REVIEW ARTICLE

Hyperthermostable cellulolytic and hemicellulolytic enzymes and their biotechnological applications

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Abstract

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Hyperthermal cellulases and hemicellulases have been intensively studied due to their highly potential applications at extreme temperatures, which mimic industrial processes involving cellulose and hemicellulose degradation. More than 50 species of hyperthermophiles have been isolated, many of which possess hyperthermal enzymes required for hydrolyzing cellulose and hemicelluloses. Endoglucanases, exoglucanases, cellobiohydrolases, xylanases, β-glucosidase and β-galactosidase, which are produced by the hyperthermophiles, are resistant to boiling temperature. The characteristics of these enzymes and the ability to maintain their functional integrity at high temperature as well as their biotechnological application are discussed.

Key words : hyperthermophilic, cellulases, hemicellulases, extremophilic

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บทคัดย่อ

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เอนไซม์ย่อยเซลลูโลสและเฮมิเซลลูโลสที่ทนความร้อนสูงและการประยุกต์ใช้ทางเทคโนโลยีชีวภาพ

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การศึกษาค้นคว้าเกี่ยวกับเอนไซม์ย่อยเซลลูโลสและเฮมิเซลลูโลสที่ทนความร้อนสูงได้กระทำกันอย่างมากและจริงจัง เนื่องจากเป็นกลุ่มเอนไซม์ที่มีศักยภาพสูงในการนำไปประยุกต์ใช้ที่อุณหภูมิสูงซึ่งใช้ในกระบวนการทางอุตสาหกรรมดังเช่นที่ใช้ในกระบวนการย่อยสลายเซลลูโลสและเฮมิเซลลูโลส ปัจจุบันได้มีการแยก และจำแนกจุลินทรีย์ที่ทนความร้อนสูงได้มากกว่า 50 ชนิด จุลินทรีย์เหล่านี้นอกจากจะเจริญได้ที่อุณหภูมิสูงแล้วยังสร้างเอนไซม์ที่สามารถทำงานได้ดีที่อุณหภูมิสูงอีกด้วย ในกลุ่มนี้มีหลายชนิดที่สามารถสร้างเอนไซม์กลุ่มที่ย่อยเซลลูโลสและเฮมิเซลลูโลสซึ่งได้แก่ เอนโดกลูคาเนส เอกโซกลูคาเนส เซลโลไบโอไฮโดรเลส ไซแลนเนส เบต้า-กลูโคซิเดส และ เบต้า-กาแลคโตซิเดส ที่สามารถทำงานได้ดีที่อุณหภูมิน้ำเดือด ในบทความนี้ได้กล่าวถึงคุณลักษณะสำคัญของเอนไซม์กลุ่มนี้ ปัจจัยที่มีผลให้เอนไซม์คงตัวที่อุณหภูมิสูง รวมถึงการนำเอนไซม์กลุ่มนี้ไปประยุกต์ใช้ทางเทคโนโลยีชีวภาพ

ภาควิชาเทคโนโลยีชีวภาพอุตสาหกรรม คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Cellulose and hemicelluloses are the most abundant polysaccharides in nature. They build structural components of plant cell walls and are associated with lignin and other polysaccharides. Therefore, microbial degradation of cellulose and hemicelluloses has enormous economic potential for the conversion of plant biomass into fuels and chemicals. Cellulolytic microorganisms play an important role in the biosphere by recycling cellulose. Cellulose is an unbranched linear homopolymer of glucose units linked by β-1,4-D-glucosidic bonds, that forms insoluble, crystalline microfibrils which are highly resistant to enzymatic hydrolysis. The cellulolytic enzyme system is composed of at least three different enzymes: endoglucanase, cellobiohydrolase and β-glucosidase, which act together in synergism. Xylans, major components of the hemicelluloses and the second most abundant polysaccharides in nature, are branched heteroglycans with a backbone of β-1,4-linked D-xylose residues. Branches consist of α-1,3-linked L-arabinofuranosyl and α-1,2-linked 4-O-methyl-glucuronic acid residues. Complete hydrolysis of these complex molecules requires the interaction of a number of main chain-and side-chain-cleaving enzyme activities (Beguin and Aubert, 1994; Coughlan and Hazlewood, 1993; Leuschner and Antranikian, 1995).

Both cellulolytic and hemicellulolytic enzymes have been found in fungal species, especially in *Trichoderma reesei* and various bacteria. Several applications of these enzymes are being developed for textile, food and paper pulp processing. These applications are based on the modification of cellulose and hemicelluloses by partial hydrolysis. Total hydrolysis of cellulose into glucose, which could be fermented into ethanol, isopropanol or butanol, is not yet economically feasible (Beguin and Aubert, 1994; Coughlan and Hazlewood, 1993). Developments of pretreatment process and fermentation efficiency including improvements of enzyme properties are necessary. One of major drawbacks in industrial terms is that most cellulases and hemicellulases apparently lack thermostability. Currently, considerable interest is focused on the use of hemicellulases in the processing of paper pulp. Use of xylanases in bleaching step has been shown to reduce the amount of chlorine required to achieve comparable levels of paper brightness. However, the mesophilic enzymes currently in use have limitations due to high temperature used in

bleaching (Gibbs *et al*., 1995; Morris *et al*., 1995; Morris *et al*., 1998; Saul *et al*., 1995; Sunna *et al*., 2000, Viikari *et al*., 1994).

Thermophilic bacteria have received considerable attention as sources of highly active and thermostable cellulolytic and xylanolytic enzymes. There are thermostable cellulolytic enzymes characterized from thermophilic cellulolytic bacteria such as *Clostridium thermocellum, Caldocellum saccharolyticum* and *Acidothermus cellulolyticus* (Gibbs *et al*., 1995; Te'o *et al*., 1995; Bergquist *et al*., 1999). The most stable of these, from *Acidothermus*, has a half-life of less than 20 min. at 85 °C (Sakon, *et al*., 1996). Recently, hyperthermal cellulases and hemicellulases have been isolated and some of them have been purified and characterized. Most of the enzymes isolated from hyperthermophiles genus *Thermotoga* have temperature optima at 90 °C or higher and some, such as cellobiohydrolase from *Thermotoga* sp. strain FjSS3-B.1, are even active at 108 °C. The unusual properties of these enzymes have resulted in the development of the novel biotechnology processes that can be accomplished within a wide range of conditions, including the reactions that are beyond the capability of biologically based systems (Bronnenmeier *et al*., 1995; Leuschner and Antranikian, 1995; Ruttersmith and Daniel, 1991; Saul *et a*l., 1995; Winterhalter and Liebl, 1995).

Cellulose and hemicelluloses

Biomass composes on average 23% lignin, 40% cellulose and 33% hemicellulose by dry weight. Cellulose is an unbranched glucose polymer composed of D-glucose units linked by 1, 4-β-D-glucosidic bonds. Chain length varies between 100 and 14,000 residues. Cellulose chains form numerous intra- and intermolecular hydrogen bonds, which account for the formation of rigid, insoluble, crystalline microfibrils. Its enzymatic hydrolysis is considered to require action of both endoglucanases (1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) and exoglucanases (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91). These enzymes have different specificity and act together in synergism. Endoglucanase acts randomly and produces oligosaccharides with variable chain length. Cellobiohydrolase only attacks polymers from the non-reducing end. The disaccharides form is finally hydrolyzed to glucose by β-glucosidase (Beguin and Aubert, 1994; Leuschner and Antranikian, 1995).

Xylans are the major component of plant hemicelluloses and are the second most abundant polysaccharides in nature. They are branched heteroglycans with a backbone of β-linked xylopyranose residues. Branches consist of α-1,3-linked L-arabinofuranosyl and α-1,2-linked 4-O-methyl-glucuronic acid residues. The complete hydrolysis of xylan also requires the action of several enzymes. Enzymatic hydrolysis of xylan backbone involves endo-β-1,4-xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), β-D-xylosidases (1,4-β-D-xyloside xylohydrolase, EC 3.2.1.37), and possibly, exo-β-1,4-xylanases (1, 4-α-D-xylan xylohydrolase). Removal of side groups is catalyzed by α-L-arabinofuranosidases (EC 3.2.1.55), and α-D-glucuronidases (EC 3.2.1). Esterase activities are responsible for the liberation of acetyl, coumaryl, and feruloyl substituents (Beguin and Aubert, 1994; 3, 5, 12).

Apart from xylan, the second abundant constituent of hemicelluloses is galactoglucomannan and glucomannan (low-galactosemannans). Galactoglucomannan comprises a β-1,4-linked heteropolymer of mannose and glucose with galactose and acetyl side group. Enzymatic hydrolysis of β-mannan is accomplished by endomannanase (endo-1,4-β-D-mannan manohydrolase, EC3.2.1.78) and β-1,4-mannasidase (β-D-mannoside mannohydrolase, EC 3.2.1.25). The concerted action of these enzymes including β-glucosidase and α-galactosidase has been required to hydrolyze galactoglucomannans. Endomannanases cleave the mannan backbone to release short- and long-chain oligomannosides and β-1,4-mannosidases then cleave D-mannose residues from the short chain oligomannosides and mannobiose (Coughlan and Hazlewood, 1993; Gibbs *et al*., 1992; Morris *et al*., 1995).

Cellulolytic and hemicellulolytic microorganisms in extreme temperatures

Hyperthermophilic bacteria have received considerable attention as sources of highly active and hyperthermostable cellulolytic and hemicellulolytic enzymes. The most extremely thermophilic livings being up to now are organisms growing at temperatures between 80 and 110 °C. They are able to survive at low temperatures for years although they do not grow at 60 °C or below. Hyperthermophiles or extreme thermophiles are therefore considered to be organisms whose optimal growth temperatures are above the normal range of the environment. These kinds of environments are rare in nature and occur only under special conditions, such as in compost piles or in geothermal areas that are the main 'permanently hot' places on this planet. Such areas, for example, hot soil and hot spring are the main natural habitats where hyperthermophiles have been isolated. Moreover, they can also be found in artificial environments such as the boiling outflows of geothermal power plants (Kristjansson and Hreggvidsson, 1995).

To date about 50 species, 20 genera and 11 orders of hyperthermophiles are known. Some of them belong to the eubacteria but mostly they are archaea. There are many subdivisions of hyperthermophiles being suggested, such as extremophiles for those organisms growing at temperatures of 65 to 85 °C, hyperthermophiles for those growing above 100 °C. The ability of these organisms to maintain function and stability of cell components and biomolecules under extreme temperatures are still unexplained (Kristjansson and Hreggvidsson, 1995; Leuschner and Antranikian, 1995).

Hyperthermal cellulolytic and hemicellulolytic enzymes mostly have been isolated from *Thermotoga* sp. (Table 1). Some of them are also secreted by *Pyrococcus furiosus*, such as, β-glucosidase and β-galactosidase (Kengen *et al*., 1993). *Thermotoga* sp. strain FjSS3-B.1 isolated from an intertidal hot spring on Sava-Savu beach in Fiji has been shown to secrete a highly thermostable xylanase as well as cellobiohydrolase and β-glucosidase (Leuschner and Antranikian, 1995; Ruttersmith and Daniel, 1991; Saul *et al*., 1995). Likewise, *T. maritima, Dictyoglomus turgidus* and *Caldocellum saccharolyticum* are also able to excrete hyperthermal cellulases and hemicellulases (Bergquist *et al*., 1999; Bronnenmeier *et al*., 1995; Gibbs *et al*., 1995).

Hyperthermostable cellulolytic enzymes

Endoglucanase and cellobiohydrolase

With the restriction of extremely thermophilic eubacteria which possess heat tolerant ability at temperatures greater than 65 °C, there have been only two known aerobic, cellulolytic hyperthermophiles reported so far. Meanwhile, the discoveries of anaerobic counterparts are growing in number (Table 1). The cellulolytic enzyme system, which is composed of endoglucanase and β-glucosidase, has been isolated anaerobically from *Thermotoga maritima*. It is the first hyperthermophilic archaea that has been found to produce thermo-active endoglucanase. Both endoglucanases and cellobiohydrolase known to date, have been isolated from *Thermotoga* sp. strain FjSS3-B.1 with maximal activity at 105 °C (Ruttersmith and Daniel, 1991).

β-Glucosidase/β-Galactosidase

β-Glucosidases often also show β-galactosidase activity. A β-glucosidase (*Cel*B) purified from the hyperthermophilic archaeon *Pyrococcus furiosus* growing on cellobiose is extremely thermostable, with a half-life of 85 h at 100 °C and 13 h at 110 °C and has maximal activity at 102 to 105 °C, near the physiological growth optimum of the organism. Most of the enzymes are located in the cytoplasm. *Cel*B is composed of 58 kDa subunits, which are arranged into a tetramer. This is a rather unusual structure, since most glycosidases from thermophilic bacterial sources are monomers. The enzyme is not stimulated by divalent cations as found for some glycosidases (Kengen *et al*., 1993; Lebbink *et al*., 2000). *Cel*B also shows high activity on the aryl glucosides *p*-nitrophenyl β-1,4-D-glucopyranoside and *p*-nitrophenyl β-1,4-D-galactose, as well as on β-1, 4-linked disaccharides cellobiose and lactose

Table 1. Summary of cellulolytic and hemicellulolytic enzymes produced by hyperthermophiles

Enzymes	Organisms	Enzyme properties			References
		T optimum (°C)	pH (optimum)	MW (kDa)	
	Aerobes				
Endoglucanase	*Rhodothermus marinus*	95	7.0	49	Hreggvidsson *et al*. (1996)
	Acidothermus cellulolyticus	81	5.0	72	Sakon, *et al*. (1996)
	Anaerobes				
Cellobiohydrolase	*Thermotoga* sp. FjSS3-B.1	100-105	6.8-7.8	36	Ruttersmith and Daniel (1991, 1993)
Endoglucanase	*Thermotoga neapolitana*	95, 106	6.0, 6.0-6.6	29, 30	Bok *et al*. (1998)
	Thermotoga maritima	95	6.0	27	Bronnenmeier *et al*. (1995)
	Caldocellum saccharolyticum				Te'o *et al*. (1995)
					Bergquist *et al*. (1999)
	Caldocellulosiruptor - saccharolyticus				Bergquist *et al*. (1999)
	Dictyoglomus turgidus				
Exoglucanase	*Thermotoga maritima*	95	7.5	29	Bronnenmeier *et al*. (1995)
	Caldocellulosiruptor - saccharolyticus				
Xylanase	*Thermotoga* sp. FjSS3-B.1	100	5.3	31	Leuschner and Antranikian (1995)
	Thermotoga maritima MSB8	92, 105	6.2, 5.4	120, 40	Winterhalter and Liebl (1995)
	Thermotoga thermarum	90	6.0	40	Leuschner and Antranikian (1995)
	Dictyoglomus thermophilum	85	6.5	39.8	Morris *et al*. (1998)
					Gibbs *et al*. (1995)
Arabinofurano-sidase/β-Xylo-sidase	*Thermotoga* sp. FjSS3-B.1			92	Ruttersmith and Daniel (1993)
	Pyrococcus furiosus	102-105			Kengen *et al*. (1993)
β-Glucosidase/ β-Galactosidase	*Thermotoga* sp. FjSS3-B.1		7.0	75	Ruttersmith and Daniel (1993)
	Pyrococcus furiosus	102-105	5.0	230	Kengen *et al*. (1993)
	Thermotoga maritima		6.2	95	Leuschner and Antranikian (1995)
	Sulfolobus solfataricus MT4				Leuschner and Antranikian (1995)
β-Mannanase	*Caldocellum saccharolyticum*	80	6.0		Gibbs *et al*. (1993); Morris *et al*. (1995)
	Caldibacillus cellulovorans	85	6.0	30.7	Sunna *et al*. (2000)

and on the β-1,3-linked disaccharide laminaribiose (Lebbink *et al*., 2000). Additionally, β-glucosidase from *Thermotoga maritima* has been isolated and purified. The gene has been cloned and expressed in *Escherichia coli* (Leuschner and Antranikian, 1995).

Hyperthermostable hemicellulolytic enzymes

Endo-1,4-β-xylanase

Endoxylanases isolated and purified from *Thermotoga* strain FjSS-B.1 and *T. maritima* MSB8 are optimally active at temperatures of 100 and 105 °C, respectively. Two distinct endoxylanases from *T. maritima* MSB8 have been isolated: *Xyn*A (120 kDa) and *Xyn*B (40 kDa). *Xyn*B displays maximum activity at 105 °C, maximum *Xyn*A activity has occurred at 92 °C. Moreover, *Xyn*B also has greater long-term thermostability than *Xyn*A and their thermostabilities are enhanced in the presence of high salt concentration. Both of them do not hydrolyze microcrystalline cellulose or carboxymethyl cellulose so they have an important potential for biotechnological applications such as kraft pulp prebleaching or plant fiber processing (Bronnenmeier *et al*, 1995; Saul *et al*., 1995; Winterhalter and Liebl, 1995).

Xylanase activity has also been detected in *Caldocellum sachharolyticum* and *Dictyoglomus thermophilum* Rt46B.1. Their xylanase genes have been cloned, sequenced and expressed. The temperature and pH optima of the recombinant enzymes of the latter are 85 °C and pH 6.5, respectively. However, the enzyme is active across a broad pH range, with over 50% activity between pH 5.5 and 9.5. It is able to hydrolyze xylan present in *Pinus radiata* kraft pulp, indicating that it may be used as an aid in pulp bleaching (Gibbs *et al*., 1995; Morris *et al*., 1998; Te'o *et al*., 1995).

α-L-Arabinofuranosidases/β-Xylosidase

A heat stable arabinofuranosidase from *Thermotoga* strain FjSS3-B.1, classified as β-D-xylosidase, exhibits high activity towards xylobiose and *p*-nitrophenyl α-L-arabinofuranoside. It has a molecular mass of 92 kDa and is extremely thermostable with a half-life of 8 h at 95 °C (Ruttersmith and Daniel, 1993). This enzyme also can be isolated from *T. maritima* (Bronnenmeier *et al*., 1995). The *P. furiosus* enzyme, which exhibits β-glucosidase activity, also has β-xylosidase activity (Kengen *et al*., 1993).

β-Mannanase

It has been found that hyperthermal β-glucosidase from *P. furiosus* exhibits some β-mannosidase activity (Kengen *et al*., 1993). Its optimum temperature is 102-105 °C. The complete sequence of a β-mannanase gene from an anaerobic extreme thermophile, *Caldocellum saccharolyticum*, has been determined and expressed in *E. coli*. It shows that the expressed protein consists of 2 catalytic domains showing β-mannanase and endoglucanase activity. The recombinant β-mannanase works optimally at temperatures of 80 °C pH 6.0. It has been shown that the mannanases can significantly improve the bleachability of *Pinus radiata* kraft pulp in peroxide delignification sequences and can act cooperatively and perhaps even synergistically with xylanases (Gibbs *et al*., 1995; Morris *et al*., 1998; Sunna *et al*., 2000).

Due to the requirement of extreme conditions for optimal growth, cell cultivation on a large scale cannot be achieved by the existing processes, hence the difficulty for enzyme production. However, recombination techniques which express genes encoding the hyperthermal enzymes in a host organism at conventional conditions, could provide a conventional cultivation system that is workable and lead to an effective bulk production of enzyme. Furthermore, production of a particular enzyme can be accomplished without the purification step needed for elimination of another unwanted cellulase or hemicellulase enzymes.

Many genes encoding enzymes have been cloned and expressed successfully in *E. coli* (Bronnenmeier *et al*., 1995; Leuschner and Antranikian, 1995; Libbink *et al*., 2000; Morris *et al*., 1998; Sunna *et al*., 2000; Te'0 *et al*., 1995;). This is obviously an important development, as the ability to produce recombinant versions of thermostable proteins permits the application of mutagenesis methods to modify proteins and

study fundamental issues of stability, as well as to try and modify them to have more technologically desirable properties. Recombinant technology has not been successful in every case, however. The highest thermostable, recombinant xylanases reported to date are prepared from the *Thermotoga* strain FjSS3-B.1 with half-lives of 12 h at 95 °C and 22 h at 90 °C (Saul *et al*., 1995). The enzymatic properties of recombinant xylanase expressed in *E. coli* do not differ significantly from those of authentic xylanases isolated from *T. maritima* itself (Bronnenmeier *et al*., 1995).

Factors that stabilize hyperthermostable enzymes

In general, enzymes from conventional organisms are typically inactivated at temperatures above 60 °C due to disorganization of their three dimensional structures followed by denaturation in an irreversible manner. At temperatures above 80 °C, the proteins can undergo covalent modifi cation by the hydrolysis of certain peptide bonds, deamination of glutamine and asparagine side chains, and cleavage of disulfide bonds. The issue of how enzymes from hyperthermophiles can maintain their stability at temperatures above 100 °C still needs a lot of work to be performed. Regarding the studies on recombinant proteins which are able to be expressed in *E. coli* and exhibit heat stability as native forms do, they obviously indicate that extreme thermal stability is an intrinsic property which is encoded in the gene sequence (Saul *et al*., 1995; Winterhalter and Liebl, 1995).

Despite numerous studies of biochemical characteristic and gene sequence differences between thermophilic and mesophilic proteins, the ambiguity of how enzymes that can perform optimally at 40 °C can be converted into ones that work optimally near 120 °C is not yet clarified. Prior to the availability of gene sequences for the hyperthermophilic proteins, it was thought that improvement of thermostability among similar enzymes could be explained by a few general rules, for example the increase of the number of salt bridges, the increase of the hydrophobicity index, or the decrease of flexibility both in-helices and loop regions. However, the limited analysis of homologous gene sequences done thus far suggests that (i) thermostability most likely arises from a number of sometimes subtle contributions that may be difficult to impart to less stable proteins and (ii) thermostabilization mechanisms are not the same in all protein families.

Comparisons of amino acid composition and sequence analysis have been studied in many enzymes. The comparison of different xylose isomerase sequences from *T. maritima* shows that numbers of asparagine and glutamine residues decreased with increasing enzyme thermostability (Viikari *et al*., 1994). Unexpectedly, Koch *et al*. (1991) showed that the amino acid composition of α-amylase enzyme from *P. woesei* did not show a significant difference when compared with the enzyme from other mesophilic (bacilli) and moderate thermophilic (clostridia) eubacteria. A similar comparison was performed on D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Thermotoga maritima* and various sources, including mesophiles, thermophiles and yeast, by Wrba *et al*. (1990). These investigations do not explain the phenomenon of thermal adaptation. Moreover, they found there is no major difference in the overall topology of the various homologous GAPDHs or in the physiochemical characteristics of the enzymes. On the other hand, amino acid comparison of enolases from *P. furiosus* with those from *Thermus* sp. and some yeasts show that the content of strongly hydrophilic residues is strikingly reduced in *P. furiosus* enolase. These data suggest that the difference between the 'outside' and the 'inside' of the enzyme is significantly more distinct, and may contribute to the enhancement of resistance to thermal denaturation of the hyperthermal enzymes (Koch *et al*., 1991; Peak *et al*., 1994; Vieille *et al*., 1995; Wrba *et al*., 1990).

Many studies have directly compared the sequences of various enzymes from mesophiles and hyperthermophiles but cannot draw any universal terms of strategies or specific rules to distinguish their thermostable properties even though the proteins have up to 60% homology. Apparent-

ly, three-dimensional structural information is required. To date, detailed structural information is available for only three proteins from hyperthermophilic archaea. The structural data suggest that a dramatic increase in the thermal stability of protein is the result of numerous and subtle interactions that lead to global changes in structure, for example, the decreased surface area to volume ratio while increasing interior packing. However, it would be different in other cases. From the comparison of the mesophilic GAPDH and Thermotoga enzyme, the latter shows an increased number of ionic pairs on its surface but a decreased number of intersubunit ion pairs, while the hydrophobic interaction between subunits increases. Moreover, the decrease of the ratio of surface area and volume does not correlate with the thermal stability of the enzyme, as it has been concluded in the case of aldehyde ferredoxin oxidoreductase from *P. furiosus* (Adams *et al*., 1995; Adams and Kelly, 1995; Vieille *et al.,* 1995; Wrba *et al*., 1990). The most general conclusion from these studies is that hyperthermostability can be achieved without the requirement for any new types of interactions to stabilize the folded conformation. Rather than being the consequence of any one dominant type of interaction, it appears that the increased stability of these proteins reflects a number of subtle interactions involving surface energies, electrostatic interactions, increased secondary structure stabilization and packing effects.

Biotechnological applications

The driving force behind research on hyperthermal enzymes is the promise of being able to use enzymes effectively at extreme temperatures in industrial processing. The exquisite specificity of an enzyme-mediated reaction is difficult to mimic with conventional chemical catalysis. Moreover, enzyme catalysis can eliminate the need for multistep reactions and their often-unwanted waste production from side reaction.

Considerable attention is currently focused on the use of hemicellulases in processing of paper pulp, particularly, in bleaching step. Bleaching, essentially the removal of lignin from pulp, has traditionally been carried out using toxic chemicals such as chlorine and hypochlorite. This step is necessary for aesthetic reasons and for improvement of paper properties. Additionally, bleached kraft pulp is the most important pulp produced today. The production is approximately 65-70 millions ton a year, corresponding to a value of more than 50 billion US$. Presently, bleaching of kraft pulp uses large amounts of elemental chlorine and chlorine dioxide. By-products from using these chemicals are chlorinated-organic, resulting from the substitution reaction of chlorine with lignin. It has been found that xylanases can facilitate the release of lignin so that much less chlorine needs to be used. This could have important environmental consequences since the chlorine combined with phenolics derived from lignin results in pollution of paper mill effluents (Beguin and Aubert, 1994; Farrell and Skerker, 1992).

Hyperthermostable xylanases isolated from hyperthermopiles have a high potential for biotechnological application in kraft pulp prebleaching. The first part of the process in production of kraft pulp involves debarking and chipping of the wood logs followed by a strong alkaline cooking where the main part of the lignin is dissolved and then washed away (Figure 1). The result is a strong colored pulp, which has to be bleached in order to get a white pulp for paper production. The traditional bleaching of pulps has been done in multistages, which can vary by the chemicals that are used. A typical bleaching sequence is (C+D)-(E+O)-D-(E+P)-D wherein C is elemental chlorine, D is chlorine dioxide, E is sodium hydroxide sometimes used in the presence of O, oxygen, and/or P, peroxide (Figure 2). On the other hand, the process with xylanases is very simple with a pH and temperature adjustment. After xylanases are mixed into the pulp, which is kept in a holding tank for 1-3 h, the pulp is bleached in the normal way after one wash. However, due to the removal of some of the residual lignin and an opening of the structure, the chlorine consumption is much lower. A study shows xylanase treatment of pulp enable chlorine use to be replaced

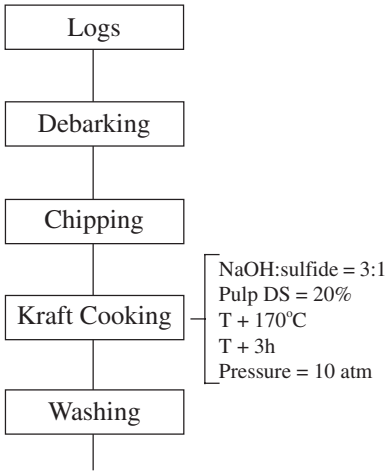

Figure 1. Kraft pulping process

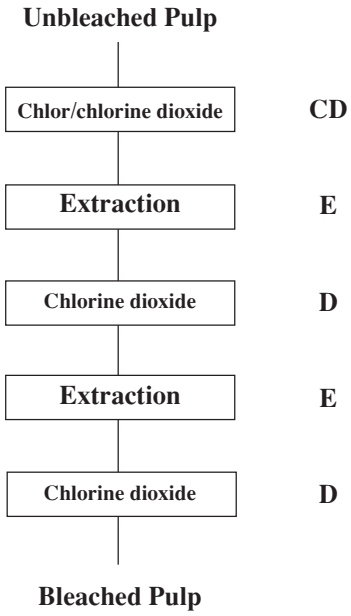

Figure 2. Kraft pulp bleaching process

completely and a higher brightness pulp to be achieved without alteration of strength properties (Farrell and Skerker, 1992; Morris *et al*., 1998; Sunna *et al*., 2000). Xylanases can remove lignin from the pulp more efficiently than lignolytic enzyme. It has been postulated that the endo-xylanases hydrolyze xylan slightly, and that the caustic extraction allows for removal of short xylan fragments, which are covalently attached to lignin fragments. Another reason is that xylanase treatment removes reprecipitated xylan on the surface of the kraft pulp fiber allowing for better chemical penetration and lignin accessibility, in turn improving lignin extractability. However, this treatment will be more advantageous if the adjustments of temperature and pH are eliminated completely. Availability of enzymes that can work at temperatures above 90 °C and pH > 9 would be very challenging. In this respect, the hyperthermostable xylanases mentioned above have a high potential since the recombinant enzymes can be overproduced by *E. coli* and possess all properties needed for the bleaching step (Gibbs, *et al*., 1995; Morris *et al*., 1995; Morris *et al*., 1998; Saul *et al*., 1995; Sunna *et al*., 2000; Viikari *et al*., 1994).

The use of xylanases has been proposed for clarifying juices, wine and beer; for extracting coffee, plant oil and starch; for improving the nutritional properties of agricultural silage; for macerating plant cell walls; for producing food thickener and for providing different textures to bakery products. Many of these applications do not strictly require pure xylanases, the presence of cellulases and/or pectinases is often desirable. Some of them have been reported to be currently in use. These enzymatic reactions usually are not carried out at moderate temperatures with conventional enzymes due to the risk of bacterial contamination. Given the availability of enzymes that function at sufficiently high temperatures, aseptic conditions could be maintained. This makes enzyme use to modify and improve food characteristics attractive. For instance, lactose in dairy products could be hydrolyzed with minimal risk of contamination. Furthermore, economical heat pretreatment can be developed for hydrolysis of cellulose and hemicelluloses since improvement of enzymatic hydrolysis at high temperature is feasible (Adams *et al*., 1995; Beguin and Aubert, 1994; Viikari *et al*., 1994).

In addition, hyperthermophilic enzymes

might also be used to enhance the flow of oil or gas in drilling operations. Practically, product flow to the well bore has been stimulated by forcing out open crevices in the surrounding bedrock which is done by flooding the well with a natural polymer (e.g., guar gum) solution and sand particles, capping the well and then pressurizing the bedrock until it fractures. The viscous polymer solution carries the sand through the fractures, propping open cracks for oil or gas flow. To facilitate product flow, the polymer solution is thinned by chemically oxidizing the gum or hydrolyzing its sugar linkages with β-1,4-mannanase and α-1,6-galactosidase. As temperature increases with depth (120 °C or higher), using conventional enzymes, which have limited stability above 80 °C, is unsuccessful. Moreover, these enzymes are active at low temperatures so they would hydrolyze the gum before the polymer solution is introduced into the well. Recently, a hyperthermophilic hemicellulase has been isolated. These enzymes are able to hydrolyze guar gum at elevated temperatures and have limited activity at lower temperatures so that implementation of oil production by these enzymes could be feasible (Adams *et al*., 1995; Winterhalter and Liebl, 1995).

Most of the hyperthermophilic enzymes have considerable potential roles in industrial processes and biological application. Not only such examples as mentioned above but also many existing industrial processes using moderately thermostable enzyme could be developed. Furthermore, chemical treatments in the processing steps, which cause environmental problems, would be replaced by using the enzymes.

References

Adams, M.W.W., Perler, F.B. and Kelly, R.M. 1995. Extremozymes: expanding the limits of biocatalysis. Bio/Technol. 13: 662-668.

Adams, M.W.W. and Kelly, R.M. 1995. Enzyme from microorganisms in extreme environments. C&EN. 18: 32-42.

Beguin, P. and Aubert, J.P. 1994. The biological degradation of cellulose. FEMS Microbiol. Rev. 13: 25-58.

Bergquist, P.L., Gibbs, M.D., Morris, D.D., Teo', V.S., Jsaul, D.J. and Morgan H.W. 1999. Molecular diversity of thermophilic cellulolytic and hemicellulolytic bacteria. FEMS Microbiol. Ecol. 28; 99-110.

Bok, J.D., Yernool, D.A. and Eveleigh, D.E. 1998. Purification, characterization, and molecular analysis of thermostable cellulases CelA and CelB from *Thermotoga neapolitana*. Appl. Environ. Microbiol. 64: 4774-4781

Bronnenmeier, K., Kern, A., Liebl, W. and Staudenbauer, W.L. 1995. Purification of *Thermotoga maritima* enzymes for the degradation of cellulosic materials. Appl. Environ. Microbiol. 61: 1339-1407.

Coughlan, M.P. and Hazlewood, G.P. 1993. β-1,4-D-Xylan-degrading enzyme systems: biochemistry, molecular biology and applications. Biotechnol. Appl. Biochem. 17: 259-289.

Erra-Pujada. M., Debeire, P., Duchiron, F. and O' Donohue, M. J. 1999. The type II pullulanase of *Thermococcus hydrothermalis* : Molecular characterization of the gene and expression of the catalytic domain. J. Bacteriol. 181: 3284-3287.

Farrell, R.L. and Skerker, P.D. 1992. Chlorine-free bleaching with Cartazyme TM HS treatment. **In** Visser, J., Beldman , G., Kusters-Van Someren, M.A. and Voragen, A.G. J. (eds.). Xylans and Xylanases. p. 315-324. Elsevier, Amsterdam.

Gibbs, M.D., Saul, D.J., Luthi, E. and Bergquist, P.L. 1992. The β-mannanases from "*Caldocellum saccharolyticum*" is part of a multidomain enzyme. Appl. Environ. Microbiol. 58: 3864-3867.

Gibbs, M.D., Reeves, R.A. and Bergquist, P.L. 1995. Cloning, sequencing and expression of a xylanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B. 1 and activity of the enzyme on fiber-bound substrate. Appl. Environ. Microbiol. 61: 4403-4408.

Hreggvidsson, G.O., Kaiste, E., Holst, O., Eggertsson, G., Palsdottir, A. and Kristjansson, J.K. 1996. An extremely thermostable cellulase from the thermophilic eubacterium *Rhodothermus marinus*. Appl. Environ. Microbiol. 62: 3047-3049.

Kengen, S.W.M., Luesink, E.J., Atams, A.J.M. and Zehnder, A.J.B. 1993. Purification and characterization of an extremely thermostable β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Eur. J. Biochem. 213: 305-312.

Koch, R., Spreinat, A., Lemke, K. and Antranikian, G. 1991. Purification and properties of a hyperthermoactive α-amylase from the archaebacterium *Pyrococcus woesei*. Arch. Microbiol. 155: 572-578.

Kristjansson, J.K. and Hreggvidsson, G.U. 1995. Ecology and habitats of extremophiles. World J. Microbiol. Biotechnol. 11: 17-25.

Lebbink, J.H.G., Kaper, T., Bron, P., Van Der Oost, J. and De Vos, W.M. 2000. Improving low-temperature catalysis in the hyperthermostable *Pyrococcus furiosus* β-glucosidase CelB by directed evolution. Biochem. 39: 3656-3665.

Leuschner, C. and Antranikian, G. 1995. Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. World J. Microbiol. Biotechnol. 11: 95-114.

Morris, D.D., Gibbs, M.D., Chin, C.W.J., Koh, M.H., Wong, K.K.Y., Allison, R.W., Nelson, P.J. and Bergquist. 1998. Cloning of the *xyn*B gene from *Dictyoglomus thermophilum* Rt46B.1 and action of the gene product on kraft pulp. Appl. Environ. Microbiol. 64: 1759-1765.

Morris, D.D., Reeves, R.A., Gibbs, M.D., Saul, D.J. and Bergquist, P.L. 1995. Correction of the β-mannanase domain of the *cel*C pseudogene from *Caldocellulosiruptor saccharolyticus* and activity of the gene product on kraft pulp. Appl. Environ. Microbiol. 61: 2262-2269.

Peak, M.J., Peak, J.G., Stevens, F.J., Blamey, J., Mai, X., Zhou, Z.H. and Adams, M.W.W. 1994. The hyperthermophilic glycolytic enzyme enolase in the archaeon *Pyrococcus furiosus*: comparison with mesophilic enolases. Arch. Biochem. Biophys. 313: 280-286.

Rees, D.C. and Adams, M.W.W. 1995. Hyperthermophiles: taking the heat and loving it. Structure. 3: 251-254.

Ruttersmith, L.D. and Daniel, R.M. 1991. Thermostable cellobiohydrolase from the thermophilic eubacterium *Thermotoga* sp. strain FjSS3-B.1. Biochem. J. 277: 887-890.

Ruttersmith, L.D. and Daniel, R.M. 1993. Thermostable β-glucosidase and β-xylosidase from *Thermotoga* sp. strain FjSS3-B.1. Biochim. Biophys. Acta. 1156: 167-172.

Sakon, J., Adney, W.S., Himmel, M.E., Thomas, S.R. and Karplus, P.A. 1996. Crystal structure of thermostable family 5 endocellulase E1 from *Acidothermus cellulolyticus* in complex with cellotetraose. Biochem. 35: 10648-10660.

Saul, D.J., Williams, L.C., Reeves, R.A., Gibbs, M.D. and Bergquist, P.L. 1995. Sequence and expression of a xylanase gene from the hyperthermophile *Thermotoga* sp. strain FjSS3-B.1 and characterization of the recombinant enzyme and its activity on kraft pulp. Appl. Environ. Microbiol. 61: 4110-4113.

Stetter, K.O., Fiala, G., Huber, G., Huber, R. and Segerer, A. 1990. Hyperthermophilic microorganisms. FEMS Microbiol. Rev. 75: 117-124.

Sunna, A., Gibbs, M.D., Chin, C.W.J., Nelson, P.J. and Bergquist, P.L. 2000. A gene encoding a novel multidomain β-1,4-Mannanase from *Caldibacillus cellulovorans* and action of the recombinant enzyme on kraft pulp. Appl. Environ. Microbiol. 66: 664-670.

Te'o, V.S.J., Saul, D.J. and Bergquist, P.L. 1995. CelA, another gene coding for a multidomain cellulases from the extreme thermophile *Caldocellum saccharolyticum*. Appl. Microbiol. Biotechnol. 43: 291-296.

Vieille, C., Hess, J.M., Kelly, R.M. and Zeikus, J.G. 1995. Cloning and sequencing and biochemical characterization of xylose isomerase from *Thermotoga neapolitana*. Appl. Environ. Microbiol. 61: 1867-1875.

Viikari, L., Kantelinen, A., Buchert, J. and Puls, J. 1994. Enzymatic accessibility of xylans in lignicellulosic materials. Appl. Microbiol. Biotechnol. 41: 124-129.

Winterhalter, C. and Liebl, W. 1995. Two extremely thermostable xylanases of the hyperthermophilic bacterium *Thermotoga maritima* MSB8. Appl. Environ. Microbiol. 61: 1810-1815.

Wrba, A., Schweiger, A., Schultes, V. and Jaenicke, R. 1990. Extremely thermostable D-glyceraldehyde-3-phosphate dehydrogenase from the eubacterium *Thermotoga maritima*. Biochemistry/ACS 29: 7584-7592.