

Cold Active Enzymes from the Marine Psychrophiles: Biotechnological Perspective

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Abstract

Psychrophiles inhabit more than 70% of Earth's surface which includes Ocean depths, polar and alpine regions. Oceans have a constant temperature of 4–5°C below a depth of 1,000 m irrespective of the latitude. Psychrophiles and oceans are one of the underutilizing resources which have great economical and environmental potential. As the organisms should withstand to strong negative effect of low temperatures they adapted modifications in their membrane components and produces anti-freeze proteins which protects from forming ice inside the cell. The enzymes from psychrophiles are cold active due to their unique structural, kinetic and activation parameters which differ from mesophiles and thermophiles. By having the properties like high specific activity at low temperatures and thermolabile nature they are having potential applications in various fields of biotechnology.

Keywords: Psychrophile; Psychrotroph; Antifreeze proteins; Cold-active enzymes; Extremophiles

Introduction

Some microorganisms are capable of growing in unusual environmental conditions, including the high temperatures of volcanic hot springs, the low temperatures of polar regions, high pressures in deep seas, very high salt concentrations, or very high and low pH values (Fujiwara, 2002). These microorganisms can be divided into three groups: psychrophiles, mesophiles, and thermophiles, depending on their optimal growth temperatures. With the exception of mesophiles, which grow at mild temperature ranges, from 20°C to 45°C, the other two types of microorganisms, thermophiles and psychrophiles, are classified as extremophiles (Cavicchioli, 2002). Psychrophiles are one of the most underutilized resources and these psychrophilic and psychrotrophic microorganisms occupy large space in global ecology as large proportion of our planet is cold and most ecosystems are exposed to temperatures that are below 5°C. Such low mean temperatures are mainly due to the fact that ~70% of the Earth's surface is covered by oceans that have a constant temperature of 4–5°C below a depth of 1,000 meters. In order to maintain metabolic rates even at low temperatures, psychrophiles contains enzymes that have a high specific activity at low temperatures. These enzymes are generally termed as cold active enzymes and are thermo labile. Due to their high specific activity and their rapid inactivation at higher temperatures, along with their producers they offer a great potential as biocatalysts in biotechnology and in food processing. From the past decade it has been found that cold adapted microorganisms and their enzymes provided a large biotechnological potential, offering numerous economical and ecological advantages over the use of organisms and their enzymes which operate at higher temperatures (Ohgiya *et al.*, 1999 and Marchi *et al.*, 2007). A wide range of cold adapted bacteria producing cold active enzymes have been described. However, most of the cold adapted enzymes that so far been

characterized originated from the Antarctic terrestrial and the Antarctic sea water (Gerday *et al.*, 1997 and Russell, 1998). Specific activity of cold enzymes from wild strains and some of their recombinant forms have been determined in organisms of Antarctic and arctic regions including alcohol dehydrogenase (Feller, 1994 (a,b)), α -amylase (Vazquez *et al.*, 1995), Aspartate transcarbamylase (Feller, 1992), Ca+Zn+2 protease, (Villeret, 1997) citratesynthetase, (Gerike *et al.*, 1997) β -lactamase (Feller, 1997), malate dehydrogenase, subtilisin (Narinx, 1997), triose phosphate isomerase (Alvarez, 1998), and xylanase (Reddy, 2003). Typically cold enzymes show higher specific activity than that of their mesophilic enzymes. By considering all unique properties of these enzymes people realize that there is a huge Biotechnological potential of enzymes from psychrophiles. So there is urgent need to exploit them for stimulating further advances in this field. In this review we focused on psychrophilic enzymes and their cold adaptations at structural and molecular level activity-parameters and their applications in various Industries.

Microorganisms that have colonized the cold environments are referred to as psychrophiles or cold adapted. They are found in deep sea to mountain and Polar Regions as well (Morita, 1975). In the strictest definition, psychrophiles do not grow above 20°C. Organisms growing well at low temperatures and also grow above 20°C are referred as psychrotolerant or psychrotrophs (Russell, 1992;1997). Thus, to avoid the difficulty of dealing with the varying maximum growth temperature, Neidhardt *et al.* (1990), defined psychrophiles as organisms that grow at ~5°C or below up to 20°C to distinguish them from mesophiles which grow best at 37°C. Based on their surroundings Psychrophiles are further divided in to Piezo-psychrophiles, lives in the ocean depths and sediments and they usually face extremely high pressures, Halo-psychrophiles which inhabit in high salt concentrations (Yayanos, 1999)

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and Troglo-psychrophiles which are evolved in poor environment in the absence of light.

More than 100 species of psychrophiles have been identified and reported which comprises of both Gram-negative and Gram-positive bacteria from various habitats ranging from soil, sandstone, fresh water and marine lakes, sea ice and oceans. Various species within the genera *Alcaligenes*, *Alteromonas*, *Aquaspirillum*, *Arthobacter*, *Bacillus*, *Bacteroides*, *Brevibacterium*, *Gelidibacter*, *Methanococcoides*, *Methanogenium*, *Methanosarcina*, *Microbacterium*, *Micrococcus*, *Moritella*, *Octandecabacter*, *Phormidium*, *Photobacterium*, *Polaribacter*, *Polaromonas*, *Psychroserpens*, *Shewanella* and *Vibrio* have been reported to be psychrophilic (Morita and Moyer, 2001). The psychrophilic and barophilic bacteria belong to α -Proteobacteria which includes *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella* and *Alteromonas haloplanktis*. For the first time, *Leifsonia aurea*, *Sporosarcina macmurdoensis* and *Kocuria polaris* (Reddy, 2003a; 2003b) have been reported from Antarctica. A psychrophilic and slightly halophilic methanogen, *Methanococcoides burtonii* was isolated from perennially cold, anoxic *hypolimnion* of Ace Lake, Antarctica. Archaea such as *Methanogenium*, *Methanococcoides* and *Halorubrum* species, yeast like *Candida* and *Cryptococcus* species, fungi such as *Penicillium* and *Cladosporium* and microalgae (*Chloromonas*) can be found in these environments and display striking cold-adaptive characteristics (Morita, 1975; Russell, 1990; Gounot, 1991; Allen, 2001; Deming *et al.*, 2002; Margesin, 2002). As the organisms should withstand to strong negative effect of low temperatures, these grow and move at rates similar to those achieved by closely related species living in temperate environments. Therefore they have developed various adaptations in the form of finely tuned structural changes in their membranes, constitutive proteins and enzymes, enabling them to compensate for the deleterious effects of low temperatures.

By incorporating unique features in their membranes, psychrophiles have developed various adaptive mechanisms to perform their metabolic functions even at low temperatures. The Psychrophilic membranes contain a higher proportion of unsaturated fatty acids, due to which their fluidity and ability to transport nutrients are maintained even under very cold conditions. Low temperature have an adverse effect on physical properties and functions of membrane which reduces the membrane fluidity, the onset of a gel-phase transition and, ultimately leads to loss of function. The lipid composition alters the physical properties of membranes and hence it is not surprising that this varies with mesophiles and thermophiles. In general, growth at lower temperatures produce a higher content of unsaturated, polyunsaturated and methyl-branched fatty acids, and/or a shorter acyl-chain length with a high proportion of *cis*-unsaturated double-bonds and *anteiso*-branched fatty acids (Chintalapati *et al.*, 2004). Change in the packing order or reducing the number of interactions in the membrane are due to altered composition which in turn has a key role in increasing membrane fluidity. The other adaptations that have been suggested to increase membrane fluidity include an increased content of large lipid head groups, proteins and non-polar carotenoid pigments (Chintalapati *et al.*, 2004). However, the different adaptations mentioned above do not seem to be widespread, and studies show more compact lipid head groups (Arthur, 1976) and decreased non-polar carotenoid pigment synthesis in some Psychrophiles.

Anti-freeze proteins (AFPs) are the proteins that have the ability to bind to ice crystals and there by create thermal hysteresis and are able to adjust the temperature at which an organism can grow (Jia and Davies, 2002). AFPs have been recently demonstrated in Antarctic lake bacteria, one of

which is from *Marinomonas primoryensis* (Gilbert *et al.*, 2005). *Pseudomonas putida* GR12-2 has trehalose and exopolysaccharides (EPSs) thus it shows both antifreeze and ice-nucleating activities (Phadtare, 2004). Trehalose having a colligative property that helps in preventing protein denaturation and aggregation (Phadtare, 2004). Antarctic marine bacteria and Arctic winter sea ice bacteria synthesize high concentrations of EPSs (Phadtare, 2004). Major functions include modifying the physico-chemical environment of bacterial cells, participate in cell adhesion to surfaces and retention of water, favour the sequestration and concentration of nutrients, retain and protect extracellular enzymes against cold degradation and also act as cryoprotectants (Georlette *et al.*, 2004).

Psychrophiles are able to continue their activities at low temperatures as they can synthesize cold-shock or AFPs and also the more efficient enzyme activity is due to alterations in enzyme kinetics (Georlette *et al.*, 2004). The following general rate equation describes the effect of temperature on enzyme activity:

$$K = k_B \times T e^{-G\#/R H}$$

where K is the rate of reaction, k_B is the transmission coefficient and is generally close to 1, k_B is the Boltzmann constant, and h is the Planck's constant, $G\#$ is the free energy of activation, R is the gas constant and T is the absolute temperature. In the above formula temperature is included in the exponent; hence any decrease in temperature results in an exponential decrease in the reaction rate, the magnitude of which is a function of $G\#$ which is the energy barrier that must be overcome by the ground-state enzyme-substrate complex to react.

The thermodynamic parameters of reactions that are catalyzed by psychrophilic enzymes show that they are characterized by lower free energy of activation ($G\#$) values than reactions that are catalyzed by mesophilic enzymes. The high-reaction rate of several cold-adapted enzymes (up to 10-fold higher than the heat-stable homolog) is due to a decrease in the activation of free-energy barrier between the ground state (substrate) and the transition state (Siddiqui and Cavicchioli, 2006). Psychrophilic enzymes are low temperature dependent than that of mesophilic enzymes hence they proceed with a low enthalpy change, H . The reduction in entropy ($T \Delta S$) contributed by retaining stability in some parts of the protein (Lonhienne *et al.*, 2000). The significance of these parameters and their implications for cold adaptation has been extensively discussed earlier (Fields and Somero, 1998; Lonhienne, 2000; Feller and Gerday, 2003(a,b,c); Nichols, 2005). The primary kinetic adaptation in cold active enzymes is achieved structurally by a requirement for the disruption of fewer enthalpy-driven interactions during the activation process (Russell, 1997; Lonhienne, 2000) This has been related to the large changes in conformation at the active site which follows that the active site of psychrophilic enzymes less stable than that of the mesophilic enzymes, and that it is heat labile (Fields and Somero, 1998; Lonhienne, 2000). Hence the psychrophilic enzymes have higher K_m values than their mesophilic counterparts (Xu, 2003). Apart from active site other regions of the enzyme might or might not be characterized by low stability when not involved in catalysis. The inactivation rate constants of psychrophiles are several times faster than their mesophilic or thermophilic homologues (Amico, 2003; Collins *et al.*, 2003).

Molecular basis of cold-active enzymes from psychrophiles received concerted interest in the recent times (Russell, 2000). Many features have been proposed to explain the molecular basis of cold adaptation

which include, reduction of salt bridges in intra-domain and inter-domain positions, increased hydrophobicity in core regions lengthy loops carrying more charge and less proline residues, increase in solvent-exposed hydrophobic residues (Russell, 1998), poorer Vander-Waals packing interactions to the molecule (Schroder, 2000), increase in flexibility in small areas of the molecule which affects the mobility of adjacent active-site structures (Fields and Somero, 1998), and so on. Hence, it can be accepted that more flexible structure is the main structural adaptation of the organisms producing cold-active enzymes along with kinetic parameters. Psychrophilic proteins show decreased ionic interactions and hydrogen bonds, possess less hydrophobic groups and more charged groups on their surface and longer surface loops compared to proteins from mesophiles (Deming, 2002; Margesin, 2002; Feller and Gerday, 2003(a,b,c); Duilio *et al.*, 2004; Georlette *et al.*, 2005). Due to decreased levels of prolyl and arginyl residues and increased levels of glycylyl residues psychrophilic proteins lose their rigidity and gain increased structural flexibility for enhanced catalytic function at low temperatures. Crystallographic structural analyses of psychrophilic proteins indicate that these do not have unusual conformations but share a high similarity with their meso and thermophilic homologues. Common trends include the reduction of the number of ion pairs, hydrogen bonds and hydrophobic interactions; decreased inter subunit interactions; increased interaction with the solvent; a reduced apolar fraction in the core; higher accessibility to the active site; increased exposure of apolar residues to the solvent; decreased cofactor binding; clustering of glycine residues; and a lower proline and arginine content (Violot, 2003).

In view of the diverse capabilities of these enzymes like lower stability and unusual specificity they offer great industrial and biotechnological potential (Georlette *et al.*, 2004). These properties of cold-adapted enzymes make them potentially valuable alternatives to their mesophilic counterparts. Apart from high specific activity at low temperatures which is obvious, the interest given relatively high to thermo sensitivity nature which provides the possibility of rapidly inactivating these enzymes by mild heat treatment in complex mixtures. In this context they are useful in preserving product quality of those industrial processes in which the contact of the enzyme with the substrates to be transformed into products should be recovered limited in time so as to prevent excessive or deleterious action. Examples include meat tenderizing with proteases and stonewashing in the textile industry, in which the excessive action of cellulases could lead to the loss of mechanical resistance of the cotton fibers. Accordingly, large-scale production of cold active enzymes by psychrophilic bacteria should be feasible only at low temperature. However, psychrophilic gene expression in a mesophilic host could represent an interesting alternative because the properties of the wild enzyme are conserved (Feller, 1991).

This industry should be mentioned first, as the market for enzymes such as proteases, lipases, amylases, and cellulases, which are commonly used as additives in detergents, represents about 40% of the total sale of enzymes. Cold-active detergents are commonly advertised because washing is now frequently carried out at environmental temperatures. Cold active proteases, lipases, amylases, and cellulases have advantages like reduction in energy consumption and a reduction in wear and tear. Cold adapted enzymes offer therefore a high potential in this type of application but production costs, their instability when added to their final product and their storage finds a major draw back of using these enzymes. But this can be overcome by using recombinant enzymes and it seems possible to improve the stability of cold adapted enzymes (Narinx, 1997).

Cold adapted cellulose is used for biopolishing and stone washing processes. In fabric production, tissue often has cotton fiber ends

protruding from the main fibers, which reduces smoothness and alter the appearance of the garment. These properties are worsened by successive washes. Pretreatment with cold active cellulases, under the appropriate conditions, by excising protruding ends, reduces the pill formation and increase the durability and softness of the cotton fibre. Cold adapted cellulase enables in decreasing the temperature of the processes and the concentration of the enzyme required.

Numerous Cold adapted enzymes are widely used in the food industry. In milk industry -galactosidase is used to reduce or removal of lactose at low temperature which is a potential alternative for lactose removal and also to enhance digestibility and sweetness. Their high specific activities at low temperature are their main advantage and they can be used during transport and storage of milk at low temperatures. And in fruit juice industry pectinase helps the juice extraction process which in turn reduces the viscosity and helps to clarify the final product and also increases the taste and yield. Where as in baking, enzymes such as amylase, proteases and Xylanases can be used to reduce the dough formation, fermentation time and also to improve the properties of the dough and also help in the retention of the aromas and moisture levels. Xylanases are directly acts on starch, gluten and hemicellulases. Proteases are used for tenderization of meat or taste improvement of refrigerated meat (Orange, 1994). Other cold enzymes can also be good alternative to mesophilic enzymes in brewing and wine industries and in cheese manufacturing. Cold-active lipases could be useful for the development of various tastes and flavor.

The idea of using microorganisms to reduce environmental pollution is not new more over a feasible alternative to physico chemical methods (Timmis, 1999). The regions where large seasonal variation occurs in temperature will reduce the effectiveness of microorganisms in degrading organic pollutants such as oils and lipids. However, bioaugmentation and inoculation of contaminated environments with cold adapted microorganisms in mixed cultures should improve the biodegradation of recalcitrant chemicals.

Bioremediation processes utilizing these organisms for the degradation of soils and waters contaminated by hydrocarbons, heavy metals, and other xenobiotic compounds have already been investigated (Margesin, 1999). Due to their unique specificity at low and moderate temperatures and catalytic efficiency, cold adapted microorganisms are ideal for bioremediation purposes and would enable low energy treatment (Margesin, 1998; Margesin, 1999; Timmis, 1999). The treatment of waste water, contaminated as a result of human activities would probably be the easiest way to start studying the potential applications of cold adapted microorganisms in biotransformation of chemicals (Vazquez *et al.*, 1995) and lowering the amount of toxic compounds like nitrates, hydrocarbons, aromatic compounds, heavy metals and biopolymers such as cellulose, chitin, lignin, proteins and triglycerols. The use of bacterial pectinases, which selectively remove pectic substances from the wastewater and thus facilitate decomposition by activated sludge treatment, represents a cost-effective and environmentally friendly alternative treatment method. An extracellular alkaline phospholipid from *Bacillus* sp. was used effectively to remove pectic substances from industrial wastewater (Tanabe, 1987).

In the Industrial and commercial production of bio chemicals like fatty acid esters, synthesis of various valuable peptides, and oligosaccharide derivatives by enzymatic methods they often use the substrates which are less soluble in water and in this context the catalytic activity is inhibited by the hydration state of the substrate which is solved by taking advantage of the high flexibility of these enzymes which will operate at low water conditions. This process can also be extended to the pharmaceutical and chemical industries for the production of compounds with high added value (Timmis, 1999).

It is worth mentioning that the first cold-adapted enzyme from Antarctic microorganisms to have been fully characterized is an alkaline phosphatase catalyzing the hydrolysis of the 5'-phosphate of oligonucleotides. The cold-adapted phosphatase offers the unique advantage, contrary to its mesophilic counterparts, of being rapidly inactivated by mild heat treatment prior to the use of the kinase, thereby allowing higher yields and preserving product integrity (Kobori, 1984). The coupling of oligonucleotides using cold-adapted DNA ligases also seems promising as the ligation yield is much higher at low temperatures. Cold-adapted DNA ligases (Georlette *et al.*, 2004), could offer a significant advantage over mesophilic enzymes poorly active at low temperatures. In addition to this the research is going on psychrophilic hosts for using them as low-temperature expression systems, which reduces the formation of inclusion bodies and an increase in the expression of correctly folded proteins under soluble form due to the weakening of hydrophobic interactions at low temperatures (Duilio *et al.*, 2004).

Psychrophiles belonging to the genus *Pseudoalteromonas* may be good sources of novel therapeutic agents by possessing antibiotic activity (Feller, 1994). Generally in cosmetic industry Lipases and esterases are widely used, especially cold-adapted lipases (Pandey, 1999; Joseph *et al.*, 2008). The exploitation of these extremophilic organisms and their enzymes in the production of biofuels, particularly bioethanol has been practiced. Cold active lipase from *Pseudomonas* strain P38 is widely used in non-aqueous biotransformation for the synthesis of n-heptane of the flavoring compound butyl caprylate. Cold active lipases could be a good alternative to mesophilic enzymes in brewing industry and wine industries (Collins *et al.*, 2002), and also used in perfumery and optically active ester synthesis (Cavicchioli *et al.*, 2002). Cold active lipases have lately attracted attention as a result of their increasing use in the organic synthesis of chiral intermediates. Due to their low optimum temperature and high activity at very low temperatures, which are favorable properties for the production of relatively frail compounds. A cold-adapted purine nucleoside phosphorylase (PNP) in ribavirin (a broad-spectrum antiviral drug) bioconversion showed approximately 15°C lower optimum temperature and 1.80-fold higher catalytic efficiency (kcat/Km) at 37°C within substrate inosine than homolog in *E. coli*.

Conclusion

Cold adaptive enzymes have huge importance in the present day. The wide application in industrial scale the enzyme found from psychrophilics are the recently exploiting, and till is a lot to find the future aspect in various application. Further researchers to tailor new enzymes active at low temperatures for biotechnological applications. Wide and constant screening of new microorganisms for their cold active enzymes at low temperature will open novel and simpler routes for the synthetic processes. Consequently, this may pave new ways to solve biotechnological and environmental problems.

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