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Review Paper: Thermozymes And Their Industrial Application

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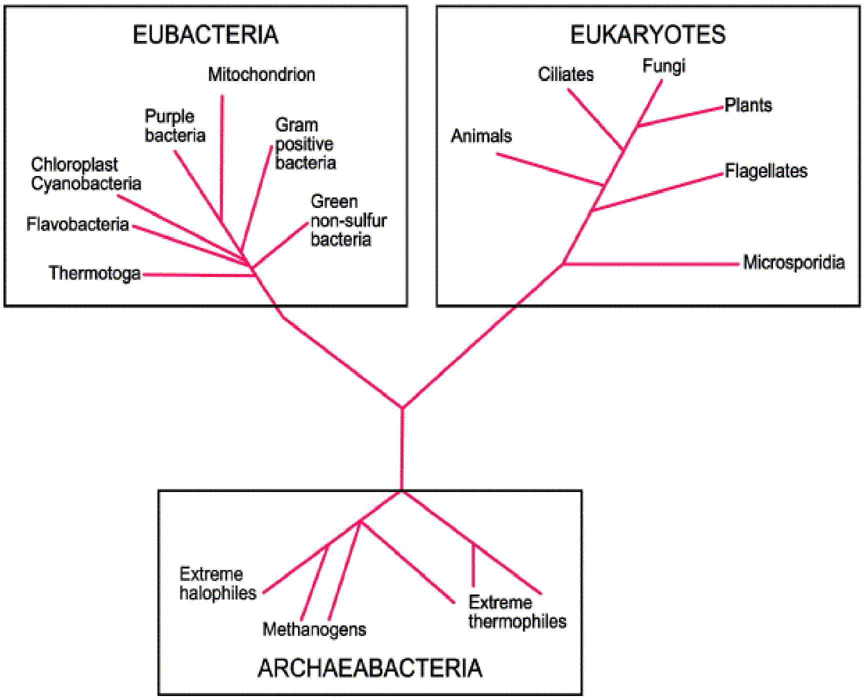
1. INTRODUCTION:

In the beginning of the 21st century, we knew that existence of life in the extreme environment in planet earth is not merely a fiction than reality. The unprecedented progress achieved in the life science, particularly in application of molecular biology techniques has discovered a third domain of living organisms: Archaea which is now a prominent object of research (Woese et al. 1990). Phylogenetic diversity of the archeacian world from other life forms is given in Fig. 1.



Nevertheless the domain comprises of Eubacteria and Archaea displays by far the widest spectrum of metabolic and ecological diversity. In fact organisms that thrives in one or poly extreme environment become the centre of attraction and they are known as extremophiles. Obviously the domain of extreme environment includes various physicochemical parameters such as Temperature, pH, pressure, salinity, desiccation, oxygen, redox potential and radiation. Investigations have revealed that numerous extremophiles generally prefer to live in extreme conditions although they tolerate milder environmental conditions. The microbial diversity exists under extreme environment has been classified (Rothschild et. al. 2001) and some examples are summarized in Table 1.

TABLE 1. EXTREMOPHILES AND THEIR HABITAT:

Environment	Type	Example
Temperature		
>80°C	Hyperthermophiles	Pyrolobus fumarii
		Methanococcus jannaschii
60 – 80°C	Thermophiles	Sulpholobus acidocaldarius
< 15°C	Psychrophiles	Psychrobacter
PH		
> 9	Alkaliphile	Natronobacterium
		Bacillus firmus OF_4
< 4	Acidophiles	Thiobacillus sp.
		Ferroplasma
		Sulfolobus acidocaldarius
Pressure		
130 Mpa	Barophile	Methanococcus jannaschii
Salinity		
2 – 5 M	Halophiles	Halobacteriaceae
		Dunaliella salina
Radiation		
γ - Rays (20K Gy)		Deinococcus radiodurans
UV (1000 J/m^{-2})		-Do-
Desiccation		
Anhydrobiotic	Xerophiles	Artemia salina

Among the various extremophiles exist in nature; much attention has been paid towards thermophiles because they often found great biochemical promise for the application of thermozymes in various industrial processes. Heat tolerant enzymes posses several advantages over their mesophilic counterpart in overall performance of a biocatalytic process. A higher rate of biochemical reaction involving organic solvents has been reported (Krahe et al. 1996; Becker et al. 1997) which may be attributed to the favorable parameters like higher diffusion coefficient of substrate and gas, lower viscosity, higher stability in wide temperature range and less chances of contamination. A variety of extremozymes have been characterized from extreme thermophiles include both archaeal and bacterial species and their prospect of application in the industries is highly promising (Eichler 2001). However an insight into the molecular mechanism of stability of the different macromolecules in a cellular organism, expression level of the thermozymes and their stability under extreme conditions might be of much interest to us in order to decipher the mystery of propagation of life under different environmental conditions and utilization of the knowledge for the welfare of mankind and environment.

2. CHALLENGES TO ORGANISMS LIVING AT HIGH TEMPERATURE:

In general, our perception on interaction of high temperature with living organisms is in antagonistic mode. Pasteurization of milk, sterilization of media or equipment etc are the common practice based on the fact that protein and nucleic acids, the backbone of living organisms get denatured at higher temperature. On the contrary, microorganisms survive at the extreme temperature defying constrains of cell membrane fluidity, osmotic pressure, low dissolved oxygen concentration, high ionic concentration and sometimes sustenance of metabolic activity under desiccation apart from the possible denaturation of proteins and nucleic acids. Pictures taken from the natural habitats of thermophiles are shown in Figs. 2 and 3.

Fig. 2. Octopus spring: Yellow Stone National Park USA

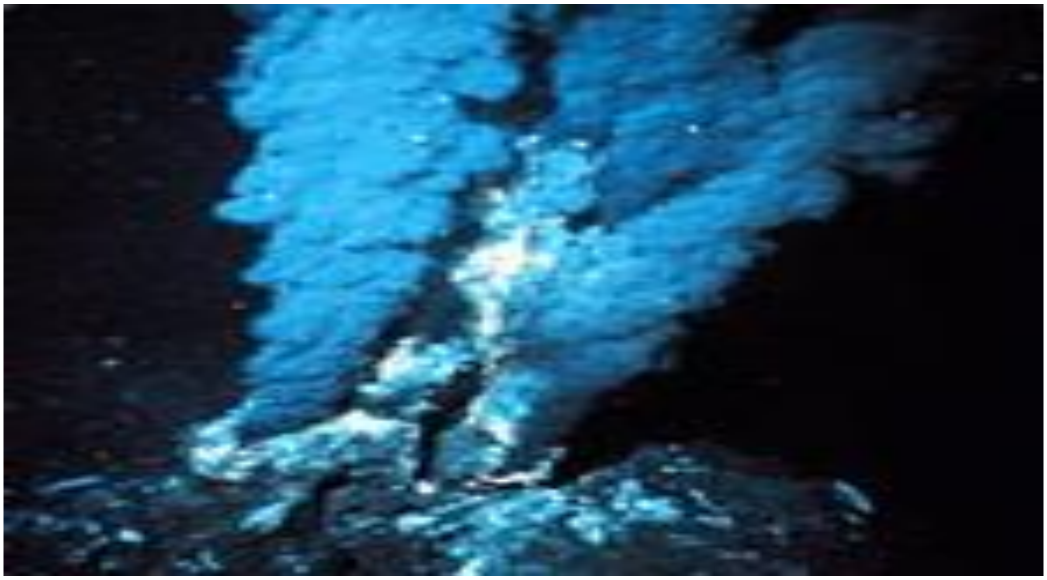


Fig. 3. An undersea hydrothermal vent

2.1 GENOMIC ADAPTATION:

The central issue of evolutionary genomics focused on the adaptive strategy of stable nucleic acid of various microorganisms having higher optimum growth temperature. Adaptation to grow at higher temperature requires a coordinated set of evolutionary changes that affect:

i) Nucleic acid thermo-stability
ii) Stability of codon-anticodon interactions.

Nevertheless, 'Thermal Adaptation hypothesis', as proposed by Bernardi (1986) provides a link between higher guanine (G) and cytosine (C) pairs than that of Adenine (A) and Thymine (T) in a double stranded DNA molecule of a thermophile (Elferink et al. 1994; Krahe et al. 1996; Becker et al. 1997). In spite of the fact that thermal stability of G:C pairs due to three pairs of hydrogen bonds is higher as compared to two pairs of hydrogen bonds in A:T pairs, yet several exceptions have been reported (Reizer et al. 1985). More over at the transcription level, a direct correlation between G+C content of RNA, both ribosomal and transfer RNA, and growth temperature of thermophiles was observed by Fang et al. (2003). Interestingly G- and C-ending codones of rRNA coding duet amino acids was in abundance in thermophiles. However no explanation for the preference of G- and C- ending codones expressing duet amino acids in thermophiles is available. A compilation of database on GC content and optimum growth temperature of numerous prokaryotes failed to demonstrate the predicted correlation. By contrast, the GC content of structural RNA is higher at higher growth temperature (Rosa and Gambacorta 1988). Yet, variation in GC content between orthologous genes of prokaryotes was profoundly observed at codon third sites (G-C3). Simple analysis of completely sequenced prokaryotic genomes showed that G-C3, but not the entire genomic GC, is higher on average in thermophilic species (Gromiha et al. 1999).

'Thermoreduction hypothesis', as proposed by Forterre (1996), describes thermophile are derived from mesophilic ancestors and much of the prokaryotic organization results from adaptations to thermophily. Thus processing time for both mRNA and rRNA in thermophiles is much faster than any mesophilic organism. In addition, post transcriptional modification of individual tRNA bases in Hyperthermophiles makes these molecules more resistant to degradation at high temperature (Singer and Hickey 2003). However, Forterre (1996) suggests that such modifications and even thermostability were not possible until the discovery of tRNA modifying enzymes. Besides, hydrophobicity of the RNA molecule was observed by methylation of ribose in rRNA ribosome assembly by small nucleolar RNAs (Sno RNAs) (Berend et al. 1997). Increase in stability of RNA molecule is evident by increase no of H- bonds in helices, additional base pairing at bases of stem loops, shortened connections between helices, minimization of irregularities and non Watson-Crick pairings in helices.

2.2 HEAT STABILITY OF PROTEINS:

In contrast to the challenges of nucleic acid stabilization, proteins could be stabilized easily at higher temperature. Additional salt bridges, hydrophobic interactions, H- bonds, increased proline content, reduced asparagine content are the some of the factors responsible for the protein stability at higher temperature (Galtier and lobry 1997; Musto et al. 2004). Enzymes from thermophiles often showed similar structural features such as amino acid sequence in and around the active sites, as their mesophilic counterpart. However thermostable proteins do tend to have highly hydrophobic cores, which probably increases internal 'sticking'. Further analysis of the thermostable proteins has revealed that the folding of the proteins that would ultimately affect its heat resistance (Fig. 4) (Sueoka 1962). Therefore minor changes in amino acid sequence are apparently sufficient to render heat stability on an otherwise heat-labile protein. Hyperthermophiles also produce protein-folding proteins, called chaperonins that act on partially denatured proteins to restore the conformity of the active sites of the enzymes. One of such chaperonins, known as thermosomes, was found in very high concentration (80%) in the cells of the thermophiles, Pyrodictium when grown at 108°C (Jonathan et al. 1991).

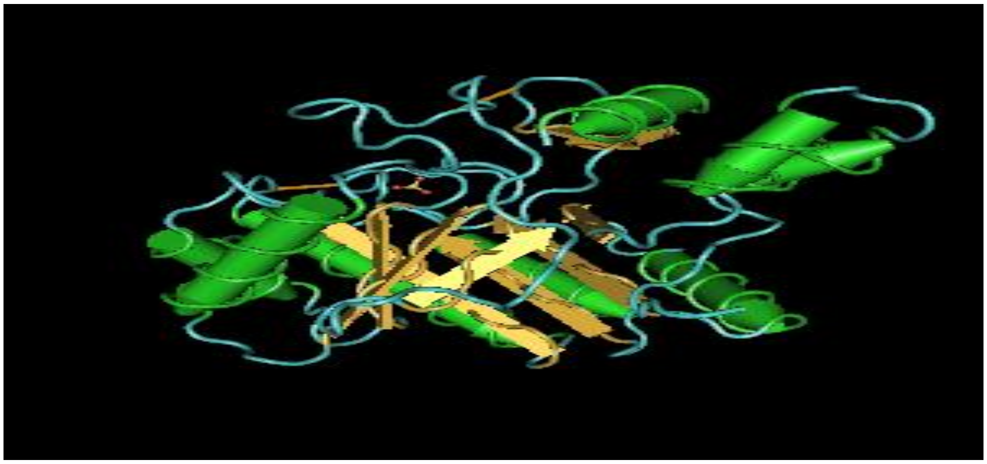
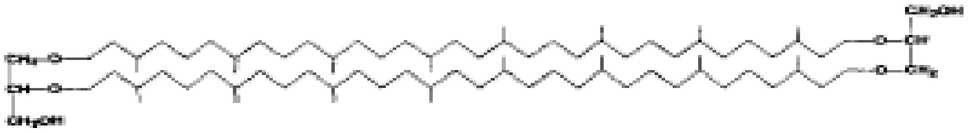


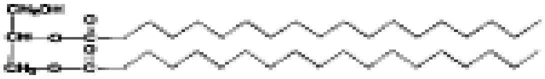
Fig. 4. Crystal structure of catalytic domain of a thermophilic endocellulase produced by Thermobifida fusca

2.3 ADAPTATION THROUGH ALTERATION OF TRANSPORT SYSTEM:

Transport of nutrients, metabolites and ions across the cell membrane is the most important physiological activity on any microorganism. The greatest challenge of survival by thermophiles probably is the maintenance of their membrane fluidity, energy transduction and ion permeability properties at high temperature. Thermophiles differ markedly from mesophiles with respect to the structure of cytoplasmic membrane (Basak and Ghosh 2005). The cytoplasmic membrane of mesophiles consists of lipid bilayer of mainly diacyl-glycerol diesters that have a 1,2-Sn stereochemistry. In contrast, an Archaean membrane consists of ether-lipids, predominantly 'diethers' in the cytoplasmic membranes. Some of the Hyperthermophiles membrane is constituted by tetraethers (Fig. 5). Thus lipid proteins interaction influences the catalytic activity, tertiary structure, movement and state of aggregation, which in turn affect thermostability of overall membrane proteins including the membrane associated enzymes.



tetra-O-di(biphytanyl) diglycerol



diacyl glycerol

Fig. 5. Membrane organization in thermophiles

Generally bacteria alter their lipid composition in such a manner that the membrane fluidity remains relative constant at different growth temperature. Such adaptive mechanism is referred as "Homeoviscous Adaptation" **(**Sinensky 1975**)**. Maintenance of membrane fluidity in lipid bilayer of the thermophiles is involved with the increase in acyl-chain length, altering anti iso to iso branching, and/ or cyclization of the fatty acids with the increase in growth temperature (Reizer et al. 1985; Russel and Fukunaga 1990; Suutari and Laakso 1994).

2.4 ENERGY TRANSDUCTION AND PROTON LEAKAGE:

Thermophiles can grow at elevated temperatures due to thermostable / active enzymes and structural adaptations of the membranes. However, often a low growth yield and high demand for maintenance energy is observed. Such cellular physiological condition may be explained by an increased ion-permeability of the membrane at high temperature forcing thermophiles to divert more metabolic energy in generating an ion motive force than mesophilic counterpart. Protons and sodium ions are the only coupling ions that used in energy transduction in bacteria and archaea. They may sustain the electrochemical gradient of protons by increasing the rate of proton pumping to compensate for the increased permeability at higher temperature. Consequently, an increased fraction of the metabolic energy is used for maintenance. Alternatively, they may alter their membrane composition such that the membrane becomes less permeable to ions. Possibly as a last resort, cells may entirely change their energy transducing mechanism by coupling these processes to an ion that is less permeable than protons, such as sodium ions mechanisms (Tolner et al. 1997; DeVrij et al. 1988; Vossenberg 1995).

3 THERMOZYMES AND ITS APPLICATION IN BIOREFINERY:

The potential applications of thermophilic microorganisms in industrial applications have been widely reported. Currently, only 1–2 % of the microorganisms on the earth have been commercially exploited and amongst these there are only a few examples of thermophiles. However, the renewed interest that is currently emerging as a result of new developments in the cultivation and production of thermophiles and success in the cloning and expression of their genes in mesophilic hosts will increase the biocatalytic applications of thermozymes. The basic advantage of stability and activity of thermozymes in one hand and a number of process advantages which could be exploited through the use of thermophilic microorganisms on the other hand project thermozymes as future biocatalyst. Energy savings through reduced cooling costs, higher saccharification and fermentation rates, continuous product removal, less energy requirement for mixing, increase in solubility of organic compounds and reduced risk of contamination are major advantages from the angle of process technology (Lee et al. 1997). 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 (Vohra and Satyanarana 2002; Haki and Rakshit 2003). Thus the need to supplement the feed with an external source of phosphorous is reduced. Indeed, the application of thermophiles in industrial processes has opened a new era in biotechnology Table 2.

TABLE 2. IMPORTANT THERMOZYMES AND THEIR INDUSTRIAL APPLICATION:

Enzyme	Temp °C	Bioconversions	Industrial application
Amylase	80-100	Starch to dextrose	Biofuel, Baking, Brewing & Health care
Pullulanase	50-60	Starch to dextrose	Food & Beverage
Cellulase	55-65	Cellulose to dextrose	Biofuel, Detergent, Textile
Xylanase	45-65	Hemicellulose to xylose	Paper & pulp
Protease	65-85	Peptide to amino acids	Detergent, Pharmaceuticals, Food & beverage, Leather
Lipase	40-70	Transesterification, fat hydrolysis	Detergent, Pharmaceutical, Cosmetic, leather, Dairy
DNA Polymerase	90-100	DNA amplification	Biotechnology, PCR

A thermophilic strain from hot springs situated in western Himalayas in India. The strain was identified as Geobacilus sp. by 16s RNA sequence in the nucleotide database and the sequence was submitted to Gene bank as Geobacillus sp. IIPTN (Accession No. DQ323407) (Dheeran et al. 2010). The thrmophilic strain produced extracellular amylases at 60ºC and pH 7.0 and the molecular weight of the amylase was estimated as 66Kd by gel electrophoresis (SDS-PAGE). The specific activity of the enzyme was observed 8 IU/mg protein at 80^{o}C and pH 5. The same enzyme showed improvement in color and turbidity when applied in apple juice (Adhikari et al. 2007).

4 CONCLUSIONS:

Ever since the existence of thermophiles has been discovered scientists belonging to different disciplines such as microbiology, molecular biology and biotechnology have shown their interest to decipher the mystery of life under extreme environmental conditions. Exciting results of their investigations have revealed evolutionary links of our ancestors and hope for existence of extra terrestrial life. Thermozymes produced by the thermophiles showed excellent biocatalytic activities that are highly desirable for different industrial applications. Such thermozymes may help to resolve present day challenges on energy and environment management in near future.

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