ELSEVIER

Biotechnology Advances 19 (2001) 261–278

BIOTECHNOLOGY ADVANCES

Research review paper

Biotechnological uses of archaeal extremozymes

Jerry Eichler*

Department of Life Sciences, Ben Gurion University, P.O. Box 653, Beersheva 84105, Israel

Abstract

Archaea have developed a variety of molecular strategies to survive the often harsh environments in which they exist. Although the rules that allow archaeal enzymes to fulfill their catalytic functions under extremes of salinity, temperature or pressure are not completely understood, the stability of these extremophilic enzymes, or extremozymes, in the face of adverse conditions has led to their use in a variety of biotechnological applications in which such tolerances are advantageous. In the following, examples of commercially important archaeal extremozymes are presented, potentially useful archaeal extremozyme sources are identified and solutions to obstacles currently hindering wider use of archaeal extremozymes are discussed. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Archaea; Extremophiles; Enzymes; Halophiles; Thermophiles; Hyperthermophiles

1. Introduction

It has become increasingly clear that life thrives in the most challenging environmental conditions found on the planet. Microbial communities can be found at extremes of salinity, acidity, alkalinity, temperature or pressure as well as in other seemingly drastic surroundings. Often, the most extremotolerant of these microorganisms are members of the domain Archaea, the most recently described yet least well understood domain of life (Woese et al., 1990; Rothschild and Manicinelli, 2001). Thus, in addition to the insight that better understanding of the genetics, biochemistry and physiology of Archaea will provide into a variety of basic research questions, a clearer comprehension of how Archaea have evolved to survive the harsh conditions in which they are often found holds great biotechnological promise.

* Tel.: +972-8646-1343; fax: +972-8647-2890.
E-mail address: jeichler@bgumail.bgu.ac.il (J. Eichler).

0734-9750/01/$ – see front matter © 2001 Elsevier Science Inc. All rights reserved.
PII: S0734-9750(01)00061-1

Presently, applied uses exist or have been proposed for a variety of archaeally derived materials. The extremely chemically stable lipids of archaeal membranes represent a novel drug delivery system (Patel and Sprott, 1999). Self-assembling archaeal components such as the S-layer glycoprotein and bacterioopsin have drawn interest for their nanotechnological potential (Oesterhelt et al., 1991; Sleytr et al., 1997). Polysaccharides secreted from halophilic archaea could find use in oil exploration efforts (Rodriguez-Valera, 1992) while secreted haloarchaeal polymers have been considered as a raw material for biodegradable plastics (Fernández-Castillo et al., 1986). Often, the mere presence of archaeal communities carries considerable economic value. Methanogenic archaea are the focus of much attention in their capacity as clean and inexpensive energy sources (Reeve et al., 1997), while acidophilic archaea have been identified at several acid mine drainage sites where their mineral–sulfide oxidizing abilities play an important role in the geochemical sulfur cycle (Vaquez et al., 1999; Edwards et al., 2000; Golyshina et al., 2000).

Although a variety of archaeal-related products carry significant commercial value, biotechnologically useful archaeal enzymes represent the main focus of industrial interest. As a result of their abilities to function at the current temperature, salinity and pH limits of life (Danson and Hough, 1998), extremophilic enzymes, or extremozymes, from Archaea currently find use or can be considered for use in a variety of biotechnological endeavours. In the following, examples of advances in the identification, characterization, purification and applied usage of archaeal extremozymes will be presented, potential sources and additional applications of novel archaeal extremozymes will be identified and future prospects for the use of archaeal extremozymes will be presented. For discussion of extremozymes obtained from non-archaeal sources, the reader is directed to recent reviews by Niehaus et al. (1999), Horikoshi (1999), Hough and Danson (1999), and Gerday et al. (2000).

2. Thermoarchaeal extremozymes

Heat-tolerant enzymes are currently the most investigated of all extremozymes. Such extremozymes not only serve as excellent models for understanding protein stability but also carry significant biotechnological potential. Applied interest in thermophilic extremozymes is related to the fact that performing biotechnologically related processes at higher temperatures is often advantageous. For instance, in chemical reactions involving organic solvents, the decrease in viscosity and increase in diffusion coefficient that occur at elevated temperatures result in higher reaction rates (Krahe et al., 1996; Becker et al., 1997). Such considerations are relevant to a variety of processes including those involving hydrophobic compounds that normally display low solubilities. Increased temperatures can also enhance the availability of such compounds for biodegradation efforts. Furthermore, performing reactions at higher temperatures reduces the possibility of complications resulting from contamination.

It is thought that thermophilic extremozymes rely on the same catalytic mechanisms as do their mesophilic counterparts (Cowan, 1992; Adams, 1993; Danson and Hough, 1998). Indeed, heterologous expression of thermostable enzymes in mesophilic hosts generally does not compromise the ability of these enzymes to function at elevated temperatures (Niehaus et al., 1999). Thus, the various molecular strategies adopted by thermophilic extremozymes in

adapting to extremely high temperatures are apparently genetically encoded. As revealed by biophysical and structural studies (cf. Jaenicke and Bohm, 1998), such adaptations (relative to their mesophilic counterparts) include sequence modifications, such as replacement of conformationally strained residues by glycines, addition of salt bridges, increased hydrophobic interactions, additional ion pairing and hydrogen bonding, improved core packing and shortening of loops. These strategies, used to differing extents by different thermophilic proteins, confer not only higher thermal stability to proteins but also enhanced rigidity and resistance to chemical denaturation.

Heat-stable extremozymes are obtained from either extreme thermophiles, which grow optimally at temperatures above 60°C, or from hyperthermophiles, with optimal growth temperatures above 90°C. Extreme thermophiles include both archaeal and bacterial species, while the vast majority of hyperthermophiles are members of the Archaea (Adams, 1993). Species found at the highest temperatures (103–113°C) are exclusively archaeal (Stetter, 1996). Due to their ability to function at such high temperatures, hyperthermophilic extremozymes have drawn the main focus of biotechnological interest. As hyperthermophiles predominately rely on the reduction of elemental sulfur for energy generation, resulting, in most cases, in the generation of H_2S, cultivation of hyperthermophiles is problematic. Accordingly, the bulk of study into the extremozymes of hyperthermophilic archaea has focused on strains belonging to the genera *Pyrococcus* and *Thermococcus*, which are also capable of growth in the absence of sulfur (Adams, 1993). Large-scale protocols for growing H_2S-producing and other hyperthermophilic strains using specialized growth vessels and conditions, however, are becoming available (Nelson et al., 1992; Rüdiger et al., 1992; Aguilar et al., 1998). This situation will encourage discovery of novel hyperthermophilic extremozymes.

2.1. Glycosyl hydrolases

To date, a large number of α- and β-glycosyl hydrolases from thermophilic and hyperthermophilic archaea have been characterized (Table 1). Glycosyl hydrolases are defined as enzymes able to hydrolyze glycosidic bonds between two or more carbohydrates or between carbohydrate and non-carbohydrate moieties. Although glycosyl hydrolases are divided among 85 families along sequence lines (Henrissat, 1998), it is convenient to group these enzymes according to their abilities to degrade starch, xylan or cellulose, polymers containing either α-1,4- and α-1,6-glycosidic bonds (starch) or β-1,4-glycosidic bonds (xylan and cellulose).

2.1.1. Starch-degrading enzymes

α-Amylases randomly hydrolyze α-1,4-glycosidic linkages in starch, yielding glucose and oligosaccharides. The α-amylases of *Pyrococcus woesi* (Koch et al., 1991) and *P. furiosus* (Brown et al., 1990; Koch et al., 1990) are the most thermophilic versions of the protein known, possessing optimal temperatures of 100°C each. Unlike mesophilic α-amylases, the *Pyrococcus* enzymes do not require the presence of calcium. An additional α-amylase from *P. furiosus* has been cloned, sequenced and expressed in *Escherichia coli* and *Bacillus subtilis* and shown to differ from the intracellular enzyme discussed above (Laderman et al., 1993;

Table 1
Starch-degrading enzymes of hyperthermophilic and thermophilic archaea

Organism	Enzyme	$T_{optimal}$	$pH_{optimal}$	Reference
D. mucosus	α-amylase	100	5.5	Canganella et al., 1994
	pullulanase type II	100	5.0	Canganella et al., 1994
P. furiosus	α-amylase	100	6.5–7.5	Laderman et al., 1993
	α-amylase	100	7.0	Jorgensen et al., 1997
	pullulanase type II	105	6.0	Dong et al., 1997
	pullulanase type II	98	5.5	Brown and Kelly, 1993
	α-glucosidase	115	5.5	Costantino et al., 1990
Pyrococcus sp. KOD1	α-amylase	90	6.5	Tachibana et al., 1996
P. woesi	α-amylase	100	5.5	Koch et al., 1991
	pullulanase type II	100	6.0	Rüdiger et al., 1995
	α-glucosidase	110	5.0–5.5	Linke et al., 1992
Pyrodictium abyssi	α-amylase	100	5.0	Niehaus et al., 1999
	pullulanase type II	100	9.0	Niehaus et al., 1999
St. marinus	α-amylase	100	5.0	Canganella et al., 1994
S. solfataricus	α-amylase			Haseltine et al., 1996
S. solfataricus 98/2	α-glucosidase	105	4.5	Rolfsmeier and Blum, 1995
S. solfataricus MT-4	glucoamylase	70	5.5	Lama et al., 1991
	pullulanase type II	90	5.5	Canganella et al., 1994
T. celer	α-amylase	90	5.5	Canganella et al., 1994
	pullulanase type II	90	5.5	Canganella et al., 1994
T. hydrothermalis	pullulanase type II	95	5.5	Gantelet and Duchiron, 1998
T. litoralis	pullulanase type II	98	5.5	Brown and Kelly, 1993
T. profundus DT5432	α-amylase (Amy S)	80	5.5–6.0	Chung et al., 1995
	α-amylase (Amy L)	80	4.0–5.0	Kwak et al., 1998
Thermococcus strain AN1	α-glucosidase	75	7.0	Pillar et al., 1996
Thermococcus strain TY	α-amylase	100	5.5	Canganella et al., 1994
	pullulanase type II	100	6.5	Canganella et al., 1994
Thermococcus strain TYS	α-amylase	100	6.5	Canganella et al., 1994
	pullulanase type II	100	6.5	Canganella et al., 1994

Jorgensen et al., 1997). *Thermococcus profundus* DT5432 also produces thermostable α-amylases, such as amylase S, which has been purified and shown to possess a temperature optimum of 80°C and to rely on calcium ions for stabilization at elevated temperatures (Chung et al., 1995), or amylase L, which is effective in more acidic conditions (Kwak et al., 1998). Thermophilic α-amylases have also been purified or partially purified from different *Sulfolobus solfataricus* strains (Lama et al., 1991; Haseltine et al., 1996). In addition to the limited number of thermo- and hyperthermoarchaeal amylases that have been purified, amylase activity with temperature optima between 90°C and 100°C has been reported in the crude extracts of a variety of strains encompassing the orders Thermoproteales, Thermococcales and Pyrodictiales. In all cases, the activity was thermostable in the absence of either calcium or substrate (Canganella et al., 1994).

Most thermo- and hyperthermoarchaeal pullulanases belong to the type II class, able to hydrolyze both α-1,4- and α-1,6-glycosidic bonds in branched polymers. Type I pullulanases, which exclusively cleave α-1,6-glycosidic bonds in branched polymers, are less common in Archaea (Sunna et al., 1997). As in the case of α-amylases, the type II pullulanases of *P.*

woesi (Rüdiger et al., 1995) and *P. furiosus* (Brown et al., 1990) are the most thermoactive versions of the protein reported. The *P. furiosus* and *P. woesi* pullulanase genes have been cloned and expressed in *E. coli* (Rüdiger et al., 1995; Dong et al., 1997). The purified recombinant *P. woesi* protein functions optimally at 100°C, pH 6, and is activated by either calcium or reducing agents. The enzymes of *P. furiosus* and *T. litoralis* are also optimally active at 100°C and are stimulated by calcium (Brown and Kelly, 1993). Type II pullulanases have also been detected in *T. celer*, *Thermococcus* strain TSY, *Thermococcus* strain TY, *Desulfurococcus mucosus* and *Staphylothermus marinus* (Canganella et al., 1994; Gantelet and Duchiron, 1998).

α-Glucosidases are involved in the final step of glycogen degradation, hydrolyzing terminal glucose residues. Various thermo- and hyperthermoarchaeal strains contain α-glucosidases, which exist in a variety of molecular sizes, subcellular localizations and oligomeric states. As with other thermostable starch-degrading enzymes of Archaea, the α-glucosidases of *P. woesi* (Linke et al., 1992) and *P. furiosus* (Costantino et al., 1990) are the best characterized. Expression of the *P. furiosus* enzyme is, like other starch-degrading enzymes in this microorganism, induced by carbohydrates containing α-1,4-linkages, by an unknown mechanism (Costantino et al., 1990). The enzyme functions optimally between 105°C and 115°C and apparently requires the presence of metal ions. An intracellular α-glucosidase has also been purified from *S. solfataricus* strain 98/2 (Rolfsmeier and Blum, 1995). Surprisingly, this enzyme possesses an optimal temperature of 105°C, over 20°C higher than the optimal growth temperature of the strain. Furthermore, the thermal activity profile and substrate specificity of the enzyme suggest that it is structurally different from its *P. furiosus* counterpart. An α-glucosidase that also differs from the *P. furiosus* enzyme has been isolated from *S. shibatae* (Di Lernia et al., 1998). Finally, an extracellular α-glucosidase has been purified from *Thermococcus* strain AN1 (Pillar et al., 1996).

Trehalose is a non-reducing dissacharide, which has found broad use as a stabilizing agent in numerous industries. Due to the expensive process of trehalose extraction from baker's yeast, there has been much interest in describing organisms capable of trehalose biosynthesis. The recent report of thermophilic trehalose-synthesizing enzymes from *S. shibatae* could extend the industrial synthesis and applicability of trehalose (Di Lernia et al., 1998).

2.1.1.1. Xylan-degrading enzymes. Xylans are a heterogeneous group of polysaccharides based on a main chain formed from β-1,4 linked D-xylopyranosyl subunits. Thermostable xylanases have gained interest of late given their roles in enzyme-assisted paper bleaching, an attractive alternative to current chlorine bleaching techniques (Viikari et al., 1994). Xylanase treatment at elevated temperatures offers various procedural advantages and, as such, considerable effort has been devoted towards describing thermostable xylanases. Hyperthermoarchaeal xylanolytic activity was first reported in two uncharacterized *Thermofilum* strains (Bragger et al., 1989). The crenarcheote *Thermosphaera aggregans* sp. was shown to grow on heat-treated, but not native, xylan (Huber et al., 1998). It was believed, however, that Archaea do not produce true thermostable xylanases, since no thermophilic or hyperthermophilic strains capable of utilizing untreated xylan as a carbon source had been reported (Sunna et al., 1997). Recently, degradation of untreated

xylan was detected in *P. furiosus*, *Thermococcus* sp. Wai21S.1, and *T. zilligii* (Uhl and Daniel, 1999). The *T. zilligii* xylanase was purified and characterized and shown to possess a half-life of 8 min at 100°C, pH 6.

2.1.2. Cellulose-degrading enzymes

Cellulose, consisting of up to 14 000 β-1,4-linked glucose residues, is the most abundant natural polymer on earth. While cellulases are widespread in Fungi and Bacteria, only one archaeal cellulase, an endoglucanase from *P. furiosus*, has been reported (Bauer et al., 1999). Various thermo- and hyperthermoarchaea, however, contain β-glycosidic enzymes. In *P. furiosus* cells growing on cellobiose (a product of cellulose breakdown), β-glucoside activity accounts for 5% of the total cell protein, with two different enzymes contributing to the activity (Kengen et al., 1993). The major activity in *P. furiosus* is catalyzed by Pfuβ-gly, which has been expressed in *E. coli* (Voorhorst et al., 1995). The role of this intracellular enzyme in Archaea is unclear, given that Archaea are apparently unable to grow on cellulose. Thermostable β-glucosidases have also been detected in different *Sulfolobales* strains (Grogan, 1991), but again, the physiological function of such enzymes is not understood.

2.2. Proteinases

The quantity of proteases produced on a commercial scale worldwide is greater than any other enzymic group of biotechnological relevance (Niehaus et al., 1999). As such, the isolation and characterization of heat-stable archaeal proteases are the focus of much interest. Various proteolytic enzymes from thermophilic and hyperthermophilic archaea, mostly of the serine protease type, have been identified (Table 2). In many cases, these enzymes retain their catalytic activity not only at elevated temperatures, but also in the presence of detergents or other denaturing agents. These properties are well-illustrated by the extracellular 68-kDa protease of *T. stetteri*, which displays a half-life of 2.5 h at 100°C and is able to retain 70% of its catalytic activity even in the presence of 1% SDS (Klingeberg et al., 1991). A cell-associated serine protease with a half-life of 4.3 h at 95°C has been detected in *Desulfurococcus* strain SY (Hanzawa et al., 1996), while archaelysin, a heat-stable serine protease, was isolated from the cell-free supernatant of *Desulfurococcus* strain $Tok_{12}S_1$ (Cowan et al., 1987). An extremely thermoresistant serine protease from *St. marinus* maintains catalytic activity even after a 10-min, 135°C treatment (Mayr et al., 1996). Subtilisin-type serine proteases have been detected in both *Pyrobaculum aerophilum* (aerolysin) and *P. furiosus* (Eggen et al., 1990; Völkl et al., 1994). The latter, called pyrolysin, is associated with the cellular envelope and retains 50% of its activity after a 20-min, 105°C challenge (Eggen et al., 1990). The gene encoding the pre-pro form of the enzyme has been cloned and sequenced (Voorhorst et al., 1996).

Other subclasses of thermostable proteases are synthesized by a variety of archaeal strains. A thiol protease has been identified in *Pyrococcus* sp. KOD1 (Morikawa et al., 1994). An acidic protease from *S. acidocaldarius* has been identified and cloned (Fusek et al., 1990), while carboxy peptidase activity has been observed in crude extracts of *S. solfataricus* cells (Fusi et al., 1991). *P. furiosus* contains both a propyl peptidase (Halio et al., 1996) as well as a novel type of protease, a prolyl endopeptidase, which has been cloned and overexpressed in

Table 2
Proteases from hyperthermophilic and thermophilic archaea

Organism	Type	$T_{optimal}$	$pH_{optimal}$	Reference
A. pernix K1	serine protease (pernilase)	90	6.5–10.5	Chavez Croocker et al., 1999
Desulfurococcus strain $Tok_{12}S_1$	serine protease	95	7.5	Cowan et al., 1987
Desulfurococcus strain SY	serine protease			Hanzawa et al., 1996
Py. aerophilum	serine protease (aerolysin)			Völkl et al., 1994
P. furiosus	serine protease	85	6.3	Eggen et al., 1990
	serine protease (pyrolysin)			Voorhoorst et al., 1996
	serine protease	85–90		Harwood et al., 1997
Pyrococcus sp. KOD1	thiol protease	110	7	Morikawa et al., 1994
St. marinus	serine protease		9.0	Mayr et al., 1996
S. acidocaldarius	acidic protease	90	2.0	Fusek et al., 1990
S. solfataricus	carboxy-peptidase			Fusi et al., 1991
	serine protease		6.5–8.0	Burlini et al., 1992
T. aggregans	serine protease	90	7.0	Klingeberg et al., 1991
T. celer	serine protease	95	7.5	Klingeberg et al., 1991
T. litoralis	serine protease	95	9.5	Klingeberg et al., 1991
T. stetteri	serine protease	85	8.5	Klingeberg et al., 1991

E. coli (Harwood et al., 1997). Indeed, *P. furiosus* contains at least 13 different proteins with proteolytic activity (Connaris et al., 1991).

All the thermo- and hyperthermostable proteases discussed above originate from anaerobic archaea. As such, cultivation of such strains on a scale required to meet industrial needs is problematic. This logistic obstacle may be overcome by the recent isolation of proteases from the aerobic hyperthermophile *Aeropyrum pernix* K1. *A. pernix* K1 grows at 90°C with a doubling time of 200 min and is amenable to large-scale cultivation techniques, making the strain an attractive source of proteases and other extremozymes of biotechnological interest (Sako et al., 1996). Indeed, this strain contains one of the most heat-stable extracellular metalloproteinases reported to date, aeropyrolysin (Sako et al., 1997), while pernilase, an intracellular proteinase purified from this species, functions optimally at 90°C, yet possesses a half-life of 12 min at 110°C (Chavez Crooker et al., 1999).

2.3. DNA-processing enzymes

Thermophilic DNA polymerases, responsible for elongation of the primer strand of a growing DNA molecule, lie at the heart of polymerase chain reaction for DNA amplification. DNA polymerases from various hyperthermoarchaea have drawn much interest, based on their stringent proofreading abilities. *P. woesi* Pwo polymerase (Frey and Suppmann, 1995), *P. furiosus* Pfu polymerase (Lundberg et al., 1991), Deep Vent polymerase from *Pyrococcus* strain GB-D (Perler et al., 1996), Vent polymerase from *T. litoralis* (Cariello et al., 1991; Mattila et al., 1991) as well as a DNA polymerase from *Thermococcus* sp. strain 9°N-7 (Southworth et al., 1996) all possess error rates that are

five- to tenfold lower than that of the widely used thermobacterial *Taq* polymerase (Chien et al., 1976). Although commercially available, the highly precise archaeal enzymes have not replaced the *Thermus aquaticus* enzyme in general use for a variety of reasons, including low extension rates and unsuitability for the amplification of longer DNA fragments. These problems may be overcome by the DNA polymerase of *Pyrococcus* sp. strain KOD1, which possesses a low error rate and high processivity and extension rates (Takagi et al., 1997). Mutation of the *Thermococcus* sp. strain 9°N-7 enzyme has been attempted in order to reduce the exonuclease activity of the enzyme without diminishing polymerase activity (Southworth et al., 1996). Interestingly, the *T. litoralis* DNA polymerase was the first example of an archaeal intein, translated protein sequences which lie between two parts of a protein precursor, yet which are not found in the final product (Perler et al., 1992; Perler and Adam, 2000). Protein purification strategies based on inteins are commercially available.

In addition to DNA polymerases, hyperthermal archaea are the source of additional potential DNA-modifying extremozymes. A DNA ligase has been identified in *Desulfurolobus* (*Acidianus*) *ambivalens* (Kletzin, 1992). Interestingly, like its eukaryal homologues, the archaeal enzyme is ATP-dependent, yet does not require NAD^+. *Thermoplasma acidophilum* expresses a thermostable pyrophosphatase that has found use in DNA-sequencing protocols (Vander et al., 1997).

2.4. Other enzymes of applied interest

2.4.1. Alcohol dehydrogenases

Alcohol dehydrogenases, able to catalyze the oxidation of alcohols to ketones as well as the reverse reaction, have attracted much biotechnological interest. A hyperthermoarchaeal version of the enzyme has been examined in *T. stetteri* (Ma et al., 1994). In contrast to bacterial or eukaryal alcohol dehydrogenases, the *T. stetteri* enzyme does not contain metal ions. Furthermore, the thermoarchaeal enzyme preferentially oxidizes primary alcohols, requires NADP and is thermostable. The *S. solfataricus* alcohol dehydrogenase has also been studied and was shown to contain zinc ions and require NAD as a cofactor (Ammendola et al., 1992). Recently, significant quantities of purified *S. solfataricus* and *Sulfolobus* sp. strain RC3 enzymes have been obtained upon heterologous expression in *E. coli* (Cannio et al., 1996).

2.4.2. Esterases

Esterases represent a family of enzymes of considerable potential in various industrial processes, finding use in stereospecific hydrolysis, *trans*-esterification, ester synthesis and other organic biosynthesis reactions. Indeed, the range of reactions in which esterases are employed has been extended due to the discovery of extremophilic versions of these enzymes. The esterase of *S. acidocaldarius* has been purified and shown to be solvent-stable and thermophilic (Sobek and Gorisch, 1988). The esterase gene of *P. furiosus* has been heterologously expressed in *E. coli* cells, resulting in the production of the most thermostable and thermoactive esterase available to date, displaying an optimal temperature of 100°C and a half-life of 50 min at 126°C (Ikeda and Clark, 1998).

2.4.3. Chitinases

After cellulose, chitin is the most abundant polymer on earth. The first examples of archaeal chitinases and *N*-acetylglucosaminidases, enzymes involved in chitin degradation, were reported in the hyperthermophilic strain *T. chitonophagus* (Huber et al., 1995).

3. Psychrophilic archaeal extremozymes

To date, the majority of interest in temperature-sensitive extremozymes have focused on the products of thermophilic or hyperthermophilic species. Although understanding the basis of thermostability carries significant biotechnological potential, thermophilic environments are not abundant. By contrast, permanently cold habitats such as Antarctica, where temperatures never exceed 5°C, are widespread. In fact, deep oceans, which cover over 70% of the Earth's surface, represent the major biosphere on the planet. Able to function with reduced energy requirements, extremozymes from psychrophilic or 'cold-loving' microorganisms could find use in numerous applications. Addition of psychrophilic polymer-hydrolyzing extremozymes such as β glycanases to detergents would allow for efficient washing in cold water. Psychrophilic polymer-degrading enzymes could also find use in the paper industry, assisting in manipulation of pulp or in bioremediation efforts (Cummings and Black, 1999). The food industry could exploit pectinases that act at lower temperatures in the processing of fruit juices or cheeses.

Cold environments, however, present proteins with a number of physical challenges. Although molecular studies of the adaptations adopted by psychrophilic extremozymes are in their infancy, several properties are apparent. Relative to their mesophilic counterparts, cold-adapted proteins contain specific sequence modifications, fewer disulfide bonds and salt bridges, helix dipole structures of lower net charge, increased solvent interactions, a decreased number of hydrogen bonds at domain interfaces and a lower degree of hydrophobic interactions in the core of the protein (Russell, 2000). Together, these modifications yield proteins that are structurally flexible and hence catalytically effective in the cold. Indeed, these adaptations are essentially the opposite of those modifications that impart heat stability to thermophilic proteins.

Archaea have been detected in many low-temperature aquatic environments. 16S ribosomal RNA gene sequence analysis of Pacific Ocean plankton samples taken from depths of 100 and 500 m revealed the presence of various, currently unidentified archaeal strains (Fuhrman et al., 1992). Archaea account for over a third of the prokaryotic biomass in coastal Antarctic surface waters (DeLong et al., 1994). Interestingly, these include members of the archaeal subdomain Crenarchaeota, previously thought to include only hyperthermophilic species (Hershberger et al., 1996). A novel crenarchaeal strain, *Cenarchaeum symbiosum*, lives symbiotically in marine sponge tissues and grows well at 10°C (Preston et al., 1996). To date, however, only three psychrophilic archaeal strains, all free-living and members of the eukaryarchaeotal archaeal subdomain, are available in pure culture. *Methanococcoides burtonii* (T_{minimum} − 2.5°C, T_{optimal} 23°C) (Franzmann et al., 1992) and *Methanogenium frigidum* (T_{minimum} − 10°C, T_{optimal} 15°C) (Franzmann et al., 1997) were originally isolated

from the bottom of Ace Lake, Antarctica, where the methane-saturated waters invariably remain between 1°C and 2°C (Franzmann et al., 1992, 1997). The haloarchaeon *Halorubrum lacusprofundii* (T_{minimum} 2°C, T_{optimal} 33°C) also originates from Antarctica (Schleper et al., 1997). Examination of enzymes from these species is only now beginning.

4. Haloarchaeal extremozymes

Highly saline environments, such as the Dead Sea, Great Salt Lake or saltern pools, represent another habitat occupied by extremophilic microorganisms including two groups of halophilic archaea: aerobic haloarchaea and anaerobic halophilic methanoarchaea (Kamekura, 1998). Fundamentally different approaches are employed by each in order to survive the osmotic challenges associated with life in saline environments (Madigan and Oren, 1999). In the latter, the cytoplasm contains low concentrations of salt due to the presence of high organic solute levels, while the former have developed an entire biochemistry that functions at near-saturating salt concentrations. To date, the bulk of applied interest in products derived from halophilic archaea has focused on stabilizing agents such as betaines or ectoines, on polysaccharides, on secreted polymers for use as biodegradable plastics or on salt-tolerant lipids from these strains (Rodriguez-Valera, 1992). In some cases, the high salt tolerance of haloarchaeal compounds is exploited, whereas in other instances, this property is secondary. For instance, retinal proteins such as bacteriorhodopsin have found uses in holographic films or in other light-sensitive or 'bioelectric' applications (Hong, 1986; Oesterhelt et al., 1991).

Despite advances in understanding the biophysical basis of halophilic enzyme stability, activity and solubility (Madern et al., 2000), potential uses of haloarchaeal extremozymes have, by contrast, received considerably less attention. This is unfortunate, given the potential advantages of halophilic enzymes over their mesophilic counterparts. Not only are haloarchaeal extremozymes extremely salt-tolerant, they are capable of retaining stability at ambient temperatures for prolonged periods and are often considerably thermotolerant. Furthermore, given that halophilic enzymes possess salt-enriched solvation shells (Madern et al., 2000), they are likely to retain catalytic activity in environments of low water activity, such as in the presence of high levels of organic solvents. Such properties could be of interest in a variety of applications. To date, however, relatively few haloarchaeal extremozymes have been adopted for applied purposes. In part, this is due to the relatively limited demand for salt-tolerant enzymes in current manufacturing or related processes. Examples of extremozymes from haloarchaea of commercial value have, however, been reported. A patent for production of a novel restriction enzyme of unusual specificity from a species of the genus *Halococcus* has been filed (Obayashi et al., 1988). A chymotrypsinogen B-like protease has been isolated from the haloalkaliphilic archaeon *Natronomonas pharaonis* and shown to act optimally at 61°C and pH 10 (Stan-Lotter et al., 1999). Unlike the numerous previously reported haloarchaeal proteases which, like the vast majority of other halophilic enzymes, lose their catalytic activity at lowered salt concentrations (Eisenberg and Wachtel, 1987; Kim and Dordick, 1997), the alkaliphilic *N. pharaonis* protease can function in salt concentrations as low as 3 mM. This suggests the usefulness of this extremozyme as a detergent additive, currently a major role of alkaliphilic enzymes (Horikoshi, 1999).

5. Piezophilic archaeal extremozymes

With an average pressure of 38 MPa, the world's oceans are home to piezophilic (formerly called barophilic) microorganisms, including a variety of thermophilic and hyperthermophilic archaeal strains (Abe and Horikoshi, 2001). Given the use of high-pressure conditions in various industries (e.g. the food industry) (Hayashi, 1996), the availability of extremozymes capable of catalysis at high pressures would offer a novel biotechnological alternative to currently employed processes. Despite the potential, there are few current applications of piezophiles or enzymes derived therefrom. In part, this is due to difficulties in cultivating piezophilic strains. As solutions to technical problems involved in laboratory growth of such strains appear and more is learned about piezophilic extremozymes, applied use of such enzymes will grow.

It has been shown that high pressure can modulate extremozyme activity. Hydrogenases, glyceraldehyde-3-phosphatases and α-glucosidases from *Methanococcus jannaschii* and *M. igneus*, originally isolated from depths of 2600 and 100 m, respectively, revealed enhanced stabilities at elevated pressures (Hei and Clark, 1994). In contrast, enzymes from related mesophilic methanoarchaeal strains were destabilized under the same high-pressure conditions. Similarly, DNA polymerases from *P. furiosus* and *Pyrococcus* sp. ES4 were also shown to be stabilized by high pressure and elevated temperatures (Summit et al., 1998). These findings suggest a relation between the protein properties responsible for thermophilicity and piezophilicity, although the two behaviours are not synonymous (Konisky et al., 1995). Thus, understanding the basis of pressure-mediated stability could enhance the usefulness of currently employed thermo- and hyperthermoarchaeal extremozymes through protein engineering efforts.

6. Archaeal vs. bacterial extremozymes

Apart from hyperthermophilic extremozymes, which are exclusively archaeal in origin, extremozymes that currently find applied uses originate from both bacterial and archaeal sources. Of these, the majority come from extremophilic bacteria. This bias is related to the fact that molecular tools for working with Bacteria are better developed than are tools for working with Archaea. However, with increased understanding of Archaea and with the ongoing development of improved techniques for studying these microorganisms, this situation will soon be remedied. Thus, not only will archaeal extremozymes experience increasing demand, but it will also be possible to address whether archaeal extremozymes offer unique advantages over their bacterial counterparts. Apart from archaeal hyperthermophilic extremozymes, which offer much greater thermostability than any bacterial enzyme, the answer to this question currently remains open. Indeed, it is generally unclear whether archaeal and bacterial extremozymes rely on the same physical properties in order to attain stability in response to a given environmental challenge. With versions of a given enzyme available from both mesophilic and extremophilic bacterial and archaeal strains, it will be possible to assess whether similar protein modification strategies are employed in both domains of life (Danson and Hough, 1998).

7. Future prospects

The near future promises a major increase in the use of extremozymes of archaeal origin in a variety of applied fields. The reasons for this optimism are several-fold. Firstly, the availability of a growing number of completed archaeal genomes as well as improved annotation techniques will help reveal novel archaeal extremozymes of commercial interest. Moreover, structural, rather than sequence-based, homologues of biotechnologically relevant enzymes will be identified in Archaea based on advances in bioinformatics and structural genomics. The identification of novel archaeal strains also promises the discovery of new archaeal extremozymes. 16S ribosomal RNA analysis has revealed the rich biodiversity of the archaeal world (DeLong, 1998). Unfortunately, it is estimated that less than 1% of these strains is currently amenable to growth in culture and hence available for study (Hugenholtz and Pace, 1996). Thus, the ability to cultivate currently non-culturable archaeal strains represents a major research goal. Improvements in growth techniques as well as advances in the development of molecular strategies and tools for working with Archaea (Sowers and Schreier, 1999) suggest that technical limitations may no longer hinder our exploitation of archaeal biodiversity in the search for novel archaeal extremozymes.

Understanding the mechanism of protein translocation in Archaea will also stimulate the realization of the enormous commercial potential of extremozymes from these microorganisms. For instance, large-scale production of archaeal extremozymes genetically targeted for secretion will be made more efficient through exploitation of a well-characterized protein export machinery. In contrast to the well-studied bacterial (Duong et al., 1997) and eukaryal (Rapoport et al., 1996) protein export systems, little is known of how proteins cross the plasma membrane of Archaea. Current understanding of archaeal protein translocation suggests that the process represents a hybrid of the bacterial and eukaryal systems, yet invokes archaeal-specific elements (Pöhlschroder et al., 1997; Eichler, 2000; Eichler and Moll, 2001).

Current concerns regarding the ability to produce or purify archaeal extremozymes in sufficient quantities for industrial use will also be alleviated in the near future. Presently, heterologous expression of thermo- and hyperthermoarchaeal extremozymes has shown that, in general, the intrinsic extremophilic properties of the enzymes are preserved upon transfer to elevated temperatures (Morana et al., 1995), although examples in which thermotolerance was not preserved following heterologous expression have been reported (Purcarea et al., 1997). Still, industrial-scale production of archaeal extremozymes in heterologous hosts and their subsequent purification is possible (Morana et al., 1995; Miura et al., 1999). As improved molecular biology tools for working with Archaea become available, it will become possible to overexpress archaeal extremozymes in their native hosts. Combined with an improved ability to grow Archaea on the reactor-scale (Krahe et al., 1996; Mukhopadhyay et al., 1999; Schiraldi et al., 1999), the availability of archaeal extremozymes at levels required by industry will be improved. Finally, as the structure–function rules that impart stability to enzymes under various extreme conditions become better understood (Danson and Hough, 1998), it will be possible to tailor specific extremophilic traits into any protein of interest by protein engineering or directed evolution (Arnold et al., 2001), possibly improving upon nature.

8. Concluding remarks

While substantial progress has been realized of late, understanding of the biochemistry, genetics and physiology of Archaea is only just beginning. Accordingly, despite their enormous economic potential, the applied potential of archaeal extremozymes remains largely unrealized. With continued basic research, development of appropriate molecular tools as well as better insight into structure–function principles, a new battery of archaeal extremozymes will become available to meet the growing industrial and biotechnological interest in these proteins.

Acknowledgments

Support came from the Israel Ministry of Absorption and the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities. The author is the incumbent of the Murray Shusterman Career Development Chair in Microbiology.

References

Abe F, Horikoshi K. The biotechnological potential of piezophiles. Trends Biotechnol 2001;19:102–8.

Adams MW. Enzymes and proteins from organisms that grow near and above 100 degrees C. Annu Rev Microbiol 1993;47:627–58.

Aguilar A, Ingemansson T, Magnien E. Extremophile microorganisms as cell factories: support from the European Union. Extremophiles 1998;2:367–73.

Ammendola S, Raia CA, Caruso C, Camardella L, D'Auria S, De Rosa M, Rossi M. Thermostable NAD(+)-dependent alcohol dehydrogenase from *Sulfolobus solfataricus*: gene and protein sequence determination and relationship to other alcohol dehydrogenases. Biochemistry 1992;31:12514–23.

Arnold FH, Wintrode PL, Miyazaki K, Gershenson A. How enzymes adapt: lessons from directed evolution. Trends Biochem Sci 2001;26:100–6.

Bauer MW, Driskill LE, Callen W, Snead MA, Mathur EJ, Kelly RM. An endoglucanase, EglA, from the hyperthermophilic archaeon *Pyrococcus furiosus* hydrolyzes beta-1,4 bonds in mixed linkage $(1 \rightarrow 3),(1 \rightarrow 4)$-beta-D-glucans and cellulose. J Bacteriol 1999;181:284–90.

Becker P, Abu-Reesh I, Markossian S, Antranikian G, Märkl H. Determination of the kinetic parameters during continuous cultivation of the lipase-producing thermophile *Bacillus* sp. IHI-91 on olive oil. Appl Microbiol Biotechnol 1997;48:184–90.

Bragger JM, Daniel RM, Coolbear T, Morgan HW. Very stable enzymes from extremely thermophilic archaebacteria and eubacteria. Appl Microbiol Biotechnol 1989;31:556–61.

Brown SH, Kelly RM. Characterization of amylolytic enzymes having both α-1,4 and α-1,6 hydrolytic activity from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. Appl Environ Microbiol 1993;59:2614–21.

Brown SH, Costantino HR, Kelly RM. Characterization of amylolytic enzyme activities associated with the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl Environ Microbiol 1990;56:1985–91.

Burlini N, Magnani P, Villa A, Macchi F, Tortora P, Guerritore A. A heat-stable serine proteinase from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*. Biochim Biophys Acta 1992;112:283–92.

Canganella F, Andrade CM, Antranikian G. Characterization of amylolytic and pullulytic enzymes from thermophilic archaea and from a new *Fervidobacterium* species. Appl Microbiol Biotechnol 1994;42:239–45.

Cannio R, Fiorentino G, Carpinelli P, Rossi M, Bartolucci S. Cloning and overexpression in *Escherichia coli* of the genes encoding NAD-dependent alcohol dehydrogenase from two *Sulfolobus* species. J Bacteriol 1996;178:301–5.

Cariello NF, Swenberg JA, Skopek TR. Fidelity of *Thermococcus litoralis* DNA polymerase (Vent) in PCR determined by denaturing gradient gel electrophoresis. Nucleic Acids Res 1991;19:4193–8.

Chavez Croocker P, Sako Y, Uchida A. Purification and characterization of an intracellular heat-stable proteinase (pernilase) from the marine hyperthermophilic archaeon *Aeropyrum pernix* K1. Extremophiles 1999;3:3–9.

Chien A, Edgar DB, Trela JM. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. J Bacteriol 1976;127:1550–7.

Chung YC, Kobayashi T, Kanai H, Akiba T, Kudo T. Purification and properties of an extracellular amylase for the hyperthermophilic archaeon *Thermococcus profundus* DT5432. Appl Environ Microbiol 1995;61:1502–6.

Connaris H, Cowan DA, Sharp RJ. Heterogeneity of proteinases from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. J Gen Microbiol 1991;137:1193–9.

Costantino HR, Brown SH, Kelly RM. Purification and characterization of an alpha-glucosidase from a hyperthermophilic archaebacterium, *Pyrococcus furiosus*, exhibiting a temperature optimum of 105 to 115 degrees C. J Bacteriol 1990;172:3654–60.

Cowan DA. Biochemistry and molecular biology of the extremely thermophilic archaeobacteria. In: Herbert RA, Sharp RJ, editors. Molecular biology and biotechnology of extremophiles. London: Blackie and Son, 1992. pp. 1–43.

Cowan DA, Smolenski KA, Daniel RM, Morgan HW. An extremely thermostable extracellular proteinase from a strain of the archaebacterium *Desulfurococcus* growing at 88 degrees C. Biochem J 1987;247:121–33.

Cummings SP, Black GW. Polymer hydrolysis in a cold climate. Extremophiles 1999;3:81–7.

Danson MJ, Hough DW. Structure, function and stability of enzymes from the Archaea. Trends Microbiol 1998;6:307–14.

DeLong EF. Everything in moderation: Archaea as 'non-extremophiles'. Curr Opin Genet Dev 1998;8:649–54.

DeLong EF, Wu KY, Prezelin BB, Jovine RV. High abundance of Archaea in Antarctic marine picoplankton. Nature 1994;371:695–7.

Di Lernia I, Morana A, Ottombrino A, Fusco S, Rossi M, De Rosa M. Enzymes from *Sulfolobus shibatae* for the production of trehalose and glucose from starch. Extremophiles 1998;2:409–16.

Dong G, Vieille , Zeikus JG. Cloning, sequencing and expression of the gene amylopullulanase from *Pyrococcus furiosus* and biochemical characterisation of the recombinant enzyme. Appl Environ Microbiol 1997;63:3577–84.

Duong F, Eichler J, Price A, Leonard MR, Wickner W. Biogenesis of the Gram-negative bacterial envelope. Cell 1997;91:567–73.

Edwards KJ, Bond PL, Gihring TM, Banfield JF. An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. Science 2000;287:1796–9.

Eggen HIL, Geerling A, Watts J, de Vos WM. Characterization of pyrolysin, a hyperthermoactive serine protease from the archaeobacterium *Pyrococcus furiosus*. FEMS Microbiol Lett 1990;71:17–20.

Eichler J. Archaeal protein translocation crossing membranes in the third domain of life. Eur J Biochem 2000;267:3402–12.

Eichler J, Moll R. The signal recognition particle of Archaea. Trends Microbiol 2001;9:130–6.

Eisenberg H, Wachtel EJ. Structural studies of halophilic proteins, ribosomes, and organelles of bacteria adapted to extreme salt concentrations. Annu Rev Biophys Biophys Chem 1987;16:69–92.

Fernández-Castillo RF, Rodriguez-Valera F, Gonzalez-Ramos J, Ruiz-Berraquero F. Accumulation of poly(β-hydroxybutyrate) by halobacteria. Appl Environ Microbiol 1986;51:214–6.

Franzmann PD, Springer N, Ludwig W, De MEC, Rohde M. A methanogenic archaeon from Ace Lake, Antarctica *Methanococcoides burtonii* sp-nov. Syst Appl Microbiol 1992;15:573–81.

Franzmann PD, Liu Y, Balkwill DL, Aldrich HC, Conway de Macario E, Boone DR. *Methanogenium frigidum* sp. nov., a psychrophilic, H-2-using methanogen from Ace Lake, Antarctica. Int J Syst Bacteriol 1997;47: 1068–72.

Frey B, Suppmann B. Demonstration of the Expand PCR System's greater fidelity and higher yields with a *lac*I-based fidelity assay. Biochemica 1995;2:34–5.

Fuhrman JA, McCallum K, Davis AA. Novel major archaebacterial group from marine plankton. Nature 1992;356:148–9.

Fusek M, Lin XL, Tang J. Enzymic properties of thermopsin. J Biol Chem 1990;265:1496–501.

Fusi P, Villa M, Burlini N, Tortora P, Guerritore A. Intracellular proteases from the extremely thermophilic archaebacterium *Sulfolobus solfataricus*. Experientia 1991;47:1057–60.

Gantelet H, Duchiron F. Purification and properties of a thermoactive and thermostable pullulanase from *Thermococcus hydrothermalis*, a hyperthermophilic archaeon isolated from a deep sea hydrothermal vent. Appl Microbiol Biotechnol 1998;49:770–7.

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. Cold-adapted enzymes: from fundamentals to biotechnology. Trends Biotechnol 2000;18:103–7.

Golyshina OV, Pivovarova TA, Karavaiko GI, Kondrateva TF, Moore ER, Abraham WR, Lunsdorf H, Timmis KN, Yakimov MM, Golyshin PN. *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the Archaea. Int J Syst Evol Microbiol 2000;50:997–1006.

Grogan DW. Evidence that β-galactosidase of *Sulfolobus solfataricus* is only one of several activities of a thermostable β-D-glycosidase. Appl Environ Microbiol 1991;57:1644–9.

Halio SB, Blumentals II, Short SA, Merrill BM, Kelly RM. Sequence, expression in *Escherichia coli*, and analysis of the gene encoding a novel intracellular protease (*Pfp*I) from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Bacteriol 1996;178:2605–12.

Hanzawa S, Hoaki T, Jannasch HW, Maruyama T. An extremely thermostable serine protease from a hyperthermophilic archaeon *Desulfurococcus* stain SY, isolated from a deep-sea hydrothermal vent. J Mar Biotechnol 1996;4:121–6.

Harwood VJ, Denson JD, Robinson-Bidle KA, Schreier HJ. Overexpression and characterization of a prolyl endopeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Bacteriol 1997;179:3613–8.

Haseltine C, Rolfsmeier M, Blum P. The glucose effect and regulation of alpha-amylase synthesis in the hyperthermophilic archaeon *Sulfolobus solfataricus*. J Bacteriol 1996;178:945–50.

Hayashi R. Use of high-pressure in bioscience and biotechnology. In: Hayashi R, Balny C, editors. High-pressure bioscience and biotechnology. New York: Elsevier, 1996. pp. 1–6.

Hei DJ, Clark DS. Pressure stabilization of proteins from extreme thermophiles. Appl Environ Microbiol 1994;60:932–9.

Henrissat B. Glycosidase families. Biochem Soc Trans 1998;26:153–6.

Hershberger KL, Barns SM, Reysenbach AL, Dawson SC, Pace NR. Wide diversity of Crenarchaeota. Nature 1996;384:420.

Hong FT. The bacteriorhodopsin model membrane system as a prototype molecular computing element. Biosystems 1986;19:223–36.

Horikoshi K. Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev 1999;63:735–50.

Hough DW, Danson MJ. Extremozymes. Curr Opin Chem Biol 1999;3:39–46.

Huber R, Stööhr H, Hohenhaus S, Rachel R, Burggraf S, Jannsch HW, Stetter KO. *Thermococcus chitonophagus* sp. nov., a novel chitin degrading, hyperthermophilic archeum from the deep-sea hydrothermal vent environment. Arch Microbiol 1995;164:255–64.

Huber R, Dyba D, Huber H, Burggraf S, Rachel R. Sulfur-inhibited *Thermosphaera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences. Int J Syst Bacteriol 1998;48:31–8.

Hugenholtz P, Pace NR. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. Trends Biotechnol 1996;14:190–7.

Ikeda M, Clark DS. Molecular cloning of extremely thermostable esterase gene from hyperthermophilic archaeon *Pyrococcus furiosus* in *Escherichia coli*. Biotechnol Bioeng 1998;57:624–9.

Jaenicke R, Bohm G. The stability of proteins in extreme environments. Curr Opin Struct Biol 1998;8:738–48.

Jorgensen S, Vorgias CE, Antranikian G. Cloning, sequencing, characterization, and expression of an extracellular

alpha-amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* in *Escherichia coli* and *Bacillus subtilis*. J Biol Chem 1997;272:16335–42.

Kamekura M. Diversity of extremely halophilic bacteria. Extremophiles 1998;2:289–95.

Kengen SW, Luesink EJ, Stams AJ, Zehnder AJ. Purification and characterization of an extremely thermostable beta-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Eur J Biochem 1993;213:305–12.

Kim J, Dordick JS. Unusual salt and solvent dependence of a protease from an extreme halophile. Biotech Bioeng 1997;755:471–9.

Kletzin A. Molecular characterisation of a DNA ligase gene of the extremely thermophilic archaeon *Desulfurolobus ambivalens* shows close phylogenetic relationship to eukaryotic ligases. Nucleic Acids Res 1992;20:5389–96.

Klingeberg M, Hashwa F, Antranikian G. Properties of extremely thermostable proteases from anaerobic hyperthermophilic bacteria. Appl Microbiol Biotechnol 1991;34:715–9.

Koch R, Zablowski P, Spreinat A, Antranikian G. Extremely thermostable amylolytic enzyme from the archaebacterium *Pyrococcus furiosus*. FEMS Microbiol Lett 1990;71:21–6.

Koch R, Spreinat A, Lemke K, Antranikian G. Purification and properties of a hyperthermoactive α-amylase from the archaebacterium *Pyrococcus woesei*. Arch Microbiol 1991;155:572–8.

Konisky J, Michels PC, Clark DS. Pressure stabilization is not a general property of thermophilic enzymes: the adenylate kinases of *Methanococcus voltae*, *Methanococcus maripaludis*, *Methanococcus thermolithotrophicus*, and *Methanococcus jannaschii*. Appl Environ Microbiol 1995;61:2762–4.

Krahe M, Antranikian G, Märkl H. Fermentation of extremophilic microorganisms. FEMS Microbiol Rev 1996;18:271–85.

Kwak YS, Akeba T, Kudo T. Purification and characterization from hyperthermophilic archaeon *Thermococcus profundus*, which hydrolyzes both α-1,4 and α-1,6 glucosidic linkages. J Ferment Bioeng 1998;86:363–7.

Laderman KA, Davis BR, Krutzsch HC, Lewis MS, Griko YV, Privalov PL, Anfinsen CB. The purification and characterization of an extremely thermostable α-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. J Biol Chem 1993;268:24394–401.

Lama L, Nicolaus B, Trincone A, Morzillo R, Calandrelli V, Gambacorta A. Thermostable amylolytic activity from *Sulfolobus solfataricus*. Biotech Forum Eur 1991;8:201–3.

Linke B, Rüdiger A, Wittenberg G, Jorgensen PL, Antranikian G. Production of heat-stable pullulanase and α-glucosidase from the extreme thermophilic archaeon *Pyrococcus woesi*. DECHEMA Biotech Conf 1992;5:161–3.

Lundberg KS, Shoemaker DD, Adams MW, Short JM, Sorge JA, Mathur EJ. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. Gene 1991;108:1–6.

Ma K, Robb FT, Adams MW. Purification and characterization of NADP-specific alcohol dehydrogenase and glutamate dehydrogenase from the hyperthermophilic archaeon *Thermococcus litoralis*. Appl Environ Microbiol 1994;60:562–8.

Madern D, Ebel C, Zaccai G. Halophilic adaptation of enzymes. Extremophiles 2000;4:91–8.

Madigan MT, Oren A. Thermophilic and halophilic extremophiles. Curr Opin Microbiol 1999;2:265–9.

Mattila P, Korpela J, Tenkanen T, Pitkanen K. Fidelity of DNA synthesis by the *Thermococcus litoralis* DNA polymerase — an extremely heat stable enzyme with proofreading activity. Nucleic Acids Res 1991;19:4967–73.

Mayr J, Lupas A, Kellermann J, Eckerskorn C, Baumeister W, Peters J. A hyperthermostable protease of the subtilisin family bound to the surface layer of the archaeon *Staphylothermus marinus*. Curr Biol 1996;6:739–49.

Miura Y, Kettoku M, Kato M, Kobayashi K, Kondo K. High level production of thermostable alpha-amylase from *Sulfolobus solfataricus* in high-cell density culture of the food yeast *Candida utilis*. J Mol Microbiol Biotechnol 1999;1:129–34.

Morana A, Moracci M, Ottombrino A, Ciaramella M, Rossi M, De Rosa M. Industrial-scale production and rapid purification of an archaeal beta-glycosidase expressed in *Saccharomyces cerevisiae*. Biotechnol Appl Biochem 1995;22:261–8.

Morikawa M, Izawa Y, Rashid N, Hoaki T, Imanaka T. Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. Appl Environ Microbiol 1994;60:4559–66.

Mukhopadhyay B, Johnson EF, Wolfe RS. Reactor-scale cultivation of the hyperthermophilic methanarchaeon *Methanococcus jannaschii* to high cell densities. Appl Environ Microbiol 1999;65:5059–65.

Nelson CM, Schuppenhauer MR, Clark DS. High-pressure, high-temperature bioreactor for comparing effects of hyperbaric and hydrostatic pressure on bacterial growth. Appl Environ Microbiol 1992;58:1789–93.

Niehaus F, Bertoldo C, Kähler M, Antranikian G. Extremophiles as a source of novel enzymes for industrial application. Appl Microbiol Biotechnol 1999;51:711–29.

Obayashi A, Hiraoka N, Kita K, Nakajima H, Shuzo T. US Patent 4: 724,209, US Cl. 435/199, 1988.

Oesterhelt D, Brauchle C, Hampp N. Bacteriorhodopsin: a biological material for information processing. Q Rev Biophys 1991;24:425–78.

Patel GB, Sprott GD. Archaeobacterial ether lipid liposomes (archaeosomes) as novel vaccine and drug delivery systems. Crit Rev Biotechnol 1999;194:317–57.

Perler FB, Adam E. Protein splicing and its applications. Curr Opin Biotechnol 2000;11:377–83.

Perler FB, Comb DG, Jack WE, Moran LS, Qiang B, Kucera RB, Benner J, Slatko BE, Nwankwo DO, Hempstead SK, Carlow CKS, Jannasch H. Intervening sequences in an Archaea DNA polymerase gene. Proc Natl Acad Sci USA 1992;89:5577–81.

Perler FB, Kumar S, Kong H. Thermostable DNA polymerases. Adv Protein Chem 1996;48:377–435.

Pillar K, Daniel RM, Petach HH. Properties and stabilization of an extracellular α-glucosidase from the extremely thermophilic archaebacteria *Thermococcus* strain AN1:activity at 130°C. Biochim Biophys Acta 1996;1292:197–205.

Pöhlschroder M, Prinz WA, Hartmann E, Beckwith J. Protein translocation in the three domains of life: variations on a theme. Cell 1997;91:563–6.

Preston CM, Wu KY, Molinski TF, DeLong EF. A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. Proc Natl Acad Sci USA 1996;93:6241–6.

Purcarea C, Herve G, Ladjimi MM, Cunin R. Aspartate transcarbamylase from the deep-sea hyperthermophilic archaeon *Pyrococcus abyssi*: genetic organization, structure, and expression in *Escherichia coli*. J Bacteriol 1997;179:4143–57.

Rapoport TA, Jungnickel B, Kutay U. Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. Annu Rev Biochem 1996;65:271–303.

Reeve JN, Nolling J, Morgan RM, Smith DR. Methanogenesis: genes, genomes, and who's on first? J Bacteriol 1997;179: 1997;179:5975–86.

Rodriguez-Valera F. Biotechnological potential of halobacteria. Biochem Soc Symp 1992;58:135–47.

Rolfsmeier M, Blum P. Purification and characterization of a maltase from the extremely thermophilic crenarchaeote *Sulfolobus solfataricus*. J Bacteriol 1995;177:482–5.

Rothschild LJ, Manicinelli RL. Life in extreme environments. Nature 2001;409:1092–101.

Rüdiger A, Ogbonna JC, Märkl H, Antranikian G. Effect of gassing, agitation, substrate supplementation and dialysis on the growth of an extremely thermophilic archaeon *Pyrococcus woesei*. Appl Microbiol Biotechnol 1992;37:501–4.

Rüdiger A, Jorgensen PL, Antranikian G. Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*. Appl Environ Microbiol 1995;61:567–75.

Russell NJ. Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles 2000;4:83–90.

Sako Y, Nomura N, Uchida A, Ishida Y, Morii H, Koga Y, Hoaki T, Maruyama T. *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100 degrees C. Int J Syst Bacteriol 1996;46:1070–7.

Sako Y, Croocker PC, Ishida Y. An extremely heat-stable extracellular proteinase (aeropyrolysin) from the hyperthermophilic archaeon *Aeropyrum pernix* K1. FEBS Lett 1997;415:329–34.

Schiraldi C, Marulli F, Di Lernia I, Martino A, De Rosa M. A microfiltration bioreactor to achieve high cell density in *Sulfolobus solfataricus* fermentation. Extremophiles 1999;3:199–204.

Schleper C, Swanson RV, Mathur EJ, DeLong EF. Characterization of a DNA polymerase from the uncultivated psychrophilic archaeon *Cenarchaeum symbiosum*. J Bacteriol 1997;179:7803–11.

Sleytr UB, Pum D, Sara M. Advances in S-layer nanotechnology and biomimetics. Adv Biophys 1997;34:71–9.

Sobek H, Gorisch H. Purification and characterization of a heat-stable esterase from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. Biochem J 1988;250:453–8.

Southworth MW, Kong H, Kucera RB, Ware J, Jannasch HW, Perler FB. Cloning of thermostable DNA polymerases from hyperthermophilic marine Archaea with emphasis on *Thermococcus* sp. 9 degrees N-7 and mutations affecting 3′-5′ exonuclease activity. Proc Natl Acad Sci USA 1996;93:5281–5.

Sowers KR, Schreier HJ. Gene transfer systems for the Archaea. Trends Microbiol 1999;7:212–9.

Stan-Lotter H, Doppler E, Jarosch M, Radax C, Gruber C, Inatomi KI. Isolation of a chymotrypsinogen B-like enzyme from the archaeon *Natronomonas pharaonis* and other halobacteria. Extremophiles 1999;3:153–61.

Stetter KO. Hyperthermophiles in the history of life. Ciba Found Symp 1996;202:1–18.

Summit M, Scott B, Nielson K, Mathur E, Baross J. Pressure enhances thermal stability of DNA polymerase from three thermophilic organisms. Extremophiles 1998;2:339–45.

Sunna A, Moracci M, Rossi M, Antranikian G. Glycosyl hydrolases from hyperthermophiles. Extremophiles 1997;1:2–1997;1:2-- 1997;1:2–13.

Tachibana Y, Mendez LM, Fujiwara S, Takagi M, Imanaka T. Cloning and expression of the a amylase gene from the hyperthermophilic archeon *Pyrococcus* sp. KOD1 and characterization of the enzyme. J Ferment Bioeng 1996;82:224–32.

Takagi M, Nishioka M, Kakihara H, Kitabayashi M, Inoue H, Kawakami B, Oka M, Imanaka T. Characterization of DNA polymerase from *Pyrococcus* sp. strain KOD1 and its application to PCR. Appl Environ Microbiol 1997;63:4504–10.

Uhl AM, Daniel RM. The first description of an archaeal hemicellulase: the xylanase from *Thermococcus zilligii* strain AN1. Extremophiles 1999;3:263–7.

Vander HPB, Davis MC, Cunniff JJ, Ruan C, McArdle BF, Samols SB, Szasz J, Hu G, Hujer KM, Domke ST, Brummet SR, Szasz J, Moffett RB, Fuller CW. Theromo Sequenase DNA polymerase and *T. acidophilum* pyrophosphatase: new thermostable enzymes for DNA sequencing. Biotechniques 1997;22:758–62.

Vaquez M, Moore ERB, Espejo RT. Detection by polymerase chain reaction amplification and sequencing of an archaeon in a commercial-scale copper bioleaching plant. FEMS Microbiol Lett 1999;173:183–7.

Viikari L, Kantelinen A, Sundquist J, Linko M. Xylanases in bleaching. From an idea to industry. FEMS Microbiol Lett 1994;13:335–50.

Völkl P, Markiewicz P, Stetter KO, Miller JH. The sequence of a subtilisin-type protease (aerolysin) from the hyperthermophilic archaeum *Pyrobaculum aerophilum* reveals sites important to thermostability. Protein Sci 1994;3:1329–40.

Voorhorst WG, Eggen RI, Luesink EJ, de Vos WM. Characterization of the *celB* gene coding for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and site-directed mutation in *Escherichia coli*. J Bacteriol 1995;177:7105–11.

Voorhorst WG, Eggen RI, Geerling AC, Platteeuw C, Siezen RJ, Vos WM. Isolation and characterization of the hyperthermostable serine protease, pyrolysin, and its gene from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Biol Chem 1996;271:20426–31.

Woese CR, Kandler O, Wheelis ML. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 1990;87:4576–9.