# MARINE MICROBIAL ENZYMES

**M. Chandrasekaran** and **S. Rajeev Kumar**
*Microbial Technology Laboratory, Department of Biotechnology, Cochin University of Science and Technology, Cochin-682022, Kerala, India*

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**Summary**

Several industrial enzymes are derived from terrestrial sources. Whereas, marine environment, which encompass about 71 percent of the earth's surface and a vast resource for useful enzymes, remain unexplored. Marine microorganisms take active part in the mineralization of complex organic matter through degradative pathways of their metabolism in the marine environments and contribute to the secondary production in sea. Microorganisms as the fastest means to search for food may often use cell-free extracellular enzymes.

Bacteria and fungi from marine environments secrete different enzymes based on their habitat and their ecological functions. Marine microbial enzymes have become the focal

point of interest and several enzymes have drawn the attention of the microbial prospectors and a few enzymes were isolated from seawater and marine sediments, purified and characterized for their properties and applications.

Enzymes known so far from marine microorganisms include α-Amylase, α-glucosidase, pullulanase, Cyclomaltodextrin-glucanotransferase, Agarase, alginate-lyase, κ-carrageenanase, α-galactosidase, beta-galactoside-alpha-2, 6-sialyltransferase, Cellulases, endo-1, 4-β-xylanase, β-galactosidase, β-glucosidase, β-fucosidase, endo-1,3-β-D-glucanase (I), endo-1,6-β-D-glucanase, chitinase, chitobiose-deacetylase, N-acetylglucosamine-deacetylase, N-acetylglucosamine-6-phosphate-deacetylase, lignin-modifying enzymes (LMEs): manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), and laccase, Catechol-oxidase, Cresolase (monophenol-monooxygenase), beta-1,4-mannanases, Poly(3-hydroxybutyrate)-depolymerase, Poly-beta-hydroxyalkanoate (PHA)-depolymerase, alkaline protease, Subtilisin (EC-3.4.21.62)-like serine protease, neutral protease, metal neutral proteinases, thiol protease, Collagenase, lipase, L-asparaginase**,** L-glutaminase, Tyrosinase, Hydrogenases, Superoxide-dismutase, Glucose-dehydrogenase etc.

Extremophiles are the primary source of enzymes that are active at extreme conditions of life. Harsh marine environments, such as deep-ocean hydrothermal vents, polar oceans, and extremely saline bodies of water, have yielded valuable extremophilic microorganisms. Of particular interest are the enzymes that help extremophiles to function in markedly hot, cold, acidic, basic, pressurized, saline or mineral rich environments.

Extremozymes from extremophiles isolated so far include thermostable DNA-polymerases- *Taq* polymerase, and *Pfu* polymerase, Glutamate-dehydrogenase, beta-glucosidase, thermostable esterase, thiol protease, Adenylate-kinase, hydrogenases, alpha-glucosidase, lipase, alpha-amylase, beta-lactamase, protease, endo-1,4-beta-D-xylanase, cellulase and xylanase, extremely thermostable protease, thermostable serine protease etc. Psychrostable metallo protease (almelysin), protease, chitinase, glucanase, esterase, lipase, phospholipase and DNA-degrading enzymes have been obtained from marine bacteria isolated from different sites in permanently cold arctic and antarctic habitats. Some of the cold adapted enzymes from bacteria include α- amylase, isocitrate dehydrogenase, lipase, β- lactamase, triose phosphate isomerase, subtilisin. Alkaline protease, alkaline cyclomaltodextrin-glucanotransferase, lipases, alkaline cellulases, alkaline phosphatase, endo-1, 3-β-D-glucanase, and alkaline metallo endopeptidase; and halotolerant amylases, proteases lipases, Nuclease H, cyclophilin-type peptidyl-prolyl-cis-trans-isomerase (PPIase) are also known from extremophiles.

Enzymes that are capable of carrying out very specific molecular tasks, usually related to the modification of DNA or RNA, for the creation of genetically modified organisms or for diagnostic procedures include restriction endonucleases, RNA and DNA polymerases, DNA ligases, alkaline phosphatases, kinases, reverse transcriptases, Several such enzymes have been isolated.

In general traditional microbial enzyme technology ventures are undergoing rapid transformation through the process of evolution of innovative technologies facilitated

through techniques like molecular gene cloning, protein and enzyme engineering, metabolic engineering and immobilization of enzymes.

Despite the fact that more and more novel enzymes are yet to be discovered from marine microorganisms from diverse marine environments around the world, the prospects of isolating the genes coding for the novel enzymes with potential applications have already received the attention of the genetic engineers and biotechnologists. The art of gene cloning have paved the way for easy isolation of the concerned genes, characterization and cloning and expression in hosts such as *E.coli* successfully.

Extremozymes can be produced through recombinant DNA technology without massive culturing of the source extremophiles. Some of the success stories of cloning and expression of few genes of novel enzymes of industrial importance include alkaline serine protease (*apr*II, subtilase), endo-1,4-beta-D-mannanase, salt-tolerant glutaminase-I, agarase (*pja*A), *Age*I methylase, DNA-polymerase, *lux* genes encoding luciferase, tyrosinase, α-Amyalse, pullulanase type II, β-galactosidase, β-glucosidase, acid protease (Thermopsin), S-Adenosyl-homocysteine hydrolase, Glyceraldehyde-3-P-dehydrogenase, and Glutamine synthetase. Nitrite reductase genes (*nir*K and *nir*S), structurally different but functionally equivalent single-copy genes coding for nitrite reductases, a key enzyme of the denitrification process, were used as a molecular marker for denitrifying bacteria in pacific northwest marine sediment communities.

While considering the enormous microbial diversity native to the vast marine environments of this planet earth the efforts channeled into discovery of novel enzymes from marine microbes are inadequate and warrant launching of intensive screening programs by the scientists at global level. Such a mammoth attempt alone can return large number of novel enzymes for varied purposes and services of humanity, for the simple reason that marine environments are rich treasure of novel enzymes which probably may even avoid the need for enzyme engineering or molecular cloning for designing novel enzymes for specific requirements.

In the long run, probably, marine microbial enzyme based processes would substitute several of the current chemical processes under practice.

## 1. Introduction

Microbial enzymes have several advantage over the enzymes derived from plant or animal sources by virtue of their great variety of catalytic activities, cheaper in cost, regular abundant supplies at even quantity and relatively more stability [see also - *Enzyme production*]. Major targets of modern enzyme technology continue to be preservation of foods and food components, efficient use of raw materials, improvement of food quality such as texture and taste, manufacture of dietetic foods, eliminating antinutritive substances from certain nutritional raw materials, utilization of raw materials for preparation of animal feed, and optimization of process to reduce process costs. Enzymes are used as cost -effective and environmentally sensitive substitutes for chemical processing in several industries including pharmaceuticals, food, starch, laundry, detergents, for processing textiles, leather, wood pulp and paper, and for the

production of fine and specialty chemicals, and industrial catalysis, organic synthesis and transformation of compounds and bioremediation [see also - Enzyme production]. Further, enzymes are finding applications as research tools in biotechnology and molecular biology. As such the worldwide enzyme market is estimated at $2 billion and is expected to expand rapidly in the new millennium.

Further, several industrial applications demand that industrial enzymes must be stable at extremes of temperature, pH, and salt concentration. For this reason, the isolation and characterization of enzymes from microorganisms known as "extremophiles" [see also - Biotechnological potential of the Archae] may yield useful new enzymes for this purposes. For instance, the deep seabed provides many of these extremophiles, and consequently may be of interest to companies involved in developing enzymes for this sector.

Despite the fact that several industrial enzymes are derived from terrestrial sources, marine microorganisms are yet to be exploited to its full potential, and consequently warrant immediate attention for industrial exploitation. Nevertheless, marine microorganisms have drawn the attention of investigators in the last decade and there is lot of interest on microbial enzymes now.

Marine environment, which encompass about 71 percent of the earth's surface, is not only rich with biodiversity but also a vast resource for potential microorganisms of useful applications. Microbes inhabit various habitats of marine environment that include neuston, plankton, nekton, seston, and epibiotic, endobiotic, pelagic and benthic environments. These habitats harbor a diverse range of microbes including archaebacteria, cyanobacteria, eubacteria, actinomycetes, yeasts, filamentous fungi, microalgae, algae, and protozoa. Almost all of these groups are potential source of useful enzymes that remain unexplored.

Enzymes catalyze not only biochemical reactions in living cells but also in the mineralization processes and cycling of elements in various environments. Hence, every marine microorganism should act as a dependable source of useful enzymes such as protease, amylase, lipases, chitinase, cellulase, ligninase, pectinase, xylanase, nucleases (DNAses, RNAses, restriction enzymes), etc. Production of copious quantities of amylase, alginate lyases, chitinases, glutaminase, asparaginase, arylsulphatase, phosphatase and beta lactamase by marine bacteria are known. With the advent of biotechnology, enzyme engineering and introduction of other innovative technologies there is plenty of scope for efficient management of our rich marine microbial biodiversity towards deriving novel enzymes could also be recovered from marine microorganisms and efficiently exploited not only as a cost effective biocatalyst but also as an ecofriendly reagent in the coming years.

## 2. Role of Microbial Enzymes in Marine Environment

Marine microorganisms take active part in the mineralization of complex organic matter through degradative pathways of their metabolism in the marine environments and contribute to the secondary production in sea. Complex polysaccharides such as cellulose, lignin, pectin, xylan, starch, proteins, fats, sugar, urea, aromatic and aliphatic hydrocarbons, and several other organic compounds which reach marine environments,

besides the dead plants and animal residues, are degraded by marine microorganisms. Their participation in the degradation of organic compounds and retting of ropes and fibers testify their potential as rich source of hydrolytic enzymes of industrial importance.

Microorganisms as the fastest means to search for food may often use cell-free enzymes. Heterotrophic bacteria living in particle aggregates and sediments therefore must depend largely on extracellular enzymolysis and (or) desorption of particulate and particle-sorbed organic material (OM) to generate the dissolved, low-molecular-weight compounds required for uptake and metabolism. Transport of OM into bacterial cells is limited by cell permeability, probably to molecules smaller than about 600 Daltons.

Both substrate uptake and bacterial growth may be coupled with extracellular hydrolysis. Bacterial extracellular enzymes (EE) can effect partitioning of OM between particulate and dissolved pools in the pelagic ocean and probably contribute to significant dissolved OM fluxes out of the sediment.

Studies with size-fractionated samples and with radio labeled substrates suggest that cell-attached EE dominate extracellular hydrolysis by free-living marine bacteria, perhaps due to dilution of cell-free EE below detection limits. However, significant dissolved EE activity is sometimes observed in environmental samples, and for some aquatic and sedimentary environments, cell-free EE are produced by most of the cultivable bacteria.

Cell-free EE foraging should be a powerful bacterial feeding mechanism in high-surface-area, organic-rich, liquid-bathed environments, with the potential to support maximal growth rates. Where net energetic gain is the purpose of producing cell-free EE, bacteria can usually be expected to release enzyme at high rates. Many organisms may use cell-free EE to search for food, even in environments in which actual feeding is optimized by some other mechanism.

Polysaccharases release microorganisms from their natural seat, marine sediments for example. The enzymatic activity works both on the microbial adherence polysaccharides and on the support surfaces (cellulose, pectin, etc.). Dosages of glucose confirm polysaccharase activity. An association of bacitracine, thiophenicol and a few enzymes: cellulase, pectinase, amyloglucosidase, alpha amylase, hyaluronidase, release a considerable number of bacteria. High activities of exoenzyme aminopeptidase and alkaline phosphatase were detected even in a 124,000-year-old sapropel layer**.**



### 3. Enzymes from Marine Microorganisms

Bacteria and fungi from marine environments secrete different enzymes based on their habitat and their ecological functions. Marine microbial enzymes have become the focal point of interest and several enzymes have drawn the attention of the microbial prospectors and a few enzymes were isolated from seawater and marine sediments, purified and characterized for their properties and applications. Interestingly the enzymes reported from marine environments belonged to one or more of the major

classes of enzymes viz: Oxidoreductase, Transferases, Hydrolases, Lyases, Isomerases and Ligases.

### 3.1. Polysaccharases

#### 3.1.1. Starch Hydrolyzing Enzymes

The enzymes involved in the conversion of starch to low molecular-weight compounds such as glucose, maltose and oligosaccharides are α-amylase, β-amylase, glucoamylase, debranching enzymes (pullulanase) and α-glucosidase.

α-Amylase, pullulanase and α-glucosidase from archaea are all active in the same pH and high temperature range and hence they could be used in a one-step process for the industrial bioconversion of starch [see also - Biotechnology of Archaea]. Improvement of the starch-conversion process using new efficient and thermoactive enzymes would significantly lower the cost of sugar syrup production.

##### 3.1.1.1. α-Amylase (EC-3.2.1.1)

α-Amylase has a wide range of industrial applications particularly in the manufacture of alcoholic beverages like beer, alcohol, preparation of animal feed, laundry, detergent, in starch industry, confectioneries, as desizing agent in textile industry and in the production of sugar syrup. This is one among the few major industrial enzymes, which is in great demand and mainly produced using microbial sources [see also - Industrial Biotechnology].

α-Amylase has been obtained from thermophilic archaea Pyrococcus woesei, Pyrococcus furiosus, Thermococcus celer, Fervidobacterium pennavorans, Desulfurococcus mucosus and Thermotoga maritime; psychrotrophic Vibrio isolated from a deep-sea mud, Vibrio gazogenes, Alteromonas rubra, and Mucor sp. Pseudomonas-like strain MS300, from Deep-sea, produce two major and two miner maltotetraohydrolases (G4-amylase). Under high hydrostatic pressure, the strain MS300 produced more amylase than under atmospheric pressure.

##### 3.1.1.2. α-Glucosidase (EC-3.2.1.2)

Alpha -Glucosidase (EC-3.2.1.2) is mainly used in the starch industry along with α-amylase**.** An extremely thermostable α-glucosidase (EC-3.2.1.2) was isolated from the hyperthermophilic marine archaebacterium, *Pyrococcus furiosus* and *Pyrococcus wesei*. The enzymes from these microorganisms appear to be the most thermostable α-glucosidases described.

##### 3.1.1.3. Pullulanases (EC-3.2.1.41)-Debranching Enzymes

Similar to α-amylases and α-glucosidase, pullulanase are mainly used in starch industry. Pullulanase type I is a typical bacterial enzyme that is specific for α-1, 6 linkages in branched oligosaccharides. It is unable to attack α-1,4-linkages in a-glucan.

The production of pullulanases type I seem to be very rare amongst thermophilic microorganisms that produce a thermoactive pullulanase with a temperature optimum of 90°C. This enzyme has been reported only *in Fervidobacterium pullulanolyticum.* Pullulanase type II (amylo pullulanase), which is capable of hydrolysing the α-1,4-linkages and branching points (α-1,6-linkages) in polysaccharides and limit dextrins is mainly found in anaerobic bacteria and is widely distributed among thermophilic bacteria and archaea. Marine thermophilic *Thermococcus litoralis* (Tl) DSM 5473 (optimal growth temp. 90°C) and *Pyrococcus furiosus* (Pf) DSM 3638 (98°C) produce pullulanase type II whose thermoactivity and thermostability were enhanced in the presence of 5 mM $Ca^{2+}$, and under these conditions, enzyme activity could be measured at temperature of up to 130-140°C. Each of these enzymes was able to hydrolyze, in addition to the alpha-1, 6 linkages in pullulan, alpha-1, 4 linkages in amylose and soluble starch. The enzymes appear to represent highly thermostable amylopullulanase versions of those which have been isolated from less thermophilic organisms. *Pyrococcus woesei, Thermococcus celer, Fervidobacterium pennavorans,* and *Desulfurococcus mucosus* have also been reported to produce pullulanase type II.

### 3.1.1.4. Cyclomaltodextrin-glucanotransferase (CGase EC-2.4.1.19)

Cyclomaltodextrin-glucanotransferase (CGase EC-2.4.1.19), which has potential application in cyclodextrin production, was isolated from alkalophilic *Bacillus subtilis* sp. from a deep-sea mud.

### 3.1.2. Agarase (EC-3.2.1.81*)*

Agarase has been the subject of investigations for quite some time owing to its immediate applications in gene technology for the elution and isolation of DNA fragments from agarose gels after electrophoresis; in the preparation of algal protoplasts such as from red alga *Gelidium robustum;* seaweed polysaccharide characterization; in the production of simple sugars, including neoagarobiose, neoagarotetraose and neoagarohexaose; degradation of agarose to oligosaccharides, facilitating the liquefaction of agar and agarose gels; and in the defouling of fermentors and bioreactors. Moreover, the purified enzyme could effectively control red algae bloom contaminations, prevent the biofouling of submerged marine surfaces or pipes by contaminating complex polysaccharide layers, or treat such biofouled surfaces after contamination. Marine Bacterial agarase has a high level of activity for the depolymerization of complex polysaccharides, including agar and agarose. Agarase is obtained from few bacteria. Agarase, named agarase-0107, an endo-type β-agarase that hydrolyzed the β-1, 4-linkage of agarose to yield neoagarotetraose and neoagarobiose at a pH of around 8, have been isolated from *Vibrio* sp. JT0107, that requires seawater salts for growth. *Pseudomonas stutzeri*, *Aeromonas* sp and *Vibrio* sp, isolated from sea produce agarase, which have been characterized.

### 3.1.3. Alginate-lyase (EC-4.2.2.3)

The alginate-lyase may be useful for the conversion of brown algal biomass to methane. This observation has led to the isolation and characterization of an intracellular inducible alginate-lyase from marine *Bacillus* sp., *Alteromonas and Photobacterium*.

### 3.1.4. κ-carrageenanase (EC-3.2.1.83)

A Cytophaga-like, carrageenan-degrading bacterium, referred to as strain Dsij, isolated from the marine red alga *Delesseria sanguinea*, simultaneously produce extracellular κ-carrageenanase (EC-3.2.1.83) and iota-carrageenanase in the presence of crude λ-carrageenan.

### 3.1.5. α-Galactosidase (EC-3.2.1.22)

α-galactosidase is used in the sugar industry for enhancing the yield of sucrose by the hydrolysis of raffinose, an α-galactoside. α-galactosidase was obtained from marine *Alteromonas spp***.,** isolated from sponges and alga *Polysiphonia* sp., and from bacteria associated with mussel (*Crenomytilus grayanus)* and scallop (*Patinopecten jessoensis)***.**

A novel beta-galactoside-alpha-2, 6-sialyltransferase (EC-2.4.99.1) is produced by *Photobacterium damsela* JT0160 (FERM BP-4900), ATCC 33539 or ATCC 35083. The enzyme should be useful in modification of glycoproteins and industrial-scale production of sialosides.

### 3.1.6. Cellulases and Related Enzymes

Cellulases are used in the manufacture of alcohol, flavors, maize gluten, silage, in laundry & detergents, and in wastewater treatment. A symbiotic bacterium found in the gland of *Deshayes* of the marine shipworm and *Aspergillus terreus* isolated from the seawater produce cellulase. Thermophilic *Thermotoga* sp produces thermostable exo-1, 4-β-cellobiohydrolase (105$^{o}$C).

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**Biographical Sketches**

**Dr. M. Chandrasekaran** is professor of Biotechnology, in the Department of Biotechnology, Cochin University of Science and Technology. He graduated in marine biology from Annamalai University and took his PhD in marine microbiology from the Cochin University of Science and Technology in 1985. He is a career awardee in microbiology, of the University Grants Commission, Government of India. He was an overseas associate of the Department of Biotechnology, Govt. of India during 1997-98 at Hiroshima Universtity, Department of Fermentation Technology. He has 23 years of research experience in the field

of marine microbiology. He has been working on marine microbial enzymes since 1988 and has contributed significantly to the growth of marine microbiology, through several publications in peer reviewed international journals. He is one among the pioneers who have introduced bioprocess technologies particularly solid state fermentation for the production of marine microbial enzymes.

He has a total of 95 publications including presentations in symposia/seminars. He had supervised 15 doctoral theses, of which 10 theses were on microbial enzymes. He is also the founder head of the Department of Biotechnology of the Cochin University of Science and Technology, which is offering masters degree program in Biotechnology. He has served as subject expert in several academic /assessment and selection committees at various levels. He has also served as referee for reviewing of papers for several international journals. He is a consultant several industries for microbial bioprocesses. He founded the society for Biotechnologists of India in the year 1995. His current research interest includes marine microbial enzymes, extremozymes, strain improvement and enzyme engineering.

**S Rajeev Kumar** is a research associate working with Prof. M. Chandrasekaran. He took his masters degree in Biotechnology from Cochin University of Science and Technology, Cochin, India and is submitting his doctoral thesis on marine bacterial enzymes. He has been specializing in use of immobilized whole cell processes for production of marine bacterial enzyme. He has over five years of research experience in the field of marine microbial enzymes.