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MINI-REVIEW

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Extremophiles as a source of novel enzymes for industrial application

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Abstract Extremophilic microorganisms are adapted to survive in ecological niches such as at high temperatures, extremes of pH, high salt concentrations and high pressure. These microorganisms produce unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes. Some of the enzymes from extremophiles have already been purified and their genes successfully cloned in mesophilic hosts. In this review we will briefly discuss the biotechnological significance of extreme thermophilic (optimal growth 70–80 °C) and hyperthermophilic (optimal growth 85–100 °C) archaea and bacteria. In particular, we will focus on selected extracellular-polymer-degrading enzymes, such as amylases, pullulanases, cyclodextrin glycosyltransferases, cellulases, xylanases, chitinases, proteinases and other enzymes such as esterases, glucose isomerases, alcohol dehydrogenases and DNA-modifying enzymes with potential use in food, chemical and pharmaceutical industries and in environmental biotechnology.

Introduction

Extremophilic microorganisms are adapted to live at high temperatures in volcanic springs, at low temperatures in the cold polar regions, at high pressure in the deep sea, at very low and high pH values (pH 0–3 or pH 10–12), or at very high salt concentrations (5%–30%). In the last decade a number of hyperthermophilic archaea have been isolated that are able to grow around the boiling point of water. The organisms with the highest growth temperatures (103–110 °C) are members of the genera *Pyrobaculum*, *Pyrodictium*, *Pyrococcus* and *Methanopyrus* (Stetter 1996). Within the bacteria, *Thermotoga maritima* and *Aquifex pyrophilus* exhibit the highest growth temperatures of 90 °C and 95 °C respectively. So far, more than 60 species of hyperthermophilic bacteria and archaea are known. They consist of anaerobic and aerobic chemolithoautotrophs and heterotrophs. The latter are able to utilize various polymeric substrates such as starch, hemicellulose, proteins and peptides. Metabolic processes and specific biological functions of these microorganisms are mediated by enzymes and proteins that function under extreme conditions. The enzymes that have been isolated recently from these exotic microorganisms show unique features, are extremely thermostable and usually resistant against chemical denaturants such as detergents, chaotropic agents, organic solvents and extremes of pH (Friedrich and Antranikian 1996; Jørgensen et al. 1997; Leuschner and Antranikian 1995; Rüdiger et al. 1995). They can hence be used as a model for designing and constructing proteins with new properties that are of interest for industrial applications.

Running biotechnological processes at elevated temperature has many advantages. The increase of temperature has a significant influence on the bioavailability and solubility of organic compounds. The elevation of temperature is accompanied by a decrease in viscosity and an increase in the diffusion coefficient of organic compounds. Consequently, higher reaction rates due to smaller boundary layers are expected (Becker et al. 1997; Krahe et al. 1996). Of special interest are reactions involving less soluble hydrophobic substrates such as polyaromatic, aliphatic hydrocarbons and fats, and polymeric compounds such as starch, cellulose, hemicellulose and proteins. The bioavailability of hardly biodegradable and insoluble environmental pollutants can also be improved dramatically at elevated temperatures allowing efficient bioremediation. Furthermore, by performing biological processes at temperatures

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above 60 °C the risk of contamination is reduced and controlled processes under strict conditions can be carried out. The number of genes from thermophiles that have been cloned and expressed in mesophiles is increasing sharply (Ciaramella et al. 1995). The majority of proteins produced in mesophilic hosts are able to maintain their thermostability, are correctly folded at low temperature, are not hydrolyzed by host proteases and can be purified by using thermal denaturation of the mesophilic host proteins. The degree of enzyme purity obtained is generally adequate for most industrial applications.

In this review we will briefly discuss the biotechnological significance of extreme thermophilic (optimal growth 70–80 °C) and hyperthermophilic (optimal growth 85–100 °C) archaea and bacteria. In particular we will focus on selected extracellular-polymer-degrading enzymes (such as amylases, pullulanases, cellulases, chitinases, xylanases, pectinases), isomerases, esterases, dehydrogenases and DNA-modifying enzymes with potential use in food, chemical and pharmaceutical industries and environmental biotechnology. Some of these aspects have already been presented in recent publications (Antranikian 1992; Ladenstein and Antranikian 1998; Moracci et al. 1998; Müller et al. 1998; Niehaus and Antranikian 1997; Rüdiger et al. 1994; Sunna and Antranikian 1997b).

Starch-degrading enzymes: biochemistry at the boiling point of water

Starch is composed exclusively of α-glucose units that are linked by α-1,4- or α-1,6-glycosidic bonds, forming two high-molecular-mass components: amylose (15%–25%), a linear polymer consisting of α-1,4-linked glucopyranose residues and amylopectin (75%–85%), a branched polymer containing α-1,6-glycosidic linkages at the branching points. Owing to the complex structure of starch, a number of enzymes are needed for its degradation (Antranikian 1992; Rüdiger et al. 1994). They can be simply classified into two groups: endo-acting and exo-acting enzymes. Endo-acting enzymes, such as α-amylase, hydrolyse linkages in the interior of the starch polymer in a random fashion, which leads to the formation of linear and branched oligosaccharides. Exo-acting enzymes (β-amylases, glucoamylases and α-glucosidases) attack the substrate from the non-reducing end, producing oligo- and/or monosaccharides. Enzymes capable of hydrolysing α-1,6-glycosidic bonds in pullulan and amylopectin are defined as debranching enzymes or pullulanases. On the basis of their substrate specificity, the pullulanases have been classified into two groups: pullulanase type I, which specifically hydrolyses the α-1,6-linkages in pullulan and in branched oligosaccharides, and pullulanase type II or amylopullulanase, which attacks both α-1,6-glycosidic linkages in pullulan and α-1,4-linkages in other oligosaccharides and polysaccharides.

The finding of extremely thermostable starch-hydrolysing enzymes such as amylases and pullulanases that are active under similar conditions will significantly improve the industrial starch bioconversion process, i.e. liquefaction, saccharification and isomerization. Owing to the lack of novel thermostable enzymes that are active and stable above 100 °C and at acidic pH values, the bioconversion of starch to glucose and fructose has to be performed under various conditions. This multistage process (step 1: pH 6.0–6.5, 95–105 °C; step 2: pH 4.5, 60–62 °C; step 3: pH 7.0–8.5, 55–60 °C) is accompained by the formation of undesirable high concentrations of salts. In the final step, where high-fructose syrup is produced, salts have to be removed by expensive ion exchangers (Crabb and Mitchinson 1997).

Heat-stable amylases and glucoamylases

Enzymes from hyperthermophilic microorganisms, which are active above 100 °C and in the compatible pH range, are regarded as interesting candidates for use in the starch bioconversion process. Intensive research has been performed aimed at the isolation of thermostable and thermoactive amylases from hyperthermophiles (Table 1). The α-amylase (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1) family consists of a large group of starch hydrolases and related enzymes, currently known as glycosyl hydrolase family 13 (Henrissat 1991). Thermostable α-amylases have been characterized from *Pyrococcus woesei*, *Pyrococcus furiosus* (Koch et al. 1991) and *Thermococcus profundus* (Chung et al. 1995; Kwak et al. 1998; Lee et al. 1996). The optimum temperatures for the activity of these enzymes are 100 °C, 100 °C and 80 °C respectively. Amylolytic activity has been also observed in the hyperthermophilic archaea of the genera *Sulfolobus*, *Desulfurococcus*, *Thermococcus* and *Staphylothermus* (Bragger et al. 1989; Canganella et al. 1994). The molecular cloning of the corresponding genes and their expression in heterologous hosts allowed circumvention of the problem of insufficient expression in the authentic host. The gene encoding an extracellular α-amylase from *P. furiosus* has been recently cloned and the recombinant enzyme expressed in *Bacillus subtilis* and in *Escherichia coli* (Dong et al. 1997a; Jorgensen et al. 1997). The high thermostability of the pyrococcal extracellular α-amylase (thermal activity even at 130 °C) in the absence of metal ions, together with its unique product pattern and substrate specificity, makes this enzyme an interesting candidate for industrial application. The gene encoding an intracellular α-amylase from *P. furiosus* has been cloned and sequenced (Laderman et al. 1993). Interestingly, the four highly conserved regions usually found in amylases are not present in this enzyme. Less thermoactive α-amylases have been characterized from the archaea *Thermococcus profundus* and *Pyrococcus* sp. KOD1 and the bacterium *Thermotoga maritima*. The genes encoding these enzymes were successfully expressed in *E. coli* (Lee et al. 1996; Tachibana

Table 1 Starch-hydrolysing enzymes from extremely thermophilic and hyperthermophilic archaea and bacteria

Enzymes	Organism[a]	Enzyme properties				Reference
		Optimal temperature (°C)	Optimal pH	mW[b] (kDa)	Remarks	
α-Amylase	*Desulfurococcus mucosus* (85)	100	5.5	–	Crude extract	Canganella et al. 1994
	Pyrococcus furiosus (100)	100	6.5–7.5	129	Pur./cloned/intracellular	Ladermann et al. 1993a, b
		100	7.0	68	Pur./cloned/extracellular	Jorgensen et al. 1997 Dong et al. 1997a
	Pyrococcus sp. KOD1	90	6.5	49.5	Pur./cloned/extracellular	Tachibana et al. 1996
	Pyrococcus woesei (100)	100	5.5	68	Pur./extracellular	Koch et al. 1991
	Pyrodictium abyssi (98)	100	5.0	–	Crude extract	Unpublished
	Staphylothermus marinus (90)	100	5.0	–	Crude extract	Canganella et al. 1994
	Sulfolobus solfataricus (88)	–	–	240	Extracellular	Haseltine et al. 1996
	Thermococcus celer (85)	90	5.5	–	Crude extract	Canganella et al. 1994
	Thermococcus profundus (80)	80	5.5	42	Pur./cloned/"Amy S"	Chung et al. 1995 Lee et al. 1996
	Thermococcus profundus (80)	80	4.0–5.0	43	Purified/"Amy L"	Kwak et al. 1998
	Thermococcus aggregans (85)	100	5.5	–	Crude extract	Canganella et al. 1994
	Dictyoglomus thermophilum Rt46B.1 (73)	90	5.5	75	Pur./cloned/cytoplasmic fraction	Fukusumi et al. 1998
	Thermotoga maritima MSB8 (90)	85–90	7.0	61	Pur./cloned/lipoprotein	Schumann et al. 1991
Pullulanase type I	*Fervidobacterium pennavorans* Ven5 (75)	85	6.0	190 (93)	Pur./cloned	Koch et al. 1997 Bertoldo et al. (1999)
	Thermotoga maritima MSB8 (90)	90	6.0	(93)	Cloned/type I[c]	Bibel et al. 1998
	Thermus caldophilus GK24 (75)	75	5.5	65	Pur./cell-associated	Kim et al. 1996
Pullulanase type II	*Desulfurococcus mucosus*	100	5.0	–	Crude extract	Canganella et al. 1994
	Pyrococcus woesei (100)	100	6.0	90	Pur./cloned	Rüdiger et al. 1995
	Pyrococcus furiosus (100)	105	6.0	90	Pur./cloned	Dong et al. 1997b
	Pyrodictium abyssi (98)	100	9.0	–	Crude extract	Unpublished
	Thermococcus celer (85)	90	5.5	–	Crude extract	Canganella et al. 1994
	Thermococcus litoralis (90)	98	5.5	119	Pur./extracell./glycoprotein	Brown and Kelly 1993
	Thermococcus hydrothermalis (80)	95	5.5	128	Purified	Gantelet and Duchiron 1998
	Thermococcus aggregans (85)	100	6.5	–	Crude extract	Canganella et al. 1994

[a] Values in parentheses give the optimal growth temperature for each organism in °C
[b] Values in parentheses give the molecular mass of each subunit
[c] Unpublished results
– Not determined

et al. 1996; Liebl et al. 1997). Like the amylase from *Bacillus licheniformis*, which is commonly used in liquefaction, the enzyme from *T. maritima* requires the presence of Ca^{2+} for its activity (Liebl et al. 1997). Further investigations have shown that the hyperthermophilic archaeon *Pyrodictium abyssi* can also grow anaerobically on various polymeric substrates and secrete a heat-stable amylase that is active even above 100 °C (unpublished data).

Glucoamylase (1,4-glucanohydrolase; EC 3.2.1.3) is an exo-acting enzyme that attacks α-1,4- and α-1,6-glycosidic linkages of α-glucan from the non-reducing ends. The action of this enzyme liberates one molecule of β-D-glucose at a time, causing the complete conversion of polysaccharides to glucose. The branching points, however, are hydrolyzed at a very slow rate. Glucoamylases are typical fungal enzymes and are among the most important industrial enzymes that are used for the production of glucose syrups. For the saccharification of dextrin, the glucoamylases from *Aspergillus niger* and *Aspergillus oryzae* are generally used. Ca^{2+} ions were found even to decrease the stability of the enzyme from this latter organism (Antranikian 1992). Interestingly, the production of glucoamylases seems to be very rare in extreme thermophilic and hyperthermophilic bacteria and archaea. Among the thermoanaerobic bacteria, glucoamylases have been purified and characterized from *Clostridium thermohydrosulfuricum* 39E (Hyun and Zeikus 1985) and *Clostridium thermosaccharolyticum* (Specka et al. 1991). The latter enzyme is optimally active at 70 °C and pH 5. Recently, a glucoamylase has been purified from *Thermoanaerobacterium thermosaccharolyticum* DSM 571 (Ganghofner et al. 1998).

Thermoactive pullulanases

Thermostable and thermoactive pullulanases (pullulan 6-glucanohydrolase; EC 3.2.1.41) from extremophilic microorganisms have been detected in *Thermococcus celer*, *Desulfurococcus mucosus*, *Staphylothermus marinus* and *Thermococcus aggregans*. Temperature optima between 90 °C and 105 °C, as well as remarkable thermostability even in the absence of substrate and calcium ions, have been observed (Canganella et al. 1994). Most of the enzymes studied to date belong to the pullulanase type II group. They have been purified from *P. furiosus* and *Thermococcus litoralis* (Brown and Kelly 1993), *Thermococcus hydrothermalis* (Gantelet and Duchiron 1998) and strain ES4 (Schuliger et al. 1993). The extreme thermostability of these enzymes, coupled with their ability to attack both α-1,6- and α-1,4-glycosidic linkages, may improve the industrial starch hydrolysis process. Pullulanase type II from *P. woesei* has been expressed in *E. coli*. The purified recombinant enzyme is optimally active at 100 °C and extremely thermostable with a half-life of 7 min at 110 °C (Rüdiger et al. 1995). The gene encoding the same enzyme from *P. furiosus* was also cloned and expressed in *E. coli*. (Dong et al. 1997b).

The aerobic thermophilic bacterium *Thermus caldophilus* GK-24 produces a thermostable pullulanase of type I. The pullulanase is optimally active at 75 °C and pH 5.5, stable up to 90 °C and does not require Ca^{2+} ions for either activity or stability. The first starch-debranching enzyme (pullulanase type I) from an anaerobe was identified in the thermophilic bacterium *Fervidobacterium pennavorans* Ven5 (Koch et al. 1997). The corresponding gene has been recently cloned and expressed in *E. coli* (Bertoldo et al. 1999). In contrast to the pullulanase from *P. woesei*, the enzyme from *F. pennavorans* Ven5 attacks exclusively the α-1,6-glycosidic linkages in polysaccharides. This is the only thermostable debranching enzyme known to date that attacks amylopectin, leading to the formation of long-chain linear polysaccharides, which are the ideal substrates for the action of glucoamylase (Table 1).

Cyclodextrin glycosyltransferases (CGTases)

CGTases (EC 2. 4.1.19) attack α-1,4-linkages in polysaccharides in a random fashion and convert starch by an intramolecular transglycosylation reaction. The non-reducing cyclization products of this reaction are α-, β- or γ-cyclodextrins, consisting of six, seven or eight glucose molecules respectively. The predominant application of CGTase is in the industrial production of cyclodextrins. The ability of cyclodextrins to form inclusion complexes with a variety of organic molecules means that they improve the solubility of hydrophobic compounds in aqueous solutions. Cyclodextrin production occurs in a multistage process in which, in the first step, starch is liquefied by a heat-stable amylase and, in the second step, the cyclization reaction with a CGTase from *Bacillus* sp. takes places. Because of the low stability of the latter enzyme, the process must run at two different temperatures. The finding of heat-stable and more specific CGTases from extremophiles will solve this problem. The application of heat-stable CGTase in jet cooking, where temperatures up to 105 °C are used, will allow the liquefaction and cyclization to take place in one step. Thermostable CGTases have been already found in *Thermoanaerobacter* sp. (Norman and Jørgensen 1992; Pedersen et al. 1995) and *Thermoanaerobacterium thermosulfurogenes* (Wind et al. 1995). Recently a heat- and alkali-stable CGTase (optimal activity 65 °C, pH 10) was purified from a newly identified strain "*Anaerobranca bogoriae*", which was isolated from Lake Bogoria, Kenya (Prowe et al. 1996).

Degradation of cellulose, the most abundant polymer in nature

Cellulose commonly accounts for up to 40% of the plant biomass. It consists of glucose units linked by β-1, 4-glycosidic bonds with a polymerisation grade of up to 15 000 glucose units in an absolutely linear mode.

Although cellulose has a high affinity to water, it is completely insoluble. Natural cellulose compounds are structurally heterogeneous and have both amorphous and highly ordered crystalline regions. The degree of crystallinity depends on the source of the cellulose and the higher crystalline regions are more resistant to enzymatic hydrolysis. Cellulose can be hydrolysed into glucose by the synergistic action of at least three different enzymes: endoglucanase, exoglucanase (cellobiohydrolase) and β-glucosidase. Synonyms for cellulases (EC 3.2.1.4) are β-1,4-D-glucan glucanohydrolases, endo-β-1,4-glucanases or carboxymethylcellulases. This enzyme is an endoglucanase that hydrolyses cellulose in a random manner as an endohydrolase, producing various oligosaccharides, cellobiose and glucose. The enzyme catalyses the hydrolysis of β-1,4-D-glucosidic linkages in cellulose but can also hydrolyse 1,4-linkages in β-D-glucans containing 1,3-linkages. Cellulases belong to the family 12 of the glucosyl hydrolases (Henrissat 1991).

Exoglucanases or β-1,4-cellobiosidases or exo-cellobiohydrolases or β-1,4-cellobiohydrolases (EC 3.2.1.91) hydrolyse β-1,4 D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing end of the chain. They belong to family 6 of the glycosyl hydrolases.

β-Glucosidases (EC 3.2.1.21) or gentobiases or cellobiases or amygdalases catalyse the hydrolysis of terminal, non-reducing β-D-glucose residues releasing β-D-glucose. These enzymes belong to family 3 of the glycosyl hydrolases and have a wide specificity for β-D-glucosides. They are able to hydrolyse β-D-galactosides, α-L-arabinosides, β-D-xylosides, and β-D-fucosides. Cellulose-hydrolysing enzymes are widespread in fungi and bacteria. Such enzymes have found various biotechnological applications. The most effective enzyme of commercial interest is the cellulase produced by *Trichoderma* sp. (Teeri et al. 1998). Cellulases were also obtained from strains of *Aspergillus*, *Penicillium* and *Basidiomycetes* (Tomme et al. 1995). Cellulolytic enzymes can be used in alcohol production to improve juice yields and effective colour extractions of juices. The presence of cellulases in detergents causes colour brightening and softens and improves particulate soil removal. Cellulase (Denimax Novo Nordisk) is also used for the "biostoning" of jeans instead of the classical stones in stonewashed jeans. Other applications of cellulases include the pretreatment of cellulosic biomass and forage crops to improve nutritional quality and digestibility, enzymatic saccharification of agricultural and industrial wastes and the production of fine chemicals.

Thermoactive cellulases

Thermostable cellulases active towards crystalline cellulose are of great biotechnologial interest. Several cellulose-degrading enzymes from various thermophilic organisms have been cloned, purified and characterized. A thermostable cellulase from *T. maritima* MSB8 has been characterized (Bronnenmeier et al. 1995). The enzyme is rather small with a molecular mass of 27 kDa and it is optimally active at 95 °C and between pH 6.0 and 7.0. Two themostable endocellulases, CelA and CelB, were purified from *Thermotoga neapolitana*. CelA (29 kDa) is optimally active at pH 6 at 95 °C, while CelB (30 kDa) has a broader optimal pH range (pH 6–6.6) at 106 °C. The genes encoding these two endocellulases have been identified (Bok et al. 1998).

Cellulase and hemicellulase genes have been found clustered together on the genome of the thermophilic anaerobic bacterium *Caldocellum saccharolyticum*, which grows on cellulose and hemicellulose as sole carbon sources. The gene for one of the cellulases (*celA*) was isolated and was found to code for 1751 amino acids. This is the largest known cellulase gene to date (Teo et al. 1995).

A large cellulolytic enzyme (CelA) with the ability to hydrolyse microcrystalline cellulose was isolated from the extremely thermophilic bacterium *Anaerocellum thermophilum* (Zverlov et al. 1998). The enzyme has an apparent molecular mass of 230 kDa, exhibits significant activity towards Avicel and is most active towards soluble substrates such as carboxymethyl(CM)-cellulose and β-glucan. Maximal activity was observed at pH 5–6 and 85–95 °C. The thermostable exoacting cellobiohydrolase from *T. maritima* MSB8 is 29 kDa and is optimally active at 95 °C at pH 6.0–7.5 with a half-life of 2 h at 95 °C. The enzyme hydrolyses Avicel, CM-cellulose and β-glucan forming cellobiose and cellotriose (Bronnenmeier et al. 1995). A thermostable cellobiase is produced by *Thermotoga* sp. FjSS3-B1 (Ruttersmith and Daniel 1991). The enzyme is highly thermostable and shows maximal activity at 115 °C at pH 6.8–7.8. The thermostability of this enzyme is salt-dependent. This cellobiase is active against amorphous cellulose and CM-cellulose.

Recently a thermostable endoglucanase that is capable of degrading β-1,4 bonds of β-glucans and cellulose has been identified in the archaeon *P. furiosus*. The gene encoding this enzyme has been cloned and sequenced in *E. coli* and has significant amino acid sequence similarities with endoglucanases from glucosylhydrolases family 12. The purified recombinant endoglucanase hydrolyses β-1,4 but not β-1,3-glucosidic linkages and has the highest specific activity with cellopentaose and cellohexaose as substrates (Bauer et al. 1999). In contrast to this, several β-glucosidases have been detected in archaea. In fact, archeal β-glucosidases have been found in *Sulfolobus solfataricus* MT4, *Sulfolobus acidocaldarius*, *Sulfolobus shibatae* (Grogan 1991) and *P. furiosus* (Kengen et al. 1993). The enzyme from the latter microorganism (homotetramer/56-kDa subunits) is very stable and shows optimal activity at 102–105 °C with a half-life of 3.5 days at 100 °C and 13 h at 110 °C (Kengen et al. 1993; Voorhorst et al. 1995). The β-glucosidase from *S. solfataricus* MT4 has been purified and characterized (Nucci et al. 1993). The enzyme is a ho-

motetramer (56 kDa/subunit) and very resistant to various denaturants with activity up to 85 °C (Pisani et al. 1990). The gene for this β-glucosidase has been cloned and overexpressed in *E. coli* (Cubellis et al. 1990; Moracci et al. 1993; Prisco et al. 1994).

Xylan-degrading enzymes

Xylan is a heterogeneous molecule that constitutes the main polymeric compound of hemicellulose, a fraction of the plant cell wall that is a major reservoir of fixed carbon in nature. The main chain of the heteropolymer is composed of xylose residues linked by β-1,4-glycosidic bonds. Approximately half of the xylose residues are substituted at the O-2 or O-3 positions of acetyl, arabinosyl and glucuronosyl groups. The complete degradation of xylan requires the action of several enzymes (for a detailed description see reviews Sunna and Antranikian 1997a; Sunna et al. 1996a). The endo-β-1,4-xylanase (EC 3.2.1.8), or β-1,4-xylan xylanohydrolase, hydrolyses β-1,4-xylosydic linkages in xylans, while β-1,4-xylosidase or β-xylosidase or β-1,4-xylan xylohydrolase or xylobiase or exo-β-1,4-xylosidase (EC 3.2.1.37) hydrolyses β-1,4-xylans and xylobiose by removing the successive xylose residues from the non-reducing termini. α-Arabinofuranosidase or arabinosidase (EC 3.2.1.55) hydrolyses the terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides. The enzyme also acts on α-L-arabinofuranosides, α-L-arabinans containing either 1,3 or 1,5 linkages. Glucuronoarabinoxylan endo-β-1,4-xylanase or feraxan endoxylanase or glucuronoarabinoxylan β-1,4-xylanohydrolase (EC 3.2.1.136) attacks β-1,4-xylosyl linkages in some glucuronoarabinoxylans. This enzyme also shows high activity towards feruloylated arabinoxylans from cereal plant cell walls. Acetylxylanesterase (EC 3.1.1.6) removes acetyl groups from xylan. Xylanases from bacteria and eukarya comprise families 10 and 11 of the glycosyl hydrolases and have a wide range of potential biotechnological applications. They are already produced on an industrial scale and are used as food additives in poultry, for increasing feed efficiency (Annison 1992; Classen 1996) and in wheat flour for improving dough handling and the quality of baked products (Maat et al. 1992).

In recent years, the major interest in thermostable xylanases lay in enzyme-aided bleaching of paper (Viikari et al. 1994). More than 2×10^6 tonnes of chlorine and chlorine derivatives are used annually in the United States for pulp bleaching. The chlorinated lignin derivatives generated by this process constitute a major environmental problem caused by the pulp and paper industry (McDonough 1992). Recent investigations have demonstrated the feasibility of enzymatic treatments as alternatives to chlorine bleaching for the removal of residual lignin from pulp (Viikari et al. 1994). Treatment of craft pulp with xylanase leads to a release of xylan and residual lignin without undue loss of other pulp components. Xylanase treatment at elevated temperatures opens up the cell wall structure, thereby facilitating lignin removal in subsequent bleaching stages. Candidate xylanases for this important, potential market would have to satisfy several criteria: (1) they must lack cellulolytic activity to avoid hydrolysis of the cellulose fibres, (2) their molecular mass should be low enough to facilitate their diffusion in the pulp fibres, (3) they must be stable and active at high temperature and at alkaline pH, and (4) one must be able to obtain high yields of enzyme at very low cost. All of the xylanases currently available from commercial suppliers can only partially fulfil these criteria. Xylanases from moderate thermophilic microorganisms are rapidly denatured at temperatures above 70 °C. Several of the non-chlorine bleaching stages used in commercial operations are performed well above this temperature; consequently, pulp must be cooled before treatment with the available enzymes and reheated for subsequent processing steps (Chen et al. 1997).

Thermostable xylanases

So far, only a few extreme thermophilic microorganisms are able to grow on xylan and secrete thermoactive xylanolytic enzymes (Table 2). Members of the order Thermotogales and *Dictyoglomus thermophilum* Rt46B.1 have been described to produce xylanases that are active and stable at high temperatures (Gibbs et al. 1995; Sunna and Antranikian 1997a). The most thermostable endoxylanases that have been described so far are those derived from *Thermotoga* sp. strain FjSS3-B.1 (Simpson et al. 1991), *T. maritima* (Winterhalter and Liebl 1995), *T. neapolitana* (Bok et al. 1994) and *Thermotoga thermarum* (Sunna et al. 1996b). These enzymes, which are active between 80 °C and 105 °C, are mainly cell-associated and most probably localized within the toga (Ruttersmith et al. 1992; Schumann et al. 1991; Sunna et al. 1996a; Winterhalter and Liebl 1995). Several genes encoding xylanases have been already cloned and sequenced. The gene from *T. maritima*, encoding a thermostable xylanase, has been cloned and expressed in *E. coli*. Comparison between the *T. maritima* recombinant xylanase and the commercially available enzyme, Pulpenzyme (Yang and Eriksson 1992) indicates that the thermostable xylanase could be of interest for application in the pulp and paper industry (Chen et al. 1997). Only recently an archaeal xylanase with a temperature optimum of 110 °C was found in the hyperthermophilic archaeon *Pyrodictium abyssi* (unpublished data).

Chitin degradation

Chitin is a linear β-1,4 homopolymer of *N*-acetylglucosamine residues and is the second most abundant natural biopolymer after cellulose on earth. Particularly in the marine environment, chitin is produced in enormous amounts and its turnover is due to the action of

Table 2 Production of heat-stable xylanases by extreme thermophilic and hyperthermophilic archaea and bacteria

Enzymes	Organism[a]	Enzyme properties				Reference
		Optimal temperature (°C)	Optimal pH	*m*W[b] (kDa)	Remarks	
Endoxylanases	*Pyrodictium abyssi* (98)	110	5.5	–	Crude extract	Unpublished
	Dictyoglomus thermophilum Rt46B.1 (73)	85	6.5	31	Pur./cloned	Gibbs et al. 1995
	Thermotoga maritima MSB8 (80)	92	6.2	120	Pur./toga-assoc./XynA	Winterhalter and Liebl
		105	5.4	40	Pur./toga-assoc./XynB	1995
	Thermotoga sp. strain FjSS3-B.1 (80)	105	5.3	31	Pur./cloned/toga-assoc.	Simpson et al. 1991
		85	6.3	40	Pur./cloned	Saul et al. 1995
	Thermotoga neapolitana (80)	85	5.5	37	Purified	Bok et al. 1994
		102	5.5–6.0	119	Pur./cloned	Zverlov et al. 1996
	Thermotoga thermarum (77)	80	6.0	105/150	Pur./toga-assoc./Endo1	Sunna et al. 1996a
		90–100	7.0	35	Pur./Endo2	
Endoglucanase	*Thermotoga maritima* MSB8 (80)	95	6.0–7.5	27	Pur./cloned/cellulase I	Bronnenmeier et al. 1995
	Thermotoga neapolitana (80)	95	6.0	29	Purif./cloned/CellA	Bok et al. 1998
		106	6.0–6.6	30	Purif./cloned/CellB	
Exoglucanase	*Thermotoga maritima* MSB8 (80)	95	6.0–7.5	29	Purified/cellulase II	Bronnenmeier et al. 1995
	Thermotoga sp. strain FjSS3-B.1 (80)	115	6.8–7.8	36	Pur./cell-associated	Ruttersmith et al. 1991
	Anaerocellum thermophilum (100)	95	5.0–6.0	230	Cloned	Zverlov et al. 1998
	Pyrococcus furiosus (100)	100	6.0	35.9	Cloned	Bauer et al. 1999
β-Glucosidase	*Pyrococcus furiosus* (100)	102–105	–	230/58	Purified/cloned	Kengen et al. 1993
						Voorhorst et al. 1995
	Sulfolobus solfataricus (88)	105	5.3	240/56	Pur./cloned	Nucci et al. 1991
						Moracci et al. 1993
	Thermotoga maritima MSB8 (80)	75	6.2	95 (47)	Cloned	Gabelsberger et al. 1993
	Thermotoga sp. strain FjSS3-B.1 (80)	80	7.0	100 (75)	Pur./toga-associated	Ruttersmith and Daniel 1991

[a] Values in parentheses give the optimal growth temperature for each organism in °C
[b] Values in parentheses show the size of subunits
– Not determined

chitinolytic enzymes. Chitin is the major structural component of most fungi and invertebrates (Gooday 1990, 1994), while for soil or marine bacteria chitin serves as a nutrient. Chitin degradation is known to proceed with the endo-acting chitin hydrolase chitinase A (EC 3.2.1.14) and the chitin oligomer exo-acting hydrolases chitinase B and *N*-acetyl-D-glycosaminidase (trivial name: chitobiase, EC 3.2.1.52).

Endo- and exo-chitinases comprise three glycosyl hydrolase families, i.e. families 18, 19 and 20. Chitinases, endo-β-*N*-acetyl-D-glucosaminidases (EC 3.2.1.96) and di-*N*-acetylchitobiases from eukarya, bacteria and viruses belong to family 18. The *N*-acetyl-D-glucosamine oligomeric product retains its C1 anomeric configuration. Family 19 contains only chitinases from eukarya and bacteria and, in contrast to family 18, the product has inverted anomeric configuration. Family 20 contains β-hexosaminidases and chitobiases. Chitobiase degrades only small *N*-acetyl-D-glucosamine oligomers (up to pentamers) and the released *N*-acetyl-D-glucosamine monomers retain their C1 anomeric configuration.

Chitin exhibits interesting properties that make it a valuable raw material for several applications (Chandy and Sharma 1990; Cohen-Kupiec and Chet 1998; Georgopapadakou and Tkacz 1995; Kas 1997; Muzzarelli 1997; Spindler et al. 1990). Although a large number of chitin-hydrolysing enzymes have been isolated and their corresponding genes have been cloned and characterized, only a few thermostable chitin-hydrolysing enzymes are known. These enzymes have been isolated from the thermophilic bacterium *B. licheniformis* X-7u (Takayanagi et al. 1991), *Bacillus* sp. BG-11 (Bharat and Hoondal 1998) and *Streptomyces thermoviolaceus* OPC-520 (Tsujibo et al. 1995).

The extreme thermophilic anaerobic archaeon *Thermococcus chitonophagus* has been reported to hydrolyse chitin (Huber et al. 1995). This is the first extremophilic archeon found that produces chitinase(s) and *N*-acetyl-glucosaminidase(s).

Protein degradation

Proteases are involved in the conversion of proteins to amino acids and peptides. They have been classified according to the nature of their catalytic site into the following groups: serine, cysteine or aspartic proteases or metalloproteases (Table 3).

The amount of proteolytic enzymes produced worldwide on a commercial scale is larger than that of any of the other biotechnologically used enzymes. Serine alkaline proteases are used as additives to household detergents for laundering, where they have to resist denaturation by detergents and alkaline conditions. Proteinases showing high keratinolytic and elastolytic activities are used for soaking in the leather industry. Proteinases are also used as catalysts for peptide synthesis, using their reverse reaction. The exploration of proteases that can catalyse reactions under extreme conditions (high temperatures

Table 3 Production of heat-stable proteolytic enzymes

Enzymes	Organism[a]	Enzyme properties				Reference
		Optimal temperature (°C)	Optimal pH	mW^b (kDa)	Remarks	
Serine protease	*Desulfurococcus mucosus* (85)	95	7.5	52	Purified	Cowan et al. 1987
	Pyrococcus furiosus (100)	85	6.3	124 (19)	Protease I/purified	Eggen et al. 1990
				105/80	Pyrolysin/pur./cloned	Voorhorst et al. 1996
	Pyrobaculum aerophilum (95)	–	–	–		Völkl et al. 1995
	Thermococcus aggregans (75)	90	7.0	–	Crude extract	Klingeberg et al. 1991
	Thermococcus celer (85)	95	7.5	–	Crude extract	Klingeberg et al. 1991
	Thermococcus litoralis (90)	95	9.5	–	Crude extract	Klingeberg et al. 1991
	Thermococcus stetteri (75)	85	8.5	68	Pur./cloned	Klingeberg et al. 1995
	Staphylothermus marinus (90)	–	9.0	140	Stable up to 135 °C	Mayr et al. 1996
	Sulfolobus solfataricus (88)	–	6.5–8.0	118 (52)	Purified	Burlini et al. 1992
	Fervidobacterium pennavorans (70)	80	10	130	Purified/keratin hydrolysis	Friedrich and Antranikian 1996
Thiol protease	*Pyrococcus* sp. KOD1 (95)	110	7	44	Purified	Fujiwara et al. 1996 Morikawa et al. 1994
Acidic protease	*Sulfolobus acidocaldarius* (70)	90	2.0	–	Cloned	Fusek et al. 1990
Carboxy-peptidase	*Sulfolobus solfataricus* (88)	–	–	160	Crude extract	Fusi et al. 1991

[a] Values in parentheses give the optimal growth temperature for each organism in °C
[b] Values in parentheses show the size of the subunits
– Not determined

and extremes of pH) will be valuable for industrial applications (Ladenstein and Antranikian 1998).

A variety of heat-stable proteases have been identified in hyperthermophilic archaea belonging to the genera *Desulfurococcus*, *Sulfolobus*, *Staphylothermus*, *Thermococcus*, *Pyrobaculum* and *Pyrococcus*. It has been found that most proteases from extremophiles belong to the serine type and are stable at high temperatures even in the presence of high concentrations of detergents and denaturing agents. A heat-stable serine protease was isolated from cell-free supernatants of the hyperthermophilic archaeon *Desulfurococcus* strain $Tok_{12}S_1$ (Cowan et al. 1987). Recently, a cell-associated serine protease was characterized from *Desulfurococcus* strain SY, that showed a half-life of 4.3 h at 95 °C (Hanzawa et al. 1996). A globular serine protease from *Staphylothermus marinus* was found to be extremely thermostable. This enzyme, which is bound to the stalk of a filiform glycoprotein complex, named tetrabrachion, has residual activity even after 10 min of incubation at 135 °C (Mayr et al. 1996). The properties of extracellular serine proteases from a number of *Thermococcus* species have been analysed (Klingeberg et al. 1991). The extracellular enzyme from *T. stetteri* has a molecular mass of 68 kDa and is highly stable and resistant to chemical denaturation, as illustrated by a half-life of 2.5 h at 100 °C and retention of 70% of its activity in the presence of 1% sodium dodecyl sulfate (Klingeberg et al. 1995). Another gene encoding a subtilisin-like serine protease, named aereolysin, has been cloned from *Pyrobaculum aerophilum* and the protein was modelled on the basis of structures of subtilisin-type proteases (Völkl et al. 1995). Multiple proteolytic activities have been observed in *P. furiosus*. The cell-envelope-associated serine protease of *P. furiosus,* called pyrolysin, was found to be highly stable with a half-life of 20 min at 105 °C (Eggen et al. 1990). The pyrolysin gene was cloned and sequenced and it was shown that this enzyme is a subtilisin-like serine protease (Voorhorst et al. 1996).

Proteases have also been characterized from the thermoacidophilic archaeon *S. solfataricus* (Burlini et al. 1992) and *S. acidocaldarius* (Fusek et al. 1990; Lin and Tang 1990). In addition to the serine proteases, other types of enzymes have been identified in extremophiles: a thiol protease from *Pyrococcus* sp. KOD1 (Fujiwara et al. 1996; Morikawa et al. 1994), a propylpeptidase and a new type of protease from *P. furiosus* (Blumentals et al. 1992; Halio et al. 1996; Harwood et al. 1997; Robinson et al. 1995). A thermostable serine protease was also detected in the extreme thermophilic bacterium *Fervidobacterium pennavorans*. Interestingly, this enzyme is able to hydrolyse feather keratin, forming amino acids and peptides. The enzyme is optimally active at 80 °C and pH 10.0 (Friedrich and Antranikian 1996).

DNA-processing enzymes: DNA polymerases

DNA polymerases (EC 2.7.7.7) are the key enzymes in the replication of cellular information present in all life forms. They catalyse, in the presence of Mg^{2+} ions, the addition of a deoxyribonucleoside 5′-triphosphate onto the growing 3′-OH end of a primer strand, forming complementary base pairs to a second strand. More than 100 DNA polymerase genes have been cloned and sequenced from various organisms, including thermophilic bacteria and archaea. Several native and recombinant enzymes have been purified and characterized (Perler et al. 1996). Thermostable DNA polymerases play a major role in a variety of molecular biological applications, e.g. DNA amplification, sequencing or labelling (Table 4).

One of the most important advances in molecular biology during the last 10 years is the development of the polymerase chain reaction (PCR, Erlich et al. 1988; Mullis et al. 1986; Saiki et al. 1988). The first PCR procedure described utilized the Klenow fragment of *E. coli* DNA polymerase I, which was heat-labile and had to be added during each cycle following the denaturation and primer hybridization steps. Introduction of thermostable DNA polymerases in PCR facilitated the automation of the thermal cycling part of the procedure. DNA polymerase I from the bacterium *Thermus aquaticus*, called *Taq* polymerase, was the first thermostable DNA polymerases characterized (Chien et al. 1976; Kaledin et al. 1980) and applied in PCR.

Taq polymerase has a 5′–3′-exonuclease activity, but no detectable 3′–5′-exonuclease activity (Longley et al. 1990). Owing to the absence of a 3′–5′-exonuclease activity, this enzyme is unable to excise mismatches and, as a result, the base-insertion fidelity is low (Dunning et al. 1988; Keohavong and Thilly 1989; Ling et al. 1991; Tindall and Kunkel 1988). The use of high-fidelity DNA polymerases is essential for reducing the increase of amplification errors in PCR products that will be cloned, sequenced and expressed. Several thermostable DNA polymerases with 3′–5′-exonuclease-dependent proofreading activity have been described, and the error rates (number of misincorporated nucleotides per base synthesized) for these enzymes have been determined. A thermostable DNA polymerase from *T. maritima* (Huber et al. 1986) was reported to have a 3′–5′-exonuclease activity (Bost et al. 1994). Archaeal proofreading polymerases such as *Pwo* pol (Frey and Suppmann 1995) from *P. woesei* (Zillig et al. 1987), *Pfu* pol (Lundberg et al. 1991) from *P. furiosus* (Fiala and Stetter 1986), Deep Vent pol (Perler et al. 1996) from *Pyrococcus* strain GB-D (Jannasch et al. 1992) or Vent pol (Cariello et al. 1991; Mattila et al. 1991) from *T. litoralis* (Neuner et al. 1990) have an error rate that is up to tenfold lower than that of *Taq* polymerase. The 9°N-7 DNA polymerase from *Thermococcus* sp. strain 9°N-7 has a fivefold higher 3′–5′-exonuclease activity than *T. litoralis* DNA polymerase (Southworth et al. 1996). However, *Taq* polymerase was not replaced by these DNA polymerases because of their low extension rates among other factors. DNA polymerases with higher fidelity are not necessarily suitable for amplification of long DNA fragments because of their poten-

Table 4 Applications of thermostable DNA polymerases. Data are derived from references cited or from commercial sources

Polymerase	Organism	Application				References
		PCR[a]	High-fidelity PCR[b]	Reverse transcription	DNA sequencing	
Bacterial DNA polymerases						
Taq pol I	*Thermus aquaticus*	+	–	+ (weak; Mn^{2+})[c]	+[e]	Longley et al. 1990 Tse and Forget 1990 Barnes 1992 Jones and Foulkes 1989 Lawyer et al. 1993 Reeve and Fuller 1995
Tth pol	*Thermus thermophilus*	+	–	+ (Mn^{2+})	+	Rüttimann et al. 1985 Myers and Gelfand 1991 Auer et al. 1995
Tfi pol	*Thermus filiformis*	+	NI	+ (Mg^{2+})	NI	Perler et al. 1996
Tfl pol	*Thermus flavus*	+	NI	–	+	Kaledin et al. 1981 Rao and Saunders 1992
Tca pol	*Thermus caldophilus* GK24	+	–	+ (weak; Mn^{2+})	NI	Park et al. 1993
*Bst*I pol	*Bacillus stearothermophilus*	+	–	–	+	Mead et al. 1991
Tma pol	*Thermotoga maritima*	+	+	NI	+	Bost et al. 1994
Archaeal DNA polymerases						
Pwo pol	*Pyrococcus woesei*	+	+	–	–	Frey and Suppmann 1995
Pfu pol	*Pyrococcus furiosus*	+	+	–	–/+[d]	Lundberg et al. 1991
Deep Vent pol	*Pyrococcus* sp. GB-D	+	+	–	–/+[d]	Cline et al. 1996
KOD1 pol	*Pyrococcus* sp. KOD1	+	+	–	–	Takagi et al. 1997
Vent pol	*Thermococcus litoralis*	+	+	–	–/+[d]	Kong et al. 1993 Perler et al. 1996
9°N-7 pol	*Thermococcus* sp. 9°N-7	+	+	–	–/+[d]	Southworth et al. 1996

[a] Polymerase chain reaction (*PCR*) amplificates up to 40 kb could be obtained by applying a combination of a DNA polymerase with high extension rate (e.g. *Taq* pol or *Tth* pol) and a proof-reading DNA polymerase with 3′–5′ exonuclease activity (e.g. *Pwo* pol or *Pfu* pol)

[b] Fidelity is enhanced by a proof-reading DNA polymerase with 3′–5′ exonuclease activity

[c] Efficiency of reverse transcription is more than 100-fold weaker than with *Tth* pol

[d] Only applicable as an exo^{-} mutation with deleted 3′–5′-exonuclease activity

[e] *Taq* pol I has undergone several modifications to enhance the sequencing procedure as there is elimination of 5′–3′-exonuclease activity either by N-terminal deletion or point mutation and reduced discrimination against dideoxy-NTP also by point mutation

\+ Suitable, – not suitable, NI no information available

tially strong exonuclease activity (Barnes 1994). The recombinant KOD1 DNA polymerase from *Pyrococcus* sp. strain KOD1 has been reported to show low error rates (similar values to those of *Pfu*), high processivity (persistence of sequential nucleotide polymerization) and high extension rates, resulting in a very fast, accurate amplification of target DNA sequences up to 6 kb (Takagi et al. 1997). In order to optimize the delicate competition of polymerase and exonuclease activity, the exo-motif 1 (Blanco et al. 1991; Morrison et al. 1991) of the 9°N-7 DNA polymerase was mutated in an attempt to reduce the level of exonuclease activity without totally eliminating it (Southworth et al. 1996).

An additional problem in the performance of PCR is the generation of non-specific templates prior to thermal cycling. Several approaches have been made to prevent the elongation of polymerase before cycling temperatures are reached. Following the use of wax as a mechanical barrier between DNA and the enzyme, more sophisticated methods were invented like the inhibition of *Taq* polymerase by a neutralizing antibody at mesophilic temperatures (Kellogg et al. 1994; Scalice et al. 1994; Sharkey et al. 1994) or heat-mediated activation of the immobilized enzyme (Nilsson et al. 1997).

Recently, the PCR technique has been improved to allow low-error synthesis of long amplificates (20–40 kb) by adding small amounts of thermostable, archaeal proof-reading DNA polymerases, containing 3′–5′-exonuclease activity, to *Taq* or other non-proof-reading DNA polymerases (Barnes 1994; Cheng et al. 1994; Cohen 1994). In this long PCR, the reaction conditions are optimized for long extension by adding different components such as gelatine, Triton X-100 or bovine serum albumin to stabilize the enzymes and mineral oil to prevent evaporation of water in the reaction mixture. In order to enhance specificity, glycerol (Cha et al. 1992) or formamide (Sarkar et al. 1990) is added.

High-temperature reverse transcription

The technique of DNA amplification has been extended to include RNA as the starting template by first converting RNA to cDNA, employing either avian myeloblastosis virus reverse transcriptase or moloney murine leukemia virus RT (Frohman et al. 1988; Kawasaki et al. 1988; Powell et al. 1987). The resultant first-strand complementary DNA (cDNA) can be used for generating cDNA libraries, quantifying the levels of gene expression, or determining unknown sequences of either the 3′- or the 5′-ends of messenger RNA strands. The latter applications are often referred to as RACE (rapid amplification of cDNA ends) "anchored" PCR (Loh et al. 1989) or "one-sided" PCR (Ohara et al. 1989). A significant problem in using mesophilic viral reverse transcriptases is the occurrence of stable secondary RNA structures at low temperatures (Kotewicz et al. 1988). Many thermostable DNA polymerases, e.g. *Taq* polymerase (Jones and Foulkes 1989; Kaledin et al. 1980; Tse and Forget 1990) and the DNA polymerases from *Thermus thermophilus* (Auer et al. 1995; Myers and Gelfand 1991; Rüttimann et al. 1985) or *T. caldophilus* (Park et al. 1993) can use RNA as a template in the presence of Mn^{2+} instead of Mg^{2+}. The DNA polymerase from *T. thermophilus* was reported to be 100-fold more efficient in a coupled RT-PCR than *Taq* polymerase (Myers and Gelfand 1991). Although we could not find any fidelity values, it is probable that the use of Mn^{2+}, as in the case of mesophilic DNA polymerases (Dong et al. 1993; El-Deiry et al. 1984), may increase the error rate and reduce the fidelity of thermostable DNA polymerases. The DNA polymerase from *Thermus filiformis* has been reported to use Mg^{2+} in RT-PCR, yielding products comparable to those synthesized by *T. thermophilus* DNA polymerase in the presence of Mn^{2+} (Perler et al. 1996).

DNA sequencing

DNA sequencing by the Sanger method (Sanger et al. 1977) has undergone countless refinements in the last 20 years. A major step forward was the introduction of thermostable DNA polymerases, leading to the cycle sequencing procedure. This method uses repeated cycles of temperature denaturation, annealing and extension with dideoxy-DNA termination to increase the amount of sequencing product by recycling the template DNA. Because of this "PCR-like" amplification of the sequencing products, several problems have been overcome. The cycle denaturation means that, only a few femtomoles of template DNA are required, no separate primer annealing step is needed and unwanted secondary structures within the template are resolved by high-temperature elongation.

The first enzyme used for cycle sequencing was the thermostable DNA polymerase I from *T. aquaticus* (Gyllensten 1989; Innis et al. 1988; Murray 1989). As described by Longley et al. (1990), the enzyme displays 5′–3′-exonuclease activity, which is undesirable because of the degradation of sequencing fragments. This enzymatic activity could be deleted by the construction of N-terminal truncated variants. One of them, which lacks the N-terminal 289 amino acids, was termed the Stoffel fragment (Lawyer et al. 1993). Two other variants lacked the first 235 and 278 amino acids (Barnes 1992, 1994). This deletion goes along with an improvement in the fidelity of polymerization (Barnes 1992). One of the disadvantages over conventional sequencing with T7 polymerase was the inefficient incorporation of chain-terminating dideoxynucleotides by *Taq* polymerase into DNA (Innis et al. 1988). Mutagenetic analysis of the dNTP binding site revealed that only a single residue is critical for the selectivity. Therefore, $Phe^{667}\rightarrow$ Tyr exchange in *Taq* polymerase decreased the discrimination against dideoxy-NTP several thousandfold (Tabor and Richardson 1995) resulting in longer reads (Reeve and Fuller 1995) and improved signals (Fan et al. 1996).

Another drawback in sequencing efficiency is the ability of DNA polymerases to catalyse pyrophosphorolysis, resulting in removal of dideoxynucleotides by pyrophosphate. This backward reaction has been suppressed by adding a thermostable pyrophosphatase from *Thermoplasma acidophilum* (Vander et al. 1997). Degradation of the inorganic pyrophosphate, therefore, results in a more efficient termination reaction. Several other bacterial thermostable polymerases have been described for use in cycle sequencing (Table 4), namely *Bst* DNA polymerase from *Bacillus stearothermophilus* (Mead et al. 1991) or *Tfl* DNA polymerase from *Thermus flavus* (Rao and Saunders 1992). The most thermostable DNA polymerases are derived from hyperthermophilic archaea and are therefore highly desirable for application in cycle reactions. Unlike the above-mentioned PolI-like polymerases, these archaeal α-like DNA polymerases exhibit strong 3′–5′-exonuclease activity, which is detrimental for DNA sequencing. As a result of this intrinsic proof-reading activity, the incorporation of dideoxynucleotides is inhibited almost completely. By comparing the primary sequence of several α-type polymerases, active sites responsible for 3′–5′-exonucleolytic activity were detected and altered by site-specific mutagenesis producing polymerases suitable for cycle sequencing reactions (Kong et al. 1993; Perler et al. 1996; Sears et al. 1992; Southworth et al. 1996).

Ligase chain reaction

A variety of analytical methods are based on the use of thermostable ligases. Of considerable potential is the construction of sequencing primers by high-temperature ligation of hexameric primers (Szybalski 1990), the detection of trinucleotide repeats through repeat expansion detection (Schalling et al. 1993) or DNA detection by circularization of oligonucleotides (Nilsson et al. 1994).

Tremendous improvements have been made in the field of heritable diseases. A powerful analytical method for detecting single base mutations in specific nucleotide sequences utilizes DNA ligases (Landegren et al. 1988). Two oligonucleotides are hybridized to a DNA template, so that the 3′ end of the first one is adjacent to the 5′ end of the second one. In the event that the two oligonucleotides are perfectly base-paired, a DNA ligase can link them covalently (Wu and Wallace 1989). A major drawback of this method is the detection of relative small amounts of ligated product and the high background due to unspecific ligation by the applied T4 DNA ligase. These problems have been overcome by the invention of the ligase chain reaction (Barany 1991). In a preceding step a thermostable ligase links two adjacent primers at a temperature above 60 °C. This product is amplified exponentially in the presence of a second set of complementary oligonucleotides when denaturation, annealing and ligation are repeated several times, as in the polymerase chain reaction. The specificity of the ligation reaction is dramatically enhanced by performing the reaction near the melting point of the primers. Remarkably, the first thermostable DNA ligase was described in 1984. It was derived from *T. thermophilus* HB8 (Takahashi et al. 1984) and displayed a wide temperature range between 15 °C and 85 °C with an optimum at 70 °C. This enzyme was cloned and overexpressed 7 years later independently by two different groups (Barany and Gelfand 1991; Lauer et al. 1991). Over the years several additional thermostable DNA ligases have been discovered. Bacterial enzymes were derived and cloned from *Thermus scotoductus* (Jonsson et al. 1994) and *Rhodothermus marinus* (Thorbjarnardottir et al. 1995). Recent studies in the crude extract of 103 strains of the genera *Thermus*, *Bacillus*, *Rhodothermus* and *Hydrogenobacter* have revealed the presence of thermostable DNA ligases in 23 of the *Thermus* strains (Hjörleifsdottir et al. 1997). Up to now only one archaeal DNA ligase from *Desulfurolobus ambivalens* has been described (Kletzin 1992). Unlike bacterial enzymes, this ligase is NAD^+-independent but ATP-dependent, similar to the enzymes from bacteriophages, eukaryotes and viruses.

Other enzymes of biotechnological interest

In addition to polymer-degrading and DNA-modifying enzymes, other enzymes from extremophiles are expected to play a role in industrial processes involving reactions like transesterification and peptide, oligosaccharide and phospholipid synthesis (Table 5).

Glucose isomerases

Glucose isomerase or xylose isomerase (D-xylose ketol-isomerase; EC 5.3.1.5) catalyses the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose respectively. The enzyme has the largest market in the food industry because of its application in the production of high-fructose corn syrup. This equilibrium mixture of glucose and fructose is 1.3 times sweeter than sucrose. Glucose isomerase is widely distributed in mesophilic microorganisms, and intensive research efforts are being directed towards improving its suitability for industrial application. In order to achieve a fructose concentration of 55% the reaction must approach 110 °C. Improved thermostable glucose isomerases have been engineered from mesophilic enzymes (Crabb and Mitchinson 1997). The gene encoding a xylose isomerase (XylA) of *Thermus flavus* AT62 was cloned and the DNA sequence was determined. XylA (185 kDa; 45 kDa/subunit) has its optimum activity at 90 °C and pH 7.0; divalent cations such as Mn^{2+}, Co^{2+} and Mg^{2+} are required for the enzyme's activity (Park et al. 1997). *Thermoanaerobacterium* strain JW/SL-YS 489 forms a xylose isomerase (200 kDa; 50 kDa/subunit) that is optimally active at pH 6.4 (60 °C) or pH 6.8

Table 5 Other thermoactive enzymes of biotechnological interest

Enzymes	Organism[a]	Enzyme properties				Reference
		Optimal temperature (°C)	Optimal pH	mW^b (kDa)	Remarks	
Glucose isomerase	*Thermus flavus* AT62 (75)	90	7.0	185 (45)	Cloned	Park et al. 1997
	Thermoanaerobacterium Strain JW/SL-YS-489	80	6.8	200 (50)	Cloned	Liu et al. 1996
	Thermotoga maritima (80)	105	6.5–7.5	180 (45)	Purified	Brown et al. 1993
	Thermotoga neapolitana MSB8 (80)	95	7.1	200 (51)	Cloned	Vielle et al. 1995
Alcohol-deydrogenase	*Thermoanaerobacterium ethanolicus* 39E (70)	90	–	160 (37.7)	Cloned	Burdette et al. 1997
	Sulfolobus solfataricus (88)	95	7.0	140 (40)	Pur./cloned	Rella et al. 1987 Cannio et al. 1996
	Thermococcus litoralis (90)	80	8.8	200 (48)	Purified	Ma et al. 1994
Esterase	*Pyrococcus furiosus* (100)	100	7.6	–	Cloned	Ikeda and Clark 1998

[a] Values in parentheses give the optimal growth temperature for each organism in °C
[b] Value in parenthesis show the molecular mass of each subunit
– Not determined

(80 °C). Like other xylose isomerases, this enzyme required Mn^{2+}, Co^{2+} or Mg^{2+} for thermal stability (stable for 1 h at 82 °C in the absence of substrate). The gene encoding the xylose isomerase of *Thermus* strain JW/SL-YS 489 was cloned and expressed in *E. coli*, and the complete nucleotide sequence was determined. Comparison of the deduced amino acid sequence with sequences of other xylose isomerases showed that the enzyme has 98% homology with a xylose isomerase from a closely related bacterium, *T. saccharolyticum* B6A-RI (Liu et al. 1996). A thermostable glucose isomerase was purified and characterized from *T. maritima*. The enzyme is stable up to 100 °C, with a half-life of 10 min at 115 °C (Brown et al. 1993). Interestingly, the glucose isomerase from *T. neapolitana* displays a catalytic efficiency at 90 °C which is 2–14 times higher than any other thermoactive glucose isomerases at temperatures between 60 °C and 90 °C (Vieille et al. 1995).

Alcohol dehydrogenases

The secondary-specific alcohol dehydrogenase, which catalyses the oxidation of secondary alcohols and, less readily, the reverse reaction (the reduction of ketones), has a promising future in biotechnology. Although these enzymes are widely distributed among microorganisms, only few examples derived from hyperthemophilic microorganisms are currently known. Among the extreme thermophilic bacteria, *Thermoanaerobacter ethanolicus* 39E was shown to produce an alcohol dehydrogenase and its gene was cloned and expressed in *E. coli* (Burdette et al. 1997). Interestingly, a mutant has been found to possess an advantage over the wild-type enzyme by using the more stable cofactor NAD instead of NADP. In extreme thermophilic archaea, alcohol dehydrogenases have been studied from *S. solfataricus* (Ammendola et al. 1992; Pearl et al. 1993; Rella et al. 1987) and from *Thermococcus stetteri* (Ma et al. 1994). The enzyme from *S. solfataricus* requires NAD as cofactor and contains Zn ions. In contrast to alcohol dehydrogenases from bacteria and eukarya, the enzyme from *T. stetteri* lacks metal ions. The enzyme catalyses preferentially the oxidation of primary alcohols, using NADP as cofactor, and it is very thermostable, showing half-lives of 15 min at 98 °C and 2 h at 85 °C. Compared to mesophilic enzymes, the alcohol dehydrogenase from *T. litoralis* represents a new type of alcohol-oxidizing enzyme system. Recently, the genes of alcohol dehydrogenases from *S. solfataricus* and *Sulfolobus* sp. strain RC3 were expressed at high level in *E. coli* and the recombinant enzymes were purified and characterized (Cannio et al. 1996).

Esterases

In the field of biotechnology, esterases are receiving increasing attention because of their application in organic

biosynthesis. In aqueous solution, esterases catalyse the hydrolytic cleavage of esters to form the constituent acid and alcohol whereas, in organic solutions, the transesterification reaction is promoted. Both the reactants and the products of transesterification are usually highly soluble in the organic phase and the reactants may even form the organic phase themselves. Clearly, solvent-stable esterases are formed by the extreme thermophilic bacterium *Clostridium saccharolyticum* (Luthi et al. 1990) and the archaeon *S. acidocaldarius* (Sobek and Gorisch 1988). Recently, the *P. furiosus* esterase gene has been cloned in *E. coli* and the functional properties have been determined. The archaeal enzyme is the most thermostable (a half-life of 50 min at 126 °C) and thermoactive (optimum temperature of 100 °C) esterase known to date (Ikeda and Clark 1998).

Conclusion

The steady increase in the number of newly isolated thermophilic and hyperthermophilic microorganisms and the related discovery of their enzymes document the enormous potential within this scientific field. Although major advances have been made in the last decade, our knowledge of the physiology, metabolism, enzymology and genetics of this fascinating group of organisms is still limited. In-depth information on the molecular properties of the enzymes and their genes, however, has to be obtained in order to analyse the structure and function of proteins that are catalytically active around the boiling point of water. There is little doubt that extremophiles will supply novel catalysts with unique properties. Furthermore, modern techniques like mutagenesis and gene shuffling will lead to in vitro tailored enzymes that are highly specific for countless industrial applications. Owing to the unusual properties of this class of enzymes, they are expected to fill the gap between biological and chemical industrial processes.

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