tamaru, Y.; Doi, R.H. *J. Bacteriol.* **2000** 182, 244-7.
24. Thomas, L.; Crawford, D.L. *Can. J. Microbiol.* **1998** 44, 364-72.

25. Thomas, S.R.; Laymon, R.A.; Chou, Y.-C.; Tucker, M.P.; Vinzant, T.B.; Adney, W.S.; Baker, J.O.; Nieves, R.A.; Mielenz, J.R.; Himmel, M.E. In *Enzymatic Degradation of Insoluble Polysaccharides*; Saddler, J.N.; Penner, M.H., Eds., ACS Series 618, American Chemical Society, Washington, DC. **1995** 208-236.
26. Garnier, J.; Gibrat, J.-F.; Robson, B. *Methods Enzymol.* **1996** 266, 540-553.
27. Nielsen, H.; Engelbrecht, J.; Brunak, S.; von Heijne, G. *Protein Eng.* **1997** 10, 1-6.

Chapter 21

Diversity of Microbial Endo- β -1,4-Xylanases

Peter Biely

Institute of Chemistry, Slovak Academy of Sciences, 84238 Bratislava,
Slovak Republic

Endo- β -1,4-xylanases (EC 3.2.1.8, EXs) are the principal xylan-depolymerizing enzymes produced by microorganisms and plants. Extensive studies of EXs in the past two decades, stimulated by their successful application in the pulp and paper and bakery industries, and as animal feed additives, has greatly increased knowledge on their structure and catalytic properties. This work is briefly reviewed in this article. The best known are EXs of glycosyl hydrolase families 10 and 11, however, new types of EXs are emerging. New important findings can be expected from studies of the plant-microbial interactions focusing on xylanase toxicity for plants and the plant xylanase inhibitors.

The past two decades have seen a growing interest in microbial enzyme systems that degrade plant xylan, which is, after cellulose, the most abundant polysaccharide in nature. Xylan is a heteropolysaccharide composed mainly of the five-carbon sugar D-xylose. Xylose can be converted to a variety of useful products, including ethanol (1,2). Enzymatic saccharification of agricultural, industrial and municipal wastes may provide sugar syrups for human and animal consumption, and carbon sources for industrial fermentations. Xylooligosaccharides have a variety of uses as food additives (3). Plant structural polysaccharides provide the major source of nutrients for ruminant livestock and also play an important role in animal fodder. Pretreatment of forage crops with polysaccharide-degrading enzymes improves the nutritional quality and digestibility of ruminant fodder and facilitates composting (4,5).

Enzymatic hydrolysis of highly viscous arabinoxylans originating in cereal endosperms improves nutrient uptake and digestion in broiler chickens (6-9). Other applications of xylanolytic enzymes include improvement of the baking process and modification of baked products (10-16). Xylanases are also important components of enzyme systems used for liquifaction of vegetables and fruit, and for clarification of juices (4,17). In all of the above listed processes, microbial xylanolytic systems can be applied together with other enzymes hydrolyzing plant polysaccharides such as amylases, cellulases and pectinases. There are, however, applications in which xylanases should not be contaminated by cellulases to preserve the polymerization degree of cellulose.

Xylanases free of cellulases have applications with important ecological implications in pulp and paper industry. They facilitate lignin extraction, reducing the consumption of toxic chemicals required for pulp bleaching (18-21). The mechanism of this process, called enzymic prebleaching or bleachbusting, is not completely understood. However, it is believed that xylanase attacks the xylan moiety of lignin-carbohydrate complexes (18). Xylanases also have potential in the purification of dissolving pulp from hemicellulose (22,23), in the recovery of cellulose textile fibres (24) and in paper recycling (25). However, it was mainly the discovery of Liisa Viikari and her coworkers (18) that xylanases can be useful in pulp bleaching which made these enzymes so recognized and promoted their extensive research worldwide. Xylanolytic enzymes have become one of the best known hydrolases. They also have been extensively reviewed in the past (26-33) and books covering exclusively xylan and xylanases have also been published (34,35). In this article we would like to summarize the knowledge that has accumulated on the principal xylan-depolymerizing enzymes, endo- β -1,4-xylanases (EC 3.2.1.8) (further as EXs).

Cooperation of EXs with Debranching Enzymes

Xylan is structurally similar to cellulose, but instead of D-glucose, its main chain is built from β -1,4-linked xylopyranosyl residues. The main chain is usually substituted to various degree by residues of 4-O-methyl-D-glucuronic acid, D-glucuronic acid, or L-arabinofuranose, and in some cases is also esterified by acetyl groups (36-40). The substituents may be also represented by oligosaccharides and esterified by cinnamic (phenolic) acids, of which ferulic acid is the most abundant. Dimerization of feruloyl residues to form diferuloyl bridges is believed to be an important cross-linking between xylan and other cell wall polymers (41,42). A hypothetical fragment of a plant xylan showing the major structural features found in this group of hemicelluloses and the enzymes required for its hydrolysis are depicted in Figure 1.

The crucial enzyme for xylan depolymerization is endo- β -1,4-xylanase (β -1,4-xylan xylanohydrolase; EC 3.2.1.8, abbreviated EX). EX attacks the main chain, most easily and therefore most rapidly at non-substituted regions, generating non-substituted and branched or esterified oligosaccharides. The main chain substituents are liberated by corresponding glycosidases or esterases, so called debranching or accessory enzymes: α -L-arabinosyl residues by α -L-arabinofuranosidases (α -L-arabinofuranoside arabinofuranosidase; EC 3.2.1.55), D-glucuronosyl and 4-O-methylglucuronosyl residues by α -glucuronidase (EC 3.2.1.139), acetic acid, ferulic acid and p-coumaric acid residues by corresponding esterases (EC numbers not assigned). β -Xylosidase (xylobiase or exo- β -1,4-xylanase) (β -1,4-xylan xylohydrolase; EC 3.2.1.37) attacks xylooligosaccharides from the non-reducing end, liberating D-xylose.

Two categories of debranching enzymes can be recognized: i) those that operate on both polymeric and oligomeric substrates and ii) those that are active only on branched or substituted oligosaccharides generated by EXs. There is usually no sharp boundary between these groups. The enzymes operating on polymeric substrates can be distinguished from those of the second group by their ability to catalyze precipitation of the originally highly soluble branched polymeric xylan. During xylan hydrolysis EX acts synergistically with accessory enzymes (43-46). EX releases substituted xylooligosaccharides, which are more readily diffusible and more favorable substrates for accessory enzymes. On the other hand, the removal of side chains substituents by accessory enzymes creates new sites on the main chain for productive binding with EX.

Classification

A comparison of microbial EXs based on their different physicochemical properties, such as molecular mass and isoelectric point by Wong et al. (47) indicated that these enzymes can be divided into two groups, one consisting of high-molecular-mass enzymes with low pI values, and the other consisting of low-molecular-mass enzymes with high pI values. As usual, there are exceptions, particularly concerning pI values. Interestingly, this grouping of EXs (47) was found to be in agreement with the general classification of glycanases on the basis of hydrophobic cluster analysis and sequence similarities (48-51). Hydrophobic cluster analysis is designed to predict protein folding based on hydrophobic/hydrophilic patterns and is used to compare members of a protein group of similar functions (52). Acidic high-molecular-mass EXs (> 30 kDa) were assigned to glycanase family 10 (51) (formerly family F), and basic, low-molecular-mass EXs (<30 kDa), were assigned to glycanase family 11 (formerly family G). While family 10 also contains other glycosyl hydrolases, family 11 is exclusively made up of EXs.

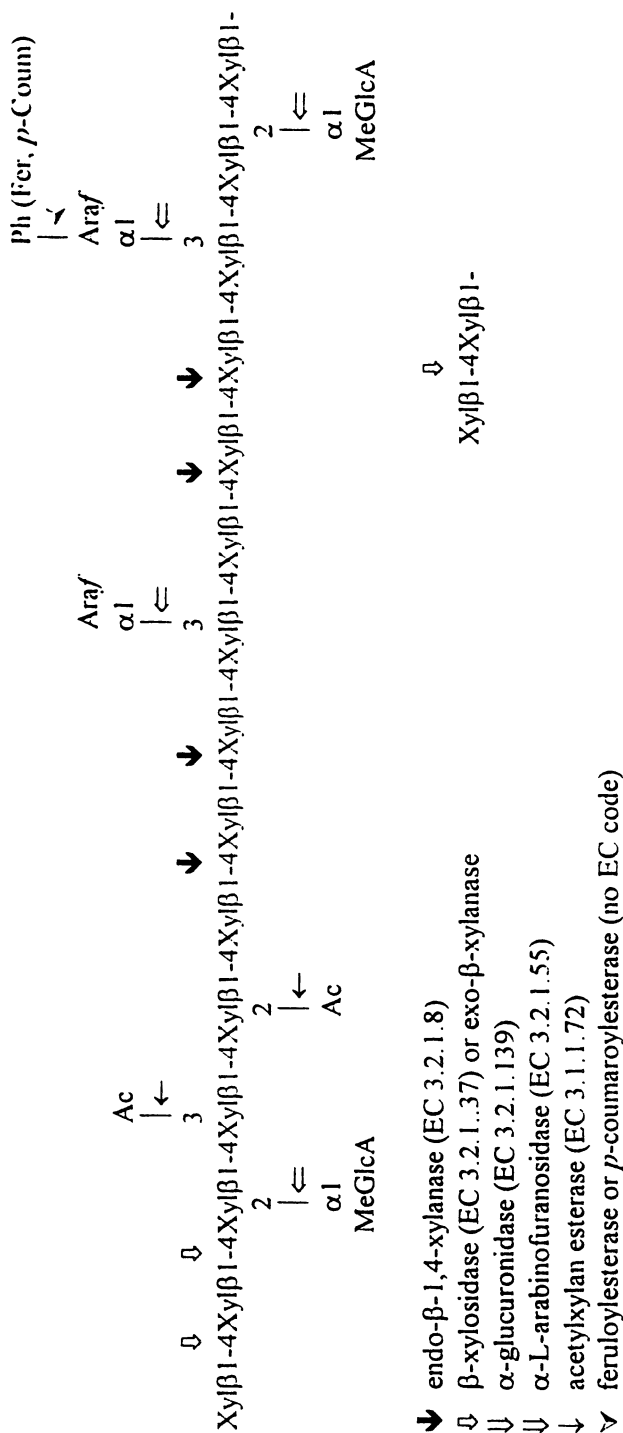


Figure 1. Structure of hypothetical plant xylan comprising the known structural features and the enzymes involved in its complete hydrolysis. EX is the only enzyme attacking the xylan main chain randomly.

New classes of EXs emerge which do not show any homology with EXs from either family 10 or 11. One type of enzymes have been reported in *Erwinia chrysanthemi* (xylanase A) (53,54), *Aeromonas caviae* (xylanase VI) (55) and *Trichoderma reesei* (xylanase IV) (56,57). The enzymes from all three sources show the highest homology with glycosyl hydrolases of family 5, however, they seem to exhibit different catalytic properties. Another type of novel EX was discovered in psychrophilic bacterium *Pseudoalteromonas haloplanktis* (58,59). The enzyme has a molecular mass of 46 kDa and belongs to glycosyl hydrolase family 8. Functionally it is similar to EXs of family 11, however, it is an inverting EX, the first such example among EXs (59).

Protein Fold

EXs belonging to the two glycosyl hydrolase families show remarkable differences in their tertiary structures as established by crystallography (Figure 2). Members of individual families have a similar protein folding pattern, i.e. a similar three-dimensional structure. These tertiary structures are more conserved than amino acid sequences. The most conserved region within a family appears to be the catalytic domain. EXs of family 11 appear to be smaller, well-packed molecules, formed mainly of β -sheets (60-63). These enzymes appear very small in their native states (64,65). The catalytic groups are present in a cleft that accommodates a chain of five to seven xylopyranosyl residues.

The tertiary structure of EXs of family 10 (Figure 2) is typically an eightfold α/β barrel [$(\alpha/\beta)_8$], resulting in a "salad bowl" shape (66-69). The substrate binds to a shallow groove on the bottom of the "bowl", which is shallower than the substrate binding cleft of family 11 EXs (31). This feature together with the generally greater conformational flexibility of larger enzymes, may account for the lower substrate specificity and greater catalytic versatility of family 10 EXs.

A comparison of the tertiary structure of EXs with other classified glycosyl hydrolases reveals that the two EX families evolved from different ancestors. EXs of family 10 are closely related to the endo- β -1,4-glucanases of family 5, with which they share some common catalytic properties (see below). Both enzyme families 5 and 10 belong to a larger clan of enzymes possessing the 8-fold α/β barrel architecture (68,70), known as clan GH-A (51). EXs of family 11 show a certain degree of similarity to the low molecular mass endo- β -1,4-glucanases of family 12 (formerly H) (71).

Little knowledge is available on protein folding of EXs belonging to family 5. Interesting that while proteins belonging to family 5 show 39 to 45 % α -helical structure, *E. chrysanthemi* xylanase A was estimated to contain only 10-15% of α -helices (54). Classification of the novel psychrophilic EX in family 8 suggests an 6-fold α/α barrel structure, distinct from that of all currently known EXs (59).

Molecular Architecture

The molecular architectures of EXs range from a single catalytic domain to a complex arrangement of catalytic and non-catalytic domains (72-75), recently named modules (72,73). Non-catalytic modules of EXs act independently of the catalytic modules as polysaccharide-binding domains (carbohydrate-binding modules, CBMs). Catalytic modules are connected to CBMs to which they are linked by linker sequences rich in proline and hydroxy amino acids. The linkers function as flexible, extended hinge regions between functional modules (49). Originally, it was believed that CBMs increased the catalytic rate against insoluble substrates. However, recent observations suggested that binding modules may also facilitate the hydrolysis of soluble polysaccharides (74,76). The CBMs have also been reported to function as thermostabilizing domains (77,78).

Surprisingly, there are several EXs that contain binding modules that bind exclusively to cellulose (cellulose binding domain, CBD) and not to xylan (49,73). This is likely related to the biological role of these enzymes in degrading plant cell walls which are predominantly built from cellulose closely associated with hemicellulose. Targeting of EX to cellulose may bring the enzyme in proximity of its substrate. There is a family of CBMs (CBD9) that are found only in EXs (72). CBMs of EX B (XlnB) from *Streptomyces lividans* and EX D from *Cellulomonas fimi* appear to be specific for soluble and insoluble xylan (74,76). The 3D structure of this xylan-binding domain (XBD) is very similar to that of CBD. A single amino acid change converts the protein from a XBD to a CBD (74). An example of an EX containing two CBMs has been found in *Pseudomonas fluorescens* subsp. *cellulose* (79).

Nature also has designed multifunctional enzymes with two catalytic domains connected by linker sequences. Some bacteria produce bifunctional EXs containing two identical (80) or two different EX domains (81,82). Other

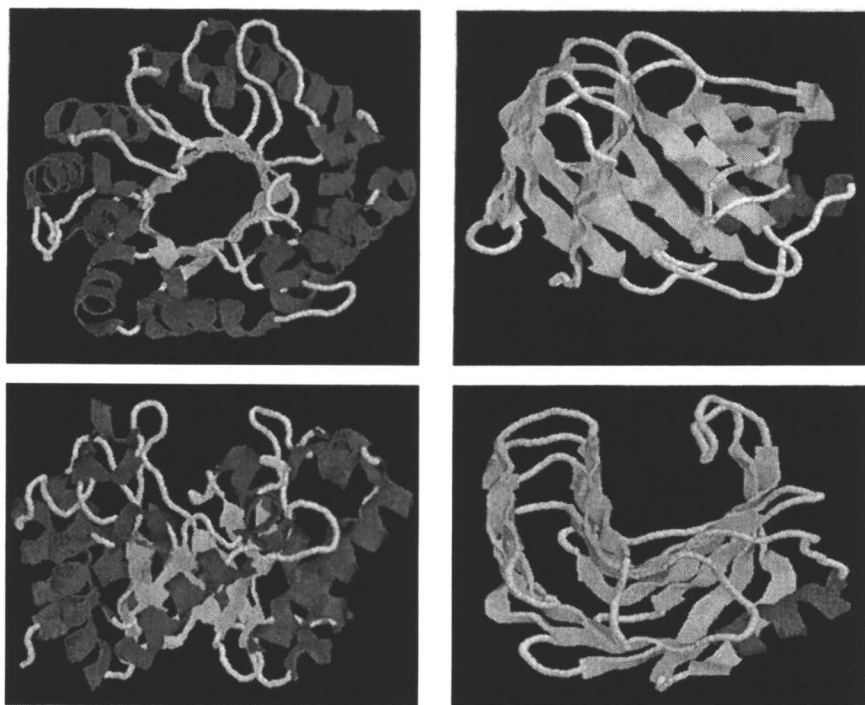


Figure 2. A ribbon representation of three-dimensional structures of EXs of family 10 (left panels, top and side view) and family 11 (right panels, top and side view)

enzymes possess catalytic domains of two different xylanolytic enzymes, e.g. one catalyzing the xylan depolymerization and the other deacetylation of the xylan main chain (83-85). Such bifunctional enzymes may have an advantage over enzymes containing a single catalytic domain in the hydrolysis of native acetylated xylan. Multidomain xylanases also occur as subunits of cellulosomes and xylanosomes. These supramolecular extracellular cell-associated protein complexes of thermophilic clostridia can efficiently degrade cellulose, xylan and related plant cell wall polysaccharides (86,87).

Catalytic Properties

EXs belonging to glycanase family 10 hydrolyze heteroxylans and rhodymenan (a linear algal β -1,3- β -1,4-xylan) to a greater extent than EXs of family 11 (88,89). The EXs of family 10 are better able to cleave glycosidic linkages in the xylan main chain that are near substituents, such as MeGlcA and acetic acid. The alteration of the xylan main chain by replacing β -1,4-linkages by β -1,3-linkages, like it is in rhodymenan, represents a more serious steric barrier for EXs of family 11 than for EXs of family 10. Consequently, EXs of family 10 liberate smaller products from 4-O-methyl-D-glucurono-D-xylan, rhodymenan and, with some exceptions, also from acetylxylan, than do EXs of family 11. EXs of family 10 can further hydrolyze the shortest branched or isomeric xylooligosaccharides which are released by EXs of family 11 (88).

Characterization of the shortest acidic fragments released from 4-O-methyl-D-glucurono-D-xylan after a long-term hydrolysis (89,90) suggests the linkages accessible to hydrolysis by the two types of EXs. EXs of family 10, unlike EXs of family 11, are capable of attacking the glycosidic linkage next to the branch and towards the non-reducing end. While EXs of family 10 require two unsubstituted xylopyranosyl residues between the branches, EXs of family 11 require three unsubstituted consecutive xylopyranosyl residues (Figure 3).

Kinetic data for cleavage of individual glycosidic linkages of the xylan main chain are not available, but time course of product analyses suggest that linkages closer to substituents are hydrolyzed more slowly than more distant linkages. Proposed sites of hydrolysis of glucuronoxylan by *E. chrysanthemi* enzyme (EXs 5) (54) are also shown in Figure 3. However, these do not correspond to the products released by other EXs of family 5 (55-57).

The degradation of a cereal L-arabino-D-xylan with two EXs of *Aspergillus awamori* (EXI, 39 kDa, pI 5.7-6.7, most probably a member of family 10, and EXIII, 26 kDa, pI 3.3-3.5, most probably a member of family 11) (91) is shown in Figure 4. The cleavage site specificities on L-arabino-D-xylan

resemble those on 4-O-methyl-D- glucurono-D-xylan. The L-arabinosyl substituents represent a more serious steric hindrance for the formation of productive complexes of the polysaccharide with EXIII. Only the larger type of the enzyme is capable of attacking the linkages between substituted and unsubstituted xylopyranosyl residues.

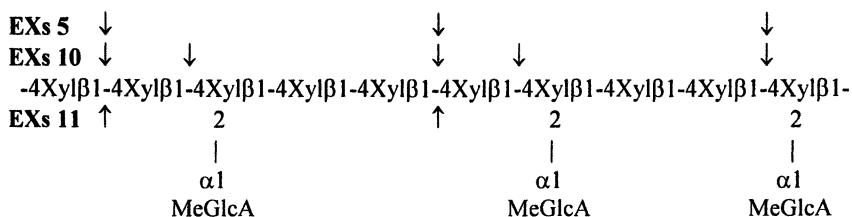


Figure 3. Sites of 4-O-methyl-D-glucurono-D-xylan cleavage by EXs of three different families.

Studies of the action of EXs on rhodymenan are complicated by the fact that EXs of family 10, are also capable of cleaving β -1,3-linkages (92,93). Generally, EXs of family 10 do not attack the polysaccharide at β -1,4-linkages which follow β -1,3-linkages towards the reducing end (92), liberating xylotriose, $\text{Xyl}\beta 1-3\text{Xyl}\beta 1-4\text{Xyl}$, as the shortest isomeric product. EXs of family 11 liberate from rhodymenan isomeric products larger than xylotriose.

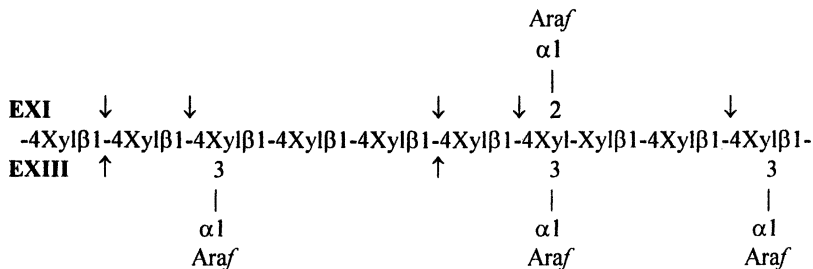


Figure 4. Cleavage sites of L-arabino-D-xylan by two different EXs from *Aspergillus awamori* (Ref. 91).

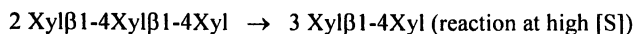
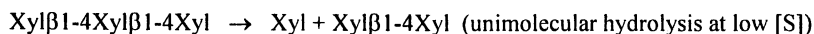
Consistent with the fact that EXs of family 10 are related to endo- β -1,4-glucanases and some glycosidases belonging to the clan of glycosyl hydrolases GH-A (51), EXs of family 10 catalyze hydrolysis of aryl β -D-xylopyranosides and low molecular mass cellulase substrates. These include aryl β -D-cellobiosides, aryl β -D-lactosides (94, 89), and some cellooligosaccharides (95). The degradation of aryl xylosides, e.g. of 4-nitrophenyl β -D-xylopyranoside, also involves a complex reaction pathway involving glycosyl transfer reactions leading to xylooligosaccharides and their glycosides (96). The EXs of family 11 do not possess these catalytic abilities. They catalyze glycosyl transfer reactions with xylooligosaccharides.

EXs of families 10 and 11 can also be differentiated on the basis of their reaction with ω -epoxyalkyl glycosides of xylose and xylooligosaccharides (97). These potential site-directed inhibitors of EXs inactivate only EXs of family 11. EXs of family 10 are resistant to electrophilic attack of the epoxides (97).

Mechanism of Substrate Degradation

EXs of families 10 and 11 catalyze the hydrolysis of glycosidic linkages with the retention of anomeric configuration (98,99). The reaction involves a double displacement mechanism leading to a new reducing end with β -anomeric configuration (100). The catalytic residues have been identified as two conserved glutamate residues (in a few cases an aspartate replaces a glutamate), which are located opposite of each other in the active site. One residue functions as a general acid catalyst protonating the glycosidic oxygen, while the other functions as a nucleophile, attacking the anomeric center to form a covalent enzyme-glycosyl intermediate (101). In the second step a water molecule attacks the intermediate in a general base-catalyzed process to yield the product of retained anomeric configuration (101). The catalytic residues have been identified by chemical modification, site-directed mutagenesis, and by use of stabilized enzyme-glycosyl intermediates (75,101). Glycosyl transfer reactions catalyzed by both types of EXs at high substrate concentrations are also evidence for the enzyme-glycosyl intermediate. Using linear β -1,4- xylooligosaccharides as substrates, it has been established that EXs utilize multiple pathways of substrate degradation (102, 103). Unimolecular hydrolysis takes place at low substrate concentrations (Figure 5). Different cleavage of the substrate occurs at so called shifted binding, which is observed at higher substrate concentrations, and which is, generally, accompanied by formation of products larger than the

substrate. At sufficiently high concentrations of xylotriose, xylobiose initially is generated as the only product of xylotriose degradation (Figure 5). Analogous reaction pathways are also observed with longer oligosaccharides.



Partial reactions at high [S] (E,enzyme; E-Xyl, enzyme-glycosyl intermediate):

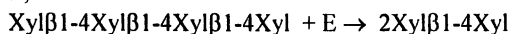
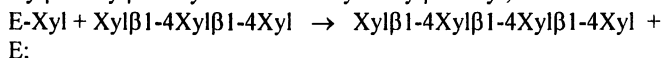
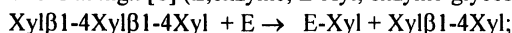


Figure 5. Different pathways of β -1,4-xylotriose degradation by EXs observed at low and high substrate concentrations

We also should mention that EXs of family 10 and 11 differ in stereochemistry of protonation of the glycosidic oxygen. The protonation can take place from two different sites involving one of the two electron pairs of the oxygen which are stereochemically not equivalent (104). Then one type of protonation occurs as *anti* and the other as *syn*. EXs of family 10 similarly as the whole glycosyl hydrolase clan A use *anti* protonation, while EXs of family 11 use *syn* protonation (104).

The main molecular and catalytic properties of EXs of families 10 and 11 are summarized in Table I. The knowledge of properties of EXs fit into families 5 and 8 is limited and does not allow such a generalization. We should mention again that the psychrophilic EX classified as a member of family 8, uses a single displacement mechanism of hydrolysis of glycosidic linkage, which means the enzyme is an inverting glycosyl hydrolase (59).

Xylan-Degrading Endo- β -1,4-glucoanases

It has been unambiguously established that at least one type of endo- β -1,4-glucoanases exhibits activity on both cellulose and xylan (105, 106). The best known enzyme in this regard is endoglucanase I from *Trichoderma reesei*, a member of family 5 glycosyl hydrolases. The enzyme catalyzes the hydrolysis of

both polysaccharides in the same active site (107). Although the enzyme belongs to the same clan as EXs of family 10, the products the enzyme generates from glucuronoxylan and rhodymenan are more similar to those liberated by EXs of family 11 (90). In *T. reesei* the enzyme is coinduced as a component of the cellulolytic and not of the xylanolytic system (17).

Physiological Role – Microbial *versus* Plant EXs

With steadily increasing evidence that one microorganism produces several types of EXs, in few cases even more than the two enzymes belonging to glycosyl hydrolase families 10 and 11, the question on the physiological role of the variety of EXs still remains open and awaits explanation. We realize that diversity of EXs could be a consequence of different evolutionary history during which microorganisms followed perhaps different routes to grow at the expense of xylan (30). However, we do not stop asking: do EXs play a different nutritional role in microorganisms, do they play a specific role during plant invasion, or, do they differ in toxicity to plants? As we have seen, EXs differ not as much in substrate specificity and the extent of xylan digestion as in their physico-chemical properties. Some EXs of family 11 were shown to act as elicitors of plant defence mechanisms (64). A remarkable observation associated with this effect of microbial EXs in plants is, that plants do not seem to produce EXs of family 11. The genome *Arabidopsis thaliana*, which has been sequenced, does not contain any sequence that would code for a protein belonging to glycosyl hydrolase family 11 which, as we know, contains exclusively EXs. The studies of EXs in barley (108) and maize (109) also provided evidence for only the presence of EXs of family 10. Will be the information on the lack of EXs of family 11 in plants confirmed in future studies? Will be this finding relevant to reports on occurrence of xylanase inhibitors in plants (110-113)?

Industrial Effectiveness of Different EXs

There is a lack of information which of the EXs are more efficient in industrial applications. The reason is that reliable data can be obtained only with purified enzymes, which is rarely the case when used on a larger scale. Everywhere,

Table I. Properties of EXs of families 10 and 11

<i>Properties of EXs</i>	<i>Family 10</i>	<i>Family 11</i>
Molecular mass	usually >30 kDa	usually <30 kDa
Isoelectric points	usually < 7	usually >7
Protein fold (catalytic module)	(α/β) ₈	β -sheets and one α -helix
Substrate binding site	shallow groove	deep cleft
Catalytic amino acids	two glutamic acids (occasionally one glutamate and one aspartate)	two glutamic acids (occasionally one glutamate and one aspartate)
Number of subsites	4-5	5-7
Substrate specificity	less specific	more specific
heteroxylans	active	active
aryl- β -xylosides	active	inactive
aryl- β -cellobiosides	active	inactive
aryl- β -lactosides	active	inactive
Shortest acidic oligosaccharide released from glucuronoxylan	Aldotetrauronic acid α 1-2MeGlcA-Xyl β 1-4Xyl β 1-4Xyl	Aldopentauronic acid Xyl β 1-4(α 1-2MeGlcA)Xyl β 1-4Xyl β 1-4Xyl
Stereochemistry of hydrolysis	retaining	retaining
Protonation of glycosidic oxygen	<i>anti</i>	<i>syn</i>
Multiple reaction pathway at high substrate concentrations	yes	yes
Effect of epoxyalkyl glycosides of Xyl, Xyl ₂ and Xyl ₃	no effect	inactivation

where depolymerization of xylan is to be accomplished using enzymes, all types of EXs could be equally efficient, unless the substrate main chain is not too heavily substituted or, unless the enzyme does not have an electric charge that influences the enzyme-substrate interaction, or unless the enzyme does not stick to an insoluble substrate or cellulose by means of its CBM. Studies devoted to application of pure EXs of family 10 and 11 on kraft pulp bleaching (114) and removal of hemicellulose from sulfite pulp (115) did not lead to straightforward data that would give preference for members of a particular EX family. The efficiency of enzymes was found more or less unrelated to the type of enzyme family. The efficiency could be more influenced by the presence of cellulose-binding module or enzyme charge given by its pI value. Buchert et al. (116) have demonstrated that the action of alkaline EXs in pulp may be severely hampered if they are applied at pH values lower than their pI values. The reason is the presence of positively charged groups in the pulp glucuronoxylan.

According to recent studies, more important than the EX family are the enzyme properties that are compatible with the process of pulp bleaching. In this process EXs with a high pH and temperature optima are of great importance (117,21,32,33).

Conclusions

Despite considerable progress in the area of xylanases, there are still many interesting tasks in front of the scientific community. One task is to elucidate the multiplicity of EXs in relation to heterogeneous nature of the substrate. This point is particularly important in relation to our effort to strengthen the overall economics of utilization of lignocellulosic biomass. There is challenge for construction of chimeric bifunctional or multifunctional enzymes that would operate efficiently on highly substituted xylans. Specially designed cellulosomes or xylanosomes could be one of the options. Directed evolution studies and environmental genomics could lead to new highly efficient and robust enzymes with desired substrate specificity, thermal stability and optimum operation pH values. New important findings may emerge from studies of the plant-microbial interactions focusing on xylanase toxicity for plants and the presence of xylanase inhibitors in xylanase-treated plant products and plant residues.

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References

1. Gong, C.-S.; Chen, L.F.; Flickinger, M.C.; Tsao, G.T. *Adv. Biochem. Engin.* **1981**, *20*, 93-118.
2. Hahn-Hägerdahl, B.; Jeppsson, H.; Skoog, K.; Prior, B.A. *Enzyme Microb. Technol.* **1994**, *16*, 933-943.
3. Koga, K.; Fujikawa, S. In: *Oligosaccharides: Production, Properties, and Applications*; Nakakuki, T., Ed.; Gordon and Breach Science Publishers, Switzerland, 1993; pp. 130-143.
4. Wong, K.K.Y.; Saddler, J.N. In *Hemicellulose and hemicellulases*; Coughlan, M.P.; Hazlewood, G.P., Eds.; Portland Press, London and Chapel Hill, 1993; pp.127-143.
5. Gilbert, H.J.; Hazlewood, G.P. *J. Gen. Microbiol.* **1993**, *139*, 187-194.
6. Annison, G. *Aust. J. Agric. Res.* **1993**, *44*, 405-422.
7. Annison, G.; Choct, M. In *Biotechnology in the Feed Industry*; Lyons, T. P.; Jacques, K.A., Eds.; Alltech Inc., Nottingham, 1994; pp. 51-66.
8. Bedford, M.R.; Classen, H.L. In *Xylans and Xylanases*; Visser, J.; Beldman, G.; Kusters-van Someren, M.A.; Voragen, A.G.J., Eds. Elsevier Science Publishers, Amsterdam, 1992; p. 361-370.
9. Morgan, A.J.; Graham, H.; Bedford, M.R., In *Enzymes in Animal Nutrition* (Proceedings of the 1st symposium, Kartause, Ittingen, Switzerland). Wenk, C.; Boessinger, M., Eds.; Zurich, 1993; pp. 73-77.
10. McCleary, B. *Inst. J. Biol. Macromol.* **1986**, *8*, 349-354.
11. Maat, J.; Roza, M.; Verbakel, J.; Stam, H.; Santos da Silva, M.J.; Bosse, M.; Egmond, M.R.; Hagemans, M.L.D.; Gorcom, R.F.M.v.; Hessing, J.G.M.; Hondel, C.A.M.J.J.v.d.; Rotterdam, C.v. In *Xylans and Xylanases*; Visser, J.; Beldman, G.; Kusters-van Someren, M.A.; Voragen, A.G.J., Eds. Elsevier Science Publishers, Amsterdam, 1992; p. 349-360.
12. Rouau, X. *J. Cereal Sci.* **1993**, *18*, 145-157.
13. Rouau, X.; Moreau, D. *Cereal Chem.* **1993**, *70*, 626-632.

14. Rouau, X.; El-Hayek, M.-L.; Moreau, D. *J. Cereal Sci.* **1994**, *19*, 259-272.
15. Autio, K.; Härkönen, H.; Aman, P.; Parkkonen, T.; Frigard, T.; Siika-aho, M.; Poutanen, K. *Lebensmittel, Wissenschaft und Technologie* **1996**, *29*, 18-27.
16. Monfort, A.; Blasco, A.; Prieto, J.A.; Sanz, P. *Appl. Environ. Microbiol.* **1996**, *62*, 3712-3715.
17. Biely, P. *Trends Biotechnol.* **1985**, *3*, 286-290.
18. Viikari, L.; Kantelinen, A.; Sundquist, J.; Linko, M. *FEMS Microbiol. Rev.* **1994**, *113*, 335-350.
19. Buchert, J.; Salminen, J.; Siika-aho, M.; Ranua, M.; Viikari, L. *Holzforchung* **1993**, *47*, 473-478.
20. Kirk, T.K.; Jeffries, T.W. *ACS Symp. Series* **1996**, *655*, 2-14.
21. Baipai, P. *Biotechnol. Prog.* **1999**, *15*, 147-157.
22. Paice, M.G.; Jurasek, L. *J. Wood Chem. Technol.* **1984**, *4*, 187-198.
23. Christov, L.P.; Prior, B.A. *Biotechnol. Prog.* **1997**, *13*, 695-698.
24. Milagres, A.M.F.; Prade, R.A. *Enzyme Microb. Technol.* **1994**, *15*, 627-632.
25. Baipai, P. *Adv. Appl. Microbiol.* **1996**, *45*, 242-269.
26. Dekker, R.F.H.; Richards, G.N. *Adv. Carbohydr. Chem. Biochem.* **1976**, *32*, 277-352.
27. Reilly, P.J. *Basic Life Sc.* **1981**, *18*, 11-129.
28. Eriksson, K-E.L.; Blanchette, R.A.; Ander, P., In *Microbial and Enzymatic Degradation of Wood and Wood Components*; Timell, T.E., Ed.; Springer-Verlag, Berlin, 1990; pp. 181-224.
29. Coughlan, M.P.; Hazelwood, G.P. *Biotechnol. Appl. Biochem.* **1993**, *17*, 259-289.
30. Uffen, R.L. *J. Ind. Microbiol. Biotechnol.* **1997**, *19*, 1-6.
31. Prade, R.A. *Biotechnol. Gen. Engin. Rev.* **1995**, *13*, 101-131.
32. Kulkarni, N.; Shendye, A.; Rao, M. *FEMS Microbiol. Rev.* **1999**, *23*, 411-456.
33. Beg, Q.K.; Kapoor, M.; Mahajan, L.; Hoondal, G.S. *Appl. Microbiol. Biotechnol.* **2000**, *56*, 326-338.
34. *Xylans and Xylanases*; Visser, J.; Beldman, G.; Kusters-van Someren, M.A.; Voragen, A.G.J., Eds.; Elsevier Science Publishers, Amsterdam, 1992; 576 pages.
35. *Hemicellulose and Hemicellulases*; Coughlan, M.P.; Hazelwood, G.P., Eds. Portland Press, London, 1993; 152 pages.
36. Timmel, T.E. *Wood Sci. Technol.* **1967**, *1*, 45-70.
37. Wilkie, K.C.B. *Adv. Carbohydr. Chem. Biochem.* **1979**, *36*, 215-264.
38. Wilkie, K.C.B. *Chem. Tech.* **1983**, *13*, 306-319.

39. Joseleau, J.P.; Comtat, J.; Ruel, K. In *Xylans and Xylanases*; Visser, J.; Beldman, G.; Kusters-van Someren, M.A.; Voragen, A.G.J., Eds.; Elsevier Science Publishers, Amsterdam, 1992; p. 1-15.
40. Puls, J.; Schuseil, J. In *Hemicellulose and Hemicellulases*; Coughlan, M.P.; Hazlewood, G.P., Eds.; Portland Press, London, 1993; pp. 1-27.
41. Chesson, A. In *Biotechnology in the Feed Industry*; Lyons, T.P.; Jacques, K.A., Eds.; Alltech Inc., Nottingham, 1994; pp. 83-98.
42. Watanabe, T.; Koshijima, T. *Agric. Biol. Chem.* **1988**, *52*, 2953-2955.
43. Biely, P.; MacKenzie, C.R.; Puls, J.; Schneider, H. *Bio/Technology* **1986**, *4*, 731-733.
44. Coughlan, M.P.; Tuohy, M.G.; Filho, E.X.F.; Puls, J.; Clayessens, M.; Vršanská, M.; Hughes, M.H. In *Hemicellulose and Hemicellulases*; Coughlan, M.P.; Hazlewood, G.P., Eds.; Portland Press, London, 1993; pp. 53-84.
45. Kormelink, F.J.M.; Voragen, A.G.J. *Appl. Microbiol. Biotechnol.* **1993**, *38*, 688-695.
46. de Vries, R.P.; Kester, H.C.M.; Poulsen, C.H.; Benen, J.A.E.; Visser, J. *Carbohydr. Res.* **2000**, *327*, 401-410.
47. Wong, K.K.Y.; Tan, L.U.L.; Saddler, J.N. *Microbiol. Rev.* **1988**, *52*, 305-317.
48. Gilkes, N.R.; Claeysens, M.; Aebersold, R.; Henrissat, B.; Meinke, A.; Morrison, H.; Kilburn, D.G.; Warren, R.A.J.; Miller-Jr., R.C. *Eur. J. Biochem.* **1991**, *202*, 367-377.
49. Gilkes, N.R.; Henrissat, B.; Kilburn, D.G.; Miller-Jr., R.C.; Warren, R.A.J. *Microbiol. Rev.* **1991**, *55*, 303-315.
50. Henrissat, B.; Bairoch, A. *Biochem. J.* **1993**, *293*, 781-788.
51. Henrissat, B.; Bairoch, A. *Biochem. J.* **1996**, *316*, 695-696.
52. Gaboriaud, C.; Bissey, V.; Benchetrit, T.; Mornon, J.P. *FEBS Lett.* **1987**, *224*, 149-155.
53. Keen, N.T.; Boyd, B.; Henrissat, B. *Mol. Plant-Microbe Interact.* **1996**, *9*, 651-657.
54. Hurlbert, J.; Preston III, J.F. *J. Bacteriol.* **2001**, *183*, 2093-2100.
55. Suzuki, T.; Ibata, K.; Hatsu, M.; Takamizawa, K.; Kawai, K. *J. Ferment. Bioengin.* **1997**, *84*, 86-89.
56. Vršanská, M.; Tenkanen, M.; Siika-aho, M.; Saloheimo, M.; Biely, P. *Book of Abstracts, 11th Eur. Carbohydr. Symp.*, Lisabon, Portugal, 2001; p.386.
57. Tenkanen, M.; Siika-aho, M.; Saloheimo, M.; Vršanská, M.; Biely, P. *Abstracts of the 223rd American Chemical Society Meeting*, Orlando, Florida, 2002.
58. Van Petegem, F.; Collins, T.; Meuwis, M.A.; Gerday, C.; Feller, G.; VanBeeumen, J. *Acta Crystallogr. D, Biol. Crystallogr.* **2002**, *58*, 1494-1496.

59. Collins, T.; Meuwis, M.A.; Stals, I.; Claeysens, M.; Feller, G.; Gerday, C. *J. Biol. Chem.* **2002**, *277*, 35133-35139.
60. Kusakabe, I.; Ohgushi, S.; Yasui, T.; Kobayashi, T. *Agr. Biol. Chem.* **1983**, *47*, 2713-2723.
61. Campbell, R.L.; Rose, D.R.; Wakarchuk, W.W.; To, R.; Sung, W.; Yaguchi, M. In *Trichoderma Cellulases and Other Hydrolases*; Pouminen, P.; Reinikainen, T. Eds.; Fagepaino Oy, Helsinki, 1993; pp. 63-72.
62. Wakarchuk, W.; Campbell, R.L.; Sung, W.L.; Davoodi, J.; Yaguchi, M.. *Protein Science* **1994**, *3*, 467-475.
63. Törrönen, A.; Harkki, A.; Rouvinen, J. *EMBO Journal* **1994**, *13*, 2493-2501.
64. Dean, J.F.D.; Anderson, J.D. *Plant Physiol.* **1991**, *95*, 316-323.
65. Grabski, A.C.; Jeffries, T.W. *Appl. Environ. Microbiol.* **1991**, *57*, 987-992.
66. Derewenda U.; Swenson, L.; Green, R.; Wei, Y.; Morosoli, R.; Shareck, F.; Kluepfel, D.; Derewenda, Z.S. *J. Biol. Chem.* **1994**, *269*, 20811-20814.
67. Harris, G.W.; Jenkins, J.A.; Connerton, I.; Cummings, N.; Lo Leggio, L.; Scott, M.; Hazlewood, G.P.; Laurie, J.I.; Gilbert, H.J.; Pickersgill, R.W. *Structure* **1994**, *2*, 1107-1116.
68. Dominguez, R.; Souchon, H.; Spinelli, S.; Dauter, Z.; Wilson, K.S.; Chauvaux, S.; Beguin, P.; Alzari, A.M. *Nature Struct. Biol.* **1995**, *2*, 569-576.
69. Schmidt, A.; Schlacher, A.; Steiner, W.; Schwab, H.; Kratky, C. *Protein Science* **1998**, *7*, 2081-2088.
70. Jenkins, J.A.; Legio, L.L.; Harris, G.; Pickersgill, R. *FEBS Lett.* **1995**, *362*, 281-285.
71. Törrönen, A.; Kubicek, C.P.; Henrissat, B. *FEBS Lett.* **1993**, *321*, 135-139.
72. Coutinho, P.M.; Henrissat, B. In *Recent Advances in Carbohydrate Bioengineering*; Gilbert, H.J.; Davies, G.J.; Henrissat, B.; Svensson, S., Eds.; Royal Society of Chemistry, Cambridge, 1999; pp. 3-12.
73. Boraston, A.B.; McLean, B.W.; Kormos, J.M.; Alam, M.; Gilkes, N.R.; Haynes, C.A.; Tomme, P.; Kilburn, D.G.; Warren, R.A.J. In *Recent Advances in Carbohydrate Bioengineering*; Gilbert, H.J.; Davies, G.J.; Henrissat, B.; Svensson, S., Eds.; Royal Society of Chemistry, Cambridge, 1999; pp. 202-211.
74. Williamson, M.P.; Simpson, P.J.; Bolam, D.N.; Hazlewood G.P.; Ciruela, A.; Cooper, A.; Gilbert, H.J. In *Recent Advances in Carbohydrate Bioengineering*; Gilbert, H.J.; Davies, G.J.; Henrissat, B.; Svensson, S., Eds.; Royal Society of Chemistry, Cambridge, 1999; pp. 212-220.
75. McAllister, K.; Marrone, L.; Clarke, A.J. In *Recent Advances in Carbohydrate Bioengineering*; Gilbert, H.J.; Davies, G.J.; Henrissat, B.; Svensson, S., Eds.; Royal Society of Chemistry, Cambridge, 1999; pp. 89-98.

76. Dupond, C.; Roberge, M.; Shareck, F.; Morosoli, R.; Kluepfel, D. *Biochem. J.* **1998**, *330*, 41-45.
77. Charnock, S.J.; Bolam, D.N.; Turkenburg, J.P.; Gilbert, H.J.; Ferreira, L.M.A.; Davies, G.J.; Fontes, C.M.G.A. *Biochemistry* **2000**, *39*, 5013-5021.
78. Sunna, A.; Gibbs, M.G.; Bergquist, P.L. *Microbiology* **2000**, *146*, 2947-2955.
79. Gill, J.; Rixon, J.E.; Bolan, D.N.; McQueen-Maasn, S.; Simpson, P.J.; Williamson, M.P.; Hazlewood, G.P.; Gilbert, H.J. *Biochem. J.* **1999**, *342*, 473-480.
80. Flint, H.J.; Forsberg, C.F. In *Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction*; Engelhardt, W.V.; Leonhard-Merek, S.; Breves, G.; Giesicke, D., Eds.; F. Enke Verlag, Stuttgart, 1995; pp. 43-70.
81. Zhang, J.X.; Flint, H.J. *Mol. Microbiol.* **1992**, *6*, 1013-1023.
82. Gilbert, H.J.; Hazlewood, G.P.; Laurie, J.I.; Orpin, C.G.; Xue, G.P. *Mol. Microbiol.* **1992**, *6*, 2065-2072.
83. Laurie, J.I.; Clarke, J.H.; Ciruela, A.; Faulds, C.B.; Williamson, G.; Gilbert, H.J.; Rixon, J.E.; Millward-Sadler, J.; Hazlewood, G.P. *FEMS Microbiol. Lett.* **1997**, *148*, 261-264.
84. Fernandes, A.C.; Fontes, C.M.G.A.; Gilbert, H.J.; Hazlewood, G.P.; Fernandes, T.H.; Ferreira, L.M.A. *Biochem. J.* **1999**, *342*, 105-110.
85. Hayashi, H.; Takehara, M.; Hattori, T.; Kimura, T.; Karita, S.; Sakka, K.; Ohmiya, K. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 348-357.
86. Shoham, Y.; Lamed, R.; Bayer, E.A. *Trends Microbiol.* **1999**, *7*, 275-281.
87. Sunna, A.; Antranikian, G. *Crit. Rev. Biotechnol.* **1997**, *17*, 39-67.
88. Biely, P.; Kluepfel, D.; Morosoli, R.; Shareck, F. *Biochim. Biophys. Acta* **1993**, *1162*, 246-254.
89. Biely, P.; Vršanská, M.; Tenkanen, M.; Kluepfel, D. *J. Biotechnol.* **1997**, *57*, 151-166.
90. Biely, P.; Vršanská, M.; Kremnický, L.; Tenkanen, M.; Poutanen, K.; Hayn, M. In *Trichoderma Cellulases and Other Hydrolases*; Souminen, P.; Reinikainen, T., Eds.; Fagepaino Oy, Helsinki, 1993; pp. 125-135.
91. Kormelink, F.J.M.; Searle-van Leewen, M.J.F.; Wood, T.M.; Voragen A.G.J. *J. Biotechnol.* **1993**, *27*, 249-265.
92. Biely, P.; Vršanská, M. *Eur. J. Biochem.* **1983**, *129*, 645-561.
93. Chen, W.P.; Matuo, M.; Yasui, T. *Agric. Biol. Chem.* **1986**, *50*, 1195-1200.
94. van Tilbeurgh, H.; Claeysens, M. *FEBS Lett.* **1985**, *187*, 283-288.
95. Biely, P.; Vršanská, M.; Bhat, M.K. In *Carbohydrases from Trichoderma and Other Microorganisms: Structure, Biochemistry, Genetics*;

- Claeyssens, M.; Nerinckx, W.; Piens, K., Eds.; Royal Society of Chemistry, Cambridge, 1998; pp. 94-101.
96. Biely, P.; Vršanská, M.; Krátký, Z. *Eur. J. Biochem.* **1980**, *112*, 375-381.
 97. Ntarima, P.; Nerinckx, W.; Klarskov, K.; Devreese, B.; Bhat, M.K.; van Beeumen, J.; Claeyssens, M. *Biochem. J.* **2000**, *347*, 865-873.
 98. Gebler, J.; Gilkes, N.R.; Claeyssens, M.; Wilson, D.B.; Beguin, P.; Wakarchuk, W.W.; Kilburn, D.G.; Miller Jr., R.C.; Warren, R.A.; Withers, S.G. *J. Biol. Chem.* **1992**, *267*, 12559-12561.
 99. Biely, P.; Kremnický, L.; Alföldi, J.; Tenkanen, M. *FEBS Lett.* **1994**, *356*, 137-140.
 100. Sinnott, M.L. *Chemical Reviews* **1990**, *90*, 1171-1202.
 101. Withers, S.G. *Pure & Appl. Chem.* **1995**, *67*, 1673-1682.
 102. Biely, P.; Vršanská, M.; Krátký, Z. *Eur. J. Biochem.* **1981**, *119*, 565-571.
 103. Biely, P.; Vršanská, M.; Gorbacheva, I.V. *Biochim. Biophys. Acta* **1982**, *704*, 114-122.
 104. Heightman, T.D.; Vasella, A. *Angew. Chem., Int. Ed.* **1999**, *38*, 750-770.
 105. Hakansson, U.; Fagerstam, L.G.; Pettersson, G.; Anderson, L. *Biochem. J.* **1979**, *179*, 141-149.
 106. Shomaker, S.; Watt, K.; Tsisovsky, G.; Cox, R. *Bio/Technology* **1983**, *1*, 687-690.
 107. Biely, P.; Vršanská, M.; Claeyssens, M. *Eur. J. Biochem.* **1991**, *200*, 157-163.
 108. Banik, M.; Garrett, T.; Fincher, G. *Plant Mol. Biol.* **1996**, *31*, 1163-1172.
 109. Bih, F.Y.; Wu, S.S.H.; Ratnayake, C.; Walling, L.L.; Nothnagel, E.A.; Huang, A.H.C. *J. Biol. Chem.* **1999**, *274*, 22884-22894.
 110. Debyser, W.; Derdelinckx, G.; Delcour, J.A. *J. Am. Soc. Brew. Chem.* **1997**, *55*, 153-156.
 111. Gebruers, K.; Debyser, W.; Goesaert, H.; Proost, P.; Van Damme, J.; Delcour, J.A. *Biochem. J.* **2001**, *353*, 239-244.
 112. McLauchlan, W.R.; Garcia-Conesa, M.T.; Williamson, G.; Rosa, M.; Ravenstein, P.; Maat, J. *Biochem. J.* **1999**, *338*, 441-446.
 113. Flatman, R.; McLauchlan, W.R.; Juge, N.; Furniss, C.; Berrin, J.-G.; Hughes, R.K.; Manzanares, P.; Ladbury, J.E.; O'Brien, R.; Williamson, G. *Biochem. J.* **2002**, *365*, 773-781.
 114. Clarke, J.H.; Rixon, J.E.; Cirula, A.; Gilbert, H.J.; Hazlewood, G.P. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 177-183.
 115. Christov, L.; Biely, P.; Kalogeris, E.; Christakopoulos, P.; Prior, B.A.; Bhat, M.K. *J. Biotechnol.* **2000**, *83*, 231-244.
 116. Buchert, J.; Ranua, M.; Kantelinen, A.; Viikari, L. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 825-829.
 117. Sunna, A.; Moracci, M.; Rossi, M.; Antranikian, G. *Extremophiles* **1997**, *1*, 2-13.

Chapter 22

Fusion Proteins Containing *Coprinus cinereus* Peroxidase and the Cellulose-Binding Domain of *Humicola insolens* Family 45 Endoglucanase

Feng Xu¹, Aubrey Jones¹, Michael H. Lamsa¹,
Claus C. Fuglsang², Lars S. Conrad², Jesper V. Kierulff²,
Stephen H. Brown¹, and Joel R. Cherry^{1,*}

¹Novozymes Biotech Inc., 1445 Drew Avenue, Davis, CA 95616

²Novozymes A/S, Bagsvaerd 2800, Denmark

Two fusion proteins were constructed to attach the cellulose-binding domain of *Humicola insolens* family 45 endo-1,4-glucanase, via the linker of the endoglucanase, to *Coprinus cinereus* heme peroxidase. One fusion protein utilized the wild type peroxidase, and another utilized a peroxidase mutant resistant to alkaline pH, high temperature, and H₂O₂. The fusion proteins were expressed in *Aspergillus oryzae* and characterized. Their peroxidase activity was similar to that of the donor peroxidase, but their cellulose-binding affinity was 100-fold less than the donor endoglucanase. Neutralizing the surface negative charge enhanced their affinity to cotton fabric. Although the fusion proteins could bind to cellulose, they failed to oxidize cellulose-adsorbed dyes that were their substrates in dissolved state.

Introduction

Efficient hydrolysis of insoluble, polymeric carbohydrates is essential for many organisms that rely on the biomaterials as energy/nutrient source. To accomplish the task, microbes build multi-module carbohydrate hydrolases or assemble multi-protein cellulolytic cellulosomes (1-4). Besides the catalytic core, one or more polysaccharide-binding modules are often incorporated into the holoenzyme/ensemble. Many cellulases have so-called cellulose-binding domains (CBD) capable of anchoring the enzymes tightly to insoluble cellulose to facilitate the hydrolysis. In general, CBDs are 4 to 20 kD in size, locate either terminally or internally in parental proteins, and bind cellulose tightly with μM^{-1} range affinity. The 3-D structures of CBD belonging to different families (including the fungal family 1) have been determined, and more than 200 CBD sequences belonging to 13 families are known (2, 5-9).

As a discrete protein module, CBD isolated from the parental hydrolase or cellulosome scaffoldin can maintain its cellulose-binding capability. Such a function of the CBD is being exploited as a powerful biotechnology/protein-engineering tool for a wide range of applications (2, 10, 11). In comparison to other industrial/medicinal matrix materials, cellulose holds many highly desirable properties, such as human safety, environment-friendliness, low cost, inertness to many agents, and diverse physical states (powder, membrane, fabric, bead, hydrogel, solution, etc). Benefiting from the excellent properties of cellulose, CBD-tagging can allow a protein/biomolecule/cell to be: (1) readily isolated through cellulose-affinity chromatography (2, 11); (2) immobilized to cellulose-based bioreactor/display/microarray/matrix (12, 13); (3) transported by cellulosic carrier (14); or (4) transformed to a heterogeneous biocatalyst for cellulose-adsorbed substrates. Various CBD fusions involving more than 20 proteins and biomolecules have been constructed for the first three applications (2, 15-18, 38).

Oxidoreductases, including peroxidases, have great potential for a wide range of applications, such as pulp delignification, polysaccharide degradation, biopolymerization, biosynthesis, fuel desulfurization, bioremediation, textile colorization, and biosensor/diagnostics (19, 20). Equipping these enzymes with cellulose-binding capability could tremendously improve/expand their biocatalytic function. Recently glucose oxidase has been chemically linked to a bacterial CBD, and horseradish peroxidase has been immobilized to cellulose through the interaction between a tripeptide affinity tag and cellulose-bound CBD (2, 21). In this report, we describe the construction of two fusion proteins from a family 1 fungal CBD and a fungal heme-containing peroxidase. The 37 amino acid CBD and the 35 amino acid linker are from family 45 *Humicola insolens* endo-1,4-glucanase (HiC45) (22, 23, 39, 40). The peroxidase is from

Coprinus cinereus (CiP) (24-26). One fusion protein, CBD-CiP, involved the wild type CiP. Another fusion protein, CBD-mCiP, involved an alkaline/thermal-stable, H₂O₂-resistant mutant (mCiP) having the mutations V53A, E239G, M242I, Q270R, and Y272F (27, 28). We intended to test the construction as the first example of CBD-peroxidase fusion proteins as well as engineered oxidoreductases able to activate insoluble, cellulose-adsorbed substrates.

Experimental Methods

Materials and instruments

Chemicals used were commercial products of at least reagent grade. Britton and Robinson (B&R) buffer was made by mixing 0.1 M phosphoric acid, 0.1 M acetic acid, and 0.1 M boric acid with 0.5 M NaOH to desired pH. Purified HiC45 was obtained as previously described (23). The CBD from HiC45 was obtained by digesting HiC45 overnight with Alcalase (from Novozymes), followed by C18 reverse-phase chromatography purification. Congo Red-adsorbed Avicel (~0.14 mmol/g) was prepared by mixing 5 g of Avicel with 27 mL of 27 mM dye for 20 min, followed by exhaustive washing of the dyed pellets with water (via cycles of suspension and centrifugation until the supernatant showed a ~0.06 absorption at 486 nm at 1 cm pathlength) and drying (180 °C, 4 days). Cotton fabrics (stoned-washed/desized Dakota denim, white Veitex, bleached/desized plain cotton Style #400, black currant-stained #400) were from Novozymes' textile research laboratory.

Chromatography was performed on a Pharmacia FPLC. Ultrafiltration/diafiltration was performed using an Amicon Spiral Concentrator and an H1-P30-43 hollow fiber cartridge. Electrophoresis was performed with a Bio-Rad Minicell apparatus and precast gels. Spectral data were recorded on either a Shimadzu UV160U spectrophotometer with 1-cm quartz cuvette, or a Molecular Devices Thermomax microplate reader with Costar 96-well microplate. N-terminal amino acid sequencing was performed on an Applied Biosystems 476A Protein Sequencer, with either purified sample or electroblotted PVDF membrane. DNA sequencing was performed on an Applied Biosystems 377 DNA sequencer. Oligonucleotides were synthesized by an Applied Biosystems 294 DNA/RNA synthesizer. The protocols for molecular biology experiments (including restriction digests, DNA ligations, gel electrophoresis, and DNA preparations) were adapted from either the instructions of the manufacturer or standard procedures. Radioactive decay was

measured using a HP 1900 TR liquid scintillation analyzer and OptiPhase scintillation liquid. Bleaching of denim was assayed by a Minolta ChromaMeter CR 300 with a CIE standard C light source.

Enzyme assays

Specific peroxidase activity was assayed at 30 °C with 0.1 M Na-phosphate of pH 7, 0.9 mM H₂O₂, and 1.7 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), by monitoring the absorption increase at 418 nm. The molar absorptivity of 36 mM⁻¹cm⁻¹ and a stoichiometry of 2 were used to calculate the rate. For selecting *S. cerevisiae* transformants, a nitrocellulose or cellulose acetate filter with bound CBD-CiP was coated with a solution of 100 mM Na-phosphate, pH 7, 50 mg/L diaminobenzidine, and 1 mM H₂O₂. A brown precipitate appeared on the filter due to the peroxidase activity where positive.

Oxidation of soluble Congo Red was monitored at 486 nm in a 96-well microplate with 0.1 mL solutions containing 45 mM Na-phosphate, pH 7, 0.25 mM H₂O₂, 1 to 160 nM peroxidase, and 88 μM dye (yielding an initial absorption of ~1 at 486 nm). Oxidation of Avicel-adsorbed Congo Red was studied in 0.65-mL tubes with 0.13 mL suspensions containing 0.5 mg dyed Avicel, 0.24 or 2.6 or 70 mM H₂O₂, and 0.14 or 1.5 μM peroxidase, under gentle shaking at 20 °C. Solutions/suspensions without peroxidase, H₂O₂, or peroxidase/H₂O₂ served as controls.

Denim bleaching was tested on two pieces of fabric (12 g each) soaked in 0.24 L solution containing 5.5 mM K-phosphate, pH 6.5, 4 nM peroxidase, 1 mM H₂O₂, 0 or 0.1 mM 10-phenothiazine-propionic acid. The reaction was carried out at 30 °C for 30 min. The fabric discoloration was measured by a Minolta Chroma Meter in the L*a*b* color space (L*: change in black (0)/white (100), a*: change in green (-)/red (+), b*: change in blue (-)/yellow (+)) and calculated as the difference, Δ(L*a*b*), before and after the bleaching.

Thermal stability was assayed by first incubating 0.02 g/L protein in B&R buffer of pH 10.5 at 50 °C for 20 min, followed by measuring the oxidation of 1.4 mM ABTS with 1 mM H₂O₂ in 0.1 M Na-phosphate of pH 7. Resistance to H₂O₂ was assayed by including 0.2 mM H₂O₂ in the incubation solution.

Cellulose binding

Avicel, denim, Style #400 fabric, and black currant-stained Style #400 fabric were used to study the binding by CBD-CiP. For black currant-stained fabric, the cloth was washed, prior to use, until no brown color was released.

Cellulose-binding was studied in 5.5 mM Na-phosphate, pH 7, with 0.2 mg/L protein and 100 g/L cellulosic material; or in 10 mM Tris, 5 mM CaCl₂, pH 7.6, with 0.6 mg/L peroxidase and 250 g/L cellulosic material. The incubation was carried out in either glass vials (2-mL) or plastic tubes (1.7-mL) with gentle shaking at 20 °C for 15-90 min. After incubation, the supernatant was assayed by peroxidase activity and SDS-PAGE.

The binding was also measured with ¹⁴C-labelled proteins. Incubations were made in 5 mM Na-phosphate, pH 7, 0.1 µg/L protein, and 100 g/L cellulosic materials at 30 °C for 30 min. The abundance of the soluble protein was quantitated by the disintegration per min reading relative to that in solution prior to the mixing with the cellulosic materials.

Langmuir isotherms of HiC45 binding by cellulose were established by incubating ¹⁴C-labelled HiC45 with pre-swollen Avicel or Veitex fabric in 50 mM Na-phosphate of pH 7 or 50 mM B&R buffer of pH 10 for 30 min. Binding equilibrium was reached within 5 min for both Avicel and Veitex. Data were fitted, by non-linear regression, to Langmuir equation $B = B_m P_e / (P_e + 1/K_a)$ or $P_e^2 - (P_i - 1/K_a - S_i B_m) P_e - P_i / K_a = 0$, in which P_i and P_e were the initial and equilibrated protein amount in solution, respectively, S_i was the initial cellulose amount, B and B_m were the equilibrium and maximal binding capacity, respectively, and K_a was the affinity constant (29).

Chemical modification

Solvent-accessible carboxyl groups of CiP or CBD-CiP were amidated by glycine methyl ester according to a published method (30). For instance, 0.31 µmoles (21 mg in 1 mL) CBD-CiP, previously dialyzed in 2 L H₂O, was mixed with 10 mmoles glycine methyl ester dissolved in 9 mL H₂O with pH adjusted to 4.8. One mmole 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide was added and the pH was maintained at 4.75 by adding HCl. After 2 h and the addition of 12 µmoles HCl, the pH was stabilized and the reaction stopped. The modified protein, CBD-CiP_{cm}, was dialyzed in H₂O, concentrated, and buffer-exchanged (on a B io-Rad B ioSpin P 6 c column) to 0.1 M Na-phosphate, pH 7. Based on their structure, CiP and CBD-CiP had 37 and 39 -COOH.

Radioactive labeling of desalted proteins was performed with ¹⁴CH₂O, through their exposed lysines and N-termini, in NaBH₃CN containing 50 mM Na-phosphate, pH 7, by a reductive methylation (31). After 4 h of incubation at room temperature, the labeled protein was purified on a Pharmacia Superdex 75 column, and the specific labeling was established by co-precipitating the protein with 1 % bovine serum albumin in cold 20 % trichloroacetic acid.

Construction of fusion protein genes

The CBD-CiP fusion was constructed by amplifying four separate gene fragments using PCR: (1) The CiP 5'-untranslated region and the region encoding the amino acid sequence 1-22 (for CiP signal), (2) the HiC45 region encoding the amino acid sequence 248-305 (for the CBD), (3) the HiC45 region encoding the amino acid sequence 213-247 (for the linker), and (4) the CiP region encoding the amino acid sequence 21-344 (for mature CiP). The primers used in the four amplifications were: (1a) 5'CCCCCTTCCCTGGCGAATTCCGCATGAGG3', (1b) 5'ACCTTGGGGTAGAGCGAGGGCACCGATG3', (2a) 5'TGCACTGCTGAGAGGTGGGC3', (2b) 5'CAGGCACTGATGATACCAGT3', (3a) 5'CCCTCCAGCAGCACCAGCTCT3', (3b) 5'TCCTCCAGGACCCTGACCGCTCGGAGTCGTAGGCTG3', (4a) 5'TACGACTCCGAGCGGTCAGGGTCCTGGAGGAGGCCGGG3', and (4b) 5'GGGAGGGCGTGAATGTAAG3'. The amplified fragments (1) and (2) were purified, phosphorylated by T4 polynucleotide kinase, ligated to one another (for 15 min at room temperature), and amplified with primers 1a and 2b to generate the ligated fragment (1+2). Similarly, the amplified fragments (3) and (4) were ligated to (3+4), and the fragments (1+2) and (3+4) were ligated to generate the fused gene. The gene was integrated into plasmid JC106 (27, 28), in which the CiP gene was previously removed by digestion with *Bam*HI and *Xho*I. The resulting plasmids contained the CiP or H₂O₂-resistant CiP variant gene, GAL1 promoter and *URA3* marker.

To express CBD-CiP in *Aspergillus oryzae*, the synthetic genes were subcloned from pJC106 to an *A. oryzae* expression vector. This was accomplished by digesting pJC106 with *Bam*HI followed by blunt ending with Klenow, then digesting with *Not*I, and finally subcloning into the *A. oryzae* vector as a *Swa*I and *Not*I fragment. The resulting vectors, pAJ11 and pAJ12, contained maltose-inducible *A. oryzae* α -amylase (TAKA) promoter, CBD-CiP or CBD-mCiP coding region, *A. niger* glucoamylase (AMG) terminator, and *amdS* selectable marker (Figure 1).

Heterologous expression

S. cerevisiae transformation was carried out as previously reported (27, 28). Transformants were grown on minimal URA⁻ media plates containing 2% galactose (to induce the GAL1 promoter). The plates were covered with a nitrocellulose filter topped by a cellulose acetate filter. After growth for 48 to 72 h, both filters were washed twice with 100 mL of 20 mM Na-phosphate buffer,

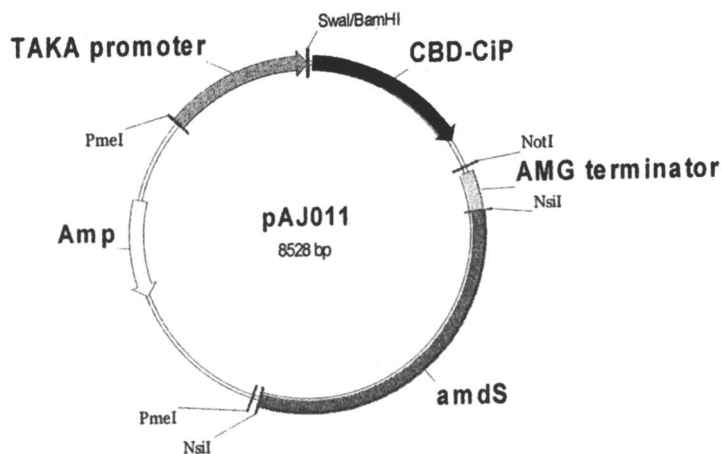


Figure 1. Vector expressing CBD-CiP. For CBD-mCiP-expressing pAuJo12, the coding sequence of CiP was replaced by that of mCiP.

pH 7 to remove colony debris, and then assayed by the diaminobenzidine assay. The liquid cultures of selected transformants were grown overnight in minimal media containing 2% galactose.

pAJ011 and pAJ012 (10 μ g each) were used to transform an *A. oryzae* recipient strain JaL142 (from J. Lehmbeck of Novozymes) using a previously described method (32) with two modifications: protoplasting was made with 10 g/L Novozyme 234 in the absence of bovine serum albumin; 1 mL of “SPTC” solution (0.8 M sorbitol, 40% polyethylene glycol 4000, 50 mM Tris, pH 8, 50 mM CaCl_2) was used to replace the PEG solution. Transformants were selected on COVE media (20 mL/L COVE salts (26 g/L KCl, 26 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 76 g/L KH_2PO_4 , 50 mL/L COVE trace elements (0.04 g/L $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.4 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.2 g/L $\text{FeSO}_4 \cdot \text{H}_2\text{O}$, 0.7 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.8 g/L $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$, 10 g/L $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$)), 1 M sucrose, 25 g/L Noble agar, and 20 mM acetamide). Selected spores were inoculated in 24-well plates containing 1 mL “half-strength” MY50 medium (1 \times MY salts (2g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2g/L K_2PO_4 , 10g/L KH_2PO_4 , 2g/L citric acid, 0.5 mL/L AMG trace elements (13.9 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 8.5 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 14.28 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.63 g/L CuSO_4 , 0.24 g/L $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 3.0 g/L citric Acid)), 5% maltodextrin, 1% yeast extract, 0.2% urea,

pH 6.5). Cultures were grown for three days at 30 °C under 100 rpm shaking before being assayed for CiP activity. The most active transformants were spore purified and grown in a 2-L fermentation under conditions similar those previously reported (27, 33).

Protein purification

The purification scheme comprised ultrafiltration and anion-exchange chromatography. The cell-free broth (pH 7.7, 11 mS conductivity) was filtered with Whatman #2 paper and ultrafiltered with a polysulfone membrane (30 kD molecular weight (MW) cutoff). The washed and concentrated broth (pH 7.7, 1 mS) was then loaded onto a Q-Sepharose column pre-equilibrated with 10 mM Tris-HCl, pH 7.6, and 5 mM CaCl₂ (Buffer A). The active fraction eluted by 5% Buffer B (Buffer A plus 2 M NaCl) was washed (with 5 mM CaCl₂) to 1 mS, then applied to a Mono-Q column equilibrated with Buffer A. Buffer B was used again for the elution. Fractions were analyzed by the peroxidase assay and SDS-PAGE.

Results

Construction and expression of CBD-CiP

In HiC45, the N-terminus of the CBD is linked to the C-terminus of the linker, whose N-terminus is in turn linked to the C-terminus of the catalytic domain (22). We tried to replace the catalytic domain of HiC45 with CiP to produce a "CiP-CBD" fusion, with the CBD fused (via the linker) to the C-terminus of CiP. However, no catalytically active CiP-CBD was observed. Because the C-terminus of CiP is in intimate contact with the protein surface (25), an attachment of HiC45's linker-CBD (~20 kD) might disrupt the polypeptide conformation, leading to an inactivation.

We connected the N-terminus of CiP to the C-terminus of HiC45's linker, whose N-terminus was in turn linked to the C-terminus of HiC45's CBD, in a reversed arrangement to that in HiC45. Two genes were constructed to encode two fusion proteins composed of four discrete domains: CiP signal sequence, HiC45 CBD, HiC45 linker, mature CiP or a H₂O₂-resistant mutant mCiP (Figure 2). The first two N-terminal amino acid residues (Gln-Gly) of the mature CiP were placed between the CiP signal and HiC45 CBD to prevent any "abnormal" signal cleavage that might be detrimental to the integrity of the CBD. N-terminal

M K L S L L S

BamHI

1 GGATCCACTA GTAACGGCG CCAGTGTGCT CTAAGACTA TGAAGCTCTC GCTTTTGTC
 T F A A V I I G A L A L P Q G C T A E R
 ----- *Cip* Signal Sequence -----<>

61 ACCTTGCTG CTGTCAATCAT CGGTGCCCTC GCTTACCCCG AGGGTTGCAC TGCTGAGAGG
 W A Q C G G N G W S G C T T C V A G S T
 ----- *Hic45 CBD* -----

121 TGGGTCAGT GGGGGGCAA TGGTGGAGC GGCTGCACCA CCTGCGTGGC TGGCAGCACT
 C T K I N D W Y H Q C L P S S T S S P
 -----<>-----

181 TGCAGAAGA TTAATGACTG GTACCATCAG TGCCTGCCCT CCAGCAGCAC CAGCTCTCG
 V N Q P T S T S T T S T S T T S S P P V
 ----- *Hic45 Linker* -----

241 GTCAACCAGC CTACCAGCAC CAGACCAGC TCCACCTCCA CCACCTCGAG CCGGCCAGTC
 Q P T T P S G Q G P G G G S V T C P G
 -----<>-----

301 CAGCCTACGA CTCCCAGCG CCAGGTCTCT GGAGGAGCG GGTCAGTCCAC TTGCCCCCGT
 G Q S T S N S Q C C V W F D V L D D L Q
 ----- *Mature Cip* -----

361 GGACAGTCCA CTTCGAACAG CCAGTGTGC GTCTGGTTG ACGTTCTAGA CGATCTTCAG
 T N F Y Q G S K C E S P V R K I L R I V/A

421 ACCAACTTCT ACCAAGGTC CAAGTGTGAG AGCCCTGTTT GCAAGATTCT TAGAATTGTT
 F H D A I G F S P A L T A A G Q F G G G

481 TTCCATGAG CGATCGGATT TTCCGGCGG TTGACTGCTG CTGGTCAAIT CGGTGGTGA
 G A D G S I I A H S N I E L A F A N G

541 GGAGCTGATG GCTCCATCAT TGGCGATTG AACATCGAAT TGGCCTTCCC GGCTAATGGC
 G L T D T V E A L R A V G I N H G V S F

601 GGCCTCACCG ACACGGTGA AGCCCTCGC GGGTGGTA TCAACCACCG TGCTCTCTTC
 G D L I Q F A T A V G M S N C P G S P R

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661  GGCATCTCA TCCAAATCGC CACTGCCGTC GGCATGTCCA ACTGCCCTGG CTCTCCCGA
    L E F L T G R S N S S Q P S P P S L I P
721  CTTGAGTTCT TGACGGCAG GAGCAACAGT TCCCAACCT CCCCTCCTTC GTTGATCCCC
    G P G N T V T A I L D R M G D A G F S P
781  GGTCCGGAA ACACGGTAC CGCTATCTTG GATCGTATGG GCGATGCAGG CTTCAGCCCT
    D E V V D L L A A H S L A S Q E G L N S
841  GATGAAGTAG TCGACTTGCT TGCTGGCAT AGTTGGGTT CTCAGGAGGG TTTGAATCG
    A I F R S P L D S T P Q V F D T Q F Y I
901  GCCATCTTCA GATCTCCTTT GGACTCGACC CCTCAAGTTT TCGATACCCA GTTCTACATT
    E T L L K G T T Q P G P S L G F A E E L
961  GAGACCTTGC TCAAGGTAC CACTCAGCCT GGCCTTCTC TCGGCTTTGC AGAGGAGTTC
    S P F P G E/G F R M/I R S D A L L A R D S R
1021 TCCCCTTCC CTGGCGAATT CCGCATGAGG TCCGATGCTC TCTGGGCTCG CGACTCCGGA
    T A C R W Q S M T S S N E V M G Q/P R V/F R

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Figure 2. Fusion gene and deduced amino acid sequence of CBD-CiP. A dipeptide (Gln-Gly), corresponding to the N-terminus of matured CiP, was inserted between CiP signal sequence and HiC45 CBD. Experimentally determined N-termini of purified CBD-CiP and its cleaved product are underlined. Boxed are amino acid residue changes in mCiP, made by changing the codons GTT for V53 to GCT, GAA for E239 to GGA, ATG for M242 to ATA, CAG for Q270 to CCG, and TAC for Y272 to TTC.

Continued on next page.

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1081  -----
      A C C G C T G C C   G A T G G C A A T C   C A T G A C C A G C   A C C A A T G A A G   T T A T G G G C C A   G C G A T A C C G C
      A   A   M   A   K   M   S   V   L   G   F   D   R   N   A   L   T   D   C   S
      -----
1141  G C C G C C A T G G   C C A A G A T G T C   T G T T C T C G G C   T T C G A C A G G A   A C G C C C T C A C   C G A T T G C T C T
      D   V   I   P   S   A   V   S   N   N   A   A   P   V   I   P   G   G   L   T
      -----
1201  G A C G T T A T T C   C T T C T G C T G T   G T C C A A C A A C   G C T G C T C C T G   T T A T C C C T G G   T G G C C T T A C T
      V   D   D   I   E   V   S   C   P   S   E   P   F   P   E   I   A   T   A   S
      -----
1261  G T C G A T G A T A   T C G A G G T T T C   G T G C C G A G C   G A G C C T T T C C   C T G A A A T T G C   T A C C G C C T C A
      G   P   L   P   S   L   A   P   A   P
      -----
1321  G G C C C T C C C   C C T C C C T O G C   T C C T G C T C C T   T G A T C T G G T G   A G A T G G T A C   A T C C T G C T C T
1381  C T C A T C A T C C   C T C T A G C T A   T T T A T C C A A T   C T A T C T A C C T   A T C T A T G C A G   T T T C I G T T C T
1441  A T C A C C A C A G   G A A G C A A G A A   A G A A A A C A A   C A A T G C A A C G   T G A C A G A A A   T C A G C A A A A
1501  A A T A A A T C A G   T A T A C T A C A G   T A A T G A G G C C   A G T T T G C G T G   G T G T C A G A A G   T A A G T A C C A C
      /NotI
1561  T C G G C T T T A C   A C A C T G G C G G   C C G C

```

Figure 2. *Continued.*

sequencing showed that the signal was cleaved in the *A. oryzae* host between the Gln and Gly, leading to an intact CBD in the secreted CBD-CiP.

The transformed *S. cerevisiae* colonies were grown on selective plates covered with a cellulose acetate filter topped with a nitrocellulose filter. Secreted CiP and mCiP passed through the cellulose acetate, but bound to the nitrocellulose, whereas CBD-CiP and CBD-mCiP showed significant binding to the cellulose acetate filter, as visualized by the diaminobenzidine assay. The binding by cellulose acetate greatly facilitated the selection of CBD-CiP transformants.

Expression of CBD-CiP in *A. oryzae* was comparable to that of CiP. However, significant cleavage by the host between the CBD/linker and CiP domains was observed. As revealed by SDS-PAGE, the ratio of the intact to the cleaved CBD-CiP was about 2:3 (data not shown).

Purification of the fusion proteins

To avoid potential binding of the fusion protein to cellulosic materials, a polysulfone membrane was used for ultrafiltration, which led to ~90% recovery of the initial peroxidase activity. Q-Sepharose chromatography yielded two active fractions, eluted by 5 and 20% Buffer B. SDS-PAGE showed that the first fraction, containing ~30% recovery of the initial peroxidase activity, was enriched in a 66 kD band, and the second fraction was enriched in a 45 kD band. Mono-Q chromatography of the first fraction led to the purification (to apparent homogeneity) of the 66 kD protein, which did not bind to the column, from the 45 kD protein, which was eluted with 5% buffer B. The final CBD-CiP preparation had a recovery of 13% (of the initial peroxidase activity in broth) and a 12-fold purification. The 45 kD protein had an N-terminus of GPGGGGSVTC-, which matched the 2nd to 11th amino acids in the N-terminus of the mature wild type CiP, indicating a cleavage between the linker and CiP of the fusion protein.

Molecular and enzymatic properties of the fusion proteins

The CBD-CiP showed an N-terminus of GCTAERWAQ-, one residue short of the predicted N-terminus (which would be QG- plus the N-terminus of the CBD). Like CiP, the fusion proteins had a pI of 3.5. On SDS-PAGE, they had a MW of ~66 kD, in comparison to the deduced 43 kD value, likely due to a ~53% glycosylation. The observed and deduced MW of the CiP expressed by the same host was 36 and ~45 kD, respectively, indicating a ~26% glycosylation. The

higher glycosylation extent of CBD-CiP could be attributed to the Thr/Ser-rich linker, well known for its extensive glycosylation in its parental HiC45 (22, 23).

The purified fusion proteins both showed a ratio of the absorption at 405 nm to that at 280 nm equal to 2.2, in comparison to the 2.5 observed for the CiP. The absorption increase at 280 nm for CBD-CiP, relative to CiP, was consistent with the contribution from the Tyr (1), Trp (3), and Cys (6) in the CBD and linker (Figure 2). Thus, the fusion proteins had one heme loaded per protein.

With ABTS as the reducing substrate, the CBD-CiP had a specific activity of 2400 IU/mg or 1.1×10^5 turnover/min, and CBD-mCiP had a specific activity of 2700 IU/mg or 1.2×10^5 turnover/min, in comparison to the 3000 IU/mg or 1.1×10^5 turnover/min value observed for CiP. Thus the fused CBD/linker did not affect the catalysis at the substrate pocket of CiP.

As a thermally stable and H_2O_2 -resistant CiP mutant, mCiP could retain, after being incubated at pH 10.5 and 50 °C for 20 min, ~95 or 74% activity in the presence of 0 or 0.2 mM H_2O_2 , respectively (28). CBD-mCiP showed, however, drastically decreased stability. After 20 min incubation at pH 10.5 and 50 °C, CBD-mCiP lost all its peroxidase activity, similar to CBD-CiP, even in the absence of H_2O_2 .

Surface charge modification of CiP and CBD-CiP

The modified CiP (CiP_{cm}) showed a UV-visible spectrum and SDS-PAGE identical to those of CiP. However, CiP_{cm} and CBD-CiP_{cm} had a pI of 8.2 and 9, respectively, significantly higher than the 3.5 of CiP and CBD-CiP. The amino acid analysis showed ~20 more glycines in CiP_{cm} than in CiP. With ABTS as the substrate, CiP_{cm} had an optimal pH = 8, shifted one unit higher than that of CiP. The specific activity of CiP_{cm} was 70% of that of CiP, probably due to amidations at the vicinity of the heme site or on the heme's two propionate groups. Above pH 8, CiP_{cm} showed lower H_2O_2 resistance than that of CiP.

Cellulose-binding

For its binding by Avicel at pH 10, Veitex at pH 10, or Veitex at pH 7, HiC45 showed a Langmuir isotherm with a maximal binding capacity B_m of 710 ± 110 , 32 ± 5 , or 34 ± 3 nmol/g, and an affinity constant K_a of 5.4 ± 2.1 , 3.8 ± 1.2 , or $4.8 \pm 1.1 \mu M^{-1}$, respectively. The affinity of isolated CBD appeared similar to that of HiC45. The K_a was comparable to that of the Cel7A cellobiohydrolase from *Trichoderma reesei* (2, 29).

During the screening of *S. cerevisiae* transformants, CBD-CiP was bound by cellulose acetate, but not Whatman #1 paper filter. Towards Avicel, Style #400

fabric, denim, and black currant-stained #400 fabric, the ^{14}C -labeled CBD-CiP showed 17, 0, 1, and 38% adsorption respectively, whereas the ^{14}C -labeled CiP showed 3, 1, 1, and 46% adsorption, respectively.

Under the conditions of 5.5 mM Na-phosphate, pH 7, 0.1 mg/L protein, and 100 g/L denim, essentially no CBD-CiP bound to denim after 30 min incubation, based on the peroxidase activity in the supernatant. With 100 g/L Avicel, however, ~50 and 70% CBD-CiP were bound after 30 and 50 min incubations, respectively, in contrast to the <6% binding for CiP. Assuming a B_m of 0.7 $\mu\text{mol/g}$, as that observed for HiC45, a $K_a \sim 0.03 \mu\text{M}^{-1}$ was estimated for the Avicel-binding of CBD-CiP.

The adsorption was also studied in 10 mM Tris, 5 mM CaCl_2 , pH 7.6, with 250 g/L cellulosic material and 0.6 mg/L peroxidase. Figure 3 shows the relative activity of the peroxidases free in solution after incubation. With Avicel, only 10% of CBD-CiP was bound, in comparison with the 35% binding for CBD-CiP_{cm}. With Style #400 fabric, only CBD-CiP_{cm} showed a significant binding (50%). With denim, neither CiP nor CBD-CiP showed detectable binding, whereas 40% of CBD-CiP_{cm} was bound. With the black currant-stained fabric, the binding was 10, 20, 40, and 80% for CiP, CBD-CiP, CiP_{cm}, and CBD-CiP_{cm}, respectively.

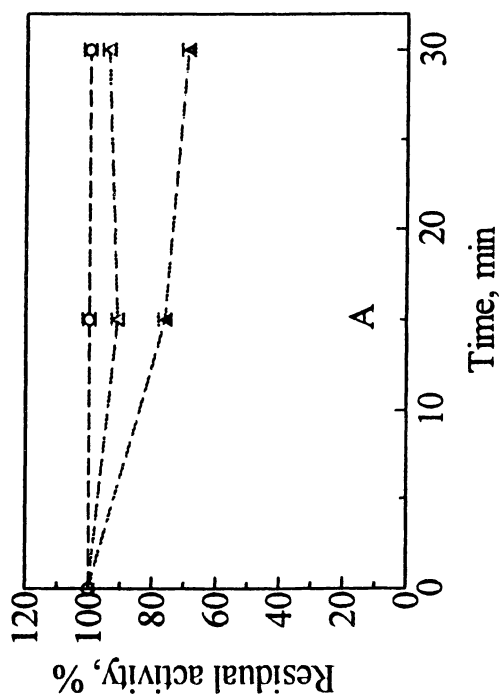
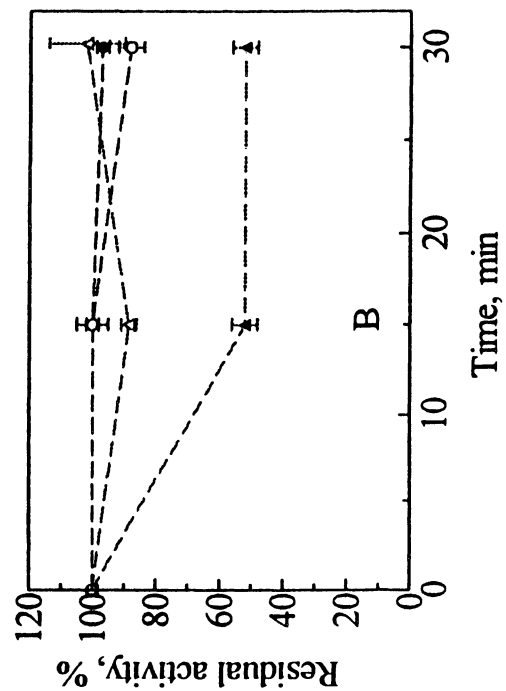
Oxidation of soluble and Avicel-adsorbed Congo Red

CiP-catalyzed homogenous oxidation of Congo Red by H_2O_2 led to a bleach of the intense red color in solution into faint yellow-brown color, corresponding to a decrease of the absorption at 486 nm. A linear relationship was observed between the initial rate and the enzyme concentration at the range of 1 to 160 nM. A turnover/min of 130, 160, 340, or 160 was observed for CiP, CBD-CiP, CiP_{cm}, or CBD-CiP_{cm}, respectively.

To oxidize Avicel-adsorbed Congo Red, 0.5 mg dyed Avicel was mixed in 0.13 mL solution with 0.24 mM H_2O_2 and 0.14 μM peroxidase, under gentle shaking at 20 °C for 17 h. Another 6 h incubation was made with 2.4 mM newly added H_2O_2 , followed by a two-day incubation with 1.4 μM newly added peroxidase, and a one-week incubation with newly added 70 mM H_2O_2 . After all these incubations, no visual red color bleaching was detected for either CiP, CBD-CiP, CiP_{cm}, or CBD-CiP_{cm}, indicating insignificant peroxidase action of these enzymes on the adsorbed dye.

Denim bleaching

In the absence of phenothiazine, treating denim with CiP and H_2O_2 led to a $\Delta(L^*a^*b^*)$ of 0.97, indicating a poor bleaching. In the presence of 0.1 mM phenothiazine, however, the treatment led to a $\Delta(L^*a^*b^*)$ of 6.82 (corresponding to a visual change from intense blue to pale blue), indicating a strong bleaching



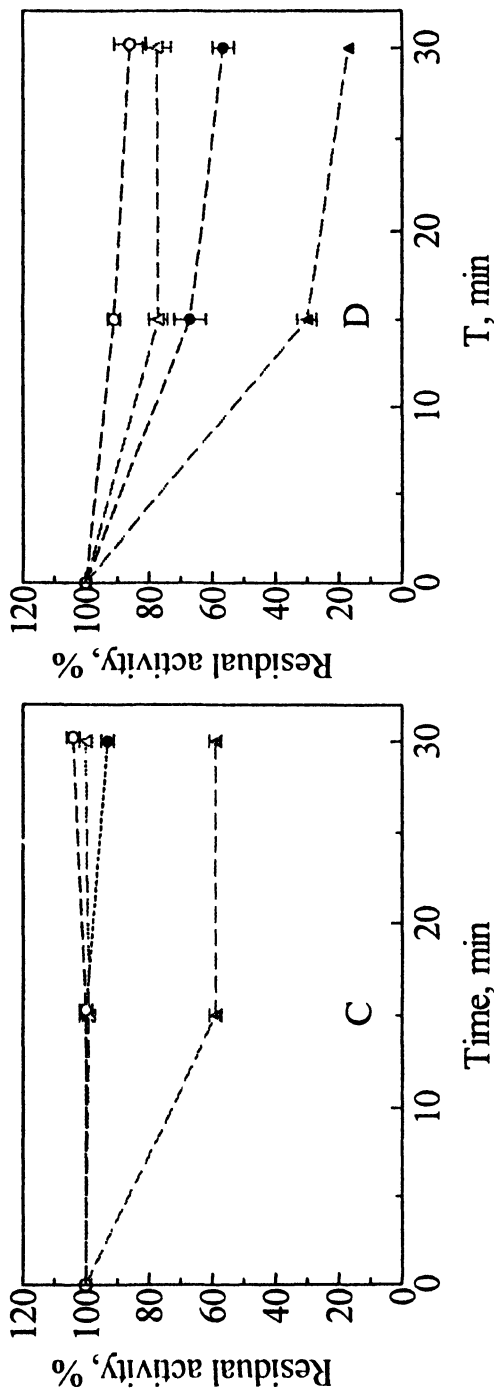


Figure 3. Binding by cellulosic materials. The CiP or CBD-CiP free in solution was measured by the peroxidase activity in the solution phase. Cellulosic materials: (A) Avicel, (B) Style #400 fabric, (C) denim, (D) black currant-stained #400 fabric. Open symbols: (o) CiP, (Δ) CBD-CiP; filled symbols: (o) CiP_{cm}, (Δ) CBD-CiP_{cm}.

under the mediation of phenothiazine, which shuttled electrons from cellulose-adsorbed indigo dye to soluble CiP (20, 34). When CiP was replaced by CBD-CiP, a $\Delta(L^*a^*b^*)$ of 1.48 or 6.27 was observed with 0 or 0.1 mM phenothiazine, respectively. Increasing [CBD-CiP] 10-fold or pre-incubating CBD-CiP with denim led to comparable results. Thus the ability of CBD-CiP to bind to cellulose did not improve the denim bleaching.

Discussion

Construction of CBD-CiP

In HiC45, the N-terminus of the CBD is linked (via the linker) to the C-terminus of the catalytic domain (22). We connected the N-terminus of CiP (via the linker) to the C-terminus of HiC45's CBD, in an arrangement "reversal" to that in HiC45. The resulting fusion protein CBD-CiP was readily secreted by *S. cerevisiae* and *A. oryzae* hosts, was active as a peroxidase, and functional as a cellulose-binder. We purified CBD-CiP by ion exchange chromatography at neutral pH, rather than cellulose affinity chromatography, because the elevated pH (~11) needed to break CBD-cellulose binding was known to destroy the peroxidase activity of CiP. Although CiP and CBD-CiP had similar pI of 3.5, they were readily separated by our procedure, likely due to the heavy glycosylation of the linker in CBD-CiP.

The CBD-CiP showed an Avicel affinity ~100-fold weaker than that of HiC45 and isolated CBD. This weakened cellulose-binding of the CBD in CBD-CiP may be attributable to the C-terminally attached CiP moiety (via the linker), whose thermal movement/freedom could interfere with the CBD's cellulose-binding, which aligns the flat binding surface of the wedge-shaped CBD with cellulose polymer (29, 35, 36). Both CBD-CiP and CBD-mCiP showed comparable peroxidase activity at pH 7 and 20 °C, indicating an insignificant effect of the CBD moiety on the heme site of CiP. However, the attachment of the CBD abolished the stability of mCiP at pH 10 and 50 °C. It has been postulated that the mutations in mCiP enhance the inter/intra helix H bonds, leading to higher thermal stability (28). Attaching a 20 kD CBD-linker moiety may weaken the interhelix interactions in CiP, resulting in a decreased CiP's stability.

Effect of surface charge on the cellulose-binding by CBD-CiP

Perfect cellulose has no net charge in its polymeric structure. However, the cellulose matrix in cotton fabric generally carries negative charges at neutral pH,

due to various treatments during manufacturing. Thus, electrostatic repulsion could contribute to the weak binding of CBD-CiP, which was negatively charged at $\text{pH} \geq 4$. The chemically modified CBD-CiP_{cm} was positively charged at $\text{pH} \leq 9$, and its improved cellulose-binding could be attributed to its favorable electrostatic attraction to the fabric. Similar electrostatic effect has been observed for the cellulase binding on charged hemicellulose (37).

The staining of black currant, which contains positively charged anthocyanidines, embedded positive charges onto the negative cotton fabric. The electrostatic attraction by embedded anthocyanidines could contribute to the binding of CiP and CBD-CiP to the black currant-stained fabric, which was significantly enhanced in comparison to that to the unstained fabric. Both CiP_{cm} and CBD-CiP_{cm} showed better binding to the black currant-stained fabric than CiP and CBD-CiP, likely due to the ability of CiP_{cm} and CBD-CiP_{cm} to bind the larger cellulosic matrix area unembedded by anthocyanidines.

CBD-CiP activity on cellulose-adsorbed substrate

One of the potential applications for CBD-CiP is in the field of textiles: to bleach cellulose-adsorbed dye. In general, the adsorption by insoluble cellulose makes a substrate inaccessible for a soluble enzyme, as exemplified by the Congo Red and CiP of this study. The fused CBD in CBD-CiP could anchor CiP on cellulose, thus creating close proximity between adsorbed Congo Red and CiP. However, no significant oxidation of the adsorbed Congo Red by the anchored CiP was observed, although the soluble Congo Red was readily oxidized by CBD-CiP. No beneficial effect from the cellulose-binding of CBD-CiP was observed either in CiP's oxidative bleaching of indigo dye in denim. The inability of the bound CBD-CiP to oxidize the adsorbed dye indicated that the heme:substrate interaction was still too weak, likely due to unfavorable steric effects. It is known that different dyes have different activity towards the decolorization by peroxidases. Thus it would be of interest to test whether CBD-CiP could decolorize other dyes being used by the textile industry other than Congo Red and indigo.

Conclusions

We have successfully prepared two CBD-fused peroxidases. Although the fusion enzymes retained their peroxidase activity, the cellulose-binding affinity of the CBD moiety and the alkaline/thermal stability of the CiP moiety were significantly compromised. The fusion could immobilize the peroxidase on a cellulosic matrix, enabling it to be delivered/transported by a cellulosic carrier or

to function in a multi-phasic bioreactor for soluble substrate. However, CBD-CiP failed to activate cellulose-adsorbed, insoluble substrate. More protein engineering is needed to transform the peroxidase from a homogenous biocatalyst into a heterogeneous biocatalyst for interfacial biocatalyses such as bleaching dyed fabric.

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References

1. Linder, M.; Teeri, T. T. *J. Biotechnol.* **1997**, *57*, 15-28.
2. Tomme, P.; Boraston, A.; McLean, B.; Kormos, J.; Creagh, A. L.; Sturch, K.; Gilkes, N. R.; Haynes, C. A.; Warren, R. A. J.; Kilburn, D. G. *J. Chromatogr. B* **1998**, *715*, 283-296.
3. Carrard, G.; Koivula, A.; Soderlund, H.; Beguin, P. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10342-10347.
4. Esteghlalian, A. R.; Srivastava, V.; Gilkes, N. R.; Kilburn, D. G.; Warren, R. A. J.; Saddler, J. N. *Appl. Biochem. Biotechnol.* **2001**, *91-93*, 575-592.
5. Xu, G.; Ong, E.; Gilkes, N. R.; Kilburn, D. G.; Muhandiram, D. R.; Harris-Brandts, M.; Carver, J. P.; Kay, L. E.; Harvey, T. S. *Biochemistry* **1995**, *34*, 6993-7009.
6. Tormo, J.; Lamed, R.; Chirino, A. J.; Morag, E.; Bayer, E. A.; Shoham, Y.; Steitz, T. A. *EMBO J.* **1996**, *15*, 5739-5751.
7. Brun, E.; Moriaud, F.; Gans, P.; Blackledge, M. J.; Barras, F., Marion, D. *Biochemistry* **1997**, *36*, 16074-16086.
8. Mattinen, M.; Linder, M.; Drakenberg, T.; Annala, A. *Eur. J. Biochem.* **1998**, *256*, 279-286.
9. Shimon, L. J. W.; Pages, S.; Belaich, A.; Belaich, J.; Bayer, E. A.; Lamed, R.; Shoham, Y.; Frolow, F. *Acta Crystall. D Biol. Crystall.* **2000**, *56*, 1560-1568.
10. Goldstein, M. A.; Shoseyov, O.; Shpiegl, I.; Doi, R. H. PCT International Patent WO9424158A1, 1994.
11. Park, J. S.; Shin, H. S.; Doi, R. H. *Appl. Chimeric Genes Hybrid Prot. A* **2000**, *326*, 418-429.
12. Linder, M.; Nevanen, T.; Soderholm, L.; Bengs, O.; Teeri, T. T. *Biotechnol. Bioengineer.* **1998**, *60*, 642-647.
13. Wang, A. A.; Mulchandani, A.; Chen, W. *Biotechnol. Prog.* **2001**, *17*, 407-411.

14. Gunder, W.; Lippold, B. H.; Lippold, B. C. *Eur. J. Pharma. Sci.* **1995**, *3*, 203-214.
15. Berdichevsky, Y.; Lamed, R.; Frenkel, D.; Gophna, U.; Bayer, E. A.; Yaron, S.; Shoham, Y.; Benhar, I. *Prot. Expression Purif.* **1999**, *17*, 249-259.
16. K auffmann, C.; Shoseyov, O.; Shpigel, E.; Bayer, E. A.; Lamed, R.; Shoham, Y.; Mandelbaum, R. T. *Environ. Sci. Technol.* **2000**, *34*, 1292-1296.
17. Richins, R. D.; Mulchandani, A.; Chen, W. *Biotechnol. Bioengineer.* **2000**, *69*, 591-596.
18. Gustavsson, M.; Lehtio, J.; Denman, S.; Teeri, T. T.; Hult, K.; Martinelle, M. *Prot. Engineer.* **2001**, *14*, 711-715.
19. Sheldon, W. M. *Curr. Opinion Biotechnol.* **1999**, *10*, 370-375.
20. Xu, F. In *The Encyclopedia of Bioprocessing Technology: Fermentation, Biocatalysis, and Bioseparation*; Flickinger, M. C. and Drew, S. W. Ed.; John Wiley & Sons: New York, NY, 1999; pp. 1545-1554.
21. Levy, I.; Shoseyov, O. *J. Peptide Sci.* **2001**, *7*, 50-57.
22. Rasmussen, G.; Mikkelsen, J. M.; Schülein, M.; Patkar, S. A.; Hagen, F.; Hjort, C. M.; Hastrup, S. PCT International Patent WO9117243A1, 1991.
23. Schülein, M. *J. Biotechnol.* **1997**, *57*, 71-81.
24. Baunsgaard, L.; Dalbøge, H.; Houen, G.; Rasmussen, E. M.; Welinder, K. G. *Eur. J. Biochem.* **1993**, *213*, 605-611.
25. Petersen, J.; Kadziola, A.; Larsen, S. *FEBS Lett.* **1994**, *339*, 291-296.
26. Abelskov, A. K.; Smith, A. T.; Rasmussen, C. B.; Dunford, H. B.; Welinder, K. G. *Biochemistry* **1997**, *36*, 9453-9463.
27. Petersen, A. H.; Vind, J.; Svendsen, A.; Cherry, J. R.; Schneider, P.; Jensen, B. R. PCT International Patent WO9510602A1, 1995.
28. Cherry, J. R.; Lamsa, M. H.; Schneider, P.; Vind, J.; Svendsen, A.; Jones, A.; Pedersen, A. H. *Nature Biotechnol.* **1999**, *17*, 379-384.
29. Linder, M.; Mattinen, M.; Kontteli, M.; Lindeberg, G.; Stahlberg, J.; Drakenberg, T.; Reinikainen, T.; Pettersson, G.; Annala, A. *Prot. Sci.* **1995**, *4*, 1056-1064.
30. Bechtold, R.; Bosshard, H. R. *J. Biol. Chem.* **1985**, *260*, 5191-5200.
31. Means, G. E.; Feeney, R. E. *Anal. Biochem.* **1995**, *224*, 1-16.
32. Christensen, T.; Wøldike, H.; Boel, E.; Mortensen, S. B.; Hjortshøj, K.; Thim, L.; Hansen, M. T. *Biotechnol.* **1988**, *6*, 1419-1422.
33. Andersen, H. D.; Jensen, E. B.; Welinder, K. G. PCT International Patent WO9216634A1, 1992.
34. Kierulff, J. V. *Textile Horizon* **1997**, *10/11*, 33-36.
35. Kraulis, P. J.; Clore, G. M.; Nilges, M.; Jones, T. A.; Pettersson, G.; Knowles, J.; Gronenborn, A. M. *Biochemistry* **1989**, *28*, 7241-7257.
36. Hoffrén, A.; Teeri, T. T.; Teleman, O. *Prot. Engineer.* **1995**, *8*, 443-450.
37. Reinikainen, T.; Teleman, O.; Teeri, T. T. *Prot. Struc. Func. Genet.* **1995**, *22*, 392-403.

38. Bolam, D. N.; Ciruela, A.; McQueen-Mason, S.; Simpsons, P.; Williamson, M. P.; Rixon, J. E.; Boraston, A.; Hazlewood, G. P.; Gilbert, H. J. *Biochem. J.* **1998**, *331*, 775-781.
39. Schülein, M. *Biochem. Soc. Trans.* **1998**, *26*, 164-167.
40. Karlsson, J.; Momcilovic, D.; Wittgren, B.; Schülein, M.; Tjerneld, F.; Brinkmalm, G. *Biopolymers* **2001**, *63*, 32-40.

Chapter 23

Heterologous Expression of *Trichoderma reesei* 1,4- β -D-Glucan Cellobiohydrolase (Cel 7A)

**William S. Adney, Yat-Chen Chou, Stephen R. Decker,
Shi-You Ding, John O. Baker, Glenna Kunkel, Todd B. Vinzant,
and Michael E. Himmel**

**Biotechnology Division for Fuels and Chemicals, National Bioenergy
Center, National Renewable Energy Laboratory, 1617 Cole Boulevard,
Golden, CO 80401**

Cellobiohydrolase I (Cel7A) from *Trichoderma reesei* is generally recognized as being the most important enzyme in the construction of engineered component cellulase systems designed for hydrolysis of microcrystalline cellulose. We previously reported that full-length *T. reesei* Cel7A can be expressed from *E. coli* or *P. pastoris*, however the enzyme was either produced as insoluble inclusion bodies or hyperglycosylated respectively. In this study, we report the expression of active and stable full length Cel7A from transformed *A. awamori* and insect cells, and additional attempts to produce active soluble enzyme in *E. coli*. From this and previous work, we have concluded that *E. coli* and *P. pastoris* are unsuitable for expression of full length or the catalytic domain of Cel7A for the purpose of conducting site-directed-mutagenesis. We compare the activity kinetics and thermal denaturation properties of two forms of recombinant and the wild type *T. reesei* Cel7A.

Many members of the phylum Dikaryomycota (both Ascomycetes and Basidiomycetes) are known for their role in the biodegradation and recycling of organic matter in nature (1). Their ability to digest cellulosic biomass (e.g. leaf litter and wood), and in the case of many Basidiomycetes both cellulose and lignin, is of great interest to the emerging bioenergy industry, since biomass represents an enormous renewable resource for the production of fuels and chemicals. Among the notable genera are a number of industrially important fungi and agricultural mycoparasites. These fungi are known to secrete a variety of hydrolytic enzymes, many of which have been exploited industrially. These industrial enzymes include cellulases, amylases, invertases, proteases, pectinases, and lipases.

The anamorphic filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*) secretes several hydrolytic and cellulolytic enzymes known to participate synergistically in the degradation of biomass. One of the key enzymes from this group is the structural glycoside hydrolase family 7 enzyme cellobiohydrolase I (Cel7A), an exoglucanase that constitutes as much as 60% of the total secreted protein produced by the fungus (2). Cel7A is a 65 kDa glycoprotein that cleaves the cellulose chain from the reducing end to release cellobiose subunits. The enzyme consists of three domains: a relatively large 46 kDa *N*-glycosylated catalytic domain, a smaller 3.7 kDa cellulose-binding domain, and a flexible 30 amino acid *O*-glycosylated peptide linking the other two domains. During the process of secretion the protein undergoes several important posttranslational modifications that contribute both to the biological function and to the ultimate structure and stability of the protein (3). These posttranslational modifications include the oxidation of cysteine sulfhydryl groups to form 10 disulfide bonds in the catalytic domain which undoubtedly play an important role in the folding and maintaining of the three dimensional structure of the enzyme (Table I). *N*-terminal modification of the mature protein by formation of a pyroglutamate also occurs, a modification whose function is not clearly understood. The protein is targeted for secretion with a 17-residue amino acid *N*-terminal signal peptide that specifically targets the protein to the endoplasmic reticulum and subsequent secretory pathway, and may also be responsible for the initiation of folding, and may serve as an intra-molecular chaperone itself. In addition, the specific cleavage of the signal peptide by host cell proteases may or may not be specific to *T. reesei*.

The catalytic domain of Cel7A is *N*-glycosylated to various extents depending on *Trichoderma* strain and storage history (3). As in the case of most secretory proteins, glycosylation is thought to occur as the nascent peptide enters the endoplasmic reticulum and before the final native secondary structure is acquired (4). It is probable that the combination of these essential posttranslational modifications and the microheterogeneity of glycosylation by various heterologous hosts play a critical role in dictating the ultimate structure, stability, and activity of the expressed enzyme (5). Considering the potential

impact these modifications have on the function of secreted proteins, it became apparent that the assessment of advanced heterologous expression systems was critical for work to improve fungal cellulases through protein engineering.

Table I. Specific Disulfide Bonds Assignments in *T. reesei* Cel7A

<i>Cysteine 1</i>	<i>Cysteine 2</i>	<i>Type</i>
A4	A72	
A19	A25	SRH
A50	A71	LHS
AA61	A67	RHH
A 138	A 397	LHS
A 172	A 210	RHH
A 176	A 209	SRH
A 230	A 256	SRH
A 238	A 243	RHH
A 261	A 331	

The cost of producing ethanol from biomass can be divided into three areas: pretreatment costs, fermentation costs, and other costs. Pretreatment costs include capital construction costs, biomass milling, pretreatment reagents, equipment maintenance, power and water, and waste neutralization and disposal. Fermentation costs include capital, enzymes, nutrient supplements, yeast maintenance and scale-up, and waste disposal. Other costs include biomass purchase, transportation and storage, plant labor, plant utilities, ethanol distillation, and administration (which may include technology-use licenses). One of the major expenses incurred in the biomass conversion process is the cost of enzymes (6).

Our primary goal in support of the United States Department of Energy Biofuels Program remains the reduction in cost of cellulases acting on pretreated biomass. A logical approach to this task is to increase the specific activity and possibly the thermal tolerance of the enzymes in the cellulase complex. Development of an effective and relevant heterologous expression system for Cel7A therefore became a critical objective.

Materials and Methods

Acquisition of the *T. reesei cel7a* Gene.

Cel7a cDNA was previously isolated from a *T. reesei* cDNA library constructed using a PCR-generated probe based on published *cel7a* gene sequences (7). The resulting plasmid, pB210-5a, contains the entire *cel7a* coding sequence including the native signal sequence and was used to construct

expression vectors for *E. coli*, *Aspergillus awamori*, and insect cells. PCR was used to isolate and/or modify the *cel7a* gene from plasmid pB210-5a for all expression vectors used in this study. Using this approach, we designed primers that first isolated the translated portion of the *cel7a* gene sequence, secondly we added convenient restriction enzyme sites for cloning, and then we placed the coding region in the proper reading frame. *Pfu* polymerase, a polymerase with proofreading ability and high fidelity, was obtained from Stratagene and used for all PCR reactions. Inserts containing the *cel7a* gene were then prepared by gel purification of the PCR products followed by restriction digestion and directional cloning into the appropriate expression vectors. For the expression of Cel7A in *E. coli*, the gene was truncated so that only the catalytic domain of protein was expressed. After the *cel7a* gene was inserted into the appropriate expression vector, the insert was sequenced to ensure no errors in the primary sequence were introduced by PCR.

Expression of *T. reesei* Cel7A in *E. coli*.

Expression Vectors and Strains.

The DE3 lysogen of strain BL21, and variations of this strain, were used as the primary hosts for the expression of the *cel7a* gene in *E. coli*. As an *E. coli* B strain, BL21 is deficient in the *lon* protease and lacks the *ompT* outer membrane protease that can degrade proteins during purification (8). In this host, the target protein should be more stable than in those host strains that contain these proteases. Additionally, the thioredoxin reductase-deficient strain BL21*trxB* (DE3) was used to attempt to maximize soluble protein expression with the pET vectors. The *trxB*⁻ cells have been shown to permit disulfide bond formation in the cytoplasm of *E. coli* (8-11), which is dependent on the presence of an oxidized form of thioredoxin (9). The Origami™ (DE3) and Origami B (DE3) strains used to express Cel7A catalytic domain in this study contain an additional mutation in the glutathione reductase gene (*gor*), which is also involved in the reduction of cytoplasmic disulfide bonds (11). The Origami and Origami B strains were used to attempt to express Cel7A, since it is thought that this enzyme requires disulfide bonds to achieve an active, properly folded conformation.

Another approach used during this study to address issues of protein insolubility when expressed from *E. coli*, was the use of the tightly regulated pBAD expression system developed by Invitrogen. The use of this system permitted tight regulation of *cel7a* expression utilizing dose dependent induction with arabinose. The pBAD expression system is based on the *araBAD* operon, which controls the arabinose metabolic pathway in *E. coli* (12). With this system

expression levels can be tightly controlled to reduce insolubility of the recombinant protein.

Genotypes of E. coli Strains Used for Protein Production.

***E. coli* XL1-Blue:** *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' *proAB lacIqZ.M15 Tn10(Tet^r)*] (Stratagene, La Jolla, CA).

BL21*trxB*(DE3)pLysS: genotype: *E. coli* B strain derived, F – *ompT hsdSB(rB– mB–) gal dcm trxB15*; Antibiotic resistance: kan (DE3) pLysS (CmR).

This strain is a high-stringency expression host allowing disulfide bond formation in the *E. coli* cytoplasm, has thioredoxin reductase mutation (*trxB*), and has resistance to kanamycin (15 µg/ml) and chloramphenicol (34 µg/ml) (Novagen, Inc., Madison, WI).

Origami(DE3)pLysS: genotype: K-12 derived, *ara–leu7697 lacX74 phoAPvuII phoR araD139 galE galK rspL F⁺[lac+(lacIq)pro] gor522 ::Tn10* (TcR) *trxB::kan* (DE3) pLysS (CmR).

This strain is a high-stringency expression host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in *E. coli* cytoplasm

Antibiotic resistance: Tetracycline (12.5 µg/ml), Kanamycin (15 µg/ml), Chloramphenicol (34 µg/ml) (Novagen, Inc., Madison, WI).

BL21-CodonPlus(DE3)-RP: *E. coli* B F– *ompT hsdS(rB– mB–) dcm+ Tetr gal I* (DE3) *endA Hte [argU proL Camr]*.

This strain, a derivative of *E. coli* B, is a general protein expression strain that lacks both the Lon protease and the OmpT protease, which can degrade proteins during purification (Novagen, Inc., Madison, WI).

Construction of MBP-Cel7A E. coli Fusion Proteins.

To improve the solubility of Cel7A expressed in *E. coli* we utilized the pMAL™-2 protein fusion and purification system from New England Biolabs (NEB) to successfully express and purify soluble rCel7A-cd. Following the NEB protocols the cloned *cel7a* gene was inserted downstream from the *ale* gene of *E. coli*, which encodes maltose-binding protein (MBP), resulting in the expression of an MBP fusion protein (13) shown in Figure 1. The method uses the strong “tac” promoter and the *ale* translation initiation signals to give high-level expression of the cloned sequence, and also allows for a one-step purification of the fusion protein the affinity of MBP for maltose (14).

The constructed vector expressed the *mal* gene (with or without its signal sequence) fused to the *lacZ* gene. When present using the pMal-p2X vector, the signal peptide on pre-MBP directs fusion proteins to the periplasm. Since Cel7A is secreted it was thought that periplasmic targeting would allow folding and disulfide bond formation to take place in the periplasm of *E. coli*, plus allow rapid purification of the protein from the periplasm. The vectors as supplied by New England BioLabs carry the *lacIq* gene, which codes for the Lac repressor. This keeps expression from Ptac low in the absence of IPTG induction. The pMAL-2 vector also contains the sequence coding for the recognition site of a specific protease, located just 5' to the polylinker insertion sites. This allows MBP to be cleaved from the fusion protein after purification (14). We utilized the specific protease Factor Xa that cleaves at the C-terminal side of a specific four amino acid recognition sequence (IleGluGlyArg). The cleavage of the fusion at this site resulted in a cellobiohydrolase product with no vector-derived residues attached to the final protein product.

Selection of E. coli Hosts for the Formation of Disulfide Bonds.

An attempt was made to improve the solubility of Cel7A and to produce correctly folded enzyme in *E. coli* by using the vector pET-32, a vector designed to produce a thioredoxin fusion protein and to increase the yield of soluble product in the cytoplasm. Expression of Cel7A was attempted in the host strains Origami, Origami B, BL21trxB, and BL21 (DE3) pLySs, which allow for the formation of disulfide bonds in the *E. coli* cytoplasm (11). Since the Cel7A protein contains multiple essential disulfide bonds, the combination of a pET-32 vector and a trxB or trxB/gor host was used to examine the expression of Cel7A linked to the presence of thioredoxins.

Targeted expression of the Cel7A protein to the periplasm of *E. coli* was also attempted using the following methods. The periplasm of *E. coli* is known to be an oxidizing environment that contains enzymes that catalyze the formation and isomerization of disulfide bonds (15-17). The directing of heterologous proteins to the periplasm is a common strategy employed when attempting to isolate active, folded proteins containing disulfide bonds (18). Cel7A was fused to a *pelB* signal sequence enabling potential localization of the expressed protein to the periplasm using the vector pET-40b (+)(Novogen). The vector pET-40b (+) contains signal sequences to create fusions of the target gene to the enzyme that catalyze the formation (DsbA) or isomerization (DsbC), respectively, of disulfide bonds in the periplasm of *E. coli* (18,19). This construct was designed to produce a Cel7A fusion protein that would be localized to the periplasm, so that its direct association with the catalytic enzyme would enhance its solubility and facilitate disulfide bond formation. The various vectors used for *E. coli* expression are illustrated in Table II and in Figure 1.

Purification and Cleavage of Cel7a-MBD E. coli Fusion Proteins.

MBP-Cel7A fusion proteins were purified by affinity chromatography using amylose resin from New England BioLabs. Cells from an overnight culture were induced using IPTG, harvested and lysed by sonication. The resulting cell debris was removed by centrifugation and the cell lysate loaded on to the amylose resin column. The column was then washed extensively with 20 mM acetate, 200 mM NaCl, 1 mM EDTA pH 5.0 buffer. The bound fusion protein was then eluted from the column using the same buffer with the addition of 10 mM maltose. Following cleavage by the specific protease Factor Xa, the rCel7A protein was purified from the MBP via anion exchange chromatography on a Pharmacia HiPrep 16/10 DEAE FF column.

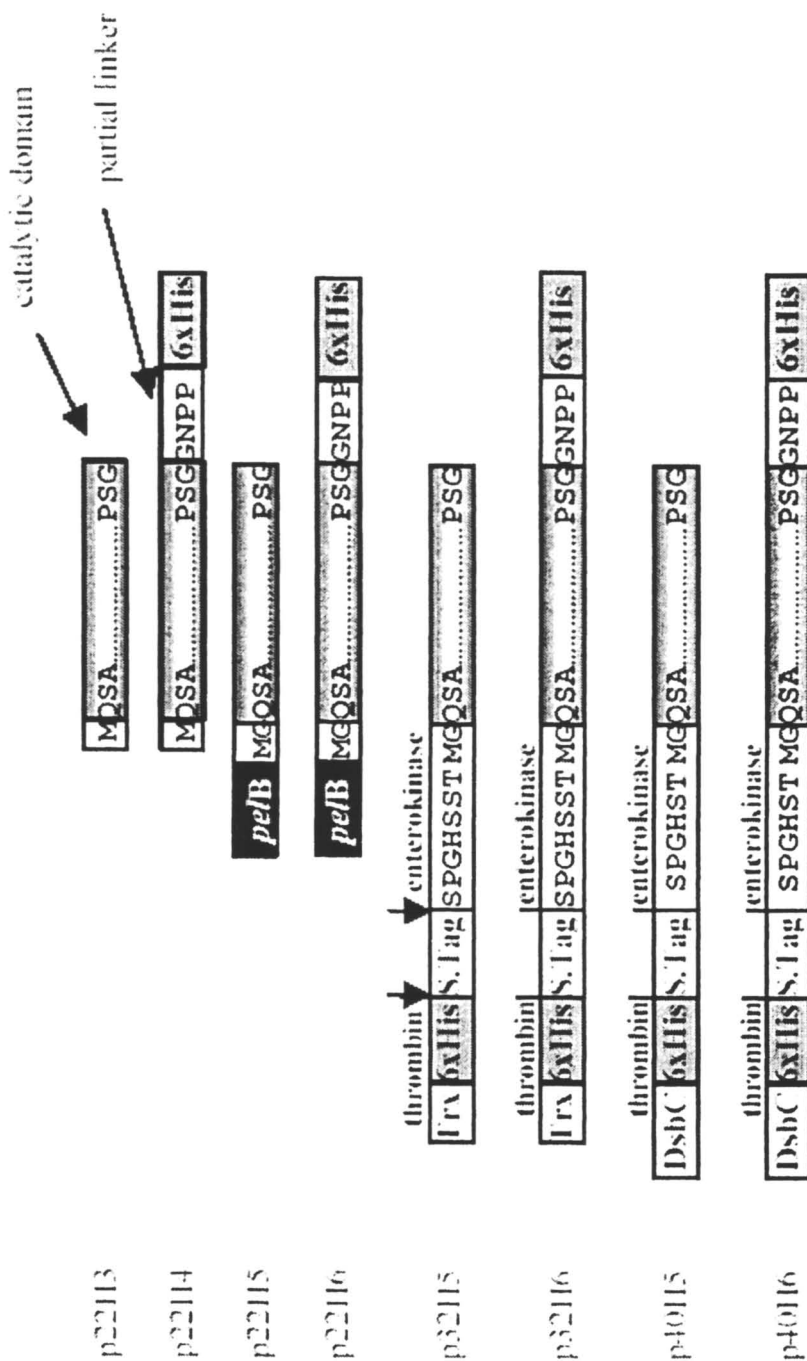
Fusion protein cleavage was carried out at 1% (w/w) the amount of fusion protein using the specific protease Factor Xa. The reaction mixture was incubated for several days at 4°C and the cleavage followed by SDS-PAGE.

Expression of *T. reesei* Cel7A in Insect Cells.

Construction of Recombinant Baculovirus.

To generate recombinant baculovirus containing the *cel7a* gene from *T. reesei*, a recombinant donor plasmid was first constructed. This vector was constructed using PCR, which generated a 1.57kb PCR fragment, from a cDNA template containing the *cbh1* gene plus its signal sequence (pB210-5a). This PCR product was also modified by the addition of BssH II and Xho I linkers and cloned into the Bac-to-Bac vector from Life Technologies. In addition, the translation initiation start signal was optimized for insect cells. Baculoviruses were constructed by transposon-directed integration of the *cel7a* gene into the baculovirus genome using competent DH10Bac *E. coli* cells. Transposition and isolation of recombinant bacmid DNA was carried out as described in the Bac-to-Bac Baculovirus expression Systems Manual (Life Technologies) using the vector pFast-*cel7a*.

The insertion of the *cbh1* gene was confirmed using the following methods. PCR amplification of the bacmid DNA preparation using M13F and M13R primers resulted in amplification of a 3.8kb fragment, the size predicted if transposition was successful (~1.5 kb gene, 2.3 kb transposition region). PCR using M13F and *cel7a* gene-specific antisense primer P261-A4645 (5'-AGATCGCGGGGACACTGG-3') amplified a 2.2kb product, the size predicted. This result indicates the presence of the *cel7a* gene in the bacmid DNA.





catalytic domain partial linker *pelB* = signal peptide from *E. coli* to send protein to periplasm

malE = same peptide from *E. coli*

MBP = maltose binding protein (fusion protein)

Trx = thioredoxin protein to assist protein folding (facilitate disulfide bond formation)

S. Tag = small antigenic tag for detection

6xHis = tag for detection and purification

Factor Xa = protease

PDI = protein disulfide isomerase (produced by yeast and fungi)

Figure 1. Vector constructs and diagrams of gene products for E. coli expression of cel7a gene used in this study.

Table II. Plasmids and *E. coli* Strains Used in This Study

<i>Plasmid*</i>	<i>Comments**</i>	<i>E. coli host</i>
p22H3	Cel7a-cd in pET22b	BL21(DE3) pLySs BL21trxB(DE3)pLySs Origami(DE3)pLySs
p22H4	Cel7a-cd with C-term 6x His tag in pET22b	
p22H5	Cel7a-cd with pelB leader in pET22b	
p22H6	Cel7a-cd with pelB leader and C-terminal 6x His tag in pET22b	
p32H5	Cel7a-cd with N-terminal thioredoxin tag in pET32b	
p32H6	Cel7a-cd with N-terminal thioredoxin and C-terminal 6x His tag in pET32b	
p40H5	Cel7a-cd with N-terminal DsbC tag in pET40b	
p40H6	Cel7a-cd with N-terminal DsbC and C-term 6x His tag in pET40b	
p22H3/pET32b	Co-expression of Cel7a-cd and thioredoxin	
p22H3/pET40b	Co-expression of Cel7a-cd and DsbC	
p22H4/pFA3	co-expression of Cel7a-cd and TrxA	
p22H4/pFA5	co-expression of Cel7a-cd and TrxA grx-like	
p22H4/pBADdsbA	co-expression of Cel7a-cd and DsbA	
p22H4/pBADdsbC	co-expression of Cel7a-cd and DsbC	
p22H4/pBAD.ssdsbA	co-expression of Cel7a-cd and DsbA (.2-19)	
p22H3/pBAD.ssdsbC	co-expression of Cel7a-cd and DsbC (.2-20)	
p22H5/pBAD.ssdsbC	DsbC (d2-20) in pBAD33 with optimized RBS	
pMALH7	Cel7a-cd in pMAL-p2	BL21-CodonPlus(DE3)-RP
pMALH8	Cel7a-cd with 6x His tag in pMAL-p2	

*pFA3, pFA5, pBADdsbA, pBADdsbC, pBAD.ssdsbA and pBAD.ssdsbC were kindly provided by Prof. G. Georgiou (PNAS, Nov23, 1999, vol 96(24):13703-13708).

**pET 22b(+), pET32b(+), pET40b(+) were purchased from Novagen (Madison, WI). pMAL-p2 was purchased from New England Biolab.

Transfection and Screening of Insect Cells.

The *cel7a* bacmid DNA was transfected into *Sf21* and *Tni* insect cells and cytopathogenesis was visible at harvest. Viral plaques were purified and five plaques were selected for expression of *T. reesei* Cel7A and production of protein biochemical analysis. Pilot expression experiments using 1.8×10^6 *Sf21* cells tested for active rCel7A and confirmed secretion into the culture media demonstrated in Table III and Figure 2.

Table III. Initial Rate Kinetics of Outgrowth Supernatants from Insect Cells Transfected With pFast-Cel7a Baculovirus on p-nitrophenol β -D-lactopyranoside.

<i>pFast-Cel7A</i> plaque	Initial rate on pNPL ($\mu\text{mol/mL min}^{-1}$)
1	9.72×10^{-4}
2	2.31×10^{-4}
3	1.50×10^{-4}
4	3.77×10^{-4}
5	1.86×10^{-4}
Control	Not detected

Expression of *T. reesei* Cel7A in *Aspergillus awamori*.

The coding sequence for the *cel7a* gene was inserted and expressed in *A. awamori* using the fungal expression vector pPFE2. The pPFE2 vector is an *E. coli* - *Aspergillus* shuttle vector, and contains elements required for maintenance in both hosts. The pPFE2 vector directs the expression and secretion of the protein of interests as a fusion protein with a portion of the glucoamylase gene fused to the *cel7a* gene as shown in Figure 3. In this case, the rCel7A protein was expressed as a fusion protein with the secretion signal peptide for glucoamylase present under the control of the *Aspergillus niger* glucoamylase (GA) promoter.

The major features of the pPFE2-*cel7a* construct are shown in Figure 4. The sequence immediately upstream of the N-terminus of the Cel7A catalytic domain encodes a LysArg dipeptide, which is recognized by a host Kex2 like protease during the secretion process (Figure 3). During secretion the fusion



Figure 2. Western blot analysis demonstrating successful expression of *Cel7A* from the transfection of *Sf21* insect cells. Lane 1 = See Blue Plus2 molecular weight standards; Lane 2 = native *T. reesei* expressed *Cel7A*; Lane 3 = pFast-*Cel7A* plaque 1 outgrowth supernatant; Lane 4 = pFast-*Cel7A* plaque 2 outgrowth supernatant; Lane 5 = pFast-*Cel7A* plaque 3 outgrowth supernatant; Lane 6 = pFast-*Cel7A* plaque 4 outgrowth supernatant; Lane 7 = pFast-*Cel7A* plaque 5 outgrowth supernatant; Lane 8 = Uninfected *Sf21* outgrowth supernatant; Lane 9 = BacPAK6 infected *Sf21* cell outgrowth supernatant expressing β -gal; Lane 10 = native *T. reesei* expressed *CEL7A*.

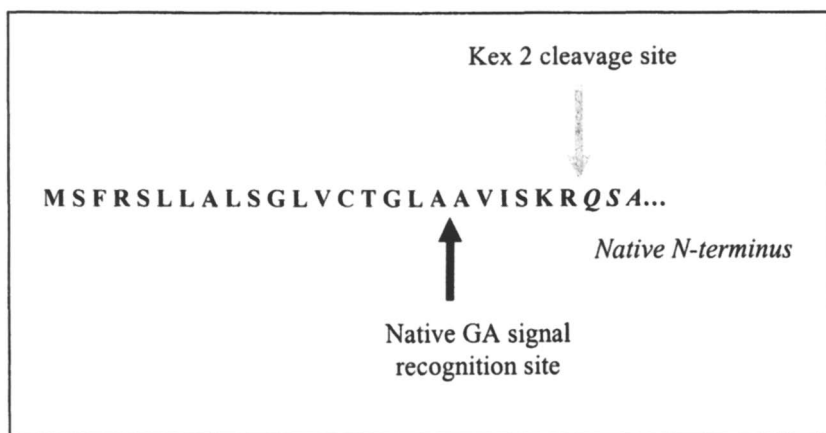


Figure 3. Glucoamylase signal sequence used to express *Cel7A* in *A. awamori* showing the native GA signal recognition site, the engineered Kex2 site, and the N-terminus of the expressed and secreted r*Cel7A*. Note that when processed with the GA signal sequence, the formation of the N-terminal pyroglutamate did not occur.

peptide is cleaved, removing the glucoamylase signal peptide. In this way, the rCel7A protein experiences an “efficient ride” through the *A. awamori* secretion system and is expressed and secreted with the native N-terminal protein. The net result is that the rCel7A is processed so that it can accumulate in the medium without its glucoamylase secretion signal fusion partner. The vector also contains the *Streptoalloteichus hindustanus* phleomycin resistance gene, under the control of the *A. niger* β -tubulin promoter, for positive selection of *Aspergillus* transformants. The vector also contains a β -lactamase gene for positive selection using ampicillin in *E. coli*, and the *A. niger* trpC terminator. Insertion of the *cel7a* coding sequence into the pPFE2 vector was accomplished by designing primers to give a Not I site on the 5' end, and an engineered Xba I site on the 3' end of the PCR fragment. The full-length coding sequence for the *cel7a* gene without the native signal sequence was obtained by PCR using Pfu DNA polymerase and using the cDNA construct pB210-5a as the template. The pPFE vectors and the PCR product were digested using Not I and Xba I then directionally cloned into the pPFE2 vector using T4 DNA ligase and used to transform *E. coli* XL-1 Blue. The insertion of the *cel7a* coding sequence into the pPFE2 vector was confirmed using PCR, restriction digest analysis, and DNA sequencing through the insertion sites. The entire coding sequence of the *cel7a* insert was also confirmed by DNA sequencing.

Expression of *T. reesei* Cel7A in *A. awamori* was also accomplished using its native signal sequence (Figure 5) by first constructing a plasmid in which a DNA fragment containing the structural gene of *cel7a* was cloned precisely under the control of an *A. niger* GA promoter by polymerase chain reaction (PCR)-mediated overlap extension (20). PCR was conducted using two sets of primers to individually amplify fragments of GA promoter and *cel7a* structural gene. The two fragments were joined together by the second round of PCR.

The final fragment was then cloned in pFE2 vector and used to transform *E. coli*. The recombinant plasmid was purified and subsequently used to transform *A. awamori*.

A 1-kb DNA fragment containing *A. niger* GA promoter was isolated by PCR using pFE2 DNA as the template using Pfu polymerase (Stratagene, CA). The reverse primer was designed so that the 5' sequence of *cel7a* structural gene was included and joined with the 3' end of the promoter sequence. The PCR product contained the GA promoter with a 15-bp “tail” of *cel7a* sequence. A SacI site was incorporated in the forward primer for the purpose of PCR fragment cloning. The primers used for synthesis of GA promoter include:

Forward 5'-AATTCGAGCTCTCCCCTGACC

Reverse 5'-CAACTTCCGATACATTGCTGAGGTGTAATGATGC

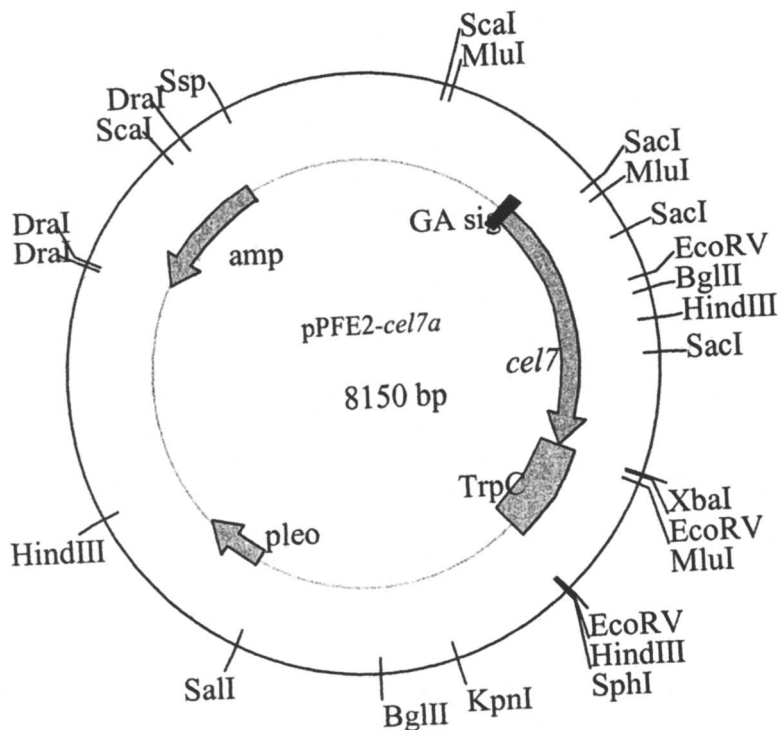


Figure 4. Plasmid map of the *E. coli-Aspergillus* shuttle vector *pPFE2-cel7a*. The *cel7a* gene was directionally cloned as a *Not*I-*Xba*I fragment. The *Not*I site was subsequently engineered out by site-directed mutagenesis to obtain the native N-terminus of *Cel7A*.

The structural gene of *cel7a* (1545 bp) was isolated by PCR using the plasmid pB210-5a as a template. The forward primer was designed so that the 3' sequence of the GA promoter is fused to the 5' end of *cel7a* structural gene. The PCR product contained a 19-bp "tail" of GA promoter sequence at the 5' end of the *cel7a* structural gene. A *Not*I restriction site was incorporated in the reverse primer for cloning purposes. The primers used for the synthesis of *cel7a* structural gene include:

Forward 5'- GCATCATTACACCTCAGCAATGTATCGGAAGTTG

Reverse 5'- GAATGCGGCCGCGGAGCTTTACAGGCACTG

Because the “tails” from the two PCR fragments above are complementary and can anneal to each other in a second round of PCR, the GA promoter can be combined with *cel7a* structural gene at the “ATG” start codon. This eliminates the potential effect on expression due to extra bases between the promoter and the gene.

The final PCR product (2.6 kb) was digested with NotI, which generated a cohesive terminus at the 3' end of *cel7a* gene, and ligated to pFE2 digested with SacI (and treated with T4 DNA polymerase for blunt-ending) and NotI. The ligation mixture was used to transform *E. coli* DH5a. The correct recombinant plasmid DNA was isolated from DH5a and used for transformation in *A. awamori* host.

Transformation of *Aspergillus awamori* with *cel7a*.

Aspergillus awamori (ATCC22342) spore stocks were stored at -70°C in 20% glycerol, 10% lactose. After thawing, 200 μL of spores were inoculated into 50 mL CM-glucose broth (5 $\text{g}\cdot\text{L}^{-1}$ Yeast Extract; 5 $\text{g}\cdot\text{L}^{-1}$ Tryptone; 10 $\text{g}\cdot\text{L}^{-1}$ Glucose; 20X Clutterbuck's Salts (120.0 $\text{g}\cdot\text{L}^{-1}$ Na_2NO_3 ; 10.4 $\text{g}\cdot\text{L}^{-1}$ KCl; 10.4 $\text{g}\cdot\text{L}^{-1}$ $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$; 30.4 $\text{g}\cdot\text{L}^{-1}$ KH_2PO_4) 50 $\text{mL}\cdot\text{L}^{-1}$) at pH 7.5 in each of eight-baffled 250 mL Erlenmeyer flask. The cultures were grown at 28°C , 225 rpm for 48 h. The mycelial balls were removed by filtration through sterile Miracloth (Calbiochem, San Diego, CA) and washed thoroughly with sterile KCM (0.7M KCl, 10mM MOPS, pH 5.8) to remove ungerminated spores. Approximately 10 g wet weight of washed mycelia were transferred to 50 mL KCM + 250 mg Lysing Enzyme from *Trichoderma harzianum* (Sigma-Aldrich, St. Louis, MO) in a 250 mL baffled Erlenmeyer flask. The digestion mixture was incubated overnight at 30°C , 80 rpm. Following digestion, the mycelia were titrated with a 25 mL disposable pipette to loosen the hyphal cells and filtered through sterile Miracloth into 50 mL conical centrifuge tubes. The spheroplasts were pelleted at 1500 x g for 12 min and resuspended in 0.7M KCl by gentle titration with a 25 mL pipette. This was repeated once. After a third pelleting, the spheroplasts were resuspended in 10 mL KC (0.7M KCl; 50mM CaCl_2), pelleted and resuspended in 1.0 mL KC using a wide-bore pipet tip. The washed spheroplasts were transformed by adding 12.5 μL PCM (40% PEG 8000; 50mM CaCl_2 ; 10mM MOPS pH 5.8) and 5 μL DNA (~ 0.5 $\mu\text{g}/\mu\text{L}$) to 50 μL of spheroplasts in sterile 1.5 mL Eppendorf tubes. After incubation on ice for 45 minutes, 0.5 mL of room temperature PCM was added to the transformation mixture and was mixed by inversion and gentle vortexing. The mixture was incubated at room temperature for 45 minutes. One milliliter of KC was added and mixed. Selection of transformants was by zeocin resistance. The mixture was allocated between four tubes (10 mL each) of CM top agar at 55°C , which were each poured over a 15 mL CM plate with 170 $\mu\text{g}/\text{mL}$ zeocin. The plates were incubated at 28°C for 2-3 days. Subsurface colonies were partially picked with a sterile wide bore pipet tip, exposing the remaining part of the colony to air and

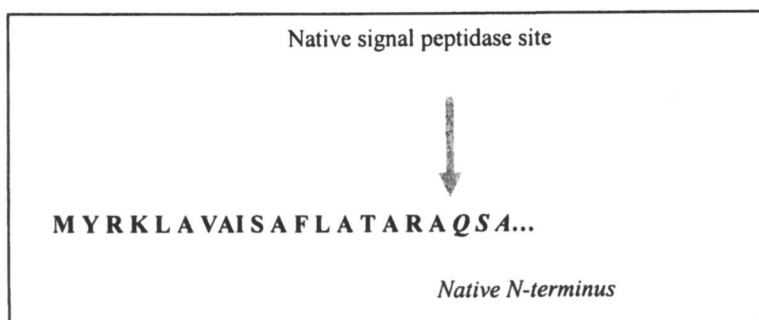


Figure 5. Native *T. reesei* signal peptide for Cel7A used for the expression and secretion of rCel7A in *Aspergillus awamori* and insect cells.

promoting rapid sporulation. After sporulation, spores were streaked onto several successive CM plates with either 100 or 300 $\mu\text{g}/\text{mL}$ zeocin added. After a monoculture was established, heavily sporulated plates were flooded with sterile spore suspension medium (20% glycerol, 10% lactose), the spores were suspended and aliquots were frozen at -70°C . Working spore stocks were stored on CM slants in screw cap tubes at 4°C . Protein production was confirmed and followed by western blot using anti-Cel7A monoclonal antibodies and the Novex Western Breeze anti-mouse chromogenic detection kit (Invitrogen, Carlsbad, CA). The recombination event was through random integration of the plasmid into the *A. awamori* genome.

For enzyme production, spores were inoculated into 50 mL CM basal fermentation medium (5.0 $\text{g}\cdot\text{L}^{-1}$ Enzymatic Casein Hydrolysate; 5.0 $\text{g}\cdot\text{L}^{-1}$ NH_4Cl ; 10.0 $\text{g}\cdot\text{L}^{-1}$ Yeast Extract; 10.0 $\text{g}\cdot\text{L}^{-1}$ Tryptone; 2.0 $\text{g}\cdot\text{L}^{-1}$ $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$; 50.0 $\text{g}\cdot\text{L}^{-1}$ Soluble Starch; 50 mM Bis-Tris-Propane, pH 7.0), and grown at 32°C , 225 rpm in 250 mL baffled flasks. The cultures were transferred to 1.0 L of basal fermentation medium in 2800 mL Fernbach flasks and grown under similar conditions. The flasks were harvested by filtration through Miracloth after 4-5 days of growth.

Purification of rCel7A.

The purification of rCel7a from insect cells and fungal broths was started by adjusting the culture filtrate to pH 5.8 by dilution into 50 mM Bis-Tris pH 5.8 buffer to a point at which the conductivity of the solution was less than 2 mS/cm. The sample was then applied to a HiPrep 16/10 DEAE FF column (Amersham Biosciences) equilibrated with 50 mM Bis-Tris, pH 5.8 buffer with a flow rate of

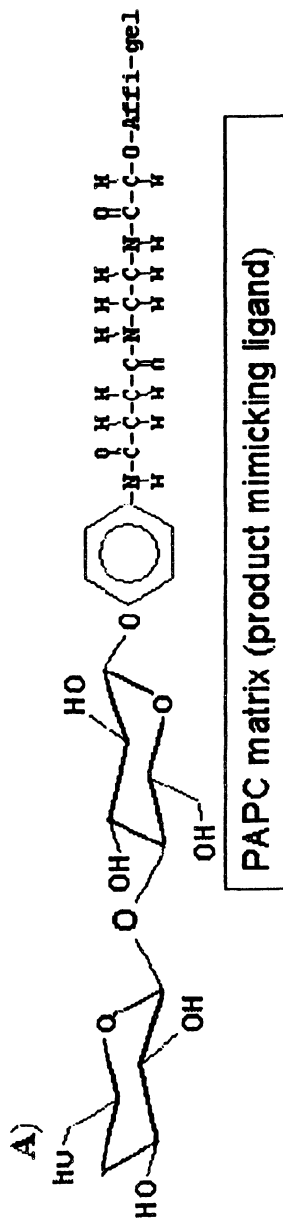
10 mL/min at 4°C. After the sample was loaded and the column washed extensively with equilibration buffer the bound fraction was eluted with a linear gradient of 0 to 1.0 M NaCl in the same equilibration buffer. The fractions containing activity on *p*-nitrophenol β -D lactopyranoside were pooled and concentrated to a final volume of 50 mL using Amicon stirred cell concentrators and PM-10 cutoff filters (10,000 kDa nominal molecular weight cutoff).

Purification of the recombinant enzyme was continued by use of *p*-aminophenyl- β -D-cellobioside (*p*APC) affinity chromatography. The basic chemistry of this affinity method is the linkage of an amino group to a succinimide ester to immobilize *p*-aminophenyl- β -D-cellobioside to an Affi-gel 10 chromatography matrix (BioRad, Catalogue# 153-6099). The *p*APC mimics a cellobiose molecule and enters the active site tunnel of Cel7A or other reducing-end exocellulases and binds to the enzyme, retaining it on the column (Figure 6). Because the bond between the two glucoses is still a glycosidic linkage and not a thio-linkage, care must be taken when using this matrix in the presence of β -glucosidase, which will cleave the cellobiose moiety and render the column ineffective. If β -glucosidase is known or suspected to be in the loading material, 1mM gluconolactone is added to inhibit the β -glucosidase and prevent column degradation. Elution of specifically-bound enzyme is accomplished by adding cellobiose to competitively remove the Cel7A from the *p*APC.

Because *p*APC is not commercially available, *p*-nitrophenyl- β -D-cellobioside must first be reduced to the amino form in order to fix it to the column. This is done by hydrogenation using hydrogen gas and a palladium catalyst. The *p*NPC is dissolved in methanol and flushed with hydrogen to remove oxygen. In an anaerobic chamber, the catalyst, 10% palladium on carbon (Pd/C) is added and H₂ is bubbled through the reactor for 15 minutes. After the reaction is complete, the catalyst is removed by filtration and the *p*APC is ready for coupling to the column matrix (21). Care must be taken with the removed catalyst, as it will react with O₂ upon drying and spontaneously combust.

The combined and concentrated fractions from the DEAE anion exchange chromatography were loaded onto a 1 x 6 cm packed bed *p*APC affinity column at a flow rate of 0.5 mL/min pre-equilibrated with 50 mM acetate, 1.0 mM gluconolactone pH 5.0 buffer. After the sample was loaded and the column washed extensively with equilibration buffer the sample was eluted with 10 mM cellobiose in the same equilibration buffer.

Following the *p*APC affinity chromatography step the recombinant enzyme was further purified and the buffer exchanged by means of size exclusion chromatography using a HiLoad 26/60 Superdex 200 column (Amersham Biosciences) in 20 mM acetate, 100 mM NaCl, pH 5.0 buffer. At this point the protein eluted as a single, symmetrical peak and the purity was confirmed as a single band using a NuPage 4-12 % Bis-Tris gradient gel using MOPS-SDS buffer (Invitrogen). Concentrations of purified proteins were determined by absorbance at 280 nm using the extinction coefficient and molecular weight



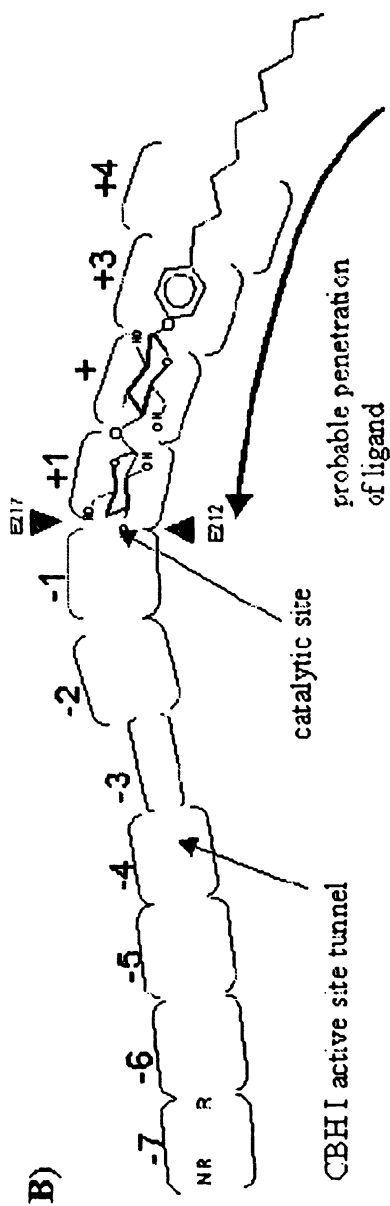


Figure 6. Affinity chromatography using p-amino- β -D-cellobioside affinity matrix chromatography. A) Shows the ligand as it is attached to the support matrix via the reducing end. B) Indicates the probable penetration of the ligand into the active site tunnel exit of Cel7A.

calculated for Cel7A by the ProtParam tool on the ExPASy website (<http://www.expasy.ch/tools/protparam.html>)

Circular Dichroism.

CD spectra were obtained using a Jasco 715 spectropolarimeter (Easton, MD) equipped with water bath temperature control and J-700 data collection software. The parameters for the circular dichroism spectroscopy of the recombinant and native Cel7A protein were as follows: protein concentration 100 $\mu\text{g/mL}$ in 20 mM sodium acetate, 100 mM NaCl, pH 5.0; temperature, 30°C; wavelength range, 190-250 nm; path length 0.01 cm; bandwidth, 1.0 nm, resolution, 0.1 nm; accumulation, 1; sensitivity, 200 mdeg; response, 8s speed, 20 nm/min. A buffer background scan was done under identical experimental conditions and was subtracted from the protein scan before obtaining the final spectrum.

Diafiltration Saccharification Assay (DSA).

Diafiltration saccharification assays were carried out as described earlier (22,23), with the modifications that the membrane installed in the cells was a BioMax-5 (5,000 Da nom. MWCO, Millipore Corp.) rather than a PM-10 (10,000 Da nom. MWCO, Amicon, Inc.), and the buffer flow rate through the membrane was 0.020 mL/min. All assays were at pH 5.0 in acetate buffer with 0.02%(w/v) sodium azide added. Assays were run at 38°C as a compromise between the higher activities of the cellulases at still higher temperatures and the temperature-tolerance of *S. cerevisiae* D5A, the organism used in companion simultaneous saccharification and fermentation (SSF) assays. Pretreated corn stover was prepared for use as a DSA substrate as described earlier (4,5). Substrate loadings averaged 96.4 mg (dry wt.) biomass (standard deviation, n = 5, of 0.8% or less) per DSA cell loading, for cellulose loadings of 55.5 mg glucose per assay. Substrate loadings thus amounted to 4.3% (w/v, solids) or 2.5% (w/v, cellulose). Because the effective saccharification of crystalline cellulose requires the synergistic action of both endoglucanases and exoglucanases, the activities of Cel7A and rCel7A proteins were assayed in combination with the catalytic domain of *A. cellulolyticus* endoglucanase I (EI-cd), used here as a standard endoglucanase. In all DSA protocols, the particular Cel7A species being tested was loaded at 27.8 mg per g cellulose, and the endoglucanase (EI-cd) was loaded at 1.11 mg per g cellulose, resulting in a 95:5 molar ratio of exoglucanase to endoglucanase. Quantitation of product sugars in effluent fractions was by HPLC using an Aminex HPX-87H column operated at 65°C with 0.01 N H₂SO₄ (0.6 mL/min) as mobile phase, in an Agilent Model 1100 chromatograph.

Protein Stability Measurements.

The overall protein stability was measured by differential scanning microcalorimetry using a Microcal model VP-DSC calorimeter (Microcal, Inc., Northampton, MA), with data analysis by means of Origin for DSC software (Microcal). Thermograms were collected for samples containing 50 $\mu\text{g/mL}$ protein at pH 5.0 in 20 mM sodium acetate with 100 mM NaCl. Calorimeter scan rate was 60°C/h.

Results

Expression of *T. reesei* Cel7A in *E. coli*.

The formation of inclusion bodies has remained a significant barrier to gene expression of cellobiohydrolases, specifically Cel7A from *T. reesei*, in the cytosol of *E. coli*. In this study, we used several approaches to minimize the formation of inclusion bodies and to improve folding and solubility of Cel7A expressed in *E. coli*. These efforts included the growth of the bacterial cultures at lower temperatures (15,20,25, and 30°C), the testing of different *E. coli* strains, the co-production of chaperones, and the use of *E. coli* thioredoxin as a fusion partner and a protein co-produced with the cellobiohydrolase. Additionally, we examined *E. coli* strains that are known to be deficient in thioredoxin reductase. The combinations of vectors built during the study and the resultant gene product are shown in Table II and in Figure 1.

A second approach to the expression of functional Cel7A in *E. coli* and problems associated with protein insolubility was also undertaken. Periplasmic targeting of expressed proteins in *E. coli* offers several advantages over cytoplasmic expression. In general, the oxidizing environment of the periplasm facilitates the proper folding of proteins expressed by *E. coli*, and protein degradation in general is less extensive. Periplasmic expression may also offer advantages for building a high throughput suitable host when the complexity of the target substrate for cellobiohydrolases is taken into consideration. To enable the periplasmic expression of Cel7A in *E. coli*, we took the approach of building fusion proteins with various signal peptides and co-expression of the Cel7A catalytic domain with protein disulfide isomerases and molecular chaperones. All of these attempts to express Cel7A in *E. coli* were met with marginal success. In general, Cel7A-cd can be expressed in *E. coli* with significant levels of protein produced as indicated by western blots and SDS-PAGE. Unfortunately, all of these constructs result in supernatants/cells showing limited activity on soluble substrates and inclusion bodies of cellulose inactive, non-functional protein. Interestingly, most forms of rCel7A-cd protein produced were recognized by monoclonal anti-Cel7A.

The pET System was chosen for this study because it is one of the most powerful systems available for the cloning and expression of recombinant proteins in *E. coli*. Using this system, we were able to express rCel7A cloned in pET plasmids under control of bacteriophage T7 transcription and expression regulation. The T7 RNA polymerase system is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein a few hours after induction. In our study, rCel7A-cd protein could be expressed by induction following the addition of IPTG to the bacterial culture at reduced culture temperatures.

During the course of this study, we attempted the expression of Cel7A-cd in *E. coli* as a fusion protein with the *E. coli* derived maltose-binding protein (MBP). Eukaryotic proteins and proteins that tend to form inclusion bodies in *E. coli* have been reported to be soluble when expressed with the MBP as a fusion partner (13). In the large majority of these cases, fusion proteins expressed from a pMAL-c2 plasmid, which lacks the *malE* signal sequence, can constitute 20–40% of the total cellular protein. When expressed from a pMAL-p2 plasmid containing the *malE* signal, when targeted to the periplasm, can constitute 1 to 5% of the total cellular protein. In the case of Cel7A, we were able to produce relatively large quantities of soluble rCel7A-cd/MBP in *E. coli*, with a yield of 20 to 30 mg purified protein per L of culture broth which was not active on synthetic substrates. The rCel7A-cd/MBP could be easily purified using amylose affinity chromatography and cleaved from its fusion partner using the specific protease Factor Xa. When released, the rCel7A-cd tended to become insoluble, presumably due to unsatisfied cysteines forming random disulfide bonds and subsequently forming insoluble protein complexes. A significant amount of the recombinant protein did however remain soluble and was subsequently purified using anion exchange chromatography. This purified protein when tested for activity versus soluble and insoluble substrates was found to be inactive, indicating improper protein secondary structure.

The similarities reported thus far between eukaryotic and prokaryotic disulfide-bond-forming systems suggest that commonalities might be found between disulfide bond isomerization pathways in eukaryotes and the DsbC–DsbD system in prokaryotes. Eukaryotic secretory proteins typically contain more disulfide bonds than their prokaryotic counterparts, indicating that there might be an even greater need for disulfide bond reducing or reshuffling functions in the ER than in the periplasm. However, genetic tests for the requirements for the oxidative folding of proteins with multiple disulphide bonds have not yet been developed in eukaryotes, so the components of isomerization pathways in the ER remain unclear. During this study we obtained several PDI homologues that could be produced in *E. coli* from the laboratory of Dr. George Georgiou. Variations of the vectors employed in an attempt to achieve proper disulfide bond formation in *E. coli* are outlined in Table II. These included PDI homologues that are likely to serve as catalysts for disulfide reshuffling in *E. coli*. Variations of expression included cytosolic and periplasmic targeting. Our

conclusions from these studies was that proper formation of disulfide bonds using *E. coli* chaperones and co-expression of PDI proteins was not possible, or the resultant proteins were highly susceptible to host cell proteases or inclusion body formation. While the production of rCel7A-cd protein could be confirmed by western blot, very little activity could be detected using the most sensitive assays available.

Expression of *T. reesei* Cel7A in Insect Cells.

We report the successful transfection of insect cells using recombinant bacmids containing the *cbh1* gene, and the production of recombinant baculovirus. Activity assays on five outgrowth supernatants from the pilot expression studies using *p*-nitrophenol β -D-lactopyranoside (2 mg/mL) as the substrate indicates that the rCel7A expressed in insect cells is functional, although expression levels were somewhat variable between each outgrowth. The supernatant from plaque 1 showed the highest level of activity (9.72×10^{-4} $\mu\text{mol/mL min}^{-1}$ as measured by *p*-nitrophenol release) and presumably has the highest level of expression (Table III). Close observation of the western blot in Figure 2 also supports this conclusion. The presence of activity in the culture supernatant is also an indication that insect cells by means of the native Cel7A signal sequence secrete the rCel7A. Western blot analysis of the outgrowth supernatants using monoclonal antibody specific to Cel7A also demonstrates the successful expression of full length Cel7A by insect cells (Figure 2).

Functional rCel7A was also produced and secreted in both the insect cell lines *Sf21* and *Tni*. Western blot analysis of culture supernatants indicates that the rCel7A is secreted, and shows no evidence of proteolysis. In addition, no evidence of contaminating enzyme activities could be detected in control insect cell cultures using methylumbelliferyl β -D lactopyranoside. In order to study the activity of Cel7A produced from insect cells on crystalline cellulose substrates, we produced and purified 11.25 mg of rCel7A produced from infection of both *Spodoptera frugiperda* and *Trichoplusia ni* lines of insect cells. The biochemical evaluation of the expressed and purified rCel7A produced in insect cells on a relevant biomass (corn stover) using the dialysis saccharification assay and the demonstration of similar kinetics to native enzyme on both soluble and insoluble cellulose (Figure 7). Differential scanning microcalorimetry analysis also showed identical thermal denaturation properties when compared to the native enzyme (Figure 8). Additionally, the rCel7A protein was found to be glycosylated to a lesser extent than fungal heterologous expression systems (Figure 9) as demonstrated by SDS-PAGE. The absence of contaminating enzyme activities, the yields from insect cultures, the biochemical characteristics of the expressed enzyme all indicate that insect cell expression is suitable for rapid screening of CBH I mutants for protein engineering and improvement efforts.

The activity of the purified rCel7A expressed in insect cells was compared to the native enzyme on corn stover using the diafiltration saccharification assay (DSA). The DSA assay measures the progression of cellulose hydrolysis by direct measurement of product formation (cellobiose and glucose) in the presence of *A. niger* β -glucosidase. The assay is our best measure of enzyme performance on a process relevant substrate at enzymes loadings specifically designed to detect small differences between the individual enzyme components. The insect cell expressed Cel7A performance when measured using DSA was comparable to the rate and extent of total cellulose hydrolysis of native enzyme purified from *T. reesei* culture broth. The level of cellulose (pretreated corn stover) hydrolysis achieved using the insect cell expressed Cel7A was approximately 56% at 120 hours compared to a 60% level of conversion achieved with the native enzyme a difference that is not considered to be significant (Figure 7).

Expression of *T. reesei* Cel7A in *A. awamori* Using the Glucoamylase Signal Peptide.

Cel7A when fused to a glucoamylase signal sequence exhibited near-native activity (about 92%) when compared to *T. reesei* expressed Cel7A using the Diafiltration Saccharification Assay on pre-treated yellow poplar (Figure 10). However, DSC comparisons showed a dramatic difference between native *T. reesei* Cel7A and *Aspergillus* expressed Cel7A when expressed utilizing the GA signal (Figure 11). The native *T. reesei* protein shows a single thermal transition with a maximum of 64.5°C; whereas the heterologous protein shows a bimodal transition, with one at about 61°C, and the other at about 65.5°C. The two-transition melting curve for *Aspergillus*-expressed rCel7A indicated differences existed in either the secondary structure or possibly in the glycosylation of the rCel7A compared to native enzyme. Either there were two forms of rCel7A being expressed or heterologous expression had altered the native structure in a non-critical portion of the protein. Analysis of the N-terminal peptide of the expressed protein by Edman degradation (Protein and Peptide Sequencing Center, Virginia Medical School, Charlottesville, VA) indicated that the N-terminal residue was not a pyroglutamate, but was a glutamine. The expressed protein also tended to be sensitive to host cell proteases as demonstrated by western blot analysis during growth of the recombinant *A. awamori* and the loss of measurable activity after 72 hours.

Expression of *T. reesei* Cel7A in *A. awamori* Using its Native Signal Peptide.

The saccharification performance of the *A. awamori* expressed rCel7A enzyme was measured using the DSA and was found to be comparable in rate and extent of total cellulose hydrolysis to that found for the native enzyme purified from *T. reesei* culture broth (Figure 7). The level of cellulose hydrolysis

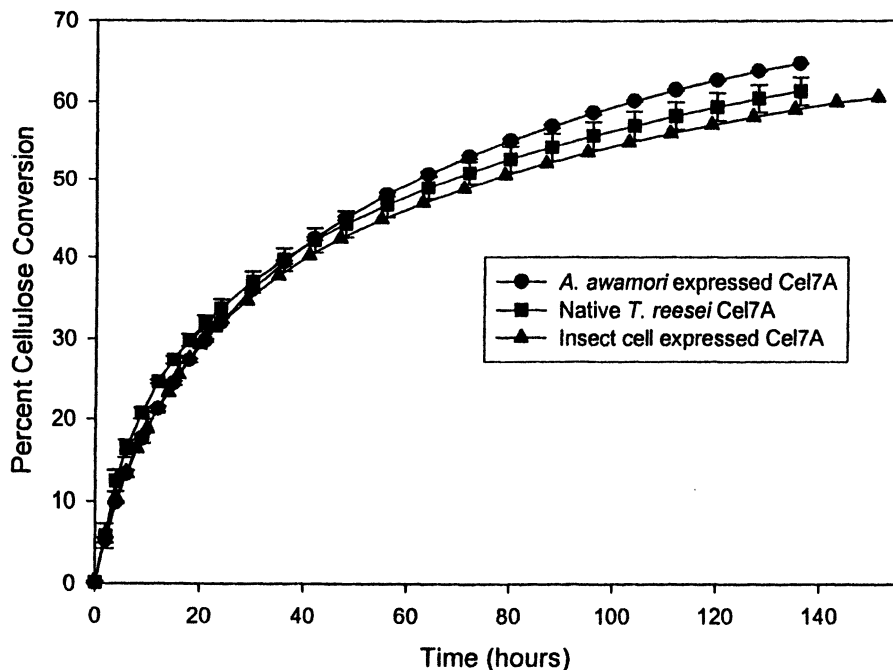


Figure 7. Dialysis saccharification assay (DSA) comparison of recombinant and native Cel7A on dilute acid pretreated cornstover at 38°C. All reactions were performed in 20 mM acetate, pH 5.0 buffer with the following protein loadings: Cel7A (27.8 mg/g cellulose), *A. cellulolyticus* EI-cd (1.11 mg/g cellulose) in a 95:5 mole ratio of Cel7A to EI-cd. The reaction buffer flux rate was 0.02 mL/min and the curves are corrected for product hold-up in the cell.

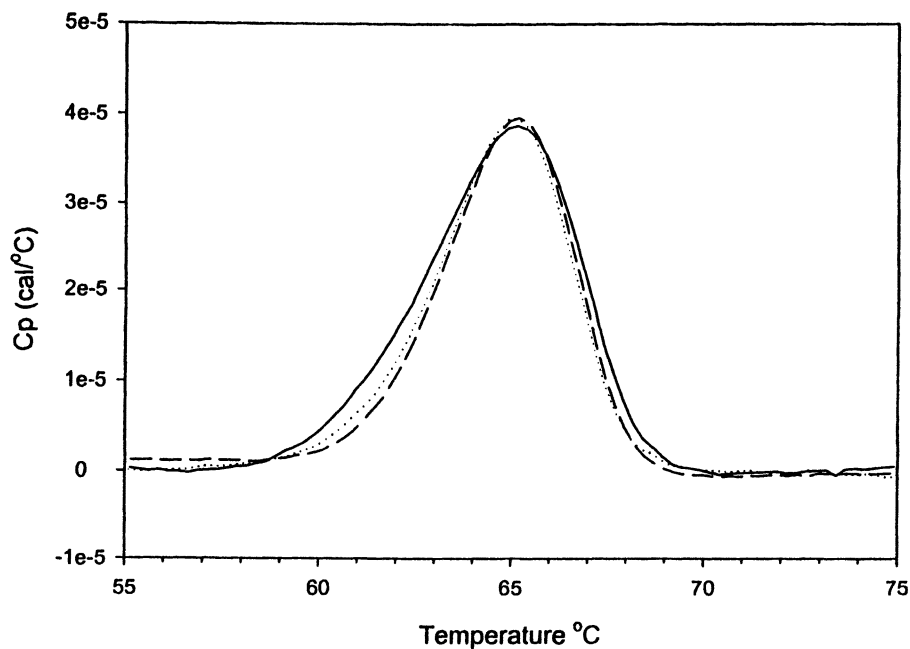


Figure 8. Co-plotted DSC thermograms of native *T. reesei* Cel7A (solid line), insect-cell-expressed rCel7A (dashed line), and *A. awamori*-expressed rCel7A (circles). Conditions: pH 5.0 in 20 mM acetate, 100 mM NaCl, 50 $\mu\text{g/mL}$ protein, scanned at 60°C/h.

achieved was approximately 62% at 120 hours, compared to a 60% level of conversion achieved with the native enzyme. The apparent molecular weight of the purified *A. awamori* expressed rCel7A was compared to the native enzyme using NuPage (Invitrogen) SDS-PAGE (Figure 9). This enzyme form appeared to be glycosylated to a greater extent than the enzyme expressed from insect cells and the native enzyme.

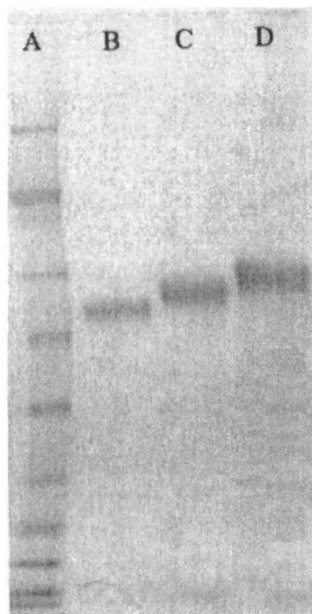


Figure 9. SDS-PAGE of native and recombinant CBH I enzymes, approximately 10 ng each lane. Lane A = MW standards, Lane B = native *T. reesei* Cel 7A, Lane C = rCel 7A from *tni* insect cells, Lane D = rCel 7A from *A. awamori*.

Discussion

Previously, we demonstrated that the endoglucanase EI (Cel 5A) from *A. cellulolyticus* functions with a high degree of synergism with fungal exoglucanases and that a 90:10:2 mixture of Cel7A, EI, and *A. niger* β -D-glucosidase is capable of releasing nearly as much reducing sugar from

pretreated yellow poplar after 120 h as the native *T. reesei* ternary system (24). This result is encouraging for the ultimate success of engineered cellulase systems, because the artificial enzyme system described above was tested at 50°C, a temperature far below that considered optimal for one key component, EI, in order to spare the more heat labile enzymes, Cel7A and β -D-glucosidase. Binary cellulase systems acting on microcrystalline cellulose have been shown to follow the Arrhenius-like relationship over a temperature range accessible to the best enzymes currently known (i.e., 35 to 50°C) for saccharification rates measured over the first 20% of conversion, and with the assumption that neither component is inactivated by the higher temperatures, this study predicted that the rate of hydrolysis may be doubled by increasing the digestion temperature from 50 to 70°C (25). An obvious research objective, then, is to increase the thermal tolerance of *T. reesei* Cel7A by protein engineering.

Site-directed-mutagenesis is a one possible tool for improving the thermal tolerance of *T. reesei* Cel7A; however this requires an efficient expression system that produces enzyme active on crystalline cellulose. Another approach is the integration of site-directed-mutagenesis with non-informational mutagenesis techniques (referred to generically as “directed evolution”) for the purpose of engineering a more thermal stable Cel7A. Directed evolution, in conjunction with high-throughput screening, allows testing of statistically meaningful variations in protein conformation (26). Directed evolution technology has undergone significant refinement from initial error-prone PCR methodology and now includes Gene Shuffling (27,28). Key factors necessary for conducting a directed evolution campaign to improve the characteristics of a protein include the requirement that the gene encoding for the protein to be modified is functionally expressed in a microbial host with high transformation efficiencies. Additionally there must be an effective screening procedure available to detect the desired activity and to differentiate incremental improvements.

Prokaryotic Expression Systems.

The structure of Cel7A indicates a distinct pattern of disulfide bond formation with ten pairs of disulfide bonds that are required to form, in the correct pairs, in order for the Cel7A catalytic domain to be folded properly (Table I). *Trichoderma* has apparently developed the cellular machinery to accomplish this, either through the use of chaperone proteins, maintenance of a compartmentalized, oxidizing environment for correct disulfide bond formation and folding, or through some other means as yet unclear. *E. coli* has a difficult time with the formation of disulfides, as the intracellular environment of *E. coli* is strongly reducing (29). This may explain why Cel7A so readily forms inclusion bodies when expressed in *E. coli*. Several new strains of *E. coli*

designed through reductase knockout mutations to have a more reduced interior have recently become available, and were tested in this study for improved expression of Cel7A. As an alternative to obtaining a reducing chemistry cytosol, secretion of the Cel7A into the extracellular environment may encourage correct folding. *E. coli* is not a good candidate for secretion, as proteins tend to pool in the periplasm, where they are subjected to relatively high concentrations of proteases. *Bacillus* and *Streptomyces* species readily secrete proteins directly to the culture media. Although *Bacillus* expression of Cel7A has been attempted, the work was not extensive and should be explored with more effort. *Streptomyces* has been shown in our lab to be an excellent producer of heterologous cellulase and should also be tested as an expression host for Cel7A.

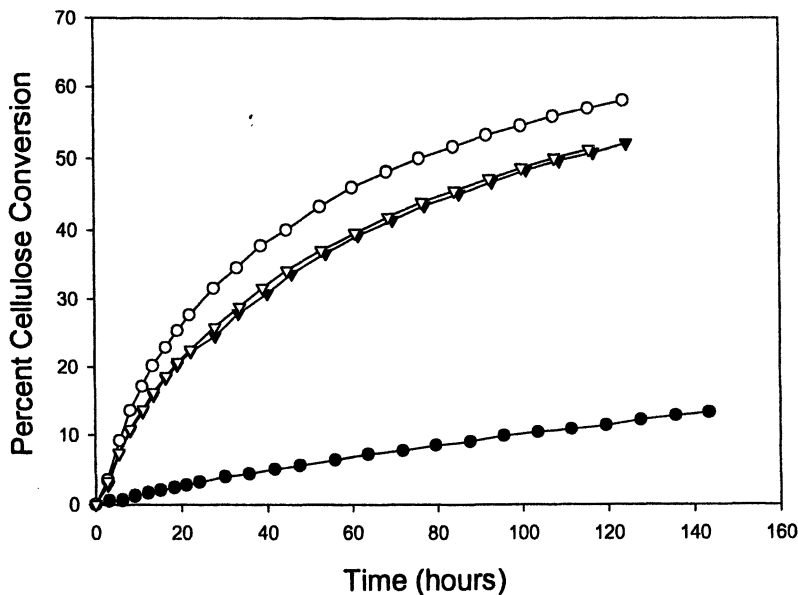


Figure 10. DSA was used to determine the activity of *A. awamori* expressed *rCel7A* (open and closed inverted triangles) on pretreated yellow poplar in the presence of the *A. cellulolyticus* endoglucanase EI. Native *T. reesei* Cel7A with EI (open circles) was used as a control for comparison, while EI alone (closed circles) was used to show the relative activity from each cellobiohydrolase.

The formation of inclusion bodies has remained a significant barrier to gene expression of cellobiohydrolases, specifically Cel7a from *T. reesei*, in the cytosol of *E. coli*. A number of physicochemical factors have been reported to contribute to the formation of inclusion bodies (15) including: charge average,

the number of turn forming residues, the number of cysteine residues and subsequent disulfide bonds, the number of proline residues, the protein's inherent hydrophilicity, and finally the total number of residues (15). In this study we have used several approaches to minimize the formation of inclusion bodies and to improve folding of Cel7A. These efforts have included the growth of the bacterial cultures at lower temperatures, the selection of different *E. coli* strains, the co-production of chaperones, the use of *E. coli* thioredoxin as a fusion partner and as a protein co-produced with the cellobiohydrolase. Additionally, we have tested *E. coli* strains that are deficient in thioredoxin reductase in an attempt to achieve disulfide bond formation.

Eukaryotic Expression Systems.

Another problem associated with heterologous cellulase expression is glycosylation and posttranslational processing. Bacterial exocellulases do not, apparently, exhibit the relatively high activity rates observed for fungal cellobiohydrolases. This may, in part, be explained by the glycosylation of fungal proteins, especially the linker peptide (30), permitting more extensive interaction with the cellulose residues and water. Since most bacteria do not glycosylate proteins, this potentially critical step is missing in prokaryotes. One solution to these problems lies in the selection of an appropriate eukaryotic expression host. We have in the past attempted expression using the methylotrophic yeast *Pichia pastoris* with very limited success (31,32). The best host for *T. reesei* Cel7A would obviously be a *T. reesei* strain. *Trichoderma reesei*, however, produces significant amounts of Cel7A as well as other cellulases, presenting a very difficult background activity challenge.

The selection of *T. reesei* as an expression system for protein engineering of Cel7A and other fungal cellobiohydrolases would be the first logical choice due to its capability of high expression levels and ability to produce correctly folded active enzyme. However, this system has several disadvantages including low transformation efficiencies and is of limited use for protein engineering studies of cellobiohydrolases, due to multiple interfering background activities. The expression and production of fungal cellulases in prokaryotic systems has not been successful due to the extent and types of posttranslational modifications required for the native enzyme. The issue of massive hyperglycosylation of the enzyme has plagued the expression of Cel7a in yeast such as *Saccharomyces cerevisiae* (33) and *Pichia pastoris* (32). The fungus, *Aspergillus awamori* is generally a good candidate for the heterologous expression of enzymes from other fungi and was thus our first choice for heterologous expression using a filamentous host. Previous work by von Ossowski and coworkers indicated that Cel7A may also be expressed from insect cells; however, no thermal denaturation data was reported (34).

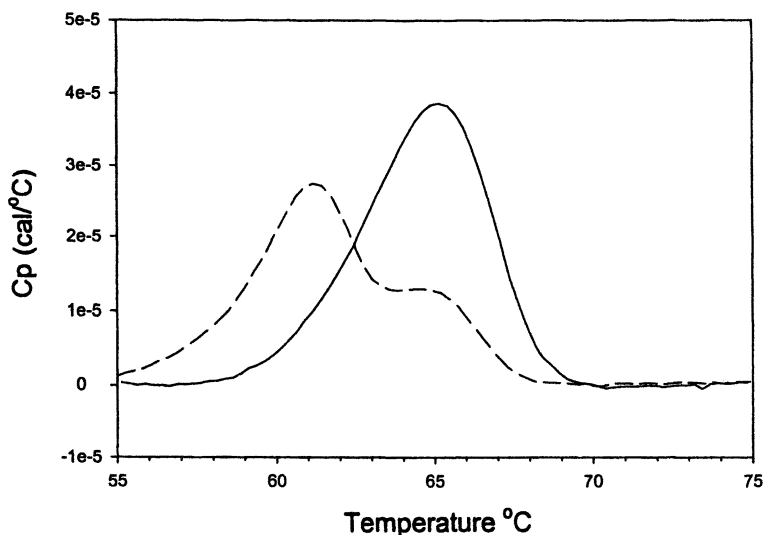


Figure 11. Co-plotted DSC thermograms of native *T. reesei* Cel7A (solid line) and *A. awamori*-expressed rCel7A secreted using the glucoamylase signal sequence (dashed line). Conditions: pH 5.0 in 20 mM acetate, 100 mM NaCl, 50 $\mu\text{g}/\text{mL}$ protein, scanned at 60°C/h.

Comparison of Active rCel7A Forms.

In this study, the thermal stability and conformational changes in Cel7A expressed in different host systems was measured by differential scanning microcalorimetry and circular dichroism spectroscopy. Scanning microcalorimetry gives a direct measurement of the overall thermodynamic properties of a protein such as transition enthalpies, entropies and temperature induced transitions, and serves as a sensitive and direct method to compare protein secondary structure (35). Also of interest in the comparison of different expression hosts is the extent and apparent differences in the degree of glycosylation of rCel7A, and the effect that glycosylation microheterogeneity has on protein conformation and stability. We found that *A. awamori* tends to glycosylate Cel7A more than when this enzyme is expressed in insect cells, and that both enzymes are apparently more heavily glycosylated than the native *T. reesei* enzyme as demonstrated by SDS-PAGE. In general, glycosylation is thought to have a non-specific but positive effect on the thermal stability of proteins, since glycoproteins tend to be destabilized following deglycosylation

(4). The reverse may also be true; the addition of increased oligosaccharide content in proteins expressed in heterologous hosts may confer higher thermal stability, as was observed for β -1,4-glucanases by Olsen and coworkers (36). In our study, there were no apparent differences in the thermal stability of the rCel7A forms examined by scanning microcalorimetry (Figure 8), even though the oligosaccharide contents varied slightly by SDS-PAGE. The far UV circular dichroism spectra for the rCel7A expressed in insect cells and *A. awamori* were nearly identical to spectra obtained for the native protein and are similar to previously reported spectra for the native Cel7A (31). The CD spectra shown in Figure 12 show a broad negative peak from 210 to 218 nm, consistent with the structural features of a protein dominated by random coil. These spectra suggest that there is no significant deviation in the secondary structure conformation of rCel7A expressed in *A. awamori* and insect cells compared to the native protein when processed with the native (*T. reesei*) signal sequence.

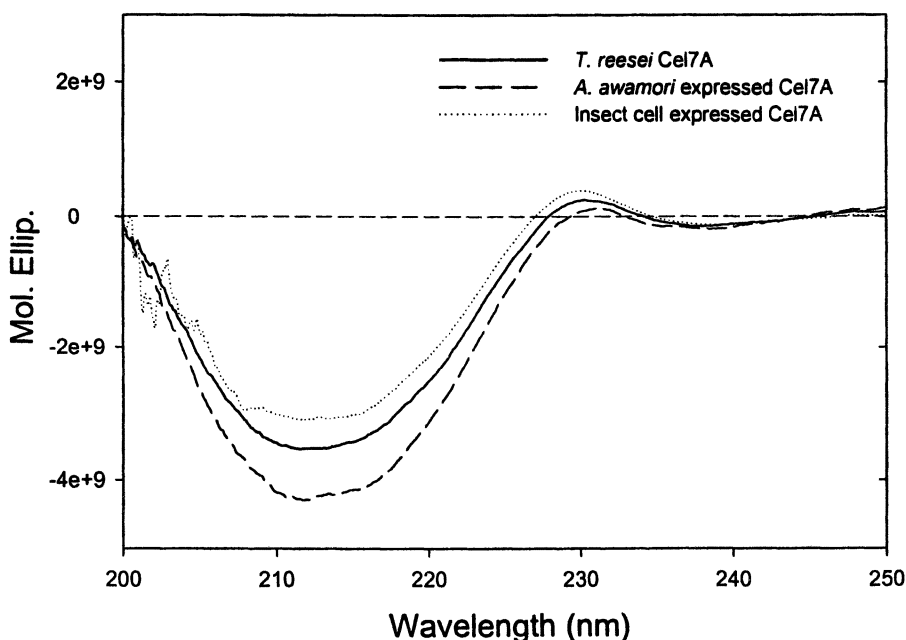


Figure 12. Comparison of CD spectra for native *T. reesei* Cel7A, *A. awamori* expressed rCel7A, and insect expressed rCel7A.

Conclusions

In this study, *T. reesei* rCel7A was successfully expressed from *A. awamori* cells using the native *T. reesei* signal sequence and the *Aspergillus* GA promotor. This rCel7A was found to be indistinguishable from native Cel7A by DSA activity on cellulose, differential scanning microcalorimetry, and circular dichroism analysis. The construct using the GA signal peptide and promotor resulted in an active, but structurally anomalous form or possibly two chromatographically indistinguishable forms. rCel7A expressed from insect cells as also found consistent with all parameters tested to the native *T. reesei* enzyme. SDS-PAGE analysis of these enzyme forms showed that the insect cell expressed enzyme was closest to the native *T. reesei* enzyme in apparent MW; however, the *A. awamori* enzymes were also very similar (i.e., within 10%). The *E. coli* rCelA constructs all resulted in inactive enzymes when tested on cellulose, although some forms were produced with marginal solubility.

Acknowledgments

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References

1. Kendrick, B. *The Fifth Kingdom*; 3rd Edition; Focus Publishing: Newburyport, MA, 2000 pp 38-107.
2. Henrissat, B. *Biochem J* **1991**, *280*, 309-316.
3. Nevalainen, H.; Jardine, M. H. D.; Zachara, N.; Paloheimo, M.; Suominen, P.; In *Carbohydrases from Trichoderma reesei and other microorganisms*; Claeysens, M., Piens, K., Eds.; Royal Society of Chemistry: Cambridge, 1998, pp 335-344.
4. Wang, C.; Eufemi, M.; Turano, C.; Giartosio, A. *Biochemistry* **1996**, *35*, 7299-7307.

5. Klarskov, K.; Piens, K.; Stahlberg, J.; Hoj, P.B.; Beeumen, J.V.; Claeysens, M. *Carbohydrate Research* **1997**, *304*, 143-154.
6. Bergeron, P. In *Handbook on Bioethanol: Production and Utilization*; Wyman, C.E., Ed.; Taylor & Francis: Washington, DC, 1996, pp 90-103.
7. Laymon, R. A.; Adney, W. S.; Mohagheghi, A.; Himmel, M. E.; Thomas, S. R. *Applied biochemistry and biotechnology* **1996**, *57/58*, 389-397.
8. Weiner, M. P.; Anderson, C.; Jerpseth, B.; Wells, S.; Johnson-Browne, B. et al. *Strategies* **1994**, *7*, 41-43.
9. Grodberg, J.; Dunn, J. J. *J Bacteriol* **1989**, *171*, 2903-2905.
10. Murphy, C.; Prinz, W.; Pohlschroder, M.; Derman, A.; Beckwith, J. *Cold Spring Harb Symp Quant Biol* **1995**, *60*, 277-283.
11. Derman, A. I.; Prinz, W. A.; Belin, D.; Beckwith, J. *Science* **1993**, *262*, 1744-1747.
12. Guzman, L. M.; Belin, D.; Carson, M. J.; Beckwith, J. *J Bacteriol* **1995**, *177*, 4121-4130.
13. Maina, C. V.; Riggs, P. D.; Grandea, A. G., 3rd; Slatko, B. E.; Moran, L. S.; Tagliamonte, J. A.; McReynolds, L. A.; Guan, C. D. *Gene* **1988**, *74*, 365-373.
14. LaVallie, E.R.; McCoy, J.M. In *Current Protocols in Molecular Biology*; Ausebel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J.A.; Struhl/John, K., Eds.; Wiley & Sons, Inc.: New Your, NY 1990.
15. Raina, S.; Missiakas, D. *Annu Rev Microbiol* **1997**, *51*, 179-202.
16. Rietsch, A.; Belin, D.; Martin, N.; Beckwith, J. *Proc Natl Acad Sci U S A* **1996**, *93*, 13048-13053.
17. Sone, M.; Akiyama, Y.; Ito, K. *J Biol Chem* **1997**, *272*, 10349-10352.
18. Missiakas, D.; Georgopoulos, C.; Raina, S. *Embo J* **1994**, *13*, 2013-2020.
19. Zapun, A.; Missiakas, D.; Raina, S.; Creighton, T. E. *Biochemistry* **1995**, *34*, 5075-5089.
20. Ho, S. N.; Hunt, H. D.; Horton, R. M.; Pullen, J. K.; Pease, L. R. *Gene* **1989**, *77*, 51-59.
21. Piyachomkwan, K.; Penner, M. H. *Anal Biochem* **1998**, *255*, 223-235.
22. Baker, J. O.; Ehrman, C. I.; Adney, W. S.; Thomas, S. R.; Himmel, M. E. *Appl Biochem Biotechnol* **1998**, *70-72*, 395-403.
23. Himmel, M.E.; Adney, W. S.; Baker, J.O.; Decker, S.R.; Vinzant, T.B.; Nieves, R.A.; Godbole, S.; Thomas, S.R. In *The 98th General Meeting of the American Society for Microbiology*: Atlanta, GA, 1998.
24. Arnold, F. H. *Nat Biotechnol* **1998**, *16*, 617-618.
25. Stemmer, W. P. *Nature* **1994**, *370*, 389-391.
26. Stemmer, W. P. *Proc Natl Acad Sci U S A* **1994**, *91*, 10747-10751.
27. Makrides, S. C. *Microbiol Rev* **1996**, *60*, 512-538.
28. Srisodsuk, M.; Reinikainen, T.; Penttila, M.; Teeri, T. T. *J Biol Chem* **1993**, *268*, 20756-20761.

29. Godbole, S.; Decker, S. R.; Nieves, R. A.; Adney, W. S.; Vinzant, T. B.; Baker, J. O.; Thomas, S. R.; Himmel, M. E. *Biotechnol Prog* **1999**, *15*, 828-833.
30. Boer, H.; Teeri, T. T.; Koivula, A. *Biotechnol Bioeng* **2000**, *69*, 486-494.
31. Penttila, M. E.; Andre, L.; Lehtovaara, P.; Bailey, M.; Teeri, T. T.; Knowles, J. K. *Gene* **1988**, *63*, 103-112.
32. von Ossowski, I.; Teeri, T.; Kalkkinen, N.; Oker-Blom, C. *Biochem Biophys Res Commun* **1997**, *233*, 25-29.
33. Shnyrov, V.L.; J. Boiko, B.N.; Zhadan, G.G.; Permyakov, E.A. *Thermochimica Acta* **1997**, *302*, 165-180.
34. Olsen, O.; Borriss, R.; Simon, O.; Thomsen, K. K. . *Mol Gen Genet* **1991**, *225*, 177-185.

Chapter 24

Recombinant Bleaching Enzymes from Thermophiles Expressed in Fungal Hosts

Peter Bergquist^{1,2}, Valentino Te'o¹, Moreland Gibbs¹,
Natalie Curach¹, and Helena Nevalainen¹

¹Department of Biological Sciences, Macquarie University, Sydney,
Australia

²Division of Molecular Medicine and Pathology, University of Auckland
Medical School, Auckland, New Zealand

Bulk production of hemicellulases from thermophilic microorganisms is a prerequisite for their use in industrial processes. As effective secretors of gene products, fungal expression systems provide a promising, industrially relevant alternative to bacteria for heterologous enzyme production. We are currently developing the yeast *Kluyveromyces lactis* and the filamentous fungus *Trichoderma reesei* for the extracellular production of thermophilic enzymes for the pulp and paper industry. The *K. lactis* system has been tested with two thermophilic xylanases, and produces gram amounts of almost pure xylanase A from *Dictyoglomus thermophilum* in the medium in a chemostat culture. Both fungal and bacterial thermostable xylanases have been successfully expressed in *T. reesei*. Efficient expression of the AT-rich *xynB* gene of *Dictyoglomus thermophilum* required reconstruction of the gene according to *T. reesei* codon preferences. Unlike the situation found in *K. lactis*, a variety of post-translationally-modified species of XynB were visualized on gels. Removal of the N-linked glycosylation sites did not remove all of the iso-forms of XynB, suggesting that other post-translational modifications such as O-linked glycosylation also may be responsible for the multiple protein bands. In a protease-deficient strain of *T. reesei*, removal of all the N-linked glycosylation sites appeared to result in increased yields of XynB secreted into the culture medium. A glucose non-repressible promoter has been isolated to complement the use of the *cbh1* promoter, and combined with a fluorescent protein marker to aid in the identification of transformants and facilitate promoter comparison.

Cost-effective production of heterologous enzymes for commercial or industrial processes makes the appropriate selection of the host-vector expression system critical. However, it has been difficult to achieve effective expression of foreign proteins at high levels to attain the full potential of a particular system. We have chosen to explore the potential of fungi as hosts for heterologous protein production to allow significant scale-up, and to simplify down-stream processing with the objective of being able to use enzymes secreted into the growth medium without further treatment.

Protein production (transcription, translation and secretion) in uni- and multicellular fungi follows the same principles and uses the same basic mechanisms as in other eukaryotes. The yeast *Kluyveromyces lactis* and the filamentous fungus *Trichoderma reesei* are relatively simple organisms that possess the eukaryotic machinery for protein processing. However, there are also differences. *K. lactis* is a unicellular organism related to *Saccharomyces cerevisiae* that has the advantage that does not hyperglycosylate heterologous proteins to the extent seen with *S. cerevisiae* (1). *T. reesei* is an industrially-exploited multicellular organism and one of the most powerful secretors of extracellular proteins, being able to excrete in the order of 40 grams per liter of protein into the culture medium.

The economics required for a particular application varies according to the volume and final product of the process. For industrial processes of large volume, such as pulp bleaching, enzymes must be produced at several grams per liter range to provide a competitive alternative/additive to eliminate or decrease the amount of chlorine dioxide used in pulp delignification. Most commercial xylanases are mesophilic enzymes produced by the filamentous fungi *T. reesei* and *Aspergillus niger*. This reflects the fact that filamentous fungi are naturally excellent protein secretors, and can produce enzymes at industrially-feasible amounts. However, endogenous fungal xylanases may not be efficient enough at all conditions used in paper mills, where enzymes active at high temperatures (up to 85°C and pH 9) would be desirable. Consequently, enzymes from thermophilic microorganisms have attracted attention and a number of genes encoding hemicellulases and cellulases have been isolated from *Bacillus* species, other Bacteria and Archaea (2) and some thermophilic fungi (3-5). However, low productivity, considerable technical problems and unfavorable production economics associated with the cultivation of thermophilic microorganisms on a large scale (6,7) has led to a search for other, industrially-exploited microorganisms as production hosts for thermophilic proteins. Genes of bacterial origin are usually expressed in heterologous bacterial systems, typically *B. licheniformis* or *B. amyloliquefaciens*, which are currently used for the efficient secretion of gene products for industrial applications (8). Even though yields of some secreted enzymes from genetically improved *Bacillus* systems can reach several grams per liter (9), many heterologous gene products suffer from proteases produced by the host organism (10,11). Mutant strains of filamentous fungi can excrete tens of grams of endogenous extracellular protein into their growth medium (12,13). However, there are a few examples of successful expression of bacterial genes in fungal hosts. Many bacterial enzymes

produced in fungi seem to remain trapped in the fungal mycelium (14). With some exceptions, the best yields reported for bacterial enzymes secreted by fungi have been of the order of 10-20 mg/l (15). While fungal systems possess great potential for protein secretion, more research is needed to overcome the bottlenecks of heterologous gene expression.

In this paper, we review our recent experiments on the expression of thermophilic xylanases in the two fungal hosts, *K. lactis* and *T. reesei*.

Materials and Methods

Kluyveromyces lactis system

The construction of the vector pCWK1 and the insertion of the *Dictyoglomus thermophilum* and *Thermotoga maritima* FjSS3B.1 *xynA* genes has been described previously (16-18). The vector is composed of the 2 μ plasmid of *K. drosophilum* for replication in *K. lactis* (19); the origin and ampicillin-resistance gene of pUC19 for replication in *E. coli*, the pLAC4 promoter in front of the killer toxin signal sequence with a single *MluI* (or *AatII*) site for cloning the heterologous gene as a fusion, a LAC4 terminator, the kanamycin-resistance gene from Tn903 (conferring resistance to Geneticin in yeasts) and the URA3 gene of *S. cerevisiae* for integration and selection in antibiotic-free medium (Fig. 1). Other deletion derivatives have been developed, but they were unstable and rapidly segregated as a result of deletion of two inverted repeat sequences believed to be required for stable partition (20).

Trichoderma reesei system

A series of vectors have been constructed by us and others using the strong main cellobiohydrolase *cbh1* promoter (21) with provision for in-frame fusions of the heterologous genes to either the *cbh1* signal sequence or downstream of the *cbh1* core-linker region. These vectors, which are shown in Fig. 2 as stylised representations, may include either a N- or C-terminal 6 x HIS-tag for subsequent purification of the foreign protein, and a KEX2 site (PMDKR or RDKR) has been inserted for correct cleavage to regenerate the mature protein from the CBHI core-linker. Glycine 477 and glutamine 478 of the CBHI cellulose binding domain have been retained to allow cleavage by an uncharacterised *T. reesei* proteinase. Suitable restriction sites have been introduced into the vectors to allow linearization for targeted integration at the *cbh1* locus after the removal of the pUC19 portion of the vector (gapped vector). All vectors have the pUC19 replicon for replication in *E. coli* and utilize the hygromycin B gene as a selective marker in *Trichoderma*.

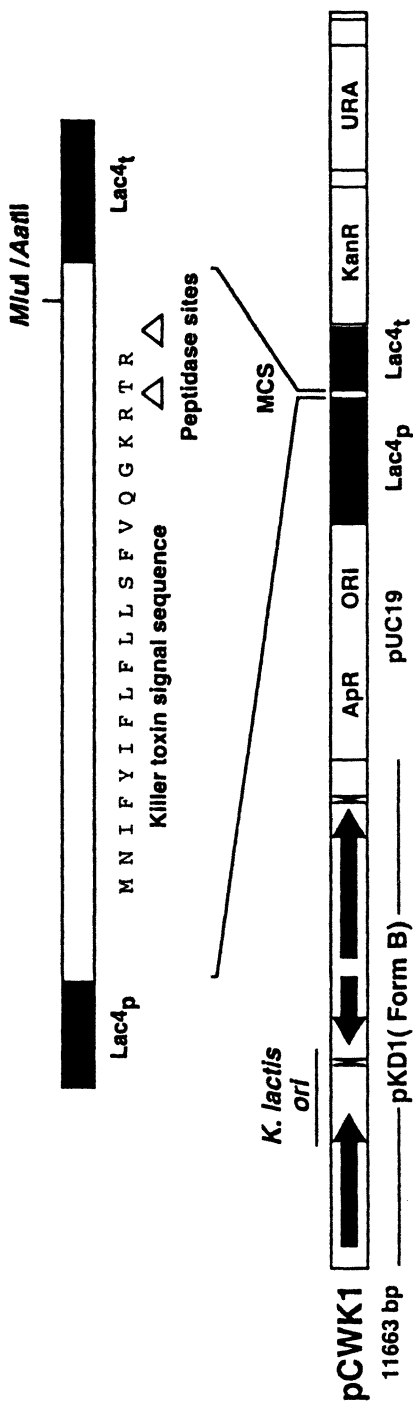
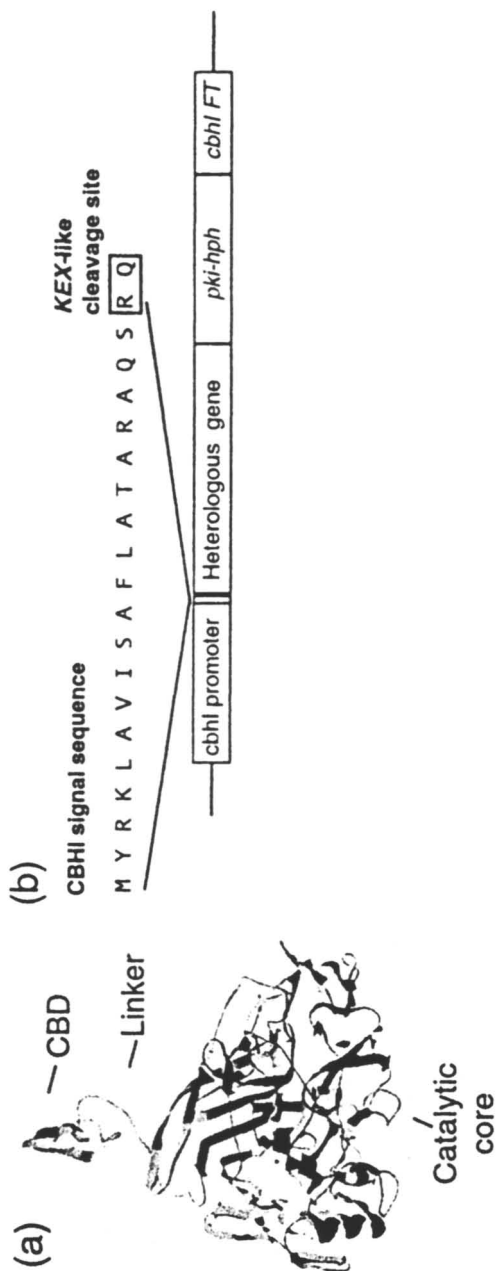


Figure 1. Schematic of the *Kluyveromyces lactis* expression vector *pCWK1*. The 11.66kb vector consists of the 2 μ plasmid of *K. drosophilum*, the *pUC19* plasmid and the *LAC4* promoter and terminator region from *K. lactis*. The killer toxin signal sequence ends in an *AatII* or *MluI* cloning site.



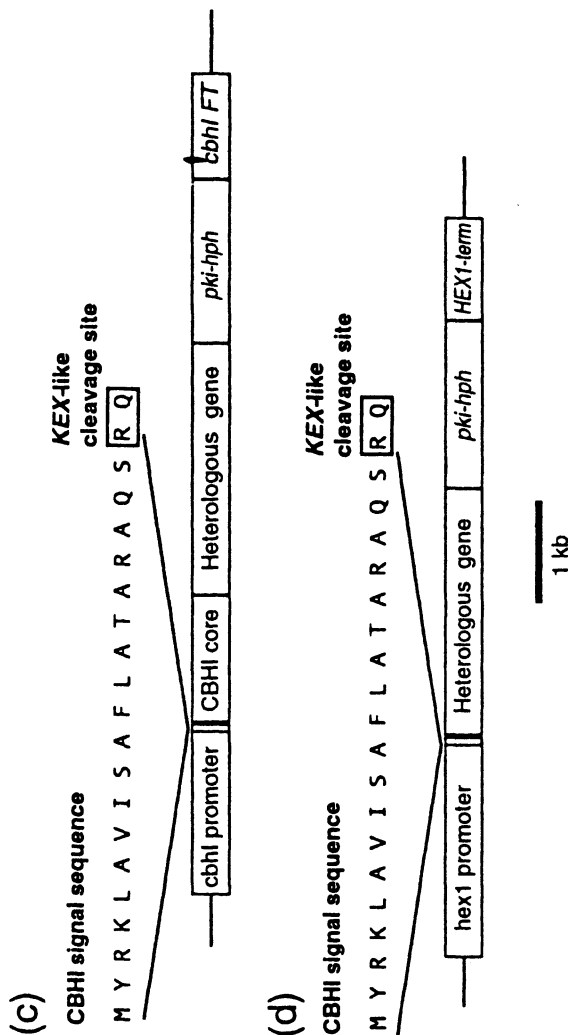


Figure 2. Ribbon diagram of cellobiohydrolase I (CBHI) from *T. reesei* (a) and schematic of *T. reesei* expression vectors (b-d). Vector b has only the CBHI signal sequence fused to the heterologous protein e vector c has the foreign protein fused to the CBHI core-linker sequences. Vector d shows the cbhl and hex1 promoter constructions fused to a heterologous gene sequence.

Synthesis of the *Dictyoglomus xynB* gene with altered codon usage

XynB from the bacterium *Dictyoglomus thermophilum* has been shown to be very effective in the bleaching of kraft pulp (22). Preliminary experiments showed that the native gene was not expressed in *Trichoderma* and that it was necessary to change its codon usage to resemble that of the fungus. A primer extension-PCR strategy was employed to change 20 codons of *xynB* (23). The synthetic gene was first ligated into the pUC19 vector and its functionality tested in *Escherichia coli* using the xylan-overlay assay (24) at 70°C. The synthetic *xynB* insert was isolated from an *E. coli* recombinant found positive for xylanase B production, and inserted into a *T. reesei* expression vector under the *cbh1* promoter. Xylanase production by the transformants was verified in the xylan-overlay assay carried out following overnight incubation at 70°C to inactivate endogenous xylanases. Full details of the experimental methods are published elsewhere (23).

Removal of potential N-glycosylation sites from codon-optimized *xynB*

A PCR site-directed mutagenesis strategy was employed to introduce changes to the three asparagine (Asn) amino acids at positions 9 (changed to Asp), 27 (changed to Asp or Gln) and 193 (changed to Arg), in accordance with the sites described for N-linked glycosylation. This replacement was carried out using primer extension PCR as previously described (23). An expression cassette containing the modified *xynB* gene sequence fused to the *cbh1* signal sequence was constructed and introduced back into *T. reesei*.

Biolistic bombardment of *Trichoderma*

Details of the biolistic transformation using a single barrel procedure, developed for conidia, have been published (25). In this work, we also applied the seven barrel system allowing for more efficient bombardment and therefore higher transformation frequencies (26).

Fusion proteins with Ds-Red in *Trichoderma* vectors

The red fluorescent protein DsRed1-E5 was used as a reporter gene for the activity of *cbh1* and *hex1* promoters. The gene was amplified by PCR from the pTimer plasmid (BD Biosciences Clontech, Palo Alto, CA) with *Kpn1* and *Sall* restriction enzyme sites added to allow insertion into the multiple cloning site of the pHEN54-RQ vector (Fig. 2b) allowing the construction of N-terminal fusions of the gene coding for DsRed with the two promoters.

Proteomic displays of *Trichoderma* cell wall proteins under repressing and inducing conditions

Cell wall fractions were isolated from *T. reesei* cultures grown on a medium inducing cellulase production and secretion, and on a glucose medium that represses cellulase biosynthesis where there is considerably less secretion. The differential analysis of *Trichoderma* cell wall proteins of mycelia grown in the presence of glucose and cellulose has been published (27). In this work, the data was applied to identify dominant proteins encoded by genes featuring strong promoters.

Analytical techniques

Standard protein analysis procedures on polyacrylamide gels have been described, as have enzyme assay procedures (17,22).

Pulp bleaching

ECF bleaching involved a D-EO-D-D procedure as described previously (22).

Results and Discussion

We have reported significant gains in brightness in standard chlorine dioxide bleaching routines for both the *Dictyoglomus* XynB and the *Caldibacillus* ManA enzymes (22,28), but these tests were limited to small-scale bench operations and the enzymes were prepared in *Escherichia coli*. It was evident from the outset that such an expression system was neither efficient nor cost-competitive despite the attractive features of the proteins in terms of stability and operating temperature. Accordingly, we have devoted a substantial effort towards achieving high-level expression in fungi.

K. lactis strains harboring a pKD1-based expression vector efficiently secrete the recombinant *Thermotoga* xylanase at high levels (17,18). All strains examined were able to promote the secretion of xylanase with the same electrophoretic mobility as the control enzyme produced in *E. coli* (approx. 33 kDa, ref. 29). Recombinant xylanase is by far the most abundant protein present in transformed *K. lactis* culture supernatants and the *Thermotoga* XynA protein comprises over 95% of the total proteins secreted from *K. lactis* strain CBS1065 as determined by densitometry of scanned gels (Fig. 3).

Several parameters influence xylanase secretion levels, including plasmid architecture and the composition of growth medium. The carbon source influenced secretion levels by modulating promoter strength, which in turn affects plasmid mitotic stability (17). In non-inducing glucose medium, *xynA* expression directed by LAC4 is partially repressed in CBS1065, although plasmid stability remains high. Full induction of the LAC4 promoter on

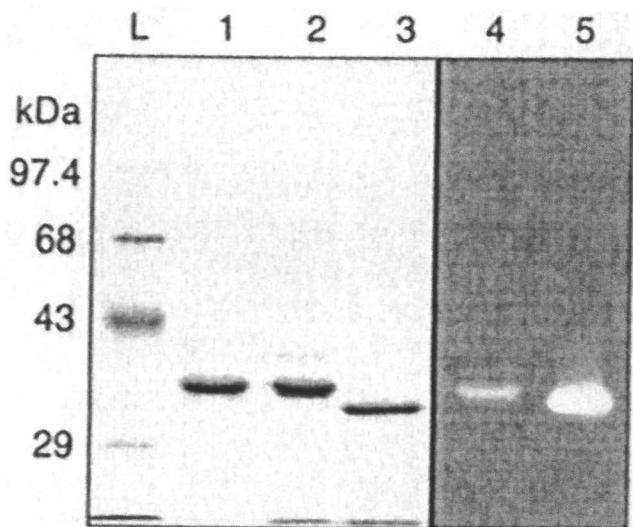


Figure 3. Polyacrylamide gel electrophoresis of supernatants from *K. lactis* strain CBS1065 with pCWK-xynA of *Dictyoglomus thermophilum* Rt46B.1 (lanes 2, 4) or *Thermotoga maritima* FjSS3B.1 (lanes 3, 5). Lanes 4 and 5 show an activity gel exhibiting xylanase activity after renaturation and Congo Red staining of pCWK-xynA supernatant fractions. Lane L; molecular weight markers; Lane 1, purified XynB produced in *E. coli*. For more details, see (17).

galactose results in a drastic reduction in plasmid stability in strain CBS1065, and consequently, lower levels of xylanase secretion are observed (17).

The killer toxin secretion signal (30) is processed by *K. lactis* signal peptidase and cleaves after Gln-Gly in the killer toxin signal sequence and release of correctly processed XynA (17). Recombinant xylanases produced in *K. lactis* are biologically active as shown by activity gels (16,17), and are not hyperglycosylated, as found previously for expression of a thermophilic xylanase in *Saccharomyces cerevisiae* (1). Since little is known of glycosylation patterns in *K. lactis* strains (31) further investigation would help future studies of expression by this organism.

The XynA enzyme from *Thermotoga*, produced in *K. lactis*, has optimal activity at 90°C and a half-life comparable to the results reported for this enzyme produced in *E. coli* (29). Similarly, the *Dictyoglomus* XynA protein produced in *K. lactis* also was shown to have identical biochemical characteristics to its counterpart produced in *E. coli* (15). The next step involves the scale-up of the production system in fermenters for the production of substantial amounts of enzyme for larger-scale bleaching trials that require kilogram quantities of enzyme. It will be necessary to develop an appropriate induction strategy to minimize the effects of plasmid instability with pCWK1 in the absence of Geneticin selection, as well as the rapid metabolism of the

inducer, galactose, in wild-type strains. The galactose concentration falls rapidly, limiting the level of heterologous enzyme produced (Sunna, Gibbs and Bergquist, unpublished). This phenomenon may be overcome by introducing the mutant galactokinase gene *gal1-209* to provide a gratuitous induction system (32).

Transformation of *Trichoderma*

T. reesei is an asexually-reproducing filamentous fungus in which the vegetative growth is mycelial and conidiation is induced by light. *T. reesei* conidia are haploid and uninucleate; intact conidia present excellent material for their direct transformation by particle bombardment. This procedure provides simplicity, savings in time and stable recombinants. Accordingly, we have developed a technique in which DNA is introduced into *T. reesei* conidia using bombardment with tungsten microprojectiles carrying precipitated plasmid, gapped plasmid DNA of one or two different plasmids (25). The cotransformation frequencies obtained are sufficient to avoid the need for cloning the gene of interest in the same plasmid as the selection marker. The procedure is convenient as there is no requirement for osmotic stabilizers or elaborate transformant purification, and the transformants are mitotically stable. The copy numbers of the integrated hygromycin resistance gene varied from 1-6 and integration occurred at several different places on the genome (25). A modification of the procedure in which the 'gene gun' is adapted to carry seven instead of one barrel allows an entire standard petri dish to be bombarded (33). Bombardment conditions have been optimized and up to 50 well-defined *Trichoderma* colonies can be accommodated on a standard petri dish (26). Biolistic transformation with single plasmids has been applied to *T. harzianum* (34) and *T. longibrachiatum* (35) by others.

Heterologous bacterial thermophile xylanase genes expressed in *Trichoderma*

It is necessary to express the *xynB* gene of *D. thermophilum* in a high-yielding organism familiar to the fermentation industry for commercial exploitation. We introduced this gene into *T. reesei*, but preliminary experiments showed no expression. This result led us to compare the codon usage of the *xynB* gene to that of *T. reesei* (Table 1). It appeared that efficiently expressed *Trichoderma* genes exhibit a strong bias against A or T at the anticodon wobble position (36,) whereas *D. thermophilum xynB* prefers A or T at the third codon position (22). In addition, the overall A-T content of *xynB* is 61 % compared to less than 40 % in a typical *T. reesei* cellulase gene. These observations may be a source of potential problems for the expression of genes with high A-T content, such as the *D. thermophilum xynB*, in the fungal host as the result of the formation of truncated mRNA transcripts due to incorrect processing of A-U-rich elements and potential under-representation of isoacceptor tRNAs for effective peptide synthesis (37).

We have reconstructed the 603 bp catalytic domain of the *D. thermophilum xynB* gene to accommodate the codon usage pattern of *Trichoderma*. Altogether 20 codons were changed; but the changes did not alter the amino acid sequence of the gene product. A similar strategy has been successfully applied for the expression of mammalian gene products in *E. coli* (38), but published reports on the synthesis and expression of heterologous genes exhibiting the codon preference of highly expressed genes in filamentous fungi have appeared only recently (23). *T. reesei* transformants carrying the synthetic *xynB* gene produce up to 12 times more thermotolerant xylanase activity (assayed at 70°C) than the nontransformed host.

Our results with the *D. thermophilum xynB* in *T. reesei* indicate that extreme differences in the codon usage between the incoming foreign gene and the expression host could arrest expression at the transcriptional level. Modification of the codon usage of the *xynB* has proved instrumental in restoring effective transcription for the production of the thermophile enzyme in the mesophilic fungal host.

Table I. Major codon usage differences between *T. reesei cbh1* and *D. thermophilum xynB*

Amino acid	Codon	% Usage in <i>T. reesei cbh1</i>	% usage in XynB	New codon
Gly	GAG	82	25	GAG
Gly	GAA	18	75	GAG
Arg	AGA	7	47	CGC
Ile	ATA	4	42	ATC
Thr	ACA	10	47	ACC
Thr	ACT	20	33	ACC
Cys	TGT	24	80	TGC
Tyr	TAT	25	62	TAC
Leu	TTA	1	43	CTG
Leu	CTT	14	30	CTC
Phe	TTT	38	33	TTC
Gln	CAA	19	60	CAG
Pro	CCA	12	50	CCC

Multiple protein species secreted into the supernatant by *T. reesei* expressing XynB

Expression of the thermophilic XynB from *D. thermophilum* in *T. reesei* appears to produce multiple forms of enzyme secreted into the culture medium, which may be glycosylated (Fig. 4). A detailed analysis of the XynB peptide sequence revealed the presence of three potential N-linked glycosylation sites ('N-X-S/T', where N (Asn) is the potential sugar attachment site and X can be

any other amino acid except for Pro). Thus, a PCR-site-directed mutagenesis strategy was employed to introduce changes to the three asparagine (Asn) amino acids at positions 9 (changed to Asp), 27 (changed to Asp and Gln) and 193 (changed to Arg), thus removing the sites described for N-linked glycosylation. An expression cassette containing the modified *xynB* gene sequence fused to the *cbh1* signal sequence was constructed and introduced back into *T. reesei* via biolistic bombardment (26). Transformed cultures revealed the absence of some of the high molecular weight multiple forms present in XynB transformants with the three asparagine sites intact. Other post-translational modifications such as O-linked glycosylation could also contribute to the multiple forms of XynB.

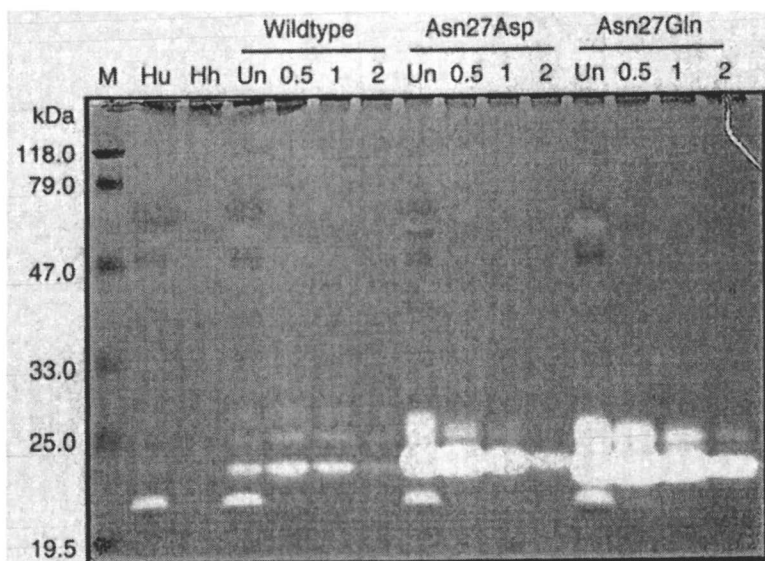


Figure 4. Activity gel showing multiple protein bands in the supernatant of cultures of the proteinase-deficient *T. reesei* strain HEP-1 expressing XynB carrying wild-type and modified N-glycosylation site sequences. The transformant supernatants were heated at 80°C for 0.5, 1.0 and 2.0 hours before gel electrophoresis to inactivate endogenous *Trichoderma* proteins. Heating times are shown on the top of the gel. Un: non-heated supernatant. Hu and Hh indicate non-transformed HEP-1 supernatants unheated and heated, respectively. The band at ~21 kDa represents *Trichoderma* xylanase activity, which is precipitated by heating. The first set of transformants (Wild type Un, 0.5, 1, 2) shows XynB with unchanged N-glycosylation sites. The second and third set represent supernatants from transformants with all three Asn sites changed, expressing XynB where Asn27Asp and Asn27 Gln sequences at the second N-glycosylation site have been changed to either aspartic acid or to glutamine. In these transformants, the asparagines at sites 9 and 197 have been replaced by aspartic acid and arginine, respectively.

An alternative promoter to *cbh1* for gene expression

Until now there has not been an alternative promoter to the strong *cbh1* for heterologous protein expression in filamentous fungi. Cell wall fractions isolated from *T. reesei* cultures grown on cellulase induction and glucose-containing (repressing) medium showed the presence of a dominant HEX1 protein in both preparations (27). The appearance of such high yields indicated HEX1 protein to have been transcribed from a powerful promoter. Thus, the gene coding for HEX1, as well as its upstream promoter fragment, has been isolated using genomic walking PCR, and an expression vector has been constructed such that the fluorescent reporter gene *dsRed* has been fused to the *hex1* promoter for comparative studies between the *hex1* gene promoter and that of the *cbh1* promoter (Fig.5). Preliminary experiments have shown that fungal mycelia expressing DsRed show bright fluorescence on plate and in liquid cultures.

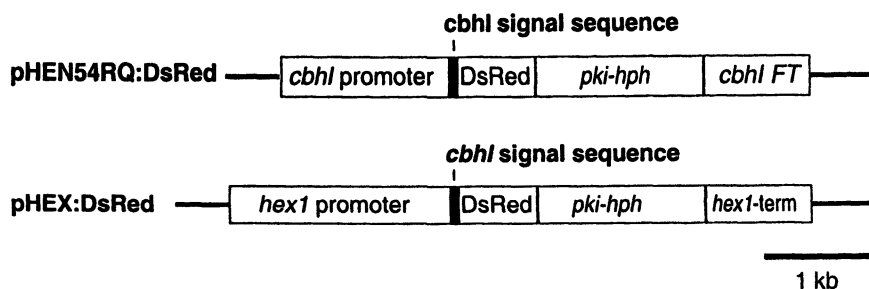


Figure 5. Diagrammatic representation of vectors constructed to compare the *T. reesei* *cbh1* promoter with the *T. reesei* *hex1* promoter as determined by expression levels of *DsRed*.

Conclusions

The *Kluyveromyces* system uses a multicopy plasmid vector and is convenient because of its relative ease of transformation, genetic manipulation and verification and rapid growth. *K. lactis* does not hyperglycosylate proteins in the same manner as *S. cerevisiae* (16,17). Although protein concentration is usually around 100 mg/liter in shake flask culture, we achieved much higher levels in fermentor culture where the pH can be kept constant. However, conditions have not been fully optimized. Using the PLAC promoter has resulted in some problems as this sequence has several *E. coli*-like promoter sequences present. Consequently, there tends to be leakage in steps involving

plasmid DNA replication in the bacterium and it has been difficult to clone and maintain other genes encoding products toxic to the cell in the shuttle vector (Gibbs, Curach and Bergquist, unpublished). Another disadvantage is the instability of the plasmid vector in the absence of Geneticin selection. Although this effect is host strain-dependent, it is possible to prevent segregation of plasmid-less cells in the absence of selection by careful attention to physiological conditions during growth. We have yet to explore enzyme production from genes recombined into the chromosome.

The *T. reesei* system relies on the integration of the transforming DNA into the fungal genome, resulting in a better stability of transformants than when using autonomously replicating plasmids. Our biolistic transformation procedure allows relatively simple and reproducible transformation of industrial strains with a complex history of mutational events aimed at increasing enzyme secretion. The vectors constructed provide a variety of N- and C-terminal modifications for ease of purification and processing. Indirect evidence suggests that the heterologous gene products expressed in *Trichoderma* are being glycosylated, and this modification contributes to the yield of proteins in the supernatant. A combination of proteomic displays and genomic walking PCR has allowed us to identify a protein (HEX1) that is expressed in large amounts in the presence of glucose. The associated glucose non-repressible promoter has considerable potential as an alternative to the *cbhl* promoter for high-level expression of proteins to be secreted into the growth medium.

The basic requirements for a host suitable for large-scale protein production with good economics are the cost and simplicity of cultivation and minimal downstream processing. This can be achieved with a production system where the enzyme is effectively secreted into the cultivation medium. In this regard, fungi provide an attractive option to industrial bacterial systems. Current strategies to improve the processing and yields of biotechnically-relevant heterologous proteins in filamentous fungi include studies into gene regulation, protein glycosylation, occurrence of intra- and extracellular proteases, manipulation of chaperones and foldases assisting other proteins in the secretory pathway, and initiatives in fungal genomics and proteomics. However, there is no single way to success. In each case, such as with bacterial xylanases discussed here, the end result seems to depend on the matching of the gene to be expressed in a particular heterologous host at molecular level. Therefore, making the incoming DNA "interpretable" to the production host is of fundamental importance. For example, each gene can be modified in terms of codon usage and by introducing or deleting signals for a particular cellular location. In addition, it is possible to add post-translational processing sites for the gene product, change the biochemical properties of the molecule to be expressed to facilitate translocation or delete sequences recognized by host proteases. It is evident that further research is needed into the expression biology of the chosen host organism, especially regarding conditions for effective product fermentation. It is reasonable to assume that the yields of recombinant xylanases produced in fungal hosts will be considerably improved over the next few years following the rationale discussed here.

Acknowledgements

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References

1. Donald, K. A. G., Carle, A., Gibbs, M. D. and Bergquist, P. L. *Appl Microbiol Biotechnol.*, **1994**, *42*, 309.
2. Bauer, M. W., Driskell, L. E. and Kelly, R. M.. *Curr Opin Biotechnol.*, **1998**, *9*, 141.
3. Schülein, M., Tikhomirov, D. and Schou, C. In: Suominen, P. and Reinikainen, T. (eds) *Proceedings of the second TRICEL symposium on Trichoderma reesei cellulases and other related hydrolases, Espoo, Finland. Foundation for Biotechnical and Industrial Fermentation Research. 1993*, Vol 8, pp. 109-116
4. Paloheimo, M., Mäntylä, A., Vehmaanperä, J., Hakola, S., Lantto, R., Lahtinen, T., Parkkinen, E., Fagerström, R. and Suominen, P. In: Claeysens, M., Nerinckx, W. and Piens, K. (eds) *Carbohydrates from Trichoderma reesei and other microorganisms. Structures, biochemistry, genetics and applications*. The Royal Society of Chemistry, Cambridge UK. **1998**, pp. 255-264.
5. Faria, F.P., Te'o, V. S. J., Bergquist, P. L., Azevedo, M. O. and Nevalainen, K. M. H.. *Lett. Appl. Microbiol.* **2002**, *34*, 119.
6. Robb, F. T. and Place, A. R. (eds). *Archaea. A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor NY. **1995**, pp 1-217
7. Adams, M. W. W, (ed). *Adv. Prot. Chem.* **1996**, *48*, 1.
8. Debadov, V. G. In: Dubnau, D. A. (ed) *The Molecular Biology of the Bacilli, Vol I: Bacillus subtilis*. Academic Press, New York. **1982**, pp. 331.
9. Yoneda, Y. In: Ganesan, A. T., Chang, S. and Hoch, J. A. (Eds). *Molecular Cloning and Gene Regulation in Bacilli*. Academic Press, New York. pp111.
10. Lundström, K. *FEMS Microbiol. Lett.* **1984**, *23*, 65.
11. Kallio, P., Simonen, M., Palva, I. and Sarvas, M. *J. Gen. Microbiol.* **1986**, *132*, 677.
12. Nevalainen, H., Suominen, P. and Taimisto, K. *J. Biotechnol.* **1994**, *37*, 193.
13. Berka, R. M., Bayliss, F. T., Bloebaum, P., Cullen, D., Dunn-Coleman, N. S., Kodama, K., Hayenga, K., Hitzeman, R., Lamsa, M., Przetak, M. M., Rey, M., Wilson, L. and Ward, M. In: Keely, J. W. and Baldwin, T. O. (eds). *Applications of Enzyme Biotechnology*, Plenum Press, New York, **1991**, pp. 273-292.
14. Jeenes, D. J., MacKenzie, D. A., Roberts, I. N. and Archer, D. B. *Biotech. Genet. Eng. Rev.* **1991**, *9*, 327.

15. Gwynne, D. I., Buxton, F. P., Williams, S. S. A., Garven, S. and Davies, R.W. *Bio/Technol.* **1987**, 5, 713.
16. Walsh, D. J. and Bergquist, P. L. *Appl. Environ. Microbiol.* **1997**, 63, 3297.
17. Walsh, D. J., Gibbs, M. D. and Bergquist, P. L. In: Eriksson K.-E. and Cavaco-Paulo, A. (eds), *Enzyme Applications in Fiber Processing. Am. Chem. Soc. Symp.* **1998**, 687, 155.
18. Walsh, D. J., Gibbs, M. D. and Bergquist, P. L. *Extremophiles*, **1998**, 2, 2.
19. Bianchi, M. M., Falcone, C., Chen, X. J., Wésolowski-Louvel, M., Frontali, L. and Fukuhara, H. *Curr. Genet.* **1987**, 16, 95.
20. Bianchi, M. M. *J. Bacteriol.* **1992**, 174, 6703.
21. Penttilä, M. E. In: *Trichoderma and Gliocladium*, vol. 2, Harman, G. E. and Kubicek, C. P., eds., Taylor and Francis Ltd., London and Bristol, **1998**, pp. 365-382.
22. Morris, D. D., Gibbs, M. D., Chin, C., Koh, M.H., Wong, K. K., Allison, R. W., Nelson P. J. and Bergquist, P. L. *Appl. Environ. Microbiol.* **1998**, 64, 1759.
23. Te'o, V. S. J., Cziferszky, A. E., Bergquist, P. L. and Nevalainen, K. M. H. *FEMS Microbiol. Lett.* **2000**, 190, 13.
24. Teather, R. M. and Wood, P. J. *Appl. Environ. Microbiol.* **1982**, 43, 777.
25. Hazell, B. W., Te'o, V. S. J., Bradner, J. R., Bergquist, P. L. and Nevalainen, K. M. H. *Let. Appl. Microbiol.* **2000**, 30, 282.
26. Te'o, V.S.J., Bergquist, P.L., and Nevalainen, K.M.H. *J. Microbiol. Meth.*, **2002**, Accepted. **51**, 393.
27. Lim, D., Hains, P., Walsh, B., Bergquist, P. and Nevalainen, H. *Proteomics* **2001**, 1, 899.
28. Sunna, A., Gibbs, M. D., Chin, C. W., Nelson P. J. and Bergquist, P. L. *Appl. Environ. Microbiol.* **2000**, 66, 664.
29. Saul, D. J., Williams, L. C., Reeves, R. A., Gibbs, M.D. and Bergquist, P. L. *Appl. Environ. Microbiol.* **1995**, 61, 4110.
30. Tanguy-Rougeau, C., Wésolowski-Louvel, M. and Fukuhara, H. *FEBS Lett.* **1988**, 234, 464.
31. Dominguez, A., Ferminan, E., Sanchez, M., Gonzalez, F. J., Perez-Campo, F. M., Garcia, S., Herrero, A. B., San Vicente, A., Cabello, J., Prado, M., Iglesias, F. J., Choupina, A., Burguillo, F. J., Fernandez-Lago, L. and Lopez, M. C. *Internat. Microbiol.* **1998**, 1, 131.
32. Hsieh, H. B. and Da Silva, N. A. *Biotechnol. Bioeng.* **2000**, 67, 408.
33. Lorito, M., Hayes, C. K., Di Pietro, A. and Harman, G. E. *Curr. Genet.* **1993**, 24, 329.
34. White, T. C. and Hinde, C. in: *Thermophiles '96 Conference*, Athens, Georgia, Sept 4-9, **1996**, Abstract P2 K4.
35. MacKenzie, D. A., Jenes, D. J., Belshaw, N. J. and Archer, D. B. *J. Gen. Microbiol.* **1993**, 139, 2295.
36. Goller, S.P., Gorfer, M., Mach, R. L. and Kubicek, C. P. In: *Applications of PCR in Mycology*, Bridge, P. C., Arora, D. K., Reddy, C. A. and Elander, R. P. (eds.), CABI Publishing, CAB International, New York, **1998**, pp. 21-45.
37. Gouka, R.J., Punt, J., Hessing, J. G. M. and van den Hondel, C. A. M. J. J. *Appl. Environ. Microbiol.* **1996**, 62, 1951.
38. Au, L.C., Yang, W. J., Lo, S. H. and Kao, C. F. *Biochem. Biophys. Res. Commun.* **1999**, 248, 200.

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