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Harnessing the Potential of Thermophiles: The Variants of Extremophiles

Madan Lal Verma[1,2] • Shamsher Singh Kanwar[1*]

[1] Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla-171 005, India
[2] Present address: Centre for Biotechnology and Interdisciplinary Sciences, Institute for Technology Research and Innovation, Deakin University, Victoria 3217, Australia
Corresponding author: * **kanwarss2000@yahoo.com**

ABSTRACT

Extremophiles are life-forms that thrive under some of the harshest conditions found on earth. In recent years, extremophiles have been discovered in such inhospitable places as active volcanoes, deep sea vents, and the ultra-saline remnants of extinct inland seas. Extensive studies of extremophiles ecology, physiology, and molecular biology have yielded valuable information about life processes at every level, with a number of important industrial applications. This review articles summarize the most outstanding features of thermophilic microorganisms that can survive under extreme conditions. The latest findings on the thermophiles, the protein structure of these exotic organisms, potential applications of extremophiles in biotechnology industries including the production of enzymes, are discussed in the present research study.

Keywords: applications, enzyme, function, stability, structure, temperature

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INTRODUCTION

The term 'extremophiles' refers to bacteria/archaea that are able to exist and thrive in environments that are extremely harsh, in terms of those environments classically envisioned as hospitable to the growth of bacteria. The vast majority of extremophiles are microbes, representing numerous and diverse genetic lineages from across the three domains of life: Bacteria, Archaea and Eukarya. Organisms that live at the extremes of pH (> 8.5, < 5.0), temperature (> 45°C, < 15°C), pressure (> 500 atmospheres), salinity (> 1.0 M NaCl) and in high concentrations of recalcitrant substances or heavy metals (extremophiles) represent one of the last frontiers for biotechnological and industrial discovery (Cavicchioli *et al.* 2011; Kumar *et al.* 2011; Wackett 2011). The discovery of extremophiles, beginning in the 1970s, has had three major influences on the biotechnology industry (Rothschild and Mancinelli 2001; Satyanarayana *et al.* 2005; Rampelotto 2010; Atomi *et al.* 2011).

Firstly, the discovery of bacteria growing in environments such as the hot springs of Yellowstone National Park and around the hydrothermal vents located on the ocean

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Invited Review

Table 1 Applications of extremophiles in biotechnology.

Industrial process	Extremozyme	Salient feature	Source organism
Hydrolysis of starch to produce soluble dextrins, maltodextrins and corn syrup	α-Amylase	High stability, aciduric, bacterial, amylase	*Bacillus stearothermophilus*
Paper bleaching	Xylanases	Decreases amount of bleach needed	Thermophiles
Prevent stalling in range of baked products	α-Amylase	Gives boost to yeast fermentation	*Bacillus stearothermophilus*
Food processing, baking, brewing, detergents	Proteases	Stable at high temperatures	Thermophiles
Cheese maturation, dairy production	Neutral proteases	Stable at low temperatures	Psychrophiles
Degradation of polymers in detergents	Proteases, amylases, lipases	Improved performance of detergent	Psychrophiles
Degradation of polymers in detergents	Cellulases, proteases, amylases, lipases	Stable at high pH	Alkaliphiles
Mariculture	Polyunsaturated fatty acids	Produced in cold temperatures	Psychrophiles
Bioremediation	Reduction of oil spills	Works efficiently in cold waters	Psychrophiles
Pharmaceuticals	Polyunsaturated fatty acids		Psychrophiles
Biosensors	Dehydrogenases		Psychrophiles
Desulphurication of coal	Sulphur oxidation		Acidophiles
Antibiotic production	Antibiotics		Alkaliphiles
Food colouring	Carotene	Inexpensive to produce	Halophiles/*Dunaliella*
Pharmaceuticals	Glycerol, compatible solutes	Inexpensive to produce	Halophiles
Surfactants for pharmaceuticals	Membranes		Halophiles

floor (where the bacteria are in fact the fundamental basis of the specialized ecosystem that is fueled by the vents) has greatly increased the awareness of the possibilities for bacterial life on earth and elsewhere. Indeed, the growth of some extremophiles occurs in environments that by all indications could exist on planets such as Mars and other stellar bodies. Thus, extremophilic bacteria might conceivably not be confined to earth (Demirjian *et al.* 2001; Pakchung *et al.* 2006; Cavicchioli *et al.* 2011).

The second major influence of extremophiles has been the broadening of the classification of the evolutionary development of life on Earth. With the advent of molecular means of comparing the genetic sequences of highly conserved regions from various life forms, it became clear that extremophiles were not simply offshoots of bacteria, but rather had diverged from both bacteria and eukaryotic cells early in evolutionary history. Extremophilic bacteria are grouped together in a domain called archaea. Archae share similarities with bacteria and with eukaryotes. Thirdly, extremophiles are continuing to prove to be a rich source of enzymes that are useful in biotechnological processes. The hardiness of the enzymes, such as their ability to maintain function at high temperatures, has been crucial to the development of biotechnology (Burg 2003; Gomes and Steiner 2004; Ratnaparkhe and Tiwari 2011; Xu *et al.* 2011).

In many cases, biotechnological applications of extremophiles and their extremozymes have been the driving force in both academic and industrial research of these organisms. These extremozymes occupy an important place in the multibillion dollar environmental biotechnology industry, with applications spanning agricultural, biomedical and industrial sectors (**Table 1**). In this review, we have focused on the biotechnological applications of extremophiles (Egorova and Antranikian 2005; Atomi 2005; Antranikian *et al.* 2005; Harris *et al.* 2010; Zhang and Kim 2010; Castellano *et al.* 2011; Tayyab *et al.* 2011).

CLASSIFICATION

Extremophiles are classified on the basis of their extreme climate conditions (Stetter 1996; Burg *et al.* 2003). Extreme conditions can refer to physical as well as geochemical extremes (e.g. temperature, pressure, radiation, salinity and pH). Thermophiles can be generally classified into moderate thermophiles (optimum growth optimum 50-60°C), extreme thermophiles (optimum growth optimum 60-80°C) and hyperthermophiles (optimum growth optimum 80-110°C) (Rothschild and Manicinelli 2001; Fujiwara 2002; Haki and Rikshit 2003; Kang *et al.* 2006; Atomi *et al.* 2011).

Psychrophiles are organisms adapted to life at temperatures below 15°C. They are isolated from Antarctica, the Arctic/Antarctic oceans, and the oceanic abyss. Halophiles are organisms that inhabit hypersaline environments (e.g. 2-5 M NaCl). They can be isolated from the Dead Sea, Africa, Europe, and the USA and have even been found in Antarctic lakes. Microorganisms that like high-pressure conditions for growth are termed piezophiles (formerly known as barophiles). With an average pressure of 38 MPa, the world's oceans are home to piezophiles, including various thermophiles and hyperthermophiles (Jenney and Adams 2008). Microorganisms that are highly resistant to high levels of ionizing and ultraviolet radiation are called radiophiles. Acidothermophiles thrive under conditions of low pH and high temperature. For example, the acidothermophile *Sulfolobus solfataricus* grows at pH = 3 and 80°C. Alkalithermophilic microorganisms grow optimally under two extreme conditions: at pH values of 8 or above and at high temperatures i.e. 50-85°C (Khalil 2011).

THERMOPHILES

Thermophilic microorganisms have rekindled attention over the last couple of years. They are amongst the most studied extremophiles (Irwin and Baird 2004; Dheeran *et al.* 2010; Wackett 2011). The stability of the enzymes, such as their ability to maintain function at high temperatures, has been crucial to the development of biotechnology. Such enzymes are of great industrial and biotechnological interest due to the fact that the enzymes are better suited for harsh industrial processes. There are many advantages of conducting industrial processes at high temperature, such as the increased solubility of many polymeric substrates, resulting in decreased viscosity, increased bioavailability, faster reaction rate and the decreased risk of microbial contamination. These proteins have subsequently been derived from hundreds of different organisms, extremophiles and particularly thermophiles have been specifically targeted due to the increased stability, ease of handling of their proteins and tendency to easily form better protein crystals, relative to those from mesophiles. These enzymes have also been used as models for the understanding of thermostability and thermoactivity, which is useful for protein engineering (Sterner and Liebl 2001; Kumar and Nussinov 2001; Paiardini *et al.* 2002; Jenney and Adams 2008; Atomi *et al*. 2011; Wackett 2011).

Thermostable '*Taq* polymerase' enzyme isolated from the extremophile *Thermus aquaticus* is fundamental to the procedures of the polymerase chain reaction (PCR) that has revolutionized biotechnology. More than 50 species of thermophiles have been discovered to date (Hicks and Kelly 2002). Such bacteria tolerate temperatures far above the tolerable limits known for any animal, plant, or other bacteria. Some thermophiles, such as *Sulfolobus acidocaldarius*, are capable of growth and reproduction in water temperatures that exceed 100°C. The most heat-tolerant thermophile known so far is *Pyrolobus fumarii* that grows in the

walls of the hydrothermal vents where temperatures exceed 93°C. In fact, the bacterium requires a temperature above 90°C to sustain growth (Blochl *et al*. 1997).

Representative of thermophilic extremophiles

Among the extreme thermophiles, multi-cellular animals or plants cannot tolerate temperatures above about 50°C and the eukaryotic microbes that have been discovered so far cannot survive at temperatures higher than 60°C (Rothschild and Manicinelli 2001). Extreme thermophiles, growing optimally at 60-80°C, are widely distributed among the genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Rhodothermus*, *Thermotoga* and *Aquifex* (Demijian *et al.* 2001; Vieille and Zeikus 2001; Bertoldo and Antranikian 2002; Haki and Rikshit 2003; Littlechild *et al.* 2007; Levisson *et al.* 2009; Palm *et al.* 2011).

Phylogeny

Thermophiles are more common than hyper-thermophiles. Short branches in the unrooted phylogenetic tree indicate a rather slow clock in the biological evolution and all hyper-thermophiles that can grow at temperatures above 90°C are represented as the deepest and shortest lineage, suggesting that hyper-thermophiles are very primitive and their common ancestor is a hyper-thermophile (Fujiwara 2002; Cava *et al.* 2009). The genome sizes of hyper-thermophiles are comparable to those of mesophiles and are in fact often somewhat smaller. Generally, hyper-thermophiles have a smaller size genome than well-studied mesophiles (Fujiwara *et al.* 1996; Ambily and Loka 2011). The small size genomes of hyper-thermophiles may define the lower limit of their genetic capacity. Chromosomes of hyper-thermophiles are densely packed with genes, most of which are required for essential functions (Fujiwara *et al.* 1998; Jenney and Adams 2008; Cai *et al.* 2011). This indicates that the earliest life forms may have had small genomes.

Structural and functional relationship of thermophilic enzymes

Structural features of thermophilic extremozymes have attracted much attention. One of the most striking features of thermostable enzymes, when compared with mesophilic enzymes, is the decrease in the number of Lys (Lysine) residues, which are mainly replaced with Arg (Arginine) and Glu (Glutamic acid) residues (Shirai *et al.* 1997). Several three-dimensional structures have been resolved and compared with those of mesophilic counterparts, with the ultimate goal of elucidating the mechanisms underlying thermostability (Sterner and Liebl 2001; Jenney and Adams 2008; Burg *et al.* 2011). A large number of sequence and structural factors are thought to contribute toward higher intrinsic thermal stability of proteins from thermophiles. Thermophiles produce special proteins known as 'chaperonins', which are thermostable and resistant to denaturation and proteolysis (Hemmingsen *et al.* 1988). Proteins of thermophiles, denatured at high temperature, are refolded by the chaperonins, thus restoring their native form and function (Paiardini *et al.* 2002; Luke *et al.* 2011). The cell membrane of thermophiles consists of saturated fatty acids, which increase protein core hydrophobicity and keep the cell rigid enough to survive at high temperatures. Moreover, hyper-thermophiles have membranes containing lipids linked with ether to their cell walls. This layer is much more heat resistant than a membrane formed of fatty acids. In addition, proteins of thermophiles have increased surface charge and less exposed thermolabile amino acids. Thus, increased ionic interaction and hydrogen bonds, increased hydrophobicity, decreased flexibility and smaller surface loops confer stability on the thermophilic protein (Unsworth *et al.* 2007; Burg *et al.* 2011).

HYPERTHERMOPHILES

Hyperthermophiles belong to a group of mostly archaea and a few bacteria with an optimum growth temperature of at least 80°C (Takano *et al.* 2011). The majority of the known genera are present in marine origin, continental hot springs and solfataric fields. The most common hot biotopes are associated with tectonically active zones on earth. The terrestrial biotopes are mainly solfataric fields which consist of soils, mud holes and surface waters heated by volcanic exhaustion from magma chambers below. The surface of the so-called "solfataras" is rich in sulfate and is acidic (pH < 4). Marine hydrothermal systems are situated in shallow and abyssal depths and consist of hot fumaroles, springs and deep sea vents with temperatures up to 380°C (Atomi *et al.* 2011). A variety of micro-organisms have been isolated from these exotic habitats such as the black smokers at the bottom of the Pacific Ocean.

Representatives of hyperthermophilic extremophiles

Most of hyperthermophiles belong to the archaea, which consists of four phyla: Crenarchaeota, Euryarchaeota, Korarchaeota and Nanoarchaeota. Some genera belonging to Crenarchaeota are *Sulfolobus*, *Acidianus*, *Pyrodictium*, *Pyrolobus*, *Pyrobaculum*, *Desulfurococcus*, *Thermoproteus*, *Thermofilum* and *Staphylothermus*. Euryarchaeota include extreme halophiles (*e.g. Halobacterium*, *Halobaculum*, *Halococcus*, *Haloferax*, *Halorubrum*), methanogens (*e.g. Methanobacterium*, *Methanosphaera*, *Methanococcus*, *Methanobrevibacter* and *Methanothermus*), extreme acidophiles (*e.g. Picrophilus*, *Thermoplasma*) and extreme thermophiles (*e.g. Thermococcus*, *Pyrococcus*, *Methanopyrus*, *Archaeoglobus* and *Ferroglobus*) (Dheeran *et al.* 2010; Atomi *et al.* 2011). The so-called phylum Korarchaeota has been postulated on the basis of PCR amplification of 16S rRNA genes from environmental DNA, but has not been confirmed by the pure cultivation of any organisms (Barns *et al.* 1996). The novel phylum Nanoarchaeota is represented by the hyperthermophilic anaerobic nano-sized coccus *Nanoarchaeum equitans*. It grows only in co-culture with a new chemolithoautotrophic *Ignicoccus* species (Huber *et al.* 2002, 2003). Among the hyperthermophiles, a strain dubbed strain 121 (Kashefi and Lovely 2003) and *Pyrolobus fumarii* (Blöchl *et al.* 1997), capable of growing at 121 and 113°C, respectively, hold the record for the most thermophilic microorganisms. These strains cannot grow below 90°C. Strain 121 grows chemoautotrophically using formate as an electron donor and Fe^{3+}, while *Pyrolobus fumarii* is a nitrate-reducing chemolithoautotroph.

Phylogeny

Nearly all these microorganisms are classified as Archaea including highly thermophilic organisms such as *Pyrococcus*, *Methanopyrus* and *Pyrobaculum* species which grow at a temperature above 100°C. To date, only two bacterial genera are represented among hyperthermophiles: *Thermotoga* and *Aquifex*. It has been shown by analysis of 16S rRNA that the hyperthermophiles are the most slowly evolving organisms within the archaeal and bacterial domains (Woese *et al.* 1990; Burg *et al.* 2011). Hyperthermophiles may be the closest living descendants of ancestral life forms which evolved under conditions of high temperature. Therefore, hyperthermophiles are generally found close to the root of the phylogenetic tree, which suggests that they preceded their mesophilic counterparts in the course of evolution (Sawle and Ghosh 2011). The maximum growth temperature which has been observed for a hyperthermophile (*Pyrodictium occultum*) is around 110°C (Stetter 1982). It is still unknown whether this temperature represents the upper limit. It should be noted, however, that some of the building blocks of proteins, the amino acids arginine, cysteine, aspartate and glutamate undergo hydrothermal decomposition

which becomes significant at temperatures around 120-130°C (Bernhardt *et al.* 1984). Proteins isolated from hyperthermophiles show considerable thermal stability and seem to become increasingly important for the investigation of fundamental problems in structure biology such as the stability and folding of proteins (Burg *et al.* 2011). Extremely thermostable enzymes are more frequently used in biotechnological applications that require proteins with increased stability at high temperatures (Unsworth *et al.* 2007; Takano *et al.* 2011).

Structural and functional relationship of hyperthermophilic proteins

Hyperthermostable proteins are composed of the common twenty amino acids. Thus all the information needed to create high thermotolerance is encoded in the gene sequence. This thermotolerance is an intrinsic property. Their size, complexity and functional properties are comparable to the analogous proteins from conventional sources, and for those proteins with known amino acid sequence there is usually a high degree of (sequence and structure) homology between the mesophilic and thermophilic versions. The necessary changes, however, are not readily apparent from sequence comparisons of mesophilic and thermophilic proteins. It seems to be quite clear that specific rules for "thermostability" or generalizations can hardly be derived by systematic analyses of the sequences of homologous mesophilic and thermophilic proteins (Jaenicke 1991; Bohm and Jaenicke 1994; Sawle and Ghosh 2011).

Nevertheless, certain amino acid changes do occur and seem to be characteristic for thermostable proteins. The effect of hydrophobic substitutions in the protein core and their influence on stability has been investigated by site-directed mutations (Pace 1992; Nishitani *et al.* 2010). Substitutions of isoleucine with valine residues resulted in less stable mutants: the average effect on the free stabilization energy was AAG = - 1.3 + 0.4 kcal $mo1^{-1}$. The decrease of the free energy of unfolding, however, was not only a result of the reduced hydrophobic contact surface but also dependent on the created cavity volume. In a comparison of the sequences of Glutamate dehydrogenases (GluDH) from *Pyrococcus furiosus* (Pf) (T_m = 105°C) and *Thermotoga maritima* (T_m = 95°C and *Clostridium symbiosum* (T_m = 55°C); Pf GluDH contained 35 isoleucines, whereas only 21 and 20 isoleucines were found in the sequences of Tm and Cs GluDH, respectively. Eleven of the 35 isoleucines in Pf are conserved in T_m; 10 of them resulted from valine to isoleucine mutations and 6 from leucine to isoleucine mutations (Knapp *et al.* 1996; Burg *et al.* 2011). The structure-based sequence comparison on the three GluDHs has furthermore pointed out that the number of glycines is reduced drastically in the two hyperthermostable enzymes: the sequence of Cs GluDH contains 48 glycine residues; the sequences from T_m and Pf contained only 39 and 34 glycines, respectively. In a structure comparison of ferredoxins, it has been found, that residues in strained conformations of the mesophilic members are exchanged with glycines in the thermophilic members (Macebo-Ribeiro *et al.* 1996; Nishitani *et al*. 2010; Palm *et al.* 2011).

Stability of hyperthermophilic protein(s)

The free energy of stabilization of a globular protein either from mesophilic or thermophilic microbes is quite small (~7-15 kcal/mol) which is equivalent to the energy that results from the formation of a few hydrogen bonds, ion pairs or hydrophobic interactions (Pfeil 1986). Hyperthermophilic proteins are therefore not expected to differ greatly from mesophilic proteins. The energetic stabilization of any protein is due to the cumulative effect of a large number of attractive and repulsive interactions at different locations within the molecule. Their contributions to the free energy term are quite large, if taken into account separately.

Their actual superposition in a protein, however, yields only a small difference between big numbers and thus only a marginal stabilization free energy which is attributable to the equivalent of a few non-covalent interactions. The average contributions of a hydrogen bond and a surface ion pair have been estimated to be -1.6 and -1.0 kcal/mol, respectively (Pace *et al.* 1996). Whereas equilibrium studies on chymotrypsin have indicated that the free energy of formation for a buried ion pair is roughly -3 kcal/mol (Fersht 1972). However, the overall stability of a protein with a stability increment per residue, which is one order of magnitude below the thermal energy kT, can only be explained by taking into account high cooperativity of the interactions (Jaenicke 1991). Basic proteins stabilization mechanisms are optimization of atomic packing equivalent to the minimization of the relation surface/volume, optimization of the charge distribution, and minimization of the accessible hydrophobic surface area. It follows that no dramatic changes in stabilization energies can be expected, not even for proteins from the most extreme environments from thermodynamic stability measurements. Rather, a decrease and broadening of the stability maximum, which shifts the T_m to higher values, seems to be characteristic at least for hyperthermophilic small proteins.

Molecular adaptation to extreme temperatures depends in the first line only on mutational changes of the local and global distribution of the amino acids in the sequence of a protein molecule. However, in the second line, stabilization by extrinsic factors, like compatible solutes, the high protein concentration in a cell and the action of chaperonins seems to be of considerable importance, too. There is a third line of importance, suggested by the observation that a charge-charge interaction. Its actual strength, depending mainly on the distance of the charges, the solvent accessibility and the electrostatic field produced by the overall charge distribution, can be a target for optimization in a protein (Spassov *et al.* 1994).

Theoretical and experimental analyses of proteins have shown that the 3D structure of a folded protein and the resulting properties, including thermostability, are a result of the delicate balance of different interactions: van der Waals interactions, hydrogen bonds, charge-charge interactions, protein-solvent interactions (hydrophobic effect). Comparisons of amino acid sequences (Argos *et al.* 1979) and of 3D structures have suggested that thermal stability is largely achieved by an additive series of small improvements at many locations in the macromolecule without significant changes in the tertiary structure. Their overall effect is to increase the fraction of buried hydrophobic residues, to optimize charge-charge interactions by removal of repulsive interactions and possibly organizing them in networks and to improve packing by decreasing the ratio of surface area to volume (Stellwagen and Wilgus 1978). Protein-solvent interactions, in particular the hydrophobic effect, are considered to be important factors responsible for protein stability and represent presumably the most important part of the driving force for protein folding and protein stability (Ponnuswamy 1993; Privalov and Makhadatze 1993).

IMPACT OF THERMOSTABLE ENZYMES ON BIOTECHNOLOGY INDUSTRY

Various kinds of thermostable enzymes are required by industries (**Tables 2, 3**). The biotechnology industries are the major users of thermostable enzymes (Vieille and Zeikus 2001; Bertoldo and Antranikian 2002; Irwin and Baird 2004; Barnard *et al.* 2010). To date, a large number of polysaccharide degrading enzymes (*e.g.* cellulases, amylases, pullulanases, xylanases, mannanase, pectinases and chitinases), proteases, lipases, esterases and phytases have been characterized from extremely thermophilic microorganisms (Yeoman *et al.* 2010; Takemasa *et al.* 2011).

Thermostable DNA polymerases, isolated from hyperthermophiles, have led to a tremendous advance in molecular biology due to their capacity to amplify DNA, in the

Table 2 Production of extremozymes by thermophiles.

Thermophiles	Thermophilic enzymes	*T*opt/°C	Stability[#]	Reference
Bacillus cereus	Proteases	50	90°C/40 min/75°C	Sidler *et al*. 1986
B. thuringiensis	Protease	70	50°C/30 min/60°C	Kuniatate *et al*. 1989
Thermomonospora fusca	Xylanases	70	90°C/60 min/70°C	McCarthy *et al.* 1985
Thermomonospora chromogena	Xylanases	75	98°C/60 min/70°C	McCarthy *et al.* 1985
Clostridium stercorarium	Xylanases	75	50ºC/90 min/80°C	Berenger *et al.* 1985
Sporotrichum thermophile	Polygalacturonate hydrolase	55	65°C/4 h	Kaur *et al.* 2004
Bacillus subtilis	α-Amylase	50	60°C/1 h/55°C	Asgher *et al.* 2007
Thermoanaerobacter thermohydrosulfuricus SOL1 and *Caldanaerobacter subterraneus*	Lipase	70	75°C/48 h/70°C	Royter *et al*. 2009
Bacillus cereus	Lipase	55	55°C /50 min/55°C	Verma *et al.* 2010

[#] Stability at stated temperature for a given time, and the optimal temperature for growth.

Table 3 Production of extremozymes by hyperthermophiles.

Hyperthermophiles	Thermophilic enzymes	*T*opt/°C	Stability	Reference
Alicyclobacillus acidocaldarius	Endoglucanase (CelB)	80	Retains 60% activity after 1 h at 80°C	Eckert and Schneider 2003
Rhodothermus marinus	β-1,4-Endoglucanase (β-1,(3)4-Endoglucanase	90°C	$t_{1/2}$ (90°C): 3 h, T_m at 120°C	Turner *et al.* 2007
Thermus thermophilus	Mn-superoxide dismutase	90°C	Retains 57% activity after 1 h at 100°C	Liu et al. 2011
Bacillus cereus	Lipase	80°C	Retains activity after 30 min at 80°C	Akanbi *et al.* 2010
Environmental DNA	β-Xylanase	100	Stable at 90°C	Sunna and Bergquist 2003
Methanococcus jannaschii	α-Amylase	120	Stable against denaturants	Kim *et al.* 2001
Pyrobaculum calidifontis	Carboxylesterase	90	½ life: 2 h at 100°C	Hotta *et al.* 2002
Pyrococcus furiosus	Chitinase a and b	90-95	NA	Gao *et al.* 2003
Rhodothermus marinus	Amylase	85	½ life: 3 h at 85°C	Gomes *et al*. 2003
	Pullulanase	80	30 min at 85°C	Gomes *et al.* 2003
	α-L-Arabinofuranosidase	85	8.3 h at 85°C	Gomes *et al*. 2000
	β-Mannanase	85	45.3 h at 85°C	Gomes and Steiner 1998
Sulfolobus solfataricus	Xylanase	100	½ life: 47 min at 90°C	Cannio *et al.* 2004
Sulfolobus solfataricus	α-Glucosidase	120	Highly thermostable (whole cells used)	Giuliano *et al.* 2004
Sulfolobus shibatae	Glucosidase	98	Retained 67% activity after 5 h at 80°C	Woosowska and Synowiecki 2004
Thermococcus litoralis	L-Aminoacylase	85	½ life: 25 h at 70°C, 1.7 h at 85°C	Taylor *et al.* 2004
Thermoplasma acidophilum	Glucoamylases	90	½ life: 24 h at 90°C for *P. torridus* and *T. acidophilum*, 20 h for *P. oshimae*	Serour and Antranikian 2002
Picrophilus torridus		90		
Picrophilus oshimae		90		

so-called polymerase chain reaction (Rothschild and Manicinelli 2001; Fujiwara 2002).

MOLECULAR ADAPTATION OF THERMOPHILES TO EXTREME TEMPERATURES

It is well established from the experimental data that proteins from psychro- or thermophilic organisms do not exhibit any peculiarities with respect to composition, size, or complexities as compared to their counterparts adapted to moderate temperatures. Extremophilic temperature adaptation is therefore an intrinsic property of the proteins, and the reasons for the striking differences observed both in stability and function at different temperatures are encoded in their gene sequences (Atomi *et al.* 2011). A number of studies have therefore been carried out in attempts to elucidate the molecular causes for their high thermal stability to correlate different structural factors, at all levels of the structural hierarchy of the proteins, to differences in temperature-adaptive properties (mainly thermal stability) in comparative studies of homologous proteins from organisms isolated from different temperature habitats.

Thermophiles are either bacteria or Archaea, and most hyperthermophiles are Archaea (Vielle and Zeikus 1996; Sawle and Ghosh 2011). In earlier comparative studies on thermostabilization, attempts were made to correlate structural factors based on the content of certain amino acids and derived parameters such as hydrophobic and aliphatic indices (Kristjansson and Kinsella 1991), to thermal stability of relatively small number of meso- and thermophilic proteins. Argos *et al.* (1979) carried out the first systematic study on sequence comparison of homologous proteins from meso- and thermophiles and identified certain amino acid exchanges that occurred more frequently in going from the meso- to thermophilic representative by compared the three protein families. These studies has been done to identify certain "traffic rules" used for thermostabilization of proteins in nature (Mendez-Arias and Argos 1991). The effect of the thermostabilizing amino acid exchanges identified in these studies showed a tendency toward increasing surface: hydrophilicity, decreased flexibility, and increased hydrophobicity, mainly in α-helical regions of the proteins (Argos *et al.* 1979). These include higher content of charged amino acids, especially Arg and Glu in hyperthermophiles, often occurring concomitantly with lower content of polar uncharged amino acids (Vogt and Argos 1997; Deckert *et al.* 1998; Haney *et al.* 1999; Szilagyi and Zavodsky 2000; Atomi *et al.* 2011).

A similar trend was observed in sequences deduced from the complete genome of the hyperthermophilic bacterium *Aquifex aeolicus* (Deckert *et al.* 1998) and 12 mesophiles (Chakravarty and Varadarajan 2000). Moreover, Asparagine (Asn) and Glutamine (Gln) seem to be more discriminated against in proteins from hyperthermophiles, as compared to those from moderate thermophiles, while the decrease in Serine (Ser) was apparently significant in moderate thermophiles (Szilagyi and Zavodsky 2000). The trend to lower content of Asn and Gln likely reflects molecular adaptation in thermophilic proteins as it would tend to minimize deamidation, a reaction that may limit the thermal stability of the proteins as a result of irreversible denaturation (Klibanov and Ahern 1987). A decrease in Cysteine (Cys), another thermolabile amino acid residue, has also been reported for thermophilic proteins when compared to their mesophilic counterparts, which may also be indicating an evolutionary pressure to reduce the number of residues that can cause irreversible thermal denaturation of the proteins (Vielle *et al.* 1996).

Some of the most frequent amino acid replacements that lead to a decrease in polar uncharged residues in the proteins of *Methanococcus jannaschii* contribute significantly to increased hydrophobicity (Ser → Ala (Alanine), Thr

(Threonine) → Ile (Isoleucine), Ser → Pro (Proline), Thr → Val (Valine)). Moreover, most frequent nonpolar to nonpolar replacements (*e.g.*, Leu (Leucine) → Ile, Gly (Glycine) → Ala, and Met (Methionine) → Leu) tended toward bulkier and more hydrophobic residues (Haney *et al.* 1999). The hydrophobic interaction has been emphasized as the major stabilizing force for the folded state of proteins (Honig and Yang 1995). Strengthening of hydrophobic interactions and improved packing in the hydrophobic core has indeed been suggested to play an important stabilizing role in thermophilic proteins (Ladenstein and Antranikian 1998). Mutation studies have shown that increasing the bulkiness of buried nonpolar side chains can significantly increase thermal stability of proteins (by ~1.3 kcal mol^{-1} per $–CH_2–$ residue buried), by a direct hydrophobic effect and by reducing the size of cavities, and hence optimized core packing through, e.g., more favorable van der Waals interactions (Eriksson *et al.* 1992; Lipscomb *et al.* 1998; Malhauskas and Mayo 1998). Indeed, increased residue volume (Haney *et al.* 1999) and smaller cavities (Szilagyi and Zavodsky 2000) have been found to correlate well with hyperthermophilic adaptation.

The increased number of high-resolution crystal structures has allowed more direct structural comparisons, where 3D structures of thermophilic proteins are being compared to corresponding structures of one or more of its mesophilic counterparts (Tahirov *et al.* 1998; Chang *et al.* 1999; Knapp *et al.* 1999; Jenney and Adams 2008; Sawle and Ghosh 2011). More detailed structural data have also facilitated systematic studies where various structural parameters have been calculated on the basis of known crystal structures or computer generated homology models from related sequences, and compared within or across protein families (Xiao and Honig 1999; Kumar *et al.* 2000; Ambily and Loka 2011).

Additional hydrogen bonds have also been pointed out as a major stabilizing factor from comparisons of known crystal structures of meso- and thermophiles (Tanner *et al.* 1996; Wallon *et al.* 1997). An increase in the fractional polar surface area, reflecting added hydrogen bonding density to water, was also found to increase with the thermostability of the proteins compared. In other extensive comparative studies of meso- and thermophilic protein structures, an increase in the number of residues involved in hydrogen bonding and an increase in side chain-side chain hydrogen bonds (Xiao and Honig 1999) were found to correlate with thermostability. The most significant difference between meso- and thermophilic proteins was found in the number of ion pairs (salt bridges), a property that also correlated well with growth temperatures.

TEMPERATURE ADAPTATION BY SITE-DIRECTED MUTAGENESIS AND DIRECTED EVOLUTION

Site-directed mutagenesis is often employed in tandem with rational design ideas in order to understand the functioning of enzymes. Theories derived from the study of a three-dimensional model of a particular enzyme are utilized to select particular residues for mutation and predict the possible outcome. Thus, experiments with T4 lysozyme showed that even a single mutation in the hydrophobic core of an enzyme can have dramatic effects on stability (Eriksson *et al.* 1993). The thermostability is not systematically inversely related to specific activity using site-directed mutagenesis. The enzyme subtilisin excreted by an Antarctic *Bacillus* TA39 displays the usual characteristics of cold-active enzymes *i.e.* a high catalytic efficiency at low temperatures and an increased thermosensitivity (Narinx *et al.* 1998). The affinity for calcium is quite lower (~ 3 orders of magnitude) than that of mesophilic subtilisins. An important stabilization of the molecular structure was achieved through a modification of one residue acting as a calcium ligand. The thermostability of the mutated product expressed in a mesophilic *Bacillus* reached that of mesophilic subtilisin, and this mutation further enhanced the specific activity (~ 2 fold) when compared to the wild-type enzyme. Single-base mutations do not cover a large fraction of protein sequence space since they often produce conservative substitutions. Directed evolution is another approach that increases non-conservative substitutions in which rapid screening procedures are combined with random mutagensis and *in vitro* recombination (Cava *et al.* 2009; Ambily and Loka 2011). Structural analysis of selected mutants can bring about understanding of the observed phenotype, be it stability or catalytic activity, in terms of chemical function. The vast majority of possible evolutionary paths lead to poorer enzymes, so for successful directed evolution, the strategic challenge is to choose the right path that will eventually improve the desired features (Kuchner and Arnold 1997).

A moderately stable thermolysin-like metalloprotease from *Bacillus stearothermophilus* was made hyperstable by a limited number of mutations. An eightfold mutant enzyme had a half-life of 2.8 h at 100°C, but still displayed wild-type-like activity at 37°C (Eijsink *et al.* 1995). Subtilisin E from a mesophilic *Bacillus subtilis* was converted into an enzyme functionally equivalent to its thermophilic counterparts by directed evolution (Zhao and Arnold 1999). Subtilisin E differs from thermitase at 157 amino acid positions. However, only eight amino acid substitutions were sufficient to convert subtilisin E into an enzyme equally thermostable, a result from five generations of DNA alterations. Interestingly, the eight substitutions were distributed over the surface of the enzyme.

In another study, thermoresistance was engineered into bacterial β-glucosidase by a directed evolution strategy (Gonzalez-Blasco *et al.* 2000), whereas no significant alterations in kinetic parameters were observed. The main factors for increasing the thermostability were a combination of one extra salt bridge, replacement of a solvent exposed asparagine residue, stabilization of the hydrophobic core, and stabilization of the quaternary structure. An earlier study used *in vitro* evolution to probe the relationship between stability and activity in a mesophilic esterase. Six generations of random mutagenesis, recombination, and screening, stabilized *Bacillus subtilis p*-nitrobenzyl esterase significantly without compromising its catalytic activity at lower temperatures. The results suggested that stability at high temperatures was not incompatible with high catalytic activity at low temperatures because of perceived mutually exclusive demands on enzyme flexibility (Giver *et al.* 1998).

DNA shuffling was used to mutate indoleglycerol phosphate synthase from the hyperthermophile *Sulfolobus solfataricus* (Merz *et al.* 2000). The parental enzyme's turnover number at room temperature was limited by the dissociation of the enzyme-product complex, apparently because the loops that obstruct the active site were not flexible enough at low temperatures. In the variants, the binding and release of product was much more rapid, and this shifted the rate-determining step to a preceding chemical step. Similarly, multiple random mutants of β-glucosidase from the hyperthermophile *Pyrococcus furiosus* were screened for increased activity at room temperature (Lebbink *et al.* 2000). Multiple variants were identified with up to threefold increased rates of substrate hydrolysis. Amino acid substitutions were widespread, occurring in the active-site region, at the enzyme surface, buried in the interior of the monomers, and at subunits interfaces. Single amino acid substitutions were sufficient to drastically alter the kinetic properties, as would be expected, if evolutionary processes are to work. The enzyme was able to accommodate in its interior amino acids with larger or smaller side chains, and with different properties without affecting thermostability. Substrate specificity was also determined by substitutions distant to the active site. It was concluded that enzymes most likely reflects the effects of evolution rather than any intrinsic physico-chemical limitations on proteins (Miyazaki *et al.* 2000). However, the dependence of slower thermal inactivation on calcium concentration indicated that enhanced calcium binding was largely responsible for the increased stability.

APPLICATIONS OF THERMOPHILES/ HYPER-THERMOPHILES IN BIOTECHNOLGY INDUSTRIES

It is generally advantageous to run industrial processes at elevated temperatures, as long as sensitive components in the reaction are not damaged under such conditions. So many of the biotechnological processes involving enzymes are carried out at relatively high temperatures, and most of the enzymes used are quite thermostable, despite them being usually of mesophilic origin (Kristjansson and Kristjansson 1993). A useful industrial enzyme must have several specific properties depending on the particular application, but thermostability is usually an absolute necessity in biotechnology processes. Despite the potential benefits that may be achieved by using more thermostable enzymes in several of these processes, the industrial uses of thermophilic enzymes are still very limited. In some industrial sectors (including the food industry), which use ''bulk'' industrial enzymes, cost is the major concern, as introducing a new enzyme is worthwhile only if the cost improvement provided by the new enzyme justifies the research and development costs as well as the required changes to production equipment (Vielle *et al.* 1996). At present, by far the most important large-scale application of thermophilic enzymes is in the use of DNA polymerases in the polymerase chain reaction (PCR). The most commonly used enzyme in these applications is the so-called *Taq* polymerase from *Thermus aquaticus*, but several other DNA polymerases from other thermo- or hyper-thermophilic bacteria and Archaea have been characterized and are commercially available (Niehaus *et al.* 1999).

Due to the high biodiversity of extremophiles and their ability to produce a large number of novel enzymes they have attracted the attention of academia and industry. Compared to mesophilic bacteria growth and productivity of enzymes, however, is often severely limited. Genetic engineering has been possible to produce enzymes from extremophiles at high concentration and analyse their structure and function extensively. The cloning and expression of these enzymes in mesophilic hosts opens new possibilities for novel industrial processes (Sunna and Antranikian 1997; *Antranikian et al.* 2005). Few examples of extremozymes of thermophilic and hyperthermophilic origin are discussed below.

Lipases

Lipases catalyse a plethora of reactions like hydrolysis of lipids; acidolysis (replacement of a esterified fatty acid with a free fatty acid); transesterification (exchange of fatty acids between triglycerides); and ester synthesis (Verma *et al.* 2008; Verma and Kanwar 2008; Kanwar and Verma 2010; Cai *et al.* 2011). Most of these reactions are preferably done at low water activity to prevent hydrolysis of the desired ester (Verma *et al.* 2011). In order to avoid the use of solvents, lipase reactions are often carried out in mixtures of the reactants. For this to be feasible, the reaction mixture has to be heated from 50 to 80°C for the fat to be liquid. Lipases therefore have to be thermostable for optimal performance. Recently a very thermostable lipase has been discovered from *Bacillus cereus* (Verma and Kanwar 2010).

Other applications of lipase include the removal of pitch from pulp produced in the paper industry, the hydrolysis of milk fat in the dairy industry, the removal of non-cellulosic impurities from raw cotton before further processing into dyed and finished products, the removal of subcutaneous fat in the leather industry and manufacturing of drugs in the pharmaceutical industry (Sharma *et al.* 2011).

Amylase and Pullulanase

The starch industry is one of the largest users of thermostable amylolytic enzymes for the hydrolysis and modification of starch to produce glucose and various other products (Leveque *et al.* 2000; Bertoldo and Antranikian 2002; Gomes *et al.* 2003). Amylolytic enzymes are also used in the textile, paper and baking industries. Cellulolytic enzymes are employed in the removal of polyphenolic substances from juices, in detergents for color brightening and softening of textiles, in the bio-stoning of jeans, in the pulp and paper industries and in the pretreatment of plant biomass. The importance of the starch industry is demonstrated by the large amount of sugars and sweeteners produced worldwide every year. Several successive steps involving different microbial enzymes are required during the manufacture of sugars (Antranikian 1992; Dheeran *et al*. 2010). The process is initially carried out at extremely high temperature (95-105°C) and at pH 6.0-6.5. In the second step all process conditions have to be changed to a pH of 4.5 and temperatures of 60°C due to the absence of more suitable enzymes.

The discovery of more stable and specific enzymes would be significant for the improvement of the starch conversion process. Using thermostable enzymes (α-amylase, pullulanase, glucoamylase and xylose isomerase) from hyperthermophiles, it will be possible to run the liquefaction and saccharification process in one step and under the same conditions. This will also reduce the costs by avoiding the use of expensive ion exchangers. The most thermostable amylases and pullulanases (temperature optimum 100-105°C) have been found in the archaea *Pyrococcus woesei*, *Pyrococcus furiosus*, *Desulfurococcus mucosus*, *Pyrodictium abyssi* and *Staphylotherrnus marinus* (Sunna *et al.* 1997). The genes for the extracellular amylase and pullulanase from *Pyrococcus* sp. have been already cloned and expressed in *Escherichia coli* and *Bacillus subtilis* (Jorgensen *et al.* 1997). *Pyrococcus woesei* pullulanase has been crystallized by vapor diffusion (Knapp *et al.* 1995).

Improved thermal stability is a highly desirable property for the enzymes α-amylase, glucoamylase, pullulanases and glucose isomerase) that are used in the commercial processing of starch to glucose and fructose in the production of high-fructose corn syrup. In this process the starch is typically gelatinized at 105°C for 5 min and then α-amylase is used at 95°C and pH 6-6.5 to partially cleave the α-1-4-glycosidic linkages in the interior of the polymer, leading to liquefaction of the starch. For the second (saccharification) step, the temperature must be lowered to 60-62°C and the pH to 4.5, to adjust to the stability and the pH optimum of the exo-acting glucoamylase and pullulanase that further cleave the chain to smaller saccharides (95- 96% glucose in glucose syrups) (Crabb and Mitchinson 1997; Niehaus *et al.* 1999). In the final step the concentrated glucose syrup is again pH adjusted to 7-8.5, and is passed through a column with immobilized glucose isomerase (xylose isomerase) at 55-65°C, which converts it preferably to 55% fructose. The limited thermal stability of the currently used glucose isomerases determines the moderate temperatures used in this process.

The use of more thermostable enzymes which are active under similar temperature and pH conditions could significantly improve the starch conversion process, as it would be possible to run the liquefaction and saccharification process in one step (Vielle *et al.* 1996). Finding thermostable enzymes with similar pH characteristics (activity and stability) would also reduce costs in the process as the use of ion exchanger step could be avoided, but that is necessary to carry out to remove salts that accumulate as a result of the pH adjustments between different steps. In this respect hyperthermophilic α-amylases with lower pH optima are promising candidates (Leveque *et al.* 2000).

Studies have also shown that isomerization of glucose to fructose can be improved considerably by running this step at elevated temperatures (Crabb and Mitchinson 1997). A search for more thermostable glucoamylases (or α-glucosidases), pullulanases, and glucose isomerases from hyperthermophilic Archaea and bacteria is now actively being pursued with the goal of finding enzymes with more desirable properties in the starch conversion process. Furthermore, protein engineering has increasingly been used to

improve thermal stability (Mrabet *et al.* 1992), or catalytic properties (Meng *et al.* 1991) of glucose isomerases.

Mutant enzymes obtained with protein engineering techniques may prove to be important candidates for future industrial applications, in these and other processes.

Glycosidases

Enzyme catalyzed synthesis of carbohydrates is now well established. A series of thermophilic glycosidases, sold commercially as CLONEZYMEs™, can be used to catalyze the formation of a range of linkages, including Galβ(1→4), Galβ(1→6), Galα(1→6), D-Fucβ(1→3) and D-Fuc β(1→2) (Li *et al.* 1999; Barnard *et al.* 2010; Yeoman *et al.* 2010). The synthetic ability of thermophilic enzymes was further confirmed by using β(1→3)-galactosidase from *Thermus thermophilus*, which showed high regioselectivity during the transfer of galactosyl, glucosyl and D-fucosyl residues from *p*-nitrophenyl donors to a variety of saccharide acceptors (Lee *et al.* 2010; Sherief *et al.* 2010). Industrial production of fine chemicals and their respective intermediates as final products, especially in the form of enantiomerically pure compounds, will receive much attention and find increasing applications in biocatalytic industries in the near future.

Cyclodextrin glycosyl transferase

Cyclodextrins are useful in the food industry in specific separation processes, in flavor stabilization, and in controlled release and exclusion of unwanted compounds from the bulk phase (Whitaker 1990). They are non-reducing cyclic products of six to eight glucose units. At the industrial level they are made from starch that first is liquefied at high temperature by a thermostable α-amylase, followed by transglycosylation reaction leading to the cyclized product, a reaction catalyzed by cyclodextrin glycosyl transferase (CGTase; Niehaus *et al.* 1999). The mesophilic CTGases that are conventionally used in the process are heat-labile and the second step in the process must be run at lower temperatures. Using a thermostable CGTase would greatly improve the process, as liquefaction and cyclization could be carried out in one step.

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. Cyclodextrin production is a multistage process in which starch is first liquefied by a heat-stable amylase and in the second step a heatlabile CGTase from *Bacillus* sp. is used. Due to the low stability of the enzyme the process must be run at lower temperatures. The finding of heat stable and more specific CGTases from extremophiles will significantly improve the process. The application of heat-stable CGTases in the jet cooking, where temperatures up to 105°C are achieved, will allow that the liquefaction and cyclization take place in one step. Thermostable CGTases are produced by the members of the genus *Thermoanaerobacter*, *Thermoanaerobacterium thermosulfurogenes* and *Thermalkalibacter bogoriae* (Petersen *et al.* 1995; Prowe *et al.* 1996).

Phytases

Thermostable phytases are added to animal feeds in order to hydrolyze phytic acid (phytate), an antinutritional factor present in cereals and oil seeds, thereby releasing digestible phosphorous (Eichler 2001; Haki and Rikshit 2003). Thus the need to supplement the feed with an external source of phosphorous is reduced. Adding phosphorous to feeds results in excessive excretion of phosphorous in the manure of the animals, which creates an environmental problem. Phytase also releases minerals (including Ca, Mg, Zn and K), amino acids and proteins that are complexed with phytate and therefore improves dietary absorption of minerals.

Xylanases

Cellulase-free thermostable hemicellulases like xylanases have offered a major step forward in the biobleaching of pulp and paper, thus lowering the environmental pollution by halogens. Xylan is the major component of plant hemicelluloses. It is consisting of a main chain of β-1,4-linked D-xylopyranosyl residues. The depolymerization action of endoxylanase results in the conversion of the polymeric substrate into xylooligosaccharides. One possible use of heat-stable xylanases is the production of animal food. Thermoactive xylanases in combination with cellulases can be used for the effective conversion of these polymers to xylose and glucose (Atomi *et al.* 2011). The other area of application involves the use of thermostable xylanolytic enzymes as pre-bleaching agents for kraft pulp. Here the use of xylanases will help in reducing the kappa numbers (measure of residual lignin content) of the pulp, thus reducing the requirements for chlorine during pulp bleaching. Xylanases that are optimally active at temperatures above 100°C were detected in *Pyrodictium abyssi*, *Therrnotoga thermarum*, *Thermotoga neapolitana*, *Thermotoga maritima* and *Thermotoga* sp. strain FjSS3-B.1 (Sunna *et al.* 1997).

Proteolytic enzymes

Several proteases from thermophiles and hyperthermophiles have been characterized (Ryo *et al.* 2011; Kumar *et al.* 2011). The major large-scale use of proteases as industrial enzymes is in the detergent industry, as additives in household detergents, with alkaline serine proteinases from mesophilic *Bacillus* species (subtilisins) as the main enzymes. These enzymes are generally quite stable at moderately high temperatures (60-65°C) and under alkaline conditions (pH 9-11). A number of subtilisin-like serine proteinases from extreme thermophiles have been characterized (Voorhorst *et al.* 1997) and have properties more optimized for detergent applications, but that have not been used for that purpose. A major reason is that changing the large bulk production lines for the present detergent proteases for the more thermophilic enzymes may not be cost-effective. An advantage of doing proteolysis at high temperatures is that the protein substrates are denatured, which makes them generally more accessible to proteolytic attack than when in their native states. This may be particularly important when hydrolyzing difficult proteinaceous waste materials.

Because of their high thermal activity and stability under conditions that denature most other proteins, thermophilic proteases are good candidates for applications aimed at utilizing different protein waste materials for making protein hydrolyzates for different purposes. High temperature treatment of such waste materials would also contribute to maintaining aseptic conditions and to prevent the growth of spoilage bacteria and pathogens during processing. Proteases have increasingly been used in peptide synthesis, where advantage is taken of their reverse reaction under modified solvent conditions. Studies have shown that peptide synthesis is favored under controlled conditions at higher temperatures. Owing to their optimal activity at high temperatures and higher resistance to organic solvents than their mesophilic counterparts, thermophilic proteases should prove excellent candidates for enzymatic peptide synthesis (Vielle *et al.* 1996). Indeed, the use of the thermophilic neutral metalloprotease thermolysin for the synthesis of the artifical sweetener aspartame (a dipeptide) has been developed into a large-scale application, and is currently the only industrial application that uses a thermophilic protease (Kristjansson and Kristjansson 1993).

It has been found that most enzymes from extremophiles are stable even under the presence of high concentrations of detergents and denaturing agents. A variety of thermoactive proteinases have been identified from thermophilic archaea which belong to the genera *Pyrococcus*, *Thermococcus*, *Staphylothermus*, *Desulfurococcus* and *Sulfolobus.* Optimal activities have been detected between 90 and 110°C and pH

2.0 to 10 (Leuschner and Antranikian 1995). A keratin degrading thermophilic bacterium (*Fervidobacterium pennavorans*) was isolated from hot springs of the Azores Island and is able to convert chicken feather completely to amino acids and peptides at 80°C within 48 h (Friedrich and Antranikian 1996). A few examples of genetic engineering of thermophilic extremozymes to their mesophilic counterparts are discussed below.

Complete 16S rRNA gene sequence of a thermophilic microorganism, SBS-4S that grew on various sugars, carboxylic acids and hydrocarbons at temperatures between 45 and 75°C exhibited highest homology of 99.8% to various species of genus *Geobacillus* (Tayyab *et al.* 2011). A partial (0.7 kbp) chaperonin gene sequence also showed a highest homology of 99.4% to that of *G. kaustophilus*. Based on biochemical characterization, 16S rRNA and chaperonin gene sequences, SBS-4S was identified as a strain of genus *Geobacillus*. Strain SBS-4S produced several extracellular enzymes including amylase, protease and lipase. The lipase encoding gene was cloned, expressed in *Escherichia coli* and the gene product was characterized. The recombinant lipase was optimally active at 60°C with stability at wide pH range (6-12). The K_m and the V_{max} for the hydrolysis of *p*-nitrophenyl acetate were 3.8 mM and 2273 μmol min^{-1} mg^{-1}, respectively. The ability of the recombinant enzyme to be stable at a wide pH range makes it a potential candidate for use in industry (Harris *et al.* 2010).

A gene, *tayI*, encoding a novel subtilisin-like protease, designated thermicin, from the extremely thermophilic bacterium *Thermoanaerobacter yonseiensis* KB-1 (DSM 13777) was cloned by using a sequence tag containing the consensus sequence of proteases. Thermicin was overproduced in *E. coli* as a fusion protein with a histidine tag and purified by nickel nitrilotriacetic acid affinity chromatography. Thermicin from *E. coli* showed maximum proteolytic activity at 92.5° C and pH 9.0, and its half-life was 30 h at 80°C (Jang *et al.* 2002).

The cloning, expression and purification of two members of γ-glutamyltranspeptidase (γ-GT) family from two different extremophilic species, *Thermus thermophilus* (*Tt*GT) and *Deinococcus radiodurans* (*Dr*GT); the first is an aerobic eubacterium, growing at high temperatures (50-82°C), the second is a polyextremophile, as it tolerates radiations, cold, dehydration, vacuum, and acid. *Tt*GT and *Dr*GT were both synthesized as precursor proteins of 59-60 kDa, undergoing an intramolecular auto-cleavage to yield two subunits of 40 and 19-20 kDa, respectively (Castellano *et al.* 2011). However, like the γ-GT from *Geobacillus thermodenitrificans*, but differently from the other characterized bacterial and eukaryotic γ-GTs, the two new extremophilic enzymes displayed γ-glutamyl hydrolase, but not transpeptidase activity in the 37-50°C temperature range, pH 8.0. The comparison of sequences and structural models of these two proteins with experimental-determined structures of other known mesophilic γ-GTs suggests that the extremophilic members of this protein family have found a common strategy to adapt to different hostile environments (Castellano *et al.* 2011). Moreover, a phylogenetic analysis suggests that γ-GTs displaying only γ-glutamyl hydrolase activity could represent the progenitors of the bacterial and eukaryotic counterparts.

CONCLUSION AND PERSPECTIVES

The study of thermophilic/hyperthermophilic extremophiles have added greatly to our understanding of protein folding, stability, structure and function. Hyperthermophilic extremozymes have great economic potential in biotechnology industries. Discoveries of new extremophiles and genetic engineering of the newly isolated as well as of the currently available extreme microbes will offer novel opportunities for biocatalysis and biotransformations.

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