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Extremophiles and extremozymes: Importance in current biotechnology

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Abstract. The use of biocatalysts in different biochemical reactions is generally restricted to mild ambient conditions. However, the enzymes produced by different living organisms inhabiting ecosystems with extreme conditions of temperature, pH, pressure and solvent are not constrained with mild conditions. It is now known that enzymes can function at temperatures as high as 140°C and as low as below freezing point. Organisms living in these conditions are described as extremophiles and the enzymes that function under these environmental situations are called extremozymes. Extremo-thermophiles are generally known as the thermophiles. This review is discussing about the existence and potential applications of thermophiles and thermostable enzymes. Applications of such enzymes in maximizing reactions accomplished in the food, paper, detergent and pharmaceutical industries, toxic wastes removal is being studied.
Key words: thermophiles, extremophiles, cellulose, xylanase chitinase, amylase, protease.

Rezumat. Utilizarea biocatalizatorilor în diferite reacţii biochimice este limitată în general la condiţii ambientale moderate. Totuşi, enzimele produse de diferite organisme ce traiesc în ecosisteme cu condiţii extreme de temperatură, pH, presiune şi solvent nu sunt limitate la condiţii ambientale moderate. Se ştie acum că enzimele pot funcţiona la temperaturi ridicate de până la 140°C şi la temperaturi scăzute până sub punctul de îngheţ. Organismele care trăiesc în aceste condiţii sunt considerate extremofile şi enzimele ce funcţionează în astfel de situaţii ambientale sunt denumite extremozime. Extremo-termofilele sunt în general cunoscute ca şi termofile. Această lucrare îşi propune o trecere în revistă a existenţei şi posibilelor aplicaţii ale termofilelor şi ale enzimelor termostabile. Sunt studiate aplicaţiile acestor enzime în maximizarea reacţiilor ce au loc în industria alimentară, farmaceutică, a hartiei, detergenţilor şi în tratarea reziduurilor toxice.
Cuvinte cheie: termofile, extremofile, celuloză, xilanază chitinază, amilază, protează.

Introduction. Thomas Brock in 1966, made the remarkable discovery that he noticed microorganisms were growing in the boiling hot springs of Yellowstone National Park (USA). Since Brock's discovery, thermopiles have been discovered in geothermal features all over the world including areas in Iceland, Kamchatka, New Zealand, Italy, Mt. Lassen, and other locations. While boiling hot springs are far beyond the comfort zone of humans and other animal's life especially prokaryotic life is able to adapt to environments that would prove fatal to most other life forms. Terrestrial geothermal areas are located at various regions of our earth and most particularly around the borders of tectonic plates and in areas where the Earth's crust is relatively thin. In these sites thermal springs, fumaroles and geysers are commonplace. Probably the most famous and well-studied geothermal area is Yellowstone National Park (Wyoming, USA). Scientists in the biotechnology field are among many groups of researchers taking an interest in thermophiles and their thermozymes. Astrobiologists, including researchers from NASA, suggested that hot springs all over the world provided some of the best "doorways into early Earth." Many scientists believe that life might have begun roughly 3 billion years ago in high temperature environments and that the first organisms might therefore have been thermophiles. Not only does this give insight into the origin of life on Earth, but opens up a new realm of possibilities for life elsewhere in the universe.

The role of enzymes in many processes has been known for a long time. Their existence was associated with the history of ancient Greece where they were using

enzymes from microorganisms in baking, brewing, alcohol production, cheese making etc. With better knowledge and purification of enzymes the number of applications has increased many fold and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged. Thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Demirijan et al 2001; Haki & Rakshit 2003; Bhoria et al 2009). Advances in this area have been possible with the isolation of a large number of beneficial thermophilic microorganisms from different exotic ecological zones of the earth and the subsequent extraction of useful enzymes from them (Kohilu et al 2001; Bhoria et al 2009; Rastogi et al 2010; Meenakshi et al 2010). While the most widely used thermostable enzymes are the amylases in the starch and beverage industry (Sarikaya et al 2000; Fernandes et al 2010), a number of other applications are in various stages of development. In the petroleum, chemical and pulp and paper industries, for example, thermostable enzymes have been used for the elimination of sulphur containing pollutants through the biodegradation of compounds like dibenzothiophene (Bahrami et al 2001), in the production of 1,3-propanediol from glycerol and in replacing polluting chemical reagents causing toxic products. Within the bacteria *Thermo toga maritima* and *Aquifex pyrophilus* exhibit the highest growth temperatures of 90 and 95ºC respectively (Herbert & Sharp 1992). These properties imply extremely important industrial and biotechnological implications due to the fact that enzymes from such microorganisms can be employed for use in harsh industrial conditions where their specific catalytic activity is retained. Efforts to grow the uncultured bacterial majority have employed new enrichment and cultivation protocols, such as diffusion chambers that allow growth *in situ*, and high-throughput systems using flow cytometry or dilution techniques to separate mixed cultures into individual cells that can be incubated under wide range of conditions (Connon & Giovannoni 2002; Zengler et al 2002; Burns et al 2003). Geothermal environments are particularly appealing for biodiversity research because they are geochemically diverse and rare habitats, and are rich in deeply rooting phylogenetic groups. Early molecular diversity studies identified many novel bacterial groups in geothermal hot springs, including several candidate divisions (Hugenholtz et al 1998). A recent survey on world sales of enzymes ascribes 31% for food enzymes, 6% for feed enzymes and the remaining for technical enzymes. A relatively large number of companies are involved in enzyme manufacture, but major producers are located in Europe, USA and Japan. Denmark is dominating, with Novozymes (45%) and Danisco (17%), moreover after the latter taking over Genencor (USA), with DSM (The Netherlands) and BASF (Germany) lagging behind, with 5% and 4%. The pace of development in emerging markets is suggestive that companies from India and China can join this restricted party in a very near future (Fernandes et al 2010).

Natural Sources of Thermophiles on Earth. Temperature range between 80 and 115ºC is generally optimum for the many of hyper thermophilic bacteria (Huber & Stetter 1998; Miyazaki 2005). Thermophiles are also adapted to live at low temperatures in the cold polar regions, at high pressure in the deep sea and at a very low and high pH values (pH 0–3 or 10–12), or at a very high (5–30%) salt concentration (Herbert & Sharp 1992; Haki & Rakshit 2003). In the last more than ten years, a number of hyperthermophilic archaea the least understood domain of life (Woese et al 1990; Yang et al 2007) have been isolated and are able to grow around the boiling point of water (Niehaus et al 1999). The organisms with the highest growth temperatures (103–110ºC) are members of the genera *Pyrobaculum, Pyrodictium, Pyrococcus and Methanopyrus.* Within the bacteria, *Thermotoga maritima and Aquifex pyrophilus* exhibit the highest growth temperatures of 90 and 95 ºC respectively (Herbert & Sharp 1992). These properties imply extremely important industrial and biotechnological implications due to the fact that enzymes from such microorganisms can be employed for use in harsh industrial conditions where their specific catalytic activity is retained. Microorganisms specially bacteria are more like as comparison to all living beings adapted to the environmental condition in which they have to live and survive. Thermophiles are reported to contain

proteins which are thermostable and resist denaturation and proteolysis (Kumar & Nussinov 2001; Rastogi et al 2010; Meenakshi et al 2010). These specialized proteins known as chaperonins are produced by these organisms, which helps, after their denaturation to refold the proteins to their native form and restore their functions (Everly & Alberto 2000). The cell membrane of thermophiles is made up of saturated fatty acids. The fatty acid provides a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures (Herbert & Sharp 1992). The archae, which compose most of the hyperthermophiles, have lipids linked with ether on the cell wall. This layer is much more heat resistant than a membrane formed of fatty acids. The DNA of thermophiles contains a reverse DNA gyrase which produces positive super coils in the DNA (Lopez 1999). This raises the melting point of the DNA (the temperature at which the strands of the double helix separate). Thermophiles can also tolerate high temperature by using increased interactions that non-thermotolerant organisms use, namely, electrostatic, disulphide bridge and hydrophobic interactions (Kumar & Nussinov 2001). Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms (Saboto et al 1999; Singh et al 2007). These authors indicated that relatively few studies have been carried out in this regard and the only available information is that thermophilic enzymes are more rigid proteins than their mesophilic counterparts. A clearer understanding of this capacity should be possible with new methodologies that clearly indicate changes in protein structure. Table 1 is showing the recent discoveries in the isolation of thermophilic bacteria and production of thermozymes.

Table 1

Thermophilic organisms and their thermozymes

Organisms	Growth temp.	Enzyme	References
Bacillus and *Geobacillus* strains	60ºC	Cellulase	Rastogi et al (2010)
Geobacillus pallidus	60ºC	Cellulase	Baharuddin et al (2010)
Paenibacillus ehemensis, Bacillus cereus, *B. thuringiensis* and *B. subtilis*	>50ºC	Xylanase	Singh et al (2010)
Bacillus sp. KYJ963	NA	β-Amylase	Young et al (2001)
Pyrococcus abyssi	NA	Alkaline phosphatase	Sebastien et al (2001)
Thermus thermophilus HB27	72ºC	Laccase	Miyazaki (2005)
Bacillus licheniformis strain JS	NA	Chitinase	Waghmare et al (2010)
Streptomyces roseolilacinus and *Silanimonas lenta*	>50ºC	Chitinase	Manucharova et al (2008)
Bacillus sp	70ºC	α-Amylase	Fooladi & Sajjadian (2010)
Pyrococcus furiosus	90ºC	α-Amylase	Dong et al (1997)
Pyrococcus horikoshii	95ºC	Endoglucanase	Ando et al (2002)

NA: Not available

Cellulases. Cellulose is a homopolysaccharide composed of β-D-glucopyranose units, linked by β-(1→4)-glycosidic bonds. The smallest repetitive unit is cellobiose, as the successive glucose residues are rotated 180° relative to each other (Turner et al 2007). Recently Rastogi et al (2010) reported the cellulose degrading bacterial strains WSUCF1 and DUSELR13. Remarkably these bacteria retained 89% and 78% of the initial CMCase activities respectively when incubated at 70°C for 1 day. These thermostable cellulases may be facilitate the development of more efficient and cost-effective forms of the simultaneous saccharification and fermentation process to convert lignocellulosic biomass into biofuels. An alkaline thermoactive cellulase from thermophilic *Actinomycete* was purified 90 KDa, enzyme was optimally active at pH 8 and 60°C and was stable from pH

6 to 9 with more than 80% activity remaining after incubation at room temperature for 12 hour (Aboul et al 2010). A significant industrial importance for cellulases was reached during the last 15-20 years, mainly within detergent, pulp and paper technology like deinking of recycled paper. Several thermostable enzymes have been characterized and there has been many trials in these areas as thermostability is highly relevant for the performance of the enzymes. Degradation of cellulose into fermentable sugars for commodity product production is a biorefining area that has invested enormous research efforts as it is a prerequisite for the subsequent production of energy. It is likely to be performed at least partly at high temperatures to facilitate the degradation, thus making thermostable enzymes (or thermophilic microorganisms) desirable. Although cellulases cleave a single type of bond, the crystalline substrates with their extensive bonding pattern necessitate the action of a consortium of free enzymes or alternatively multi-component complexes called cellulosomes (Bayer et al 1998; Turner et al 2007).

Xylanases. These are the groups of biocatalysts that depolymerized xylan molecules into xylose units and microbial populations consume xylose as a primary carbon source (Nath & Rao 2001). The xylan is a major hemicellulose component of agro-industrial residues, is advantageous for the recovery of hexose and pentose sugars to be used as raw materials in a wide number of biotechnological applications. Microbial xylanases represented one of the largest group of industrial enzymes and they have attracted a great deal of attention during the past few decades. Their potential biotechnological applications in various industries include the food, feed, fuel, textile, detergents, paper and pulp industries and in waste treatment (Azeri et al 2010). A thermo stable xylanase was purified and characterized from the cladodes of *Cereus pterogonus* plant species. The enzyme showed a final specific activity of 216.2 U/mg and the molecular mass of the protein was 80 KDa when purified. The optimum pH and temperature for xylanase activity were 5.0 and 80°C respectively. The substrate specificity of xylanase yielded maximum activity with oat spelt xylan (Vikramathithan et al 2010). Recently Azeri et al (2010) applied the xylanases from different prokaryotic sources in the biobleaching studies of kraft pulp and subsequent treatment with 1.0% EDTA (30 min at 50°C) and peroxide (80 min at 70°C), showed that the enzymes reduced the kappa number and enhanced the brightness significantly. An extracellular xylanase produced by a thermophilic strain of *Bacillus* sp. XTR-10 and was applied in the bio bleaching of Kraft pulp. Eight hours pretreatment with 40 IU of xylanase/g of dry pulp resulted in 16.2% reduction of kappa number with 25.94% ISO increase in brightness as compared to the control. The same treatment slightly lowered the tensile strength and burst index, however. Enzyme pretreatment of the pulp saved 15% active chlorine charges in single step and 18.7% in multiple steps chemical bleaching with attainment of brightness at the level of the control. These results indicate the potential of enzymatic pretreatment of pulp for reduction in environmental discharge of hazardous waste from the pulp and paper industry (Saleem et al 2009).

Chitinases. Chitin is a polysaccharide composed of N-acetyl-D-glucosamine units. It is highly distributed in nature as a constituent of insect exoskeleton, shells of crustaceans living in sea and fungal cell walls. Chitinases are the enzymes capable of hydrolyzing insoluble chitin in to its oligo and monomeric components of amino sugars. This biocatalyst is present in bacteria and plants with a diversity of roles such as chitin metabolism in growing hyphae, defense mechanisms in response to pathogens, abiotic stress, and in nutrition and parasitism. Recently the thermophilic *Bacillus licheniformis* strain JS was isolated from a bed of mushrooms, *Pleurotus sajor-caju*. The organism could produce a novel, single-component, thermostable chitinase that was purified by ion-exchange chromatography using DEAE-cellulose in 7.64% yield and in an 8.1-fold enhancement in purity. Its molecular weight is 22 kDa. The enzyme is a chitobiosidase, since the chitin hydrolysate is N^{I},N^{II}-diacetylchitobiose. The optimum temperature for enzyme activity is 55 °C, and the optimum pH is 8.0. It was completely inhibited by Hg^{2+} ions whereas Co^{2+} ions served as an activator. The thermostability of this enzyme is important in the bioconversion of chitinous waste and for the production of

chitooligosaccharides (Shailesh et al 2010). A moderately thermophilic bacterium, strain A-471, capable of degrading chitin was isolated from a composting system of chitin-containing waste. Analysis of the 16S rDNA sequence revealed that the bacterium belongs to the genus *Ralstonia*. A thermostable chitinase A (Ra-ChiA) was purified from culture fluid of the bacterium grown in colloidal chitin medium. The pH and temperature optima were determined to be 5.0 and 70ºC, respectively. The enzyme was classified as a retaining glycosyl hydrolase and was most active against partially N-acetylated chitosan. Its activities towards the partially N-acetylated chitosan, i.e. chitosan 7B, chitosan 8B, and chitosan 9B, were about 11- fold, 9-fold, and 5-fold higher than towards colloidal chitin, respectively. Ra-ChiA cleaved (GlcNAc)6 almost exclusively into (GlcNAc)2. Activation of Ra-ChiA was observed by the addition of 1 mM Cu^{2+}, Mn^{2+}, Ca^{2+}, or Mg^{2+}. Degradation of the partially N-acetylated chitosan produced oligosaccharides with a degree of polymerization ranging from 1–8; these are products that offer potential application for functional oligosaccharide production.

Amylases. Amylases are among the most important enzymes used in biotechnology, particularly in process involving starch hydrolysis. Though amylases originate from different sources (plants, animals and microorganisms), the microbial amylases are the most produced and used in industry, due to their productivity and thermo stability (Burhan et al 2003). Natural fermented media (foods and soils) offer the substrates for isolation of microorganism strains producing amylases. In this respect, many strains used in food industry originate from fermented food media, while soils, particularly wastes and mud offer strains used mainly in chemical industry. The optimum amylase activity was found at 70°C and pH 5.5 and 6.5 produced by ascomycetes yeast strain isolated from starchy soils (Fossi et al 2004). Thermophilic processes appear more stable, rapid and less expensive and facilitate reactant activity and product recovery. Amylases have a quarter of the world enzyme market and thermostable a-amylases possess extensive commercial applications. Since little work has been done on strain isolation, growth and enzyme yield optimization, the level of thermophilic enzyme production remains relatively low. To all these factors keep in mind Rasooli et al (2008) reportd the *Bcillus licheniformis* -07. The maximum enzyme production after 26 h of cultivation at pH 7.0 and at 50°C, 0.5% tryptophan in production medium enhanced the enzyme productivity to two fold, whereas peptone and lysin at 0.5% level showed a strong repression. Crude a-amylase characterization revealed that optimum activity was at pH 7.5 and 70°C. The crude enzyme was stable for 24 h at pH range of 6-7h at 70°C. Enzyme activity increased with temperature within the range of 40-70°C. The *Bacillus licheniformis* -07 strain produced thermostable a-amylase with characteristics suitable for application in starch processing and food industries.

Proteases. Proteolytic enzymes produced by thermophiles are of considerable interest because they are stable and active at elevated temperatures. Moreover, they are resistant to organic solvents, detergents, low and high pH and other denaturants. Such properties allow many technological processes. Advantages of thermozymes applications are reduced risk of microbial contamination, increased mass transfer, lower viscosity and improved susceptibility of some proteins to enzyme molecules (Synowiecki et al 2010). Proteases are physiologically important molecules and their synthesis is widespread in plants, animals and different strains of bacteria, fungi and yeasts. An excellent source of proteases are microorganisms, and among them the great attention received thermophilic bacteria and archaea which are classified into moderate- or extreme thermophiles and hyperthermophiles, growing optimally at 50 - 60°C, 60 - 80°C or 80 - 113°C, respectively (Fujiwara 2002). In some cases, decomposition of proteinaceous by-products is achieved during cultivation of microorganisms with strong proteolytic activity. Unfortunately such mesophiles can often invade the human and animal tissues which cause their pathogenicity (Suzuki et al 2006). However, there have been no reports of the pathogenicity of obligate thermophiles. Thus thermophiles have a great advantage in terms of their safe use. Thermophilic and hyperthermophilic microorganisms studied during the recent years fulfill demand on different proteolytic enzymes that have

optimum pH up to 12.0 and temperatures ranged from 45 - 110°C (Antranikian et al 1995). At present, industrially important thermostable proteases are usually produced using thermophilic strains belonging to the genus *Bacillus* (Haaki and Rakshit, 2003). An example of such enzyme is thermolysin, a neutral metalloprotease isolated from *Bacillus stearothermophilus* with half-life of 1 h at 80°C (Rahman et al 1994; Rao et al 1998). The pyrolysin, serine protease which reach maximal activity at 100°C was isolated from hyperthermophilic archaeon *Pyrococcus furiosus* (Antranikian et al 1995). Currently only few thermostable proteases are commercially available at industrial level. One of them is alcalase isolated from *Bacillus licheniformis*. The major ingredient of this preparation is subtilisin, which is an endoprotease of serine type, exhibiting highest activity at 60°C and pH of 8.3. Alcalase found many applications in the food industry, e.g., by reason of their low specificity towards different proteins from plant and animal sources. For example, this enzyme is important in the processing of soy meal which results in soluble, non-bitter hydrolyzate, used as component of protein-fortified soft drinks and dietetic food (Synowiecki 2008). Alcalase is also useful for recovery of proteins from by-products of the meat and fish industry and from crustacean shell waste during chitin production. Furthermore, thermostable proteases that are resistant to anionic or non-ionic surfactants and are active at temperatures above 60°C found application as component of dishwashing detergents (Banerjee et al 1999). Such enzymes can be also used for cleaning ultrafiltration membranes at high temperatures, increasing the efficiency of this process (Bruins et al 2001). The other potential application of heat-resistant proteases is meat tenderizing. It is due to the great difference of enzymatic activity at moderate and high temperatures. The mesophilic proteases injected into the tissue show a residual activity during the whole period of post-slaughter storage of the meat cuts leading to an excessive fragmentation of the protein molecules.

Conclusion. Thermophilic bacteria and their enzymes have to gained an excessive demand of both as analytical tools and as well as biocatalysts for the application in large scale and at laboratory scale too. Application of these catalysts is however still today often limited due to the cost of the enzymes production at high temperatures and media cost. With an increasing market for the enzymes leading to production in higher volumes, the cost is however predicted to reduce. Moreover with a paradigm shift in industry moving from fossils towards renewable resource utilization the need of microbial catalysts is speculating to increase and certainly there will be a continued and increased need of thermostable selective biocatalysts in the future. The integration of enzymes in food and feed processes is a well-established approach but evidence clearly shows that dedicated research efforts are consistently being made as to make this application of biological agents more effective and diversified. These endeavors have been anchoring in innovative approaches for the design of new and improved biocatalysts that will be more stable to higher temperatures.

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