Review Article

Marine Fridge, Micro Lives and Mega Hopes - A Critique on the Potentiality of Psychrophilic Bacteria

Nabil Mohammed K.K*, Remya .S, Rajendran N
School of Bio Sciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India.
***Corresponding author's E-mail:** kknabil@gmail.com

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ABSTRACT

Oceans are indispensable for supporting life on the planet and it covers a vast expanse of the earth's surface that shades around 70% of the globe. The occurrence of psychrophilic organisms in the marine environment suggests that there exists a favorable condition for the survival of life particularly in the deeper region where the temperature is below 5˚C and more or less isothermal, thus providing cold natural milieu on a consistent basis in a way similar to a man-made refrigerator. Due to their innate power for resisting cold and acclimating to extremely low climates, they find a wide variety of applications in many fields mainly because of the cold adapted cellular components derived from them. These applications include different strategies to preserve food, usage in reaction as a cold-adapted enzyme, bioremediation of oil spills, additives in detergents and in textile industries etc. The biotechnological exploration of psychrophilic bacteria came into limelight with the application of rDNA technology, as these extremophiles can produce anti-freeze proteins that protect them from ice crystal damage which can be explored in rDNA experiments like gene transfer to plants. Thus the extremophilic micro lives present in the marine fridge are a potential source from which the colossal hopes for life science emanates. This review focuses on the potentiality of psychrophilic bacteria derived from marine sources in various biotechnological applications along with the impediments embedded with these explorations.

Keywords: rDNA, psychrophiles, bioremediation, cryoprotectants, marine.

INTRODUCTION

When we see from the shore, oceans are never ending and roughly uniform. But it's not the case, when we go deeper in to the ocean where a wide variation in physical parameters is discernible. When there is variation in the environment, the organisms present there have to adapt to it since adaptation is the prime basis for survival. When we take ocean water as 100 parts, then around 90 of it has a temperature of 5 ˚C or less and most of it comes beyond a depth of 3km or more where the temperature is isothermal at 3 – 5˚C.[1] This consistently cold temperature is in a way similar to the condition provided by a refrigerator. And hence this atmosphere can be penned as a natural Refrigerator or more literally as a 'Marine Fridge'. With depth comes high hydrostatic pressure which is coupled with a wicked cold temperature, so that the condition becomes extreme and the survival becomes extremity. But still there is life, in fact abundant life and thus we call the possessor's of life in those extreme environments as extremophiles, which are barophilic (pressure loving) and at the same time psychrophilic (cold loving). Since all research prefer a bottom-up approach, studies on the micro lives present in these cold extreme environments emanate our cognition of how they have been adapted to the conditions and in what way it can be turned to benefit the life science.[1]

Life under oceans, as we know, is a repertoire of resources. In the course of exploration searching for more resources with high magnitude of hopes, the scientific community is intensively exploring the microorganisms present in the consistently cold natural environment or marine fridge. One could deduce the contradiction that the cause of exploring the organisms in micro size is because of the view of hopes in mega size, in an ironical manner 'Micro' and 'Mega' are the two extremes on the scale of magnitudes. This critical attempt in turn explores the yesteryear developments, the current improvements and the succeeding exploitation of scientific knowledge about the micro lives present on the natural marine environment of low temperature.

Requisition for Extreme Environmental Exploitation

Anything that deviates from the normal by a considerable level can be cited as 'Extremes' and to dig in the extreme environment facing all the obstacles, needs a clear cut reason that justifies the huge amount of investment in the form of money and man power spent for the purpose. The reason for looking in to organisms present in extreme environment is because of the fact that the enzymes and other biomolecules derived from them will be stable at that extreme condition. If a reaction has to be carried out in such a condition of extremity, the enzymes derived from these organisms would play a pivotal role. The revolution created by the heat stable Taq polymerase enzyme derived from *Thermus aquaticus*, which is an extremophile living in hydrothermal vents exemplifies the significance of these extremophiles.[2] When it comes to cold stable enzyme, the thought will be directed towards organisms living in extreme cold marine fridge.

Benefits of Cold-Stable Enzymes Educed from Psychrophiles

The energy consumption will be reduced to a considerable level by the use of psychrophilic bacteria derived enzymes in biological applications because of the fact that expensive heating steps can be skipped.[3] This will aid energy saving along with great economic benefits as the isolated enzyme functions optimally in a cold environment and thus eliminating the undesirable chemical reaction that might occur at higher temperature. Other than that, cold-stable enzymes has an increased reaction yield and capable of accommodating high level of stereo specificity. They have a clear cut advantage over the usage of mesophilic enzyme at cold temperature since a very low concentration is required to bring a high level of efficacy. The inactivation of cold-adapted enzyme is also an easy task which can be done by increasing the temperature, since characteristically they are highly thermolabile and thus rapid and spontaneous inactivation is possible by a mild heating. Mild heating does not alter the quality of the product and it also provides room for the selective inactivation of enzyme in a complex media without any sophisticated heating or cooling system. This heat inactivation of cold enzyme is having a greater usefulness in the food industry where the modification of heat sensitive substrates and products can be prevented. This is also useful in sequential processes such as molecular biology where the actions of an enzyme must be terminated before the next process is undertaken.[3]

Physiological Adaptation of Psychrophilic Bacteria

The diversity of the microbial population present in the psychrophilic environment is enormously large despite the extreme condition and it even covers microbial population from major phylogenetic groups.[4] Microbes which are adapted to live in cold condition will have both structural and functional components that support the adaptation for a low temperature existence so as to maintain a complete thermal equilibrium with the environment.[5, 6] Even though the exact genetic basis of psychrophily is still unknown, it is apparent that the cardinal temperature for growth is determined by the genetic constituents.[7] From the genome sequenced of 4 psychrophilic organisms, a distinct genomic trait which connects all of them is absent, but still they share a few characteristics in common.[2] Out of these 4 organisms, two of them were isolated from arctic sediments namely, *Desulfotaleas psychrophila*[8] and *Colwellia psycherythraea*[9], one from coastal Antarctic water called *Pseudoalteromonas haloplankis*[10], and the remaining one from the deep sea called*Photobacterium profundum*[11]. The major focus on the genetic level research on psychrophilic bacteria is concentrated in the enzymes and it's only in the recent years that the other cellular components caught the attention.[5] Thus, psychrophilic micro lives became a hot candidate for Gene prospecting. Cell membrane, which stands as the barrier between the cells and the outer environment, is in one gumption can be defined as a molecular sensor which monitors and perceive the change in temperature which is key for adaptation in psychrophilic environment. As the temperature becomes cooler, the membrane becomes more rigid, and thus activates a sensor associated with the membrane. This sensor acts as a signal transducer to a response regulator that induces up regulation of membrane fluidity modulation genes that are involved in cold adaptation of bacteria.[4] Unsaturation of fatty acid is a typical outcome with cold temperature, along with a reduction in chain length and a leap in methyl branching and all these forms the characteristic of membrane fluidity in psychrophilic microbes.[2, 6] Cold shock proteins present in the cold loving microorganisms are believed to destabilize secondary structure in mRNA and thus a high translational efficiency. The nutrient uptake in them is increased by the presence of cryoprotectants.[2] Oxygen has a high level of solubility at low temperature and hence the problem with the formation reactive oxygen species that cause oxidative damage is present in the marine fridge. But this threat to the cells of psychrophilic microbes is eliminated by the presence of numerous catalase and superoxide dismutase gene. The products of these genes have the capability of cleaving the reactive oxygen species.[8]

Shifting the focus to the metabolism of psychrophiles, there are two predominant types; organoheterotrophic and phototrophic organisms. Some rare species of psychotolerant methanogenic archae comes under lithoautotrophs, which are capable of deriving energy from reduced compounds of mineral origin.[12, 24]

On the analysis of molecular level adaptation of cold loving bacteria, the presence of unique genes or elements involved were brought out.[6] The unsaturated fatty acid synthesis genes, desaturases and dioxygen lipid desaturases are responsible for the elevated cell membrane fluidity in psychrophiles, while the protection against the frost is brought by the action of certain genes coding for compatible solutes. Ice Binding Proteins (IBP) and anti-freeze proteins are the other two elements that causes decline in cytoplasmic freezing point and cell organelle stabilization.[6]

By the accumulation of compatible solutes particularly like glycine, betaine, glycerol, mannitol, trehalose and sorbitol in the cell, cold-adapted bacteria are able to withstand hyper-osmolality and desiccation. This compatible solute accumulation is also one of the common methods of freeze protection. The result of the accumulation of these highly soluble poly-hydroxylated compounds is a reduction in the freezing point of the aqueous phase of the cytoplasm. It might also stabilize cytoplasmic macromolecules including enzymes in a direct manner.[6]

Cytoplasmic freezing and it deleterious effects can be controlled by other mechanisms apart from compatible solutes, like antifreeze proteins and ice nucleating protein, as these mechanisms are more specific in nature. Antifreeze proteins and Ice-Binding Proteins (IBPs) bind to ice and thereby inhibiting the ice-crystal growth and recrystallization process. The characterizations of antifreeze protein have been done in a reasonably well manner. In the case of ice binding proteins, their presence has been identified in a number of psychrophilic microorganisms in deep marine environment. Ice-nucleating proteins have the capability of inducing the crystallization of ice at temperatures close to the melting point so as to prevent supercooling of water, and they also seem to be involved in the survival of organisms at low temperatures. A freezing point depression can be produced by these proteins of over a range of 2˚C and they are strongly implicated in cellular cryopreservation.[6]

Prior Exploration of Psychrophiles

With the advent of stupendous technology, it is even possible for exploring outer space which is light years apart. So it was not a big deal for the scientific community to dig the extreme environments enshrouded on planet Earth and to unwrap the floral and faunal existence in those areas. In the fields of routine novel bacterial exploration stages like isolation, collection of culture, screening of bioactivity, taxonomy, bioremediation and production of polyunsaturated fatty acids (PUFA) along with cold-adapted enzymes, south frigid zone prokaryotes had rendered a greater developments in recent researches.[13]

The heat labile proteins inherent to the cytoplasm of psychrophiles are stable at around 0˚C and 15˚C, above which the inactivation occurs.[1] The low thermal stability of psychrophilic bacterial protein was extensively studied by the extracted glycolytic enzymes. High catalytic efficiency and an elevated turnover number are the characters which provide an upper hand to cold-adapted enzymes that compensate for the reaction rate reduction at low temperatures in order to maintain adequate metabolic fluxes.[14]

An enzyme which cannot withstand the conditions of industrial reaction has a limited application and this limitation is the major factor in exploring the extremophilic microbes for novel enzymes. The enzyme which degrades polymer at lower temperature has a predominant application in paper and pulp industry, so as in food process industry where the enzyme active at low temperature finds numerous applications. Most enzymes from psychrophiles are correlated to a greater catalytic activity which is amalgamated with low thermal stability and high flexibility.[12]

Due to the high activity at low temperature of lipases derived from psychrophiles, they are one of the hotspots of research. Lipases catalyze reactions that include esterification, acidolysis, alkalolysis along with the hydrolytic activity of triglycerides and certain others. Cold active lipases, obtained from are largely distributed in microorganisms surviving at low temperatures near 5˚C.[25] Dehydrogenases obtained from psychrophiles are used in biosensors.[15]

Antarctic and Artic seawaters are the major sources of microbes from which the cold-adapted enzyme have been extracted and characterized. Since the temperature of the Antarctic region is constant (~1˚C), a high selective pressure is exerted on the endemic microbes. Nevertheless, high bacterial density, in the range of 10^7 ml^{-1} can be found in Antarctic region, a count comparable to that of temperate water. Hence the laboratory cultivation of Antarctic strain is usually done at a temperature of ~5 ˚C that allows biochemical machinery to function optimally which leads to high cell density and production of extracellular enzyme efficiently. Depending on the bacterial species, the generation time will vary from two to ten hours under cold condition. Shortening of generation time is possible if the microbe is cultivated at higher temperature (~20˚C) but the limitation is that, stress will be induced on the cell. This in fact will leads to low density of cell coupled to a low production of extracellular enzyme.[16]

The specific activity of wild type and recombinant form of several enzymes derived from the Arctic and Antarctic microorganisms have been determined and these enzymes include α-amylase, alcohol dehydrogenase, aspartate transcarbamylase, malate dehydrogenase, subtilisin, triose phosphate isomerase and xylanase. Some of these enzymes are involved in the glycolytic pathway and hence are indispensable in the metabolism. The specific activity of these enzymes is in a range which is higher than their mesophilic counter parts. Denaturation of cold enzymes occurs at higher temperature.[16]

Thermostable enzyme is approximately a 250 million dollar market owing to the diverse potential of cold-adapted enzymes along with the offering of unusual specificity, specific activity and low stability. All these properties enable them to be incorporated in wide range of formulations shading extensive range of applications.[16]

Additive in detergent

A-amylase, cellulose, protease and lipase are the important enzymes used as detergent additives as the respectively aids in removing the stains of the nature starch, cellulose, protein and fat. Cold-washing helps to reduce the energy consumption and wear and tear to a considerable extend. But the storage of these enzymes is a drawback for this sort of washing compared to the normal washing where the detergent can be kept at room temperature. Another drawback is the instability while adding to the final product. But the adversity of stability will not stay as an impediment for longer time period due to the advent of rDNA technology.[16]

Textile industry

Cold-adapted cellulose can be used in bio-polishing and stone-washing process. The protruding cotton fibre ends of the garments is a major problem faced in textile industry as it alters the appearance of the garment at the same time reduces its smoothness too and thus refraining the customer from buying these goods. The case gets even worse with the subsequent washing of the garments as more thread will get protruded. Here comes the protogonistic role of cellulase enzyme which helps in excising the protruding ends and thus reducing the pill formation, increasing the durability and softness of the tissue. The pre-treatment with cellulase enzyme has to be carried out under appropriate condition but one problem associated with this is the loss of mechanical resistance of the garments because of the resistance of enzyme to inactivation. The concentration of the enzyme can be reduced if we're using a cold-adapted enzyme and also the process can be carried out at low temperature. As a result of this replacement in enzyme, the spontaneous inactivation of the enzyme is possible and the mechanical strength of the fabric will be high.[15, 16]

Food Industry

A myriad of application is obvious for cold-adapted enzyme in food industry.

- Lactose intolerance among humans can be reduced by the use of β-galactosidase at low temperature.
- Use of pectinase helps in the extraction of fruit juices by reducing the viscosity and clarifying the final product.
- Meat tenderization by protease.
- Amylase, protease and xylanase reduce the dough fermentation time and thus find application in the baking industry. In addition to that it, retains the level of moisture and aroma.[16]

The replacement of normal enzyme with cold-adapted enzymes helps in reducing the amount of enzyme to be used and also for the spontaneous inactivation of the enzyme, thus reducing the prolonged action of enzyme that causes changes to the texture of the food item. In cheese manufacturing, brewing and wine industry, animal feed and in certain other applications also, cold-adapted enzymes can be used as an alternative to the mesophilic enzyme that is currently used.[15]

Bioremediation

As a feasible alternative to physicochemical methods in reducing environmental contamination, microbes can be exploited mainly in the treatment of soil and waste water. The large seasonal variation of climate in temperate region limits the use of microbes in the degradation of organic pollutants such as lipids and oils, due to reduced effectiveness. However, a mixed culture of cold-adapted microorganisms helps in improving the recalcitrant chemical biodegradation. As a result of the high catalytic efficiency of their enzymes and their unique specificity at low and moderate temperatures, cold-adapted microorganisms should be ideal for bioremediation purposes. But the optimum condition for the operation of the organisms has to be carefully evaluated by research since they're less characterized. The whole bacterium finds use in the bioremediation of oil spills, toxic wastes and contaminated ground water.[9]

Margesin *et al*. (2000) explained the bioremediation of oil disgorges by an inexpensive and environmentally friendly cleanup tool with the assistance of cold-adapted microorganisms. Even though the studies were conducted on oil contaminated alpine soil, they can be potentially expanded and employed in the cleanup of marine oil spills in the future. Oil biodegradation by psychrophilic bacteria can be heightened to a significant level by the use of inorganic nutrients. But despite this heightened activity, the bio-stimulation with inorganic nutrient supply cannot solely bring complete oil elimination.[18]

Low water condition biocatalysis

Fatty acid esters, peptides, oligosaccharide derivatives and certain other compounds derived from poor aqueous soluble substrates can be synthesized commercially using enzymes operating under low water condition. The catalytic capability of these biocatalysts is a strong function of hydration state in these systems.

In the work published by Marianna *et al.,* 1999[19], it was mentioned that from the stomach of an Antarctic krill named *Euphausia superba* Dana, a marine bacterium of the species *Sphingomonas paucimobilis* Strain 116 was isolated and from this microorganism an extracellular protease was extracted. Following the purification and characterization it was found out that the protease voiding was at the peak in a temperature range 5˚C to 10˚C, which is even below the optimal growth temperature of the species which is at 15˚C. The subsequent high level of refinement revealed that the enzyme is an ethylene diamine tetra acetic acid sensitive metalloprotease which showed maximal activity against proteins at 20–30˚C and pH range of 6.5–7.0. 47% of maximal activity was retained by the enzyme at 0˚ C in urea denatured hemoglobin (Hb) hydrolysis at pH 7.0. But as the temperature goes on, a decline in the activity was apparent as it reaches 37% at 5˚C and 30% at 10˚C. The metalloprotease retained stability for 15 minutes at 30˚ C and 60 minutes at 20˚C, so that this cold-adapted enzyme could be useful in the reactions carried out at these temperature's.[19]

In another research[20], psychotolerant strains were isolated from Finnish Lapland (68-691N) which grows optimally at 0 to 30 ˚C. As a whole 331 heterotrophic bacterial strains were isolated which are aerobic in nature, out of which few of them were isolated from marine sources and around 82% of them grow at a temperature around 0˚C. More than half of the strains exhibited protease and lipase activity at 50 ˚C, and 20% of the strain possessed amylase or cellulase activity or even both the activities together.[20]

In the work done by [Hailun *et al.,* (2004)], the high activity of the cold-adapted protease derived from the deep-sea-cold bacterium *Pseudoaltermonas* sp. SM98011 in releasing free amino acid from various animals like shrimp meat, marine fish and pork were reported. The activity was assessed in comparison with a mesophilic protease produced by a mesophilic bacterium *Bacillus* sp. SM98011. Both the protease were sprayed onto the surfaces of marine fish, pork and shrimp meat, respectively, and then stored at 0˚C for 6 days. The results showed that the samples treated with cold-adapted protease released more free amino acids than those treated with mesophilic protease at 0˚C. The refrigerated meat samples treated with cold-adapted protease, released more taste amino acids and essential amino acids than those treated with mesophilic protease. Therefore, the cold-adapted protease had potential in improving the taste of refrigerated meat.[21]

Psychrophiles Derived Products Other Than Enzymes

Notwithstanding the major rivet is on the enzymes, other compounds derived from psychrophiles have also found prominent application in many fields of life science. The polyunsaturated fatty acids (PUFA) derived from the psychrophiles are used as dietary supplements are incorporated into sustenance for human beings, livestock and fish.[9] Food industries also avail the use of ice nucleation proteins educed from psychrophilic bacteria.[9] The antifreeze proteins and solutes find application in being used as cryoprotectants and as catalysts active under frigid condition.[17] Aside these applications which relies on the product derived from the psychrophiles, the whole bacteria itself find use in certain applications such as manufacturing of cheese and yoghurt, tenderizing of meat, remotion of lactose from milk and in modification of flavor.

Novel waste Water Treatment Employing Psychrophiles

Water is regarded as the elixir of life which cannot be superseded by any other material as an alternate. With the increasing demand of water in a pure form many researches are concentrated on treating the effluent water which is heavily fouled, but all of them are having certain recessions on practical application and hence the hunger for finding a better treatment of waste water is always an issue of challenge. Waste water that is discharged at anambit of moderate to low temperature for which, a treatment with psychrophilic anaerobic microorganisms is found to be attractive. A feasible anaerobic system is developed using expanded granular sludge bed (EGSB) for the treatment of pre-acidified and soluble waste water at a temperature range of 5 to 10˚C. An organic loading rate of 10 to12 kg chemical oxygen demand (COD) per cubic meter reactor per day can be achieved at 10–12˚C with a removal efficiency of 90%. At a near freezing temperature range of 4-5 ˚C, Stabile methanogenesis was observed.[22] It was reported [Gatze *et al*., (2001)] that under low temperature condition an elevation in the specific activity of mesophilic granular sludge is ascertained, indicating the growth and enrichment of acetogens and methanogens in the anaerobic system. Since there is a high fraction of particulate COD, the sewage belongs to a complex waste water category so that at moderate climate, the anaerobic sewage treatment becomes highly tedious and a real challenge. This challenge is overpowered by a two-step system consisting of either an anaerobic up-flow sludge bed (UASB) reactor combined with an EGSB reactor or an anaerobic filter (AF) combined with an anaerobic hybrid reactor (AH) is successful for anaerobic treatment of sewage at 13˚C with a total COD removal efficiency of 50% and 70%, respectively.[22]

Prospective Exploration

While looking on to the prospective application of psychrophilic bacteria, the primary focus is the exploration of rDNA technology. Many attempts have been made on this regard but only a few came to limelight. One among those is the generation of ice-minus mutant strain from the bacterium *Pseudomonas syringae*. The ice plus wild strain causes ice formation and by knocking out this gene, the formation of ice can be prevented and if this gene is incorporated into plants, they can be grown under low temperature.[17] Using rDNA technology the stability of cold-adaptive enzymes used in detergents can be improved so that it will be a leap in its application in cold-washing.[16] Expression of cold-stable enzyme derived from psychrophiles surviving in marine fridge is now possible by the construction of a host-vector system that allows the overexpression of genes in psychrophilic bacteria. The studies [Anne *et al*., (2004)] revealed that the new biotechnological potential of psychrophilic strains prevents the formation of inclusion bodies and protects heat-sensitive gene products due to their expression at low temperature. Other host-vector systems for a temperature-regulated gene expression which allowing the overproduction of thermolabile enzymes originating from psychrophiles is also under consideration. In other respects, the improvement of the competencies of psychrophilic enzymes, according to the requirement of a biotechnological process, could be obtained by directed mutagenesis or perhaps more quickly by the directed evolution of the recombinant enzyme. Possible applications of cold-adapted enzymes can be found in numerous biotechnological and industrial fields as tools in molecular biology, food, drinks, textile and detergent industries, and bioremediation.[3]

Impediments associated with psychrophilic bacteria

Despite a large number of feasible biotechnological applications, only a few cold-adapted enzymes are in commercial use. The costs of production and processing at low temperatures remain probably higher than those for the commercial enzymes that are presently in use.[3]

The enzymes derived from the micro lives present in cold habitats, have a molecular adaptation to perform optimally under low temperature alone and because of that it is extremely sensitive to various denaturing agents. This is a consequence of the weakening of the intramolecular forces and, in certain cases, of the reinforcement of molecular interactions with the solvent, both contributing to the instability of the cold enzymes.[23]

The specific activity of enzymes obtained from psychrophiles is ascertained to be lower at environmental temperature when equated with enzymes obtained from mesophiles. It is difficult to make a perfect balance between the stability and activity of cold-adapted enzyme to work in moderate temperature. When stability is concerned, it is always correlated with flexibility in such a way that an acceptable flexibility can only be achieved by compromising it with an unacceptable stability. Apart from that the stiffness of the molecular structure that is essential for achieving a reasonable stability is foreclosed in most cases so as to make the possibility to reach a high catalytic efficiency.[13]Hence the mega hopes based on the micro lives in cold marine conditions can only reach to reality, if we could successfully surmount these obstacles.

CONCLUSION

Cold-stable enzymes and other products derived from psychrophilic microorganisms have a wide range of applications in life sciences. The organism itself can be utilized for many applications like bioremediation. The micro lives that are present in extreme environment have adapted to those conditions in order for survival, and the entities which support these adaptation strategies can be utilized for the benefit of mankind. Economy is the major aspect that has to be focused on the exploration of these organisms from deep marine habitats, for making it as a feasible alternative to the existing system.

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