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Industrial Applications of Lignin-Transforming Enzymes [and Discussion]

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Industrial applications of lignin-transforming enzymes

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Lignin-degrading and modifying enzymes are produced under specific culture conditions by white-rot fungi. Most of these enzymes are excreted into the extracellular environment and can be purified from culture supernatant. At RepliGen we have characterized many of the extracellular proteins from the white-rot fungus *Phanerochaete chrysosporium*. Industrial application potentials for these enzymes are predicted to be in the chemical industry, pulp and paper industry, and perhaps in pollution control.

The isolation and characterization of enzymes that catalyse specific reactions on kraft lignins and lignosulphonates will be discussed. These reactions include (1) a polymerizing–depolymerizing activity that changes the size of the lignin substrate and (2) a decolorizing reaction that reduces chromophoric groups in lignin. Kraft lignin and lignosulphonates have many diverse commercial applications because of their dual properties of hydrophobicity and hydrophilicity. They could have broad use if modified, as in the above reactions, in an efficient manner. The development of enzyme systems may provide just such efficient reactions.

Introduction

New methods of advanced biotechnology are now being used to develop products for industrial application. One of the most exciting of these is the development of lignin-transforming enzymes, utilizing state-of-the-art techniques of recombinant-DNA technology, protein, organic and inorganic chemistries, genetic and chemical engineering. The successful industrial application of lignin transforming, e.g. degrading and modifying enzymes, has the following objectives.

1. Efficacious application of the enzymes to appropriate industrial operations, i.e. in the pulp and paper or chemical industries. The application of the enzymes should provide some advantage over other means for the process or its product.
2. Economically viable production of the enzymes and their relative high stability.
3. Amenable integration into existing processes.

The industrial application of lignin-transforming enzymes requires both basic and applied research: some basic science is crucial for commercialization, and that which is not crucial is very interesting and fundamental in several fields of science. The goal of this paper is to describe some of RepliGen's current research on lignin-transforming enzymes and to further elaborate on possible industrial applications.

Biochemistry of lignin-transforming enzymes

Tien & Kirk (1983) first discovered and characterized a ligninolytic enzymatic activity that could be purified from the extracellular growth medium of a *Phanerochaete chrysosporium* culture.

In this symposium, Dr Kirk has discussed the biochemistry of ligninase and probable reaction mechanisms. RepliGen began a collaboration with Kent Kirk and Ming Tien of the U.S.D.A. Forest Products Laboratory in 1984.

Feasability testing for industrial applications requires that enough enzymes be produced for structural and functional characterization as well as pilot testing. Approaches for increasing enzyme production involved (1) surveying the effects of growth conditions, (2) selection of mutant ligninase 'over-producer' strains, and (3) ligninase gene cloning.

A survey of growth-medium additives was carried out on the *Phanerochaete chrysosporium* BKM wild-type strain (BKM) (ATCC no. 24725) (Kirk *et al.* 1986). Faison & Kirk (1983) had shown that ligninase activity was increased in cultures of *Phanerochaete chrysosporium* ME446 by the addition of veratryl alcohol, a secondary metabolite of the organism. This was tested on cultures of BKM and results are shown in table 1 (Kirk *et al.* 1986).

TABLE 1. EFFECT OF GROWTH MEDIUM ADDITIVES ON TOTAL LIGNINASE ACTIVITY (STRAIN BKM)

condition	activity (unit l^{-1})	increase in activity	specific activity (unit mg^{-1})	increase in specific activity
(*a*) control	20.2±0.3	1.0	5.4	1.0
(*b*) +0.4 mM veratryl alcohol	42.6±10.4	2.1	10.4	1.9
(*c*) +6-fold trace elements	34.9±1.5	1.7	8.3	1.5
(*d*) +0.4 mM veratryl alcohol+ 6-fold trace elements	95.5±16.2	4.7	22.2	4.1
(*e*) +6-fold Cu^{2+}	33.8±1.4	1.7	10.2	1.8
(*f*) +6-fold Mn^{2+}	36.4±5.8	1.8	11.3	2.1

Addition of 0.4 mM veratryl alcohol resulted in a 1.9-fold increase in specific activity on day 5. Increasing the concentrations of a trace element solution (containing magnesium, manganese, cobalt, copper, iron, aluminum, zinc and molybdenum salts) to 7-fold (+6-fold over basal level), without veratryl alcohol addition, also resulted in 1.5-fold higher ligninase activity. The addition of veratryl alcohol and increased trace element solution resulted in an additive increase in ligninase activity (4.1-fold). Further testing indicated that addition of 7 times the basal-level concentration of either $CuSO_4$ or $MnSO_4$ (respectively 4×10^{-5} or 3×10^{-3} M) causes an increase in activity equal to that observed with the complete trace element solution.

The extracellular growth medium of ligninolytic cultures can be analysed by high-performance liquid chromatography (HPLC) with an FPLC anion exchange column. The resulting HPLC profiles of the concentrated extracellular fluids from the cultures (sample (d), table 1) is shown in figure 1. There are more than 13 peaks with 280 nm absorbance and at least 10 with 410 nm absorbance. Absorbance at 410 nm indicates a haem-containing peak, i.e. that of a haem protein.

Peaks designated H1, H2, H6, H8, and H10 all have veratryl alcohol-oxidizing activity. H8 appears to be the original ligninase isolated by Tien & Kirk (1983). The proportions of the haem proteins can vary depending on culture conditions, including media composition, time of culture and culture protocol, including whether cultures are grown in stationary or shake flasks, in rotating biological contactors (RBCs) or fermentors. Proteins from the HPLC-purified peaks have been analysed by SDS-polyacrylamide gel electrophoresis; H1 and H2 show an

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apparent molecular size of 38 kDa; H6, although isolated from the HPLC as a single eluting peak, shows two bands at apparent molecular size of 39 and 41 kDa; H8 and H10 both demonstrate an apparent size of 41 kDa. Apparent sizes are approximate molecular masses because all the enzymes are glycosylated.

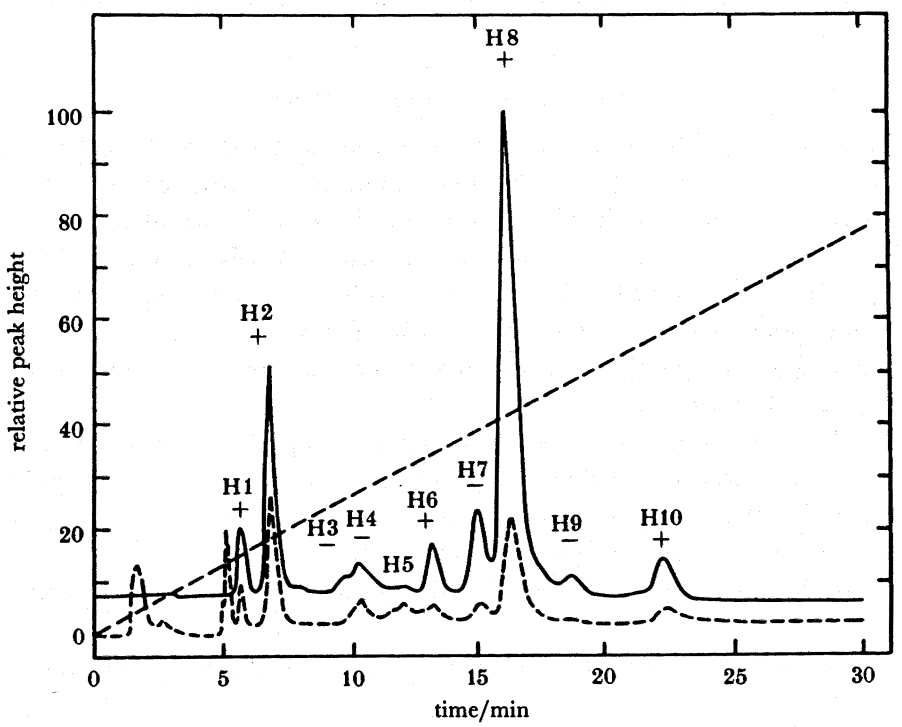

FIGURE 1. High-performance liquid chromatogram of five-day old *P. chrysosporium* BKM concentrated extracellular growth medium. The solid line represents absorption of 410 nm and the broken line represents absorption at 280 nm. Peak designations are indicated. A plus (+) sign indicates a positive reaction catalysed by the purified peak in a veratryl alcohol oxidation (Kirk *et al.* 1986).

Selection of ligninase 'over-producer' mutant strains of *P. chrysosporium* can facilitate enhanced production of lignin-transforming enzymes. Ultraviolet mutagenesis of BKM and selection for enhanced decolorization of E1 effluent, the effluent from the first alkali extraction of a kraft pulp bleach plant, resulted in the isolation of the mutant SC26. It has similar growth requirements to the wild type, although its hyphae have a greater polysaccharide sheath as evidenced by phosphotungstic acid staining. An additional property of SC26 is a higher production of total ligninase activity per millilitre of growth medium (Kirk *et al.* 1986). The haem proteins produced by SC26 appear to be the same as those produced by BKM. The higher ligninase activity in SC26 is caused by their greater accumulation in the growth medium (Kirk *et al.* 1986).

Large-scale production of ligninases from either wild-type or mutant over-producer strains of *P. chrysosporium* are unlikely, though, to be cost effective. The induction of enzymic activity is under secondary metabolic control, which is difficult to regulate and requires a culture time of 6–8 days. A screen for constitutive ligninolytic *P. chrysosporium* cultures is underway, but if successful it will only solve part of the fungal culture problems (M. Tien, personal communi-

cation). With constitutive ligninolytic cultures it would still be necessary to have large-scale fermentation for industrial application. Fermentation success at this time is only at the 10 l scale.

RepliGen has therefore cloned ligninase, and is expressing the recombinant-ligninase gene in a variety of hosts. With recombinant-DNA engineering techniques one can conceivably modify the ligninase genes, and thus the proteins, to increase either enzyme efficacy or stability.

Industrial applications

Applications for lignin-transforming enzymes have been suggested from fungal treatment work by K.-E. Eriksson of the Swedish Forest Products Laboratory, whose recent work is described in this symposium, T. K. Kirk, of the U.S.D.A. Forest Products Laboratory, H.-m. Chang and T. Joyce of North Carolina State University, and S. Aust of Michigan State University among others (Eriksson & Vallander 1980; Kirk & Chang 1981; Bumpus & Aust 1985).

The industrial applications may be to the following fields.

1. The bleaching of kraft pulp, specifically to displace chlorine-based oxidizing agents in the bleaching of chemical pulps.
2. Partial delignification of coarse thermomechanical pulp. Also, the enzymes may significantly reduce the brightness reversion and increase strength of mechanical pulps without significantly affecting the high yield contributed by lignin.
3. Decolorization of the effluent from bleaching plants.
4. Lignin-transforming enzymes may be detoxification agents by degrading pollutants such as dioxin and DDT.
5. Lignin-transforming enzymes may be used to enhance the utility of kraft lignin and lignosulphonates.

Kraft lignin and lignosulphonates are by-products of pulping and are isolated respectively from the black liquor of the kraft or sulphite cook processes in wood pulping. The majority of black liquor is currently burned as fuel, after which inorganic chemicals are recovered for recycling. Kraft lignin and lignosulphonates have many diverse commercial applications because of their dual properties of hydrophobicity and hydrophilicity. To accomplish the goal of conversion of kraft lignin and lignosulphonates into more usable products, specific modification and degradation reactions need to be employed. Chemical reactions for modifying the kraft lignins and lignosulphonates exist, but they are costly, non-specific and require extensive purification of the desired end products from side-reaction products (Glasser 1981). We have demonstrated with lignin-transforming and other enzymic activities depolymerization and decolorization reactions on lignin substrates.

In conclusion, the industrial application of the lignin-transforming enzymes may account for improved product quality and yield, decreased operating costs regarding the chemical, waste treatment or energy costs, and possibly decreased capital costs in the pulp and paper industry. With application to lignin by-products, there may be expanded and created products for chemical production, binders, adhesives, and perhaps petrochemical substitutes.

This paper is dedicated to the memory of Dr Robert J. Farrell II, who delighted in all scientific knowledge. I thank Judith Hooper and Ted Maione for technical assistance and helpful discussions, and Ms Kelly Beckman for preparation of this manuscript.

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Discussion

D. A. Wood (*Glasshouse Crops Research Institute, Littlehampton, West Sussex, U.K.*). It would be interesting to know the likely cost of using 'ligninase' to treat pulp and other products given the current knowledge of its molecular specific activity and its stability.

Currently, bulk enzymes are used in food and beverage processing, where relatively high-value human foodstuffs are produced. To use enzymes in high-volume, low-value bulk applications will represent a novel departure from the normal economics of bulk enzyme use.

Roberta L. Farrell. At this point it is premature to estimate a likely cost of using 'ligninase' in any application. The cost will be dependent upon several factors including, but not limited to, production costs of recombinant ligninase (dependent upon host organism, fermentation capacity, process handling etc.), the amount and recyclability of recombinant ligninase used for application, and the value added to the final product as a result of ligninase applications. Likely cost estimates will only be meaningful after pilot-plant testing of production of the enzyme and its industrial application.

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