

Extremophilic microbes: Diversity and perspectives

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A variety of microbes inhabit extreme environments. Extreme is a relative term, which is viewed compared to what is normal for human beings. Extreme environments include high temperature, pH, pressure, salt concentration, and low temperature, pH, nutrient concentration and water availability, and also conditions having high levels of radiation, harmful heavy metals and toxic compounds (organic solvents). Culture-dependent and culture-independent (molecular) methods have been employed for understanding the diversity of microbes in these environments. Extensive global research efforts have revealed the novel diversity of extremophilic microbes. These organisms have evolved several structural and chemical adaptations, which allow them to survive and grow in extreme environments. The enzymes of these microbes, which function in extreme environments (extremozymes), have several biotechnological applications. Antibiotics, compatible solutes and other compounds obtainable from these microbes are also finding a variety of uses.

MODERATE environments are important to sustain life. Moderate means environments with pH near neutral, temperature between 20 and 40°C, air pressure 1 atm and adequate levels of available water, nutrients and salts. Many extreme environments, such as acidic or hot springs, saline and/or alkaline lakes, deserts and the ocean beds are also found in nature, which are too harsh for normal life to exist. Any environmental condition that can be perceived as beyond the normal acceptable range is an extreme condition. A variety of microbes, however, survive and grow in such environments. These organisms, known as extremophiles, not only tolerate specific extreme condition(s), but usually require these for survival and growth. Most extremophiles are found in microbial world. The range of environmental extremes tolerated by microbes is much broader than other life forms. The limits of growth and reproduction of microbes are, -12° to more than +100°C, pH 0 to 13, hydrostatic pressures up to 1400 atm and salt concentrations of saturated brines. Besides natural extreme environments, there are man-made extreme conditions such as coolhouses, steam-heated buildings and acid mine waters.

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Life in extreme environments has been studied intensively focusing attention on the diversity of organisms and molecular and regulatory mechanisms involved. The products obtainable from extremophiles such as proteins, enzymes (extremozymes) and compatible solutes are of great interest to biotechnology. This field of research has also attracted attention because of its impact on the possible existence of life on other planets.

The progress achieved in research on extremophiles/thermophiles is discussed in international conferences held every alternate year in different countries. The last conference on extremophiles was held in USA in 2004, and that on thermophiles in England in 2003, and the forthcoming one is due in Australia in 2005. In this article, an attempt has been made to review the diversity of microorganisms that occur in extreme environments, their adaptations and potential biotechnological applications. The 'Great Plate Count Anomaly' wherein only a miniscule fraction of microorganisms (<5%) are detected using the culture-dependent methods as compared to *in situ* detection methods and culture independent methods, such as the 'rRNA approach' is now well known. The latter approach has indeed revealed a greater degree of biodiversity but suffers from the fact that other characteristics of living organisms cannot be ascertained. Therefore, there is a need to have methods, which not only reflect the phylogeny of the bacteria, but also their biotechnological potential and this indeed is possible using the 'metagenome' approach discussed elsewhere in this special section.

Metagenome represents the genomes of the total microbiota found in nature, and involves cloning of total DNA isolated from an environmental sample in a bacterial artificial chromosome (BAC) vector and expressing the genes in a suitable host. Thus the metagenome approach, apart from providing phylogenetic data based on 16S rRNA gene sequence analysis and enumerating the extent of microbial diversity, also provides an access to genetic information of uncultured microorganisms. In fact, metagenome libraries could form the basis for identification of novel genes from uncultured microorganisms. Recognizing the potential of this approach, molecular biologists and chemists have undertaken a project to establish the metagenome of 'Whole earth' (soil) to catalogue genes for useful natural products such as enzymes, antibiotics, immunosup-

pressants and anticancer agents. Thus it may not be too long before this approach is also customized to hunt for genes of interest in metagenomes from various extreme environments.

Extreme environments

Low temperature (cold) environments prevail in fresh and marine waters, polar and high alpine soils and waters, glaciers, plants and animals. The major man-made cold environments are refrigerators and freezers of industrial and domestic food storage. Oceans represent 71% of earth's surface and 90% by volume, which are at 5°C or colder. At altitudes >3000 m, the temperature of the atmosphere is consistently <5°C. As the altitude increases, the temperature decreases progressively and temperatures below -40°C have been recorded. Low temperatures are characteristic of mountains where snow or ice remains year-round. The temperature is cold, part of the year, on mountains where snow or ice melts. Thus, cold environment dominates the biosphere. According to Morita¹, cold environments can be divided into two categories: psychrophilic (permanently cold) and psychrotrophic (seasonally cold or where temperature fluxes into mesophilic range) environments.

Although habitats with elevated temperatures are not as widespread as temperate or cold habitats, a variety of high temperature, natural and man-made habitats exist. These include volcanic and geothermal areas with temperatures often greater than boiling, sun-heated litter and soil or sediments reaching 70°C, and biological self-heated environments such as compost, hay, saw dust and coal refuse piles. In thermal springs, the temperature is above 60°C, and it is kept constant by continual volcanic activity. Besides temperature, other environmental parameters such as pH, available energy sources, ionic strength and nutrients influence the diversity of thermophilic microbial populations. The best known and well-studied geothermal areas are in North America (Yellowstone National Park), Iceland, New Zealand, Japan, Italy and the Soviet Union. Hot water springs are situated throughout the length and breadth of India, at places with boiling water (e.g. Manikaran, Himachal Pradesh). Geothermal areas are characterized by high or low pH.

Fresh water alkaline hot springs and geysers with neutral/alkaline pH are located outside the volcanically active zones. Solfatara fields, with sulphur acidic soils, acidic hot springs and boiling mud pots, characterize other types of geothermal areas. These fields are located within active volcanic zones that are termed 'high temperature fields'. Because of elevated temperatures, little liquid water comes out to the surface, and the hot springs are often associated with steam holes called fumaroles.

With increase in temperature, two major problems are encountered, keeping water in liquid state and managing the decrease in solubility of oxygen. Therefore, the microbes

growing above boiling point of water have been isolated from hydrothermal vents, where hydrostatic pressure keeps the water in liquid state; however, majority of them are anaerobes. Thermal vent sites have recently been found in the Indian Ocean.

The primary areas with pH lower than 3 are those where relatively large amounts of sulphur or pyrite are exposed to oxygen. Both sulphur and pyrite are oxidized abiotically through an exothermic reaction where the former is oxidized to sulphuric acid, and the ferrous iron in the latter to ferric form. Both these processes occur abiotically, but are increased 10⁶ times through the activity of acidophiles. Most of the acidic pyrite areas have been created by mining and are commonly formed around coal, lignite or sulphur mines. All such areas have very high sulphide concentrations, and pH values as low as 1. These are very low in organic matter, and are quite toxic due to high concentrations of heavy metals. In all acidic niches, the acidity is mostly due to sulphuric acid. Due to spontaneous combustion, the refuse piles are self-heating and provide the high-temperature environment required to sustain thermophiles. The illuminated regions, such as mining outflows and tailings dams, support phototrophic algae.

In alkaline environments such as soils, increase in pH is due to microbial ammonification and sulphate reduction, and by water derived from leached silicate minerals. The pH of these environments fluctuates due to their limited buffering capacity and therefore, alkalitolerant microbes are more abundant in these habitats than alkaliphiles. The best studied and most stable alkaline environments are soda lakes and soda deserts (e.g. East African Rift valley, Indian Sambhar Lake). These are characterized by the presence of large amounts of Na₂CO₃ but are significantly depleted in Mg²⁺ and Ca²⁺ due to their precipitation as carbonates. The salinity ranges from 5% (w/v) to saturation (33%). Industrial processes including cement manufacture, mining, disposal of blast furnace slag, electroplating, food processing and paper and pulp manufacture produce man-made unstable alkaline environments.

Soils and oceans represent typical low nutrient environments. Open oceans and seas are more homogeneous low nutrient (oligotrophic) environments than the soils. Open ocean water contains the least organic material with dissolved organic carbon (DOC) between 0.1 and 1.0 mg carbon l⁻¹. Deep water contains less DOC than the surface water. Fresh waters contain relatively high concentrations of organic matter, but the flux of DOC in lake water is similar to that in seawater. Waters are richer in polymeric material than low molecular compounds. The nutrient levels are very low in certain freshwater lakes, called oligotrophic. Soils are far more heterogeneous low-nutrient environments. Desert soils in particular, represent very low-nutrient environments, which have scant and unpredictable rainfall and poorly developed soils. Such soils are generally low in organic matter and available water and range from acidic to strongly alkaline on the surface.

In habitats with low nutrients, solid surfaces play an important role and have strong effect on colonization, resulting in the formation of microbial films and layers.

Environments with high hydrostatic pressures are typically found in deep sea and deep oil or sulphur wells. Almost all barophiles isolated to date have been recovered from the deep sea below a depth of approximately 2000 m. High-pressure condition is also met within soils where factors such as high temperature, high salinity and nutrient limitation may exert further stress on living species. Bacteria adapted to such an extreme environment are able to grow around or beyond 100°C, and 200 to 400 bar of hydrostatic pressure.

Although the oceans are the largest saline body of water, hypersaline environments are generally defined as those containing salt concentrations in excess of seawater (3.5% total dissolved salts). Many hypersaline bodies are derived from the evaporation of seawater, which are called thalassic, with salt concentration of about 3–3.5 mol l⁻¹ NaCl. The two largest and best-studied hypersaline lakes are the Great Salt Lake (USA) and the Dead Sea in the Middle East; the former is slightly alkaline, while the latter is slightly acidic. Many evaporation ponds are found near coastal areas, where seawater penetrates through seepage or via narrow inlets from the sea. Hypersaline evaporation ponds have also been found in Antarctica, several of which are stratified with respect to salinity. A number of alkaline hypersaline soda brines also exist, including Wadi Natrum (Egypt), Lake Magadi (Kenya), Great Ba-

sin lakes (USA), and Sambhar Lake (Rajasthan, India). Soda brines lack magnesium and calcium divalent cations because of their low solubility at alkaline pH. Besides natural hypersaline natural lakes, numerous artificial solar lakes have been constructed for producing sea salts. A common phenomenon in hypersaline environments is the production of gradients of salinity due to evaporation of seawater.

The a_w (water activity) of 0.85 is considered as the limit, below which microbes tolerant to high osmotic pressures are found to grow only in concentrated syrups. Desiccation results in extreme osmotic stress and low a_w values. Some microbes are very resistant to drying, e.g. bacteria in the family Deinococcaceae. This is believed to stem from their thick cell walls, which help in protecting membrane integrity. Microbes may be exposed to intense sources of radiation in the form of γ -irradiation as a means of sterilization or by being in close proximity to nuclear reactors. Environments containing high concentrations of organic solvents are those polluted with petroleum or synthetic organic solvents.

Diversity of microbes in extreme environments and their adaptations

Psychrophiles

As of now, about 100 odd new species of Gram-negative and Gram-positive bacteria from various habitats ranging

Table 1. Comparison of the bacterial diversity in different habitats of Antarctica as determined by the rRNA approach

Bacterial class	Continental shelf sediment		Cyanobacterial mat sample 3*	Subglacial ice 4*	Lake sediment 5*	Saline lake sediment 6*
	1*	2*				
a -Proteobacteria	4	4	5	+	0	+
b -Proteobacteria	1	0.5	9	+	+	+
\bar{a} -Proteobacteria	41	41	1	0	0	+
$\bar{\alpha}$ -Proteobacteria	4	16	2	0	0	+, 16
$\bar{\beta}$ -Proteobacteria	4	2	0	0	0	0
Acidobacteria	0	5.5	0	0	+	0
Gemmatimonadetes	0	0	0	0	0	0
Bacteroidetes	0	0	15	0	0	0
Actinobacteria	3	2	1	+	+	36
Chloroflexi	0	0	0	0	0	0
Chlamydiae	2	2	9	0	+	+
<i>Nitrospira</i>	0	0	0	0	0	0
<i>Clostridium</i> – <i>Bacillus</i>	0	0	34	0	0	+
<i>Cytophaga</i> – <i>Flavobacteria</i>	15	6.5	5	0	0	+
Spirochaetales	0	0.5	0	0	0	+
Green non sulphur	1	4	0	0	+	0
Planctomycetales	1.5	5	0	0	+	0
Cyanobacteria	0	0	0	0	+	+
OP11 Group/Others	0	2	0.5	0	+	0
Uncultured	0	0	0	0	0	5
Archaea	23	6	18	NA	NA	+

1, 2, Continental shelf⁷³; 3, Mat sample⁷⁴; 4, Subglacial ice⁷⁵; 5, Lake sediment⁷⁶; 6, Saline lake sediment⁷⁷. Values indicate percentage of clones. +, indicates presence but % not known and NA, data not available. For details of the identity of the clones up to the genera level, under each class, see the respective publication.

from soil, sandstone, fresh water and marine lakes, sea ice and oceans have been reported. Various species within the genera *Alcaligenes*, *Alteromonas*, *Aquaspirillum*, *Arthrobacter*, *Bacillus*, *Bacteroides*, *Brevibacterium*, *Gelidibacter*, *Methanococcoides*, *Methanogenium*, *Methanosarcina*, *Microbacterium*, *Micrococcus*, *Moritella*, *Octandecabacter*, *Phormidium*, *Photobacterium*, *Polaribacter*, *Polaromonas*, *Psychroserpens*, *Shewanella* and *Vibrio* have been reported to be psychrophilic². The genus *Moritella* appears to be composed of psychrophiles only. The psychrophilic and barophilic bacteria, which have been cultivated, belong to α -Proteobacteria, *Shewanella*, *Photobacte-*

rium, *Colwellia*, *Moritella* and *Alteromonas haloplanktis*. For the first time, *Leifsonia aurea*³, *Sporosarcina mcmurdoensis*⁴ and *Kocuria polaris*⁵ have been reported from Antarctica. A comparison of the diversity of bacteria in different habitats of Antarctica is given in Table 1 and a phylogenetic tree suggesting the relationships among psychrophilic bacteria of Antarctica is shown in Figure 1. A psychrophilic and slightly halophilic methanogen, *Methanococcoides burtonii* was isolated from perennially cold, anoxic hypolimnion of Ace Lake, Antarctica⁶. This suggests that members in the domain Archaea are also capable of psychrophilic life style and require further in-

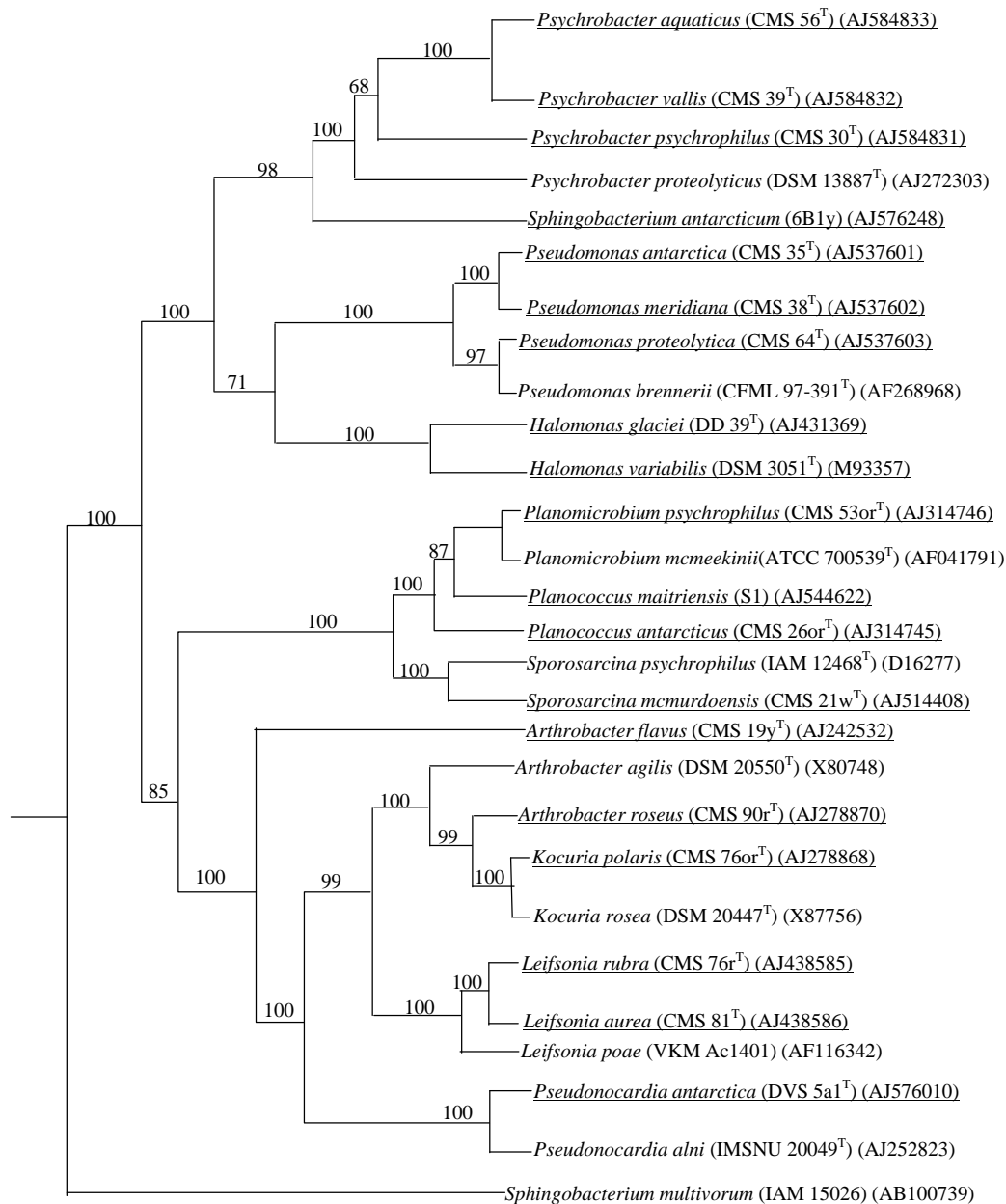


Figure 1. UPGMA phylogenetic tree showing the relationships between psychrophilic bacteria from Antarctica isolated by Indian scientists (underlined) based on 16S rRNA gene sequence analysis. The strain identification number and 16S rRNA gene accession number are given within brackets.

vestigations. As much as 30% of the marine picoplankton from both polar and temperate coastal waters are Archaea and the majority is associated with the Crenarchaeota.

In any cold environment, many of the isolates are psychrotrophs. Psychrotrophs isolated from food and dairy products include mesophilic bacteria such as *Bacillus megaterium*, *B. subtilis* and some species of *Arthrobacter* and *Corynebacterium*. Psychrotrophic bacteria have been reported in permanently cold caves in the Arctic, Lapland, the Pyrenees, the Alps and Romania. Most of the organisms belonged to the genera *Arthrobacter*, *Pseudomonas* and *Flavobacterium*, with the *Arthrobacter* predominating. Many of the psychrophiles isolated from soils of these caves resembled *Arthrobacter glacialis*. Algae are found where snow melts and snow surface is red, green or yellow. Most of these snow algae are psychrotrophs. Snow algal flagellates include, *Chloromonas brevispina*, *C. pichinchae*, *C. rubroleosa*, *C. polyptera* and *Chlamydomonas nivalis*⁷.

Numerous bacteria can be isolated from troposphere and stratosphere, where the temperatures are between -20 and -40°C. Bacteria procured from an altitude of 7000 m were typical soil forms and over the ocean may be marine. Bacterial growth could occur in clouds, and this may be responsible for the occurrence of relatively high levels of cobalamin, biotin and niacin¹. The incidence of psychrophiles in the atmosphere, however, needs confirmation.

A phylogenetic analysis of SSU rRNA sequences of the cultivated psychrophiles suggested five major lineages of cultivated obligate psychrophilic bacteria and archaea: Crenarchaeota, Euryarchaeota, *Flexibacter-Cytophaga-Bacterioides* group, Gram-positive bacteria and proteobacteria². Furthermore, it appears that psychrophiles have evolved from their mesophilic counterparts inhabiting permanently cold environments².

Psychrophilic bacteria have the unique ability to survive and grow at low temperatures and thus, could serve as excellent model systems to understand the molecular basis of low temperature adaptation. Their adaptation to low temperature is dependent on a number of survival strategies such as the ability to modulate membrane fluidity, ability to carry out biochemical reactions at low temperatures, ability to regulate gene expression at low temperatures, and capability to sense temperature^{8,9}. Unlike mesophilic bacteria, psychrophiles have increased levels of unsaturated fatty acids that further increase with reduction in temperature, so as to modulate membrane fluidity, an important strategy for cold adoption. Carotenoids are also shown to regulate membrane fluidity due to their temperature-dependent synthesis^{10,11}. The presence of cold active enzymes¹²⁻¹⁴ and the ability to support transcription and translation in psychrophiles were demonstrated at low temperatures^{15,16}. Studies have also revealed the presence of certain genes which were active at low temperature^{17,18}. In addition, genes essential for survival at low temperatures were identified. More importantly, at-

tempts have been made to understand sensing temperature and transduction of signals for specific induction of the genes. Differential phosphorylation of membrane proteins^{19,20} and probably LPS²¹ have also been implicated in temperature sensing. Recent studies of low-temperature-induced changes in the composition of LPS highlight the importance of LPS in cold adaptation²². Sea-ice microbes produce polymeric substances, which could serve as cryoprotectants both for the organisms and their enzymes²³. In response to sudden changes in environmental temperature (cold shock), psychrotrophs and psychrophiles, such as *Trichosporon pullulans*, *Bacillus psychrophilus*, *Aquaspirillum arcticum* and *Arthrobacter globiformis*, synthesize cold shock and cold-acclimation proteins, which function as transcriptional enhancers and RNA-binding proteins.

Studies on the molecular basis of cold adoption, apart from unravelling the molecular mechanisms involved, have also opened up a number of avenues with respect to our understanding of optimization of biological processes required for cell growth and survival. The challenge, therefore, is to understand and unravel the physiological and molecular basis of survival and growth of psychrophiles.

Thermophiles

Microbes capable of growth at high temperatures are a wide variety of prokaryotes as well as eukaryotes. Therefore, Brock²⁴ suggested a definition: 'a thermophile is an organism capable of living at temperatures at or near the maximum for the taxonomic group of which it is a part'. This definition has the advantage of emphasizing the taxonomic distinctions of thermophily in different groups of organisms.

A few thermophilic fungi belonging to Zygomycetes (*Rhizomucor miehei*, *R. pusillus*), Ascomycetes (*Chaetomium thermophile*, *Thermoascus aurantiacus*, *Dactylomyces thermophilus*, *Melanocarpus albomyces*, *Talaromyces thermophilus*, *T. emersonii*, *Thielavia terrestris*), Basidiomycetes (*Phanerochaete chrysosporium*) and Hyphomycetes (*Acremonium alabamensis*, *A. thermophilum*, *Myceliophthora thermophila*, *Thermomyces lanuginosus*, *Scytalidium thermophilum*, *Malbranchea cinnamomea*) have been isolated from composts, soils, nesting materials of birds, wood chips and many other sources²⁵⁻²⁷. Some algae (*Achanthes exigua*, *Mougeotia* sp. and *Cyanidium caldarium*) and protozoa (*Cothuria* sp. *Oxytricha falla*, *Cercosulcifer hamathensis*, *Tetrahymena pyriformis*, *Cyclidium citrullus*, *Naegleria fowleri*) grow at high temperatures.

Several bacteria and archaeobacteria, which are capable of growth at elevated temperatures have been classified into moderate (*Bacillus caldolyticus*, *Geobacillus stearothermophilus*, *Thermoactinomyces vulgaris*, *Clostridium thermohydrosulfuricum*, *Thermoanaerobacter ethanolicus*, *Thermoplasma acidophilum*), extreme (*Thermus aquaticus*, *T. thermophilus*, *Thermodesulfobacterium commune*, *Sulfolobus acidocaldarius*, *Thermomicrobium roseum*,

Dictyoglomus thermophilum, *Methanococcus vulcanicus*, *Sulfurococcus mirabilis*, *Thermotoga mritima*) and hyperthermophiles (*Methanococcus jannaschii*, *Acidianus infernos*, *Archaeoglobus profundus*, *Methanopyrus kandleri*, *Pyrobaculum islandicum*, *Pyrococcus furiosus*, *Pyrodictium occultum*, *Pyrolobus fumarii*, *Thermococcus littoralis*, *Ignicoccus islandicum*, *Nannoarchaeum equitans*) – based on their optimum temperature requirements^{28–32}. These have been isolated from composts, sun-heated soils, terrestrial hot springs, submarine hydrothermal vents and geothermally heated oil reserves and oil wells. The diversity of bacteria of a hot spring in Bukreshwar (West Bengal, India) was recently assessed by a culture-independent approach³³. The sediment samples were shown to contain α -proteobacteria, cyanobacteria and green non-sulphur and low-GC Gram-positive bacteria from 16S rDNA clones. The first of the phylotypes was suggested to co-branch with *Shewanella*, an iron reducer. Extremely thermophilic *Geobacillus thermooleovorans* strains were isolated from hot spring³⁴ and paper pulp³⁵ samples.

Kashefi and Lovely³⁶ have recently reported isolation of an archaeal strain 121 that reduced Fe(III) to Fe(II) from a water sample of Mothra hydrothermal vent field (Northern Pacific field) with upper temperature limit of 121°C, which is the highest upper temperature limit reported for any microbe till date. Most hyperthermophiles are anaerobic and many are chemolithotrophs. There are no particular carbon-use or energy generation pathways that are exclusively linked to growth at high temperatures.

For any microbe, lipids, nucleic acids and proteins are generally susceptible to heat and therefore, there is no single factor that enables all thermophiles to grow at elevated temperatures. The membrane lipids of thermophiles contain more saturated and straight chain fatty acids than mesophiles. This allows thermophiles to grow at higher temperatures by providing the right degree of fluidity needed for membrane function. Many archaeal species contain a paracrystalline surface layer (S-layer) with protein or glycoprotein and this is likely to function as an external protective barrier.

Histone-like proteins that bind DNA have been identified in hyperthermophiles, and these may protect DNA. Besides, hyperthermophiles have a reverse gyrase, a type I DNA topoisomerase that causes positive supercoiling and therefore, may stabilize the DNA. Heat shock proteins, chaperones, are likely to play a role in stabilizing and refolding proteins as they begin to denature. Certain properties of proteins such as a higher degree of structure in hydrophobic cores, an increased number of hydrogen bonds and salt bridges and a higher proportion of thermophilic amino acids (e.g. proline residues with fewer degrees of freedom), is also known. A higher content of arginine and lower content of lysine have been reported for thermostable proteins. Protein stability may also be assisted by polyamines, accumulation of intracellular potassium and solutes such as 2,3-diphosphoglycerate.

The hyperthermophilic extreme acidophiles, with pH optima for growth at or below 3.0, *Sulfolobus*, *Sulfurococcus*, *Desulfurolobus* and *Acidianus* produce sulphuric acid from the oxidation of elemental sulphur or sulphidic ores, in solfataras of Yellowstone National Park. Other microbes that occur in hot environments include *Metallosphaera* that oxidizes sulphidic ores and *Stygiolobus* sp., which reduces elemental sulphur. *Thermoplasma vulcanicum* that grows at pH 2 and 55°C, has also been isolated from solfataric fields. *Thermoplasma acidophilum* was isolated from self-heating coal refuse piles. *Thiobacillus caldus* was isolated from hot acidic soils, while *Bacillus acidocaldarius*, *Acidimicrobium ferrooxidans* and *Sulfobacillus* sp. have been isolated from warm springs and hot springs. The most extreme acidophiles (pH optimum 0.7) *Picrophilus oshimae* and *P. torridus* were isolated from two solfataric locations in northern Japan. The red alga *Cyanidium caldarium* (pH opt. 2–3, 45°C) was recovered from cooler streams and springs in Yellowstone National Park. The green alga *Dunaliella acidophila* is adapted to pH range, 0–3. Mesophilic and sulphur-oxidizing acidophiles such as *Thiobacillus ferrooxidans*, *T. thiooxidans* and *Leptospirillum ferrooxidans* are encountered in acidic mine drainage waters and mineral processing bioreactors.

Acidophiles

Acidophiles maintain their internal pH close to neutral and therefore, maintain a large chemical proton gradient across the membrane. Proton movement into the cell is minimized by an intracellular net positive charge; the cells have a positive inside membrane potential caused by amino acid side chains of proteins and phosphorylated groups of nucleic acids and metabolic intermediates, that act as titrable groups. As a result, the low intracellular pH leads to protonation of titrable groups and produces net intracellular positive charge. In *Dunaliella acidophila*, the surface charge and inside membrane potential are positive, which is expected to reduce influx of protons into the cells. It also overexpresses a potent cytoplasmic membrane H⁺-ATPase to facilitate efflux from the cell.

The acid stability of rusticyanin (acid-stable electron carrier) of *T. ferrooxidans* has been attributed to a high degree of inherent secondary structure and hydrophobic environment in which it is located in the cell. A relatively low level of positive charge has been linked to acid stability of secreted proteins, such as thermopsin (a protease of *Sulfolobus acidocaldarius*) and an α -amylase of *Alicyclobacillus acidocaldarius*, by minimizing electrostatic repulsion and protein folding.

Alkaliphiles

From neutral soils, alkaliphilic Gram-positive and endospore-forming *Bacillus* spp., and non-spore-forming species of

Pseudomonas, *Paracoccus*, *Micrococcus*, *Aeromonas*, *Corynebacterium* and *Actinopolyspora*, Gram-positive endospore-forming *Bacillus* sp. and alkalitolerant fungi have been isolated. Alkaliphilic *Exiguobacterium aurantiacum* was described from man-made alkaline environment such as potato processing waste, while *Ancyclobacterium* sp. was described from Kraft paper and board process effluents. Calcium springs in Oman yielded aerobic species of *Bacillus*, *Vibrio*, *Flavobacterium*, *Pseudomonas* and members of enterobacteria. Soda lakes often exhibit blooms of phototrophs such as *Cyanospira (Anabaenopsis)* sp., *Chlorococcum* sp. and *Pleurocapsa* sp. East African lakes contain *Spirulina platensis* and other lakes harbour *Spirulina maxima*. The photosynthetic yield of *Spirulina* surpasses that of others in the terrestrial environment. Significant blooms of red pigmented *Ectothiorhodospira mobilis* and *E. vacuolata* have been found together with cyanobacteria in soda lakes. They play an important role in sulphur cycle in these lakes by utilizing H_2S as an electron donor in photosynthesis. Black anoxic lake sediments, rich in methylamine-utilizing methanogens such as *Methanohalophilus*, also occur.

Highly saline and alkaline environments such as those observed in Lake Magadi in Rift Valley, Owens Lake in California, the Wadi Natrum Lake in Egypt, several saline soda lakes, and soils in Tibet, Pakistan, India and Russia harbour different populations of prokaryotes. The lakes are often coloured red due to large numbers of haloalkaliphilic archaea such as *Natronobacterium pharaonis*, *N. gregoryi* and *Natronococcus occultus*.

Moderately haloalkaliphilic methanotrophs such as *Methylobacter alcaliphilus* and *Methylomicrobium alcaliphilum* were reported from Lake Khadyn and Kenyan soda lake sediment, respectively. Alkaliphilic spirochetes such as *Spirochaeta alcalica* and haloalkaliphilic *S. asiatica* have been isolated from water of lake Magadi and lake Khadyn, respectively. An alkaliphilic sulphate-reducing bacterium *Desulfonatronovibrio hydrogenovorans* was reported from Lake Magadi. Anaerobic alkalithermophiles *Clostridium*, *Thermoanaerobacter* sp. and *Thermopallium natronophilum* were recovered from lake sediments.

Alkaliphiles maintain a neutral or slightly alkaline cytoplasm. The intracellular pH regulation is dependent on the presence of sodium, which is exchanged from cytoplasm into the medium by H^+/Na^+ antiporters. Electrogenic proton extrusion is mediated by respiratory chain activity and protons are transported back into the cells via antiporters, which are efficient at transporting H^+ into the cell at the expense of Na^+ export from it. Besides controlling protons, Na^+ -dependent pH homeostasis requires re-entry of Na^+ into the cell. Na^+ -coupled solute symporter and Na^+ -driven flagella rotation ensure a net sodium balance³⁷⁻³⁹. The combined action of antiporters coupled with respiration provides the cell with a means of controlling its internal pH, while maintaining adequate Na^+ levels through symport and flagella rotation. The peptidoglycan

layer in alkaliphiles has a higher cross-linking rate at higher pH values; this may provide shielding effect by 'tightening' the cell wall. Sodium gradient is used to energize solute transport and flagella movement, but not for ATP synthesis. Sodium-dependent ATP synthases have not been identified in alkaliphiles. Further, ATP synthases in alkaliphilic *Bacillus* species have been demonstrated to be exclusively proton translocating.

Barophiles

Organisms growing at high pressures, low and high temperatures, low and high organic nutrients are expected to thrive under deep-sea conditions. Deep-sea bacteria have been isolated from the deepest part of the ocean at 10,500 m depth and cultured at >100 Mpa at 2°C and 40 Mpa above 100°C⁴⁰. Barophilic bacteria have been isolated from several locations and identified^{41,42}. Most of the barophilic and barotolerant bacteria belong to α -Proteobacteria⁴³. The coexistence of Archaea was shown along with *Pseudomonas* in Mariana Trench⁴¹. Filamentous fungi and actinomycetes were isolated at 1 bar (0.1 Mpa) with almost same frequency as that of facultative psychrophilic bacteria. Several alkaliphilic, thermophilic and non-extremophilic microbes were also isolated from these sediments. Several filamentous fungi were isolated from deep-sea calcareous sediments at 10 MPa pressure that corresponds to 1000–3000 m depth; most of these were common terrestrial fungi and therefore, it is possible that some of these have acquired barotolerance⁴⁴. Non-sporulating filamentous fungi and yeasts have been isolated from deep-sea sediments at 0.1 MPa⁴⁵. Lorenz and Moliitoris⁴⁶ cultivated a few marine yeasts at 20–40 MPa pressure. Psychrophilic deep-sea, extremely barophilic bacteria that belong to α -proteobacteria include, *Photobacterium*, *Shewanella*, *Colwellia* and *Motiella*. Deep-sea hydrothermal communities are centered around interfacial zone, where vent fluids mix with cold bottom seawater. Microbial communities are either free-living populations associated with discharged vent fluids or those occurring as microbial mats on surfaces that are in direct contact with the discharged vent fluids. The bacteria often form dense mats and are 'grazing grounds' for some of the vent animals. Chemosynthetic exo- and endo-symbiotic bacteria provide the main source of food for several specialized vent animals. There is a plethora of microbes that inhabit hydrothermal vents, which contribute to its rich biodiversity.

The effect of pressure on cell membrane, protein and gene expression has been investigated. In response to high pressure, the relative amount of monounsaturated and polyunsaturated increases in the membrane. A barotolerant *Alteromonas* sp. exhibited increased proportion of unsaturated fatty acid in the cell membrane⁴⁷. The increase in unsaturation produces more fluid membrane and counteracts the effects of the increase in viscosity caused by

high pressure. Barophiles are extremely sensitive to UV light and therefore, require dark or light-reduced environment, as prevails in the deep sea, for growth. Pressure may stabilize proteins and retard thermal denaturation, as seen in DNA polymerases of hyperthermophiles *Pyrococcus* strain ES4, *P. furiosus* and *Thermus aquaticus*, the thermal inactivation of which is reduced by hydrostatic pressure. A moderately barophilic *Shewanella* sp. was shown to possess a pressure regulated operon, which was cloned and sequenced. This strain appeared to produce different DNA-binding proteins at different pressures⁴⁸. Photobacterium SS9 expressed two outer membrane proteins (porins) under different pressures⁴⁹. These cytoplasmic membrane proteins are thought to be pressure sensors controlled by membrane fluidity. A pressure-regulated operon was identified in a barophilic bacterium DB6705. An ORF encodes CydD protein that is required for cytochrome-bd complex in the aerobic respiratory chain, suggesting the apparent importance of membrane components in high-pressure adaptation⁵⁰.

Halophiles

At moderately high salinities (1–3.5 M NaCl), green algae of the genus *Dunaliella* (*D. salina*, *D. parva*, *D. viridis*) are ubiquitous. Green algae predominantly use polyols as compatible solutes (e.g., glycerol in *D. salina*). A variety of species of diatoms such as *Amphora coffeaeformis* and *Nitzschia* and *Navicula* have been found at 2 M NaCl. Some diatoms accumulate proline and oligosaccharides for osmoregulation. Protozoa such as *Porodon utahensis* and *Fabrea salina* have been described from saline environments. Halotolerant yeast *Debaryomyces hansenii* was isolated from seawater. *Cladosporium glycolicum* was found growing on submerged wood in the Great Salt Lake. Halophilic fungi such as *Polypaecilum pisce* and *Basipetospora halophila* were isolated from salted fish. Buchalo *et al.*⁴⁹ have reported 26 fungal species representing 13 genera of Zygomycotina (*Absidia glauca*), Ascomycotina (*Chaetomium aureum*, *C. flavigenum*, *Emericella nidulans*, *Eurotium amstelodami*, *Gymnoascella marismortui*, *Thielavia terricola*) and mitosporic fungi (*Acremonium persicinum*, *Stachybotrys chartarum*, *Ulocladium chlamydosporum*) from the Dead Sea.

In hypersaline lakes, cyanobacteria predominate planktonic biomass. Unicellular *Aphanothece halophytica* grows over a wide range of salt concentrations. This uses glycine betaine as the major compatible solute, taken up from the medium or synthesized from choline. Another cyanobacterium reported from the Great Salt Lake is, *Dactylococopsis salina*. Filamentous *Microcoleus chthonoplastes*, *Phormidium ambiguum*, *Oscillatoria neglecta*, *O. limnetica* and *O. salina* have also been described from the green second layer of mats in hypersaline lakes. However, the diversity of cyanobacteria of hypersaline environment has not been studied extensively.

Halophilic bacterial and archaeal diversity was comprehensively reviewed by Grant *et al.*⁵¹. Phototrophic bacteria occur beneath the cyanobacterial layers in anaerobic but lighted zones in hypersaline microbial mats. The moderately halophilic *Chlorobium limnicola* takes up glycine betaine from the environment and synthesizes trehalose for use as an osmolyte. *Thiocapsa halophila* synthesizes glycine betaine and *N*-acetylglutaminylglutamine amide for osmoprotection.

Aerobic Gram-negative organotrophic bacteria that are abundant in brines of medium salinity are, species of *Acinetobacter*, *Alteromonas*, *Deleya*, *Flavobacterium*, *Marinomonas*, *Pseudomonas* and *Vibrio*. In solar salterns, aerobic heterotrophs are not as common as Gram-negative bacteria, but similar species of genera *Marinococcus*, *Sporosarcina*, *Salinococcus* and *Bacillus* have been isolated from saline soils and salterns. Neutral hypersaline waters all over the world harbour halobacteria belonging to the archaeal genera, *Haloarcula*, *Halobacterium*, *Haloferax*, *Halorubrum*, *Halococcus*, *Halobaculum*, *Haloterrigena* and *Halorubrum*. Salted foods enrich proteolytic *Halobacterium salinarum*. Raghavan and Furtado⁵² have recently reported a population of $5-7 \times 10^3$ extreme archaeal halophiles per g of offshore marine sediments from the west coast of India using an MPN method. The intracellular salt concentration in halobacteria is very high and generally organic compatible solutes are not accumulated. Potassium ions are accumulated internally up to 5 mol l^{-1} concentration. Although sodium also accumulates in molar range, the ratio of cytoplasmic potassium to sodium is high. Proteins of halobacteria are either resistant to high salt concentrations or require salts for activity. Halobacteria have purple membrane that contains crystalline lattice of chromoprotein, bacteriorhodopsin, which acts as a light-dependent transmembrane proton pump. The membrane potential generated is used to drive ATP synthesis and supports a period of phototrophic growth. Halobacteria produce buoyant gas vesicles like many aquatic bacteria.

Many hypersaline environments exhibit methanogenesis. Halophilic methanogens such as *Methanohalophilus mahii*, *M. evestigatum* and *M. halophilus* have been isolated from hypersaline lakes, marine stromatolites and solar ponds, including alkaline saline environments. Low molecular-weight substances like methylamines are the likely major substrates for these methanogens.

Oligotrophs/oligophiles

Oligotroph is an organism that is capable of growth in a medium containing 0.2–16.8 mg dissolved organic carbon per liter. In natural ecosystems, oligotrophs and eutrophs (copiotrophs) coexist, and their proportion is dependent on the ability of an individual to dominate in a particular environment. Oligotrophic bacterium *Sphingomonas* sp. strain RB2256 isolated from the Resurrection Bay, Alaska

retained its ultramicrosize irrespective of the growth phase, carbon source, or carbon concentration. Another oligotroph *Cycloclasticus oligotrophicus* RB1, isolated from the Resurrection Bay, shared properties similar to *Sphingomonas* (e.g. single copy of the rRNA operon, relatively small size and genome size). Oligotrophy appears to be a growth strategy that is not inviolable but may take a long time to adopt, and a long time to lose⁵³. While investigating the diversity of these bacteria in Leh soils, Pramanik *et al.*⁵⁴ have recently reported oligophiles to be abundant in nature.

Characteristics that are considered to be important for oligotrophic microbes include a substrate uptake system that is able to acquire nutrients from its surroundings. Thus, oligotrophs would ideally have large surface area to volume ratio, high-affinity uptake systems with broad substrate specificities and an inherent resistance to environmental stresses (e.g. heat, hydrogen peroxide and ethanol). A number of microbes that are adapted to low nutrient environments produce appendages to enhance their surfaces, e.g. *Caulobacter*, *Hyphomicrobium*, *Prosthecomicrobium*, *Ancalomicrobium*, *Labrys* and *Stella*.

Radiation resistance

Radiation resistance can occur due to prevention or efficient repair of damage. In *Deinococcus radiodurans*, on exposure to g -irradiation, DNA is severely damaged and the intact chromosomal DNA replaces the fragmented DNA. However, in spite of the severe damage to DNA, the cell viability is not affected. This bacterium is also resistant to mutagenic chemicals, UV irradiation and desiccation, and is also multigenomic (e.g. 2.5–10 copies of the chromosome depending on growth rate). The ability of *D. radiodurans* to use its genome multiplicity to repair DNA damage appears to be the most fundamental reason for the extraordinary radioresistance of this species. Some hyperthermophilic Archaea such as *Thermococcus littoralis* and *Pyrococcus furiosus* are also known to survive high levels of g -irradiation.

Other extremophilic microbes

Methanogenic Archaea are often considered extremophiles. Methanogens can be isolated from diverse range of salinities, Antarctic lakes and hydrothermal vents. *Methanococoides burtonii* and *Methanogenium frigidum* were isolated from Antarctica, while *Methanopyrus kandleri*, *M. jannaschi* and *M. igneus* were isolated from hydrothermal vents. *Methanohalobium vestigatum* was recovered from hypersaline Sivash Lake. Alkaliphilic (*Methanosalvus zhilinae*, pH 8.2–10.3) and acidophilic (*Methanosarcina* sp., pH 4–5) methanogens have also been reported.

Other microbes that may be considered extremophiles include, toxitolerants (those tolerant to organic solvents,

hydrocarbons and heavy metals), xerophiles and xerotolerants (surviving very low-water activity, e.g. fungi such as *Xeromyces bisporus*, and endolithic microbes that live in rocks). Toluene-tolerant *Pseudomonas putida* IH-2000 was isolated from soil in Japan by Inoue and Horikoshi⁵⁵. Later Aono *et al.*⁵⁶ and Nakajima *et al.*⁵⁷ obtained several toluene-tolerant microbes. Sardesai and Bhosle⁵⁸ isolated *Bacillus* sp. SB1 from sediment and water samples of Mandovi estuary in Goa that was tolerant to a wide range of organic solvents. This bacterial strain was shown to grow at 2% *n*-butanol, but the growth was severely retarded at 3% level. Gram-negative bacteria generally show higher tolerance for organic solvents than Gram-positive forms. *Pseudomonas* sp. (*P. putida*, *P. chlorophilus* and *P. syringe*) show high solvent tolerance levels and this is probably based on the cell surface properties of such species⁷⁸. Increase in lipoprotein content may mechanically strengthen the outer membrane structure, which is likely to help in improving organic solvent tolerance. In *P. putida*, solvent tolerance is considered to be due to efflux pumps that remove the solvent from bacterial cell membranes.

Extremophiles in biotechnology

A major impetus that has driven extensive and intensive research efforts on extremophiles during the last decades is the potential biotechnological applications associated with these microbes and their products. The likely potential has been increasing exponentially with the isolation of new microbial strains, the identification of novel compounds and pathways, and the molecular and biochemical characterization of cellular components. Examples of extremozymes that are now commercially used include, alkaline proteases in detergents. It is a huge market, with 30% of the total worldwide production for detergents. In 1994, the total market for alkaline proteases in Japan was around 15,000 million yens. DNA polymerases have been obtained from *Thermococcus littoralis*, *Thermus aquaticus*, *Thermotoga maritime*, *Pyrococcus woessii* and *P. furiosus* for application in polymerase chain reaction (PCR). Development of an ideal starch saccharification process involves use of thermostable enzymes such as α -amylase that does not require Ca^{2+} for its activity/stability, amylopullulanase, glucoamylase and glucose isomerase that are active in a narrow pH range for the cost-effective starch hydrolysis and these can now be obtained from extremophiles^{34,59–63}. Alkali and thermostable xylanases are useful in pre-bleaching of pulps in order to reduce chlorine requirement in pulp bleaching^{64–67}. Bergquist *et al.*^{68,69} have attempted to clone and express two xylanases from an extreme thermophile, *Dictyoglomus thermophilum* in *Kluyveromyces lactis* and *Trichoderma reesei*. The immobilized thermostable chaperonins CpkA and CpkB of hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 were reported to be useful in enzyme stabiliza-

Table 2. Potential applications of extremophiles in biotechnology*

Source	Use
Thermophiles	
DNA polymerase	DNA amplification by PCR
DNA ligase	Ligase chain reaction (LCR)
Alkaline phosphatase	Diagnostics
Proteases and lipases	Dairy products
Lipases, pullulanase, amylopullulanase, and proteases	Baking and brewing and amino acid production from keratin
α -Amylases, glucoamylase, α -glucosidase, pullulanase, amylopullulanase and xylose/glucose isomerases	Starch processing, and glucose and fructose for sweeteners
Alcohol dehydrogenase	Chemical synthesis
Xylanases	Paper bleaching
Antibiotics	Pharmaceutical
S-layer proteins and lipids	Molecular sieves
Oil degrading microorganisms	Surfactants for oil recovery
Sulphur oxidizing microorganisms	Bioleaching, coal, and waste gas desulfurization
Thermophilic consortia	Waste treatment and methane production
Psychrophiles	
Alkaline phosphatase	Molecular biology
Proteases, lipases, cellulases, and amylases	Detergents
Lipases and proteases	Cheese manufacture
Proteases	Contact-lens cleaning solutions, meat tenderizing
Polyunsaturated fatty acids	Food additives, dietary supplements
Various enzymes	Modifying flavours
<i>b</i> -Galactosidase	Lactose hydrolysis in milk products
Ice nucleating proteins	Artificial snow, ice cream, other freezing applications in the food industry
Ice minus microorganisms	Frost protectants for sensitive plants
Various enzymes (e.g. dehydrogenases)	Biotransformations
Various enzymes (e.g. oxidases)	Bioremediation, environmental biosensors
Methanogens	Methane production
Halophiles	
Bacteriorhodopsin	Optical switches and photocurrent generators in bioelectronics
Polyhydroxyalkanoates	Medical plastics
Rheological polymers	Oil recovery
Eukaryotic homologues (e.g. <i>myc</i> oncogene product)	Cancer detection, screening antitumour drugs
Lipids	Liposomes for drug delivery and cosmetic packaging
Lipids	Heating oil
Compatible solutes	Protein and cell protectants in a variety of industrial uses (e.g. freezing, heating)
Various enzymes (e.g. nucleases, amylases, proteases)	Various industrial uses (e.g. flavouring agents)
<i>g</i> -Linoleic acid, <i>b</i> -carotene and cell extracts (e.g. <i>Spirulina</i> and <i>Dunaliella</i>)	Health foods, dietary supplements, food colouring, and feedstock
Microorganisms	Fermenting fish sauces and modifying food textures and flavours
Microorganisms	Waste transformation and degradation (e.g. hypersaline waste brines contaminated with a wide range of organics)
Membranes	Surfactants for pharmaceuticals
Alkaliphiles	
Proteases, cellulases, xylanases, lipases and pullulanases	Detergents
Proteases	Gelatin removal on X-ray film
Elastases, keritinases	Hide dehairing
Cyclodextrins	Foodstuffs, chemicals, and pharmaceuticals
Xylanases and proteases	Pulp bleaching
Pectinases	Fine papers, waste treatment, and degumming
Alkaliphilic halophiles	Oil recovery
Various microorganisms	Antibiotics
Acidophiles	
Sulphur-oxidizing microorganisms	Recovery of metals and desulfurification of coal
Microorganisms	Organic acids and solvents
Organic solvent tolerant microbes	
Radiation-resistant microbes	
Oligotrophs/oligophiles	
Barophiles	
	Bioassay of assimilable organic carbon in drinking water
	Microbially enhanced oil recovery process

*Modified from Cavicchioli and Thomas⁵⁰.

tion⁷⁰. An eukaryotic homologue of the *myc* oncogene product from halophilic Archaea has been used to screen the sera of cancer patients. The archaeal homologue produced a higher number of positive reactions than the recombinant protein expressed in *E. coli*. Green alga *Dunaliella bardawii* is used for the manufacture of **b**-carotene. A halotolerant *Marinobacter hydrocarbonoclasticus* was shown to degrade a variety of aliphatic and aromatic hydrocarbons⁷¹. Recently, Nicholson and Fathepure⁷² developed a highly enriched halophilic culture that was able to degrade benzene, toluene, ethylbenzene and xylene in 1–2 weeks. There is a possibility of developing cost-effective methods for remediation of brine impacted soil and aquifers. The applications of extremophiles and their products are still limited. The potential applications are, however, immense. Some examples of their uses and potential applications are listed in Table 2.

Major biotechnological advances are expected in the area of protein engineering. Identification of structural properties essential for thermal activity and stability will enable development of proteins with the required catalytic and thermal properties. A metalloprotease from *Bacillus stearothermophilus* was mutated based on a rational design in an effort to enhance its thermostability⁵⁰. The mutated protein was 340 times more stable than the wild-type protein and was functional at 100°C, even in presence of denaturing agents, while retaining activity at 37°C. Advances are likely to come from the development of recombinant microbes for specific purposes. A recombinant strain of *Deinococcus* was capable of degrading organopollutants in radioactive mixed-waste environment. The recombinant strain expressed toluene dioxygenase that enabled oxidation of toluene, chlorobenzene, 2,3-dichloro-1-butene and indole in highly irradiating environment (6000 rad/h). Furthermore, it remained tolerant to the solvent effects of toluene and trichloroethylene at levels higher than those of many radioactive waste sites. This suggests potential use of genetically engineered extremophilic microbes in bioremediation of waste sites contaminated with a variety of organopollutants plus radionuclides and heavy metals⁵⁰.

The complete genome sequences of thermophiles (*Thermoplasma acidophilum*, *Sulfolobus acidocaldarius*, *Pyrococcus furiosus*, *Methanococcus jannaschii* and others) are now being analysed to understand phylogenetic relationships, similarities and dissimilarities and to find genes for novel products.

Future perspectives

The extensive and intensive efforts world over in understanding the diversity of extremophilic microbes have indicated that what we know today is just the tip of the iceberg. Many more novel and useful extremophilic microbes are expected to be discovered in the near future. Only sporadic attempts have been made to isolate extre-

mophiles from extreme Indian environments. Extensive funding and concerted and collaborative efforts are needed for understanding the diversity of these microbes from extreme environments of the Indian subcontinent and their exploitation.

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