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Extremophiles: A Novel Source of Industrially Important Enzymes

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Abstract: Microbial life does not seem to be limited to specific environments. During the past few decades it has become clear that microbial communities can be found in the most diverse conditions, including extremes of temperature, pressure, salinity and pH. These microorganisms, called extremophiles, produce biocatalysts that are functional under extreme conditions. Consequently, the unique properties of these biocatalysts have resulted in several novel applications of enzymes in industrial processes. From recent study, major approaches to extending the range of applications of extremozymes have emerged. Both the discovery of new extremophilic species and the determination of genome sequences provide a route to new enzymes, with the possibility that these will lead to novel applications. Only a minor fraction of the microorganisms on earth have been exploited. Novel developments in the cultivation and production of extremophiles but also developments related to the cloning and expression of their genes in heterologous hosts, will increase the number of enzyme-driven transformations in chemical, food, pharmaceutical and other industrial applications. Of equal importance directed evolutions provide approaches to improve enzyme stability and modify specificity in ways that may not exist in the natural world.

Key words: Extremophiles, enzymes, industrially important, extremozymes

INTRODUCTION

The use of enzymes as biotransformation catalysts is well established and has been the subject of numerous texts and reviews. The majority of enzymes used to date, however, have been obtained from mesophilic organisms and despite their many advantages; the application of these enzymes is restricted because of their limited stability to extremes of temperature pH, ionic strength and etcetera. On the other hand, extremophiles are microorganisms that are found in environments of extreme temperature (-2 to 15, 60-110°C), ionic strength (2-5 M NaCl) or pH (<4, >9). The majority of extremophiles are members of the Archaea, one of the three phylogenetic domains of life, defined by comparison of 16 S rRNA gene sequences, although extremophilic members of the bacterial domain are also known (Brown and Doolittle, 1997). Extremophiles are a source of enzymes (extremozymes) with extreme stability and the application of these enzymes as biocatalysts is attractive because they are stable and active under conditions that were previously regarded as incompatible with biological materials. Furthermore, it is clear that some extremophiles, particularly those from the Archaea, have novel metabolic pathways and so might serve as a source of enzymes with novel activities and applications. Recent study suggests that the diversity of organisms in extreme environments is far greater than was initially suspected. The majority of extremophiles have not yet been isolated in pure culture, however, and thus it is difficult to determine the stability characteristics and precise substrate specificity and enantioelectivity of their enzymes. We have concentrated on advances in the isolation and identification of extremozymes, progress towards understanding the stability of extremozymes at a molecular level and enzyme engineering to create novel biocatalysts with enhanced stability and altered specificity.

Driven by increasing industrial demands for biocatalysts that can cope with industrial process conditions, considerable efforts have been devoted to the search for such enzymes. Compared with organic synthesis, biocatalysts often have far better chemical precision which can lead to more efficient production of single stereoisomers, fewer side reactions and a lower environmental burden (Rozzell, 1999). Despite the fact that to date more than 3000 different enzymes have been

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Table 1: Classification of extremophiles and examples of applications of some of their industrial enzymes (Ven den Burg, 2003)

Type	Growth characteristics	Enzymes	Applications
Thermophiles	Temp>80°C (Hyperthermophile) and 60-80°C (Thermophile)	proteases	Detergents, hydrolysis in food and feed, brewing, baking
		Glycosyl hydrolases (e.g. amylases, pullulanase, glucoamylases ,glucosidases, cellulases, xylanases)	Starch, cellulose, chitin, pectin processing, textiles
		Chitinases	Chitin modification for food and health products
		Xylanases	Paper bleaching
		Lipases, esterases	Detergents, stereo-specific reactions (e.g., trans-esterification, organic biosynthesis)
		DNA polymerases	Molecular biology (e.g., PCR)
		Dehydrogenases	Oxidation reactions
Psychrophiles	Temp <15°C	Proteases	Detergents, food applications (e.g., dairy products)
		Amylases	Detergents and bakery
		Cellulases	Detergents, feed and textiles
		Dehydrogenases	Biosensors
		Lipases	Detergents, food and cosmetics
Halophiles	High salt, (e.g., 2-5 M NaCl)	Proteases	Peptide synthesis
		Dehydrogenases	Biocatalysis in organic media
Alkaliphiles	pH>9	Proteases, cellulases	Detergents, food and feed
Acidophiles	pH <2-3	Amylases, glucoamylases	Starch processing
		Proteases, cellulases	Feed component
		Oxidases	Desulfurization of coal
Piezophiles	Pressure-loving; up to 130 MPa	To be defined	Food processing and antibiotic production

identified and many of these have found their way into biotechnological and industrial applications, the present enzyme toolbox is still not sufficient to present demands. A major cause for this is the fact that many available enzymes do not withstand industrial reaction conditions. As a result, the characterization of microorganisms that are able to thrive in extreme environments has received a great deal of attention: Such extremophiles are a valuable source of novel enzymes (Herbert, 1992; Madigan and Marrs, 1997). Extreme conditions can refer to physical extremes (e.g., temperature, pressure or radiation) but also to geochemical extremes such as salinity and pH (Table 1). Most of the extremophiles that have been identified to date belong to the domain of the Archaea. However, many extremophiles from the eubacterial and eukaryotic kingdoms have also been recently identified and characterized (Van den Burg, 2003).

The notion that extremophiles are capable of surviving under non-standard conditions in non-conventional environments has led to the assumption that the properties of their enzymes have been optimized for these conditions. Indeed, data for a considerable fraction of the enzymes that have been isolated and functionally characterized from extremophiles support this assumption. In this review, recent examples of the discovery, isolation and application of enzymes from extremophiles have been discussed.

Extremophiles: As illustrated in Table 1, the classification of extreme environments refers to a wide variety of different conditions to which microorganisms have adapted. The biocatalysts obtained from these microorganisms could be applicable in similarly diverse conditions (Table 1). For the degradation of polymers such as chitin, cellulose or starch, enzymes that are active at and resistant to high temperatures are often preferred. Under these conditions the solubility and, consequently, the accessibility of the substrate is improved. Alternatively, if one needs to perform a stereospecific modification of a compound for the synthesis of a pharmaceutically relevant product in organic solvents, very different prerequisites apply to the biocatalysts. As salt is known to reduce water activity, enzymes from halophilic microorganisms could be the most suitable choice for application in nonaqueous media (Sellek and Chaudhuri, 1999; Marhuenda-Egea and Bonete, 2002). This diversity of environments to which different extremophiles have adapted offers many exciting opportunities for a variety of applications.

Thermophiles: Thermophilic extremophiles have attracted most attention. In particular extremophilic proteases, lipases and polymer degrading enzymes, such as cellulases, chitinases and amylases have found their way into industrial applications (Table 1). The reasons to exploit enzymes that are stable and active at elevated temperatures are obvious. At elevated temperatures the solubility of many reaction components, in particular polymeric substrates, is significantly improved. Moreover, the risk of contamination, leading to undesired

Table 2: Production of extremophilic enzymes by hyperthermophiles

Hyperthermophiles	Thermophilic enzymes	T_{opt} °C	pH_{opt}	References
Bacteria		-	-	
Bacillius, clostridia, fervidobacterium	Cellulase	-	-	Adams *et al.* (1995)
pennavorans, rhodothermus marinus,	Amylase	-	-	Hough and Danson and (1999)
Rhodothermus obamensis, thermus	Pullulanase I	-	-	Niehaus *et al.* (1999)
caldophilus, thermoanaerobacter sp.,	Pullulanase II	-	-	Demiorjan *et al.* (2001)
Thermoplasma acidophylum, thermotoga	α-Glucosidase	-	-	Irwin and Baird (2004)
Maritima, thermotoga neapolitana,	β-Glucosidase	-	-	Irwin and Baird (2004)
Picrophilus oshimae,	Glucoamylase	-	-	Vieille and Zeikus (2001)
Picrophilus torridus	Xylanase	-	-	Haki and Rakshit (2003)
Archaea	Mannanase	-	-	Fujiwara (2002)
Desulfurococcus mucosus, pyrococcus	Pectinase	-	-	Sellek and Chaudhuri (1999)
Furiosus, pyrococcus	Chitinase			Leveque *et al.* (2000)
Woesei, pyrodictium	Protease	-	-	Bertoldo and Antranikian (2002)
Abyssi, staphylothermus marinus,	Lipase	-	-	
Sulfolobus solfataricus, Thermococcus	Esterase	-	-	Van der Maarel *et al.* (2002)
Hydrothermalis, Thermococcus litoralis,				
Thermococcus celer,				
Thermococcus profundus,				
Thermococcus aggregans	Phytase	-	-	Eckert and Schneider (2003)
Alicyclobacillus acidocaldarius	Endoglucanase (CelB)	80	4	Hatada *et al.* (2005)
Alicyclobacillus acidocaldarius	β-Glycosidase	85	5.0-5.5	Redecke *et al.* (2007)
Deinococcus radiopugnas	DNA-binding protein	90	-	Sunna and Bergquist (2003)
Environmental DNA	β-Xylanase	100	6	Chen *et al.* (2007)
Gold-bearing metagenome	3 Sulfur oxygenase reductases	75-80	-	Dheeran *et al.* (2010)
Geobacillus sp. IIPTN	α-amylase	80	5	Kim *et al.* (2001)
Methanococcus jannaschii	α-Amylase	120	5.0-8.0	Hotta *et al.* (2002)
Pyrobaculum calidifontis	Carboxylesterase	90	7	Gao *et al.* (2003)
Pyrococcus furiosus	Chitinase a and b	90-95	6	Hutcheon *et al.* (2005)
Pyrocuccus horikoshii	Cellulase	100	5.6	Fukushima *et al.* (2005)
Pyrocuccus horikoshii	Endoglucanase	95	7	Andrade *et al.* (2001)
Pyrodictium abyssi	Xylanase	105	6	Lorentzen *et al.* (2006)
Pyrococcus horikoshii	L-Threonine dehydrogenase	100	10	Tatur *et al.* (2006)
Pyrococcus horikoshii	Protein-disulfide oxidoreductase	100	8	Nakagawa *et al.* (2006)
Pyrococcus furiosus	Ferritin	120	7	Gomes *et al.* (2003)
Rhodothermus marinus	Amylase	85	6.5	Gomes *et al.* (2000)
Rhodothermus marinus	Pullulanase	80	6.5-7.0	
Rhodothermus marinus	α-L-Arabinofuranosidase	85	5.5-7.0	Gomes and Steiner (1998)
Thermus thermophilus RQ-1	β-Mannanase	85	5.0-6.5	
Mud and sediment-rich water	Trehalase	88	6.5	Jorge *et al.* (2007)
Metagenome	Family 18 chitinase	95	4.5-5.0	Merone *et al.* (2005)
Thermotoga maritima MSB8	Trehalose-6-phosphate synthase			
	and Trehalose-6-phosphate			
	phosphatase	100	6.0-7.0	
Sulfolobus solfataricus	Esterase	95	6	Silva *et al.* (2005)
Sulfolobus solfataricus	Alpha-L-arabinofuranosidase	100	7	Rhee *et al.* (2005)
Sulfolobus solfataricus	Xylanase	100	7	Kang *et al.* (2006)
Sulfolobus shibatae	α-Glucosidase	120	4.5	Cannio *et al.* (2004)
Sulfolobus solfataricus	Trehalosyl transglucoylase	75	5	Giuliano *et al.* (2004)
Thermococcus litoralis	α-Glucosidase	98	5.5	Fang *et al.* (2004)
Ralstonia sp. A-471	Phosphotriesterase	100	8	Woosowska and Synowiecki (2004)
Thermococcus chitonophagus	L-Aminoacylase	85	8	Palmieri *et al.* (2006)
Thermoplasma acidophilum	Chitinase	70	5	Taylor *et al.* (2004)
Picrophilus torridus	Chitinase	70	7	Sutrisno *et al.* (2004)
Picrophilus oshimae	Glucoamylases	90	2	Andronopoulou and Vorgias (2003)
		90	2	Serour and Antranikian (2002)
		90	2	Serour and Antranikian (2002)

complications, is reduced at higher temperatures. The structural feature of thermophilic extremozymes has attracted much attention. Several three dimensional structures have been solved and compared with those of mesophilic counterparts, with the ultimate goal of elucidating the mechanisms underlying thermostability (Sterner and Liebl, 2001; Vieille and Zeikus, 2001; Van den Burg and Eijsink, 2002). In a comprehensive study, 10 thermophilic and hyperthermophilic serine hydroxymethyltransferases were compared with 53 mesophilic homologs (Paiardini *et al.*, 2002). Structural alignment and homology modeling was applied to identify

the different mechanisms involved in thermal stability. For this enzyme class, it was concluded that stability was achieved by a combination of increased surface charge, increased protein core hydrophobicity and replacement of exposed thermolabile amino acids. The biocatalytic potential of thermophiles and their enzymes has been reviewed by Adams *et al*. (1995), Hough and Danson (1999), Niehaus *et al*. (1999), Dermiorijan *et al*. (2001), Van den Burg (2003), Irwin and Baird (2004), Eichler (2001), Vieille and Zeikus (2001), Haki and Rakshit (2003), Fujiwara (2002), Sellek and Chaudhuri (1999), Leveque *et al*. (2000) and Bertoldo and Antranikian (2002). Thermophiles can be generally classified into moderate thermophiles (growth optimum 50-60°C), extreme thermophiles (growth optimum 60-80°C) and hyperthermophiles (growth optimum 80-110°C) (Table 2). Among the extreme thermophiles, multicellular 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. Extreme thermophiles, growing optimally at 60-80°C, are widely distributed among the genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Rhodothermus*, *Thermotoga* and *Aquifex* (Table 2). On the other hand, 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* (Table 2). Euryarchaeota include extreme halophiles (e.g., *Halobacterium, Halobaculum, Halococcus, Haloferax* and *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*) (Table 2).

Psychrophiles: More recently, enzymes from psychrophiles have become interesting for industrial application, partly because of ongoing efforts to decrease energy consumption. For example, there is an increasing desire to apply psychrophilic enzymes in detergents. With such enzymes it becomes feasible to develop laundry applications that can be performed at lower temperatures. For such processes, psychrophilic proteases, amylases or lipases have great commercial potential. The pulp and paper industry is also interested in polymer-degrading enzymes that are active at lower temperatures. Several food processing applications would also benefit from the availability of low temperature enzymes like L-glutaminase and L-asparaginase. A characteristic feature of many enzymes from psychrophiles is the correlation of high catalytic activity and low thermal stability at moderate temperatures which can be partly explained by the increased flexibility of the molecule, compared with mesophilic and thermophilic enzymes. This adaptation of the enzymes to low temperatures has been the subject of several studies (Kumar *et al*., 2011a; Kamakshi *et al*., 2010; Kumar *et al*., 2009; Bentahir *et al*., 2000; Kim *et at*., 1999; Fields, 2001; Smal *et al*., 2000; Watanabe *et al*., 2002; DAmico *et al*., 2003). It has been assumed that increased flexibility is correlated with decreased stability and a delicate balance between stability and activity is indeed often observed. Nevertheless, there are an increasing number of examples from nature as well as from protein engineering studies showing that the structural features involved in stability or activity can be very different and act independently (Schoichet *et al*., 1995; Van den Burg *et al*., 1998; Beadle and Schoicet, 2002). A good overview of present knowledge on the properties of enzymes from psychrophiles and their applications can be found in recent review (Cavicchioli *et al*., 2002; Deming, 2002).

The potentials of psychrophiles and psychrophilic enzymes have been reviewed by Cavicchioli *et al.* (2002), Deming (2002), Margesin *et al.* (2002), Feller and Gerday (2003) and Georlette *et al.* (2004). In fact, deep oceans which cover over 70% of the Earths surface, represent the major ecosystem on the planet. Many psychrophiles live in biotopes having more than one stress factors, such as low temperature and high pressure in deep seas (piezo-psychrophiles), or high salt concentration and low temperature in sea ice (halo-psychrophiles). A diverse range of psychrophilic microorganisms, belonging to Gram-negative bacteria (e.g., *Pseudoalteromonas*, Moraxella, Psychrobacter, Polaromonas, Psychroflexus, *Polaribacter*, *Moritella*, *Vibrio* and *Pseudomonas*) Gram-positive bacteria (e.g., *Arthrobacter*, *Bacillus* and *Micrococcus*) archaea (e.g., *Methanogenium*, *Methanococcoides* and *Halorubrum*) yeast (*Candida* and *Cryptococcus*) and fungi (*Penicillium* and *Cladosporium*) have been isolated from these cold environments (Cavicchioli *et al*., 2002; Deming, 2002; Margesin *et al*., 2002; Feller and Gerday, 2003; Georlette *et al*., 2004). These psychrophiles are able to degrade a wide range of polymeric substances such as starch, cellulose, xylan, pectin, chitin, protein and lipid and produce enzymes like amylase, cellulase, xylanase, pectinases, chitinase, protease and lipase, respectively The ability of psychrophilic enzymes to catalyse reactions at low or moderate temperatures offers great industrial and biotechnological potential (Demiorjian *et al*., 2001;

Table 3: Production of extremophilic enzymes by psychrophiles/psychrotolerant microorganis

Psychrophiles/psychrotolerant	Cold-active enzymes	T_{opt} °C	pH_{opt}	Referances
Acinetobacter sp. strain no. 6	Novel esterase	50	7.8	Suzuki *et al.* (2001)
	Lipase	20	7.0	
Arthrobacter sp. C2-2	ß-Galactosidase	40	7.5	Karasova-Lipovova *et al.* (2003)
Arthrobacter sp. strain TAD20	Chitinases	NA	NA	Mavromatis *et al.* (2003)
Bacillus subtilis A-53	Carboxymethylcellulase	35	6.8	Lee *et al.* (2010)
Bacillus sp. 158	Protease	30	7.0	Pawar *et al.* (2009)
Bacillus, Clostridium, Actinomycetes	α-Amylase	NA	NA	Groudieva *et al.* (2004)
Cytophaga-Flexibacter-Bacteroides	β-Galactosidase			
Pedobacter cryoconitis sp. Nov	Oxidase, catalase,			
	protease, amylase,	NA	NA	Margesin *et al.* (2003)
	ß-glucosidase,	20	5.0-6.0	
	ß-galactosidase,	(for all	(for all	Akila and Chandra (2003)
	ß-lactamase	three)	three)	
Clostridium strain PXYL1	Filter paper cellulase	10-15	7.0	Coker and Brenchley (2006)
	Endocellulase	15	6.0	Skalova *et al.* (2005)
	Xylanase	5-20	6.8	Hu *et al.* (2007)
Arthrobacter sp. SB	β-Galactosidase	0-10	4.0	Nakagawa *et al.* (2006)
Arthrobacter sp. C2-2	β-Galactosidase	45-50	5.0–5.0	Gomes *et al.* (2000a)
Planococcus sp. L4	β-Galactosidase	20	7.0	Kuddus and Ramteke (2008)
Guehomyces pullulans	β-Galactosidase			
Cryptococcus adeliae	Xylanase	NA	NA	Nakagawa *et al.* (2004)
Curtobacterium luteum	Metalloprotease	24	6.5	Secades *et al.* (2003)
Cryptococcus cylindricus	Pectinase	40-60	3.0-11.0	Ferrer *et al.* (2005a)
Mrakia frigida				
Cystofilobasidium capitatum				
Flavobacterium psychrophilum	Metalloprotease	50	6.0	Kojima *et al.* (2006)
Cow rumen metagenome	9 Endoglucanases	50	7.0	Hobel *et al.* (2005)
Haloarcula hispanica	α-Amylase	15-20	6.0	Lee *et al.* (2006)
Haloarcula sp. strain S-1	α-Amylase	40	6.0-7.0	Kashima *et al.* (2005)
Environmental DNA library	Xylanase	40	5.5-9.0	Voget *et al.* (2006)
Pseudoalteromonas sp. DY3	Cellulase	25	5.3-8.0	Collins *et al.* (2002)
Soil metagenome	Cellulase	5-25	7.0-9.0	Kulakova *et al.* (2004)
Pseudoalteromonas haloplanktis	Xylanase (family 8)	NA	NA	Yumoto *et al.* (2003)
Psychrobacter sp. Ant300	Esterase	30-40	6.0-7.0	Sakamoto *et al.* (2003)
Psychrobacter okhotskensis sp. Nov	Lipase	40	10.0	Zeng *et al.* (2003)
Penicillium chrysogenum	Endo-arabinanase	40-45	10.5-11.0	Okuda *et al.* (2004)
Pseudomonas strain DY-A	Alkaline protease	60	2.5-4.0	Golyshina *et al.* (2006)
Bacillus sp.	Subtilisin	60	2.5-3.0	Ferrer *et al.* (2005b)
Ferroplasma acidiphilum	2α-Glucosidases	50	1.5	Golyshina *et al.* (2006)
Ferroplasma acidiphilum	α-Glucosidase	0-30	7.5	Zimmer *et al.* (2006)
Ferroplasma acidiphilum	Esterase	40	7.5	Yazdi *et al.* (2008)
Rhodotorula mucilaginosa	Benzoyl tartrate esterase	20		Luo *et al.* (2006)
Rhodococcus sp. PTCC 1633	Cholesterol Oxidase	-	8.0	Song *et al.* (2005)
Pseudoalteromonas haloplanktis	Lipase	70	-	Maki *et al.* (2006)
Cool-seep sediment metagenome	TEM-b-lactamase	0-10	6.0-8.8	Yoshimune *et al.* (2005)
Thermotoga maritime	Homoserine transsuccinylase	60	7.5	Beloqui *et al.* (2006)
Vibrio salmonicida	Catalase	92	3.5-9.5	Hu *et al.* (2007)
Cow rumen metagenome	Polyphenol oxidase	74	5.0	Rigano *et al.* (2006)
Thermus thermophilus	Laccase	15-30	8.0	Zimmer *et al.* (2006)
Thermotoga maritime	Sec traslocase (ATPase)	15-25	7.5	Luo *et al.* (2006)
Shewanella sp. Ac10	DnaK protein	10-40	7.5	Yoshimune *et al.* (2005)
Koliella Antarctica	Nitrate reductase		4.0-9.0	Flavobacterium frigidimaris KUC-1
	Malate dehydrogenase			

Van den Burg, 2003; Eichler, 2001; Cavicchioli *et al*., 2002; Deming, 2002; Margesin *et al*., 2002; Feller and Gerday, 2003; Georlette *et al*., 2004). In addition, some examples are shown in Table 3.

The use of cold-active hydrolytic enzymes such as proteases, lipases, amylases and cellulases in the formulation of detergents would be of great advantage for cold washing. This would reduce the energy consumption and wear and tear of textile fibers. The industrial dehairing of hides and skins at low temperatures using psychrophilic proteases or keratinase would not only save energy but also reduce the impacts of toxic chemicals used in dehairing. Apart from these examples, cold-active enzymes have potential for other interesting applications such as the hydrolysis of lactose in milk using-galactosidase, biopolishing and stone washing of textile products using cellulases, extraction and clarification of fruit juices using pectinases, tenderization of meat or taste improvement of refrigerated meat using proteases (Chen *et al*., 2007) improvement of bakery

products using glycosidases (e.g., amylases, proteases and xylanases) softening of wool or cleaning of contact lenses using proteases. Other cold active enzymes could be good alternatives to mesophilic enzymes in brewing and wine industries, cheese manufacturing, animal feed supplements and so on. Psychrophilic microorganisms as well as their enzymes (e.g., oxidase, peroxidase and catalase) have been proposed as alternatives to physicochemical methods for the bioremediation of solids and waste waters polluted by hydrocarbons, oils and lipids (Margesin *et al*., 2002).

Halophiles: Halophiles can survive in hypersaline habitats by their ability to maintain osmotic balance. They accumulate salts such as sodium or potassium chloride (NaCl or KCl), up to concentrations that are isotonic with the environment. As a result, proteins from halophiles have to cope with very high salt concentrations (e.g., KCl concentrations of 4 M and NaCl concentrations of >5 M) (Demiorijan *et al*., 2001; Danson and Hough, 1997). The enzymes have adapted to this environmental pressure by acquiring a relatively large number of negatively charged amino acid residues on their surfaces to prevent precipitation. Consequently, in surroundings with lower salt concentrations the solubility of halophilic enzymes is often very poor which could limit their applicability (Madern *et al*., 2000) However, this property has been taken advantage of by nonaqueous media (Klibanov, 2001). For example, an extracellular protease from Halobacterium halobium has been exploited for efficient peptide synthesis in water/N0-dimethylformamide (Kim and Dordick, 1997). A P-nitrophenylphosphate Phosphatase (p-NPPase) from Halobacterium salinarum was used in an organic medium at very low salt concentrations after entrapping the enzyme in reversed micelles (Marhuenda-Egea *et al*., 2002) Under these conditions p-NPPase was active and stable. Similar observations were made with a halophilic malate dehydrogenase (Piera-Velazquez *et al*., 2001). Exploitation of reversed micelles in combination with halophilic enzymes is likely to result in the development of novel applications for these enzymes (Marhuenda-Egea *et al*., 2002). Halophiles respond to increases in osmotic pressure in different ways. The extremely halophilic archaea the Halobacteriaceae accumulate K^+, while other bacteria accumulate compatible solutes (e.g., glycine, betaine, sugars, polyols, amino acids and ectoines) which help them to maintain an environment isotonic with the growth medium. These substances also help to protect cells against stresses like high temperature, desiccation and freezing. Consequently, in surroundings with lower salt concentrations, the solubility of halophilic proteins is often very low (Danson and Hough, 1997; Madern *et al*., 2000) Halophiles from the archaeal domain provide the main source of extremely halophilic enzymes. The potentials of halophiles and haloenzymes have been reviewed previously (Gomes *et al*., 2003; Van den Burg, 2003). The production of halophilic enzymes, such as xylanases, amylases, proteases and lipases has been reported for some halophiles belonging to the genera Acinetobacter, Haloferax, Halobacterium, Halorhabdus, *Marinococcus*, *Micrococcus*, *Natronococcus*, *Bacillus*, *Halobacillus* and *Halothermothrix*. Table 4 shows some recent reports on these enzymes.

Alkaliphiles/acidophiles: Enzymes from microorganisms that can survive under extreme pH could be particularly useful for applications under highly acidic or highly alkaline reaction conditions for example, in the production of detergents. However, one of the striking properties of acidophilic and alkaliphilic microorganisms is their ability to maintain a neutral pH internally and so the intracellular enzymes from these microorganisms do not need to be adapted to extreme growth conditions. However, this does not account for extracellular proteins which have to function in low or high pH environments in the case of acidophiles and alkaliphiles, respectively. Proteases, amylases, lipases and other enzymes that are resistant to

Table 4: Production of extremophilic enzymes by halophiles

Halophiles	Halophilic enzymes	T_{opt} ^{0}C	pH_{opt}	References
Halothermothrix orenii	α-Amylase	65	7.5	Mijts and Patel (2002)
Bacillus dipsosauri	α-Amylase	60	6.5	Deutch (2002)
Bacillus sp. *NTU-06*	Xylanase	40	8.0	Wang *et al*. (2010)
Weissella cibaria	glutaminase	30	7.5	Jaruwan *et al*. (2009)
Halobacillus sp. strain MA-2	α-Amylase	50	7.5-8.5	Amoozegar *et al*. (2003)
Haloferax mediterranei	α-Amylase	50-60	7-8	Perez-Pomares *et al*. (2003)
Halophilic bacterium, CL8	Xylanase 1	60	6.0	Wejse *et al*, 2003
	Xylanase 2	65	6.0	
Halorhabdus utahensis	β-Xylanase	55.70	NA	Waino and Ingvorsen (2003)
	β-Xylosidase	65	NA	
Lactobacillus reuteri KCTC3594	Glutaminase	40	7.5	Jeon *et al*. (2009)
Pseudoalteromonas sp. strain CP76	Protease CPI	55	8.5	Sanchez-Porro *et al*. (2003)
Micrococcus luteus K-3	Glutaminase	-	-	Yoshimune *et al*. (2010)

Table 5: Production of extremophilic enzymes by thermoalkaliphiles/alkaliphiles

Thermoalkaliphiles/Alkaliphiles	Thermoalkaliphilic/alkaliphilic enzymes	T_{opt} ^{0}C	pH_{opt}	References
Aspergillus fumigates	Lignocellulosic bioethanol	40	5.5	Sherief *et al.* (2010)
Aspergillus niger	α-Amylase	30	5.0	Gupta *et al.* (2008)
Streptomyces sp.	Endocellulase	50	8.0	Von Solingen *et al.* (2001)
Bacillus firmus	Xylanases (xyn10A and xyn11A)	70	5.0-9.5	Chang *et al.* (2004)
Bacillus subtilis RM-01	β-keratinase	50	8.0	Sudhir *et al.* (2009)
Bacillus mojavensis A21	Protease	60	10	Hadder *et al.* (2009)
Bacillus subtilis strain DM-04	Protease	45	10.5	Sudhir *et al.* (2009)
Bacillus halodurans strains	Amylase, pullulanase	55-65	10.0	Hashim *et al*, 2004
Bacillus sp. strain JAMB-750	Mannase	55	10.0	Zeng *et al.* (2006)
Nocardiopsis sp. strain F96	β-1.3-Glucanase	70	9.0	Miyazaki (2005)
Bacillus isolate KSM-K38	α-Amylase	55-60	8.0-9.0	Hagihara *et al.* (2001)
Nesterenkonia sp. AL-20	Alkaline protease	Tm = 74°C	10.0	Bakhtiar *et al.* (2003)
Bacillus pumilus	Alkaline protease	50-60	11.5	Kumar (2002)
Arthrobacter ramosus Bacillus alcalophilus	Alkaline protease	65	11.0-10.0	Kanekar *et al.* (2002)
Nocardiopsis sp.	Alkaline protease keratinase	70-75	11.0-11.5	Mitsuiki *et al.* (2002)
Bacillus pseudofirmus FA30-01	Feather-degrading enzyme	60	8.8-10.3	Takeda *et al.* (2006)
Thermus thermophilus	Sulfite oxidase	60	11.0	Pretz *et al.* (2005)
Cow rumen metagenome	Cyclodextrinase	75	5.5-9.0	Ferrer *et al.* (2005c)
Burkholderia cepacia strain ST-200	Cholesterol esterase	45	12.0	Shi *et al.* (2006)
Deep-sea sediment metagenome	Esterase	55	10.5	Park *et al.* (2007)
Bacillus alcalophillus	Pectate lyase	45	9.0-10.0	Zhai *et al.* (2003)
Thermomonospora(Actinomycete)	Endocellulase	50	5.0	George *et al.* (2001)
Cow rumen metagenome	12 Esterases	60	7.0-11.0	Reyes-Duarte *et al.* (2005)
Cow rumen metagenome	Esterase	50	8.0	Reyes-Duarte *et al.* (2005)
Deep-sea metagenome	5 Esterases	40-60	8.0-9.0	Ferrer *et al.* (2005d)
Rhizopus chinensis CCTCC- M201021	Lipase	30	6.0	Pogori *et al.* (2008)

and active at high pH and high chelator concentrations of modern detergents are desirable. This has prompted the screening of alkaliphilic bacteria and Archaea for their ability to produce such enzymes. By these means several useful enzymes have already been identified and obtained. Combinations of homologybased PCR and activity screening have been applied to screen for and detect alkaline proteases in a collection of thermoacidophilic archaeal and bacterial strains isolated from hot environments (Kocabiyik and Erdem, 2002). In an alternative approach, alkaliphilic bacilli that could grow at>pH 9 were used as a source for oxidation-resistant alkaline proteases (Saeki *et al.*, 2002). Polymer-hydrolysis related processes have also initiated the search for biocatalysts from acidophiles. Several enzymes used for starch-hydrolysis (e.g., amylases, pullulanases, glucoamylases and glucosidases that are active at low pH) have been isolated (Bertoldo and Antranikian, 2002; Serour and Antranikian, 2002).

One of the most striking properties of acidophilic and alkaliphilic microorganisms is their use of proton pumps to maintain a neutral pH internally and so the intracellular enzymes from these microorganisms do not need to be adapted to extreme growth conditions. However, the extracellular enzyme proteins of acidophiles have to function at low pH whereas those of alkaliphiles function at alkaline pH. How these extracellular proteins operate at high or low pH values is yet poorly understood. In order for the cells to survive in the aggressive conditions of pH, alkaliphiles and acidophiles utilize several strategies. Alkaliphiles have negatively charged cell wall polymers in addition to peptidoglycan which may reduce the charge density at the cell surface and help to stabilize the cell membrane (Wiegel and Kevbrin, 2004; Horikoshi, 1999). Cellular fatty acids in alkaliphilic bacterial strains contain predominantly saturated and mono-unsaturated straight-chain fatty acids (Ma *et al.*, 2004). In order to withstand low pH, acidophiles employ a range of mechanisms such as: a positively charged membrane surface a high internal buffer capacity, over-expression of H^+ exporting enzymes and unique transport systems (Wiegel and Kevbrin, 2004; Horikoshi, 1999). Thermoalkaliphiles and alkaliphiles are good sources of alkaliphilic enzymes like cellulases, xylanases, amylases, proteases, lipases, pectinases, chitinase, catalase, peroxidase and oxidoreductase (Wiegel and Kevbrin, 2004; Horikoshi, 1999). Some recent reports on these enzymes are shown in Table 5. Thermoalkaliphilic enzymes have great biocatalytic potential in processes that are performed at alkaline pH and higher temperatures. For example, proteases, lipases and cellulases are used as additives in laundry and dishwashing detergents, proteases are also used for dehairing of hides and skins and to improve smoothness and dye affinity of wool (Eichler, 2001; Wiegel and Kevbrin, 2004; Horikoshi, 1999) as well as in detergent industries as a additives (Kumar *et al.*, 2011b; Shukla *et al.*, 2009). Cellulase free xylanases are used for biobleaching of pulp and paper,

pectinases are used in degumming of ramie fibers and catalase and peroxidase or oxidoreductase may be used to remove residual hydrogen peroxide from effluent streams of the textile processing industry (Table 5).

Piezophiles: It is thought that pressure does not exert a major selective force on protein function (Gros and Jainicke, 1994). This assumption is based on the consideration that pressures exceeding 400 MPa are needed to induce the denaturation of single-chain proteins. In view of that, it should be noted that even microorganisms that live in the deep sea are not exposed to pressures that exceed 120 MPa. Under those conditions, their enzymes do not need specific pressure-related adaptations. However, there are some examples of the specific stabilization of proteins by increased pressure (Hei and Clark, 1994). Pressure-resistant proteins could be of use, in particular for food production, where high pressure is applied for processing and the sterilization of food materials (Hayashi, 1996). The biotechnological opportunities for piezophiles have recently been reviewed in detail (Abe and Horikoshi, 2001).

Based on the facts that pressures exceeding 400 MPa are needed to induce protein denaturation and microorganisms living in the deep sea are not exposed to pressures exceeding 120 MPa (Gros and Jainicke, 1994) it is assumed that pressure does not represent a major selective factor for protein structure and function in piezophiles and that their proteins do not need specific pressure related adaptations (Van den Burg, 2003). However, there are some examples of protein stabilization by high pressure (Pledger *et al*., 1994; Hei and Clark, 1994) during processing and sterilization of food materials, high pressures of a few hundred MPa can be used to induce the formation of gels or starch granules, the denaturation/coagulation of proteins or the transition of lipid phases. The use of high pressure leads to better flavor and color preservation than the use of high temperature to achieve the same ends (Abe and Horikoshi, 2001; Hayashi, 1996; Ludwig *et al*., 1996). Moreover, enzymes that can operate at increased pressure and temperature have great advantages in biotechnological applications. Enzymatic reactions that have a negative change in activation volume ($V<0$) are favored by increasing pressure, whereas reactions with a positive change ($V>0$) are not. The change in activation Volume (V) can be used as a method to control reaction specificity. For example, chymotrypsin catalyzes both the hydrolysis of an anilide ($V<0$) and the hydrolysis of an ester ($V>0$). A reaction mixture containing both substrates and chymotrypsin in organic media was controlled by altering the pressure to favor one or the other reaction (Mozhaev *et al*., 1994). Although there are many possible biotechnological applications of piezophiles and piezophilic enzymes, there are few known practical applications of piezophiles or piezophilic enzymes (Abe and Horikoshi, 2001). This is due to the fact that it is not easy to cultivate piezophiles under high-pressure conditions using current technology. Therefore, the properties of these enzymes and other cellular components have not yet been fully investigated.

Other extremophiles: At present, it is clear that no matter how extreme the conditions are at defined locations on Earth there is a fair chance that microbes will be able to survive. Additional examples further to those described above are microorganisms that grow in the presence of high metal concentrations (metallophiles) at high radiation levels (radiophiles) or under oxygen deprivation (microaerophiles). The biotechnological application of enzymes from such extremophiles is not always obvious. Nevertheless, in view of the great potential of biocatalysis it is very likely that new concepts will be developed that will result in the application of enzymes from these and other extremophiles in industrial processes.

CONCLUSIONS

Extremozymes have a great economic potential in many industrial processes, including agricultural, chemical and pharmaceutical applications. Many consumer products will increasingly benefit from the addition or exploitation of extremozymes. The toolbox to select and make such enzymes available is steadily expanding. It has been suggested that less than 10% of the organism in a defined environment will be cultivatable and so further improvement of gene expression technologies (e.g., by the development of novel and improved heterologous host systems) will accelerate the exploration of microbial diversity. It is now possible to construct gene expression libraries from the most diverse sources. If such libraries are screened with fast and accurate detection technologies many new extremozymes will be discovered in the years to come. These extremozymes will be used in novel biocatalytic processes that are faster, more accurate specific and environmentally friendly. Concurrent developments of protein engineering and directed evolution technologies will result in further tailoring and improving biocatalytic traits which will increase the application of enzymes from extremophiles in industry.

Our experience with extremophiles leads us to caution that it will take a lot of research to turn extremozymes into

industrial products. We strongly believe that 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 industrially important enzymes.

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