Biotechnological Application of Psychrophiles and Their Habitat to Low-Temperature

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Microorganisms living in extreme environments are divided into five categories: thermophiles, psychrophiles, alkaliphiles, acidophiles and halophiles. Environments that are considered to be extreme are colonized by these special microorganisms which are adapted to these ecological niches. The application of psychophilic microorganisms in industrial processes opens up a new era in biotechnology. Due to its unique biochemical features, it can be exploited for use in biotechnological industries busy in manufacturing food, enzymes, and value added pharmaceuticals products. Recent developments show that psychrophiles are good source of novel catalysts of industrial interest. Some of the enzymes have been isolated and the genes are successfully cloned and expressed in target hosts. Biopolymer degrading enzymes like amylase, pullulanase, xylanase, and proteases play an important role in food, detergents, and pulp industries. Cell membranes of psychrophiles contain surfactants bearing unique stability at low temperatures and that can be used in pharmaceutical formulation. The cold adaptation process of psychrophiles encompasses wide modifications in structure and molecular architecture, physiology, and biochemistry of these organisms. These are described, in detail, here.

Introduction

At low temperature, growth and development of microorganisms is drastically reduced due to the low solubility of organic and inorganic nutrients, decreased ion/solute transport, reduced diffusion, and osmotic effects on plasma membrane and cell wall. Psychrophilic bacteria are most widely distributed than other types of extremophiles for the simple reason that water covers approx. 70 per cent of the earth’s surface and a large part of it constitutes the marine environment. About 90 per cent of the marine habitat exist at < 5°C beyond the thermocline zone of oceans. Apart from the marine habitat, polar regions measure nearly 14 per cent of the earth’s surface, representing an extreme low-temperature habitats in the world. The growth characteristics of psychrophiles and psychrotrophs make them suitable to grow in various low-temperature habitats i.e., in lake sediments, as gut flora of aquatic animals, and in the form of periphytic microflora, and on rocks at low moisture conditions. Psychrophilic bacteria are highly thermosensitive and exposure to moderate room temperature could be detrimental to their activity. Most of the psychophilic bacteria thus far isolated are gram-negative and reports on the occurrence of gram positive bacteria are relatively few. To exemplify their diversity, various bacteria belonging to different genera have been isolated in the recent past.

Extremophiles and their enzymes have attracted much attention because of their wide range of biotechnological applications and also to understand their biochemical mechanisms of adaptation to extremes temperature, pH and salinity. Further, they are being utilized in various bioprocesses to be carried out at low-temperature, in addition to their role in natural decomposition of organic matter and nutrient recycling at low temperature habitats. Particularly, studies on psychophilic and psychrotrophic microorganisms are relatively sparse in comparison to thermophiles and hyperthermophiles. According to the definition of Morita2, psychrophiles show an optimum growth temperature of < 15°C with upper growth temperature of 20°C, whereas psychrotrophs can grow at 0°C although their optimum temperature is around 20-25°C. Psychrotrophs were further classified as stenopsychrophils and eurypsychrophils based on their ability to grow at 40°C (ref.4). Stenopsychrophils are able to grow sub-optimally at 40°C in contrast to eurypsychrophils. Conspicuously the characteristic growth rate of these microorganisms is not much affected at low temperature.

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The ability of psychrophilic and psychrotrophic bacteria to grow at low-temperatures is due to the stability of cell constituents such as enzymes, fatty acid composition of lipids in cell membranes, and modifications in nucleic acid constituents and control over the gene expression through the mediation of transcription and translation with the production of cold-shock proteins. In the biosphere, both prokaryotes and eukaryotes are known to exist as psychrophiles. In addition to polar regions fresh water lakes, rivers, marine sediments and deep sea water are the best source of these bacteria. In addition to the isolation of bacteria, several types of yeasts, fungi and algae have also been reported. These microorganisms were found to be responsible for mineralization and nutrient recycling at as low as -1°C in maritime Antarctica. The presence of a wide variety of hydrolyses namely amylase, protease, cellulase, nuclease, lipase, β-galactosidase, and phosphatase have been reported from Antarctic bacteria3-7. This review discusses the role and importance of psychrophilic microorganisms in the biodegradation of organic pollutants. In addition, various basic aspects of cold adaptation and biotechnological potential of metabolites and hydrolytic enzymes have also been reviewed.

**Phylogeny of Extremophiles**

The phylogeny of extremophiles is based on their 16S rRNA sequence and the universal tree has been divided into bacteria, archaee and eukarya. The archaee consist of two major kingdoms, the crenarchaeota-thermoproteales branch and the euryarchaeota. The latter kingdom consists of extremophiles and methanogens8. The starting lineage of bacteria is represented by the orders thermotogales and aquificales which again represent hyperthermophiles9. Significantly, most of the psychrophilic bacteria have been assigned to bacteria. Exceptionally, a few representative species of crenarchaeota and euryarchaeota have been reported with the help of polymerase chain reaction and gene cloning experiments10. Some of the recently isolated Antarctic bacterial isolates belongs to *Cytophaga-Flavobacterium-Bacteroides* and they have been classified as *Polaribacter franzmannii* strain 301 and *Polaribacter filamentosus* strain 215 based on their 16S rRNA sequence analysis. Most of the bacterial strains isolated from Antarctica have been classified phylogenetically based on the sequence homology of their 16S ribosomal DNA or RNA indicating the presence of many new and previously unreported organisms that include *Planococcus meneekini*, *Arthrobacter*, *Brachybacterium* and *Psychroflexus torquis* gen., nov., sp.nov.1112.

**Habitat in Relation to Structure and Molecular Architecture**

**Ecological Significance and Distribution**

It has widely been noticed that in cold habitats, metabolic role of these microorganisms is essential in the biodegradation of organic matter and for recycling various carbonaceous and nitrogenous nutrients in large quantities13,14. Most of the organic nitrogen released in natural habitats under extreme low-temperature is present in the form of high molecular weight proteins, nucleic acids along with uric acid and urea which cannot be utilized directly by the native bacterial populations. Proteins can only be utilized by bacteria after extracellular hydrolysis by proteases. Several psychrophiles that produce substantial amounts of extracellular enzymes such as proteases, lipases, amylase, nucleases, phosphatase, cellulase, and β-galactosidases have been implicated in biodegradation. In low-temperature habitats, comparatively large amount of enzyme is synthesized and secreted to compensate the decreasing enzyme activity. The phenomenon was noticed in the case of hydrolytic enzymes such as phosphatase, nuclease, amylase, and lipase15-17. Nutrient rich media with suitable inducer substrate molecules are effective to stimulate the synthesis of higher quantities of enzymes. Various enzymes produced by these bacteria are listed in Table I along with their optimum temperature and pH. Also the quantity if enzyme is known to affect the rate of biodegradation. Usually, hydrolysis by extracellular enzymes is often a rate limiting step for bacterial utilization of organic nitrogenous and carbon substrates18,19.

**Some Unique Features of Bacteria and their Enzymes**

Psychrotrophic bacterial isolate of *Vibrio* sp strain 5709 has an optimum growth at 20°C. The bacteria was isolated from a deep sea sediment and the protease from this strain was optimally active at 40°C. Retention of high enzyme activity at low temperatures is a characteristic feature of psychrophiles. For example, 16, 32, and 45 per cent of optimal activity was observed at 0°C, 10°C, and 20°C respectively.

Alkaline phosphatase (EC 3.1.3.1) is one of the hydrolytic enzymes which is required for phosphorylation in molecular biology20. Enzymes such as nitrate reductase and arginino succinate lyase from psychrophilic green algae belonging to the genus *Chloromonas* have
been investigated and their properties compared with enzymes obtained from a mesophilic *Chlamydophila reinhardtii*. The enzymes were found to be cold active and thermolabile. The enzyme arginino succinate lyase (ASL) retained 25 per cent of its original activity at 4°C, whereas mesophilic enzyme was completely inactivated at the same temperature. The study also revealed that *Chloromonas* nitrate reductase and ASL, displayed different thermal properties. The former enzyme was found to be more thermolabile with 50 per cent of its activity loss after incubation at 40°C for 30 min. More often, it was found that enzymes from an organism can display different temperature and pH optima along with varying stability.
The chitinase production is mainly attributed to the biodegradation of large quantities of chitin produced by invertebrates that inhabit the water column and sediments of oceans and estuaries. The exocellular chitinases hydrolyze chitin into microparticles, oligomers and other small molecular weight sugars which can be further metabolized by bacteria as carbon and energy source. Apart from chitin the biomass resulting from sea weeds and animal activity is continuously decomposed by microorganisms mainly by psychrophilic bacteria.

In the marine ecosystem several of chitinolytic bacteria have been reported in water and sediments of the antarctic maritime peninsula. In our earlier investigations, we have categorized antarctic psychrophilic bacteria isolated from decaying blue green algal mass as acidotolerants, alkalitolerants, and thermostolerants. The bacterial isolates were able to grow at higher salt concentrations (up to 10.0 per cent) in the growth medium signifies that these isolates are halotolerant in their nature.

Antarctica psychrophilic bacteria display remarkable activity at 0°C. Bacteria belonging to the genus Psychrobacter sp are predominantly found in antarctic orthogenic soils.

Many enzymes from psychrophiles display substantial activity at very low-temperatures. For example an acid phosphatase with maximal activity at pH 6.0 and 30°C has displayed 27 and 28 per cent of its maximal activity at 0 and 5°C, respectively. Several other enzymes have also been noticed to show high catalytic activity at low and intermediate temperatures with rapid inactivation at or above 40°C. Subtilisin from psychrophilic antarctic bacteria has been well characterized in comparison to other enzymes. Hydrolytic enzymes are essential for processing the huge quantities of organic matter, mainly in marine habitat. Bacterial consortia with different but complementary extracellular hydrolytic enzyme profiles act on organic particles to rapidly solubilize major macromolecular constituents. Hydrolytic enzyme activities such as protease, phosphatase, glucosidase and other enzyme activities are responsible for enzymatic fractionation of carbon, nitrogen, and phosphorus containing organic wastes and the production of these enzymes is mainly attributed to attached bacterial populations.

Bacteria present in marine snow have cell specific ectoenzyme activities several-fold of magnitude higher than bacteria present in the surrounding water. The phenomenon is indispensable for optimum growth even in the presence of low-quantities of nutrients in their habitat. Several enzymes are also known to be produced extracellularly by psychrophilic microorganisms. The enzyme, alkaline phosphatase is produced to supplement their metabolism with organic carbon substrate, which is also a final product of phosphoester hydrolysis. Further the high phosphatase activity of bacteria is an indication of high phosphorous flux in these microenvironments.

Effect of Low Temperature on Membrane Composition

Plants, animals, and microorganisms predominantly incorporate mono-unsaturated and polyunsaturated fatty acids into the phospholipid fraction of their cell membrane at low growth temperatures. Several detailed investigations have been undertaken to elucidate the effect of low temperatures on fatty acid unsaturation of membrane lipids in psychrophiles. Mono-unsaturated and polyunsaturated fatty acid containing lipids have been implicated mainly in the maintenance of liquid crystalline state of cell membranes. The modulation of membrane liquid crystalline state of its constituent lipids is termed as homeoviscous adaptation. The phenomenon is essential for the membrane to carry out its usual cellular functions like solute transport, nutrient uptake, assembly of transport proteins, and for the stability of membrane bound enzymes. The importance of a temperature induced synthesis of desaturase has been revealed in Bacillus megaterium. At low temperatures, fatty acid synthase II activity increases several fold in comparison to synthase I activity. Substantial amounts of short-chain mono-unsaturated and di-unsaturated fatty acid synthesis occurred in psychrophilic Vibrio isolates grown at low temperatures. Another well known PUFA 20:5 has been observed to be synthesized by a marine Flexibacter sp. In one of the previous investigations, several strains of Vibrio marinus grown at 2°C were analysed for their PUFA’s composition of cell membranes. The synthesis of docosahexaenoic acid (22:6), and eicosapentaenoic acid (20:5) in these bacteria has been found to occur predominantly in response to low growth temperatures. Bacterial strains containing docosahexaenoic acid displayed higher growth temperature but at less than 20°C. Whereas the strains with eicosapentaenoic acid have displayed a maximum growth between 20-25°C (ref. 32). Active synthesis of PUFA’s is a characteristic feature of deep-sea microorganisms. The synthesis of large amounts of di-unsatu-
\textbf{Cold-Shock Proteins and Their Role in Cold Tolerance}

The major inductible proteins such as cold-shock proteins (CspS), cold acclimation proteins (Caps), heat acclimation proteins (Haps) and heat shock proteins (HspS) are likely to play an essential role in their cold adaptation process\(^3\). The induction of cold-shock proteins namely CspA, CspB, CspC, CspD, and CspE has been studied, in detail, mainly in \textit{E. coli}. These proteins show amino acid sequence homology with each other\(^3\). The cold-shock protein CspA (110.6) protein has been designated as major cold-shock protein in bacteria due to its high level of expression (200-fold increase) following a shift down from 37\(^\circ\) to 10\(^\circ\)C (ref. 36). The cold-shock protein CspA is a 70 amino acid protein encoded by the cspA gene. In the same family, there are other genes responsible for the synthesis of cold-shock proteins namely CspB, CspC, and CspD with amino acid residues of 71, 69, and 74, respectively. Further these proteins show 79, 70, and 45 per cent sequence homology with CspA protein. The recently reported CspE protein with 69 amino acid residues has shown 70 per cent homology with the major cold-shock protein. Besides the cold stress response in several mesophilic bacteria has been shown to involve the same gene products for cell adaptation. Cold shock proteins, in general, have been proved to elicit transcriptional and translational control in prokaryotic as well as in eukaryotic organisms. Initiation of translation appears to be the most thermosensitive step but elongation of the proteins can occur uninterruptedly at low temperature as revealed by Friedman et al.\(^37,38\). It has also been demonstrated that the cell growth stops below 7.8\(^\circ\)C only due to the inability to initiate protein synthesis. The induction of cold-shock proteins increases from 2 to 10 fold after lowering the growth from 37\(^\circ\) to 27\(^\circ\)C\(^5\). These proteins also include NusA which is required for termination and anti-termination of transcription. Initiation factor 2 binds to the charged tRNA\(^5\) to the initiation site of 30S ribosomal subunit for maintaining the translation process. Its ability to combine with polynucleotide phosphor-}

\textit{lated fatty acids (18:2) has been reported in \textit{Vibrio} sp. Evidence supporting this phenomena also has been provided by cold shock experiments in mesophiles as well as in psychrophilic bacteria and yeast\(^33,34\). Microorganisms with high quantities of unsaturated fatty acids are less susceptible to damage occurring at culture storage temperature as low as minus 80\(^\circ\)C.}

\textit{Protein Folding and Intramolecular Interactions}

Several factors have been described to contribute to the flexibility of enzymes in psychrophiles. Flexibility of a protein conformation is often known to play a major role in deciding the thermosensitivity and catalytic efficiency of enzymes. The rigidity of a protein molecules is mainly reduced through changes in intramolecular interactions. According to recent investigations protein surface properties like low hydrophobicity and high hydrophilic contribute to the stability of enzymes in psychrophiles. Decrease in hydrophobicity is ascribed to the presence of low number of proline residues. However, increased hydrophilicity is caused by the incorporation of more charged amino acid residues to form an extended surface loops. Nevertheless the decreased content of isoleucine, arginine, and at times the total content of arginine-lysine suggests that hydrophilicity can also be achieved by substitution of amino acid residues other than the charged amino acids\(^40,41\). Certain psychrophilic enzymes are completely devoid of disulfide bonds, is also attributed to the protein flexibility.\(^42\). In spite of all these generalisations the data are insufficient to explain conclusively the role of various interactions in enzyme stability in psychrophiles. Aspartate transcarbamylase produced by a psychrophilic strain of \textit{antartica} displayed 26 per cent of its optimum enzyme activity at 0\(^\circ\)C. Some of the kinetic properties and thermal stability of the enzyme resembled transcarbamylase of mesophilic \textit{E. coli}. It indicates that these enzymes can achieve catalytic efficiency through specific changes in the catalytic site in addition to changes that occur in the enzyme molecule\(^43,44\).
Further, diverse type of enzymes were found to be responsible for cold-adaptation apart from fatty acid desaturases and cold shockproteins as well as cold acclimation proteins, was confirmed by the transposon mutant analysis. Goverde et al.\textsuperscript{35} have demonstrated the role of an exoribonuclease, polynucleotide phosphorylase (PNPase) in cold adaptation. The enzyme PNPase mediates gene expression through the degradation of mRNA in the cell. After an initial cleavage by other endonucleolytic enzymes the 3' to 5' exonuclease activity of PNPase is responsible for the degradation of mRNA in eubacteria. However, in E. coli, two types of 3' to 5' exonucleases i.e. RNase II and PNPase, were found to be responsible for exonuclease activity. Also, studies have revealed that the loss of this activity in conditionally lethal mutants was found to be detrimental to the growth of E. coli strain at low temperatures. \textit{Yersinia enterocolitica} a psychrotrophic isolate from a transposon mutant library was found to depend on the enhanced expression of PNPase and its mRNA for cold-adaptation at 5°C. The cold inducible promoter contained an ATGG sequence characteristic of cold promoters.\textsuperscript{46} Although inconclusive generalizations have been made by various authors some of the factors which are responsible for cold adaptation are: (i) Presence of substantial amount of polyunsaturated fatty acids in the lipid moieties of cell membranes\textsuperscript{57,48}, (ii) modifications in enzyme structure resulting in high specific activity at low-temperatures i.e., low $K_{m}$ and high substrate turnover. This phenomenon is being reflected in the activation energies of psychrophilic \textit{P. fluorescens} which showed activation energy values of 10-38.0 kJ/mol whereas the mesophilic protease showed 60.0 kJ/mol, (iii) modifications in the DNA replication, transcription, and translation machinery of the cell\textsuperscript{50,56}.

**Biotechnological Aspects**

\textit{Archaeal Enzymes as Novel Biocatalysts}

Several groups are actively involved in the exploitation of thermophilic and hyperthermophilic enzymes from archae in developed countries. Enzymes of extremophiles known as extremozymes can be employed in various fields ranging from the production of bulk chemicals to healthcare products. Extremozymes can optimally function at both extremes of temperature and pH. On the other hand, some microorganisms thrive at high salt concentrations and, therefore, their enzymes exhibit a natural tendency to work at high osmolality. Thermozymes refers to enzymes which are produced by thermophiles and hyperthermophiles. These enzymes are optimally active between 60-125°C. The temperature characteristic of these enzymes makes them attractive in various biotechnological applications. They have been used in molecular biology and one of the successful stories is attributed to DNA polymerase of \textit{Thermus aquaticus} and its wide use in polymerase chain reaction. Detergents and starch hydrolysis for the production of glucose and high fructose corn syrup using $\beta$-amyase and glucose isomerase respectively. Several of them have been proposed for the synthesis of organic chemicals in addition to diagnostic kits, waste treatment, pulp and paper-milling and in the preparation of animal feed\textsuperscript{31}. Thermostable enzymes have been shown to be resistant to various chemicals and can be functional in organic solvents in the absence of water, thereby leading to many desirable properties. Proteases of Archaea are known to hydrolyze highly resistant proteins like keratin for the production of amino acids. Alcohol dehydrogenase produced by \textit{Thermoanaerobium brockii} is currently used in biosensors for the detection of alcohol\textsuperscript{15} and in the synthesis of chiral compounds.\textsuperscript{53} The enzyme is active at high solvent concentration and 60°C. One of the great advantages of using enzymes in organic solvents is to reverse the hydrolytic reaction occurring in aqueous media. Enzymes which hydrolyze esters, peptides, and oligosaccharides in water can synthesize the same compounds in organic solvents. Biosensors prepared with thermophilic enzymes do show remarkable stability when exposed to various chemical reagents during immobilization procedure for biosensor and storage during transportation in tropical countries. Thermostable enzymes may be used as antibody conjugates in immunoassays. However the main constraint at present is their prohibitive cost of production and reluctance to use innovative methods.

Hydrogenases are predominantly found in hyperthermophilic fermentative archa. In view of the depleting energy reserves biophotolysis of water by solar energy has been investigated to liberate hydrogen. \textit{Methanothermobacterium thermoautotrophicum} produces hydrogenase\textsuperscript{55}. Immobilized enzyme processes based on these enzymes show an enormous operational stability making a bioprocess cost-effective. Higher yields of enzymes can be obtained with minimal loss of activity during purification\textsuperscript{55}. 


Use of Psychrophile Enzymes in Food Processing Industry

Psychrophilic microorganisms and their enzymes have a wide range of applications in dairy and food industry. Psychrophilic milk coagulating enzymes have the advantage of controlled casein coagulation for maintaining the quality of whey resulting from cheese industry which can be used in other processes. The enzyme activity in whey can be destroyed by pasteurization. The commercial microbial rennet available in the market in developed countries with the brand names Marzyme, Rennilase 50TL, and Modilase are products of cold active microorganisms. Another interesting application of cold active enzymes is in the form of β-galactosidase. Lactose hydrolysis in milk and whey to galactose and glucose results in increased solubility, digestibility, and sweetness of milk. Usually, β-galactosidase obtained from mesophilic strains of Kluyveromyces and Aspergillus strains are active at relatively higher temperatures, i.e., 30-40°C, and the milk has to be processed in conventional methods for at least 4 hours for complete hydrolysis of lactose. These conditions increase the chances of microbial contamination during the process. With the use of thermolabile β-galactosidases hydrolysis of lactose can be carried out at 5°C-10°C in about 16-24 h. Using the cold active β-galactosidase 70-80 per cent of product yields can be obtained, which is much higher in comparison to the processes obtained using enzyme from mesophilic microorganisms. A commercially available galactosidase is in use for the purpose. The commercial cold active neutral protease is mainly obtained from Bacillus subtilis and being marketed under the commercial name Neutrase. The enzyme is known to increase the flavour intensity with reduction in the ripening time from 4 to 1 mon.

Psychrophilic microorganisms are able to produce various enzymes of industrial importance. Neutral proteases from psychrophilic bacteria are being used in cheese maturation, Polymer degrading enzymes such as amylases, pullulanases, xylanases, and proteases are employed in food processing. Proteases with low optimum temperature and high pH are being marketed under the commercial names Savinase, Maxacal, and Opticlean.

Source of Natural Pigments

Carotenoids are present in various microorganisms and they play an important role in protecting the photosynthetic machinery of the organism from photo-oxidation. Several bacteria of antarctic origin can also produce pigments and mainly belong to the Flectobacillus⁶⁶, Pseudomonas⁶⁶, and Micrococcus⁶⁶. As there is growing tendency to use natural pigments, bacterial pigments of different hues and colours may prove to be handy and renewable source for food processing industry.

Hydrolysate of Biomasses As Feed Stock

The extracellular production of Laminaria sp decomposing enzymes were partly characterized in marine bacterial isolates belonging to the genera Alteromonas sp, Pseudomonas sp, Moraxella sp, and Flavobacterium⁶⁶. These enzymes were found to be highly active against several marine polysaccharides like alginate, fucoidan, and cellulose. The bacteria population largely consists of psychrophilic Vibrio sp whose optimum growth temperature ranges from 5°C-20°C. In the deep sea, bacteria and cyanobacteria that take part in the biodegradation of phytoplankton between 2°C-15°C and at a depth of > 4500 m⁶⁶⁶¹. The enzymes responsible for decomposition were identified as alginate and fucoidanase at 15°C. Hydrolytic activity is a common feature of marine bacterial populations as well as other microorganisms. In future, this may help in producing single cell protein and liquid fuel, after hydrolysis of enormous amounts of sea-weeds and aquatic plant biomass.

Several types of psychrophilic microalgae have been reported from antarctica and other cold habitats. Because of their and inexpensive growth requirements substrates comprising solar light and other inorganic compounds present in marine waters can be used for biochemical production such as vitamins, carotenoids, pigments polysaccharides, protein, and foods.

Lipids as Food Additives

Microbial lipids containing polyunsaturated fatty acids (PUFA’s) are recommended to increase nutritional value of food products and as additives in cosmetics and as starting substrates for the preparation of pharmaceuticals⁶³. Polyunsaturated fatty acids are commonly found in marine microorganisms⁶⁶. Probably these organisms produce PUFA’s in response to low temperature of marine habitats.

Lipids extracted from psychrophilic antarctica bacteria and marine algae mainly consist of C₁₅ and C₁₆ unsaturated fatty acids. The marine algae Anadyomene stellata can synthesis 16-22 carbon containing unsaturated fatty acids possessing as much as four conjugated
double bonds. The synthesis and modification of fatty acids mainly occurs in chloroplast and in the endoplasmic reticulum of these eukaryotic microorganisms. A group of psychrophilic sea-ice derived bacterial strains are known to produce polyunsaturated fatty acids such as eicosapentaenoic acid (20:5ω3) and arachidonic acid (20:4ω6). Bacteria belonging to the family Flavobacteriaceae are also known to synthesize a range of volatile fatty acid containing lipids in addition to algae.

**Single Cell Protein from Chitin**

Chitinase (EC 3.2.1.14) from Serratia marcescens QMB 1466 was attempted to hydrolyse large quantities of shell fish chitin into soluble monomers such as N-acetyl glucosamine and its conversion to singly cell protein. The process includes size reduction, deproteination, and demineralization. The hydrolysis of chitin may also be carried out using chitin from marine bacteria since chitinase producers are prevalent in marine waters.

**Use of Hydrolytic Enzymes in Pulp Industry**

Debleaching of paper mill pulp has extensively studied by many investigators. After cellulose xylan is the most abundant biopolymer in nature. The main sugar component of xylan is D-xylose. Hemicellulose consists of a series of heteropolysaccharides that include glucans, mannans, arabinans, and xylans. Microbial enzymes degrading hemicelluloses have great industrial applications. Cellulase free hemicellulases are useful in the pulp and paper industry for bleaching of kraft pulp. Xylanases are useful in the modification of pulp during paper making and for recycling of waste paper. With the discovery of more and more varieties of hemicellulases it would be possible to apply them commercially in the pulp industry. Alkaline xylanases are particularly preferred in pulp making process of paper industry. Increasing solubility of xylan at high pH helps in increasing the hydrolysis of xylan by these enzyme. Xylanases are largely produced by alkaliphilic of the genus Bacillus. Alkalophilic Bacillus strains optimally grow at high pH and their xylanases also display optimum activity at pH > 9.0. Bacillus subtilis xylanase hydrolyses up to 38.0 per cent of the substrate with low product inhibition constants in comparison to Trichoderma viride xylanase preparation. However, xylanase production by psycrophiles has not been attempted. Some of the alkalophilic Bacillus sp xylanase showed higher optimum pH and temperature (60°C). In spite of all these developments xylanases of thermo-phobic alkaliphilic are preferable to withstand high pulp temperature of 60°C and pH > 9.0. Zakaria et al. have reported the synthesis of β-mannanase in a psychrophilic strain of Flavobacterium. The enzyme synthesis occurs in the presence of 1 per cent guar gum and it was able to hydrolyse efficiently the same substrate optimally at 35°C. At 10°C the enzyme displayed 25 percent of its optimal activity.

Microorganisms producing acetyl esterases which are functionally and physiologically similar to lipases in combination with α-glucuronidases are required in the pulp making process to obtain maximum xylan hydrolysis. Bio-pulping/ enzymatic pulping for xylan hydrolysis and cellulose derivatization also requires the cooperative activity of esterases in the enzymatic degradation of acetyl xylans.

**Use of Enzymes in Biodetergents**

The concept of utilizing enzymes started in 1913 by Otto Rohn has developed now into a major commercial exploitation of proteases, lipases, amylases, and cellulases as detergent additives. Remarkably, most of these enzymes are being produced from microbial sources including bacteria, yeast, and fungus or genetically engineered microorganisms. These enzymes are marketed at present throughout world under several brand names. These enzymes are optimally active between pH 9-11.0. Genetic engineering is widely used to increase enzyme yields from wild microbial strains which are uneconomical for large-scale production. Subtilisin type of proteases and α- amylases have been found to be suitable for detergent industry. Alpha amylases are useful not only as additives of laundry detergents but they are incorporated into powder and liquid formulations for industrial and machine dish-washing.

Protease, lipase, amylase, and cellulases have been widely reported in psychrophilic microorganisms and they may prove very useful for low temperature washing. The principle of the so-called “greener” and milder chemicals detergents is based on enzymes displaying high activity at low temperatures. Cold washing temperatures are preferred in several countries. Amylases and other enzymes which are stable in the presence of protease prove to be preferable for kitchen ware washing. With the discovery of novel enzymes in psychrophilic, in future, it will be possible to formulate detergents for various purposes. Psychrophilic alkaline proteases reported in the antarctic Pseudomonas sp and Aeromonas hydrophila strain can be utilized as detergent additive. Enzymes with maximum activity at
alkaline pH and stable at moderately higher temperatures are highly useful as detergent additives\(^2\).

**Psychrophiles As A Source of Pharmaceuticals**

Discovery of secondary metabolites from marine microorganisms has been drawing much attention for the last three decades. As a consequence, several strains of bacteria, streptomycetes, and fungus have been reported to produce antifungal, anticancer, and anti-tumor agents. It is evident that most of these microorganisms belong to the category of psychrophiles as they have been isolated from deep-sea sediments, marine waters and gut flora of aquatic animals and plants\(^2\). The bacterial genera that produce these agents are: *Streptomyces, Alteromonas, Bacillus, Micrococcus, Aeromonas, Flavobacterium, Moraxella, Pseudomonas*, and *Vibrio* with growth temperature between -3 to 30°C. Aquatic plants and animals are highly prone to infection by pathogenic microorganisms. Surface bacterial symbiosis is an essential adaptive and protective mechanism in fresh water and marine sediments. An *Alteromonas* sp. has been reported to synthesize 2,3-indolinedione (isatin). Experiments have amply demonstrated that this compound protects the shrimp *Palaeomon macrodactylus* from a pathogenic fungus *Legenidium callinectes*. Similarly, another strain of *Alteromonas* sp. intimately associated with the marine sponge *Halichondria okada*, produces a tetracyclic alkaloid namely alterimide A\(^2\). There is a wide scope for the discovery of novel biologically active compounds in marine microorganisms. By learning their structural intricacies the development of new pharmaceutical products might be possible in future. Studies have also indicated that marine strains of *Moraxella* sp and *Flavobacterium* sp can produce antiviral and anti-tumor agents, respectively\(^2\). The anti-tumor polysaccharide has been identified as narcaim. Pathirana et al.\(^2\) isolated L-arabinose class of esters from marine actinomycetes. Unfortunately, biologically active compounds from marine microorganisms have not been exploited. Jensen and Fenical have adequately emphasized on the potential of marine microorganisms\(^2\). Similarly, antarctic bacterial isolates may yield several of these metabolites, since most of the genera also occur predominantly in antarctic habitat. To our knowledge, so far no attempt has been made to screen antarctica these bacteria for the production of biologically active compounds.

Shaver and Schiff et al.\(^2\) have demonstrated the usefulness of protease and amylase mixture from *Bacillus subtilis* in removing the dental plaque. Lipases are mainly used as stereo-specific catalysts and in the biotransformations of various high value compounds such as flavouring agents and pharmaceuticals\(^2\). Concentrates of polyunsaturated fatty acids of the type 3-3 are obtained by the hydrolysis of fish oils\(^2\). Fatty acids such as gadoleic acid, erucic, and nervonic acids are obtained by the hydrolysis of seed oils\(^2\). Lipolytic enzymes are widely employed for the extraction of these high value nutropharmaceuticals. Lipases have been studied mainly from antarctic *Moraxella*\(^9,17\), *Pseudomonas* and *Bacillus* sp and *Serratia liquefaciens* isolated from blue green algal mats\(^8\). Lipases from psychrophilic bacteria are preferred because of their unique positional specificity for fatty acids not found in mesophilic enzymes. The enzyme from a psychrotrophic *Pseudomonas* strain was found to exhibit 1,3- positional specificity towards triglyceride substrates like triolein. Owing to their low activation energies, i.e. 11.2 and 7.7 kcal/mol, cold-active lipases are catalytically more efficient at low temperatures in comparison to mesophilic enzymes. The enzyme was found to be activated by organic solvents like methanol and dimethyl sulfoxide from 0-30 per cent (vol/vol). Enzymes which show stability in organic solvents are biotechnologically important to carry out enzymatic reactions in organic solvents. This aspect of biotransformation is gaining great importance to increase reaction rate and to extend the substrate specificity of enzymes. Use of organic solvents as a medium of reaction aids in solubilising the compounds which are insoluble in water. In the biosynthesis of water insoluble compounds such enzymes are quite useful. Bacteria able to grow in the presence of high concentrations of organic solvents have been reported from deep-sea sediments with an optimum growth temperature of 10°C. They have been identified as genera *Flavobacterium, Bacillus* sp, and *Arthrobacter* and can withstand high concentrations of benzene, toluene, and p-xylene\(^8\). Recently, it has been found that immobilized enzymes in organic solvents are efficient in terms of their velocity to catalyse biochemical transformation to obtain biologically active compounds.

Trehalose (\(\alpha-D\)-glucopyranosyl \(\alpha-D\)-glucopyranoside) is a non-reducing disaccharide widespread among bacteria. The sugar is economically important because of its function as cryoprotectant to stabilize proteins and protection of animal tissues. The sugar finds various applications such as sweetener, stabilizer of frozen foods, in cosmetics and as a drug additive. Trehalose is formed by an enzyme trehalase present in
several psychrophilic and thermophilic bacteria. Psychrophilic microorganisms synthesize the sugar to protect the cell from desiccation, heat, and osmotic shock.

**Uses of Bacterial Ice Nucleating Agents**

There are several uses for ice-nucleating agents (INA) produced by bacteria. They are being used in artificial snow making, in the production of ice creams and other frozen foods. In immunodiagnostic kits as a conjugate to antibodies and as a substitute for silver iodide in cloud seeding. Among several organisms, INA from bacteria have attracted much prominence owing to their ability to form ice nuclei at relatively high temperatures in comparison to other sources. The subject has been reviewed in detail by Lindow et al.\(^\text{86,87}\). *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Pseudomonas viridiflava*, Erwinia herbicola, and Xanthomonas campestris are the well known producers of INA. Further, some marine strains of *Pseudomonas fluorescens* have been shown to produce INAs. Most of the bacterial strains used for producing INA are psychrophilic in nature. Several properties of INA resemble psychrophilic enzymes. Particularly, INAs are thermolabile molecules. For many practical uses, ice nucleating agents from bacterial sources are preferable, since microorganisms can be improved by recombinant DNA technology and they can be mass produced in bioreactors. The unique property of bacterial ice-nucleating agents is that they are least soluble even at high temperatures in comparison to plant and insect agents.\(^\text{88}\) An optimum growth temperature of 20°C is required for the synthesis of INA in bacteria such as *P.syringae*. The ice nucleating temperature as well as the synthesis of INA is highly influenced by bacterial growth temperature. INA produced at 20°C preserve the property of ice nucleating activity at higher temperature. In the case *P.fluorescens*\(^\text{89}\). *Pseudomonas syringae* is another widely occurring bacterial epiphyte. The INA is produced in the form of an outer membrane protein. Large aggregates of INA can orient water molecules into crystal lattice mimicking the natural ice particles and leading to a cascade of ice crystal formation.\(^\text{90}\).

**Use in Fermentation Industry**

Mesophilic yeasts which contain unsaturated fatty acids in membranous lipids have been found to be resistant between −80 to −20 °C, normally preferred for their storage in baking and other food processing industries.\(^\text{88}\) For wine production, fermentation at 6-8°C has been found to reduce the inhibitory effect of ethanol on cell membrane of yeast cells.\(^\text{91}\). Specifically, cryotolerant yeast strains have been selectively obtained. Sparkling wine is produced using the yeast strains which are tolerant to high alcohol concentrations.

**Application in Microbial Leaching**

Current microbial leaching operations involve oxidative solubilization of copper and uranium ores. Leaching operations in temperate countries have to be carried out at very low ambient temperature. The temperature dependent variations of microbial activity has been noticed to affect the ore leaching operations by Ahonen and Tuovinen\(^\text{92}\). Microbial leaching operation from sulphide ores was studied at low temperature between 4°-37°C. Probably the process involves psychrophilic microorganisms and improvements in this area would be possible by employing pure cultures of the bacteria of antarctica origin or from other cold habitats.

**Use in Bioremediation**

In addition to their ability to biodegrade various compounds in the habitat they can be promising in bioremediation of several pollutants at low-temperatures. The occurrence of biodegradative psychrophiles is relatively more in contaminated sites where the ambient temperature overlaps with the optimum growth temperature of these microorganisms. Psychrotrophic hydrocarbon degrading bacteria have been isolated from oil polluted sites of Ontario and Quebec and it has been noticed that the occurrence of hydrocarbon degrading microorganisms is low from relatively pristine environmental habitats.\(^\text{93}\). The bacterial strains were found to mineralize dodecane, hexadecane, naphthalene, and toluene at low temperatures and it has been evident in laboratory and field experiments, utilizing specific bacterial cultures. Some of the degradative genes responsible for catabolism of naphthalene (nadB) and toluene (xyLE, todC1) have also been detected in psychrotrophic bacteria using molecular techniques such as southern hybridization and polymerase chain reaction.\(^\text{94}\). Psychrophilic bacteria that can degrade high molecular weight hydrocarbons at low temperature were described earlier.\(^\text{95}\).

A psychrotrophic bacterium, *Rhodococcus* sp. strain Q15 has been studied for its ability to degrade n-alkanes and diesel fuel at low temperature. The strain mineralize short chain alkanes like dodecane and hexadecane at higher rates than the long chain hydrocarbons such as octacosane and dotriacontane (0°-5°C). Also the strain has been able to mineralize at both branched alkanes and substituted cyclohexanes present in diesel oil (5°C). In
addition, mineralization of hexadecane at 5°C was noticed in hydrocarbon contaminated and pristine soil microcosms using the organism *Rhodococcus*<sup>9</sup>. The psychrotrophic bacterium *Rhodococcus* sp. strain Q15 that can degrade n-alkanes and diesel fuel at 0°-5°C was shown to contain the gene responsible for the production of aromatic aldehyde dehydrogenase with DNA sequence resembling the mesophilic *R. erythropolis* theA gene.

**Anaerobic Digestion of Organic Wastes**

The obligate anaerobes which convert organic acids to CH<sub>4</sub> and CO<sub>2</sub> (methanogens) are highly sensitive to low temperature and pH levels. Psychrophilic anaerobic bacteria are not extensively investigated in comparison to aerobic bacteria, owing to their very low growth rates and cumbersome isolation procedures. However, there are some reports that describe the isolation of psychrophilic anaerobic bacteria from antarctica habitat. Variety of organic wastes can be degraded to innocuous products by anaerobic degradation. It is one of the potential routes for methane generation in cold habitats. Further, anaerobic digestion has assumed much importance in the treatment of various toxic chemicals and high strength industrial wastes. Isolation of psychrophilic anaerobic microorganisms is of immense importance to supplement the anaerobic digestion process to be carried out at low-temperatures. At present, anaerobic digestion of sewage sludge, cow dung, and other animal wastes is treated by adapted mixed microbial consortia<sup>97</sup>. Nevertheless, microbial growth and methane production is very limited at sub-ambient temperatures (35°C). The rate of methanogenesis can be increased several fold by low temperatures adaptation of methanogens. The process can be made possible by selective enrichment of psychrophilic methanogens through long term laboratory trials. Hence the basic understanding of these microorganisms is essential for improving the performance of the methanogenic processes. Besides methanogens, bacteria with the ability to reduce sulphate have been isolated and identified as *Desulfotalea vacuolata*<sup>98</sup>. Few reports have revealed the importance and the role of methanogens in antarctic habitat. In one of these studies, a psychrophilic strain of *Methanogenium frigida* sp nov., was isolated from Ace lake, antarctica which grows optimally at 15°C. The organism was found to produce methane from hydrogen and carbon dioxide. The growth rate of psychrophilic methanogens is very low (0.24 d<sup>-1</sup>) with a <i>l</i> of 2.9d<sup>0</sup>. Similarly, an acetoclastic psychrophilic strain of *Methanococcales burtoni* from Ace lake, antarctica, has been reported<sup>99</sup>.

**Denitrification of Drinking Water Sources**

The presence of high nitrate concentrations in water has been a major problem in many countries including the US, Canada, and Europe. Drinking water containing NO<sub>3</sub>- quantities exceeding 10-50 mg/l are harmful. The most widely used practice for the removal of NO<sub>3</sub>- is biological denitrification. Most of the denitrification processes are carried out at about 10°C. Hollo and Czako<sup>100</sup>, evaluated microbial denitrification process at 12°C and the maximum specific nitrate removal rate observed was 220 mg NO<sub>3</sub>-/h/g cells. The nitrate removal capacity of the reactor at 10°C was 50 to 60 kg NO<sub>3</sub>-/m<sup>3</sup>/d. To increase the treatment efficiency at these temperatures, immobilized microbial cell based bioreactors such as packed bed, and fluidized bed are being used. Also, a full scale denitrification unit using the same process has been operated between 12° and 18°C. Temperature decrease of few degrees may reduce the process efficiency substantially which emphasises the need to use cold active denitrifying bacteria. The denitrification efficiency has been found to reduce by about 33 per cent while the maximum denitrification rate at 18°C recorded was 2.24 kg NO<sub>3</sub>-/m<sup>3</sup>/d<sup>0</sup>. However, report does not conform to the presence of psychrophilic nature of these microorganisms. However, it appears that microbial population consists of cold-adapted mesophilic bacteria functioning at lower temperature probably with lower denitrification rates. The rate of denitrification in these cases can be enhanced by employing psychrophilic bacteria isolated from permanently cold habitats.

**Biological Amendment of Psychrophiles and Genetic Engineering**

With the advent of polymerase chain reaction, various genes responsible for biodegradation and metabolism have been identified and characterized from psychrophiles. Recombinant DNA techniques have been proved to be of great utility in the elucidation of properties of crenarchaeota which cannot be cultivated under laboratory conditions. Two subtilisins produced by a marine isolate of *Bacillus TA39* and TA41 strain have been purified and the genes responsible for their synthesis are identified as Sub1 and sub2. The enzyme displayed a high catalytic efficiency at low and moderate temperatures. However, the enzyme was highly ther-
mosenstive as revealed by its flexibility and 3-D protein conformation analysis. The gene responsible for α-amylase production in a psychrophilic strain of Alteromonas haloplankis has been cloned in mesophilic E.coli to probe its protein folding mechanism and effect host cell physiology on enzyme activity. Studies have shown that the enzyme expressed was similar in every aspect. The heterologous expression system provides a means for large-scale production of cold active enzymes in a cost-effective manner. The effect of intramolecular interactions that increase the rigidity of the protein backbone have been inserted by site-directed mutagenesis to assess the impact of flexibility on thermosensitivity of the enzyme. The rigidity has been increased by creating additional salt-bridges, aromatic interactions and by increasing the affinity for calcium ions.

Extremozymes of psychrophiles can be mass-produced without growing the actual culture. Gene cloning of psychophilic enzymes may enable them to produce in pure form by modern methods of protein expression in heterologous hosts such as E.coli using simple media components. Most often it is difficult to find an enzyme with properties matching with the reaction conditions. Therefore, to modify the enzyme for a specific task bio-amendment or rational design has been carried out. As a result, xerozymes or enzymes foreign to the nature have been created using chemical and protein engineering techniques.

Mesophilic bacteria can express enzymes of psychrophilic bacteria without drastic change in their catalytic properties. It has been shown that cloning of lipase genes into mesophilic E.coli preserved its low temperature activity and thermolabile property of cold active bacteria. Heterologous expression of genes responsible for cold active enzymes in mesophiles, therefore, offers a convenient means of economical production of cold enzymes in large-scale for commercial applications. On the same ground, over expression of a psychrophilic α-amylase gene was demonstrated in a mesophilic strain of E.coli. The structural investigations revealed that the 3-D conformations and activity of the enzyme were similar with the wild type enzyme. For the production of enzymes heterologous expression systems such as in E.coli, an optimum temperature has to be determined in order to obtain maximum growth of the organism and also to maintain stability of the expressed enzyme. Thus, isolation of novel psychrophilic microorganisms would widen the gene diversity required for obtaining useful chemicals through recombinant DNA technology. Methods like site-directed mutagenesis and protein engineering, have been followed to increase the catalytic efficiency of enzymes at extremes of temperature, pH, and in organic solvents. Crenarchaeum symbiosum an uncultivated psychrophilic microorganism belonging to the kingdom Crenarchaeota has been reported by Schleper et al. Culturing of this microorganism under laboratory conditions has not been possible and its phenotypic characterization was carried out solely using molecular techniques and also depending on its distribution in cold and temperate habitats. After isolation and amplification of DNA and RNA, the genetic material has been expressed in mesophilic hosts for elucidating properties of their gene products. The investigations carried out under similar lines, revealed the presence of a DNA polymerase in C. symbiosum and it was found to be thermostable with rapid loss of activity at 40°C.

Protein engineering has been most useful in understanding the complex relationship between protein structure and function at low temperature. The technique has offered an effective means to understand and predict the contribution of covalent and ionic interactions for protein stability, flexibility, and enzyme activity.

Aspartate carbamoyl transferase has been studied relatively more with respect to its molecular structure and enzymology, since this is one of the multimeric, allosteric enzyme present in psychrophile, as well as psychrotrophic bacteria. Attempts have been made to unravel the changes that could occur in enzyme activity and controlling mechanism after its cloning in heterologous expression systems. The genes responsible for ATCase in a Vibrio strain 2693 have been cloned in mesophilic E.coli strain.

Conclusion

The application of extremophiles, in general, and psychrophiles, in particular, in industrial processes opens up a new era in biotechnology. Each group has unique biochemical features which can be exploited for use in biotechnological industries. The main reason for selecting enzymes from this group of microorganisms is their high stability and reduced risk of contamination at low temperatures. Due to their unusual properties these enzymes are expected to fill the gap between biological and chemical processes. However the biotechnology of extremophiles is still in its infancy but has important and far-reaching implications.
References

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