ENVIRONMENTAL MICROBIOLOGY

Extreme Environments and Extremophiles

Satya P. Singh
Department of Biosciences
Saurashtra University, Rajkot- 360 005
E mail: satyapsingh@yahoo.com

CONTENTS

Keywords

Extremophiles; Extreme habitats; Thermophiles; Hyperthermophiles; Halophiles; Haloalkaliphiles; Macromolecular stability;, Archaebacteria; Dead Seas; Salt lakes; Adaptation strategies.

Introduction

Microbes play key roles in the life of human being since the time immemorial. We have been exploiting them much before the knowledge and realization of their existence in the universe. However, the microbes played havoc time to time in the form of infectious diseases and other forms of their detrimental activities. Therefore, they are referred as both friend and foe to the society and human being.

Systematic applications of microbes date back to the days of Louis Pasteur, when he established the relationship between chemical transformations and the existence of microbes. The active involvement of microbes in daily life is due to their versatility, diversity and fast growth. They affect almost every spheres of our life ranging from agriculture to industry and from environment to health. In view of their role in human society, the microbes have been the center of attraction for researchers and during the last century several turning points and milestones were established. This led to the better understanding of the organisms and their impact on human society.

Considering the contemporary developments towards modern biology, the elucidation of the DNA structure has been a major turning point. Further, newer applications of the microbes got into the way due to the understanding of the fundamental processes of life (Central Dogma of Life), ability to manipulate genes and genomes and availability of the tools to rapidly sequence the DNA and proteins. However, despite the significant developments in the molecular biology, microbial activities remain to be under limited set of conditions. Due to different restrictions on cultivation of the naturally occurring population, only fraction (1-10%) of microbial world is known and biocatalysts from microbes are able to function only under delicate and defined set of conditions. In the light of above considerations, exploration of newer habitats, particularly extreme ones for Environmental and Biotechnological applications become quite significant. In this context, evolving microbial potential by molecular approaches such gene shuffling and directed evolution would be another strategy.

Extreme Environments and their Microbial Life

Extreme Environments can be categorized based on the nature of their physical and chemical extremities. The major extreme conditions taken into account with respect to their microbial life include: highly saline environments with variable salinity, extreme of temperatures (cold and hot), underground environments (ice and rocks) and deep sea vent (extremely high temperature and pressure). Problems relating to the existence and survival of organisms and strategies to overcome extremities are the major issues of studies. The microbes from extreme environments are focusing major attention in recent years primarily due to the following interests of the scientific communities:

Evolution

The survival of the microbes under extreme conditions would provide important clues on the origin and evolution of life. Extremophile are the oldest living thing on earth. Since many environmental conditions on earth closely resemble to those existing on other planets, the extremophiles may be quite useful in predicting and search of life on these planets.

Biodiversity

Only fraction of microbial life (less than 10 %) is known and explored. The microbes from extreme habitats constitute important component of the vast unexplored biological potential. The limited studies on the diversity of the extreme organisms from varied habitats have indicated highly diversified population dynamics. Therefore, exploration of many more extreme habitats would be of great significance.

Biotechnological applications

The limited studies on the unique applications of extremophiles have indicated that the unique "survival strategies" available with them can be a great source of novel commercial applications. The robust biocatalysts and unique metabolic capabilities are the major points of attractions in developing processes towards bioremediation
and other biotechnological applications. The value based molecules from microbes dwelling in unexplored and extreme habitats have recently prompted interest among the scientific communities and industries.

While mesophiles require moderate growth conditions, the extremophiles need extreme conditions. On the basis of the extremity, the extreme environments can be categorized as ultra extreme and extreme. Ultra extreme habitats, such as those supporting the growth of hyperthermophiles and hyperhalophiles, are stable in nature with respect the extreme factors. The ultra extreme environments are lethal to most organisms and are dominated by extremophilic organisms, particularly belonging to domain archaea. On the other hand, extreme environments are characterized by the seasonal variation in the extreme conditions. Under the variable extremity, both extreme and moderate organisms are present.

Thermophiles

Usually microorganisms able to grow optimally at temperatures above 40^0C are referred as thermopile and are among the best studied groups of extremophiles. They are present in varying habitats including hot springs, geothermal locations, composts and sun heated soil. While majority of the thermophiles are able to grow optimally at temperatures between 50-70^0C, they can also grow, though slowly, at below 40^0C. The thermophiles belong to various taxonomic groups, such as bacteria, actinomycetes, fungi, protozoa, algae and blue green bacteria. With the advancing knowledge in this area, the boundaries have been redefined regarding the upper temperature limits supporting life. Among the long time contributors to the field of thermophiles, the work of Thomas D. Brock on microbial life in hot springs of yellow Stone National Park in Wyoming (USA) deserves attention. In 1960's, his group identified bacteria, now known as *Thermos aquaticus* which later became a star candidate for it's extremely heat stable DNA polymerase (Taq polymerase) used in PCR applications.

The studies on the thermophile were further propelled by the discovery of the eubacteria and archaeabacteria able to grow at temperatures between 80 and 110^0 C. A term, hyperthermophile, was used to group and describe these organisms, which were unable to grow below 60^0 C. The most extreme hyperthermophile described so far is *Pyrolobus fumarii,* which can not even grow at 90^0 C.While majority of such organisms belong to domain archaea, few, such as *Thermotoga* and *Aquifex* represent eubacterial hyperthermophiles. Among the archaeal hyperthermophilic organisms; *Pyrococcus, Pyrodictium, Thermoproteus* and *Sulfolobus* are the extensively investigated groups. These organisms are highly diverse with respect to their environment and, therefore, in addition to temperature, are adapted to various other factors such as acidity, oxygen, low salinity exhibited by sulfataric fields. Similarly, other extreme habitats within submarine hydrothermal systems characterized by neutrality, low redox potential, extremely high temperature and pressure are also key environment for hyperthermophilic organisms.

Some of the key habitats for moderate hyperthermophiles to hyperthermophilic organisms include fresh water alkaline hot springs, acidic solfatara fields and anaerobic geothermal mud and soil.

Fresh water alkaline hot springs and geysers

These habitats are usually located outside volcanically active zones and characterized with neutral to alkaline pH. The pH ranges from 9-10 and the temperature is around 75^0C. The representative groups belong to both primary producers (autotrophs) and consumers (heterotrophs). *Thermothrix thiopara, Methanobacterium thremoautotrophicum* and *Desulfovibrio thermophilus* are the autotrophs able to use H_2S/O_2, H_2/CO_2 and H_2/SO^{2-}_4 as energy source, respectively and can grow up to 80-85 ^{0}C, the optimum temperature and pH being 65-75^0C and 6.8-7.5. Similarly, consumers (heterotrophs) are represented by *Thermus* spp. and *Bacillus* spp. in these habitats. The heterotrophs are able to grow up to 80^0 C, with the optimum ranging from 60-80^0 C.

Acidic solfatara fields

The geothermal areas are dominated by solfatara fields containing large amount of sulfur. The habitats are also represented by boiling mud pots, acidic hot springs and acidic soil. The resident organisms from these habitats are relatively more thermally resistant as compared to those from fresh water alkaline hot springs. While the sub-surface streams are nearly neutral in pH due to weak acids, CO_2 and H_2S, the pH on the surface is highly acidic in nature due to chemical and biological oxidation of H_2S first to sulfur and then to H_2SO_4. Therefore, the organisms from both autotrophs (*Sulfolobus acidocaldarius* and *Acidianus infernos*) and heterotrophs (*Thermoplasm volcanium* and *Sulfolobus acidocaldarius*) groups can grow optimally at pH 2-2.5 and temperatures 60-90^0 C. Among the organisms dwelling in such habitats, *Sulfolobus acidocaldarius* and *Acidianus infernos* are the most thermally stable and able to grow up to 90-96^0 C.

Anaerobic geothermal mud and soil

The hyperthermophilic representatives of anaerobic geothermal mud and soil belong to both, autotrphic and heterotrophic groups. The autotrophs, such as *Methanococcus igneus* and *Thermoproteus tenax* use H_2/CO_2 and CO/S as energy source respectively and are able to grow optimally at around 90^0C. Among the heterotrophs, *Thermotoga neapolitana* and *Thermoproteus tenax* can optimally grow at 80-88^0 C and are able to use organic matter and sulphur as energy source. This type of habitats is dominated by sulphites and hydrogen.

Deep-Subsurface Extremophiles

During the recent years, exploration of diversity and genetic resources existing in the deep subsurface naturally has gained considerable momentum. The programme has particularly got impetus due to the dedicated and concerted efforts of a group of scientists in Japan under the leadership of Prof. Koki Horikoshi. Application of recent molecular tools such as DNA-chip technology will expand the horizons for investigating and exploring novel biotechnological potential of unculturable and undiscovered microorganisms from ocean. The Japanese group is quite hopeful for searching and exploring new biomolecuales for various applications, in collaborations with private enterprises. It's indicated that the subvent biosphere might have been a possible site for origin of life and possible microbial forms in such habitats would be Thermococcales type hyperthermophiles, putative hyperthermophiles and halophilic archaea. Some of the unique microbes isolated and reported by the Horikoshi group include; a cellulose-degrading extremely thermophilic bacterium isolated from the world's deepest point of the Mariana Trench, a novel thermophile isolated from >300^0 C, 1500m deep, geothermal water pool, a novel hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent environment and a harmful and poisonous metals-reducing thermophile isolated from a deep-sea hydrothermal vent.

Halophiles and Haloalkaliphiles

Halophiles are the organisms able to grow at very high salt concentrations prevailing in diverse hypersaline environments. Depending on their requirement for salt, they are classified as halotolerant (optimum growth, 0-0.3M salt; growth range, 0-1.0 M salt), moderately halophilic (optimum growth, 0.2-2.0 M salt; growth range, 0.1- 5.0 M salt) and extremely halophilic/hyperhalophilic (optimum growth, 3.0-5.0 M salt; growth range, 2.0 - 5.5 M salt). Halophilc organisms are highly diverse groups including heterotrophic and methanogenic archaea, autotrophic and heterotrophic bacteria and heterotrophic eukaryotes. Among the archaeal category, *Halobacterium and Halococcus* (from Dead Sea) and *Nitrobacter and Nitrococcus* (from Kanyan Soda lakes) can grow between 3- 5 M salt and are the most extensively studies organisms. Similarly, green alga *Dunaliella salina* is among the eukaryotic systems initially studied. Based on the studies, so far, the halophilic organisms have evolved several biochemical and molecular strategies for adaptation under extremely high salt concentrations. These strategies include salt- adapted enzymes, purple membranes enabling phototrophic growth and accumulation of variety of osmolytes into the cell. These features would be discussed in detail in following sections. The realization and exploration of many habitats which harbour halophilic microorganisms able to survive and grow under more than one extremity has attracted the attention of scientific community. The groups of bacteria able to grow at high salt under alkaline conditions are referred as haloalkaliphiles.

Earlier, it was believed that halophilic archaea thrive only in soda lakes but they can also be found in solar salterns and other hyper saline environments. Generally, extreme halophiles are representative of the group archaea but recently, an extremely halophilic, uncultured bacteria, *Candidatus Salinibacter gen. nov* from crystallizer ponds in solar salterns was described. This bacterium grows very well in 35-37% salinity. Several halophilic methanogens, fermentative halophiles and even sulphate reducers have also been isolated from sediments in hypersaline lakes. A group of 122 moderately halophilic bacteria producing various extracellular hydrolytic enzymes were recently isolated from hypersaline environments in South Spain. Many other moderate halophiles have been isolated from various saline habitats. For example, *Halobacillus karajensis* sp. nov., grows at 10–49^0C, pH 6·0–9·6 and at salinities of 1–24 % (w/v) NaCl, showing optimal growth at 10 % (w/v).

Diversity of Haloalkaliphilic Bacteria and Archaea

Haloalkaliphiles possess special adaptation mechanisms for survival in high salinity and alkaline pH. These properties make them interesting from both, fundamental research and biotechnological points of view. Studies on haloalkaliphiles started in early 1980's and during the current and past decades, studies on ecology, physiology, and taxonomy of haloalkaliphiles revealed an impressive diversity in highly saline and alkaline lakes. The occurrence of haloalkaliphiles was probably first mentioned by Tindall in 1984. He described two new genera; *Natronobacterium* gen. nov. and *Natronocococcus gen.* nov. Further, Bivin and Stoeckenius (1986) isolated 51 haloalkaliphilic strains from alkaline salt lakes in Kenya and the Wadi Natrun in Egypt. The isolates contained retinal pigments identical to halorhodopsin of *H. halobium.* Since then, many members of the archaea with haloalkalophilic nature have been reported. A novel, obligately anaerobic, Gram-negative, haloalkaliphilic spirochaete, *Spirochaeta americana sp. nov.,* was isolated from, hypersaline Mono Lake in California, USA that could growth at 37^0C, 3% NaCl w/v and pH 9.5. *Methylophaga natronica sp. nov.,* a new alkaliphilic and moderately halophilic methylotrophic bacterium was isolated from soda lake of the Southern Transbaikal region**.** Recently, a haloalkaliphilic Gram-positive bacterium, *Bacillus saliphilus* sp. nov., was isolated from algal mat from a mineral pool located in Malvizza in the Campania region (southern Italy). This strain optimally grew at 37° C and at pH 9 and was able to tolerate NaCl up to 25 %**.**

Until recently, the biological oxidation of inorganic sulfur compounds was known to occur only under neutrophilic and acidophilic conditions. However, according to some recent observations, haloalkaliphilic bacteria also possess these characteristics. Sorokin and his co-workers are extensively involved with the exploration and analysis of phylogenetic diversity of sulfur- oxidizing haloalkaliphilic bacteria. They have studied growth and inorganic sulfur oxidation among haloalkaliphilic, sulfur oxidizing, chemolithotrophic bacteria at extreme conditions of high salt and pH. They have isolated a new group of obligate chemolithoautotrophic, sulfur-oxidizing haloalkaliphilic bacteria belonging to the γ subdivision of the Proteobacteria; under new genera, *Thialkalimicrobium* spp., *Thialkalivibrio* spp. and *Thioalkalispira*. These organisms appear to play a crucial role in the natural sulfur cycle in saline alkaline environments. Similarly, a new chemolithoautotrophic, haloalkaliphilic, sulfur-oxidizing bacterium *Thialkalivibrio halophilussp. nov.,* was reported from an alkaline hypersaline lake in the Altai Steppe (Siberia, Russia). The strain was extremely salt tolerant (0.2 -5 M NaCl with an optimum at 2 M) and able to grow at pH range 7.5–9.8 (optimum pH 8.0-9.0), with an ability to use sulfur compounds as energy source and electron donors.

Haloalkaliphilic bacteria, beyond the boundaries of their conventional habitats; soda lakes and Dead Seas have been reported from India. More than 200 bacteria / strains have been isolated from the man-made and natural saline habitats along the coastal region of Gujarat in India. Our objectives are to explore microbial heterogeneity, molecular phylogeny and the stability of extracellular enzymes. Majority of the isolates are able to grow over a wide range of salt (5- 25% (w/v) and pH 7-10. Few could grow even at 30% salt. The salt and pH requirement varies among the isolates obtained from the same site indicating extensive diversity. Another significant point which emerged was the relationship between the extent of extremity and diversity. As the extremity with respect to pH and salt during the enrichment for isolation was increased, it led to decrease in diversity and number among the Haloalkaliphilic bacteria. Studies based on the 16s rRNA gene homology have revealed the occurrence of organisms related to *Nesterenkonia abyssinicia*, *Halomonas variabilis, Bacillus* *alcalophilus* and *Bacillus krulwichiae*. In addition, various biochemical and other properties have emerged as markers towards judging the population heterogeneity. The properties of some extracelluar enzymes, such as alkaline proteases are quite attractive for biotechnological applications. The novel and unique features of the enzymes as emerged from our studies can be quite useful in investigating molecular stability at extreme conditions.

Hyper saline environments consist of two primary types: one, arising from seawater and hence contain sodium chloride as the predominant salt (thalassohaline), the second one, arising from non-seawater sources and contain varying ion compositions (athalassohaline).

The athalassohaline systems are dominated by potassium, magnesium or sodium and thus, serve as the sources for potash, magnesium metal, soda and borax. Some examples of these environments are Dead Sea, alkaline soda lakes, carbonate springs and alkaline soil.

Carbonate springs and alkaline soil

Carbonates- rich springs and alkaline soils with pH 10 or above are found in many places. Such habitats provide organic matters for diverse groups of heterotrophs, the best known alkaliphiles are *Bacillus* spp. Several species of cynobacteria are also normally abundant in such habitats. Decaying proteins and hydrolysis of urea can result in high concentrations of ammonia at specific places leading to raised pH. Most alkaliphiles have, however, been isolated from soil by enrichment techniques. Horikoshi and his group in Japan have spearheaded research on alkaliphiles during the last 2 decades and described many alkaliphiles; some have been extensively investigated for various commercial applications.

Soda Lakes

Soda lakes and soda deserts are the most stable, naturally occurring, alkaline environments on earth with high salt content. The soda lakes are characterized by the presence of large amount of Na_2CO_3 and low concentration of Mg^{2+} and Ca^{2+}. The Kenyan-Tanzanian Rift Valley has a number of such lakes containing 5% (bogoria, Nakuru, Elmenteita and Sonachi) to 35% w/v salts (Magadi and Natron) with pH values of 8.5 to > 11.5. Because of the alkaline-saline environments, the organisms from these habitats, referred as Haloalkaliphiles, have characteristics of both; alkaliphiles and halophiles. Bacterial diversity associated with Baer Soda Lake in inner Mongolia of China has recently been investigated. Similarly, other such habitats from several parts of the world are also being explored.

The pH of the hyper saline lakes is primarily determined by the ionic composition of the brine. Na^+ is normally the main cation, with Na_2CO_3, being the major source of alkalinity. Alkaline pH also selectively precipitates other important cations, such as iron. The lakes are rich in specialized photosynthetic bacteria such as *Ectohiorhodospira*. The extreme halophiles, which are mainly aerobic heterotrophs, under low O_2 tension, harvest light energy via a specialized proton pump, using bacteriorhodopsin, or by an inward- directed Cl^- pump.

Soda lakes, highly alkaline extreme environments, are formed due to closed drainage basins exposed to high evaporation. Due to the scarcity of Mg^{2+} and Ca^{2+} in the water, the lakes get enriched in CO_3^{2-} and Cl^-, with pHs in the range of 8 - 12. Although there is a clear difference in prokaryotic communities between the hyper saline lakes (NaCl, > 15% w/v) and relatively dilute waters (NaCl about 5%, w/v), photosynthesis based primary production could be the basis of all nutrients recycling. However, it is quite possible that alkaliphiles evolved separately within an alkaline environment. Although present-day soda lakes are geologically quite recent, their existence may relate to archaeal time, leading to independent evolution of microbial communities in these habitats.

Highly saline environments

The main difference between the soda lakes and other hyper saline environments are the pH and ionic composition of the brine. The most notable hyper saline biotops apart from soda lakes are the great inland salt lakes, such as the Great Salt Lake and the Dead Sea. Such lakes are found in sub tropical or tropical areas where high rates of evaporation result due to high temperature and light intensities. They are formed when evaporation exceeds the input of fresh water from rivers or rain. Transient hypersaline areas are naturally formed on the seashore due to rapid evaporation. Similar biotopes of intermediate duration are formed by human activities, since much salt is commercially produced in salterns or evaporation ponds near the sea shore in many areas. The pH of these lakes depend on the relative abundance of $Ca^{2+}$and Mg^{2+}as also indicated in soda lakes. The ionic composition of the Great Salt Lake resembles that of sea water; however there is vast difference in the concentrations of various ions. The total salinity of the sea water is ten folds less as compared to that of Great Salt Lake. Though, total salinity of Great Salt Lake and Dead Sea is quite comparable, the latter is rich in $Ca^{2+}$and Mg^{2+}.While the Dead Sea is slightly acidic (pH 5.9 to 6.3), the pH of the Great Salt Lake is 7-7.5. The Na^{+} content of the three habitats varies extensively, the concentration being 10, around 40 and above 100 g/l in sea water, Dead Sea and Great Salt Lake respectively.

Solar Salterns

Solar salterns are fed by brine springs containing 5 - 20% NaCl where solar and wind power evaporate the water and thus produce salt. More commonl solar salterns are located on or near the seashore, and seawater is directed into the initial or inlet pan. At total salt concentrations around 8-10%, the calcium salts begin to precipitate, forming gypsum layers in the pans. Finally, at 40 % total salts, the brine is transferred into the crystallizers, where sodium chloride precipitates, leaving an overlying layer of water with high potassium and magnesium containing some sodium and calcium salts. Chloride is the major anion in saltern. The comparative studies on metabolic diversity of two solar salterns in different geographic locations have suggested that ponds may have communities with different microbial populations despite the similarities in salt content. The knowledge on the number and apparent diversity of the organisms from hypersaline environments has been steadily growing over the many years. These organisms have diverse requirements and tolerate NaCl concentration up to saturation. Several halophilic methanogens, fermentative halophiles and even sulphate reducers have also been isolated from sediments in hypersaline lakes.

The hypersaline environments with sodium chloride concentrations close to saturation are usually dominated by halophilic members of the domain archaea, while bacteria are quite rare in such habitats. Recently, cultivation independent 16S rDNA sequence retrieval techniques are being employed to identify and characterize extremely halophilic archaea and bacteria in crystallizer ponds from solar salterns. The high abundance and growth of a new group of hitherto- uncultured bacteria in crystallizer ponds (salinity; 30 - 37%) from multi-pond solar salterns were reported. This bacterium constituted from 5 to 25% of the total prokaryotic community and was affiliated with the Cytophaga- Flavobacterium- Bacteroides phylum. A salt loving bacterium, identifiable with a species of *Bacillus* and presumed to be 250 million year old was recently isolated from a salt crystal buried at more than 1,500 feet underground in Carlsbad (Vreeland et.al, 2000). The ancient spore was extracted from the cavity in a salt and grown. In view of some of these findings, the general perception that Archaea are the only ecologically relevant prokaryotes in hypersaline aquatic environments should be revisited.

Uncultivable Microbes

The development and availability of modern molecular tools to study *in-situ* interactions in recent years has opened new arena. Now study on the complexity and population dynamics of natural bacteria and archaeal communities has become possible due to culture independent analysis. Based on the molecular sequence, many groups of bacteria and archaea that could not be cultivated were known by *in-situ* genetic analysis. The developments in this area support the view that only fraction of microbial community (1-10%) is known and explored, thus far, primarily due to the inability to grow the majority population under the laboratory conditions. Even with reference to cultivable organisms, extrapolation of the physiological behavior under cultural conditions to the natural habitat may not be realistic due to the factors such as resource competition, chemical and biological heterogeneity and species interaction.

In general, it is reasonable to suggest that non-cultivable organisms in natural samples may be in a specific physiological state which prevents their growth in conventional cultivation media. Alternatively, their physiology may be fundamentally different and thus the employed cultivation methods do not meet the requirements for growth.

With the advent and availability of newer applications of PCR, applications of DNA sequences of the microbial communities from natural habitats has become possible, even without culturing the organisms. The sequence analyses of the 16S rRNA genes have indicated the existence of vast phylogenetic diversity among the microorganisms dwelling in a given ecosystem. However, despite the success of 16S rRNA genes sequence analyses, only limited knowledge is available regarding the ecological significance of uncultivable microbes. Towards this end, bioinformatics based approaches to study metabolic functions of uncultivable microbes are being attempted. In additions to molecular approaches, some other tools, such as fatty acid methyl ester (FAME) profile, phospholipids fatty acid (PLFA) analyses and carbon utilization patterns are also being explored to get insight into the diversity of the organisms. Besides, variation in the G+C content among the microbial community of a given ecosystem can also be useful indicator of the population heterogeneity. Metagenomics together with microarray system has become significant for exploration of microbes new genetic entities**.**

Genetic Screening for the Novel Functions of the Uncultivable Microbes

- Novel genetic screening methods for retrieval of valuable genes from uncultivable microbes.
- A multi-sequence database of enzymes will be a basis to obtain new and improved enzymes for various functions
- Designing novel genetic screening method for a particular protein family
- Retrieval of environmental sequences without creating large gene libraries avoiding high throughput expression cloning

- Exploration of the diversity of new sequences
- Selection of the most unique genes
- Expression, production and characterization. The correlation between gene sequence, structure and function of the given enzyme family

The initial findings on the exploration of genetic potential of uncultivable microbes using molecular tools have proved the usefulness of this approach beyond doubt. This area, in fact, has focused so much attention of the scientific community that a new research platform referred as metagenomics is being promoted and developed. Creation of a multi - sequence data base on various useful enzymes would lead to obtain unique and improved biocatalysts. The approach would contribute to the general understanding of the existence and diversity of sequences in nature and correlating the gene sequences with the structure and functional analysis.

Adaptation to Extremity

Halophiles And Haloalakliphiles

Problems related to high salt concentrations relate to osmotic effect leading to water loss and dehydration. Besides, high ionic strength can precipitate and denature protein. However, proteins of hyperthermophilic bacteria and archaea are well adapted to high salt where they are able to maintain their structural integrity. The organisms belonging to halophilic group have developed various strategies, as highlighted below, coping with the high salt concentrations in their environments.

Compatible Solutes

The osmotic pressure of the surrounding is very high due to high ionic composition of the hypersaline habitats as compared to the cytoplasm into the cell. The strategies for osmoregulation in a several haloalkaliphilic organisms have been studied and described. To compensate the high osmotic pressure, halotolerant and moderate halophiles either synthesize or take up specific organic molecules as their compatible solutes. *Halomonas elongata* and obligate halophilic archaeon *Methanohalophilus portucalensis* concentrate glycine betain as compatible solutes from environment. In case of *Halobacillus halophillus,* glycine betain transport depends upon the intracellular Cl^- concentrations. Haloalkaliphilic, sulfur-oxidizing bacterium *Thioalkalivibrio versutus* strain produced glycine betaine as a main organic compatible solute when grown in the presence of high NaCl concentration. Some organisms also synthesize sugars such as sucrose or trehalose as their compatible solutes. A haloalkaliphilic methanotrophs accumulated mainly sucrose and 5-oxo-1-proline, in addition to synthesis of ectoine at high NaCl concentrations.

Na^+ Pump

Extremely halophilic archaea and few bacteria have developed an active pump involving Na^+ and K^+ antiport leading to Na^+ exclusion from the cell in order to compensate high external Na^+ concentration. The H^+ gradient is also used for either the excretion of Na^+ or ATP formation.

The findings on the salt adaptation indicate that the adaptation might have been among the earliest developments during the evolution among the Halophiles. Further studies on these aspects would reveal fascinating facts relating to adaptation and evolution.

pH

Several studies have established that one of the key features among alkaliphiles is their ability to maintain a neutral cytoplasm pH. In some cases, it has been suggested that the cell wall may play a key role in protecting the cell from alkaline environments. In addition to peptidoglycan, alkaliphilic *Bacillus* spp. contain certain acidic polymers, such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, and phosphoric acid. The negative charges on the acidic non-peptidoglycan components may give the cell surface its ability to adsorb sodium and hydronium ions and repulse hydroxide ions and, as a consequence, may assist cells to grow in alkaline environments. The presence of sodium ions in the surrounding environment has proved to be essential for effective solute transport through the membranes of alkaliphilic *Bacillus* spp. These characteristics may provide better adaptive mechanisms to haloalkaliphilic bacteria, because they require high salt concentration along with high pH.

Light

Many Halophilic archaea live in shallow evaporation pond encountering high temperature and ultraviolet light. Such organisms harbour a special retinal pigments, carotenoid, which provide protective barrier against the ultraviolet light.

Plasmids from Halophiles and Haloalkaliphiles

Presence of the extrachromosomal DNA is a common phenomenon for Halophiles. However, very few reports on plasmids from haloalkaliphiles are available. While number of Plasmids from Halophiles and Haloalkaliphiles are reported, their physiological and ecological significance is yet to be established. Attempts are also being made towards developing vectors and expression systems of halophilic origin to express their genes and investigate the regulation of gene expression.

While biochemical basis of adaptation is well established in Halophiles and Haloalkaliphiles, its understanding at molecular level is yet to be progressed. However, during the last several years, the genes responsible for the synthesis of compatible solutes have been in focus as far as salt adaptation is concerned. Based on the limited knowledge in this area, it has been indicated that both solute synthesis and transport can be regulated as a function of salt. In case of a blue green bacterium, *Synchocystis* sp**.**, the synthesis of compatible solute and the regulation of the concerned genes in response to salt concentration has been studied. According to these findings with, *Synchocystis* sp., the compatible solute synthesizing enzymes are regulated by salt at both, synthesis and activity level. Apart from the limited knowledge on the regulation of gene expression by salt, the structural basis of proteins/enzymes adapted to high salt concentration is also poorly understood.

Haloalakliphilic Bacteria & Archaebacteria: Status of Research

Molecular Phylogeny

Extensive attention is being focused on Halalakliphilic bacteria and archaea, mostly from soda lakes. However, during the last few years other habitats have attracted attention.

Biochemical basis of salt tolerance & dependence

- Amino acid composition of bulk protein
- High intracellular salt levels
- Intercellular osmolytes

Protein stability & salt dependence

- Few enzymes are purified and characterized
- Salt dependence assessed- varies extensively
- Comparison of crude, purified and recombinant glucoglycerol phosphate synthase (*Synchocystis* sp.)

Regulation of gene expression

Only limited informations are available. Salt dependent Gene expression in a marine Cyanobacterium (*Synechococcus* sp) has been studied.

Denaturation & Protein folding

- Susceptibility of salt tolerant proteins to denaturants- some are highly resistant to urea denaturation
- Renaturation under *in-vitro* conditions would be interesting to investigate
- Role of molecular chaperones in salt stress cellular conditions, if any, to be established

Thermophiles and Hyperthermophiles

Molecular biology of extremophiles

Gene organization and regulation

Genes are co-liner in archaea and eubacteria and functionally related genes are organized in polycistronic transcriptional unit. However, regulation of gene expression and operons still awaits detailed investigation and available informations indicate dissimilarity with eubacteria.

Chromosome structure

Archaea have single, circular DNA molecules containing histone like proteins. The archaeal histones have shown homology with their counterparts from eukaryotes. The genomes have relatively compact structure similar to eukaryal neculosome. HTa (DNA binding protein) from *Thermoplasma acidophilium* has been studied. There appears to be sequence similarities to the HU family of eubacterial histone like protein. DNA binding proteins protect DNA against thermal denaturation and degradation.

Topoisomerase

A novel DNA topoisomerase activity (reverse gyrase) from thermophilic archaea which catalyses positive super coiling has been widely reported. The positive super coiling appears to be important in stabilizing the DNA structure at extremely high temperatures.

Mechanism of DNA replication

DNA polymerases from several thermophilc and hyperthermophilic bacteria and archaea have been extensively characterized. Many of them are being employed in molecular biological tools such PCR and sequencing reactions. Though, the process of DNA replication and its mechanisms is not established in detail in hyperthermophilic bacteria and archaea.

Adaptation to Extremely High Temperature: The structural basis

Its well accepted fact that the traits of adaptation to extremely high temperature are genetically encoded. However, sequence modifications, for instance, replacing confirmationally constrained residues, such as glycine, could also add to thermostability of proteins. In addition, certain structural considerations, as mentioned below, have been suggested to play significant role in conferring thermal stability to proteins:

Addition of Salt Bridges, Enhanced Hydrophobic Interactions, Additional Ion pairing and H-bonding, Improved core packing, Shortening of Loops

In addition to above strategies conferring the thermal stability to proteins, there are certain other considerations such as unique structure of cell wall and cell membrane, which can contribute to adaptation at high temperature. The characteristic features of the cell membrane of bacterial hyperthermophilic member, *Thermotoga maritima,* include the presence of n-fatty acids, diabolic acid and a novel glycerol ether lipid. On the other hand, the archaeal cell membrane contains diphytanyle glycerol diether or its dimmer. These structural features have remarkable resistance against thermal hydrolysis at high temperature and low pH.

Applications and New Horizons

Domesticating Microbes

The systematic and modern applications of microorganisms got impetus and newer applications were realized due to the deeper understanding of fundamental processes of life (Central Dogma of Life) and ability to manipulate genes and genomes. Biological factories were developed for the expression of foreign genes. The new molecular applications were fueled by the availability of tools to rapidly sequence the DNA and proteins. This in addition to a fresh look on the search for “New Microbial Potential” from varied habitats, particularly of extreme nature, has added new dimensions to the applications of microbial life.

Applications of Halophilic Microorganisms

Bacteriorhodopsin

The purple membranes from many halophilic archaea could be useful in fabricating biochips for new generation of computers. Artificial membrane capable of converting sun light to electricity can also be a technological possibility. In Figure 1, biochemical functions of the *Halobacterium* membrane has been depicted.

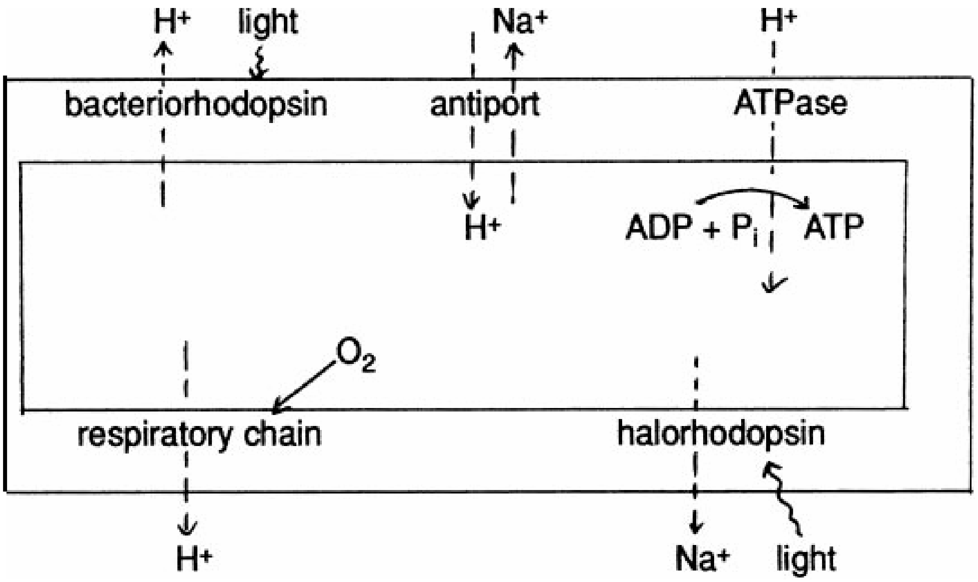

Figure 1: Biochemical functions associated with the membrane of *Halobacterium* *(Source: Bullock, 2000)*

Bio - Plastic and Polysaccharides

Biopalstics from halophilic bacteria would be biodegradable and resistant to water. Similarly, exo-polysaccharides could be used as stabilizers, thickeners, gelling agents and emulsifiers in pharmaceuticals, paints, oil recovery, paper, textiles and food industries.

Compatible solutes

Many halophilic organisms accumulate organic compounds e.g. betaine, ectoines and glycerol. These solutes could be quite useful for commercial applications. In this context, the on-going studies on the regulation of gene expression relating to the synthesis of biocompatible solutes are likely to open new area for improved and large scale production of these molecules.

Enzymes

Exoenzymes produced by these organisms includes proteases, lipases, amylases and nucleases. These biocatalysts are potentially useful in detergent, food and textile industries. Combination of properties of such enzymes at extreme conditions attracts many novel applications.

Biodegradation

Degradation of toxic industrial residue like organo phenol, heavy metals pollutant containing high salt can be a possibility using halophilic organism. Use of such organisms in bioremediation is a reality. However, processes for large scale bioremediation would have to optimized and developed.

Production of beta-carotene

Many members of the halophilic group produce beta-carotene which can be used to synthesize value based moleculaes such as vitamine A.

Some Newer Applications of Halophiles

Alternative energy in the form of hydrogen gas can be produced from biomass using cheap Sea water as media. Transgenic Plants carrying the salt tolerant genes can be developed which would be able to grow in high salt conditions. The initial attempts on the cloning of the genes for biocompatible solutes into plant have yielded encouraging results. Such plants have acquired salt resistance character and were able to grow under high salinity.

One of the strategies employed by the halophilic organisms is that their proteins compete with salt for hydration. This inherent feature of the proteins would enable them to develop resistance against environments with low in water activity. Such features would be quite useful in developing biocatalysis in organic solvents using such enzymes.

Applications of Thermophiles

Thermophilic organisms, particularly those able to grow at temperatures more than 90^0C can be excellent models for investigating and understanding protein stability at extreme temperatures. Such organisms would have significant biotechnological potential. Certain other specific advantages of such organisms are as follows:

- Higher reaction rates at elevated temperatures
- Solubility of substrate at elevated temperatures
- Reduced contamination probability
- Excellent Biocatalysts such as Glycosyl hydrolases, proteinases, Esterase.
- DNA Processing enzyme DNA Polymerase (from hyperthermophilic archaea) with High Proof reading activity useful in PCR

NASA scientists have identified proteins from hyperthermophilic microbes that live in near-boiling, acidic hot springs. These proteins could form into ordered structures to be used in nanotechnology to produce electronics much smaller than today's components. Similarly, further understanding of deep subsurface microbes may allow the search and production of new value-added chemicals and promote bioremediation of highly polluted and poisoned environments.

Applications of Psychrophiles

Psychrophiles like *Vibrio marinus* and *Vibrio psychroerythurs* are the potent producers of neutral proteases which can be very useful in food industry and cold wash laundry detergents. Enzymes can also be used as flavour modifying enzymes. Similarly, polyunsaturated fatty acids from Psychrophiles can be very useful as food additive. The enzymes are useful in food processing such as cheese manufacturing, meat tenderization and lactose hydrolysis. Biosensors for environmental applications could be developed using Psychrophiles and their macromolecules. These organisms assume significance for bioremediation in cold places.

Applications of Acidophiles

Desulphurication of coal using acidophiles involves sulphur oxidation and the process is quite useful in improving the coal quality. Similarly, these microbes can also be useful in food industries. Other potential applications of the enzymes from such organisms include degradation and synthesis of compounds under acidic conditions. Preparation of animal feed to be used in the animal stomach, where pH is acidic, is one such application.

Application of Alkaliphiles

Alkaliphiles are particularly significant for their unique enzymes such as cellulases, proteases and amylases. The ability of such enzyme to catalyze the reaction at high pH has attracted several applications and many such enzymes have already been commercialized. Alkaliphilic extremozymes are important in fabric industries where stone washed look into the denim fabric can be created by softening and fading fabric. Further proteases and amylases from alkaliphilic organisms are quite significant in detergent and leather industries.

Microbial alkaline proteases especially from the *Bacillus* sp. dominate commercial detergent market. The evaluation of detergent proteases depends on pH and ionic strength of the detergent solution, the washing temperature and pH, mechanical handling, level of soiling and the nature of textile. Recently, denaturation and autolysis of protease in a heavy-duty liquid detergent and the factors affecting its stability was studied by Stoner *et al.,* 2004. Alkaline proteases with broad range of substrate specificity can hydrolyze proteins from plants, fishes, or animals to produce hydrolysates of well-defined peptide profile with high nutritional value. Alkaline proteases may provide potential applications for the management of waste originating from various food processing industries and household activities. Proteases solubilize proteinaceous waste lowering down the biological oxygen demand of aquatic systems.

Conclusion

Exploration of newer habitats, particularly the extreme ones, would generate new horizons for their application and generation of novel and unknown value based products. In addition, directed evolution (evolving new potential in the laboratory and improving the existing potential of enzymes) has proved beneficial beyond doubt. Biocatalysts may acquire capabilities not found in naturally occurring organisms by exploring vast sequence space available in proteins. Studies on extremophiles have been propelled during the last decade or so with reference to their phylogeny, diversity and metabolic potential. The increasing attention of the scientific community on these microbes is reflected by the findings of the regularly held conferences and symposia in various parts of the world. The next conference " Extremophile – 2006" will be held in Brest (France) in September 2006.

Recently held conference "Thermophile 2005 " held in Gold Coast, Australia focused on the following aspects highlighting the dimensions of research:

- Exploration of newer habitats with the greater emphasis on the metagenomics
- Global gene expression by using micro array technology towards developing the functional genomics
- New tools for phylogeny of the thermophilic and other categories of extremophilic organisms
- Plasmids and expression systems from thermophilic and hyperthermophilic organisms
- Establishing the biochemical and structural basis of the macromolecular stability under extreme conditions
- The need for investigating biochemistry and physiology of the organisms followed by the exploration of newer/modified metabolic pathways. In this context the biochemical tools and markers towards judging the microbial diversity were also discussed

The above revelations are indicative of the fact that microbes from extreme habitats could provide unique informations on the life at extremities including facts relating to the origin of life. Besides, they can also provide unique pool of value added molecules and their unique metabolic potential would certainly open new horizons. In Figure 2, various dimensions of the current research relating to microbes from extreme environments is highlighted.

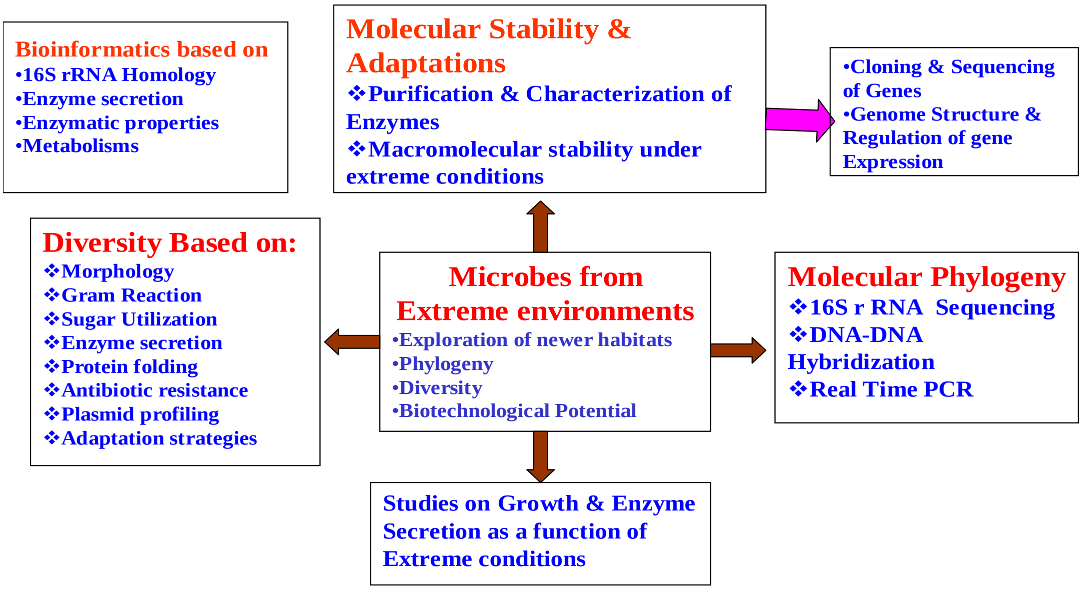

Figure 2: Research Dimensions relating to Microbes from Extreme Habitats

One of the future trends of research relating to extremophiles would be to produce recombinant enzymes after cloning and expression into suitable hosts for investigation of enzymatic properties such as stability and biochemical characterization. This in conjunction with the molecular tools available for directed evolution and tailoring intended biocatalysts would promote novel applications.

Observation of certain unique features among the extremophilic protein/enzymes, such as resistance against chemical denaturation, indicates towards their application as model system to investigate protein stability, besides attracting unique biotechnological roles.

On the gene expression, comprehensive data collection from the environmental samples still remains a difficult task. However, with the ever-increasing arrays of sophisticated technologies in the post- genomic era, the analysis of gene expression under both cultured conditions and natural environment would provide useful informations. Due to the application of modern proteomics tools, analysis of the limited gene expression data from complex microbial communities has already started yielding fruitful results.

Suggested Reading

1. Brock T.D. Thermophile. General, Molecular and Applied Microbiology, New York: John Wiley & Sons, 1986
2. Stetter K.O., Fiala G., Huber,G, Huber, R. and Segerer A.,.Hyperthermophilic microorganisms. FEMS Microbiology Rev 75: 117-124, 1990
3. Blochl E, Rachel R., Burgraff S., Hafenbradl D., Janasch HW and Stetter K.O. Pyrolobus fumarii represent a novel group archaea, extending the upper temperature limit for life to 113 ^{0}C. Extremophile1:14-21, 1997.
4. Sanchez-Porro C., Mellado E., Bertoldo C., Antranikian G., Ventosa A.,.Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium Pseudoalteromonas sp. strain CP76. Extremophiles, 7: 221–228, 2003
5. Tindall BJ., Ross HNM. and Grant W.D. Natronobacterium gen. nov., and Natronococcus gen. nov., to new genera of haloalkalophilic archaebacteria. Syst appl Micobial., 5: 41-57, 1984
6. Bivin D.B. and Stoeckenius W. Photoactive retinal pigments in Haloalkaliphilic bacteria. J Gen Microbiol., 132: 2167-77, 1986
7. Hoover RB., Pikuta EV., Bej AK., Marsic D., Whitman WB., Tang J. and Krader P. Spirochaeta americana sp. nov., a new haloalkaliphilic, obligately anaerobic spirochaete isolated from soda Mono Lake in California. Int J Syst Evol Microbiol., 53: 815-821, 2003
8. Romano I., Lama L., Nicolaus B., Gambacorta A. and Giordano A..Bacillus saliphilus sp. nov., isolated from a mineral pool in Campania, Italy. Int J Syst Evol Microbiol., 55: 159-163, 2005
9. Sorokin DY., Tourova TP., Antipov AN., Muyzer G. and Kuenen J.G..Anaerobic growth of the haloalkaliphilic denitrifying sulfur-oxidizing bacterium Thialkalivibrio thiocyanodenitrificans sp. nov., with thiocyanate. Microbiol., 150(Pt 7): 2435-2442, 2004
10. Banciu H., Sorokin D.Y., Galinski E.A., Muyzer G., Kleerebezem R. and Kuenen J.G.. Thialkalivibrio halophilus sp. nov., a novel obligately chemolithoautotrophic, facultatively alkaliphilic, and extremely salt-tolerant, sulfur-oxidizing bacterium from a hypersaline alkaline lake. Extremophiles. 8: 325–334, 2004
11. Vasavada, S. Thumar, J. and Singh, S. P. Secretion of a potent antibiotic by salt-tolerant and alkaliphilic actinomycete Streptomyces sannanensis strain RJT-1. Current Science 2006 (In Press)
12. Nowlan1B., Dodia, M.S., Singh, S.P and Patel, B. K. C. Bacillus okhensis sp. nov., a halotolerant and alkalitolerant bacterium from an Indian saltpan. Int J Syst Evol. Microbiol 56 (2006), 1073-1077
13. Dodia, M.S., Joshi, R.H., Patel, R.K. and Singh, S.P. Characterization and Stability of Extracellular Alkaline Proteases from Moderately Halophilic and alkaliphilic Bacteria Isolated from Saline Habitat of Coastal Gujarat, India. Brazilian Journal of Microbiology. 2006. 37:276-282
14. Patel, R.K., Dodia, M.S.,, Singh, S.P. Purification and characterization of alkaline protease from a newly isolated Haloalkaliphilic Bacillus sp. Process Biochemistry.. Process Biochemistry, 41(9): 2002-2004, 2006
15. Mehta, V.J., Thumar J. T., and Singh S.P. Production of alkaline protease from an alkaliphilic actinomycete. Bioresource Technology 97:1650-1654, 2005
16. Gupta, A., Roy, I., Patel, R.K. Singh, S.P.. Khare, S.K. and Gupta. M.N. One-step purification and characterization of an alkaline protease from Haloalkaliphilic Bacillus sp. Journal of Chromatography A 1075: 103-108, 2005
17. Oren A. The microbiology of the Dead Sea. Adv Microbiol Ecol., 10: 193-230, 1988
18. Grant WD. and Horikoshi K. Alkaliphiles: ecology and biotechnological applications. In Molecular Biology and Biotechnology of extremophiles, eds Herbert, R.A. & Sharp R.J. pp143-162. London: Blackie & son, 1992
19. Oren A., Heldal M., Norland S. and Galinski EA. Intracellular ion and organic solute concentrations of the extremely Halophilic Bacterium Salinibacter rubber. Extremophiles., 6: 491-498, 2002

20. Krulwich T.A. Alkaliphiles: ‘basic’ molecular problems of pH tolerance and Bioenergetics. Mol Microbiol., 15:403-410,1995
21. Stoner MR., Dale D.A., Gualfetti PJ., Becker T, Manning MC., Carpenter JF. and Randolph TW..Protease autolysis in heavy-duty liquid detergent formulations: effects of thermodynamic stabilizers and protease inhibitors. Enz Microbiol Technol. , 34: 114–125, 2004
22. Rothschild L.J. and Mancinelli R.L. Life in Extreme Environments, Nature 409: 1092-1101, 2001
23. Vreeland R.H., Rosenzweig W.D., Powers D.W. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature., 407: 897 – 900, 2000
24. Horikoshi K. Alkaliphiles: Some applications of their products for Biotechnology.Microbiol.Mol Biol Rev 63: 735-50, 1999
25. Hough D.W. and danson M.J. Extremozymes.Cur Opin Chem Biol 3:39 -46, 1999
26. Eichler J. Biotechnological uses of archaeal extremozymes. Biotechnological Advances 19: 261-278, 2001
27. Bullock, C. The Archaea a biochemical perspective Biochem. Mol Biol Edu., 28: 186-19, 2000
28. Informations on deep sea extremophiles from Prof Horikoshi Web site: http://www.jamstec.go.jp/jamstec-e/bio/en/mesubex.html