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REVIEW

Bifunctional xylanases and their potential use in biotechnology

Rakhee Khandeparker · Mondher Th. Numan

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Abstract Plant cell walls are comprised of cellulose, hemicellulose and other polymers that are intertwined. This complex structure acts as a barrier to degradation by single enzyme. Thus, a cocktail consisting of bi and multifunctional xylanases and xylan debranching enzymes is most desired combination for the efficient utilization of these complex materials. Xylanases have prospective applications in the food, animal feed, and paper and pulp industries. Furthermore, in order to enhance feed nutrient digestibility and to improve wheat flour quality xylanase along with other glycohydrolases are often used. For these applications, a bifunctional enzyme is undoubtedly much more valuable as compared to monofunctional enzyme. The natural diversity of enzymes provides some candidates with evolved bifunctional activity. Nevertheless most resulted from the in vitro fusion of individual enzymes. Here we present bifunctional xylanases, their evolution, occurrence, molecular biology and potential uses in biotechnology.

Keywords Xylanase · Bifunctional · Enzyme · Biotechnology · Hemicellulose · Cellulase

Introduction

Cellulose, hemicellulose, and lignin are the major components of plant cell walls [89]. When combined, they form the support framework for plants and defend against the invasion of aggressors. To successfully invade or live on plant tissues, microorganisms have to synthesize a number of different enzymes in order to hydrolyze cellulose or hemicellulose. Thus, to effectively degrade the plant cell wall complex, many microorganisms develop a cell associated multiprotein complex, called cellulosome [43] or xylosome [47], which contains cellulases, xylanases, and cellulose-binding factors. Another strategy is to induce the multifunctionalization of certain enzymes to hydrolyze different kinds of substrates. In addition to its importance in allowing microorganisms to invade plants and degrade plant residues, xylanase also has prospective applications for the food, animal feed, and paper and pulp industries. Furthermore, for the enhancement of feed nutrient digestibility [54, 56, 68, 93] and improvement of wheat flour quality [30] xylanases and β-(1,3-1,4)-glucanases are used at the same time. Similarly, bioethanol production requires efficient saccharification for degrading plant cell walls efficiently to fermentable sugars. Therefore, by biologically converting plant cell walls to fermentable sugars for fuel (e.g., ethanol), we could obtain not only economic but also environmental benefits, such as the reduction of greenhouse gas emission. A critical factor concerning the cost of this process is the presence of efficient, cheap cellulases and xylanases to achieve this goal in a single step.

Utilization of pentoses sugars present as hemicellulosic fraction of lignocellulosic biomass is a major step towards reducing production cost of bioethanol. Morever, presence of xylanases along with cellulases in the fermentation mixture during mashing process can solve the problem of viscosity caused by pentosans [75]. For these applications, a bifunctional enzyme is undoubtedly more valuable than a single enzyme. In this review we will discuss the recent development in bifunctional xylanases with respect to their occurrence in microorganisms, molecular biology, genetic and protein engineering, and their importance for industry.

R. Khandeparker (✉) · M. Th. Numan
National Institute of Oceanography,
Goa Dona Paula 403004, India
e-mail: rakhee@nio.org

Lignocelluloses structure and their occurrence

Lignocellulose is the major structural component of woody plants and non-woody plants such as grasses and represents a major source of renewable organic matter. Lignocellulosic materials predominantly contain a mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin. The chemical properties of these components of lignocelluloses make them a substrate of enormous biotechnological value [52]. Lignin is a biopolymer rich in phenolic components, which provides structural integrity to plants. Cellulose is unbranched linear polymer. The length of a cellulose molecule (polymer) is determined by the number of glucan units in the polymer, reffered as the degree of polymerization. The degree of polymerization of cellulose depends on the type of plants and typically is estimated to be from 2,000 to 27,000 glucan units [60]. Hemicellulose belongs to a group of heterogeneous polysaccharides. Hemicelluloses are relatively easily hydrolysed by acids to their monomer components consisting of xylose, mannose, glucose, galactose, arabinose and small amounts of rhamnose, glucuronic acid, methyl glucuronic acid and galacturonic acid [77]. The relative abundance of cellulose, hemicellulose, and lignin contents in various lignocellulosic biomass [88] are presented in Table 1.

The combination of hemicelluloses and lignin provide a protective sheath around the cellulose. Degradation of this complex structure requires a complete enzymatic system that includes cellulases and hemicelluloses. Cellulases that take part in cellulose degradation includes (1) endoglucanases (EG, endo-1,4-D-glucanohydrolase or EC 3.2.1.4.), which attacks regions of low crystallinity in the cellulose fiber, creating free chain ends [48]; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiodehydrolase or EC 3.2.1.91.), degrades the molecule further by removing cellobiose units from the free chain ends [91]; and (3) β-glucosidase (EC 3.2.1.21.), hydrolyzes cellobiose to produce glucose [18]. Furthermore, different enzymes mainly xylanase, acetylxylan-esterase, α-glucuronidase, β-xylosidase and α-arabinofuranosidase take part in degradation of hemicelluloses. For efficient degradation of plant cell walls, these hydrolyzing enzymes cooperatively work to digest polysaccharides (Fig. 1).

Table 1 Cellulose, hemicellulose, and lignin content in various sources of biomass [88]

Feedstock	Cellulose	Hemicellulose	Lignin
Corn stover	36.4	22.6	16.6
Wheat straw	38.2	24.7	23.4
Rice straw	34.2	24.5	23.4
Switchgrass	31.0	24.4	17.6
Poplar	49.9	20.4	18.1

Evolution of bifunctional enzymes

A bifunctional enzyme is an enzyme containing two distinct catalytic capacities in the same polypeptide chain. They usually catalyze two consecutive reactions [84]. Moreover, bifunctional enzymes usually catalyze complex multi-substrate reactions whose mechanisms involve a large number of intermediate enzyme forms. These features potentially allow manifestation of some new specific properties of bifunctional enzymes. First, the possible mobility of an intermediate (a product of the first reaction and at the same time, substrate of the second reaction) between two active sites without its appearance in solution [34, 55, 58]. Second, the state of the active site of the first reaction may

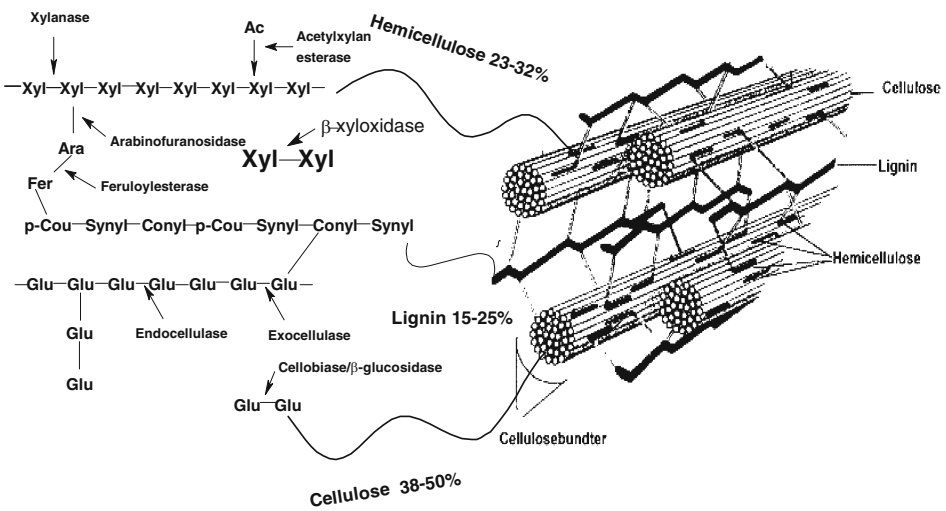

Fig. 1 Complex mixture of enzymes for degrading lignocelluloses. The complex composition and structure of lignocelluloses require multiple enzymes to break down the polymer into sugar monomers. These enzymes include: arabinofuranosidase, feruloyl esterase, acetylxylan esterase, and beta-xylosidase. Cellulose is degraded by endocellulase, exocellulase, and β-glucosidase

influence kinetic properties of the active site of the second reaction, and vice versa, the state of the active site of the second reaction may influence kinetic properties of the active site of the first reaction [46].

Bifunctional (or polyfunctional) enzymes seem to appear in evolution by the combination of genes encoding enzymes tightly bound functionally [92, 78]. The complex enzymes that are isolated and characterized are hypothesized to have evolved from more primitive precursors. At the metabolic level simple, nonfunctional enzymes are seen as an early stage of evolutionary refinement. The clustering of isolated genes that encode for enzymes with related metabolic activities represents a significant advancement since the expression of related enzymes can now be controlled by common regulatory elements. At the protein level the association of individual enzymes in the bifunctional or multifunctional enzyme complexes provides several distinct advantages. Catalytic events that take place on one enzyme can have a direct influence on the associated enzymes that are present in the complex. Single regulatory sites or regulatory subunits can control the coordinated activities of all of the enzymes in the complex. Also, the directed transfer of reactants from consecutive active sites can support more efficient metabolism. From this point of view, the most highly evolved enzyme forms are seen to be multifunctional enzymes in which gene fusion has incorporated related catalytic activities into covalently coupled domains in a single polypeptide chain [39]. Enzymes that catalyze sequential reactions tend to evolve toward bi or multifunctional enzymes for more efficient metabolism [76]. This evolution may also occur under circumstances where the substrates of microbial enzymes are proximate, e.g., in rumen microorganisms capable of exploiting plant fibers [23, 27, 29, 91], suggesting a possibility of constructing bifunctional enzymes for use in feedstuff.

Interdomain communications in bifunctional enzymes

Numerous metabolic pathways in prokaryotes and in eukaryotes include bifunctional enzymes, for example an enzyme dehydroquinate synthase has an active site capable of multistep catalysis in aromatic aminoacid biosynthesis [13]. In many cases, such enzymes contain two different active centers located within separate domains linked on a single polypeptide and encoded by a single gene. Three types of bifunctional enzyme are known: those catalyzing consecutive reactions of a metabolic pathway [58, 34, 9, 69], those which catalyze two non-consecutive reactions [39], and those catalyzing opposing reactions [66]. The role played by specific interdomain interactions in the functioning of different active centers within a macromolecule presents a problem of considerable interest. During the last decade, marked progress has been made in this area of research owing to studies performed on bifunctional enzymes catalyzing consecutive reactions and exhibiting substrate channeling. Substrate channeling is a process of direct transfer of a reaction intermediate between distinct active centers. This prevents the loss of the intermediate into solution, limits its entrance into competing processes and increases the success rate of the catalytic cycle by lowering the probability of uncoupled consecutive reactions [58].

Although bifunctional enzymes containing two different active centers located within separate domains are quite common in living systems, the significance of this bifunctionality is not always clear, and the molecular mechanisms of site-site interactions in such complex systems have come under the scrutiny of science only in recent years. According to Nagradova [62], there exist a tight coupling between active centers catalyzing consecutive reactions, whereby at a particular step of catalysis at one site, a signal is transmitted to the other site to activate it and to allow for a rapid tunneling of the intermediate from the site where it is synthesized to the site of its utilization. It is suggested that the activation process may be stepwise, involving distinct intermediate conformations of the protein, which depend on the redox and substrate ligation state.

Natural diversity of bifunctional enzymes

Bifunctional xylanase–cellulases

The natural diversity of enzymes provides some candidates that have evolved bifunctional xylanase–cellulase complex. For example, Pohlschroder et al. [71] showed the presence of a multicomplex cellulase–xylanase system in *Clostridium papyrosolvens* C7. The enzyme system of *C. papyrosolvens* C7 responsible for the hydrolysis of crystalline cellulose and xylan is a multicomplex system comprising at least seven diverse protein complexes. Two of these seven complexes are having xylanase activity in addition to cellulases activity. Another cellulose–xylanase complex has been reported by Murashima et al. [61]. Both the enzymes in this complex proved to work simultaneously for synergistic degradation of corn cell wall. Furthermore, the amounts of both xylooligosaccharides and cellooligosaccharides librated from corn cell walls were increased by the action of both enzyme XynA (xylanase) and cellulase domains. What was interesting is the finding that, sequential action of each enzyme domain alone was found to show no synergistic effects. This finding may indicate the importance and efficiency of an enzymatic system that posses both xylnases and cellulase activities. In fact, the degradation of xylan networks between cellulose microfibrils by

xylanases might allow cellulases to access and degrade cellulose microfibrils embedded in the deeper structure. Also, degradation of cellulose microfibrils in the deeper structure might help xylanase to access and degrade xylan chains further in the deeper structure. This might explain why cellulase required the presence of xylanase and how xylanase facilitate the action of cellulase and vice versa. Chemical nature and structural complexity of lignocelluloses may force microorganism to develop many enzymes, which could solve this complexity. Many other microorganisms found another way to facilitate the biodegradation of such agricultural residues. This is either by forming enzymes complexes in the form of, bifunctional, or multifunctional enzymes. Another bifunctional xylanase–cellulase has been reported by Flint et al. [23]. According to them, adjacent regions of a *Ruminococcus flavefaciens* 17 DNA fragment encoded xylanase and β-(1,3-1,4)-glucanase activities. Sequencing of this fragment showed that both activities are encoded by a single 2,406 bp open reading frame corresponding to the XynD gene. Single gene, xynD, encoded a bifunctional enzyme having separate xylanase and 1-3(1,3-1,4)-glucanase domains. This is an example of a bifunctional polysaccharidase having two separate catalytic domains within the same polypeptide chain that can act on different polymeric substrates. The N-terminal section of this polypeptide belongs to the glycosyl hydrolase 11 (cellulase G) families while the C-terminal section belongs to the glycosyl hydrolase 16 family. These two domains are connected by a region of unknown function that consists of 309 amino acids and includes a 30-amino-acid threonine-rich sequence. Furthermore, three types of xylanases (EC 3.2.1.8) were detected in the strain *Aspergillus niger* A-25 by Chen et al. [15], one of which, designated as XynIII, displayed β-(1,3-1,4)-glucanase (EC 3.2.1.72) activity, The purified XynIII could hydrolyze birchwood xylan, oat spelt xylan, lichenin, and barley β-glucan, but not CMC, avicel cellulose, or soluble starch. This indicates that this enzyme is a novel bifunctional enzyme and its xylanase and β-(1,3-1,4)-glucanase activities are catalyzed by the same active cite.

Bifunctional xylanase–arabinosidase

Xylan degradation is a multistep process involving multiple enzymatic activities. Xylanases are extracellular enzymes that hydrolyze the internal β-1,4-xylosidic linkages of the xylan backbone structure. Xylanase action is restricted by the presence of side chains. Removal of side-chain substituents requires additional enzymatic activities of arabinofuranosidase, uronidase, glucosidase, mannosidase, and acetyl esterase [65]. The xylanase gene xysA of *Streptomyces halstedii* JM8 was used to isolate a DNA fragment from a gene library of the lignocellulolytic actinomycete *Streptomyces chattanoogensis* CECT-3336. Nucleotide sequence analysis revealed a gene (xln23) encoding a bifunctional multimodular enzyme bearing two independent xylanase and α-L-arabinofuranosidase domains separated by a Ser/Gly-rich linker. The N terminus of the predicted protein showed high homology to family F xylanases. The C terminus was homologous to amino acid sequences found in enzymes included in the glycosyl hydrolase family 62 and, in particular, to those of α-L-arabinofuranosidase from *Streptomyces lividans*. PCR and RT-PCR experiments showed that the nucleotide sequences corresponding to each domain are arranged on the chromosomal DNA and that they are co-transcribed [32]. To our knowledge, this is the only report that described xylanase and arabinofuranosidase domains in the same open reading frame.

Bifunctional xylanase–deacetylase

Pseudobutyrivibrio xylanivorans has a potent xylanolytic enzyme system. Xyl 11A isolated from this bacterium belongs to the glycosyl hydrolase 11 (cellulase G) family. The gene encoding for Xyl 11A contains N-terminal CBM6 (cabohydrate binding type-6) domain, which exibit xylanolytic activity, and C-terminal domain coding for putative polysaccharide deacylase implicated in removing acetyl groups from acetylated xylan, and thus it is probably capable of hydrolyzing acetylated xylan debranching in the xylan backbone [14]. *Cytophaga hutchinsonii* is reported to produce bifunctional xylanase/esterase enzyme coded by CHU-1239 gene CHU-1240, where xylanase belongs to glycoside hydrolase family 8 proteins and esterase belong to carbohydrate esterase family 4 proteins. Furthermore, CHU-1239 gene form same bacterium is responsible for bifunctional acetylxylan esterase/xylanase enzyme where xylanase belongs to glycoside hydrolase family 10 protein and carbohydrate esterase family 6 protein [90].

Bifunctional xylanase–xylanase

The complex chemical nature and heterogeneity of xylan can account for the multiplicity of xylanases produced by microorganisms. The subtle differences in substrate specificity and mode of action of multiple xylanases improve the degradation of plant xylan in natural habitats [15]. Bifunctional xylanase–xylanase enzyme having two catalytic domains belonging to different families is reported from *R. flavefaciens*. The nucleotide sequence of the xynA gene of *R. flavefaciens* 17 was determined and found to consist of a 2,862 bp open reading frame beginning with a TTG start codon. The predicted product, XYLA, consisted of distinct amino-terminal (A) and carboxy terminal (C) domains (248 amino acids, including a putative signal sequence, and 332 amino acids, respectively) linked by a repetitive sequence

(B, 374 amino acids) extraordinarily rich in asparagine (45%) and glutamine (26%) residues. Domains A and C were shown to be capable of expressing xylanase activity independently of each other when suitably truncated derivatives of the xynA coding region were expressed as lacZ fusions. The activities associated with the two domains were shown to differ with respect to the average size of hydrolysis products formed from oat-spelt xylan, with domain C releasing relatively more xylose and domain A more xylooligosaccharides. The amino acid sequence of domain A of XYLA closely resembled that of the *Bacillus pumilus* xynA enzyme (45% identical residues). On the other hand, domain C showed significant similarity (33–40% identical residues) to a different group of bacterial xylanases and exoglucanases exemplified by the *Caldocellum saccharolyticum* xynA and celB products. The xynA product is, therefore, a bifunctional enzyme having two dissimilar catalytic domains capable of acting on xylan. Xylanase domain 1 releases more xylooligosaccharides and domain 2 more xylose. The N-terminal section belongs to the glycosyl hydrolase 11 (cellulase G) families whereas the C-terminal section belongs to the glycosyl hydrolase 10 (cellulase F) families [94].

Construction of bifunctional xylanase by gene fusion

In nature, proteins are often associates into multidomain proteins in order to perform tasks that require more than one function [5, 82]. Scientists have adopted nature's strategy to develop many applications for recombinant chimeric proteins which over the time have became indispensable tools in many of application associated with a wide variety of disciplines [10, 64]. For example, lyme vaccine is developed by genetically engineering two pieces of proteins that are normally present on the surface of the bacterium causing Lyme disease and transforming it into one chimeric protein [20]. Construction of a fusion protein involves linking two proteins or proteins domains by means of a peptide linker. The flexibility, hydrophilicity and the extended conformation of the linker were all found to be very important factors in maintaining the functionality of the individual domains by allowing controlled movement of the individual domains [28, 3, 26].

Fusion proteins are created by two different modes. One is 'end-to-end' fusion in which the N terminus of one domain is linked to the C terminus of the other domain. The second is `insertional' fusion in which one domain is inserted in-frame into the middle of the other parent domain (Fig. 2). As practical cases of the multistep sequential reaction the performance of the fusion enzyme is sometimes better than that achieved by successive action of individual enzymes, expanding the potential use of natural enzymes

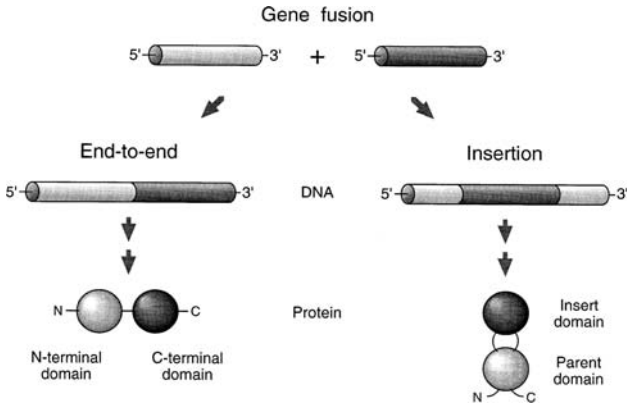

Fig. 2 Schematic representation of the two types of gene fusion approaches (adopted from Doi and Yanagawa 1999)

[10, 40, 44]. Therefore, functional fusion of two enzymes may have several advantages over individual enzymes regarding reaction kinetics and enzyme production, as well as novel properties and reactivity [45, 63, 57].

Recombinant enzymes exhibiting better activity and/or stability have been obtained as a result of mutagenesis [16] or gene fusion [86, 67]. However cleavage of protein fusions to generate free protein remains the major disadvantage of protein fusion technologies. Cleavage of the fusion is usually necessary because the fusion interferes with the structural or functional properties of the recombinant protein [4]. Moreover, misfolding, and inappropriate protein interaction are among the disadvantage that destabilize protein structure and affect fused domain activities [33] Fusion proteins produced in *E. coli* have been studied extensively [51] and some of these artificial polyfunctional chimeras were shown to facilitate consecutive catalyzed reactions via the positive proximity effects generated by the closeness of their catalytic sites [11, 12]. Mesta et al. [57] created an active chimeric enzyme, XYN3A4, by fusing two different catalytic domains exhibiting the same endoxylanase activity, XYN3A and XYN4, which originated from two different fungal endoxylanase genes, *xyn3* and *xyn4*, respectively. The hypothesis underlying this work relied on the effect of gene fusion upon activity of the new chimeric structure. However, their chimeric enzyme exhibited a better affinity and an improved rate of hydrolysis of the xylan substrate than its respective counterparts, XYN3A and XYN4. Most end-to-end fusion proteins maintain the activity of both proteins, usually providing a significant advantage [21]. Using gene fusion approach, Hong et al. [33] designed a bifunctional, thermostable enzyme with cellulase (TM1751) and xylanase (TM0061) activities from *Thermotoga maritima*. It was demonstrated that cellulase and xylanase can be fused end-to-end, via overlapping PCR, creating a bifunctional enzyme. However, the fusion protein exhibited both cellulase and xylanase activity when

xynA was fused downstream of cel5C but no activities were shown when xynA was fused upstream of cel5C. These results suggest that the activity loss could be misfolding or inappropriate protein interactions that destabilize protein structure and affect activity levels. Lu et al. [50] constructed a bifunctional enzyme by end-to-end fusion of the β-glucanase (Glu) from *Bacillus amyloliquefaciens* and the xylanase (Xyl) from *Bacillus subtilis*. Compared with parental enzymes, the Glu moiety was characterized by kinetic parameters of decreased Km (0.66-fold) and increased Kcat (2.75-fold), whereas the Xyl moiety had an increased Km (1.37-fold) and decreased Kcat (0.79-fold). These properties indicate a 3.15-fold net increase and a 31% decrease in catalytic efficiency (Kcat/Km) of the Glu and Xyl moieties. Activities and stabilities of both moieties at 40–90°C or pH 3.0–10.0 were compared with those of the parental enzymes. Despite some variations, common optima were 40°C and pH 9.0 for the Glu moiety and parent, and 50–60°C and pH 9.0 for the Xyl counterparts. Thus, the fusion enzyme Glu–Xyl was bifunctional, with greatly enhanced glucanase activity associated with a decrease in xylanase activity. The bifunctional enzyme attained here by transforming the fusion gene Glu–Xyl into *E. coli* BL21 is desirable, although not perfect, based on overall comparison of characterization and catalytic efficiency with the parents. Fusion enzymes sometimes demonstrate superiority over two individual native enzymes catalyzing the same multistep sequential reaction [44]. Fusions of cellulase and xylanase may produce a more efficient bifunctional enzyme capable of degrading xyloglucan polysaccharides. By considering this approach An et al. [1] designed xylanase–cellulase bifunctional fusion proteins by an end-to-end fusion method. They found that, the bifunctional enzyme, xylanase–cellulase, prepared by gene fusion was capable of more efficient digestion. Three chimeric genes were constructed that encoded fusion proteins of different lengths. The fusion proteins exhibited both xylanase (XynX) and cellulase (Cel5Z) activity when *cel*5Z was fused downstream of *xyn*X, but not when *xyn*X was fused downstream of *cel*5Z. Activities of bifunctional enzymes decreased when a shorter xylanase peptide was fused. These results also demonstrate the importance of a proper primary structure concerning both the length and amino acid composition of the linker to achieve autonomous folding of the fusion enzyme and to provide proper intra-chain interactions [38]. The specific enzyme activities of the fusion proteins depend on how the fusion has been made, such as the sequential order of the enzymes and the length and composition of the connecting region [31]. Studies of fusion enzymes provide a broader understanding of how enzyme structure relates to its function and what changes can be tolerated within a particular framework. The ability to engineer the properties of proteins will expand the use of enzymes in biotechnology, allowing fusion enzymes to play an important role in this endeavor.

Biotechnological applications

Bioethanol production

Enzymes which are not desirable for some industries may be of great value and benefits to other industries. For instance, a bifunctional xylanase with cellulase activity is not of value to the paper and pulp industry because of undesirable effects of cellulases on the quality of paper produced. However, such enzyme could be of great value for other applications such as bioethanol production. Tremendous amounts of cellulose are available as municipal and industrial wastes which today contribute to our pollution problems. Thus, there is great interest in the use of cellulosic biomass as a renewable source of energy via breakdown to sugars that can be then converted to liquid fuel. Bifunctionality of xylanase could results in more efficient and cheaper saccharification process of the agricultural residues, municipal and industrial wastes used for bioethanol production as it can degrade both cellulose and xylan residues. Saccharification of the cellulose and hemicellulose in biomass results in sugar-rich liquid streams useful for the production of a variety of value-added products, including ethanol, furfural, and various functional biopolymers [25]. As pentoses in hemicelloses represent 20–40% of lignocelluloses biomass, utilization of this hemicellulose fraction could reduce cost of production of bioethanol. An increased possibility of fermentation of both hexoses and pentoses sugars in lignoclluloses into methanol has been recorded [75]. Moreover, different naturally present microorganisms including bacteria, fungi and yeast were recorded to show this ability. These include *Zymomonas mobilis* [35], *Clostridium cellulolyticum* [22], and a recombinant strain of *Saccharomyces cerevisiae* [80].

Animal feedstocks

The endosperm cell walls of cereal grains are rich in polysaccharides that are usually in the forms of arabinoxylans, mixed-linked-β-glucans, celluloses, mannans, and galactans [49]. Of these, arabinoxylans and β-glucans constitute major parts. For example, wheat, triticale, and rye enrich arabinoxylans [8], whereas oats and barley contain more β-glucans [7, 19]. Generally, the viscous properties of the polysaccharides make them difficult to be digested by domestic animals. Thus, inclusion of xylanase in wheat- or rye-based diets [2, 6] or β-glucanase in barley-based diets [73] is an important measure to enhance the availability of the polysaccharides. Endo-1,4-β-xylanase and 1,3-1,4-β-glucanase

have been widely applied for breakdown of the internal β-1,4-linkages of 1,4-β-D-xylan backbone [17] and specific cleavage of 1,4-β-D-glucosidic bonds adjacent to β-1,3-linkages in the mixed-linked-β-glucans [70] respectively. However, neither the xylanase nor the β-glucanase alone fully meets the requirement for effective exploitation of the various complex sugars [49]. To enhance their biodegradation for higher availability, effort has been directed toward integration of two or more synergistic enzymes into feedstocks. Combined action of xylanase and β-glucanase results in lower intestinal viscosity of broilers and better nutrient utilization than the use of xylanase or β-glucanase alone [74, 53].

Production of xylooligosaccharides

Xylan degradation requires the interaction of several enzymatic activities, including xylanase, xylosidase and arabinosidase. The end products of this degradation include xylose, arabinose, and methyl-glucuronic acid containing oligosaccharides. Xylooligosaccharides (XO) are sugar oligomers showing potential for practical applications in a variety of fields, including pharmaceuticals, feed formulations, agricultural purposes and food applications [83]. As additives for functional foods, XO present prebiotic action [24, 72], showing positive biological effects such as improvement of the intestinal function by increasing the number of healthy *Bifidobacteria* [37]. These xylooligosaccharides if used as a dietary supplementation it may be beneficial to gastrointestinal health and may reduce the risk of colon cancer [87]. As food ingredients, XOs have an acceptable odor, and are non-cariogenic [41, 42] and low-calorie, allowing their utilization in anti-obesity diets [79, 81]. In food processing, XOs show advantages over insulin in terms of resistance to both acids and heat, allowing their utilization in low pH juices and carbonated drinks [59]. It is also suggested that dietary xylooligosaccharides may be used as a potential substitute for a dietary fiber. They can help to suppress blood cholesterol levels, especially low density lipoprotein-cholesterol, by binding to bile acids, which contain cholesterol, in the gastrointestinal tract and carrying them out of the body as waste [36, 85]. It is reported that xylooligosaccharides is half as sweet as sucrose, can be applicable to foods as a sweetener that is capable of improving diabetic symptoms [37].

Concluding remarks

This review provides the information on most of the aspects of bifunctional enzyme with special reference to bifunctional xylanases. There is no review available on bifunctionality of xylanases. This is a small effort being made to bring all possible information on bifunctional xylanases under single heading. Considering biotechnological applications of the bifunctional xylanases it is necessary in future to utilize such hybrid protein as an alternative to expensive and polluting chemical treatments or to improve already existing enzymatic processes for utilization of vegetal by-products in the agro-industries and biofuel production sectors. We believe that this is one of the aspects of enzyme production that will be improved in the next few years.

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References

1. An JM, Kim YK, Lim WJ, Hong SY, An CL, Shin EC, Cho KM, Choi BR, Kang JM, Lee SM, Kim H, Yun HD (2005) Evaluation of a novel bifunctional xylanasecellulase constructed by gene fusion. Enzyme Microb Technol 36:989–995
2. Annison G (1992) Commercial enzyme supplementation of wheat-based diets raises ileal glycanase activities and improves apparent metabolisable energy, starch and pentosan digestibilities in broiler chickens. Anim feed sci technol 38:105–121
3. Arai R, Ueda H, Kitayama A, Kamiya N, Nagamune T, (2001) Design of the linkers which effectively separate domains of a bifunctional fusion protein. Protein Eng 14:529–532
4. Balbas P (2001) Understanding the art of producing protein and nonprotein molecules in *Escherichia coli*. Mol Biotechnol 19:251–267
5. Bayer EA (1998) Cellulose, cellulases, and cellulosomes. Curr Opin Struct Biol 8:548–557
6. Bedford MR, Classen HL (1993) An in vitro assay for prediction of broiler intestinal viscosity and growth when fed rye-based diets in the presence of exogenous enzymes. Poult Sci 72:137–143
7. Beer MU, Wood PJ, Weisz J (1997) Molecular weight distribution and (1→3)(1→4)-β-d-glucan content of consecutive extracts of various oat and barley cultivars. Cereal Chem 74:476–480
8. Bonnin E, Le Goff A, Saulnier L, Chaurand M, Thibault JF (1998) Preliminary characterisation of endogenous wheat arabinoxylan-degrading enzymic extracts. J Cereal Sci 28:53–62
9. Bulock KG, Beardsley GP, Anderson KS (2002) The kinetic mechanism of the human bifunctional enzyme atic (5-amino-4-imidazolecarboxamide ribonucleotide transformylase/inosine 5′-monophosphate cyclohydrolase). J Biol Chem 277:22168–22174
10. Bülow L, Mosbach K (1991) Multienzyme systems obtained by gene fusion. Trends Biotechnol 9: 226–231
11. Bülow L, Mosbach K (1996) Fusion proteins:expression and function. Ann NY Acad Sci 99:376–382
12. Bülow L, Carlsson H, Ljungcrantz P, Persson M, Lindbladh C (1996) Togetherness between proteins generated by gene fusion. Adv Mol Cell Biol 15A:297–310
13. Carpenter EP, Hawkins AR, Frost JW, Brown KA (1998) Structure of dehydroquinate synthase reveals an active site capable of multistep catalysis. Nature 394:299–301
14. Cepeljnik T, Rincon MT, Flint HJ, Logar R (2006) Xyn11A, a multidomain multicatalytic enzyme from *Pseudobutyrivibrio xylanivorans* Mz5T. Folia Microbiol 51:263–267

15. Chen H, Yan X, Liu X, Wang M, Huang H, Jia X, Wang J (2006) Purification and characterization of novel bifunctional xylanase, XynIII, isolated from *Aspergillus niger* A-25. J Microbiol Biotechnol 16:1132–1138
16. Cherry JR, Lamsa MH, Schneider P, Vind J, Svendsen A, Jones A, Pedersen AH (1999) Directed evolution of a fungal peroxidase. Nat Biotechnol 17:333–334
17. Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23
18. Coughlan MP, Ljungdahl LG (1988) Comparative biochemistry of fungal and bacterial cellulolytic enzyme system. In: Aubert JP, Beguin P, Millet J (eds) Biochemistry and genetics of cellulose degradation. Academic Press, London, pp 11–30
19. Cui W, Wood PJ, Blackwell B, Nikiforuk J (2000) Physicochemical properties and structural characterization by two-dimensional NMR spectroscopy of wheat β-D-glucan-comparison with other cereal β-D-glucans. Carbo Poly 41:249–258
20. Dattwyler RJ, Gomes-Solecki MJC, Luft BJ, Dunn J (2007) Method for developing chimeric, or "combination," proteins that may advance the development of vaccines and diagnostic tests for Lyme disease. US. Patent 7,179,448
21. Doi N, Yanagawa H (1999) Insertional gene fusion technology. FEBS Lett 457:1–4
22. Emmanuel G, Desvaux M, Petitdemanage H (2002) Improvement of cellulolytic properties of *Clostridium cellulolyticum* by metabolic engineering. Appl Environ Microbiol 68:53–58
23. Flint HJ, Martin J, McPherson CA, Daniel AS, Zhang JX (1993) A bifunctional enzyme with separate xylanase and β-(1,3-1,4)-glucanase domains, encoded by the XynD gene of *Ruminococcus flavefaciens*. J Bacteriol 175:2943–2951
24. Fooks L, Gibson G (2002) In vitro investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbial Ecol 39:67–75
25. Fuller JJ, Ross RJ, Dramm JR (1995) Nondestructive evaluation of honeycomb and surface checks in red oak lumber. Forest Prod J 45:42–44
26. George RA, Heringa J (2002) An analysis of protein domain linkers: their classification and role in protein folding. Protein Eng 15:871–879
27. Gilbert F, Galgani F, Cadiou Y (1992) Rapid assessment of metabolic activity in marine microalgae: application in ecotoxicological tests and evaluation of water quality. Mar Biol 112:199–205
28. Gokhale R, Khosla C (2000) Role of linkers in communication between protein modules. Curr Opin Chem Biol 4:22–27
29. Gosalbes MJ, Perez-Gonzalez JA, Gonzalez R, Navarro A (1991) Two 3-glycanase genes are clustered in *Bacillus polymyxa*: molecular cloning, expression, and sequence analysis of genes encoding a xylanase and an endo-r-(1,3)-(1,4)-glucanase. J Bacteriol 173:7705–7710
30. Haros M, Rosell CM, Benedito C (2002) Improvement of flour quality through carbohydrases treatment during wheat tempering. J Agric Food Chem 50:4126–4130
31. Helen C, Sarah L, Leif B (1996) Physical and kinetic effects on introduction of various linker regions in galactosidase/galactose dehydrogenase fusion enzymes. Biochim Biophys Acta 1293:154–160
32. Hernandez M, Hernandez-Coronado MJC, Montiel MD, Rodriguez J, Arias ME, (2001) Analysis of alkali-lignin in a paper mill effluent decolourised with two *Streptomyces* strains by gas chromatography-mass spectrometry after cupric oxide degradation. J Chromatography 919:389–394
33. Hong SY, Lee JS, Cho KM, Math RK, Kim YH, Hong SJ, Cho YU, Kim H, Yun HD (2006) Assembling a novel bifunctional cellulase–xylanase from *Thermotoga maritima* by end-to-end fusion. Biotechnol Lett 28:1857–1862
34. Huang X, Holden HM, Raushel FM (2001) Channeling of substrates and intermediates in enzyme-catalyzed reactions. Annu Rev Biochem 70:149–180
35. Ingram LO, Eddy CK, Mackenzie KF, Conway T, Alterthum F (1989) Genetics of *Zymomonas mobilis* and ethanol production. Dev Ind Microbiol 30:53–69
36. Imaizumi K, Nakatsu Y, Sato M, Sedarnawati Y, Sugano M (1991) Effects of xylooligosaccharides on blood glucose, serum and liver lipids and cecum short-chain fatty acids in diabetic rats. Agric Biol Chem 55:199–205
37. Izumi Y, Kojo A (2003) Long-chain xylooligosaccharide compositions with intestinal function-improving and hypolipemic activities, and their manufacture. JP Patent 2003048901
38. Jagusztynkrynicka EK, Clarkcurtiss JE, Curtiss R (1993) *Escherichia coli* heat-labile toxin subunit B fusions with *Streptococcus sobrinus* antigens expressed by *Salmonella typhimurium* oral vaccine strains: importance of the linker for antigenicity and biological activities of the hybrid proteins. Infect Immun 61:1004–15
39. James CL, Viola RE (2002) Production and characterization of bifunctional enzymes. Domain swapping to produce new bifunctional enzymes in the aspartate pathway. Biochem 41:3720–3725
40. Jung H, Wilson DB, Walker LP (2002) Binding of *Thermobifida fusca* CDcel5A CDcel6B and CDcel48A to easily hydrolysable and recalcitrant cellulose fractions on BMCC. Enzyme Microb Technol 31:941–8
41. Kazumitsu S, Boseki I, Norio S, Yoshimasa O (1987) Production of food and drink Japanese Patent JP 62278961
42. Kazumitsu S, Boseki I, Norio S, Yoshimasa O (1997) Production of food and drink', Japanese Patent JP 9248153
43. Lamed R, Bayer EA (1988) The cellulosome concept: Exocellular enzyme reactor centers for efficient binding and cellulolysis. In: Aubert JP, Beguin P, Millet J (eds) Biochemistry and genetics of cellulose degradation. Academic Press, London, pp 101–116
44. Lemos MA, Teixeira JA, Domingues MRM, Mota M, Gama FM (2003) The enhancement of the celluloytic activity of cellobiohydrolase I and endoglucanase by the addition of cellulose binding domains derived from *Trichoderma reesei*. Enzyme Microb Technol 32:35–40
45. Levy I, Paldi T, Shoseyov O (2003) Engineering a bifunctional starchcellulase cross-bridge protein. Biomaterials 25:1841–1849
46. Liang PH, Anderson KS (1998) Substrate channeling and domain–domain interactions in bifunctional thymidylate synthase–dihydrofolate reductase. Biochem 37:12195–12205
47. Lin LL, Thomson JA (1991) An analysis of the extracellular xylanases and cellulases of *Butyrivibrio fibrisolvens* H17c. FEMS Microbiol Lett 84:197–204
48. Liu JH, Selinger LB, Hu YJ, Moloney MM, Cheng KJ, Beauchemin KA (1997) "An endoglucanase from the anaerobic fungus *Orpinomyces* joyonii: characterization of the gene and its product". Can J Microbiol 43:477–485
49. Longland AC, Theodorou MK, Sanderson R, Lister SJ, Powell CJ, Morris P (1995) Non-starch polysaccharide composition and in vitro fermentability of tropical forage legumes varying in phenolic content. Anim Feed Sci Technol 55:161–177
50. Lu P, Feng M, Li2 W, Hu C (2006) Construction and characterization of a bifunctional fusion enzyme of *Bacillus*-sourced β-glucanase and xylanase expressed in *Escherichia coli*. FEMS Microbiol Lett 261:224–230
51. Makrides S C (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol Rev 60:512–538
52. Malherbe S, Cloete TE (2003) Lignocellulose biodegradation: fundamentals and applitions. Environ Sci Biotechnol 30:425–444
53. Mathlouthi N, Lalles JP, Lepercq P, Juste C, Larbier M (2002) Xylanase and beta-glucanase supplementation improve conjugated bile acid fraction in intestinal contents and increase villus size of

small intestine wall in broiler chickens fed a rye-based diet. J Anim Sci 80:2773–2779
54. Mathlouthi N, Juin H, Larbier M (2003) Effect of xylanase and beta-glucanase supplementation of wheat- or wheat- and barley-based diets on the performance of male turkeys. Br Poult Sci 44:291–298
55. Meek TD, Garvey EP, Santi DV (1985) Meek TD, Garvey EP, Santi DV (1985) Purification and Characterization of the Bifunctional Thymidylate Synthetase-Dihydrofolate Reductase from Methotrexate-Resistant *Leishmania tropica*. Biochem 24:678–686
56. Meng X, Slominski BA, Nyachoti CM, Campbell LD, Guenter W (2005) Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. Poult Sci 84:37–47
57. Mesta L, Rascle D, Durand R, Revre M (2001) Construction of a chimeric xylanase using multidomain enzymes from *Neocallimastix frontalis*. Enzyme Microb Technol 29:456–63
58. Miles EW, Rhee S, Davies DR (1999) The molecular basis of substrate channelling. J Biol Chem 274:12193–12196
59. Modler HW (1994) Bifidogenic factors—sources, metabolism and applications. Int Dairy J 4:383–407
60. Morohoshi N (1991) Chemical characterization of wood and its components. In: Shiraishi N (eds) Wood cellulosic chemistry, Marcel Dekker, New York, pp 331–392
61. Murashima K, Kosugi A, Doi RH (2003) Synergistic Effects of cellulosomal xylanase and cellulases from *Clostridium cellulovorans* on plant cell wall degradation. J Bacteriol 185:1518–1524
62. Nagradova N (2003) Interdomain communications in bifunctional enzymes: how are different activities coordinated? IUBMB Life 55:459–466
63. Ni N, Jiang XN, Cai GP, Yang SF (1996) A novel bifunctional fusion enzyme catalyzing ethylene synthsis via 1-aminocyclopropane-1-carboxylic acid. J Biol Chem 271:25738–25741
64. Nixon AE, Ostermeier M, Benkovic SJ (1998) Hybrid enzymes: manipulating enzyme design. Trends Biotechnol 16:258–264
65. Numan MTh, Bhosle NB (2006) α-L-arabinofuranosidases: the potential applications in biotechnology. J Ind Microbiol Biotechnol 33:247–260
66. Okar DA, Manzano NA, Navarro-Sabat A, Riera LS, Bartons R, Lange AJ (2001) PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci 26:30–35
67. Olsen LS, Nielson LR, Nexø BA, Wasserman K (1996) Somatic mutation detection in human biomonitoring. Pharmacol Toxicol 78:364–373
68. Omogbenigun FO, Nyachoti CM, Slominski BA (2004) Dietary supplementation with multienzyme preparations improves nutrient utilization and growth performance in weaned pigs. J Anim Sci 82:1053–1061
69. Peters RJ, Ravn MM, Coates RM, Croteau RB (2001) Bifunctional abietadiene synthase: free diffusive transfer of the (+)-copalyl diphosphate intermediate between two distinct active sites. J Am Chem Soc 123:8974–8978
70. Planas A (2000) Bacterial 1,3-1,4-β-glucanases: structure, function, and protein engineering. Biochim Biophys Acta 1543:361–382
71. Pohlschroder M, Leschine SB, Parola EC (1994) Multicomplex cellulase–xylanase system of *Clostridium papyrosolvens* C7. J Bacteriol 176:70–76
72. Rycroft CE, Jones MR, Gibson GR, Rasta RA (2001) A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. J Appl Microbiol 91:878–887
73. Salim ME, Classen HL, Campbell GL (1991) Response of chickens fed on hull-less barley to dietary β-glucanase at different ages. Anim feed sci technol 33:139–149
74. Salobir J (1998) Effect of xylanase alone and in combination with β-glucanase on energy utilisation, nutrient utilization and intestinal viscosity of broilers fed diets based on two wheat samples. Arch Geflugelkd 5:209–213
75. Senn T, Pieper HJ (2001) The biotechnology of ethanol. In: Roehr (ed) Classical and future applications, vol 48. Wiley-VCH, Germany, pp 8–174
76. Seo M, Peeters AJM, Koiwai H, Oritani T, Marion-Poll A, Zeevaart JAD, Koornneef M, Kamiya Y, Koshiba T (2000) The *Arabidopsis* aldehyde oxidase 3 (*AA03*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc Natl Acad Sci USA 97:12908–12913
77. Sjöström E (1989) Wood chemistry: fundamentals and applications. Solar energy conversion—biomass production & conversion: wood chemistry, wood uses, anatomy, carbohydrates, cellulose, lignin, molecular structure. Academic Press, New York, p:235
78. Smith S (1994) The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. J FASEB 8:1248–1259
79. Taeko I, Koichi N, Yasushi N, Akira K, Yoshinobu K (1998) 'Food and drink effective in anti-obesity', Japanese Patent JP 10290681
80. Toivari MH, Aristidou A, Ruohonen L, Penttila M (2001) Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: importance of xylulokinase (XKS1) and oxygen availability. Met Eng 3:236–249
81. Toshio I, Noriyoshi I, Toshiaki K, Toshiyuki N, Kunimasa K (1990). 'Production of Xylobiose', Japanese Patent JP 2119790
82. Tsuji N, Suzuki K, Kasuga-Aoki H, Matsumoto Y, Arakawa T, Isobe T (2001) Intranasal immunization with recombinant *Ascaris suum* 14-kDa antigen coupled with cholera toxin B subunit induces protective immunity to *Ascaris suum* infection in mice. Infect Immun 69:7285–7292
83. Vázquez MJ, Alonso JL, Domínguez H, Parajó JC (2001) Xylooligosaccharides: manufacture and applications. Trends Food Sci Technol 11:387–393
84. Vrzheshch PV (2007) Steadystate kinetics of bifunctional enzymes.Taking into account kinetic hierarchy of fast and slow catalytic cycles in a generalized model. Biochem (mosc) 72:936–43
85. Wang B, Cheng B, Feng H (2008) Enriched arabinoxylan in corn fiber for value-added Products. Biotechnol Lett 30:275–279
86. Warren RA, Gerhard JB, Gilkes NR, Owolabi JB, Kilburn DG, Miller RC (1987) A bifunctional exoglucanase–endoglucanase fusion protein. Gene 61:421–427
87. Whitehead TR, Cotta MA (2001) Identification of a broad-specificity xylosidase/arabinosidase important for xylooligosaccharide fermentation by the ruminal anaerobe *Selenomonas ruminantium* GA192. Curr Microbial 43:293–298
88. Wiselogel ST, Johnson D (1996) Biomass feedstock resources and composition In: Wyman CE (ed) Handbook on bioethanol: production and utilization (applied energy technology series). Taylor & Francis, London pp 105–118
89. Wong KK, Tan LU, Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev 52:305–317
90. Xie G, Bruce DC, Challacombe JF, Chertkov O, Detter JC, Gilna P, Han CS, Lucas S, Misra M, Myers GL, Richardson P, Tapia R, Thayer N, Thompson LS, Brettin TS, Henrissat B, Wilson DB, McBride MJ (2007) Genome Sequence of the Cellulolytic Gliding Bacterium *Cytophaga hutchinsonii*. Appl Environ Microbiol 73:3536–3546
91. Xue GP, Kari SG, Orpin CG (1992) A novel polysaccharide hydrolase cDNA (celD) from *Neocallimastix patriciarum* encoding three multi-functional catalytic domains with high endoglucanase,

cellobiohydrolase and xylanase activities. J Gen Microbiol 138:2397–2403
92. Yourno J, Kohno T, Roth JR (1970) Enzyme evolution: Generation of a bifunctional enzyme by fusion of adjacent genes. Nat 228:820–825
93. Yu P, McKinnon JJ, Christensen DA (2005) Improving the nutritional value of oat hulls for ruminant animals with pretreatment of a multienzyme cocktail: in vitro studies. J Anim Sci 83:1133–1141
94. Zhang JX, Flint HJ (1992) A bifunctional xylanase encoded by the XynA gene of the rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 comprises two dissimilar domains linked by an asparagine/glutamine-rich sequence. Mol Microbiol 6:1013–1023