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review

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The Biocatalytic Potential of Extremophiles and Extremozymes

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Summary

Extremophiles are bizarre microorganisms that can grow and thrive in extreme environments, which were formerly considered too hostile to support life. The extreme conditions may be high or low temperature, high or low pH, high salinity, high metal concentrations, very low nutrient content, very low water activity, high radiation, high pressure and low oxygen tension. Some extremophiles are subject to multiple stress conditions. Extremophiles are structurally adapted at the molecular level to withstand these harsh conditions. The biocatalysts, called extremozymes, produced by these microorganisms, are proteins that function under extreme conditions. Due to their extreme stability, extremozymes offer new opportunities for biocatalysis and biotransformation. Examples of extremozymes include cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases, which have great potential for application in various biotechnological processes. Currently, only 1–2 % of the microorganisms on the Earth have been commercially exploited and amongst these there are only a few examples of extremophiles. However, the renewed interest that is currently emerging as a result of new developments in the cultivation and production of extremophiles and success in the cloning and expression of their genes in mesophilic hosts will increase the biocatalytic applications of extremozymes.

Key words: extremophiles, extremozymes, biocatalytic potential, thermophiles, psychrophiles, alkaliphiles, acidophiles, halophiles, piezophiles, metalophiles

Introduction

Microorganisms requiring extreme environments for growth are called extremophiles and the enzymes they produce are called extremozymes. The term extremophile was first used by MacElroy in 1974, three decades ago (1). »Extreme environment« is a relative term, since environments that are extreme for one organism may be essential for the survival of another organism. Extremophiles thrive under conditions that would kill most other creatures and many cannot survive in the normal an-

thropogenic global environments. Extreme environments include those with either high (55 to 121 °C) or low (–2 to 20 °C) temperatures, high salinity (2–5 M NaCl) and either high alkalinity (pH>8) or high acidity (pH<4), reviewed by Madigan and Marrs (2) and Rothschild and Manicynelli (3). Various extremophiles can tolerate other extreme conditions including high pressure, high levels of radiation or toxic compounds, or conditions that we consider unusual, such as living in rocks deep below the surface of the earth or living in extremely dry areas with very low water and nutrient supply (2,3). In addition,

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extremophiles may be found in environments with a combination of extreme conditions such as high temperature and high acidity, high temperature and high alkalinity or high pressure and low temperature. The limits of temperature, pressure, pH, salinity and water activity at which life can thrive have not yet been precisely defined. 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 identified recently and characterized (2,3).

The use of enzymes or microorganisms as biocatalysts for the formation of various products has been well documented in many publications and reviews (4–12). As industrial process conditions are harsh, there are demands for biocatalysts that can withstand the process conditions. The majority of the enzymes used to date originate from mesophilic organisms and, despite their many advantages, the application of these enzymes is restricted due to their limited stability at the extremes of temperature, pH and ionic strength. On the other hand, extremophiles are a potent source of extremozymes, which show utmost stability under extreme conditions. Consequently, much attention has been given to the microorganisms that are able to thrive in extreme environments. Thus, biocatalysis using extremophiles as well as extremozymes is rapidly being transformed from an academic science to an industrially viable technology. Each group of the extremophiles has unique features, which can be harnessed to provide enzymes with a wide range of application possibilities (4–29). Moreover, the dramatic advances in molecular and computational biology, new sources of enzymes, combinatorial methodologies and biochemical engineering of single and multicomponent enzyme systems have stimulated a renaissance in enzyme technology. Revolutionary changes in molecular biology are leading to the development of thousands of new uses of enzyme technology, fueling major growth in this multi-billion-dollar field. A huge amount of money is being invested worldwide in the development of industrial as well as biomedical applications of extremozymes and extremophiles.

In this review, selected examples of the discovery, isolation and application of extremophiles and their enzymes are discussed and presented. The fields of extremophiles and extremozymes have received a great deal of attention. The readers are also referred to some excellent reviews (2–29).

Thermophiles

Thermophilic microorganisms have attracted most attention and are amongst the most studied of the extremophiles. The biocatalytic potential of thermophiles and their enzymes has been reviewed by Adams *et al.* (4), Hough and Danson (5), Niehaus *et al.* (6), Demirjian *et al.* (7), Van den Burg (8), Irwin and Baird (9), Eichler (10), Vieille and Zeikus (11), Haki and Rakshit (12), Fujiwara (13), Sellek and Chaudhuri (14), Lévêque *et al.* (15) and Bertoldo and Antranikian (16). 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) (3–15). 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 (3). Extreme thermophiles, growing optimally at 60–80 °C, are widely distributed among the genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Ferroidobacterium*, *Rhodothermus*, *Thermotoga* and *Aquifex* (4–16). 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* (4–16). Euryarchaeota include extreme halophiles (e.g. *Halobacterium*, *Halobaculum*, *Halococcus*, *Haloferax*, *Halorubrum*), methanogens (e.g. *Methanobacterium*, *Methanosphaera*, *Methanococcus*, *Methanobrevibacter* and *Methanothermobacter*), extreme acidophiles (e.g. *Picrophilus*, *Thermoplasma*) and extreme thermophiles (e.g. *Thermococcus*, *Pyrococcus*, *Methanopyrus*, *Archaeoglobus* and *Ferroplasma*) (6–16). The so-called phylum »Korarchaeota« has been postulated on the basis of PCR amplification of 16S rRNA genes from environmental DNA, but has not been confirmed by the pure cultivation of any organisms (30). The novel phylum Nanoarchaeota is represented by the hyperthermophilic anaerobic nano-sized coccus »*Nanoarchaeum equitans*«. It grows only in co-culture with a new chemolithoautotrophic *Ignicoccus* species (31,32). Among the hyperthermophiles, a strain dubbed »strain 121« (33) and *Pyrolobus fumarii* (34), capable of growing at 121 and 113 °C, respectively, hold the record for the most thermophilic microorganisms. These strains cannot grow below 90 °C. »Strain 121« grows chemoautotrophically using formate as an electron donor and Fe³⁺, while *Pyrolobus fumarii* is a nitrate-reducing chemolithoautotroph. Thermophiles are more common than hyperthermophiles.

Short branches in the unrooted phylogenetic tree indicate a rather slow clock in the biological evolution and all hyperthermophiles that can grow at temperatures above 90 °C are represented as the deepest and shortest lineage, suggesting that hyperthermophiles are very primitive and their common ancestor is a hyperthermophile (13,30). The genome sizes of hyperthermophiles are comparable to those of mesophiles and are in fact often somewhat smaller. Generally, hyperthermophiles have a smaller size genome than well-studied mesophiles (13,35). The small size genomes of hyperthermophiles may define the lower limit of their genetic capacity. Chromosomes of hyperthermophiles are densely packed with genes, most of which are required for essential functions (36). This indicates that the earliest life forms may have had small genomes. The developments of functions and potential resources of extremophiles, with particular reference to gene isolation from natural environments without pure culturing, have been reviewed by Fujiwara (13).

Of all extremozymes, thermophilic enzymes have attracted most attention during the past four decades (4–16). Such enzymes are of great industrial and biotechnological interest due to the fact that the enzymes

are better suited for harsh industrial processes. There are many advantages of conducting industrial processes at high temperature, such as the increased solubility of many polymeric substrates, resulting in decreased viscosity, increased bioavailability, faster reaction rate and the decreased risk of microbial contamination. These enzymes have also been used as models for the understanding of thermostability and thermoactivity, which is useful for protein engineering (37–39). Structural features of thermophilic extremozymes have attracted much attention. Several three-dimensional structures have been resolved and compared with those of mesophilic counterparts, with the ultimate goal of elucidating the mechanisms underlying thermostability (37,38). A large number of sequence and structural factors are thought to contribute toward higher intrinsic thermal stability of proteins from thermophiles and hyperthermophiles. Thermophiles produce special proteins known as 'chaperonins', which are thermostable and resistant to denaturation and proteolysis. Proteins of thermophiles, denatured at high temperature, are refolded by the chaperonins, thus restoring their native form and function (38,39). The cell membrane of thermophiles consists of saturated fatty acids, which increase protein core hydrophobicity and keep the cell rigid enough to survive at high temperatures (37–39). Moreover, hyperthermophiles have membranes containing lipids linked with ether to their cell walls. This layer is much more heat resistant than a membrane formed of fatty acids. In addition, proteins of thermophiles have increased surface charge and less exposed thermolabile amino acids (37–39). Thus, increased ionic interaction and hydrogen bonds, increased hydrophobicity, decreased flexibility and smaller surface loops confer stability on the thermophilic protein (37–39).

To date, a large number of polysaccharide degrading enzymes (*e.g.* cellulases, amylases, pullulanases, xylanases, mannanase, pectinases and chitinases), proteases, lipases, esterases and phytases have been characterized from extremely thermophilic and hyperthermophilic microorganisms. Many of these results have been presented in some excellent reviews (4–17). Some recent publications are additionally shown in Table 1a (40–56).

Various kinds of thermostable enzymes are required by industries. The detergent, food, feed, starch, textile, leather, pulp and paper and pharmaceutical industries are the major users of enzymes (4–17,19). The starch industry is one of the largest users of thermostable amylolytic enzymes (*e.g.* α -amylases, glucoamylases and isoamylases or pullulanases) for the hydrolysis and modification of starch to produce glucose and various other products (15–17,19). Amylolytic enzymes are also used in the textile, paper and baking industries. Cellulolytic enzymes are employed in the removal of polyphenolic substances from juices, in detergents for color brightening and softening of textiles, in the bio-stoning of jeans, in the pulp and paper industries and in the pretreatment of plant biomass. Cellulase-free thermostable hemicellulases like xylanases have offered a major step forward in the biobleaching of pulp and paper, thus lowering the environmental pollution by halogens. Nowadays, biodegredients contain enzymes like amylase, protease, cellulase and lipase, using variants that are resistant to harsh conditions. Lipases are also used in various processes,

for example, fat hydrolysis, esterification, interesterification, *trans*-esterification and organic biosynthesis. Other applications of lipase include the removal of pitch from pulp produced in the paper industry, the hydrolysis of milk fat in the dairy industry, the removal of non-cellulosic impurities from raw cotton before further processing into dyed and finished products, the removal of subcutaneous fat in the leather industry and manufacturing of drugs in the pharmaceutical industry. Thermostable phytases are added to animal feeds in order to hydrolyze phytic acid (phytate), an antinutritional factor present in cereals and oil seeds, thereby releasing digestible phosphorous (10,12). Thus the need to supplement the feed with an external source of phosphorous is reduced. Adding phosphorous to feeds results in excessive excretion of phosphorous in the manure of the animals, which creates an environmental problem. Phytase also releases minerals (including Ca, Mg, Zn and K), amino acids and proteins that are complexed with phytate and therefore improves dietary absorption of minerals. Thermostable xylanases, amylases and proteases are added to animal and poultry feed in order to increase digestibility. Chitinases are used for the hydrolysis of chitin to various useful products.

Thermostable DNA polymerases, isolated from hyperthermophiles, have led to a tremendous advance in molecular biology due to their capacity to amplify DNA, in the so-called polymerase chain reaction (3,13). Enzyme-catalyzed synthesis of carbohydrates is now well established. A series of thermophilic glycosidases, sold commercially as CLONEZYMES™, can be used to catalyze the formation of a range of linkages, including Gal β (1→4), Gal β (1→6), Gal α (1→6), D-Fuc β (1→3) and D-Fuc β (1→2) (57–59). The synthetic ability of thermophilic enzymes was further confirmed by using β (1→3)-galactosidase from *Thermus thermophilus*, which showed high regioselectivity during the transfer of galactosyl, glucosyl and D-fucosyl residues from *p*-nitrophenyl donors to a variety of saccharide acceptors (60). Industrial production of fine chemicals and their respective intermediates as final products, especially in the form of enantiomerically pure compounds, will receive much attention and find increasing applications in biocatalytic industries in the near future.

Psychrophiles

Psychrophilic (cold-loving) or psychrotolerant (cold-adapted) microorganisms are found inhabiting the low temperature environments of the Earth, including polar regions, high mountains, glaciers, ocean deeps, shallow subterranean systems (*i.e.* caves), the upper atmosphere, refrigerated appliances and the surfaces of plants and animals living in cold environments, where temperatures never exceed 5 °C. The potentials of psychrophiles and psychrophilic enzymes have been reviewed by Cavicchioli *et al.* (20), Deming (21), Margesin *et al.* (22), Feller and Gerday (23) and Georlette *et al.* (24). In fact, deep oceans, which cover over 70 % of the Earth's 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 pres-

Table 1a. Production of extremophilic enzymes by hyperthermophiles

Hyperthermophiles	Thermophilic enzymes	T _{opt} /°C	pH _{opt}	Stability	Ref.
Bacteria	Cellulase				
<i>Bacillus</i> , <i>Clostridia</i> , <i>Feroidobacterium pennavorans</i> , <i>Rhodothermus marinus</i> , <i>Rhodothermus obamensis</i> , <i>Thermus caldophilus</i> , <i>Thermoanaerobacter</i> sp., <i>Thermoplasma acidophilum</i> , <i>Thermotoga maritima</i> , <i>Thermotoga neapolitana</i> , <i>Picrophilus oshimae</i> , <i>Picrophilus torridus</i>	Amylase Pullulanase I Pullulanase II α -Glucosidase β -Glucosidase Glucoamylase Xylanase				4–17
Archaea	Mannanase				
<i>Desulfurococcus mucosus</i> , <i>Pyrococcus furiosus</i> , <i>Pyrococcus woesei</i> , <i>Pyrodictium abyssi</i> , <i>Staphylothermus marinus</i> , <i>Sulfolobus solfataricus</i> , <i>Thermococcus hydrothermalis</i> , <i>Thermococcus litoralis</i> , <i>Thermococcus celer</i> , <i>Thermococcus profundus</i> , <i>Thermococcus aggregans</i>	Pectinase Chitinase Protease Lipase Esterase Phytase				
<i>Alicyclobacillus acidocaldarius</i>	Endoglucanase (CelB)	80	4.0	Stable at pH=1–7, retains 60 % activity after 1 h at 80 °C	40
Environmental DNA	β -Xylanase	100	6.0	Stable at 90 °C	41
<i>Methanococcus jannaschii</i>	α -Amylase	120	5.0–8.0	Stable against denaturants	42
<i>Pyrobaculum calidifontis</i>	Carboxylesterase	90	7.0	½ life: 2 h at 100 °C	43
<i>Pyrococcus furiosus</i>	Chitinase a and b	90–95	6.0	NA	44
<i>Pyrodictium abyssi</i>	Xylanase	105	6.0	½ life: 100 min at 105 °C	45
<i>Rhodothermus marinus</i>	Amylase	85	6.5	½ life: 3 h at 85 °C	46
	Pullulanase	80	6.5–7.0	30 min at 85 °C	47
	α -L-Arabinofuranosidase	85	5.5–7.0	8.3 h at 85 °C	48
	β -Mannanase	85	5.0–6.5	45.3 h at 85 °C	
<i>Sulfolobus solfataricus</i>	Xylanase	100	7.0	½ life: 47 min at 90 °C	49
<i>Sulfolobus solfataricus</i>	α -Glucosidase	120	4.5	Highly thermostable (whole cells used)	50
<i>Sulfolobus solfataricus</i>	Trehalosyl transglucoylase	75	5.0	Stable at pH=4.5–11.0 after 2 h at 80 °C	51
<i>Sulfolobus shibatae</i>	α -Glucosidase	98	5.5	Retained 67 % activity after 5 h at 80 °C	52
<i>Thermococcus litoralis</i>	L-Aminoacylase	85	8.0	½ life: 25 h at 70 °C, 1.7 h at 85 °C	53
<i>Ralstonia</i> sp. A-471	Chitinase	70	5.0	NA	54
<i>Thermococcus chitonophagus</i>	Chitinase	70	7.0	½ life: 1 h at 120 °C	55
<i>Thermoplasma acidophilum</i>		90	2.0		
<i>Picrophilus torridus</i>	Glucoamylases	90	2.0	½ life: 24 h at 90 °C for <i>P. torridus</i> and <i>T. acidophilum</i> , 20 h for <i>P. oshimae</i>	56
<i>Picrophilus oshimae</i>		90	2.0		

NA = not available

sure in deep seas (piezo-psychrophiles), or high salt concentration and low temperature in sea ice (halo-psychrophiles) (20–24).

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 *Halo-rubrum*), yeast (*Candida* and *Cryptococcus*) and fungi (*Penicillium* and *Cladosporium*) have been isolated from these cold environments (20–24). 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 (7,8,10,20–24). In addition, some recent examples are shown in Table 1b (61–77).

Psychrophiles have developed adaptive mechanisms to perform their metabolic functions at low temperatures by incorporating unique features in their proteins and membranes (20–24). Compared to proteins from mesophiles, psychrophilic proteins show decreased ionic interactions and hydrogen bonds, possess less hydrophobic groups and more charged groups on their surface and longer surface loops (20–24). Due to these modifications, at low temperatures psychrophilic proteins lose their rigidity and gain increased structural flexibil-

Table 1b. Production of extremophilic enzymes by psychrophiles/psychrotolerant microorganisms

Psychrophiles /psychrotolerant	Cold-active enzymes	$T_{opt}/^{\circ}\text{C}$	pH_{opt}	Stability	Ref.
<i>Acinetobacter</i> sp. strain no. 6	Novel esterase	50	7.8	Lost 75 % activity at 40 °C in 30 min	61
	Lipase	20	7.0	½ life: 30 min at 50 °C	
<i>Arthrobacter</i> sp. C2-2	β-Galactosidase	40	7.5	½ life: 8 min at 50 °C, stable after 4 h at 40 °C	62
<i>Arthrobacter</i> sp. strain TAD20	Chitinases	NA	NA		63
<i>Bacillus</i> , <i>Clostridium</i> , <i>Actinomycetes</i>	α-Amylase	NA	NA	NA	64
<i>Cytophaga-Flexibacter-Bacteroides</i>	β-Galactosidase				
<i>Pedobacter cryoconitis</i> sp. nov	Oxidase, catalase, protease, amylase, β-glucosidase, β-galactosidase, β-lactamase	NA	NA	NA	65
<i>Clostridium</i> strain PXYL1	Filter paper cellulase	20	5.0–6.0	½ life: 30 min at 40 °C for xylanase, CMCCase and FPase	66
	Endocellulase	(for all three)	(for all three)		
	Xylanase				
<i>Cystofilobasidium larimarini</i>	Polygalacturonase	40	5.0	The enzymes were very unstable at 30–40 °C	67
<i>Cystofilobasidium capitatum</i>	Polygalacturonase	40	5.0		
<i>Cryptococcus macerans</i>	Polygalacturonase	50	4.0		
<i>Cryptococcus aquaticus</i>	Polygalacturonase	50	4.0		
<i>Cryptococcus adeliae</i>	Xylanase	45–50	5.0–5.0	½ life: 78 min at 35 °C	68
<i>Cryptococcus cylindricus</i>					
<i>Mrakia frigida</i>	Pectinase	NA	NA		69
<i>Cystofilobasidium capitatum</i>					
<i>Flavobacterium psychrophilum</i>	Metalloprotease	24	6.5	Lost all activity after 5 min at 40 °C	70
<i>Pseudoalteromonas haloplanktis</i>	Cellulase	NA	NA	NA	71
<i>Pseudoalteromonas haloplanktis</i>	Xylanase (family 8)	25	5.3–8.0	Mp 52.6 °C ½ life: 1.9 min at 55 °C	72
<i>Psychrobacter</i> sp. Ant300	Esterase	5–25	7.0–9.0	16 min at 40 °C	73
<i>Psychrobacter okhotskensis</i> sp. nov	Lipase	NA	NA	NA	74
<i>Penicillium chrysogenum</i>	Endo-arabinanase	30–40	6.0–7.0	Stable up to 30 °C	75
<i>Pseudomonas</i> strain DY-A	Alkaline protease	40	10.0	NA	76
<i>Bacillus</i> spp.	Subtilisin	40–45	10.5–11.0	Thermolabile	77

NA = not available

ity for enhanced catalytic function. As the psychrophilic membranes contain a higher proportion of unsaturated fatty acids, their fluidity and ability to transport nutrients are maintained under very cold conditions. Moreover, the ability to synthesize cold-shock or antifreeze proteins as the temperature drops, the more efficient enzyme activity due to alterations in enzyme kinetics and the stabilization of microtubules enable the psychrophiles to continue their activities (24). The adaptive properties of psychrophilic enzymes are their high specific activity at low temperatures, a relatively low apparent temperature optimum for activity and a rather high thermolability.

The ability of psychrophilic enzymes to catalyse reactions at low or moderate temperatures offers great industrial and biotechnological potential (7,8,10,20–24). 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 de-

hairing 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 β-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 (78), 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 (22). Psychrophili-

lic enzymes can also be used as environmental biosensors (e.g. dehydrogenases) or in biotransformation (e.g. methylases, aminotransferases and alanine racemase). Psychrophiles may be a good source of polyunsaturated fatty acids for the pharmaceutical industry. Psychrophiles may be good sources of novel therapeutic agents, for example, many bacteria belonging to the genus *Pseudalteromonas* possess antibiotic properties (79).

Alkalithermophiles/Alkaliphiles/ Acidothermophiles/Acidophiles

Alkalithermophilic microorganisms grow optimally under two extreme conditions: at pH values of 8 or above and at high temperatures (50–85 °C). Some of the alkalithermophiles require additional salt concentrations for their growth. On the other hand, microorganisms simply classified as alkaliphiles are mesophiles and consist of two main physiological groups: alkaliphiles and haloalkaliphiles (10,25,26). Alkaliphiles require an alkaline pH of 8 or more for their growth and have an optimal growth pH of around 10, whereas haloalkaliphiles require both an alkaline pH (pH>8) and high salinity (up to *m/V* (NaCl) = 33 %). Alkalithermophiles as well as alkaliphiles have been isolated mainly from alkaliphilic and thermobiotic environments such as alkaline hot springs, the new alkaline hydrothermal vents of the

»Lost City« and alkaline lakes in Africa, Egypt and Israel. However, various alkalithermophiles have also been isolated from mesobiotic, slightly acidic to neutral environments, sometimes even from acidic soil samples and sewage. Haloalkaliphiles have been mainly found in extremely alkaline saline environments, such as the Rift Valley lakes of East Africa and the western soda lakes of the United States (25). Some of the aspects of alkalithermophiles and alkaliphiles have been presented in recent reviews by Wiegel (25) and Horikoshi (26), respectively.

Acidothermophiles thrive under conditions of low pH and high temperature. For example, the acidothermophile *Sulfolobus solfataricus* grows at pH=3 and 80 °C. True acidophiles such as the archaea *Picrophilus torridus* and *Picrophilus oshimae* grow optimally at pH values as low as 0.7 and at 60 °C and produce starch-hydrolyzing enzymes (amylases, pullulanases, glucoamylases and glucosidases) (56).

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 un-

Table 1c. Production of extremophilic enzymes by thermoalkaliphiles/alkaliphiles

Thermoalkaliphiles / alkaliphiles	Thermoalkaliphilic / alkaliphilic enzymes	$T_{opt}/^{\circ}C$	pH _{opt}	Stability	Ref.
<i>Alkalimonas amylolytica</i>	Amylase	NA	NA	NA	80
<i>Streptomyces</i> sp.	Endocellulase	50	8.0	Retained 95 % activity at opt. temp and pH for 30 min	82
<i>Bacillus firmus</i>	Xylanases (xyn10A and xyn11A)	70	5.0–9.5	Retained ca. 70 % activity after 16 h at 62 °C	83
<i>Bacillus halodurans</i> strains	Amylase, pullulanase	55–65	10.0	NA	84
<i>Bacillus subtilis</i>	α -Amylase	52–55	9.0	Lost 60 % activity after 10 min at 95 °C	85
<i>Bacillus</i> isolate KSM-K38	α -Amylase	55–60	8.0–9.0	Highly resistant to chelating reagents and chemical oxidants	86
<i>Nesterenkonia</i> sp. AL-20	Alkaline protease	$T_m=74^{\circ}C$	10.0	Highly stable against H ₂ O ₂ and sequestering agents	87
<i>Bacillus pumilus</i>	Alkaline protease	50–60	11.5	NA	88
<i>Arthrobacter ramosus</i> <i>Bacillus alcalophilus</i>	Alkaline protease	65	11.0 10.0	Both enzymes are stable at 30–65 °C and pH=7–12	89
<i>Nocardiopsis</i> sp.	Alkaline protease Keratinase	70–75	11.0–11.5	Stable below 60 °C for 10 min and pH=8	90
<i>Pseudomonas</i> sp. LBA34 <i>Halomonas</i> sp. LBB1	Lipase	NA	NA	NA	91
<i>Bacillus</i> sp.	Azoreductase	80	8.0–9.0	NA	92
<i>Bacillus</i> sp.	Catalase-peroxidase	60	8.0	½ life: 20 h at pH=9 and 60 °C	93
<i>Thermus brockianus</i>	Catalase	90	8.0	½ life: 3 h at 90 °C, 330 h at 80 °C	94
<i>Bacillus alcalophilus</i>	Pectate lyase	45	9.0–10.0	NA	95
<i>Thermomonospora</i> (actinomycete)	Endocellulase	50	5.0	Stable at pH=7–10 ½ life: 3 h at 70 °C	96

NA = not available

derstood. 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 (25,26). Cellular fatty acids in alkaliphilic bacterial strains contain predominantly saturated and mono-unsaturated straight-chain fatty acids (25,26,80). In order to withstand low pH, acidophiles employ a range of mechanisms such as: a positively charged membrane surface (81), a high internal buffer capacity, over-expression of H⁺ exporting enzymes and unique transport systems (25,26).

Thermoalkaliphiles and alkaliphiles are good sources of alkaliphilic enzymes like cellulases, xylanases, amylases, proteases, lipases, pectinases, chitinase, catalase, peroxidase and oxidoreductase (25,26). Some recent reports on these enzymes are shown in Table 1c (80, 82–96). 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 (10,25,26,97). 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 (25,26,92–94). Due to their versatile uses, alkaliphilic enzyme-producing bacteria and archaea have received great attention in recent years (25,26).

Halophiles

Halophilic microorganisms require very high salt (NaCl) concentrations for growth. They are found in salt-terns and hypersaline lakes, such as the Great Salt Lake, the Dead Sea and solar lakes in Africa, Europe and the USA. They have even been found in Antarctic lakes. They accumulate salts such as 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 (up to about 4 M KCl and over 5 M NaCl (5,10,12,27). The molecular ecology of extremely halophilic archaea and bacteria has been reviewed by Oren (27). Halophiles like *Halobacterium*, *Haloferax*, *Haloarcula*, *Halococcus*, *Natronobacterium* and *Natronococcus* belong to the archaea while *Salinibacter ruber* is a bacterium (27).

Halophilic proteins employ different adaptation mechanisms. Proteins from halophilic organisms have a biased amino acid composition in order to remain stable and active at high ionic strength. Halophilic proteins typically have an excess of acidic amino acids (*i.e.* glutamate and aspartate) on their surface (98–101), although such a high proportion of acidic amino acids is not present in the amylase from the thermophilic halophile *Halothermothrix orenii* (102). Negative charges on the halophilic proteins bind significant amounts of hydrated ions, thus reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentration.

Halophilic proteins are distinguished from their non-halophilic homologous proteins by exhibiting remarkable instability in solutions with low salt concentrations and by maintaining soluble and active conformations in high concentrations of salt, for example, up to 5 M NaCl (98–101).

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 (98,99). 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 (98–101).

Halophiles from the archaeal domain provide the main source of extremely halophilic enzymes. The potentials of halophiles and haloenzymes have been reviewed previously (5,10,14,27,98–100). 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* (4,10,14,27,98–101). Table 1d shows some recent reports on these enzymes (102–108).

However, many of these enzymes have not been investigated in detail or applied (14). Although the halophilic enzymes can perform enzymatic functions identical to those of their non-halophilic counterparts, these enzymes have been shown to exhibit substantially different properties, among them the requirement for high salt concentrations (1–4 M NaCl) for activity and stability and a high excess of acidic over basic amino residues (101). It is argued that the high negative surface charge of halophilic proteins makes them more soluble and renders them more flexible at high salt concentrations, conditions under which non-halophilic proteins tend to aggregate and become rigid. This high surface charge is neutralized mainly by tightly bound water dipoles (98–101). The requirement of high salt concentration for the stabilization of halophilic enzymes, on the other hand, is due to a low affinity binding of the salt to specific sites on the surface of the folded polypeptide, thus stabilizing the active conformation of the protein (101). The dependence of the stability and the catalytic properties of halobacterial proteins on salt concentration has been the subject of studies for many years. The property of low solubility of halophilic enzymes has been taken advantage of by applying them in aqueous/organic and non-aqueous media (109,110). To date, the use of halophilic extremozymes in organic solvents has been limited to very few enzymes (14). To explore the behaviour of these extremozymes in organic media, different approaches have been adopted, including the dispersal of the lyophilized enzyme or the use of reverse micelles (110,111). In fact, the use of reverse micelles in maintaining high activities of halophilic extremozymes under unfavourable conditions could open new fields of application such as the use of these enzymes as biocatalysts in organic media. The reverse micelles are considered as

Table 1d. Production of extremophilic enzymes by halophiles

Halophiles	Halophilic enzymes	$T_{opt}/^{\circ}\text{C}$	pH_{opt}	Stability	Ref.
<i>Halothermothrix orenii</i>	α -Amylase	65	7.5	Tolerates up to 25 % NaCl T_{opt} at 5 % NaCl	102
<i>Bacillus dipsosauri</i>	α -Amylase	60	6.5	Stable up to 60 $^{\circ}\text{C}$	103
<i>Halobacillus</i> sp. strain MA-2	Amylase	50	7.5–8.5	Maximum stable at 5 % NaCl	104
<i>Haloferax mediterranei</i>	α -Amylase	50–60	7–8	Stable at 2–4 M NaCl Optimum 3 M NaCl	105
Halophilic bacterium, CL8	Xylanase 1	60	6.0	Stable 7 min at 60 $^{\circ}\text{C}$	106
	Xylanase 2	65	6.0	Stable 192 min at 60 $^{\circ}\text{C}$ (T_{opt} at 4 M NaCl)	
<i>Halorhabdus utahensis</i>	β -Xylanase	55, 70	NA	Optimum activity at 5–15 % NaCl	107
	β -Xylosidase	65	NA	Optimum activity at 5 % NaCl	
<i>Pseudoalteromonas</i> sp. strain CP76	Protease CPI	55	8.5	Tolerates 0–4 M NaCl Optimum activity at 7.5 % NaCl	108

NA = not available

micro-reactors, which are very dynamic and change with the environment conditions (110,111). Halophilic enzymes encapsulated in reverse micelles can keep their catalytic properties in organic solvents, even at low salt concentrations. For example, the manufacture of chemicals such as pesticides, herbicides, explosives and dyes usually generates effluents containing complex mixtures of xenobiotic compounds, salt and organic solvents. Halophilic extremozymes encapsulated in reverse micelles may be used in bioremediation (*i.e.* to reduce or eliminate environmental hazards resulting from accumulation of toxic chemicals and other hazardous wastes) (110–111).

Piezophiles

Microorganisms that like high-pressure conditions for growth are termed piezophiles (formerly known as barophiles). With an average pressure of 38 MPa, the world's oceans are home to piezophiles, including various thermophiles and hyperthermophiles (28). Piezophiles are distributed among the genera *Shewanella*, *Colwellia*, *Moritella*, *Methanococcus*, *Pyrococcus* and *Thermus* (28,29).

The biotechnological potentials of piezophiles and piezoenzymes have recently been reviewed by Abe and Horikoshi (28) and Yano and Poulos (29). Piezophilic enzymes that are stable at high pressures have been isolated from a wide variety of microorganisms, most of which are either thermophilic or psychrophilic and have optimal growth conditions above one atmosphere (28,29). The molecular basis of piezophily is now being investigated extensively, focusing on aspects of gene regulation and the function of certain proteins in deep-sea isolates. Recent studies indicate that the important global factors for increased thermal stability are surface electrostatics, amino acid composition (such as a decrease in thermolabile residues), shorter loops, increased charge dipole on helices and interactions between cations and aromatic rings (cation- π interactions) (29).

Based on the facts that pressures exceeding 400 MPa are needed to induce protein denaturation, and microor-

ganisms living in the deep sea are not exposed to pressures exceeding 120 MPa (112) 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 (8,29). However, there are some examples of protein stabilization by high pressure (113,114).

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 (28,29,115,116). 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 ($\Delta V < 0$) are favored by increasing pressure, whereas reactions with a positive change ($\Delta V > 0$) are not. The change in activation volume (ΔV) can be used as a method to control reaction specificity (29). For example, α -chymotrypsin catalyzes both the hydrolysis of an anilide ($\Delta V < 0$) and the hydrolysis of an ester ($\Delta V > 0$). A reaction mixture containing both substrates and α -chymotrypsin in organic media was controlled by altering the pressure to favor one or the other reaction (117).

Although there are many possible biotechnological applications of piezophiles and piezophilic enzymes, there are few known practical applications of piezophiles or piezophilic enzymes (28,29). 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.

Radiophiles

Microorganisms that are highly resistant to high levels of ionizing and ultraviolet radiation are called radiophiles. The genetic engineering and environmental biotechnology aspects of radiophiles have been re-

viewed by Daly (118). Examples of radiophiles are *Deinococcus radiodurans* (119), *Deinococcus radiophilus* (120), *Thermococcus marinus* sp. nov. and *Thermococcus radiotolerans* (121). *Deinococcus radiodurans* is a remarkable bacterium that is highly resistant to chemicals, oxidative damage, high levels of radiation (5 Mrad, 3000 times higher than what would kill a human) and dehydration. It was first isolated in 1956 from a radiation »sterilized« can of ground meat. It contains a spectrum of genes that encode for multiple activities that repair DNA damage. The genes of three putative uracil-DNA glycosylases have been cloned and expressed to determine their biochemical function (119). DR0689 is a homologue of the *E. coli* uracil-DNA glycosylase, the product of the »ung« gene. Unlike its thermophilic relatives, the enzyme is not heat stable. The purified enzyme superoxide dismutase of another member of the genus, *Deinococcus radiophilus*, is stable at pH=5.0–11.0, but quite unstable below pH=5.0. It is thermostable up to 40 °C, but a linear reduction in activity occurs above 50 °C (120).

The presence of toxic chemicals, heavy metals, halogenated solvents and radionuclides in many nuclear waste materials presents a challenging problem for separating different species and disposing of individual contaminants. *Deinococcus radiodurans* strains and other detoxifying microorganisms may be utilized to detoxify halogenated organics and toxic metals such as mercury, and theoretically could be used to remove these classes of compounds selectively from mixed wastes under mild conditions. Several research groups are using genetic engineering techniques to develop suites of radiation resistant bacterial strains to do this (118).

Metallophilic

Microorganisms that can grow in the presence of high metal concentrations are called metallophilic. These organisms, including several members of the genus *Ralstonia*, colonize industrial sediments, soils or wastes with high contents of heavy metals. Metal-resistant *Ralstonia* have adapted well to the harsh environments created by extreme anthropogenic activities or biotopes (122). *Ralstonia metallidurans*, a gram-negative, non-spore forming *Bacillus*, thrives in millimolar concentrations of toxic heavy metals. It was first isolated in 1976 from the sludge of a zinc decantation tank in Belgium that was polluted with high concentrations of several heavy metals. A typical feature of these metal-resistant *Ralstonia* is the presence of one or two large megaplasmids that contain genes for multiple resistances to heavy metals. These plasmids confer resistance to Zn, Cd, Co, Pb, Cu, Hg, Ni and Cr.

Since pollution by heavy metals poses a threat to public health, fishery and wildlife, there has been an increased interest in developing systems that can remove or neutralize the toxic effects of heavy metals in soils, sediments and wastewaters (123). Many microorganisms, including *Ralstonia*, could be used in heavy metal bioremediation. In addition to their use as biosorbents, bacteria can be used to immobilize certain heavy metals efficiently, this being mediated by their capacity to reduce these elements to a lower redox state, producing metal species that have a lower bioactivity. Bacteria ex-

hibit a number of enzymatic activities that transform certain metal species through oxidation, reduction, methylation and alkylation. Apart from the enzymatic transformations that lead to metal precipitation and immobilisation, other biological reactions that generate less poisonous metal species have been applied to bioremediation (122,123).

Xerophiles

The driest regions in the world can support life, even dry stones and deserts. However, only specialized microbes belonging to fungi, lichens and algae have the ability to grow in such extremely dry conditions. These specialized organisms are called xerophiles (2,3). Various xerophiles are responsible for spoiling of dried foods and stored grains, spices, nuts and oilseeds. Since xerophiles live in extremely dry environments, they face the problem of reproducing and creating enzymes that will hydrolyze substrates in conditions of extremely low water contents. Some xerophiles (osmophiles) spoil salty foods, and others spoil sugary foods. As very limited studies have been conducted on xerophiles, the biocatalytic potential of these microbes and their enzymes is not yet known.

Production of Biomass and Extremophilic Enzymes

The major drawback of extremophilic biomass and enzyme production is the difficulty of cultivating them in laboratory and industrial environments. Difficulties and new developments in the production of biocatalysts from extremophiles have been presented by Schiraldi and De Rosa (18). The conditions required for the growth of extremophiles include extreme temperatures, often in an anaerobic environment, and media of extreme pH (for acidophiles and alkaliphiles) or containing up to 5 M NaCl (for halophiles). These conditions are incompatible with standard industrial fermentation and downstream processing equipment. Fermentation at high temperature using corrosive media requires the use of specially designed equipment. The lowering of gas solubility and the instability of substrates and reagents with increase in temperature are other factors of concern. Additional problems that hinder the use of these microbes and their enzymes or metabolites are low specific growth rates and the appearance of product inhibition, even at a very low concentration. Much work is still going on into developing methods by which thermophiles, extreme thermophiles and hyperthermophiles can be effectively cultivated for increased production of extremophilic biomasses, enzymes and biomolecules. To improve biomass production, different research groups have adopted different techniques such as: optimization of the medium composition (46–48,68), use of special fermentors (*e.g.* gas-lift fermentor, membrane bioreactors) (18,124,125) and use of different modes of fermentation, for example, fed-batch, cell-recycling or continuous cultivation (18). Due to the difficulties associated with large-scale culture of extremophiles and downstream processing of extremozymes, corresponding genes from extremophiles have been and are being expressed successfully in mesophilic

hosts such as *Escherichia coli* and yeasts, although difficulties arise when the expressed enzyme requires specific cofactors or metal ions that the host does not utilise (18). Most of the extremozymes that have found their way into industrial applications are produced using *E. coli* expression systems (7,10,18).

Conclusion and Perspectives

The study of extremophiles and extremozymes has added greatly to our understanding of protein folding, stability, structure and function. Extremozymes have great economic potential in many industrial processes (e.g. agriculture, food, feed and drinks, detergents, textile, leather, pulp and paper). Although there are controversial opinions about the potential of extremophiles, some companies (e.g. Diversa, Genencor International Inc., Novozymes) and several research groups are investing money and time searching for these microbes and novel applications of extremozymes and strongly believe that new discoveries will revolutionize biotechnology. This renewed confidence in enzyme biotechnology may have emerged as a result of the success of genome-based technologies that are currently in use. It is expected that many extremozymes will be discovered in the years to come and the extremozymes will be used in novel biocatalytic processes.

Since the cultivation of extremophiles is associated with many potential difficulties, the authors believe that only genetic engineering of the desired extremozymes into mesophilic hosts will allow large-scale production of these extremozymes. 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 biocatalysis and biotransformations.

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Biokatalitički potencijal ekstremofila i ekstremozima

Sažetak

Ekstremofili su iznimno nekonvencionalni organizmi koji mogu bujno rasti u ekstremnim uvjetima što su formalno nemogući za život. Ekstremni uvjeti mogu biti visoke ili niske temperature, visoki ili niski pH, velika slanost, velika koncentracija metala, vrlo mali udjel hranjivih tvari, vrlo mali aktivitet vode, visoke doze zračenja, visoki tlakovi i nedovoljna količina kisika. Neki ekstremofili žive pod različitim stresnim uvjetima. Ekstremofili su strukturno prilagođeni na molekularnoj razini kako bi preživjeli u tim uvjetima. Biokatalizatori, nazvani ekstremozimima, koje proizvode ti mikroorganizmi su proteini što funkcioniraju pod ekstremnim uvjetima. Zbog njihove specifične stabilnosti, ekstremozimi otvaraju nove mogućnosti za biokatalizu i biotransformaciju. Ekstremozimi obuhvaćaju celulaze, amilaze, ksilanaze, proteaze, pektinaze, keratinaze, lipaze, esteraze, katalaze, peroksidaze i fitaze što imaju veliku mogućnost primjene u raznim biotehnoškim procesima. Danas se komercijalno koristi 1–2 % mikroorganizama, a među njima samo nekoliko ekstremofila. Međutim, ponovno zanimanje za te ekstremofile, kao rezultat novoga načina uzgoja i proizvodnje te uspjeha u kloniranju i ekspresiji njihovih gena u mezofilnim domaćinima, povećat će biokatalitičku primjenu ekstremozima.