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Extreme environments as a resource for microorganisms and novel biocatalysts

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Abstract The steady increase in the number of newly isolated extremophilic microorganisms and the discovery of their enzymes by academic and industrial institutions underlines the enormous potential of extremophiles for application in future biotechnological processes. Enzymes from extremophilic microorganisms offer versatile tools for sustainable developments in a variety of industrial application as they show important environmental benefits due to their biodegradability, specific stability under extreme conditions, improved use of raw materials and decreased amount of waste products. Although major advances have been made in the last decade, our knowledge of the physiology, metabolism, enzymology and genetics of this fascinating group of extremophilic microorganisms and their related enzymes is still limited. In-depth information on the molecular properties of the enzymes and their genes, however, has to be obtained to analyze the structure and function of proteins that are catalytically active around the boiling and freezing points of water and extremes of pH. New techniques, such as genomics, metanogenomics, DNA evolution and gene shuffling, will lead to the production of enzymes that are highly specific for countless industrial applications. Due to the unusual properties of enzymes from extremophiles, they are expected to optimize already existing processes or even develop new sustainable technologies.

Keywords Extremophiles · Stable biocatalysts · Thermophiles · Extremes of pH · Psychrophiles · Enantioselectivity

1 Introduction

Extremophiles are unique microorganisms that are adapted to survive in ecological niches such as high or low temperatures, extremes of pH, high salt concentrations and high pressure. Accordingly biological systems and enzymes can even function at temperatures between – 5 and 130 °C, pH 0–12, salt 3–35% and 1000 bar. The majority of the organisms that grow in these extreme environments belong to a group with distinct characteristics. Carl Woese named this group archaea, and postulated the archaea as the third domain of life on earth, different form bacteria and eukarya [1, 2]. A large number of these unique microorganisms have been isolated from marine environments (Table 1). In many cases microbial biocatalysts, especially of extremophiles, are superior to traditional catalysts, because they allow the performance of industrial processes even under harsh condition, under which conventional proteins are completely denatured. By virtue of their positive properties, stability, specificity, selectivity and efficiency, enzymes already occupy a prominent position in modern biotechnology. For many processes in

Table 1 Some representatives of microorganisms living under extreme conditions

Microbial life at various temperatures	Optimal growth (°C)
Phychrophiles	
Vibrio sp	< 20 °C
Micrococcus criophilus	< 20 °C
Arthrobacter glacialis	< 20 °C
Vibrio psychroerythreus	< 20 °C
Aquaspirillum articum	< 20 °C
Moderate thermophiles (50–60 °C)	
Bacillus acidocaldarius	50
Bacillus stearothermophilus	55
Extreme thermophiles (60–80 °C)	
Thermus aquaticus	70
Thermoanaerobacter ethanolicus	65
Clostridium thermusulfurogenes	60
Fervidobacterium pennivorans	75
Hyperthermophiles (80–110 °C)	
Thermotoga maritima	90
Aquifex pyrophilus	85
Archeoglobus fulgidus	83
Methanopyrus kandleri	88
Sulfolobus sulfataricus	88
Thermococcus aggregans	88
Pyrobaculum islandicum	100
Pyrococcus furiosus	100
Pyrodictium occultum	105
Pyrolobus fumarii	106

the chemical and pharmaceutical industries, suitable microbial enzymes can be found that have the potential to optimize or even replace chemical processes. By using robust enzymes in biotechnical processes one is often able to better utilize raw materials, minimize pollutant emissions and reduce energy consumption while simultaneously improving quality and purity of products, e.g. optically pure compounds. The additional benefits in performing industrial processes at high temperature include reduced risk of contamination, improved transfer rates, lower viscosity and higher solubility of substrates. The recent exciting results in the field of extremophile research, the high demands of the biotechnology industries for tailor-made novel biocatalysts and the simultaneous rapid development of new techniques will stimulate the development of innovative processes on the basis of biocatalyst from extremophiles.

Table 1 (continued)

Microbial life at various temperatures	Optimal growth conditions Temperature	pH
Acidophilic microorganisms		
Sarcina ventriculi	37	4.0
Thiobacillus ferrooxidans	37	2.5
Alyciclobacillus acidocaldarius	55	2.0–6.0
Picrophilus oshimae	60	0.7
Picrophilus torridus	60	0.7
Thermoplasma acidophilum	60	2.0
Sulfolobus acidocaldarius	75	2.5
Acidianus infernus	75	2.0
Alkaliphilic microorganisms		
Anaerobranca gottschallki	60	9.5
Thermococcus alcaliphilus	85	9.0
Thermococcus acidoaminivorans	85	9.0
	Range of NaCl concentration in M, required for growth (optimum)	
Halophilic microorganisms		
Dunaliella spp.	0.3–5.0	
Clostridium halophilum	0.15–6.0 (0.6)	
Haloanaerobium praevalens	0.8–4.3 (2.2)	
Halobacterium denitrificans	1.5–4.5 (2.5)	
Haloferax vulcanii	1.0–3.0 (1.5)	

2
Extreme environments as a resource of unique gene and biocatalysts

Modern biotechnology has a steadily increasing demand for novel enzymes. The classical approach to the isolation of new or improved biocatalysts requires that the different microorganisms derived from an environmental sample be cultured on an appropriate growth medium and separated until individual clones are isolated. The enzymes and the corresponding genes are then recovered from the identified microorganism. This value of the classical method is substantial but it fails, however, to represent the scope of microbial diversity in nature, since only a small proportion (1–3%) of viable microorganisms in a sample have to date been recovered by culturing techniques. Ro explore the diversity and the potential of microbial communities a method was developed to analyze DNA in environmental samples that bypasses classical cultivation techniques [3]. Collectively, the term *metagenome* indicates the genome of the total microbiota found in nature and contains vastly more

genetic information than is contained in the cultivable subset. Hence, the construction of metagenomic libraries by direct extraction and cloning large fragments of DNA isolated directly from microbes in natural environments has represented an effective way of accessing the wealth of information of microbial mixed populations. This method circumvents the loss of major portions of the microbial communities in extreme environments, which derives from the different growth requirements of the different microbes. Several different laboratories have successfully isolated novel genes encoding different enzymes and secondary metabolites from microbial communities and their metagenomes without cultivation of the microbes [4]. The microbial niches studied were highly diverse and ranged from moderate environments, such as river soil [4], to extreme environments, such as the deep sea [5–9]. Studies have shown that the metagenomic approach offers a valuable pool for encountering novel genes encoding biotechnologically relevant gene products such as lipase [10] cellulases [11], amylases [12], chitinases [13, 14], and esterases [10]. Rondon et al. [15, 16] used a bacterial artificial chromosome (BAC) vector to express *Bacillus cereus* genomic DNA. The advantage of BAC vectors is that they maintain very large DNA inserts (greater than 100 kb) stably in *E. coli*, facilitating the cloning of large fragments of DNA. Their results demonstrated that expression of heterologous DNA from *B. cereus* in an *E. coli* BAC system was detectable at a reasonable frequency, validating the idea that the low-copy BAC vector (one to two per cell) could be used to express foreign DNA from foreign promoters in *E. coli* [15, 16]. However, a major difficulty associated with exploiting the metagenome directly from the environment is related to contamination of purified DNA with polyphenolic compounds that are copurified with the DNA. These compounds are difficult to remove, and it is well known that polyphenols also interfere with enzymatic modifications of isolated DNA [17, 18]. As a result, construction of environmentally derived DNA libraries with large inserts is hindered due to the poor quality of the isolated DNA. These known difficulties associated with the construction of libraries directly derived from environmental DNA samples forced researchers to isolate DNA from the metagenome of a microbial community after precultivation in the laboratory [19]. This technique, though it limits the biodiversity of laboratory enrichment cultures, has proven to be highly efficient for rapid isolation of large DNA fragments and for cloning of operons and genes with great biotechnological value. For example, Voget et al. [20] exploited the metagenome of an enrichment culture on agar plates for isolation of genes encoding a variety of different biocatalysts, including β-agarases, amylolytic enzymes, cellulases, lipases and many other enzymes with high biotechnological potential. Given the immense uncultivated and uncharacterized metabolic diversity in the environment, one would need to sequence a relatively restricted number of clones to discover fundamentally interesting sets of genes. However, this approach relies on the fortuitous expression of heterologous DNA by the library host strain. Although the speed

and effectiveness of brute-force sequencing are constantly improving, it is not yet practical to assemble a complete bacterial genome from a metagenome. There is still a need for new functional genomic approaches that systematically yield information about many of the elements in a metagenomic library. If modern genomic techniques can be used to carry out more comprehensive surveys of metagenomic libraries, the understanding of natural genetic diversity would be greatly enhanced [21]. This technology has been established by various biotechnological industries such as Diversa in San Diego and B.r.a.i.n. in Germany. By applying a high-throughput system (HTS) extreme environments have been studied for the production of stable enzymes.

2.1 Low-temperature-adapted microorganisms

The Earth's biosphere is predominantly aqueous and cold. Nearly 70% is water and a high percentage of it seldom reaches temperatures above 5 °C. The polar region provides a permanently cold environment that is surrounded by an aquatic belt of melting ice. Microorganisms able to grow at temperatures close to 0 °C have developed various adaptation mechanisms to survive and function at low temperature. These microorganisms can be divided into two main groups: psychrophiles and psychrotolerants. Pyschrophilic microorganisms grow at an optimum temperature of 15 °C, with a maximum growth temperature at about 20 °C and a minimum around 0 °C. Psychrotolerant microorganisms generally do not grow at zero but do so at 3–5 °C, and have optimum and maximum growth temperatures above 20 °C but less than 30 °C [22]. Most of the cold-adapted microorganisms have been characterized from Arctic and Antarctic seawater and, despite the harsh conditions, the density of bacterial cells in the Antarctic oceans is as high as the density reported in temperate waters (Table 1). Psychrophiles can be found in permanently cold environments such as the deep sea, glaciers, and mountain regions, in soils, in fresh or saline waters associated with cold-blooded animals such as fish or crustaceans. In general, cold-adapted enzymes have higher specific activity at low and moderate temperatures than that of their mesophilic counterparts, and are inactivated easily by a slight increase in temperature [23, 24].

2.2 Microorganisms that grow at elevated temperatures

Microorganisms capable of growing optimally at temperatures between 50 and 60 °C are designated as moderate thermophiles (Table 1). Most of these microorganisms belong to many different taxonomic groups of eu- and prokaryotic microorganisms such as protozoa, fungi, algae, streptomycetes and cyanobacteria, which comprise mainly mesophilic species. It can be as-

sumed that moderate thermophiles, which are closely related phylogenetically to mesophilic organisms, may be secondarily adapted to life in hot environments. Extreme thermophiles, which grow optimally between 60 and 80 °C, are widely distributed among the genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Thermotoga* and *Aquifex*. The relative abundance of archaea and bacteria in high-temperature environments was, until recently, mainly studied by cultivation-based techniques. Because of the frequent isolation of archaea from these habitats, it was assumed that archaea dominate the high-temperature biotopes. Recently, the application of molecular-biological methods revealed that bacterial communities are also abundant in these environments. These results suggest that archaea may generally be of lower abundance in hot environments than could be assumed from cultivation-based experiments. However, the factors that allow bacteria to dominate in high-temperature habitats, that were once believed to be the realm of archaea, remain unknown [25, 26].

Microorganisms that are adapted to grow optimally at very high temperatures (80–108 °C) have been isolated from high-temperature terrestrial and marine habitats. The most common biotopes are volcanically and geothermal-heated hydrothermal vent systems such as solfataric fields, neutral hot springs, and submarine hot vents. Submarine hydrothermal systems are situated at shallow and abyssal depth. They consist of hot fumaroles, springs, sediments, and deep-sea vents with temperatures of up to 400 °C ("black smokers") [27]. Because of their ability to convert volcanic gases and sulphur compounds at high temperatures, hyperthermophilic communities living in such hydrothermal vents are expected to play an important role in marine ecological, geochemical and volcanic processes [28]. Shallow as well as deep-sea hydrothermal systems harbour members of various genera including *Pyrococcus, Pyrodictium, Igneococcus*, *Thermococcus*, *Methanococcus*, *Archaeoglobus* and *Thermotoga*. So far, members of the genus *Methanopyrus* have been found only at greater depths, whereas *Aquifex* was isolated exclusively from shallow hydrothermal vents. Recently, interesting biotopes of extreme and hyperthermophiles were discovered in deep, geothermally heated oil reservoirs around 3500 m below the bed of the North Sea and the permafrost soil of North Alaska [29]. Interestingly, the majority of the hyperthermophiles isolated to date belong to the archaeal domain of life and no eukaryotic organism has been found that can grow at the boiling point of water. A 16S rDNA-based universal phylogenetic tree shows a tripartite division of the living world consisting of the domains Bacteria, Archaea and Eukarya [1, 30] (Fig. 1).

2.3 Life at extremes of pH

Solfataric fields are the most important biotopes of microorganisms that prefer to live under both thermophilic and acidic conditions. Solfataric soils consist of two different layers that can be easily distinguished by their characteristic colours: the upper, aerobic layer has an ochre colour due to the presence of ferric iron. The layer below, which is anaerobic, appears rather blackish-blue owing to the presence of ferrous iron. Thermophilic acidophiles, belonging to the genera *Sulfolobus* [31, 32], *Acidianus* [33], *Thermoplasma* [34], and *Picrophilus* [35], with growth optima between 60 and 90 °C and pH 0.7–5.0 are commonly found in the aerobic upper layer, whereas slightly acidophilic or neutrophilic anaerobes such as *Thermoproteus tenax* or *Methanothermus fervidus* can be isolated from the lower layer. Species of *Thermoplasma* (growth optima: pH 2.0 and 60 °C) have been found in hot springs, solfataras and coal refuse piles [36]. Their closest known phylogenetic relatives, also found in solfataras, are species of the genus *Picrophilus*, which are so far the most extreme acidophiles, with growth close to pH 0. *Picrophilus oshimae* and *P. torridus* are both aerobic, heterotrophic archaea that grow optimally at 60 °C and pH 0.7 and utilize various polymers such as starch and proteins as carbon source (Table 1) [37, 38].

Members of the genus *Sulfolobus* are strict aerobes growing either autotrophically, heterotrophically or facultative heterotrophically. During autotrophic growth, S^0, S^{2-} and H_2 are oxidized to sulphuric acid or water as end products. *Sulfolobus metallicus* [39–42] and *S. brierley* [43] are able to grow by oxidation of sulfidic ores. A dense biofilm of these microorganisms is responsible for the microbial ore leaching process, in which heavy-metal ions such as Fe^{2+}, Zn^{2+} and Cu^{2+} are solubilized. Other thermoacidophiles have been affiliated to the genera *Metallosphaera* (growth range: 50–80 °C, pH 1–4.5) [44], *Acidianus* (growth range: 60–95 °C, pH 1.5–5) [33] and *Stygioglobus* (growth range: 57–90 °C, pH 1–5.5).

The alkaliphiles that grow at high pH values are widely distributed throughout the world. They have been found in carbonate-rich springs and alkaline soils, where the pH can be around 10.0 or even higher, although the internal pH is maintained around 8.0. In such places, several species of cyanobacteria and *Bacillus* are normally abundant and provide organic matter for diverse groups of heterotrophs [45]. Alkaliphiles require alkaline environments and sodium ions not only for growth but also for sporulation and germination. Sodium-ion-dependent uptakes of nutrients have been reported in alkaliphiles. Many alkaliphiles require various nutrients for growth; few alkaliphilic *Bacillus* strains can grow in simple minimal media containing glycerol, glutamic acid, and citric acid [46]. In general, cultivation temperature is in the range of 20–55 °C. Furthermore, many haloalkaliphiles isolated from alkaline hypersaline lakes can grow in alkaline media containing 20%

NaCl. The soda lakes in the Rift Valley of Kenya and similar lakes found in a few other places on earth are highly alkaline with pH values between 11.0 or 12.0 and represent a typical habitat where alkaliphilic microorganisms can be isolated [47]. Thermophilic anaerobic spore-forming alkaliphiles, thermoalkaliphilic Clostridia, were isolated from sewage plants [48]. Very recently, two thermoalkaliphilic bacteria, *Anaerobranca gottschalkii* and *Anaerobranca horikoshii* have been isolated from Lake Bogoriae in Kenya and from Yellowstone National Park, respectively [49, 50] (Table 1). The new isolates represent a new line within the *Clostridium*/*Bacillus* subphylum. The two archaeal thermoalkaliphiles identified to date are *Thermococcus alcaliphilus* [51] and *Thermococcus acidoaminivorans* [52], both growing at 85 °C and pH 9.0.

2.4
High-salt-tolerant microorganisms

The halophiles comprise Bacteria and Archaea that grow optimally at NaCl concentrations above that of seawater (> 0.6 M NaCl). In general, halophilic microorganisms are classified as moderate halophiles if they can grow at salt concentrations between 0.85 and 1.7 M NaCl and as extreme halophiles if they require NaCl concentrations above 1.7 M for growth. Halophiles have been mainly isolated from saline lakes, such as the Great Salt Lake in Utah (salinity > 2.6 M) and from evaporated lagoons and coastal salterns with NaCl concentrations between 1 and 2.6 M [53, 54]. The term "halobacteria" refers to the red-pigmented extremely halophilic Archaea, members of the family *Halobacteriaceae*, and the only family in the order Halobacteriales (15). Most halobacteria require 1.5 M NaCl to grow and retain the structural integrity of the cell. Halobacteria can be distinguished from halophilic bacteria by their archaeal characteristic, in particular the presence of ether-linked lipids [55]. Most halobacteria are colored red or orange due to the presence of carotenoids, but some species are colourless. Halobacteria are the most halophilic organisms known so far and form the dominant microbial population when hypersaline waters approach saturation [56, 57] (Table 1).

3
Cellulases

Due to the harsh living conditions extremophiles are interesting source of stable biocatalysts. Thermostable cellulases active towards crystalline cellulose are of great biotechnological interest. Cellulose is the most abundant organic biopolymer in nature since it is the structural polysaccharide of the cell wall in the plant kingdom. It consists of glucose units linked by β-1,4-glycosidic bonds with a polymerization grade of up to 15 000 glucose units in a linear

mode. The minimal molecular weight of cellulose from different sources has been estimated to vary from about 50 000 to 2 500 000 in different species, which is equivalent to 300 to 15 000 glucose residues. Although cellulose has a high affinity to water, it is completely insoluble in it. 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 hydrolyzed into glucose by the synergistic action of at least three different enzymes: endoglucanase (cellulase), exoglucanase (cellobiohydrolase) and β-glucosidase (cellobiase). Endoglucanase (E.C. 3.2.1.4) hydrolyzes cellulose in a random manner as endo-hydrolase producing various oligosaccharides, cellobiose and glucose. Exoglucanases, (EC 3.2.1.91) hydrolyze β-1,4 D-glycosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing end of the chain. β-Glucosidases (EC 3.2.1.21) catalyze the hydrolysis of terminal, non-reducing β-D-glucose residues releasing β-D-glucose.

Several cellulose-degrading enzymes from various thermophilic organisms have been investigated (Table 2). A thermostable cellulase from *Thermotoga maritima* MSB8 has been characterized [58]. The enzyme is rather small, with a molecular weight (MW) of 27 kDa, and it is optimally active at 95 °C and between pH 6.0 and 7.0 [59]. Two themostable cellulases, CelA and CelB, with optimal activity between 95 °C and 106 °C, were purified from *Thermotoga neapolitana* [60]. 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)

Table 2 Bacterial and archaeal cellulolytic enzymes

Organism	Endoglucanase	Cellobiohydrolase	β-Glucosidase
Bacteria			
Cellulomonas fimi	+	+	–
Clostridium thermocellum	+	–	–
C. stercorarium	+	+	–
Cytophaga sp.	+	+	–
Fibrobacter succinogenes	+	–	+
Ruminococcus albus	+	+	+
Thermotoga maritima	+	+	–
Thermotoga neapolitana	+	+	–
Archaea			
Pyroccus furiosus	–	+	+
Sulfolobus solfataricus	–	–	+

was isolated and was found to consist of 1751 amino acids. This is the largest cellulase gene described to date [61–63]. A large cellulolytic enzyme (CelA) with the ability to hydrolyze microcrystalline cellulose was isolated from the extremely thermophilic bacterium *Anaerocellum thermophilum* [61–63]. The enzyme has an apparent molecular mass of 230 kDa and exhibits significant activity towards Avicel and is most active towards soluble substrates such as carboxy-methyl-cellulose (CMC) and β-glucan. Maximal activity was observed at pH 5–6 and 85–95 °C. A thermostable β-glucosidase is produced by *Thermotoga sp.* FjSS3-B1 [64]. 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 enzyme is active on amorphous cellulose and carboxy-methyl-cellulose. The thermophilic bacterium *Rhodothermus marinus* produces a hyperthermostable cellulase, with a temperature optimum of more than 90 °C [65], the structure of which was solved to 1.8 Å resolution. This is the first structure of a thermophilic member of family glycoside hydrolase 12 to have been solved. The beta-jelly roll fold observed has identical topology to those of the two mesophilic members of the family whose structures have been elucidated previously. A Hepes buffer molecule bound in the active site may have triggered a conformational change to an active configuration, as the two catalytic residues Glu124 and Glu207, together with dependent residues, are observed in a conformation similar to that seen in the structure of *Streptomyces lividans* CelB2 complexed with an inhibitor. The structural similarity between this cellulase and the mesophilic enzymes serves to highlight features that may be responsible for its thermostability, chiefly an increase in ion-pair number and the considerable stabilization of a mobile region seen in *S. lividans* CelB2. Additional aromatic residues in the active site region may also contribute to the difference in thermophilicity [66].

Recently, a thermostable endoglucanase, which is capable of degrading β-1,4 bonds of β-glucans and cellulose, has been identified in the archaeon *Pyrococcus furiosus*. The gene encoding this enzyme has been cloned and sequenced in *E. coli.* The purified recombinant endoglucanase hydrolyzes β-1,4 but not β-1,3 glycosidic linkages and has the highest specific activity with cellopentaose and cellohexaose as substrates [67]. In contrast to this, several β-glucosidases have been detected in archaea. In fact, archaeal β-glucosidases have been found in *Sulfolobus solfataricus* MT4 [68], *S. acidocaldarius, S. shibatae* and *P. furiosus* [69–71]. The enzyme from the latter microorganism is very stable and shows optimal activity at 103 °C. The β-glucosidase from *S. solfataricus* MT4 is very resistant to various denaturants with activity up to 85 °C. The gene for this β-glucosidase has been cloned and overexpressed in *E. coli* [72]. The less-thermoactive cellulases that are widespread in fungi and bacteria, have already found various biotechnological applications. The most effective enzyme of commercial interest is the cellulase produced by *Trichoderma sp.* Cellulases were also obtained from strains of *Aspergillus, Penicillium* and *Basidomycetes* [73, 74]. Cellulolytic enzymes can be used

in alcohol production to improve juice yields and effective color extraction of juices. The presence of cellulases in detergents causes color brightening, softening and improves particulate soil removal [75]. Cellulase (Denimax® Novozymes) is also used for the "biostoning" of jeans instead of using stones. Other suitable 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 production of fine chemicals [76].

4 Xylan-degrading enzymes

To date only a few extreme thermophilic microorganisms are able to grow on xylan and secrete thermoactive xylanolytic enzymes (Table 3). Xylan is a heterogeneous molecule that constitutes the main polymeric compound of hemicellulose, a fraction of the plant cell wall, which 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 have substitution at the O-2 or O-3 positions with acetyl, arabinosyl and glucuronosyl groups. The complete degradation of xylan requires the action of several enzymes. The endo-β-1,4-xylanase (E.C.3.2.1.8) hydrolyzes β-1,4-xylosydic linkages in xylans, while β-1,4-xylosidase (EC 3.2.1.37) hydrolyzes β-1,4-xylans and xylobiose by removing the successive xylose residues from the non-reducing termini [77]. Members of the order Thermotogales and *Dictyoglomus thermophilum* Rt46B.1 have been described to produce xylanases that are active and stable at high temperatures [78, 79]. The most thermostable endoxylanases that have been described so far are those derived from *Thermotoga* sp. strain FjSS3-B.1, *Thermotoga maritima* [59, 80, 81], *T. neapolitana* [82, 83] and *T. thermarum* [84]). These enzymes, which are active between 80 and 105 °C, are mainly cell-associated and most probably localized within the toga, which covers the cells. Several genes encoding xylanases have already been 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™ indicates that the thermostable xylanase could be of interest for application in pulp and paper industry [85]. Recently, *Bacillus thermantarcticus*, a thermophilic bacterium isolated from Antarctic geothermal soil near the crater of Mount Melbourne, was found to produce an extracellular xylanase and β-xylosidase. The optimum temperatures are 80 °C for xylanase at pH 5.6 and 70 °C for β-xylosidase at pH 6.0. The isoelectric points and molecular masses are 4.8 and 45 kDa for xylanase and 4.2 and 150 kDa

CE[a] Please define term ND

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Table 3 Bacterial and archaeal xylanolytic enzymes[CE][a]

Organism	Endoxylanase	β-Xylosidase	β-L-Arabinofuranosidase	β-Glucuronidase	Acetyl Xylan esterase
Bacteria					
Bacillus subtilis	+	+	+	+	+
Streptomyces olivochromogenes	+	+	+	ND	+
Thermoactinomyces vulgaris	+	ND	+	ND	+
Thermoanaerobacter saccharolyticum	+	+	+	ND	+
Thermonospora fusca	+	+	+	ND	+
Thermotoga maritima	+	ND	ND	ND	ND
Thermotoga neapolitana	+	ND	ND	ND	ND
Archaea					
Thermococcus zilligii	+	ND	ND	ND	ND

for β-xylosidase, respectively. Xylanase is stable at 60 °C for 24 h, whereas it shows a half-life at 70 °C of 24 h and at 80 °C of 50 min. β-Xylosidase activity does not decrease after 1 h at 60 °C. Interestingly, the action of two enzymes on xylan leads only to the formation of xylose [86].

The extracellular thermostable endo-1,4-β-xylanase (XT6) produced by the thermophilic bacterium *Geobacillus stearothermophilus* T-6 was shown to bleach pulp optimally at pH 9 and 338 K and was successfully used in a large-scale biobleaching mill trial. The xylanase gene was cloned and sequenced. The mature enzyme consists of 379 amino acids, with a calculated molecular weight of 43.8 kDa and a pI of 9.0. In order to study the mechanism of catalysis and to provide a structural basis for the rational introduction of enhanced thermostability by site-specific mutagenesis, the structure of wild type was refined at 2.4 Å resolution. The structure demonstrates that XT6 is made up of an eightfold TIM barrel containing a deep active-site groove, consistent with its "endo" mode of action. The two essential catalytic carboxylic residues (Glu159 and Glu265) are located at the active site within 5.5 Å of each other, as expected for "retaining" glycoside hydrolases. A unique subdomain was identified in the carboxy-terminal part of the enzyme and was suggested to have a role in xylan binding. The three-dimensional structure of XT6 is of great interest since it provides a favourable starting point for the rational improvement of its already high thermal and pH stabilities, which are required for a number of biotechnological and industrial applications [87]. Among the thermophilic Archaea, xylanase production has been demonstrated only in the hyperthermophilic archaeon *Pyrodictium abyssi* [88]. The enzyme has an optimum temperature of 110 °C, which is one of the highest reported for a xylanase. Recently, an endo-1,4-xylanase and a β-xylosidase have been characterized from the extremely halophilic archaeon, *Halorhabdus utahensis* [89]. This is the first report on hemicellulose-degrading enzymes produced by an extremely halophilic archaeon.

Xylanases from bacteria and eukarya have a wide range of potential biotechnological applications. They are already produced on industrial scale and are used as food additives in poultry, for increasing feed efficiency diets and in wheat flour for improving dough handling and the quality of baked products. In recent years, the major interest in thermostable xylanases is found in enzyme-aided bleaching of paper. The chlorinated lignin derivatives generated by this process constitute a major environmental problem caused by the pulp and paper industry. Recent investigations have demonstrated the feasibility of enzymatic treatments as alternatives to chlorine bleaching for the removal of residual lignin from pulp. 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 [90, 91].

5 Pectin-degrading enzymes

Pectin is a branched heteropolysaccharide consisting of a main chain of α-1,4-D-polygalacturonate, which is partially methyl-esterified. Along the chain, L-rhamnopyranose residues are present that are the binding sites for side chains composed of neutral sugars. Pectin is an important plant material that is present in the middle lamellae as well as in the primary cell walls. Pectin is degraded by pectinolytic enzymes that can be classified into two major groups. The first group comprises methylesterases, whose function is to remove the methoxy groups from pectin. The second group comprises the depolymerases (hydrolases and lyases), that attack both pectin and pectate (polygalacturonic acid). A great variety of pectinolytic bacteria have been isolated from various habitats such as trees, lakes, soil, tumen, mullet gut, and human intestinal track. Pectin hydrolases are predominantly synthesized by fungi whereas pectate lyases are mostly produced by bacteria and usually act at alkaline pH and are Ca^{2+}-dependent. Pectin degradation by thermophilic bacteria has been reported for *Thermoanaerobacter thermohydrosulfuricus*, *Thermoanaerobacter thermosulfurigenes*, *Clostridium thermocellum*, *Desulfurococcus amylolyticus*, *Clostridium thermosaccharolyticum* and *Bacillus stearothermophilus* [92–101]. Although many microorganisms have been screened for pectinolytic activity, little attention has been paid to pectinolytic enzymes from thermophilic and hyperthermophilic microorganisms. Previously a novel anaerobic strain from a thermal spa in Italy was isolated that produces two thermoactive lyases that have a very high affinity for polygalacturonate. This is a spore-forming anaerobic microorganism able to grow on citrus pectin and pectate optimally at 70 °C, which has been identified as *Thermoanaerobacter italicus*. After growth on citrus pectin, two pectate lyases were induced, purified and biochemically characterized [102]. Both enzymes display similar catalytic properties and can function at temperatures up to 80 °C. An increase in the enzymatic activity of both pectate lyase was observed after the addition of Ca^{+2}. The ability of the hyperthermophilic bacterium *Thermotoga maritima* to grow on pectin as a sole carbon source coincides with the secretion of a pectate lyase A (PelA) in the extracellular medium. The *pel* A gene of *T. maritima* was functionally expressed in *E. coli* as the first heterologously produced thermophilic pectinase, and purified to homogeneity [103]. Gel filtration indicated that the native form of PelA is tetrameric. Highest activity (422 u/mg, with a K_m of 0.06 mM), was demonstrated on polygalacturonic acid (PGA), whereas pectins with an increasing degree of methylation were degraded at a decreasing rate. Similar to pectate lyases, PelA demonstrated full dependency on Ca^{2+} for stability and activity. The enzyme is highly thermoactive and thermostable, operating optimally at 90 °C and pH 9.0, with a half-life for thermal

inactivation of almost 2 h at 95 °C, and an apparent melting temperature of 102.5 °C. Detailed characterization of the product formation with polygalacturonic acid indicated that PelA has a unique eliminative exo-cleavage pattern liberating unsaturated trigalacturonate as the major product, in contrast with unsaturated digalacturonate for other exopectate lyases known. To date pectin-hydrolyzing enzymes from archaea have not been identified and characterized. Enzymatic pectin degradation is widely applied in food-technology processes, as in fruit-juice extraction, to increase the juice yield, to reduce its viscosity, improve color extraction from the skin and to macerate fruit and vegetable tissues [103].

6 Chitin-degrading enzymes

Chitin is a linear β-1,4 homopolymer of N-acetyl-glucosamine residues and it is one of the most abundant natural biopolymers on earth. Particularly in the marine environment, chitin is produced in enormous amounts and its turnover is due to the action of chitinolytic enzymes. Chitin is the major structural component of most fungi and some invertebrates (crustacea and insects), while for soil or marine bacteria chitin serves as a nutrient (41). Chitin degradation is known to proceed with the endo-acting chitin hydrolase (chitinase A; EC 3.2.1.14) and the chitin-oligomer-degrading exo-acting hydrolases (chitinase B) and N-acetyl-D-glycosaminidase (trivial name: chitobiase; EC 3.2.1.52). Chitin exhibits interesting properties that make it a valuable raw material for several applications. It has been estimated that the annual worldwide formation rate and steady-state amount of chitin is of the order of 10^{10} to 10^{11} tons per year. Therefore, application of thermostable chitin-hydrolyzing enzymes (chitinases) is expected for effective utilization of this abundant biomass [104–106]. Although a large number of chitin-hydrolyzing enzymes has been isolated and their corresponding genes have been cloned and characterized, only a few thermostable chitin hydrolyzing enzymes are known. These enzymes have been isolated from the thermophilic bacterium *Bacillus licheniformis* X-7u [107], *Bacillus sp.* BG-11 [108] and *Streptomyces thermoviolaceus* OPC-520 [109]. So far, only three hyperthermophilic archaea, *Thermococcus chitonophagus* [110], *Thermococcus kodakaraensis* KOD1 [111, 112], and *Pyrococcus furiosus* [113] have been shown to grow on chitin. The extreme thermophilic anaerobic archaeon *Thermococcus chitonophagus* has been reported to posses an enzymatic system able to hydrolyze chitin. From this microorganism, a chitinase (1,4-beta-D-N-acetyl-glucosaminidase, EC 3.2.1.14) was detected and purified to homogeneity in its native form. This is the first nonrecombinant chitinase purified and characterized from archaea and also constitutes the first case of a membrane-

associated chitinase isolated from archaea. The enzyme is a monomer with an apparent molecular weight of 70 kDa and appears to be associated with the outer side of the cell membrane. The enzyme is optimally active at 70 °C and pH 7.0 and exhibits remarkable thermostability, maintaining 50% activity even after 1 h at 120 °C. The enzyme was not inhibited by allosamidin, the natural inhibitor of chitinolytic activity, and was also resistant to denaturation by urea and SDS. Chi70 shows broad substrate specificity for several chitinous substrates and derivatives and has been classified as an endochitinase due to its ability to release chitobiose from colloidal chitin [110]. Very recently, the gene encoding a chitinase from a hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 was cloned, sequenced and expressed in *E. coli*. The purified recombinant protein is optimally active at 85 °C and pH 5.0. This multidomain protein consists of two active sites with different cleavage specificities and three substrate-binding domains, which are related to two families of cellulose-binding domains. The enzyme produces chitobiose as the major end product. This thermostable chitinase, which is active in the presence of detergents and organic solvents, can be applied as useful catalyst in the industry e.g. production of N-acetyl-chitooligosaccharides with biological activity [111]. *Pyrococcus furiosus* was also found to grow on chitin, adding this polysaccharide to the inventory of carbohydrates utilized by this hyperthermophilic archaeon. Accordingly, two open reading frames (chiA [113] and chiB [Pf1233]) were identified in the genome of *P. furiosus*, which encode chitinases with sequence similarity to proteins from the glycosyl hydrolase family 18 in less-thermophilic organisms. The two chitinases share little sequence homology with each other, except in the catalytic region, where both have the catalytic glutamic acid residue that is conserved in all family 18 bacterial chitinases. The genes encoding ChiA, without its signal peptide, and ChiB were cloned and expressed in *E. coli*. The pH optima of both enzymes is about 6.0 with a broad temperature optima between 90 and 95 °C. ChiA melted at 101 °C, whereas ChiB was found to be extremely thermostable, with a melting temperature of 114 °C. ChiA exhibited no detectable activity toward chitooligomers smaller than chitotetraose, indicating that the enzyme is an endochitinase whereas ChiB is a chitobiosidase, progressively cleaving off chitobiose from the non-reducing end of chitin or other chitooligomers. Synergistic activity was observed for the two chitinases on colloidal chitin, indicating that these two enzymes work together to recruit chitin-based substrates for *P. furiosus* growth. This was supported by the observed growth on chitin as the sole carbon source in a sulfur-free media [113].

7
Starch-processing enzymes

7.1
Heat-stable amylases, glucoamylases and α-glucosidases

Extremely thermostable α-amylases have been characterized from a number of hyperthermophilic archaea belonging to the genera *Pyrococcus* [114–118] and *Thermococcus* [119, 120]. α-Amylase (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1), hydrolyzes linkages in the interior of the starch polymer in a random fashion that leads to the formation of linear and branched oligosaccharides. The sugar-reducing groups are liberated in the α-anomeric configuration. Most starch-hydrolyzing enzymes belong to the α-amylase family, which contains a characteristic catalytic $(\beta\alpha)_8$-barrel domain. Throughout the α-amylase family, only eight amino acid residues are invariant, seven at the active site and a glycine in a short turn. The optimal temperatures for the activity of these enzymes range between 80 °C and 100 °C. Thermoactive amylolytic enzymes have also been detected in hyperthermophilic archaea of the genera *Sulfolobus, Thermophilum, Desulfurococcus*, and *Staphylothermus* [121–123] (Table 4). Molecular cloning of the corresponding genes and their expression in heterologous hosts circumvent the problem of insufficient expression in the natural host. The gene encoding an extracellular α-amylase from *Pyrococcus furiosus* has recently been cloned and the recombinant enzyme has been expressed in *Bacillus subtilis* and *E. coli* (48, 49). The high thermostability of the pyrococcal extracellular α-amylase (thermal activity even at 130 °C) makes this enzyme an interesting candidate for industrial application. α-Amylases with lower thermostability have been isolated from the archaea *Thermococcus profundus* [124], *Pyrococcus kodakaraensis* [125] and the bacteria *Thermotoga maritima* [126] and *Dictioglomus thermophilum* [127, 128]. The genes encoding these enzymes were successfully expressed in *E. coli*. Similar to the amylase from *Bacillus licheniformis*, which is commonly used in liquefaction of starch in the industry, the enzyme from *T. maritima* requires Ca^{2+} for activity. Further investigations have shown that the extreme marine hyperthermophilic archaeon *Pyrodictium abyssi* can grow on various polysaccharides and also secretes a heat-stable α-amylase [88].

Unlike α-amylase, the production of glucoamylase seems to be very rare in extremely thermophilic and hyperthermophilic bacteria and archaea (Table 4). Glucoamylases (EC 3.2.1.3) hydrolyze terminal α-1,4-linked-D-glucose residues successively from non-reducing ends of the chains, releasing β-D-glucose. Among the thermophilic anaerobic bacteria, glucoamylases have been purified and characterized from *Clostridium thermohydrosulfuricum* 39, *Clostridium thermosaccharolyticum* and *Thermoanaerobacterium*

Table 4 Starch-hydrolyzing enzymes from extreme thermophilic and hyperthermophilic Archaea

Enzyme	Organism (growth temperature)	Enzyme properties	
		Optimal temperature	Optimal pH
α-Amylase	*Desulfurococcus mucosus* (85)	85	5.5
	Pyrococcus furiosus (100)	100	6.5–7.5
	Pyrococcus sp. KOD1 (100)	90	6.5
	Pyrococcus woesei (100)	100	5.5
	Pyrodictium abyssi (98)	100	5.0
	Staphylothermus marinus (90)	100	5.0
	Sulfolobus solfatatricus (88)		
	Thermococcus celer (85)	90	5.5
	Thermococcus profundus DT5432 (80)	80	5.5
	Thermococcus profundus (80)	80	4.0–5.0
	Thermococcus aggregans (85)	95	6.5
	Dictyoglomus thermophilum Rt46B.1 (73)	90	5.5
Pullulanase type II	*Desulfurococcus mucosus* (88)	100	5.0
	Pyrococcus woesei (100)	100	6.0
	Pyrodictium abyssi (98)	100	9.0
	Thermococcus celer (85)	90	5.5
	Thermococcus litoralis (90)	98	5.5
	Thermococcus hydrothermalis (80)	95	5.5
Pullulan-hydrolase type III	*Thermococcus aggregans* (85)	100	6.5
Glucoamylase	*Thermoplasma acidophilum* (60)	90	6.5
	Picrophilus oshimae (60)	90	2.0
	Picrophilus torridus (60)	90	2.0
CGTase	*Thermococcus sp.B1001* (75)	100	2.0
α-Glucosidase	*Thermococcus strain AN1* (80)	130	63
	Thermococcus hydrothermalis (80)	–	–

thermosaccharolyticum DSM 571 [129–141]. Recently, it has been shown that the thermoacidophilic archaea *Thermoplasma acidophilum*, *Picrophilus torridus* and *Picrophilus oshimae* produce heat- and acid-stable glucoamylases. The purified archaeal glucoamylases are optimally active at pH 2 and 90 °C. Catalytic activity is still detectable at pH 0.5 and 100 °C. These enzymes are more thermostable than the aforementioned glucoamylases from bacteria, yeast and fungi. This has been the first report on the production of glucoamylase in archaea [142]. However, the lack of suitable genetic methods for thermoacidophiles have precluded structural studies aimed at discovering their adaptation at very low pH. Very recently, the gene (*ssg*) encoding a putative glucoamylase from *Sulfolobus solfataricus*, was cloned and expressed

in *E. coli*, and the properties of the recombinant protein were examined in relation to the glucose production process. This represent the first successful cloning of a glucoamylase gene from a thermoacidophilic archaeon in a mesophilic host [143]. The recombinant glucoamylase is extremely thermostable, with an optimal temperature at 90 °C, however the intracellular enzyme is most active in the slightly acidophilic pH range from 5.5 to 6.0. The enzyme liberated β-D-glucose from the substrate maltotriose, and the substrate preference for maltotriose distinguishes this enzyme from fungal glucoamylases. Gel permeation chromatography and sedimentation equilibrium analytical ultracentrifugation analysis revealed that the enzyme exists as a tetramer [143]. The glucoamylase from *S. solfataricus* has a potential for improving industrial starch processing by eliminating the need to adjust both pH and temperature. However it is remarkably less acidic than the glucoamylases from *P. torridus*, *P. oshima* and *T. acidophylum*.

In addition to the glucoamylase, *S. solfataricus* produces a α-amylase, which is secreted into the culture supernatant during growth on starch as the sole carbon and energy source. The purified enzyme is a homodimer with a subunit size of 120 kDa and catalyzes the hydrolysis of starch, dextrin, and α-cyclodextrin [123]. Assimilation of starch-derived carbon in this organism is coupled to production of a cell-associated α-glucosidase, which converts maltodextrins into glucose. *S. solfataricus* employs a catabolite repression (CR) system to regulate production of glycosyl hydrolases, including synthesis of the secreted α-amylase.

α-Glucosidases (E C 3.2.1.20) attack the α-1,4 linkages of oligosaccharides that are produced by the action of other amylolytic enzymes. Unlike glucoamylase, α-glucosidase prefers smaller oligosaccharides, e.g. maltose and maltotriose, and liberates glucose with an α-anomeric configuration. α-Glucosidases are present in thermophilic archaea and bacteria. An intracellular and an extracellular α-glucosidase have been purified from *P. furiosus* and *Thermococcus* strain AN1 [144–148]. The enzyme exhibits optimal activity at pH 5.0–6.0 over a temperature range of 105 to 115 °C; the half-life at 98 °C is 48 h. An α-glucosidase (maltase) and flanking sequences from *Sulfolobus solfataricus* were cloned and characterized. *malA* is 2,083 bp and encodes a protein of 693 amino acids with a calculated mass of 80.5 kDa. It is flanked on the 5′ side by an unusual 1-kb intergenic region. The purified recombinant enzyme hydrolyzes *p*-nitrophenyl–D-glucopyranoside with a K_m of 2.16 mM and a V_{max} of 3.08 μmol of *p*-nitrophenol/min at 85 °C. It exhibited a pH optimum for maltose hydrolysis of 4.5. In contrast to its apparent greater tendency to dissociate during SDS-PAGE, the recombinant α-glucosidase exhibits greater thermostability than the native enzyme, with a half-life of 39 h at 85 °C at a pH of 6.0. Unlike maltose hydrolysis, glycogen hydrolysis is optimal at the intracellular pH of the organism. These results indicate a unique role for the *S. solfataricus* α-glucosidase in carbohydrate metabolism [149].

7.2 Thermoactive pullulanase and CGTase

Enzymes capable of hydrolyzing α-1,6 glycosidic bonds in pullulan are defined as pullulanases. Pullulan is a linear α-glucan consisting of maltotriose units joined by α-1,6 glycosidic linkages and it is produced by *Aureobasidium pullulans* with a chain length of 480 maltotriose units [150]. On the basis of substrate specificity and product formation, pullulanases have been classified into two groups: pullulanase type I and pullulanase type II. Pullulanase type I (EC 3.2.1.41) specifically hydrolyzes the α-1,6-linkages in pullulan as well as in branched oligosaccharides (debranching enzyme), and its degradation products are maltotriose and linear oligosaccharides, respectively. Pullulanase type I is unable to attack α-1,4-linkages in α-glucans. Pullulanase type II (amylopullulanase) attacks α-1,6-glycosidic linkages in pullulan and α-1,4-linkages in branched and linear oligosaccharides. The enzyme has multiple specificity and is able to fully convert polysaccharides (e.g. amylopectin) to small sugars (e.g. glucose, maltose, maltotriose) in the absence of other enzymes, such as α-amylase or β-amylase [151, 152].

Thermostable and thermoactive pullulanases from extremophilic microorganisms have been detected in *Pyrococcus furiosus* [153], *Thermococcus celer* [154], *Desulfurococcus mucosus* [155], *Staphylothermus marinus, Thermococcus hydrothermalis* [156] and *Thermococcus aggregans* [157] (Table 4). 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. Most thermoactive pullulanases identified to date belong to the type II group. Pullulanases type II from *P. furiosus* and *P. woesei* have been expressed in *E. coli* [153, 158, 159]. The unfolding and refolding of the pullulanase from *P. woesei* has been investigated using guanidinium chloride as denaturant. The monomeric enzyme (90 kDa) was found to be very resistant to chemical denaturation and the transition midpoint for guanidinium chloride-induced unfolding was determined to be 4.8. The unfolding process was reversible. Reactivation of the completely denatured enzyme (in 7.8 M guanidinium chloride) was obtained upon removal of the denaturant by stepwise dilution, 100% reactivation was observed when refolding was carried out via a guanidinium chloride concentration of 4 M in the first dilution step [160]. On the basis of the amino-acid sequence, the pullulanase type II (amylopullulanase) from *T. hydrothermalis* and *P. furiosus* belong to family 57 (GH-57) of the glycoside hydrolases. Five conserved regions were identified, which are postulated to be GH-57 consensus motifs by comparison to the 659-amino-acid-long 4–glucanotransferase from *Thermococcus litoralis*. These motifs correspond to 13_HQP (region I), 76_GQLEIV (region II), 120_WLTERV (region III), 212_HDDGEKFGVW (region IV), and 350_AQCNDAYWH (region V). The third and fourth conserved regions contain the hypothetical catalytic nucleophile E291 and the proton donor D394, respectively. To vali-

date this prediction, the characterization of catalytic sites of certain member of GH-57 has been started recently. Site-directed mutagenesis performed by Zona et al. [161] on pullulanase type II from *T. hydrothermalis* reveals that both residues are indeed critical for the pullulanolytic and amylolytic activities of the pullulanase type II [161]. The crucial role of E291 as the catalytic nucleophile has been also confirmed by Kang et al. [162], who performed similar experiment on the pullulanase type II from *P. furiosus* (PfAPU). The apparent catalytic efficiencies (k_{cat}/K_m) of mutants E291Q and D394N on pullulan were 123.0 and 24.4 times lower, respectively, than that of PfAPU. The activity of mutant E396Q on pullulan was too low to allow reliable determination of its catalytic efficiency. The apparent specific activities of these enzymes on starch also decreased 91.0 times (E291Q), 11.7 times (D394N), and 37.2 times (E396Q). The hydrolytic patterns for pullulan and starch were the same, while the hydrolysis rates differed as reported. Therefore, these data support the prediction and strongly suggest that the biofunctionality of the pullulanase type II is determined by a single catalytic centre.

Interestingly, pullulanase type I has not been isolated in Archaea so far, whereas the enzyme has been characterized in several thermophilic microorganism. The aerobic thermophilic bacterium *Thermus caldophilus* GK-24 produces a thermostable pullulanase of type I when grown on starch [163]. The pullulanase is optimally active at 75 °C and pH 5.5, is thermostable up to 90 °C, and does not require Ca^{2+} for either activity or stability. The first debranching enzyme (pullulanase type I) from an anaerobic thermophile was identified in the bacterium *Fervidobacterium pennivorans* Ven5 which was cloned and expressed in *E. coli*. The enzyme from *F. pennivorans* Ven5 attacks exclusively the α-1,6-glycosidic linkages in polysaccharides [164, 165]. This thermostable debranching enzyme leads to formation of long-chain linear polysaccharides from amylopectin [166]. The same enzyme has been also characterized from the related microorganism *T. maritima* [167]. Interestingly, data concerning the physico-chemical properties of all debranching enzymes reported so far show that they are mostly active in the acidic or neutral pH range. Until very recently, no reports have been present on the ability of thermophilic microorganisms to produce heat and alkaline stable pullulanase type I. After sequencing the whole genome of the thermoalkaliphile *A. gottschalkii*, an open reading frame with high pairwise similarity to the pullulanases from the thermophilic anaerobic bacteria *F. pennivorans* and *T. maritima* was identified and the gene (encoding 865 amino acids with a predicted molecular mass of 98 kDa) was cloned and expressed in *E. coli*. Pullulan hydrolysis activity was optimal at pH 8.0 and 70 °C, and under these physicochemical conditions the half-life of rPulAg was 22 h. The pullulanase from *A. gottschalkii*, therefore, is the first thermoalkalistable type I pullulanase that has been described [168].

Thermostable cyclodextrin glycosyltransferases (CGTases) are produced by *Thermoanaerobacter* species [169], *Thermoanaerobacterium thermosulfu-*

rigenes [170] and *Anaerobranca gottschalkii* (13, 66, 67). Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is an enzyme that is generally found in bacteria and was recently discovered in archaea. The archaeal enzyme was found in *Thermococcus* sp. and is optimally active at 100 °C (Table 4). This enzyme produces a series of non-reducing cyclic dextrins from starch, amylose, and other polysaccharides. α-, β- and γ-cyclodextrins are rings formed by 6, 7, and 8 glucose units, respectively, that are linked by α-1,4-bonds [171].

The finding of extremely thermophilic bacteria and archaea capable of producing novel thermostable starch-hydrolyzing enzymes is a valuable contribution to the starch-processing industry. By using robust starch-modifying enzymes from thermophiles, innovative and environmentally friendly processes can be developed, aiming at the formation of products of high added value for the food industry. At elevated temperatures starch is more soluble (30 to 35% w/v) and the risk of contamination is reduced. This is of advantage when starch will be converted to high-glucose and high-fructose syrups. Industrial production of fructose from starch consists of three steps: liquefaction, saccharification and isomerization. This multistage process (step 1: pH 6.5, 98 °C; step 2: pH 4.5, 60 °C: step 3; pH 8.0, 65 °C) leads to the conversion of starch to fructose with concurrent formation of high concentrations of salts that have to be removed by ion exchangers. Furthermore, high energy is required for cooling from 100 °C to 60 °C in step 2. The application of thermostable enzymes such as amylases, glucoamylases, pullulanases and glucose isomerases that are active and stable above 100 °C and at acidic pH values can simplify this complicated process. Therefore, strong efforts have been invested in the isolation of thermostable and thermoactive amylolytic enzymes from hyperthermophiles, since they could improve the starch conversion process and lower the cost of sugar-syrup production. The use of the extremely thermostable amylolytic enzymes can lead to other valuable products, which include innovative starch-based materials with gelatine-like characteristics and defined linear dextrins that can be used as fat substitutes, texturizers, aroma stabilizers and prebiotics. CGTases are used for the production of cyclodextrins that can be used as a gelling, thickening or stabilizing agent in jelly desserts, dressing, confectionery, dairy and meat products. Due to the ability of cyclodextrins to form inclusion complexes with a variety of organic molecules, cyclodextrins improve the solubility of hydrophobic compounds in aqueous solution. This is of interest for the pharmaceutical and cosmetic industries. Cyclodextrin production is a multistage process in which starch is first liquefied by a heat-stable amylase followed by a second step in which a less-thermostable CGTase from *Bacillus* sp. is used. The application of heat-stable CGTase in jet cooking, where temperatures up to 105 °C are achieved, will allow liquefaction and cyclization to take place in one step [172].

8
Proteolytic enzymes

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 in the following groups: serine, cysteine, aspartic, or metallo proteases. A variety of heat-stable proteases has been identified in hyperthermophilic archaea belonging to the genera *Desulfurococcus* [173], *Sulfolobus* [174, 175], *Staphylothermus* [176], *Thermococcus* [177, 178], *Pyrobaculum* [179] and *Pyrococcus* [180, 181] (Table 5). It has been found that most proteases from extremophiles belong to the serine type, are stable at high temperatures even in the presence of high concentrations of detergents and denaturing agents (59, 68, 69). A heat-stable serine protease was isolated from the cell-free supernatant of the hyperthermophilic archaeon *Desulfurococcus* strain $Tok_{12}S_1$. Recently, a cell-associated serine protease was characterized from the *Desulfurococcus* strain SY that has a half-life of 4.3 h at 95 °C [173]. A globular serine protease from *Staphylothermus marinus* was found to be extremely thermostable. The properties of extracellular serine proteases from a number of *Thermococcus* species have been analyzed [177, 178]. The extracellular

Table 5 Properties of thermoactive proteolytic enzymes from extreme thermophilic and hyperthermophilic Archaea

Protease	Organism (growth temperature)	Enzyme properties	
		Optimal temperature	Optimal pH
Serine protease	*Desulfurococcus mucosus* (85)	95	7.5
	Pyrococcus furiosus (100)	8.5	6.3
	Pyrobaculum aerophilum (95)	–	–
	Thermococcus aggregans (75)	90	7.0
	Thermococcus celer (85)	95	7.5
	Thermococcus litoralis (90)	95	9.5
	Thermococcus stetteri (75)	85	8.5
	Staphylothermus marinus (90)	–	9.0
	Sulfolobus solfataricus (88)	–	6.5–8
Thiol protease	*Pyrococcus sp. KOD1* (95)	110	7
Acidic protease	*Sulfolobus acidocaldarius* (70)	90	2.0
Aminopeptidase I	*Sulfolobus solfataricus* (88)	–	–
Aminopeptidase II		–	–
Endopeptidase I,II,III		–	–
Carboxypeptidase		–	–

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% SDS [182]. A novel intracellular serine protease (pernisine) from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1 was purified and characterized. At 90 °C, the enzyme has a broad pH profile and an optimum at pH 9.0 for peptide hydrolysis [183, 184]. The Pernisine, lacking the leader sequence, was expressed in *E. coli* as a fusion protein with glutathione-S-transferase. The biochemical properties of the recombinant enzyme were found to be similar to those of the native enzyme [185]. Several proteases from hyperthermophiles have been cloned and sequenced but, in general, their expression in mesophilic hosts is difficult. A gene encoding a subtilisin-like serine protease, named aereolysin has been cloned from *Pyrobaculum aerophilum* and the protein was modeled based on structures of subtilisin-type proteases [184]. 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 [186]. The pyrolysin gene was cloned and sequenced and it was shown that this enzyme is a subtilisin-like serine protease [187, 188]. A serine protease from *Aquifex pyrophilus* was cloned and weakly expressed in *E coli* as active and processed forms. The activity of the enzyme was highest at 85 °C and pH 9. The half-life of the protein (6 h at 105 °C) makes it one of the most heat-stable protease known to date [189].

Proteases have also been characterized from the thermoacidophilic archaea *Sulfolobus solfataricus* [175] and *S. acidocaldarius* [190–192]. In addition to the serine proteases other types of enzymes have been identified in extremophiles: a thiol protease from *Pyrococcus* sp. KOD1, a propylpeptidase (PEPase) and a new type of protease from *P. furiosus* [178, 181, 193]. Thermostable serine proteases were also detected in a number of extreme thermophilic bacteria blonging to the genera *Thermotoga* and *Fervidobacterium*. The enzyme system from *Fervidobacterium pennivorans* is able to hydrolyze feather keratin-forming high-value products such as amino acids and peptides. The enzyme, which has been named fervidolysin, is optimally active at 80 °C and pH 10.0 [194]. The gene encoding fervidolysin was isolated using degenerate primers combined with Southern hybridization and inverse polymerase chain reaction. Amino-terminal-sequence analysis of these bands and their comparison with that determined from biochemically purified keratinase and its predicted protein sequence identified them as a 73-kDa fervidolysin precursor, a 58-kDa mature fervidolysin, and a 14-kDa fervidolysin propeptide. Using site-directed mutagenesis, the active-site histidine residue at position 79 was replaced by an alanine residue. The resulting fervidolysin showed a single protein band corresponding in size to the 73-kDa fervidolysin precursor, indicating that its proteolytic cleavage resulted from an autoproteolytic process. Assays using keratin and other proteinaceous sub-

strates did not display fervidolysin activity, perhaps because of the tight binding of the propeptide in the substrate-binding site, where it could then function as an inhibitor. Finally, the recombinant fervidolysin has been crystallized and the structure has been determined at 1.7-Å resolution. The crystal structure shows that the protease is composed of four domains: a catalytic domain (CD), two beta-sandwich domains (SDs), and the PD domain. A structural alignment shows a distant relationship between the PD–CD substructure of fervidolysin and pro-subtilisin E. Tight binding of PD to the remaining part of the protease is mediated by hydrogen bonds along the domain surfaces and around the active cleft, and by the clamps to SD1 and SD2 [195, 196].

The amount of proteolytic enzymes produced worldwide on a commercial scale is the largest compared to the other biotechnological enzymes in use. Serine alkaline proteases are used as additives to household detergents for laundering, where they have to resist denaturation by detergents and alkaline conditions. Proteases showing high keratinolytic activity are used for soaking in the leather industry. Proteases are also used as catalysts for peptide synthesis using their reverse reaction.

9 Lipases

Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes and they find immense applications in the food, dairy, detergent and pharmaceutical industries. Lipases are produced by microbes and specifically bacterial lipases play a vital role in commercial ventures. Some important lipase-producing bacterial genera include *Bacillus, Pseudomonas* and *Burkholderia*. Lipases are generally produced on lipidic carbon, such as oils, fatty acids, glycerol or tweens in the presence of an organic nitrogen source. Bacterial lipases are mostly extracellular and are produced by submerged fermentation. The enzyme is most commonly purified by hydrophobic interaction chromatography, in addition to some modern approaches such as reverse micellar and aqueous two-phase systems.

Most lipases can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common. Bacterial lipases generally have temperature optima in the range 30–60 °C [197]. However, highly thermotolerant lipases has been reported from *B. stearothermophilus*, with a half-life of 15–25 min at 100 °C [198] and *B. thermoleovorans* [199, 200]. Lipases are serine hydrolases and have high stability in organic solvents. Besides these, some lipases exhibit chemo-, regio- and enantioselectivity. Very recently, more than five anaerobic thermophilic bacteria were found to produce extremely heat-stable lipases. They are active at a broad temperature (50–95 °C) and pH

(3–11) (unpublished results, Antranikian). The latest trend in lipase research is the development of novel and improved lipases through molecular approaches such as directed evolution and exploring natural communities by the metagenomic approach. The recent determination of structure of *Bacillus stearothermophilus* P1 lipase provides a template for other thermostable lipases, and offers insight into mechanisms used to enhance thermal stability which may be of commercial value in engineering lipases for industrial uses [201].

10 Glucose isomerases, alcohol dehydrogenases and esterases

In addition to the described extracellular enzymes, intracellular enzymes from extremophiles are of interest for various applications (Table 6). Glucose isomerase or xylose isomerase (D-xylose ketol-isomerase; EC 5.3.1.5) catalyzes 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 (HFCS). HFCS, an equilibrium mixture of glucose and fructose, is 1.3 times sweeter than sucrose. Glucose isomerase is widely distributed in mesophilic microorganisms. Intensive research efforts are directed toward improving the suitability of glucose isomerase for industrial application. To reach fructose concentration of 55% the reaction must approach 110 °C. Improved thermostable glucose isomerases have been engineered from mesophilic enzymes [202]. The gene encoding a xylose isomerase (XylA) of *Thermus flavus* AT62 was cloned and the DNA sequence was determined. XylA has an optimum temperature at 90 °C and pH 7.0; divalent cations such as Mn^{2+}, Co^{2+} and Mg^{2+} are required for enzyme activity [203]. *Thermoanaerobacterium* strain JW/SL-YS 489 forms a xylose isomerase, which is optimally active at pH 6.4 and 60 °C or pH 6.8 and 80 °C. Like other xylose isomerases, this enzyme requires Mn^{2+}, Co^{2+} and Mg^{2+} for thermal stability (stable for 1 h at 82 °C in the absence of substrate). The gene encoding the xylose isomerase the of *Thermoanaerobacterium* strain JW/SL-YS 489 was cloned and expressed in *E. coli* [204]. 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 *Thermoanaerobacterium saccharolyticum* B6A-RI. A thermostable glucose isomerase was purified and characterized from *Thermotoga maritima*. This enzyme is stable up to 100 °C, with a half-life of 10 min at 115 °C [205]. Interestingly, the glucose isomerase from *Thermotoga neapolitana* displays a catalytic efficiency at 90 °C, which is 2 to 14 times

Table 6 Enzymes with potential biotechnological application from extreme thermophilic and hyperthermophilic archaea and bacteria

Enzyme	Organism (growth temperature)	Biocatalysis	Application
α-Amylase	*Pyrococcus woesei* (100 °C)	Hydrolysis of α-1,4 glycosidic linkages in starch	Starch industry, bio-conversion of starch to glucose syrup
Debranching enzyme (pullulanase type I)	*Fervidobacterium pennivorans* Ven5 (75 °C)	Debranching of amylopectin to linear oligosaccharides	Starch bioconversion to glucose
CGTase	*Thermococcus sp.* (75 °C), *Anaerobranca gottschalkii* (60 °C)	Production of cyclodextrins	Gelling, thickening, stabilizing agents in food industry
Cellulase	*Pyrococcus furiosus* (100 °C)	Hydrolysis of cellulose to glucose	Color extraction of juice, color brightening, improving nutritional quality
Endoxylanases	*Thermotoga maritima MSB8* (80 °C)	Degradation of xylan	Bleaching of paper
Chitinase	*Thermococcus kodakaraensis* (95 °C)	Degradation of chitin	Utilization of biomass of marine environment
Serine protease	*Fervidobacterium pennivorans* (70 °C)	Keratin hydrolysis	Soaking in leather industry, production of amino acids and peptides from feathers
Glucose isomerase	*Thermotoga maritima* (80 °C)	Isomerization of glucose to fructose	Production of high-fructose corn syrup
Alcohol deydrogenase	*Sulfolobus solfataricus* (88 °C)	Oxidation of secondary alcohols	Reduction of ketones
Esterase	*Sulfolobus tokadaii* (100 °C)	Cleavage of esters	Biotransformation in organic solvents
DNA polymerase	*Thermus aquaticus* (75 °C)	DNA synthesis	Taq polymerase, polymerase chain reaction (PCR)

higher than any other thermoactive glucose isomerases at temperatures between 60 °C and 90 °C (87) [205–208].

Dehydrogenases are enzymes belonging to the class of oxidoreductases. Within this class, alcohol dehydrogenases (E.C.1.1.1.1, also named keto-

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reductases) represent an important group of biocatalysts due to their ability to stereospecifically reduce prochiral carbonyl compounds. Alcohol dehydrogenases can be used efficiently in the synthesis of optically active alcohols, which are key building blocks for the fine-chemicals industry [209]. From a practical point of view, alcohol dehydrogenases that use nicotinamide adenine dinucleotide (NADH)CE[b] as cofactor are of particular importance, because the formate/formate dehydrogenase dehydrogenase system represents an established method to regenerate NADH efficiently. By contrast, for nicotinamide adenine dinucleotide phosphate (NADP)-dependent enzymes the cofactor-recycling systems that are available are much less efficient. The secondary specific alcohol dehydrogenase (ADH), which catalyzes the oxidation of secondary alcohols and, less readily, the reverse reaction (the reduction of ketones) has a promising future in biotechnology [209]. Although ADHs are widely distributed among microorganisms, only few examples derived from hyperthermophilic microorganisms are currently known. Among the extreme thermophilic bacteria, *Thermoanaerobacter ethanolicus* 39E was shown to produce an ADH, whose gene was cloned and expressed in *E. coli*. Interestingly, a mutant has been found to posses an advantage over the wild-type enzyme by using the more-stable cofactor NADCE[c] instead of NADP [210–213]. An alcohol dehydrogenase (ADH) was purified from an extremely thermophilic bacterium, *Thermomicrobium roseum*. The native enzyme is a homodimer of 43-kDa subunits. The pI of the enzyme was determined to be 6.2, while its optimum pH is 10.0. The enzyme oxidized mainly primary aliphatic alcohols and exhibited high substrate specificity towards ethanol, n-propanol and crotyl alcohol. The highest reaction rate was observed when ethanol was used as substrate and the K_m value of the enzyme for ethanol was 24.2 mM. Pyrazole notably inhibited the enzymatic activity. The enzyme had an optimal temperature of 70 °C and is highly stable [214, 215]. In addition, a novel NADH-dependent alcohol dehydrogenase (RE-ADH) was found in *Rhodococcus erythropolis*. This ADH catalyzes the reduction of ketones to the corresponding (S)-alcohols. The enzyme was purified and its biochemical characteristics were investigated. The gene encoding for 348 amino acids was cloned in *E. coli* cells and successfully expressed [216]. The subunit molecular mass as deduced from the amino acid sequence was determined to be 36 kDa. The recombinant enzyme exhibits high thermostability, which facilitated its purification by heat treatment, followed by two column-chromatography steps. RE-ADH shows high similarity to several zinc-containing medium-chain alcohol dehydrogenases. All zinc ligands seem to be conserved except one of the catalytic zinc ligands, where Cys is probably substituted by Asp. In extreme thermophilic archaea, ADHs have been studied from *Sulfolobus solfataricus* and *Thermococcus stetteri* [217, 218]. The enzyme from *S. solfataricus* requires NAD as a cofactor and contains Zn ions. In contrast to ADHs from bacteria and Eukarya, the enzyme from *T. stetteri* lacks metal ions. The enzyme catalyzes 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 ADH from *T. litoralis* represents a new type of alcohol-oxidizing enzyme system [217, 218]. The gene of ADHs from *S. solfataricus* was expressed in *E. coli* and characterized.

In the field of biotechnology, esterases are gaining increasing attention because of their application in organic biosynthesis. In aqueous solution, esterases catalyze 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. The lipA gene encoding a thermostable esterase was cloned from *Thermoanaerobacter tengcongensis* and overexpressed in *E. coli*. The recombinant esterase, with a molecular mass of 43 kDa, hydrolyzes tributyrin but not olive oil. The esterase was optimally active at 70 °C (over 15 min) and at pH 9. It is highly thermostable, with a residual activity greater than 80% after incubation at 50 °C for more than 10 h [219]. An esterase from the putative esterase gene selected from the total genome analysis from the thermoacidophilic archaeon *Sulfolobus tokodaii* strain 7 was cloned and expressed as a fusion protein in *E. coli*. The optimum activity for ester cleavage against p-nitrophenyl esters was observed at around 70 °C and pH 7.5–8.0. The enzyme exhibits high thermostability and also shows activity in a mixture of a buffer and water-miscible organic solvents, such as acetonitrile and dimethyl sulfoxide. From the kinetic analysis, p-nitrophenyl butyrate was found to be a better substrate than caproate and caprylate [220]. The *Pyrococcus furiosus* esterase gene was cloned in *E. coli* and the functional properties were determined. The archaeal enzyme is the most thermostable (a half-life of 50 min at 126 °C) and thermoactive (temperature optimum 100 °C) esterase known to date [221].

11
Amidases and nitrilase

11.1
Amidases

Amidases [EC 3.5.1.4] catalyze the conversion of amides to the corresponding carboxylic acids and ammonia, according to the following reaction: $RCONH_2 + H_2O \rightarrow RCOOH + NH_3$. A number of amidases from bacteria have been purified and characterized and the crystal structure of the peptide amidase from *Stenotrophomonas maltophilia* [222] was also resolved recently.

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Amidases exhibit different physicochemical characteristics and a diverse substrate spectrum. The enzymes that lack the characteristic signature GGSS-(GAS)-S [223] in the primary structure are only able to hydrolyze aliphatic substrates. In contrast, amidases containing this signature are able to convert cyclic and aromatic amides as well [224–228]. These amidases are highly *S*-enantioselective, usually forming the optically pure acids with an enantiomeric excess above 99% [229]. Only in chemical and pharmaceutical industries for the production of optically pure compounds [230–232], drugs [225, 233], acrylic [234] and hydroxamic acids [235] CE[d]. In general the application of efficient biocatalysts in industrial processes allows the formation of highly pure products with a concomitant reduction of wastes [233]. Running such processes at elevated temperatures also has many advantages, including significant improvement of transfer rates, higher substrate solubility and reduced risk of contamination. Very little, however, is known about amidases that are active at high temperatures.

Three thermoactive amidases described so far were isolated from *Brevibacillus borstelensis* BCS-1 [236], *Klebsiella pneumoniae* NCTR 1 [237] and *Sulfolobus solfataricus* [238]. Very recently, the first thermoactive and thermostable amidase from the thermophilic actinomycete *Pseudonocardia thermophila* has been purified and characterized [239]. The amidase is active over a broad pH (pH 4–9) and temperature range (40–80 °C) and has a half-life of 1.2 h at 70 °C. The amidase has a broad substrate spectrum, including aliphatic, aromatic and amino acid amides. The presence of a double bond or a methyl group near the carboxamide group of aliphatic and amino acid amides enhances the enzymatic activity. The highest acyl transferase activity was detected with hexanoamide, isobutyramide and propionamide. The amidase is highly S-stereoselective for 2-phenylpropionamide; and the racemic amide was converted to the corresponding S-acid with an enantiomeric excess of > 95% at 50% conversion of the substrate. In contrast, the d,l-tryptophanamide and d,l-methioninamide were converted to the corresponding d,l-acids at the same rate.

12 DNA-processing enzymes

12.1 Polymerase chain reaction (PCR)

DNA polymerases (EC 2.7.7.7) are the key enzymes in the replication of cellular information present in all life forms. They catalyze, in the presence of Mg^{2+}-ions, the addition of a deoxyribonucleotide CE[e] 5′-triphosphate onto the growing 3′-OH end of a primer strand, forming complementary base pairs to

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a second strand. More than 100 DNA polymerase genes have been cloned and sequenced from various organisms, including thermophilic bacteria and archaea [256]. Thermostable DNA polymerases play a major role in a variety of molecular biological applications, e.g. DNA amplification, sequencing or labelling (Table 7).

One of the most important advances in molecular biology during the last ten years is the development of polymerase chain reaction (PCR) [257–259]. The first described PCR procedure 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. The DNA polymerase I from the bacterium *Thermus aquaticus*, called *Taq* polymerase, was the first thermostable DNA polymerase characterized and applied in PCR.

The *Taq* polymerase has a 5′-3′-exonuclease activity, but no detectable 3′-5′-exonuclease activity. Due 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. 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

Table 7 The most commonly used thermoactive DNA polymerases

Enzyme	Organism of origin
Bacterial DNA polymerases	
Taq pol I	*Thermus aquaticus*
Tth pol	*Thermus thermophilus*
Tfi pol	*Thermus filiformis*
Tfl pol	*Thermus flavus*
Tca pol	*Thermus caldophilus* GK24
*Bst*I pol	*Bacillus stearothermophilus*
Tma pol	*Thermotoga maritima*
Archaeal DNA polymerases	
Pwo pol	*Pyrococcus woesei*
Pfu pol	*Pyrococcus furiosus*
DeepVent pol	*Pyrococcus sp.* GB-D
KOD1 pol	*Pyrococcus sp.* KOD1
Vent pol	*Thermococcus litoralis*
9°N-7 pol	*Thermococcus* sp. 9°N-7

polymerase from *Thermotoga maritima* was reported to have a 3′-5′-exonuclease activity [260]. Archaeal proofreading polymerases such as *Pwo* pol from *Pyrococcus woesei*, *Pfu* pol from *Pyrococcus furiosus*, Deep Vent™ pol from *Pyrococcus* strain GB-D [261–268] or Vent™ pol from *Thermococcus litoralis* [269–274] have an error rate that is up to ten times lower than that of *Taq* polymerase. The 9°N-7 DNA polymerase from *Thermococcus* sp. strain The 9°N-7 has a fivefold higher 3′-5′-exonuclease activity than *T. litoralis* DNA polymerase. 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 potentially strong exonuclease activity. The recombinant KOD1 DNA polymerase from *Thermococcus kodakaraensis* 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 an accurate amplification of target DNA sequences up to 6 kb [275–278]. To optimize the delicate competition of polymerase and exonuclease activity, the exo-motif of the The 9°N-7 DNA polymerase was mutated in an attempt to reduce the level of exonuclease activity without totally eliminating it. An additional problem in the performance of PCR is the generation of nonspecific templates prior to thermal cycling. Several approaches have been made to prevent the elongation of polymerase before cycling temperatures are reached. As well as using wax as a mechanical barrier between DNA and the enzyme, more sophisticated methods were invented such as the inhibition of *Taq* polymerase by a neutralizing antibody at mesophilic temperatures or heat-mediated activation of the immobilized enzyme.

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 proofreading DNA polymerases, containing 3′-5′-exonuclease activity, to *Taq* or other non-proofreading DNA polymerases. In this long PCR, the reaction conditions are optimized for long extension by adding different components such as gelatin, 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 or formamide are added.

12.2 DNA sequencing

DNA sequencing by the Sanger method [279] has undergone countless refinements in the last 20 years. A major step forward was the introduction of thermostable DNA polymerases leading in the cycle sequencing procedure. This method uses repeated cycles of temperature denaturation, annealing and extension with dideoxy-termination to increase the amount of sequencing product by recycling the template DNA. Due to this “PCR-like” amplification

of the sequencing products several problems could be overcome. Caused by the cycle denaturation, only fmoles of template DNA are required, no separate primer annealing step is needed and unwanted secondary structures within the template are resolved at high-temperature elongation. The first enzyme used for cycle sequencing was the thermostable DNA polymerase I from *Thermus aquaticus* [280, 281]. As described by Longley et al. (118) the enzyme displays 5′-3′-exonuclease activity that is undesirable because of the degradation of sequencing fragments. A combination of thermostable enzymes has been developed that produces higher quality cycle sequences. Thermo Sequenase DNA polymerase is a thermostable enzyme engineered to catalyze the incorporation of ddNTPs with an efficiency several thousand times better than other thermostable DNA polymerases. Since the enzyme also catalyzes pyrophosphorolysis at dideoxy termini, a thermostable inorganic pyrophosphatase is needed to remove the pyrophosphate produced during sequencing reactions. *Thermoplasma acidophilum* inorganic pyrophosphatase (TAP) is thermostable and effective for converting pyrophosphate to orthophosphate. The use of the combination of Thermo Sequenase polymerase and TAP for cycle sequencing yields sequence data with uniform band intensities, allowing the determination of longer, more accurate sequence reads. Uniform band intensities also facilitate interpretation of sequence anomalies and the presence of mixed templates. Sequencing PCR products of DNA amplified from heterozygous diploid individuals results in signals of equal intensity from each allele [282].

12.3 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 [283], the detection of trinucleotide repeats through repeat expansion detection (RED) [284] or DNA detection by circularization of oligonucleotides [285]. Over the years several additional thermostable DNA ligases were discovered. Bacterial enzymes were derived and cloned from *Thermus scotoductus* and *Rhodothermus marinus* [286–289]. Recent studies in the crude extract of 103 strains of the genera *Thermus*, *Bacillus*, *Rhodothermus* and *Hydrogenobacter* revealed the presence of thermostable DNA ligases in 23 of the *Thermus* strains [Hjörleifsdottir S, 1997 #302]. To date an archaeal DNA ligase has been described from *Desulfurolobus ambivalens* [Kletzin, 1992 #298]. Unlike bacterial enzymes, this ligase is NAD^+-independent but ATP-dependent similar to the enzymes from bacteriophages, eukaryotes and viruses. A gene encoding DNA ligase (ligTk) from a hyperthermophilic archaeon, *Thermococcus kodakaraensisKOD1*, has been cloned and sequenced, and its protein product has been characterized: ligTk consists of 1686 bp, corresponding to a polypeptide of 562 amino acids

with a predicted molecular mass of 64 079 Da. Sequence comparison with previously reported DNA ligases and the presence of conserved motifs suggested that ligTk was an ATP-dependent DNA ligase. Phylogenetic analysis indicated that ligTk was closely related to the ATP-dependent DNA ligase from *Methanobacterium thermoautotrophicum* H, a moderately thermophilic archaeon, along with putative DNA ligases from Euryarchaeota and Crenarchaeota. Recombinant ligTk was monomeric, as is the case for other DNA ligases. The protein displayed DNA ligase activity in the presence of ATP and Mg^{2+}. The optimum pH of ligTk was 8.0, the optimum concentration of Mg^{2+}, which was indispensable for the enzyme activity, was 14 to 18 mM, and the optimum concentration of K^+ was 10 to 30 mM. ligTk did not display single-stranded DNA ligase activity. At enzyme concentrations of 200 nM, we observed significant DNA ligase activity even at 100 °C. Unexpectedly, ligTk displayed a relatively small, but significant, DNA ligase activity when NAD^+ was added as the cofactor. Treatment of NAD^+ with hexokinase did not affect this activity, excluding the possibility of contaminant ATP in the NAD^+ solution. This unique cofactor specificity was also supported by the observation of adenylation of ligTk with NAD^+. This was the first biochemical study of a DNA ligase from a hyperthermophilic archaeon [Nakatani, 2002 #300]. The ability of DNA ligases to use either ATP or NAD^+ as a cofactor appears to be specific to DNA ligases from Thermococcales. An archaeal DNA ligase has been cloned from *Thermococcus fumicolans* (Tfu). The optimum temperature and pH of Tfu DNA ligase were 65 °C and 7.0, respectively. The optimum concentration of MgCl2, which is indispensable for the enzyme activity, was 2 mM. Tfu DNA ligase displays nick joining and blunt-end ligation activity using either ATP or NAD^+, as a cofactor [Rolland, 2004 #299]

13 Conclusion

Owing to their properties such as activity over a wide temperature and pH range, substrate specificity, stability in organic solvents, diverse substrate range and enantioselectivity, biocatalysts from extremophilic microorganisms will represent the choice for countless future applications in industry. Their importance is increasing daily in several fields, such as food additives, detergent industry, chemicals, pharmaceuticals, etc. The growing demand for more robust biocatalysts has shifted the trend towards improving the properties of existing proteins for established industrial processes and producing new enzymes that are tailor-made for entirely new areas of application. Currently, the number of novel microbial extremophilic enzymes being cloned and biochemically characterized is steadily on the rise. New technologies such as genomics, metanogenomics, gene shuffling, and DNA evolution provide

valuable tools for improving or adapting enzyme properties to the desired requirements. However, the success of these techniques demands the production of recombinant enzymes at a high level, allowing experimental trials and application tests. Thus, the modern methods of genetic engineering combined with an increasing knowledge of structure and function and process engineering will allow further adaptation to industrial needs, the exploration of novel applications and protection of the environment.

References

1. Woese CR, Kandler O, Wheelis ML (1990) Proc Natl Acad Sci USA 87:4576
2. Woese CR, Achenbach L, Rouviere P, Mandelco L (1991) Syst Appl Microbiol 14:364
3. Lorenz P, Liebeton K, Niehaus F, Eck J (2002) Curr Opin Biotechnol 13:572
4. Kauffmann IM, Schmitt J, Schmid RD (2004) Appl Microbiol Biotechnol 64:665
5. Beja O (2004) Curr Opin Biotechnol 15:187
6. Beja O, Suzuki MT, Heidelberg JF, Nelson WC, Preston CM, Hamada T, Eisen JA, Fraser CM, DeLong EF (2002) Nature 415:630
7. Beja O, Koonin EV, Aravind L, Taylor LT, Seitz H, Stein JL, Bensen DC, Feldman RA, Swanson RV, DeLong EF (2002) Appl Environ Microbiol 68:335
8. Beja O, Suzuki MT, Koonin EV, Aravind L, Hadd A, Nguyen LP, Villacorta R, Amjadi M, Garrigues C, Jovanovich SB, Feldman RA, DeLong EF (2000) Environ Microbiol 2:516
9. Beja O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL, Spudich EN, DeLong EF (2000) Science 289:1902
10. Henne A, Schmitz RA, Bomeke M, Gottschalk G, Daniel R (2000) Appl Environ Microbiol 66:3113
11. Healy FG, Ray RM, Aldrich HC, Wilkie AC, Ingram LO, Shanmugam KT (1995) Appl Microbiol Biotechnol 43:667
12. Richardson TH, Tan X, Frey G, Callen W, Cabell M, Lam D, Macomber J, Short JM, Robertson DE, Miller C (2002) J Biol Chem 277:26 501
13. Cottrell MT, Moore JA, Kirchman DL (1999) Appl Environ Microbiol 65:2553
14. Cottrell MT, Wood DN, Yu L, Kirchman DL (2000) Appl Environ Microbiol 66:1195
15. Rondon MR, Raffel SJ, Goodman RM, Handelsman J (1999) Proc Natl Acad Sci USA 96:6451
16. Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM (2000) Appl Environ Microbiol 66:2541
17. Torsvik V, Ovreas L (2002) Curr Opin Microbiol 5:240
18. Torsvik V, Ovreas L, Thingstad TF (2002) Science 296:1064
19. Entcheva P, Liebl W, Johann A, Hartsch T, Streit WR (2001) Appl Environ Microbiol 67:89
20. Voget S, Leggewie C, Uesbeck A, Raasch C, Jaeger KE, Streit WR (2003) Appl Environ Microbiol 69:6235
21. Lorenz P, Scleper C (2002) J Mol Catal B 19–20:13
22. Morita RY (1975) Bacteriol Rev 39:144
23. Gerday C, Aittaleb M, Bentahir M, Chessa JP, Claverie P, Collins T, D'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis MA, Feller G (2000)

Trends Biotechnol 18:103
24. Lonhienne T, Gerday C, Feller G (2000) Biochim Biophys Acta 1543:1
25. Stetter KO (1996) Ciba Found Symp 202:1
26. Huber R, Huber H, Stetter KO (2000) FEMS Microbiol Rev 24:615
27. Segerer AH, Burggraf S, Fiala G, Huber G, Huber R, Pley U, Stetter KO (1993) Orig Life Evol Biosph 23:77
28. Stetter KO (1999) FEBS Lett 452:22
29. Lien T, Madsen M, Rainey FA, Birkeland NK (1998) Int J Syst Bacteriol 48 Pt 3:1007
30. Woese CR, Fox GE (1977) Proc Natl Acad Sci USA 74:5088
31. Jan RL, Wu J, Chaw SM, Tsai CW, Tsen SD (1999) Int J Syst Bacteriol 49 Pt 4:1809
32. Xiang X, Dong X, Huang L (2003) Extremophiles 7:493
33. He ZG, Zhong H, Li Y (2004) Curr Microbiol 48:159
34. Searcy DG, Stein DB (1980) Biochim Biophys Acta 609:180
35. Schleper C, Puehler G, Holz I, Gambacorta A, Janekovic D, Santarius U, Klenk HP, Zillig W (1995) J Bacteriol 177:7050
36. Smith PF (1980) Biochim Biophys Acta 619:367
37. van de V[TS f], Driessen AJ, Zillig W, Konings WN (1998) Extremophiles 2:67
38. Futterer O, Angelov A, Liesegang H, Gottschalk G, Schleper C, Schepers B, Dock C, Antranikian G, Liebl W (2004) Proc Natl Acad Sci USA 101:9091
39. Bandeiras TM, Salgueiro CA, Huber H, Gomes CM, Teixeira M (2003) Biochim Biophys Acta 1557:13
40. Nemati M, Lowenadler J, Harrison ST (2000) Appl Microbiol Biotechnol 53:173
41. Burton NP, Williams TD, Norris PR (1999) Arch Microbiol 172:349
42. Gomes CM, Huber H, Stetter KO, Teixeira M (1998) FEBS Lett 432:99
43. Brierley CL, Brierley JA (1973) Can J Microbiol 19:183
44. Kurosawa N, Itoh YH, Itoh T (2003) Int J Syst Evol Microbiol 53:1607
45. Krulwich TA, Guffanti AA, Ito M (1999) Novartis Found Symp 221:167
46. Horikoshi K (1999) Microbiol Mol Biol Rev 63:735
47. Jones BE, Grant WD, Duckworth AW, Owenson GG (1998) Extremophiles 2:191
48. Wiegel J, Kevbrin VV (2004) Biochem Soc Trans 32:193
49. Prowe SG, Antranikian G (2001) Int J Syst Evol Microbiol 51:457
50. Engle M, Li Y, Woese C, Wiegel J (1995) Int J Syst Bacteriol 45:454
51. Keller M, Braun FJ, Dirmeier R, Hafenbradl D, Burggraf S, Rachel R, Stetter KO (1995) Arch Microbiol 164:390
52. Dirmeier R, Keller M, Hafenbradl D, Braun FJ, Rachel R, Burggraf S, Stetter KO (1998) Extremophiles 2:109
53. Litchfield CD, Gillevet PM (2002) J Ind Microbiol Biotechnol 28:48
54. Sanchez-Porro C, Martin S, Mellado E, Ventosa A (2003) J Appl Microbiol 94:295
55. Ventosa A, Marquez MC, Garabito MJ, Arahal DR (1998) Extremophiles 2:297
56. Ollivier B, Caumette P, Garcia JL, Mah RA (1994) Microbiol Rev 58:27
57. Ventosa A, Nieto JJ, Oren A (1998) Microbiol Mol Biol Rev 62:504
58. Liebl W (2001) Methods Enzymol 330:290
59. Bronnenmeier K, Kern A, Liebl W, Staudenbauer WL (1995) Appl Environ Microbiol 61:1399
60. Bok JD, Yernool DA, Eveleigh DE (1998) Appl Environ Microbiol 64:4774
61. Te'o VS, Saul DJ, Bergquist PL (1995) Appl Microbiol Biotechnol 43:291
62. Saul DJ, Williams LC, Grayling RA, Chamley LW, Love DR, Bergquist PL (1990) Appl Environ Microbiol 56:3117
63. Saul DJ, Williams LC, Love DR, Chamley LW, Bergquist PL (1989) Nucleic Acids Res 17:439

[TS f] Please check this name.

Editor's or typesetter's annotations (will be removed before the final TEX run)

64. Ruttersmith LD, Daniel RM (1991) Biochem J 277 (Pt 3):887
65. Halldorsdottir S, Thorolfsdottir ET, Spilliaert R, Johansson M, Thorbjarnardottir SH, Palsdottir A, Hreggvidsson GO, Kristjansson JK, Holst O, Eggertsson G (1998) Appl Microbiol Biotechnol 49:277
66. Crennell SJ, Hreggvidsson GO, Nordberg Karlsson E (2002) J Mol Biol 320:883
67. Bauer MW, Driskill LE, Callen W, Snead MA, Mathur EJ, Kelly RM (1999) J Bacteriol 181:284
68. Moracci M, Ciaramella M, Rossi M (2001) Methods Enzymol 330:201
69. Bauer MW, Bylina EJ, Swanson RV, Kelly RM (1996) J Biol Chem 271:23 749
70. Bauer MW, Kelly RM (1998) Biochemistry 37:17 170
71. Nucci R, Moracci M, Vaccaro C, Vespa N, Rossi M (1993) Biotechnol Appl Biochem 17 (Pt 2):239
72. Moracci M, Nucci R, Febbraio F, Vaccaro C, Vespa N, La Cara F, Rossi M (1995) Enzyme Microb Technol 17:992
73. Sivori AS, Mercuri OA, Forchiassin F (1996) Rev Argent Microbiol 28:9
74. Durrant LR, Mello AB, Reginatto V (1992) Biochem Soc Trans 20:227S
75. Allen WG (1976) Biotechnol Bioeng Symp: 303 CE g
76. Schulein M (2000) Biochim Biophys Acta 1543:239
77. Jeffries TW (1996) Curr Opin Biotechnol 7:337
78. Te'o VS, Cziferszky AE, Bergquist PL, Nevalainen KM (2000) FEMS Microbiol Lett 190:13
79. McCarthy AA, Morris DD, Bergquist PL, Baker EN (2000) Acta Crystallogr D Biol Crystallogr 56 (Pt 11):1367
80. Simpson HD, Haufler UR, Daniel RM (1991) Biochem J 277 (Pt 2):413
81. Winterhalter C, Heinrich P, Candussio A, Wich G, Liebl W (1995) Mol Microbiol 15:431
82. Velikodvorskaya TV, Volkov I, Vasilevko VT, Zverlov VV, Piruzian ES (1997) Biochemistry (Mosc) 62:66
83. Zverlov V, Piotukh K, Dakhova O, Velikodvorskaya G, Borriss R (1996) Appl Microbiol Biotechnol 45:245
84. Walsh DJ, Gibbs MD, Bergquist PL (1998) Extremophiles 2:9
85. Meissner K, Wassenberg D, Liebl W (2000) Mol Microbiol 36:898
86. Lama L, Calandrelli V, Gambacorta A, Nicolaus B (2004) Res Microbiol 155:283
87. Teplitsky A, Mechaly A, Stojanoff V, Sainz G, Golan G, Feinberg H, Gilboa R, Reiland V, Zolotnitsky G, Shallom D, Thompson A, Shoham Y, Shoham G (2004) Acta Crystallogr D Biol Crystallogr 60:836
88. Andrade CM, Aguiar WB, Antranikian G (2001) Appl Biochem Biotechnol 91-93:655
89. Waino M, Ingvorsen K (2003) Extremophiles 7:87
90. Linko M, Viikari L, Suihko ML (1984) Biotechnol Adv 2:233
91. Oksanen T, Pere J, Paavilainen L, Buchert J, Viikari L (2000) J Biotechnol 78:39
92. Schink B, Zeikus JB (1983) FEMS Microbiol Lett 17:295
93. Tamaru Y, Doi RH (2001) Proc Natl Acad Sci USA 98:4125
94. Van Rijssel M, Gerwig GJ, Hansen TA (1993) Appl Environ Microbiol 59:828
95. Hoondal GS, Tiwari RP, Tewari R, Dahiya N, Beg QK (2002) Appl Microbiol Biotechnol 59:409
96. Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Bioresour Technol 77:215
97. Herron SR, Benen JA, Scavetta RD, Visser J, Jurnak F (2000) Proc Natl Acad Sci USA 97:8762
98. Prade RA, Zhan D, Ayoubi P, Mort AJ (1999) Biotechnol Genet Eng Rev 16:361
99. Lang C, Dornenburg H (2000) Appl Microbiol Biotechnol 53:366

CE g Please check page reference

Editor's or typesetter's annotations (will be removed before the final TEX run)

100. Sakai T, Sakamoto T, Hallaert J, Vandamme EJ (1993) Adv Appl Microbiol 39:213
101. Rexova-Benkova L, Markovic O (1976) Adv Carbohydr Chem Biochem 33:323
102. Kozianowski G, Canganella F, Rainey FA, Hippe H, Antranikian G (1997) Extremophiles 1:171
103. Kluskens LD, van Alebeek GJ, Voragen AG, de Vos WM, van der Oost J (2003) Biochem J 370:651
104. Cohen-Kupiec R, Chet I (1998) Curr Opin Biotechnol 9:270
105. Koga D, Mitsutomi M, Kono M, Matsumiya M (1999) EXS 87:111
106. Fukamizo T (2000) Curr Protein Pept Sci 1:105
107. Takayanagi T, Ajisaka K, Takiguchi Y, Shimahara K (1991) Biochim Biophys Acta 1078:404
108. Sakai K, Yokota A, Kurokawa H, Wakayama M, Moriguchi M (1998) Appl Environ Microbiol 64:3397
109. Tsujibo H, Minoura K, Miyamoto K, Endo H, Moriwaki M, Inamori Y (1993) Appl Environ Microbiol 59:620
110. Andronopoulou E, Vorgias CE (2004) Protein Expr Purif 35:264
111. Imanaka T, Fukui T, Fujiwara S (2001) Methods Enzymol 330:319
112. Tanaka T, Fujiwara S, Nishikori S, Fukui T, Takagi M, Imanaka T (1999) Appl Environ Microbiol 65:5338
113. Gao J, Bauer MW, Shockley KR, Pysz MA, Kelly RM (2003) Appl Environ Microbiol 69:3119
114. Grzybowska B, Szweda P, Synowiecki J (2004) Mol Biotechnol 26:101
115. Berk H, Lebbink RJ (2003) Methods Mol Biol 230:127
116. Linden A, Mayans O, Meyer-Klaucke W, Antranikian G, Wilmanns M (2003) J Biol Chem 278:9875
117. Savchenko A, Vieille C, Kang S, Zeikus JG (2002) Biochemistry 41:6193
118. Savchenko A, Vieille C, Zeikus JG (2001) Methods Enzymol 330:354
119. Leveque E, Haye B, Belarbi A (2000) FEMS Microbiol Lett 186:67
120. Canganella F, Gambacorta A, Kato C, Horikoshi K (2000) Microbiol Res 154:297
121. Kato M, Miura Y, Kettoku M, Shindo K, Iwamatsu A, Kobayashi K (1996) Biosci Biotechnol Biochem 60:546
122. Kobayashi K, Kato M, Miura Y, Kettoku M, Komeda T, Iwamatsu A (1996) Biosci Biotechnol Biochem 60:1720
123. Haseltine C, Rolfsmeier M, Blum P (1996) J Bacteriol 178:945
124. Lee J, Kanai H, Kobayashi T, Akiba T, Kudo T (1996) J Ferment Bioeng 82:432
125. Tachibana Y, Mendez L, Fujiwara S, Takagi M, Imanaka T (1996) J Ferment Bioeng 82:224
126. Liebl W, Stemplinger I, Ruile P (1997) J Bacteriol 179:941
127. Horinouchi S, Fukusumi S, Ohshima T, Beppu T (1988) Eur J Biochem 176:243
128. Fukusumi S, Kamizono A, Horinouchi S, Beppu T (1988) Eur J Biochem 174:15
129. Ducki A, Grundmann O, Konermann L, Mayer F, Hoppert M (1998) J Gen Appl Microbiol 44:327
130. Ganghofner D, Kellermann J, Staudenbauer WL, Bronnenmeier K (1998) Biosci Biotechnol Biochem 62:302
131. Coutinho PM, Reilly PJ (1994) Protein Eng 7:749
132. Coutinho PM, Reilly PJ (1994) Protein Eng 7:393
133. Ohnishi H, Matsumoto H, Sakai H, Ohta T (1994) J Biol Chem 269:3503
134. Ohnishi H, Kitamura H, Minowa T, Sakai H, Ohta T (1992) Eur J Biochem 207:413
135. Paquet V, Croux C, Goma G, Soucaille P (1991) Appl Environ Microbiol 57:212
136. Glushchenko EV, Kozlov DG, Podkovyrov SM, Mogutov MA (1990) Mol Biol (Mosk)

24:744
137. Kozlov DG, Kurepina NE, Glushchenko EV, Mogutov MA, Podkovyrov SM (1990) Mol Biol (Mosk) 24:736
138. Saha BC, Mathupala SP, Zeikus JG (1988) Biochem J 252:343
139. Hyun HH, Shen GJ, Zeikus JG (1985) J Bacteriol 164:1153
140. Hyun HH, Zeikus JG (1985) J Bacteriol 164:1146
141. Hyun HH, Zeikus JG (1985) J Bacteriol 164:1162
142. Serour E, Antranikian G (2002) Antonie Van Leeuwenhoek 81:73
143. Kim MS, Park JT, Kim YW, Lee HS, Nyawira R, Shin HS, Park CS, Yoo SH, Kim YR, Moon TW, Park KH (2004) Appl Environ Microbiol 70:3933
144. Chang ST, Parker KN, Bauer MW, Kelly RM (2001) Methods Enzymol 330:260
145. Galichet A, Belarbi A (1999) FEBS Lett 458:188
146. Xavier KB, Peist R, Kossmann M, Boos W, Santos H (1999) J Bacteriol 181:3358
147. Schicho RN, Ma K, Adams MW, Kelly RM (1993) J Bacteriol 175:1823
148. Costantino HR, Brown SH, Kelly RM (1990) J Bacteriol 172:3654
149. Miura Y, Kettoku M, Kato M, Kobayashi K, Kondo K (1999) J Mol Microbiol Biotechnol 1:129
150. Campbell BS, Siddique AB, McDougall BM, Seviour RJ (2004) FEMS Microbiol Lett 232:225
151. Bertoldo C, Antranikian G (2002) Curr Opin Chem Biol 6:151
152. Bertoldo C, Antranikian G (2001) Methods Enzymol 330:269
153. Dong G, Vieille C, Zeikus JG (1997) Appl Environ Microbiol 63:3577
154. Canganella F, Jones WJ, Gambacorta A, Antranikian G (1997) Arch Microbiol 167:233
155. Duffner F, Bertoldo C, Andersen JT, Wagner K, Antranikian G (2000) J Bacteriol 182:6331
156. Erra-Pujada M, Debeire P, Duchiron F, O'Donohue MJ (1999) J Bacteriol 181:3284
157. Niehaus F, Peters A, Groudieva T, Antranikian G (2000) FEMS Microbiol Lett 190:223
158. Knapp S, Rudiger A, Antranikian G, Jorgensen PL, Ladenstein R (1995) Proteins 23:595
159. Rudiger A, Jorgensen PL, Antranikian G (1995) Appl Environ Microbiol 61:567
160. Schwerdtfeger RM, Chiaraluce R, Consalvi V, Scandurra R, Antranikian G (1999) Eur J Biochem 264:479
161. Zona R, Chang-Pi-Hin F, O'Donohue MJ, Janecek S (2004) Eur J Biochem 271:2863
162. Kang S, Vieille C, Zeikus JG (2004) Appl Microbiol Biotechnol CE[h]
163. Kim CH, Nashiru O, Ko JH (1996) FEMS Microbiol Lett 138:147
164. Bertoldo C, Duffner F, Jorgensen PL, Antranikian G (1999) Appl Environ Microbiol 65:2084
165. Lebbink JH, Bertoldo C, Tibbelin G, Andersen JT, Duffner F, Antranikian G, Ladenstein R (2000) Acta Crystallogr D Biol Crystallogr 56 (Pt 11):1470
166. Koch R, Canganella F, Hippe H, Jahnke K, Antranikian G (1997) Appl Environ Microbiol 63:1088
167. Bibel M, Brettl C, Gosslar U, Kriegshauser G, Liebl W (1998) FEMS Microbiol Lett 158:9
168. Bertoldo C, Armbrecht M, Becker F, Schafer T, Antranikian G, Liebl W (2004) Appl Environ Microbiol 70:3407
169. Wind RD, Liebl W, Buitelaar RM, Penninga D, Spreinat A, Dijkhuizen L, Bahl H (1995) Appl Environ Microbiol 61:1257
170. Knegtel RM, Wind RD, Rozeboom HJ, Kalk KH, Buitelaar RM, Dijkhuizen L, Dijkstra BW (1996) J Mol Biol 256:611

CE[h] Please complete reference

Editor's or typesetter's annotations (will be removed before the final TEX run)

171. Tachibana Y, Kuramura A, Shirasaka N, Suzuki Y, Yamamoto T, Fujiwara S, Takagi M, Imanaka T (1999) Appl Environ Microbiol 65:1991
172. Biwer A, Antranikian G, Heinzle E (2002) Appl Microbiol Biotechnol 59:609
173. Cowan DA, Smolenski KA, Daniel RM, Morgan HW (1987) Biochem J 247:121
174. Guagliardi A, Cerchia L, Rossi M (2002) Biochem J 368:357
175. Burlini N, Magnani P, Villa A, Macchi F, Tortora P, Guerritore A (1992) Biochim Biophys Acta 1122:283
176. Mayr J, Lupas A, Kellermann J, Eckerskorn C, Baumeister W, Peters J (1996) Curr Biol 6:739
177. Kannan Y, Koga Y, Inoue Y, Haruki M, Takagi M, Imanaka T, Morikawa M, Kanaya S (2001) Appl Environ Microbiol 67:2445
178. Morikawa M, Imanaka T (2001) Methods Enzymol 330:424
179. Volkl P, Markiewicz P, Stetter KO, Miller JH (1994) Protein Sci 3:1329
180. Chang LS, Hicks PM, Kelly RM (2001) Methods Enzymol 330:403
181. Morikawa M, Izawa Y, Rashid N, Hoaki T, Imanaka T (1994) Appl Environ Microbiol 60:4559
182. Klingeberg M, Galunsky B, Sjoholm C, Kasche V, Antranikian G (1995) Appl Environ Microbiol 61:3098
183. Chavez Croocker P, Sako Y, Uchida A (1999) Extremophiles 3:3
184. Sako Y, Croocker PC, Ishida Y (1997) FEBS Lett 415:329
185. Catara G, Ruggiero G, La Cara F, Digilio FA, Capasso A, Rossi M (2003) Extremophiles 7:391
186. Voorhorst WG, Eggen RI, Geerling AC, Platteeuw C, Siezen RJ, Vos WM (1996) J Biol Chem 271:20 426
187. de Vos WM, Voorhorst WG, Dijkgraaf M, Kluskens LD, Van der Oost J, Siezen RJ (2001) Methods Enzymol 330:383
188. Voorhorst WG, Warner A, de Vos WM, Siezen RJ (1997) Protein Eng 10:905
189. Choi IG, Bang WG, Kim SH, Yu YG (1999) J Biol Chem 274:881
190. Lin X, Fusek M, Tang J (1991) Adv Exp Med Biol 306:255
191. Fusek M, Lin XL, Tang J (1990) J Biol Chem 265:1496
192. Lin X, Tang J (1990) J Biol Chem 265:1490
193. Fujiwara S, Okuyama S, Imanaka T (1996) Gene 179:165
194. Friedrich A, Antranikian G (1996) Appl Environ Microbiol 62:2875
195. Kim JS, Kluskens LD, de Vos WM, Huber R, van der Oost J (2004) J Mol Biol 335:787
196. Kluskens LD, Voorhorst WG, Siezen RJ, Schwerdtfeger RM, Antranikian G, van der Oost J, de Vos WM (2002) Extremophiles 6:185
197. Lesuisse E, Schanck K, Colson C (1993) Eur J Biochem 216:155
198. Sinchaikul S, Sookkheo B, Phutrakul S, Pan FM, Chen ST (2001) Protein Expr Purif 22:388
199. Cho AR, Yoo SK, Kim EJ (2000) FEMS Microbiol Lett 186:235
200. Lee D, Koh Y, Kim K, Kim B, Choi H, Kim D, Suhartono MT, Pyun Y (1999) FEMS Microbiol Lett 179:393
201. Tyndall JD, Sinchaikul S, Fothergill-Gilmore LA, Taylor P, Walkinshaw MD (2002) J Mol Biol 323:859
202. Crabb WD, Shetty JK (1999) Curr Opin Microbiol 2:252
203. Park BC, Koh S, Chang C, Suh SW, Lee DS, Byun SM (1997) Appl Biochem Biotechnol 62:15
204. Liu SY, Wiegel J, Gherardini FC (1996) J Bacteriol 178:5938
205. Vieille C, Hess JM, Kelly RM, Zeikus JG (1995) Appl Environ Microbiol 61:1867
206. Vieille C, Sriprapundh D, Kelly RM, Zeikus JG (2001) Methods Enzymol 330:215

207. Hess JM, Kelly RM (1999) Biotechnol Bioeng 62:509
208. Hess JM, Tchernajenko V, Vieille C, Zeikus JG, Kelly RM (1998) Appl Environ Microbiol 64:2357
209. Cowan D (1996) Trends Biotech 14:177
210. Burdette DS, Vieille C, Zeikus JG (1996) Biochem J 316 (Pt 1):115
211. Burdette DS, Jung SH, Shen GJ, Hollingsworth RI, Zeikus JG (2002) Appl Environ Microbiol 68:1914
212. Burdette DS, Tchernajencko VV, Zeikus JG (2000) Enzyme Microb Technol 27:11
213. Burdette DS, Secundo F, Phillips RS, Dong J, Scott RA, Zeikus JG (1997) Biochem J 326 (Pt 3):717
214. Yoon SY, Noh HS, Kim EH, Kong KH (2002) Comp Biochem Physiol B Biochem Mol Biol 132:415
215. Kong KH, Hong MP, Choi SS, Kim YT, Cho SH (2000) Biotechnol Appl Biochem 31 (Pt 2):113
216. Abokitse K, Hummel W (2003) Appl Microbiol Biotechnol 62:380
217. Ma K, Adams MW (2001) Methods Enzymol 331:195
218. Li D, Stevenson KJ (2001) Methods Enzymol 331:201
219. Zhang J, Liu J, Zhou J, Ren Y, Dai X, Xiang H (2003) Biotechnol Lett 25:1463
220. Suzuki Y, Miyamoto K, Ohta H (2004) FEMS Microbiol Lett 236:97
221. Ikeda M, Clark DS (1998) Biotechnol Bioeng 57:624
222. Labahn J, Neumann S, Buldt G, Kula MR, Granzin J (2002) J Mol Biol 322:1053
223. Chebrou H, Bigey F, Arnaud A, Galzy P (1996) Biochim Biophys Acta 1298:285
224. Kobayashi M, Komeda H, Nagasawa T, Nishiyama M, Horinouchi S, Beppu T, Yamada H, Shimizu S (1993) Eur J Biochem 217:327
225. Ciskanik l, Wilczek JM, Fallon RD (1995) Appl Environ Microbiol 61:998
226. Trott S, Bauer R, Knackmuss HJ, Stolz A (2001) Microbiology 147:1815
227. Mayaux J, Cerbelaud E, Soubrier F, Yeh P, Blanche F, Petre D (1991) J Bacteriol 173:6694
228. Hirrlinger B, Stolz A, Knackmuss HJ (1996) J Bacteriol 178:3501
229. Layh N, Hirrlinger B, Stolz A, Knackmuss HJ (1997) Appl Microbiol Biotechnol 47:668
230. Hermes H, Tandler RF, Sonke T, Dijkhuizen L, Meijer EM (1994) Appl Environ Microbiol 1:153
231. Sonke T, Kaptein B, Boesten WHJ, Boxterman QB, Schoemaker HE, Kamphuis J, Formaggio F, Toniolo C, Rutjes FP (2000) In: RN P (ed) Stereoselective Biocatalysis. Marcel Dekker, New York p 23
232. Kamphuis J, Boesten WHJ, Kaptein B, Hermes HFM, Sonke T, Broxterman QB, van den Tweel WJJ, Schoemaker HE (1992) in: Collins AN, Sheldrake GN, Crosby J (ed) Chirality in industry: the commercial manufacture and applications of optically active compounds. CE[1] p 187
233. Alcantara A-R, Sanchez-Montero JM, Sinisterra JV (2000) In: RN P (ed) Stereoselective biocatalysis. Marcel Dekker, New York p 659
234. Webster N, Ramsden DK, Hughes J (2001) Biotechnol Lett 23:95
235. Fournand D, Arnaud A, Galzy P (1998) J Mol Catal 4:77
236. Baek DH, Song JJ, Lee SG, Kwon SJ, Asano Y, Sung MH (2003) Enzyme Microb Technol 32:131
237. Nawaz M, Khan AA, Bhattacharayya D, Siitonen PH, Cerniglia CE (1996) J Bacteriol 178:2397
238. d'Abusco AS, Ammendola S, Scandurra R, Politi L (2001) Extremophiles 5:183
239. Egorova K, Trauthwein H, Verseck S, Antranikian G (2004) Appl Microbiol Biotech-

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Editor's or typesetter's annotations (will be removed before the final TEX run)

nol 65:38
240. Mahadevan S, Thimann KV (1964) Arch Biochem Biophys 107:62
241. Thimann KV, Mahadevan S (1964) Arch Biochem Biophys 105:133
242. Hook RH, Robinson WG (1964) J Biol Chem 239:4263
243. Robinson WG, Hook RH (1964) J Biol Chem 239:4257
244. Arnaud A, Galzy P, Jallageas JC (1976) C R Acad Sci Hebd Seances Acad Sci D 283:571
245. Harper DB (1977) Biochem J 167:685
246. Harper DB (1977) Biochem J 165:309
247. Wajant H, Effenberger F (2002) Eur J Biochem 269:680
248. Pekarsky Y, Campiglio M, Siprashvili Z, Druck T, Sedkov Y, Tillib S, Draganescu A, Wermuth P, Rothman JH, Huebner K, Buchberg AM, Mazo A, Brenner C, Croce CM (1998) Proc Natl Acad Sci USA 95:8744
249. Pace HC, Hodawadekar SC, Draganescu A, Huang J, Bieganowski P, Pekarsky Y, Croce CM, Brenner C (2000) Curr Biol 10:907
250. Harper DB (1985) Int J Biochem 17:677
251. Stevenson DE, Feng R, Dumas F, Groleau D, Mihoc A, Storer AC (1992) Biotechnol Appl Biochem 15:283
252. Nagasawa T, Mauger J, Yamada H (1990) Eur J Biochem 194:765
253. Kobayashi M, Komeda H, Yanaka N, Nagasawa T, Yamada H (1992) J Biol Chem 267:20 746
254. Kobayashi M, Yanaka N, Nagasawa T, Yamada H (1992) Biochemistry 31:9000
255. Banerjee A, Sharma R, Banerjee UC (2002) Appl Microbiol Biotechnol 60:33
256. Perler FB, Kumar S, Kong H (1996) Adv Protein Chem 48:377
257. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H (1992) Biotechnology 24:17
258. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Science 239:487
259. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H (1986) Cold Spring Harb Symp Quant Biol 51 Pt 1:263
260. Diaz RS, Sabino EC (1998) Braz J Med Biol Res 31:1239
261. Komori K, Ishino Y (2000) Protein Eng 13:41
262. Evans SJ, Fogg MJ, Mamone A, Davis M, Pearl LH, Connolly BA (2000) Nucleic Acids Res 28:1059
263. Hayashi I, Morikawa K, Ishino Y (1999) Nucleic Acids Res 27:4695
264. Goldman S, Kim R, Hung LW, Jancarik J, Kim SH (1998) Acta Crystallogr D Biol Crystallogr 54 (Pt 5):986
265. Lu C, Erickson HP (1997) Protein Expr Purif 11:179
266. Uemori T, Sato Y, Kato I, Doi H, Ishino Y (1997) Genes Cells 2:499
267. Imamura M, Uemori T, Kato I, Ishino Y (1995) Biol Pharm Bull 18:1647
268. Uemori T, Ishino Y, Toh H, Asada K, Kato I (1993) Nucleic Acids Res 21:259
269. Ogata N, Miura T (2000) Biochemistry 39:13 993
270. Ogata N, Miura T (1998) Nucleic Acids Res 26:4652
271. Keohavong P, Ling L, Dias C, Thilly WG (1993) PCR Methods Appl 2:288
272. Kong H, Kucera RB, Jack WE (1993) J Biol Chem 268:1965
273. Mattila P, Korpela J, Tenkanen T, Pitkanen K (1991) Nucleic Acids Res 19:4967
274. Cariello NF, Swenberg JA, Skopek TR (1991) Nucleic Acids Res 19:4193
275. Hashimoto H, Nishioka M, Fujiwara S, Takagi M, Imanaka T, Inoue T, Kai Y (2001) J Mol Biol 306:469
276. Hashimoto H, Matsumoto T, Nishioka M, Yuasa T, Takeuchi S, Inoue T, Fujiwara S, Takagi M, Imanaka T, Kai Y (1999) J Biochem (Tokyo) 125:983

277. Nishioka M, Fujiwara S, Takagi M, Imanaka T (1998) Nucleic Acids Res 26:4409
278. Takagi M, Nishioka M, Kakihara H, Kitabayashi M, Inoue H, Kawakami B, Oka M, Imanaka T (1997) Appl Environ Microbiol 63:4504
279. Sanger F, Nicklen S, Coulson AR (1992) Biotechnology 24:104
280. Bechtereva TA, Pavlov YI, Kramorov VI, Migunova B, Kiselev OI (1989) Nucleic Acids Res 17:10 507
281. Rao VB, Saunders NB (1992) Gene 113:17
282. Vander Horn PB, Davis MC, Cunniff JJ, Ruan C, McArdle BF, Samols SB, Szasz J, Hu G, Hujer KM, Domke ST, Brummet SR, Moffett RB, Fuller CW (1997) Biotechniques 22:758
283. Szybalski W (1990) Gene 90:177
284. Schalling M, Hudson TJ, Buetow KH, Housman DE (1993) Nat Genet 4:135
285. Landegren U, Schallmeiner E, Nilsson M, Fredriksson S, Baner J, Gullberg M, Jarvius J, Gustafsdottir S, Dahl F, Soderberg O, Ericsson O, Stenberg J (2004) J Mol Recognit 17:194
286. Georlette D, Blaise V, Bouillenne F, Damien B, Thorbjarnardottir SH, Depiereux E, Gerday C, Uversky VN, Feller G (2004) Biophys J 86:1089
287. Housby JN, Southern EM (2002) Anal Biochem 302:88
288. Jonsson ZO, Thorbjarnardottir SH, Eggertsson G, Palsdottir A (1994) Gene 151:177
289. Thorbjarnardottir SH, Jonsson ZO, Andresson OS, Kristjansson JK, Eggertsson G, Palsdottir A (1995) Gene 161:1