Research Article

Importance of Marine Thermophiles in Biotechnological Applications

Sherene Mathai*, Keerthana Rachel Roy, Rajendran N
School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India.
***Corresponding author's E-mail:** sherene.anna@gmail.com

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ABSTRACT

Biorefineries are the order of the day as they use cheap and readily available biomass to produce a variety of different chemicals. These processes employ enzymes to convert biomass to the desired products. Higher working temperatures are preferred for these reactions as they facilitate easy mixing, high mass transfer rate and diffusion coefficient as well as increase the solubility of compounds. It also decreases the risk of contamination by common mesophiles. Hence, thermostable enzymes that can withstand the harsh conditions of these industrial processes are ideal. Thermophilic organisms are also hypothesized to be the first forms of life on earth and hence, have evolutionary importance. They are being used as the source of these thermostable enzymes. Thermophilic organisms are able to grow optimally at high temperature using adaptive mechanisms and produce various valuable compounds, such as thermostable enzymes, hormones and antibiotics. The demand for thermostable enzymes, such as amylases, cellulases, lipases, proteases, DNA polymerases and xylanases, has increased enormously due to increasing industrial growth. These enzymes are used in crop biorefining, food and pharmaceutical, detergents, textile and several other biotechnological industries. Further research is being done to explore their applications in other fields like veterinary medicine and astrobiology. The importance of thermophilic organisms from marine environments has been highlighted in this review.

Keywords: Biotechnology, Polymerases, Thermophiles, Thermostable enzymes.

INTRODUCTION

Extreme environments and extremophiles

Extreme is a relative term which depends on what is considered normal. When it comes to environments normal is taken as what is normal for humans and most of the organisms. Any organism existing outside this range, either much higher or much lower is considered an extremophile. Extreme habitats can be physical extremes or geochemical extremes or both. Physical extremes include temperature, pressure, radiation while geochemical extremes comprise salinity, pH, dessication, redox potential or oxygen depletion. Extremophiles not only just survive in extreme conditions they thrive in it.

Organisms which thrive in more than one extreme are polyextremophiles. *Sulfolobus acidocaldarius* which is an archaea that flourishes at a temperature of 80°C and a pH of 3.0 is a good example of a polyextremophile. It has been proposed that extremophiles should include biological extremes as well, for example organisms which can grow in nutritional extremes, extremes of population density, etc. Although prokaryotes are the foremost members of the group, other taxonomic domains are also included. Bacteria and eukaryotes are widespread in acidophiles, pschrophiles, piezophiles, alkaliphiles, halophiles and xerophiles. Almost all the hyperthermophiles are Archaea. Pscychrophiles comprise even vertebrates.

The main interest in extremophiles is due to the enzymes which help extremophiles function under harsh circumstances. Standard enzymes become inactive or get degraded when exposed to extremes like heat or pressure. So manufactures have to take extra precautions in order to protect them during reactions or storage. By using extremozymes- enzymes from extremophiles- they can potentially eliminate the need for these added steps and hence, reduce the costs and increase efficiency of the process. Another reason for interest in this group of organisms is because of their role in geochemical processes, economic potential, threatening issues and their part in the search for extraterrestrial life.

Importance of thermophiles

Most of the biorefineries employ enzymes to produce the desired products. These processes require higher working temperatures as they facilitate easy mixing, high mass transfer rate and diffusion coefficient as well as increase the solubility of compounds. It also decreases the risk of contamination by common mesophiles. The enzymes from thermophiles unlike the normal enzymes are able to withstand these high temperatures and pressures of these bioreactors. Hence, thermophiles have become an increasingly important topic of interest due to their ability to survive at extremely high temperature and to produce enzymes that can withstand the harsh conditions of these industrial processes. Another reason for the considerable attention to this group is because it may guide us to biomolecule stability and the universal ancestor. From the perspective of evolutionary mechanisms, learning about these organisms can help us understand the evolution of life on Earth. The possibility of life elsewhere in the solar system and the universe can also be better explored as the conditions are just as harsh. Many previously unknown metabolic processes and novel compounds can also be discovered that could have commercial applications one day.[1-4]

Natural thermophilic environments

Thermophilic microbes are naturally found in shallow and deep marine hydrothermal vent environments, heated beach sediments, continental solfataric areas, geysers and hot springs. The temperatures and pressures of these habitats vary considerably. The majority of these systems are characterized by extremely low oxygen concentrations. Consequently, most of the known species of thermophiles are classified as obligate or facultative anaerobes, though aerobic and microaerophilic isolates are also known. The main aquatic environments are hot springs and hydrothermal vents; hydrothermal vents being the only marine thermophilic environment.

Hot Springs

Hot Springs are formed by the surfacing of geothermally heated ground water from the Earth's crust where the water temperature is higher than the mean air temperature. They are widespread all over the world. They usually have a high mineral content, including calcium to lithium and even radium.[5]

Hydrothermal vents

Hydrothermal vents are the underwater counterparts of hot springs. They occur in volcanically active regions, where the tectonic plates are moving apart, hotspots and ocean basins. Ocean water percolates down the fissures in the Earth's crust and gets heated by the magma and the rocks below. The water becomes superheated and very acidic as it dissolves heavy metals like zinc, cobalt, lead, copper and other chemicals like hydrogen sulphide from the surrounding rocks. The heated water rises back to the surface through openings in the crust. Vent temperatures can reach over 400ºC but the water does not boil due to the extremely high pressure of the deep ocean. Hydrothermal vents were first discovered near the Galapagos Islands in 1977 and were found to support life despite the very harsh conditions.[6, 7]

Different types of thermophiles

The vast genetic and metabolic diversity present in high temperature environments reflects the ranges of pH, oxidation-reduction states, solute concentrations, gas compositions, and mineral composition that characterize these environments. *Thermocrinis ruber,* an aerobic, facultatively chemolithotrophic bacterium, grows in alkaline hot spring in the Lower Geyser Basin of Yellowstone National Park, USA between 44 and 89ºC by oxidizing hydrogen, elemental sulfur, thiosulfate, formate, or formamide. Deep-sea hydrothermal systems at a depth of 2600 m on the East Pacific Rise support anaerobic autotrophic methanogens such as *Methanococcus jannaschii* which grows optimally 85ºC. Meanwhile, the interaction of volcanic gases and seawater at Vulcano in the Aeolian Islands and the solfatara fields of Naples, Italy that generates acid solutions host acidophilic Archaea, including *Acidianus infernus*, *Thermoplasma volcanium*, and *Metallosphaera sedula*, which grow optimally at a pH near 2.[8]

Microorganisms which inhabit these high temperature environments classified into several groups depending on their optimum temperature:

- Organisms which can survive below 45ºC are facultative thermophiles.
- Organisms which have an optimum growth temperature less than or equal to 45°C but can grow at temperatures greater than 45°C as well are known as thermotolerant.
- Organisms with an optimum growth temperature between 45 and 60ºC are moderate thermophiles.
- Strict thermophiles are those which have optimum growth at temperatures between 60 and 90ºC.
- Organisms which grow best at temperatures greater than 90ºC are extreme thermophiles or hyperthermophiles.[9]

Thermohiles in marine environments

It is increasingly apparent that surface thermal features and the organisms they support are giving researchers a glimpse of what life may be like in the deep subsurface. The metabolic diversity examined in the deep biosphere shows that chemosynthetic organisms can take advantage of many forms of energy that are sufficient to support life. These energy sources can be linked to photosynthesis at the surface, as in the case of heterotrophs that use organic compounds in sediments that are the residue of photosynthetic organisms, or they can be completely independent of photosynthesis, as in the case of autotrophs that gain energy and organic carbon by reacting CO_2 and H_2 provided by geologic processes.[10-18]

The areas around hydrothermal vents are biologically more productive when compared to the bulk of the deep sea. They host complex communities supported by the chemicals dissolved in the vent fluids. Chemosynthetic bacteria and archaea form the base of the food chain, supporting diverse organisms, including giant tube worms, clams, limpets and shrimp.

The chemosynthetic bacteria grow into a thick mat and attract other organisms, such as amphipods and copepods, which feed upon the bacteria directly. Larger organisms, such as snails, shrimp, crabs, tube worms, fish, and octopi, form a food chain of predator and prey relationships above the primary consumers. The main families of organisms found around seafloor vents are annelids, pogonophorans, gastropods and crustaceans.

Tube worms are an important part of the community around a hydrothermal vent. They may grow to over two meters tall and have no mouth or digestive tract but instead have a special organ which houses chemosynthetic bacteria. They absorb nutrients produced by the bacteria in their tissues like parasitic worms. The worm uses its plume of gills to collect the chemicals the bacteria need. In return, the bacteria provide the worm with chemosynthetically produced carbon compounds. The two species that inhabit hydrothermal vents are *Tevnia jerichonana*, and *Riftia pachyptila*. Although invertebrates dominate vent communities, some communities are known to house vertebrates like eels. For example, Eel City located near Nafanua volcanic cone, American Samoa.

Other examples of the unique fauna which inhabit this ecosystem are the scaly-foot gastropod *Crysomallon squamiferum*, a species of snail with a foot reinforced by scales made of iron and organic materials, and the Pompeii worm *Alvinella pompejana*, which is capable of withstanding temperatures up to 80°C.

Over 300 new species have been discovered at hydrothermal vents, many of them "sister species" to others found in geographically separated vent areas. The examples of convergent evolution seen between distinct hydrothermal vents is seen as major support for the theory of natural selection and of evolution as a whole.

Metabolism

Most of the life on our planet relies on plants using sunlight energy to convert water and CO_2 into sugar (photosynthesis). The sugar is then consumed by animals, moving along food chains, ultimately being decomposed back to water and CO_2. Hydrothermal vent communities are able to sustain such vast amounts of life because vent organisms depend on chemosynthetic bacteria for food. The water from the hydrothermal vent is rich in dissolved minerals and supports a large population of chemoautotrophic bacteria. These bacteria use sulfur compounds, particularly hydrogen sulfide, a chemical highly toxic to most known organisms, to produce organic material through the process of chemosynthesis.

Adaptations

The proteins of thermophilic organisms are able to maintain stable folds at high temperatures. The adaptations required to maintain stability at high temperature have been a subject of much curiosity. The mechanisms observed include an increase in secondary structure propensity, changes that decrease unfolded state entropy such as the introduction of Pro residues, reduction of Gly residues, smaller loops and the addition of disulfide bonds, an increase of hydrogen bonds and salt bridges and better optimized hydrophobicity. The most striking difference we observe between the membrane proteins in mesophiles and thermophiles is a general increase in the hydrophobicity of the thermophile transmembrane helices. One observation made by Scheider et al. is the increase in small amino acids in thermophiles. This could have two stabilizing effects: (1) packing small residues rather than large residues lowers the entropy of packing which could be particularly important at higher temperature; and (2) small residues can allow for more intimate interactions between helices.[19-35]

POTENTIAL APPLICATIONS

Novel thermostable enzymes

The thermophilic enzymes have adapted themselves to grow in extreme conditions such as high temperature, high pressure, high salt concentrations and extreme pH. They are able to survive in such harsh conditions because of certain unique biocatalysts that they produce. Some of the enzymes obtained from such thermophiles have found use in food, pharmaceutical and chemical industries and also in environmental biotechnology. A few of them are mentioned below.[36]

Starch degrading enzymes

Starch consists of α-glucose units that are joined by α-1,4 or α-1,6 glycosidic bonds thus forming two components amylase and amylopectin. Because of this complex structure it requires several enzymes for degradation. Basically two types of enzymes are used: endo acting enzymes, like α-amylase, that hydrolyse the linkages in a random fashion which produces linear and branched oligosaccharides and exo acting enzymes, like β-amylase, glucoamylases that attacks from the non reducing ends of the substrate and produces oligo or monosaccharides. The hydrolysis of starch using normal enzymes is a multi stage process and requires with various conditions so finding such thermo stable starch hydrolyzing enzyme is to improve the bioconversion process of industrial starch.[37, 38]

Xylan degrading enzymes

Xylan which is a heterogeneous molecule mainly constitutes of hemicellulose. It is mainly made up of xylose residues linked by β-1,4-glycosidic bonds. Most of these xylose residues are substituted by acetyl, arabinosyl and glucurosyl groups. Because of this the complete degradation of xylose requires many enzymes. There are several criteria that the enzymes have to meet and they are: a) they should not have any cellulolytic activity to avoid hydrolysis of cellulose fibers b)they should have a low molecular weight to help in their diffusion in the pulp fibres c) they should be stable and active at high temperatures and pH d) these enzymes must be readily available in high yields at low cost. But most of the enzymes available fulfill only half of these criteria. Only a few thermophilic organisms are able to produce thermo active xylanolytic enzymes. Most of the thermo stable endoxylanases are produced by the *Thermotoga sp.* strain, *Thermotoga maritima*, *Thermotoga Neapolitan* and *Themotoga thermarum*. These enzymes have been found to be active and stable at high temperatures. Many of the genes that encode for xylanases have been cloned and sequenced, e.g.: the gene encoding for the thermostable enzyme in *T.maritima*, has been cloned and expressed in *E.coli.*[39-43]

Chitin degradation

Chitin is a linear β-1,4homopolymer of N-acetyl glucosamine residues and is the second most abundant natural polymer on earth after cellulose. In marine environment chitin is produced in large quantities and its turnover is mainly due to chitinolytic enzymes. In marine bacteria chitin serves as a major source of nutrient. Chitin is degraded by the endo-acting chitin hydrolase, chitinase. The first enzymes involved in chitin degradation were obtained from the hyperthermophilic strain *Thermococcus chitonophagus.* Even though large number of chitin hydrolysing enzymes have been cloned and characterized only a few are thermostable. These enzymes have been isolated from thermophilic bacterium *Bacillus licheniformis* X-7u, *Bacillus sp.* BG-11 and *Streptomyces thermoviolaceus* OPC-520.[44-47]

Protein degradation

Proteases help in the conversion of proteins to amino acids and peptides. They are classified as serine, cysteine or aspartic proteases or metalloproteases depending on the nature of their catalytic site. Serine alkaline proteases are being used in the household detergents, where they are resistant to denaturation by alkaline conditions. Some are also used in the leather industry and are also used as a catalyst for peptide synthesis. Most of the proteases that are stable at high temperatures and pH find their uses in industrial applications. A wide range of heat stable proteases have been recognized in the thermophilic archea belonging to the genera *Desulfurococcus*, *Sulfolobus, Staphylothermus*, *Thermococcus*, *Pyrobaculum* and *Pyrococcus.* The proteases of the serine type are stable at high temperature and also in the presence of high concentrations of detergents and denaturing agents.[48-52]

DNA processing enzymes

The polymerase chain reaction of DNA mainly makes use of the thermophilic DNA polymerases that are used for the extension of the primer strand of DNA. The most commonly used is the thermobacterial Taq polymerase. The other polymerases like the *Pyrococcus woesei* Pwo polymerase, *Pyrococcus furiosus* Pfu polymerase and polymerase from *Thermococcus litoralis* strains are also used due to their low error rates. The much drawn interest on all these polymerases is because of their proof reading ability. None of the above mentioned enzymes have replaced the Taq polymerase because of reasons like low extension rates and unsuitability for amplification of large DNA fragments. The DNA polymerase of the *Pyrococcus* strain can overcome these problems by possessing low error rates and high processivity and extension rates. Mutations on the *Thermococcus sp.* strain has been attempted in order to reduce the exonuclease activity of the enzyme without reducing their polymerase activity.[53-56]

Other enzymes of applied interest

Alcohol dehydrogenases

These enzymes are able to catalyze the oxidation of alcohols to ketones and also the reverse reactions. The hypothermoarchaeal version of these enzymes has been examined. On comparison to the bacterial and eukaryal alcohol dehydrogenases, the hypothermoarchaeal *T.setteri* enzyme does not have metal ions. In addition to this they oxidise primary alcohol, require NADP and are also thermostable. The other enzyme studied was the *Sulfolobus solfataricus* alcohol dehydrogenase which contains zinc ions and requires a cofactor.

Esterases

These represent a family of enzymes of huge potential in various industrial processes. They have plenty of uses in the stereo specific hydrolysis, trans-esterification, ester synthesis and other organic biosynthesis reactions. Esterases are being used in such a wide range of reactions only after the discovery of their extremophilic versions. The esterase from *Sulfolobus acidocaldariusis* is solvent stable and thermophilic. The expression of the esterase gene of *P.furiosus* in *E.coli* cells, has produced the most thermostable and thermo active esterase until now.

Electricity generation

Studies have revealed that thermophilic electrode reducing bacteria can be used as catalyst in microbial fuel cells. The best source of thermophilic electrode reducing bacteria was found in the marine sediments of the temperate environments. Initially such an environment was not thought as a habitat for thermophiles but it was later found that the thermophilic electricity generating bacteria was present here. It was found that sediment fuel cells were capable of producing higher electric currents at 60˚C than at 22˚C, showing that thermophilic bacteria has a greater capability to produce electricity. The advantage of using such bacteria as catalyst in fuel cells is that they have higher rates of metabolic activity which results in more electricity and they will also be more stable under the extreme conditions normally seen in industries. The most commonly used hydrogen or methanol fuel functions at high temperatures and at low or high pH. Acetate is normally used as the source of fuel for electricity generation by thermophilic bacteria found in the marine sediments. On examination of the 16S rRNA genes of the acetate consuming colony on the anode of the fuel cells revealed that the colony was mainly made up of Gram-positive bacteria. The most common species were the *therminocola sp. T.carboxydophila,* and *T.ferriacetica*. Because of the production of higher electrical current by the thermophiles and also because of the omnipresence of the thermophiles, they have a promising future in the application of microbial fuel cells at high temperature.[57]

Herbicide metabolism

The thermophilic bacteria belonging to the bacillus group has been reclassified as the *Geobacillus* species. These *Geobacillus* species are isolated from the shallow marine hot springs and also from deep sea hydrothermal vents. These species have several applications, one being organophosphate metabolism. Organophosphonates have carbon-phosphorus bonds and are found widespread in the environment. The domestic heating system water was used to isolate *G. caldoxylosilyticus* T20 which were capable of processing a variety of organophosphonates which includes the herbicide glyphosphate. Various bacteria with related capabilities released AMPA (aminomethylphosphonate) during growth. Large amounts of AMPA and glyoxylate was detected when *G. caldoxylosilyticus* T20 cell extracts were grown on glyphosate.[57-59]

Biofuel

At present, because of the increasing cost of oil and also due to the need to reduce the emission of the green house gases and to improve the quality of air, alternate sources of biofuels from renewable sources like biogas, bioethanol, biodiesel are being examined. Bioethanol can be formed from sucrose, starch and cellulose based products. Sucrose is the most widely used source for ethanol production because direct fermentation of sucrose gives ethanol. Starch is not used much because it has to be hydrolyzed first to glucose before it can be fermented. Also it is available in limited quantity and is not a cost efficient process. For cellulose based products, because of the complex and crystalline structure of lignocellulose, it is more difficult to hydrolyze than starch. But with the use of some thermostable cellulase, hemicellulase and thermophilic organisms, the degradation of lignocellulosic material to ethanol can be obtained by a single step enzymatic process and also minimizing the risk of contamination.

Exopolysaccharides

A lot of the marine bacteria produce exopolysaccharides (EPS) for growth, to adhere to solid surfaces and for surviving in the extreme conditions. There is a lot of interest in isolating new EPS producing bacteria from marine environment, particularly from extreme marine environment such as deep-sea hydrothermal vents where there is high pressure and temperature and heavy metal concentration, are mentioned in Table 1.

Limitations

Even though the use of thermophiles at high temperatures is economically attractive, the biomass achieved by these organisms is inadequately low compared to mesophiles. Special equipments, media composition and specific processes are being developed to improve the biomass yield of thermophilic organisms. But their large scale cultivation still remains an economical challenge due to factors like low growth rate, requirement of complex, expensive media and low solubility of gases at high temperature. The high cost of production of thermophiles is justifiable for very few applications.

To reduce the production cost, many thermostable enzymes have been cloned and successfully expressed in mesophilic organisms. Tools for the over expression of these enzymes in thermophilic and mesophilic hosts are being developed to meet the increasing demand for biocatalysts. These enzymes could also serve as models to understand molecular stability under extreme conditions.

Table 1: EPSs produced by microorganisms isolated from marine hot springs and hydrothermal vents[60]

Organism	Environmental source	Applications
Pseudoalteromona sstrain721	Deep-sea hydrothermal vent	Gelling properties
Alteromonasmacle odii subsp. *fijiensis*	Deep- sea hydrothermal vent, North Fijian Basin	Thickening agent in food-processing industry, biotoxification and waste-water treatment, bone healing, treatment of cardiovascular diseases
Thermococcus litoralis	Shallow submarine thermal spring	Biofilm formation
Geobacillussp. strain 4004	Sediment in marine hot spring near the seashore of Maronti, Ischia Island, Italy	Pharmaceutical application
Bacillus thermodenitrificans strain B3-72	Water of a shallow hydrothermal vent, Vulcano Island, Italy	Immunomodulatory and antiviral activities
Bacillus licheniformis strain B3-15	Water of a shallow marine hot spring, Vulcano Island, Italy	Antiviral activity

Future Research

Veterinary medicine

Thermophiles are mainly used in veterinary medicine depending on their capacity to be pathogenic. They are used as sources of enzymes and other molecules for diagnostic and pharmaceutical purposes. Anaerobes like *Methanococcus jannaschii* cannot tolerate oxygen whereas the *Clostridium sp.* can tolerate low levels of oxygen. For the survival of many organisms anaerobic systems are very important. Animals like cows, goats and other ruminants are able to survive on fibrous vegetation because the anaerobic environment in their rumen allows fermentative reactions like methanogenesis to proceed. The ability to adapt to low oxygen tension is critical for some pathogens as well as surviving in very high temperatures. In one case *Mycobacterium xenopi,* which is a thermophilic bacterium, was found in the hot water system of a hospital and three out of eighty seven patients exposed to it developed pulmonary mycobacteriosis.[61]

Astrobiology

Thermophiles are organisms that survive at high temperatures and studying these organisms especially their protein stability is important as they can tell us what extraterrestrial life will look like. Astrobiologists study the protein structural motifs of the thermophiles to hypothesize and investigate the possibility of extraterrestrial life forms and early life. By studying the motifs they are able to say if organisms similar to the thermophiles are able to survive in planets that have a hot environment like those found in the deep-sea thermal vents. It should be kept in mind that extraterrestrial organisms may not have the same genetic make-up, metabolic process or protein structure as that of the thermophiles but still the mechanism adopted by thermophilic organism to survive the extreme conditions opens new windows to how organisms are able to survive in very extreme environments.

CONCLUSION

Extremophiles have been a topic of much interest because of their ability to survive in harsh conditions. Thermophiles, in particular, have been much studied to utilize their adaptive mechanisms for commercial purposes. Thermophilic organisms produce various valuable compounds, such as thermostable enzymes, hormones and antibiotics. These enzymes have wide ranging applications like in crop biorefining, food and pharmaceutical, detergents, textile and several other biotechnological industries. Other applications of these organisms include potential for electricity generation, herbicide metabolism and production of exo polysaccharides of commercial importance. It is believed that the study of these organisms will enhance the existing knowledge on astrobiology as well as origin of life on Earth.

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