

STUDIES ON ENZYMATIC PROPERTIES OF SELECTIVE SOUTHERN OCEAN BACTERIA

PROJECT WORK

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

by

EBSHIBA N (41300009)



**DEPARTMENT OF BIOTECHNOLOGY
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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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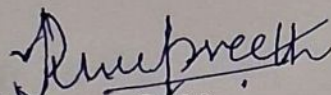
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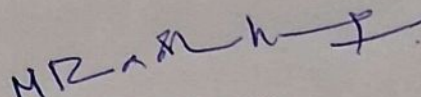
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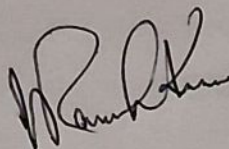
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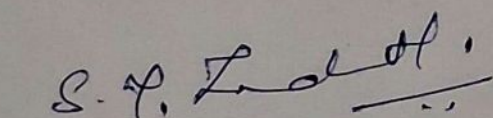
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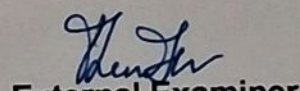


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DECLARATION

I, **EBSHIBA N (41300009)** hereby declare that the Project report entitled "**STUDIES ON ENZYMATIC PROPERTIES OF SELECTIVE SOUTHERN OCEAN BACTERIA**" done by me under the guidance of **Mrs. JESIA PERSIS PREETHI M** (internal guide) and **Dr. M. RADHAKRISHNAN** (external guide) at **CENTRE FOR DRUG DISCOVERY AND DEVELOPMENT, SATHYABAMA RESEARCH PARK, Sathyabama Institute of Science and Technology, Chennai**, is submitted in partial fulfilment of the requirements for the award of a Bachelor of Science degree in **Microbiology**.

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ABSTRACT

Bacteria offer superior biological materials like cold active enzymes with potential economic and biotechnological uses. The present study aimed to screen the enzyme properties of Southern Ocean bacteria. For this study, about 30 bacterial strains previously isolated from Southern Ocean were obtained from Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai. For this enzyme study, 30 bacterial strains were subculture and incubated in 4 °C and 30 °C. All the strains were screened for various enzymatic activities which include amylase, lipase, protease, xylanase, cellulase, urease and glutaminase. Among these, SOSIST 3, SS-M-11-5, Sea Ice 5 and Sea Ice 9 showed promising activity for at least one substrates at 30 °C. Surprisingly, Sea Ice 9 showed activity even at 4 °C. By time point assay, it revealed that Sea ice 9 showed maximum lipase activity for about 20 mm in diameter in 10 days of incubation both 4 °C and 30 °C. The quantitative assay by DNS and p-NPP method were performed to identify the unit of the sugar reduced. Further, the crude enzyme was partially purified and characterized by ammonium sulphate precipitation and molecular weight was determined by SDS-PAGE.

Keywords: *Psychrophilic bacteria, Lipase, Sea Ice, Enzyme activity*

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CHAPTER 1

INTRODUCTION

1.1 CRYOSPHERE ENVIRONMENT

Earth's surface is quite cold. Of the biosphere, around 85% is permanently exposed to temperatures below 5 °C. The Arctic and Antarctic, high mountains, and deep oceans are examples of cold habitats (Xu *et al.*, 2024). Mostly, this low temperature environment is composed of sea ice (which makes up 13% of the Earth's surface), glaciers (10% of land surface), snow (35% of land surface), permafrost (24% of land surface), and the deep sea (which makes up 90% of the ocean's volume). Caves, freezing deserts, lakes with cold water, and freezing soils especially sub surfaces are few other chilly locations (D'Amico *et al.*, 2006) In 1902, microorganisms known as "psychrophiles" were cultivated at 0 degrees Celsius by Schmidt-Nielsen (Margesin *et al.*, 2006) The fact that cold is the primary habitat of the biosphere is something that most scientists tend to overlook. The oceans make up 71% of the earth's surface, while the polar areas make up 14%. Over 90% of the oceans have a temperature of 5°C or less (by volume). The ocean is home to naturally occurring psychrophilic microorganisms. For this reason, it is quite easy to isolate psychrophilic bacteria from the ocean floor, from both polar regions, and from salt wedges, which are bays where cold oceanic water intrusion occurs (Morita, 1975).

1.2 PSYCHROPHILIC BACTERIA

The ability of a notable and distinct subset of bacteria to grow at low temperatures that is, at or close to 0°C distinguishes them from mesophiles and thermophiles. These low-temperature bacteria, which go by the name "psychrophiles," are widespread in nature. A wide range of species are found in food, water, soil, and other settings where they carry out important chemical transformations that can be beneficial or harmful, frequently at temperatures below which other bacteria cannot survive (Ingraham, 1958) A type of bacteria known as psychrophilic bacteria is one that does well in cold environments. They usually inhabit extremely cold areas. However, because there is a growing requirement for low-temperature food storage, psychrophilic bacteria have begun to multiply in cold storage and refrigerators, endangering the safety of food. The work examines the most effective strategies for

psychrophilic bacteria to cool down, considering many elements such the cell membrane, antifreeze chemicals, psychrophilic enzymes, cold shock proteins, gene regulation, and metabolic levels. There's also a succinct explanation of the psychrophilic mechanisms (Jin *et al.*, 2022).

1.3 COLD ACTIVE ENZYMES

Usually thermally unstable, low-temperature tolerant microbes produce extremely catalytically efficient cold-adapted enzymes. Since cold-adapted enzymes have special properties that make them particularly interesting for researching stability/flexibility interactions or for potential industrial process applications, researchers and industry have recently focused on these enzymes. Particularly the enzymes lipases and esterases have a plethora of possible biotechnological uses. In organic chemistry and biotechnology, this enzyme is actually the most often used biocatalysts. Present-day genetic engineering methodologies, in conjunction with growing structural and functional data, provide additional industrial requirements adaptation and innovative application research. Consequently, the study shows about the several psychrophilic lipases enzymes active in cold temperatures (De Pascale, *et al.*, 2009). Due to their increased stability and cold activity, these enzymes are becoming more and more popular in many industrial bioprocesses. The food industry has developed uses for a number of cold-active enzymes, including xylanases, lipases, amylases, and proteases. It is now feasible to express the candidate enzyme in a heterologous host for increased enzyme production and enhance the catalytic properties of interest thanks to advances in enzyme engineering techniques and our growing understanding of protein structure and function (Kumari *et al.*, 2021) Lipolytic enzymes are currently the subject of a great deal of interest due to their potential in biotechnology. Normally, lipases catalyze heterogeneous processes at the water-insoluble system interface (Eltaweel *et al.*, 2005). The hydrolysis of long-chain acylglycerols in aqueous emulsions is catalyzed by triacylglycerol hydrolases, which are also referred to by their unimportant term, lipases. Furthermore, they have the ability to catalyze other processes, such as the hydrolysis-reversed trans-esterification, inter-esterification, and esterification between an alcohol and a fatty acid. These enzymes' wide range of substrate and reaction specificity, their capacity to catalyze the reactions without the need for a cofactor, and their adaptability to a variety of industrial settings have

led to the development of several strategies for using them. The hydrolysis of fats and oils continues to be their principal industrial application today (Svetlana Laura 2010). Since the beginning of enzymology, microbial proteases which hold a crucial position in terms of their commercial applications have been the subject of intense research. Cold-adapted microorganisms that have been isolated from cold climates are a possible source of cold-active proteases. Few are known to be recognized as commercial producers of proteases, despite the fact that various microbiological sources are available for their production. Cold-active proteases and the microbes that produce them are valuable commodities with a wide range of industrial and biotechnological uses, including as detergent and food additives, environmental bioremediation, biotransformation, and molecular biology applications (Rathour *et al.*, 2020). Peptide bonds are hydrolyzed by proteases (Bruno *et al.*, 2019).

1.4 NON-MEDICAL APPLICATIONS OF PSYCHROPHILIC ENZYMES

Lipase is an attractive option for removing fatty deposits if the right enzymes are available that are active during washing. Detergents perform optimally at approximately 60 °C and pH 11. While increasing the usage of lower washing temperatures may facilitate the application of lipases at lower temperatures, lipases may still benefit from operating under comparable settings. Furthermore, lipases would need to be resistant to protease attack and able to function in the presence of known lipase inhibitors, like surfactants, an element included in washing formulations (Macrae *et al.*, 1985). In detergent industry once specific restrictions were discovered in thermo stable proteases, proteases with high activity at low temperatures were developed. In 1985, these low-temperature proteases were made available for purchase as third-generation proteases. Preserving energy and time was the primary motivation behind the development of these low-temperature proteases. Along with alkali stability, commercial detergents, oxidants and surfactants should also be present in a good detergent protease's stability profile. In comparison to detergents without enzymes, microbial cold-adapted proteases and detergents have been shown to be more successful when used for cold washing. A wide range of commercial detergents are compatible with cold-adapted proteases, which have demonstrated exceptional activity and stability in broad alkaline pH at lower washing temperatures. Furthermore, in surfactants and bleaches that are sold commercially, cold-adapted alkaline proteases have

demonstrated exceptional stability. Also, compared to enzyme-free detergents, commercial detergents and cold-adapted proteases have removed proteinous material from clothing soiled with blood, egg yolk, chocolate, tea, grass, etc. at low temperatures far more effectively. They are appropriate as detergent additives for the dishwashing and laundry sectors because of these qualities. Because of their broad substrate selectivity and generally favorable activity levels at a feeder additive, proteases Animal farm can increase their manufacturing efficiency by using environmentally friendly feed additives at physiologically appropriate pH and temperature. The keratinolytic activity of cold-adapted proteases can support and facilitate the biotechnological processing of biomaterials made of keratinous waste from the chicken and leather industries. in the textile sector Because cold-adapted proteases can lessen the negative effects of chemicals on fabrics, they may find utility in the textile industry. They prolong the life of silk and woolen materials by keeping their quality of fabric following a lower-temperature wash. According to reports, treating woolen fabrics with a cold-adapted protease treatment can enhance their surface appearance, lessen their brittleness, and maintain the silk fabric's finishing. Proteases that can resist low temperatures have potential uses in bioremediation, such as the environmental biodegradation of pollutants high in protein and the treatment of wastewater in cold climates. The capacity of anaerobic psychrophilic bacteria seen in Antarctic environments to flourish and generate proteases on a variety of substrates suggests that they may find application in the digestion of protein-rich substrates such as night soil. In a different instance, a psychrophilic *Pedobacter cryoconitis* was shown to be appropriate for treating dirty wastewater in cold temperatures and to use a variety of organic substances, including oils, carbohydrates, and proteins. Given their thermo stability and ability to be quickly and selectively activated when needed, cold-adapted proteases have demonstrated encouraging potential in the food business. Furthermore, these frigid-adapted the optimum enzymatic activity of enzymes at low temperatures reduces the possibility of microbial contamination, which makes them advantageous (Farhan., 2020). When making cosmetics, psychrophilic lipase is widely used. In the paper industry nowadays, a variety of psychrophilic enzymes are employed. The main commercial contributions are xylanase, which can withstand the pulp's high temperature (55–70 °C) and alkaline pH with appropriate bio bleaching.

Psychrophilic cellulases are used in commercial biotechnological procedures for microbial delignification and as biosensors for the examination of pulp fiber surfaces.

1.5 MEDICAL APPLICATION OF PSYCHROPHILIC ENZYMES

Pharmaceuticals use enantioselective inter esterification and trans esterification extensively for selective acylation and deacylation. Lipases are significant for use in the hydrolysis and trans esterification reactions in pharmaceutical applications. They are essential to the synthesis of digestive aids and specialty lipids. The enantiomeric values and stereo preference are significantly altered by temperature changes during the esterification procedure. When it comes to modifying monoglycerides for use as emulsifiers in pharmaceutical applications, lipases are crucial. Cold-active lipase-producing psychrophilic could be a valuable supply of polyunsaturated fatty acids for the pharmaceutical sector. Lipases are an important biocatalyst in biomedical applications due to their excellent capacity for precise region selective reactions in a variety of organic solvents with broad substrate recognition (Novak *et al.*, 2013)

CHAPTER 2

REVIEW OF LITERATURE

Polar environments with low water temperatures and seasonal pack ice movement support huge bacterial communities. Bacteria may play a key role in energy transfer within the Southern Ocean (Nichols *et al.*, 2005). The microbiological biodiversity of Antarctic environments is relatively unknown. Deep-sea hydrothermal vent settings are distinguished by tremendous pressure, intense temperatures, and heavy metals (Bowman., 2001). At low temperatures, Psychrotolerant bacteria typically thrive at the same or higher rates as psychrophiles. Psychrotolerant bacteria can be found in even the coldest settings, simply because many of them are Eco physiologically resilient and nutritionally versatile (Struvay *et al.*, 2012).

The bacterial communities linked with sea ice obtained from Antarctic coastal areas were studied using both a phenotypic and a phylogenetic method based on genes encoding 16S rRNA (16S rDNA). The diversity of bacteria associated with sea ice was compared to the bacterial diversity of seawater beneath sea ice. Psychrophilic (optimal growth temperature $< \text{or} = 15\text{ }^{\circ}\text{C}$; no growth occurs at $20\text{ }^{\circ}\text{C}$) bacterial diversity was found to be considerably enhanced in sea ice samples harboring platelets (Bowman *et al.*, 1997).

Most psychrophilic enzymes optimize their activity at low temperatures at the price of substrate affinity, lowering the transition state's free energy barrier. Furthermore, a low temperature dependence of activity ensures a moderate decline in catalytic activity in the cold. In these naturally evolved enzymes, the optimization to low temperature activity is accomplished by destabilizing the structures containing the active site or by destabilizing the entire molecule. This entails reducing the quantity and intensity of all forms of weak contacts, as well as the elimination of stability factors, which leads to enhanced dynamics of active site residues in the cold. Given the modest structural changes required for low temperature activity, directed evolution appears to be the most appropriate approach for engineering cold activity in biological catalysts. Psychrophile bacteria are extremophilic microorganisms that can thrive and multiply at low temperatures and have successfully colonized many

low-temperature settings on Earth. Cold soils play a crucial biogeochemical role in primary biomass production and nutrient cycling, but little is known about the identity and habitat of microorganisms that thrive under such conditions. Many bacteria have been shown to be resistant to adverse environmental conditions and can survive in hard environments. Because of their high catalytic activity and heat tolerance, psychrophilic bacteria produce cold-active enzymes and proteins that find utility in molecular biology, medical research, industrial food or feed technology, detergents, and cosmetics. Additionally, they are utilized for the bioremediation of polluted cold soils and waste water (Gandhi, 1997).

Lipases have the highest substrate selectivity of any known enzyme. This gives these enzymes an almost limitless number of possible applications. Lipases can be used to produce pharmaceuticals, cosmetics, leather, detergents, meals, fragrances, medical diagnostics, and other organic synthetic materials (Ray, 2012). The commercial potential of microbial lipases has prompted further research into them. Lipases are added to household and industrial laundry detergents, as well as dishwashers, to remove fatty acid residues and unclog clogged drains. Their applications in our daily lives are rising day by day (Quan-Fu Wang *et al.*, 2008). Cold-active proteases with high catalytic efficiency at low temperatures can be employed as flavor enzymes in the food sector. For example, cold-active protease can improve the flavor of refrigerated meat treatment. Antarctic microbes may be a good source of cold-active enzymes. It is of significant biological significance to investigate their cold-active enzymes, but also substantial practical value because of their potential for food-related uses (Runying Zeng *et al.*, 2003).

An extracellular protease was isolated from the deep-sea psychrophilic bacterium strain DY-A, which was recognized as a *Pseudomonas sp.* The strain's optimal growth and protease - producing temperatures were all 10 °C, with protease secretion occurring exclusively at temperatures below 20 °C. The enzyme was most active at 40 °C and a pH of 10. It was inhibited by Di isopropyl fluorophosphate and phenylmethyl sulfonyl fluoride, showing that it is a serine protease. Chelators such as EDTA, EGTA, 1,10-phenanthroline, and 2,2'-bipyridyl all decreased activity. The enzyme was susceptible to denaturing agents such as SDS, urea, and guanidine HCl, but resistant to thiol-containing reducing agents like dithiothreitol. The enzyme

was effective against N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide. The enzyme's native molecular mass was measured using native PAGE and SDS-PAGE was 25 kDa (Feller *et al.*, 1996).

Enzymes that are suited to operate at low temperatures are produced by psychrophilic organisms, which include microorganisms and other ectothermic organisms that live in polar, deep-sea, or other continuously low temperature settings. Although they are very thermolabile, these enzymes exhibit good catalytic efficiency at low and moderate temperatures. With their high specific activity and quick inactivation at as low as 30 °C, they present a huge biotechnology potential when combined with the generating micro-organisms. Researchers have examined the molecular underpinnings of the adaptability of fish living in both North Atlantic and Antarctic Sea water's trypsin, as well as Antarctic bacteria's α -amylase, subtilisin, and triose phosphate isomerase. By comparing the three-dimensional structures of North Atlantic trypsin, which were obtained through protein modeling or X-ray crystallography, with those of their mesophilic counterparts, it can be observed that the molecular modifications lead to a decrease in intra molecular interactions and an increase in solvent interactions, thereby increasing the structure's flexibility. The best approach is chosen for each enzyme so that it can accommodate the substrate with the least amount of energy expenditure. The selection pressure is mainly directed towards the synchronization of the particular activity with ambient thermal conditions, which carries a cost in terms of thermo sensibility. However, it is still possible to stabilize the structure of these enzymes without compromising their high catalytic efficiency, as evidenced by site-directed mutagenesis studies conducted on Antarctic subtilisin (Gupta *et al.*, 2023).

Psychrophilic enzymes, which are necessary for the synthesis of medicines that need low temperatures, such as antibiotics. Furthermore, as low temperatures are frequently encountered in bioremediation procedures, psychrophilic enzymes can be employed in these settings. Research on metagenomics has demonstrated the possibility of finding novel psychrophilic enzymes in cold environments from uncultivated bacteria. Psychrophilic enzymes can now be produced in huge quantities for use in industry thanks to recombinant DNA technology. Psychrophiles are a new and exciting source of commercial enzymes that are suited to the cold.

These enzymes have the potential to drastically lower energy usage and environmental impact in a variety of sectors. Future research and technological developments are expected to uncover more varied and effective cold-adapted enzymes from psychrophilic bacteria, hence broadening the range of uses for these enzymes (Qian *et al.*, 2023).

Keeping food cold is a crucial part of food quality preservation. Nonetheless, certain microbes can tolerate low temperatures, which may cause the quality of the meal to decline. These cold-tolerant microbes have developed a variety of adaptation mechanisms, and one of the most important ones for preserving their metabolic processes is the development of cold-adapted enzymes. The recent research views a lot on food-borne microbe species that have adapted to the cold and their methods. Additionally, it contrasts the enzymatic characteristics, development, and structure of microbial cold-adapted enzymes to show how they sustain function at low temperatures. Furthermore, the possible influence of these enzymes—which include proteases, lipases, amylases, decarboxylases, etc.—on food quality. Also, the development of enzyme inhibitory techniques research in recent years, including high pressure processing, dense phase carbon dioxide, cold plasma, ultraviolet light, ohmic heating, and biological resourced inactivators. These techniques may serve as a guide for reducing the activity of cold-adapted microorganisms' enzymes in food and extending its shelf life (Dhole *et al.*, 2023).

Psychrophilic and cold-tolerant bacteria, in particular, have established themselves in frigid environments that are considered uninhabitable by other creatures despite the low temperatures on Earth. This can be mostly ascribed to their innate ability to adapt and survive in the frozen environment. The diversity and existence of psychrophilic bacteria in the many Earthly spheres are reviewed here. There is disagreement over the possible uses of psychrophilic bacteria in different businesses, where they can be marketed as unique suppliers of vital enzymes (Qin *et al.*, 2023).

Popular dairy products like ultra-high temperature sterilized milk (UHT) are prized for their convenience and lengthy shelf life. However, during storage, problems with fat quality such as creaming and flavor deterioration, as well as protein gel ageing,

may occur. Psychrotrophic bacteria produce thermo stable enzymes that are the main source of these issues. Four representative strains of thermo stable psychrotrophic bacteria were chosen for the investigation in order to artificially contaminate UHT milk. The milk samples that were contaminated with *Pseudomonas fluorescens*, *Chryseobacterium carnipullorum*, *Lactococcus raffinolactis*, and *Acinetobacter guillouiae*, respectively, showed significant whey separation after 11, 11, 13, and 17 weeks of storage. As a result of the experiment, which also looked at pH, Zeta potential, and particle size in four sample groups, it was shown that UHT milk loses stability with time. Protein and fat content in the top and bottom layers of the milk were also calculated. Additionally, there was a bitter taste in the spoilt milk samples; ketones and acids were responsible for the overpowering smell. Psychrotrophic bacteria produce thermo stable enzymes by the use of three major metabolic pathways, which were identified by the metabolomics analysis: ABC transporters, butanoate metabolism, and alanine, aspartate, and glutamate metabolism. The flavor and nutritional value of UHT milk are significantly impacted by these enzymes (Bhatia *et al.*, 2021).

In order to create effective biochemical processes that will assist to shorten reaction times, require less energy, and be environmentally benign, various industries are currently searching for such innovative psychrophilic enzymes. By lowering the total cost of the finished products, these bioprocesses will contribute to an increase in the profit margin. Numerous "omics" techniques, including metagenomics, genome mining, bioinformatics, and in-silico analysis, have been used to identify, screen, and functionally characterize the majority of industrial enzymes. These techniques allow researchers to anticipate the precise enzyme function from either cultured or uncultured psychrophilic bacteria (Collins *et al.*, 2023).

By developing a wide variety of structural solutions, cold-adapted enzymes have been able to overcome the increased viscosity and decreased molecular kinetic energy that are intrinsic to their environment while maintaining high catalytic rates. Their most frequent characteristics are low substrate affinity, intrinsic structural instability, and great flexibility. This cold-adaptation paradigm is not general, though, as other cold-active enzymes have been discovered that exhibit great stability, high substrate affinity, and/or unaffected flexibility, suggesting alternative methods for

adaptation. In fact, depending on the enzyme in question, its function, structure, stability, and evolutionary background, cold-adaptation may include any of a wide variety of structural alterations, or combinations of modifications (Sunny *et al.*, 2021). Biocatalysts offer numerous benefits in comparison to conventional chemically assisted processes that are commonly used in industries. Thus, with a growing interest in thermophilic and psychrophilic bacterial species, the hunt for new enzymes has intensified over time. Many industrial uses need severe temperatures, and enzymes or extremozymes isolated from these species have demonstrated an affinity for such temperatures. Nevertheless, a significant barrier prevents these enzymes from being used (Liu *et al.*, 2023).

In contrast to cold-adapted enzymes originating from plants and animals, microorganism-derived enzymes have garnered significant attention due to their short production cycles, high yield, and ease of separation and purification (Yang *et al.*, 2023). The contemporary biotechnology sector has a need for macromolecules that are resistant to harsh conditions. Cold-adapted proteases are one type that has advantages over other types, including low energy input during synthesis and inactivation and the ability to maintain high catalytic efficiency at low temperatures. On the other hand, cold-adapted proteases are distinguished by their capacity for sustainability, preservation of the environment, and energy efficiency. As such, they are of great ecological and economic significance with respect to the use of resources and the global biogeochemical cycle. The industry has been paying more and more attention to the development and use of cold-adapted proteases of late, but their potential uses have not yet been fully realized. This has severely limited the industry's ability to promote and use these proteases (Esakkiraj *et al.*, 2022).

The prevention of microbial pathogenicity through the inhibition or removal of biofilm is a crucial approach, and the use of chemicals like antibiotics encourages the emergence of resistance. Consequently, to effectively eliminate biofilm, ecologically benign, biologically mediated techniques are required. The most successful approach for managing the infection caused by biofilm is enzyme-mediated biofilm clearance. Numerous enzymes, like glycoside hydrolases, are present (Shaw *et al.*, 2023). In the Antarctic landmass, prokaryotic life is constantly changing under a variety of harsh conditions. By creating special defense mechanisms against various

extremes such as cold stress, UV stress, metal stress, low nutrient stress, etc., microbes are able to survive in a variety of environments, including lakes, snow, and the ocean. The biological carbon pump is greatly influenced by the bacteria found in the ocean. They lessen atmospheric CO₂ by about 40%. Numerous microbial communities from Antarctica's marine and terrestrial habitats have already been documented. The extracellular enzymes protease, amylase, lipase, esterase, and others that aid in the mineralization of the organic materials contained in the snow are produced by a multitude of microorganisms that have been found in snow. Some bacteria create pigments to protect themselves against oxidative damage and damaging radiation. In order to survive in cold stress, the microorganisms also secrete cold-active proteins, which are useful in cold environments. Certain bacteria produce stress-responsive proteins, enzymes, extracellular polysaccharides, and other bioactive substances to withstand the effects of radiation and cold. Therefore, industrial and biotechnological uses such as antioxidant, photoprotective, cryoprotective, antibacterial, and emulsifying properties of the bioactive compounds are of significant interest to microbes from severe environments. These bioactive compounds also exhibit intriguing properties in the field of biological applications, such as anti-tumor and anti-proliferating agents in drug manipulation (Zhang *et al.*, 2016).

Cold-adapted enzymes originate from the cold, ultra-oligotrophic deep-sea water of the South Pacific Gyre (SPG, 20 °S – 45 °S). The distinctive qualities of psychrophilic enzymes produced from culturing microorganisms in the SPG, however, were still mostly unclear. The diversity of cold-adapted enzymes was ascertained in this work by analyzing the degradation characteristics of 174 cultures taken from the deep water of the SPG. Thus, it was determined whether the bacteria could break down DNA, proteins, lipids, and polysaccharides at 4, 16, and 28 °C. Amylase, chitinase, cellulase, lipase, lecithinase, caseinase, gelatinase, and DNase were among the extracellular enzyme activities that the majority of the isolates displayed at 4, 16, and 28 °C. Furthermore, at 4 °C, almost 85.6% of the isolates generated cold-adapted enzymes. The isolates that produced psychrophilic enzymes were mostly found in the Gammaproteobacteria genera *Alteromonas* and *Pseudoalteromonas*. *Alteromonas* released eight enzymes, excluding cellulase and chitinase, while *Pseudoalteromonas* broke down nine different types of

macromolecules but not cellulose. It's interesting to note that isolates of Gammaproteobacteria have higher enzymatic activity at 4 °C than at 16 or 28 °C. Furthermore, a gene encoding an α -amylase (Amy2235) from *Luteimonas abyssi* XH031T was cloned, produced, and its characteristics were analysed. These cold-active enzymes might be extremely useful for both industrial and scholarly applications. Furthermore, the isolates' ability to break down different kinds of organic materials would point to their distinct ecological functions in the deep biosphere's basic biogeochemical cycling (Aydemir *et al.*, 2020).

Nano flowers are newly created flower-shaped nanoparticles with several petal layers to enhance stability and surface reactivity. Because they can get beyond issues with enzymes, such as poor stability, high production costs, substrate/product inhibition, and challenging recovery, organic-inorganic hybrid Nano flowers have received special attention. The research showed that three enzymes (α -amylase, lipase, and protease) and Cu²⁺ ions were combined to create triple enzyme-inorganic hybrid Nano flowers (TrpE@ihNFs). Several techniques, such as SEM, FTIR, EDX, and XRD, were used to characterize the morphology and chemical perspective of TrpE@ihNFs. Then, using spectrophotometry, the stability and activity of TrpE@ihNFs was evaluated against free enzymes, such as lipase, amylase, and protease, at various pH and temperature ranges. According to the results, TrpE@ihNFs had much higher enzyme activity and stability than any individual free enzyme. Finally, TrpE@ihNFs, which have potential applications in the future for the treatment of wastewater, biosensors, biocatalysts, and bio-related devices (Maghraby *et al.*, 2023).

Enzymes are fundamental components of many industrial goods and play important roles in a variety of industrial sectors. Compared to their free versions, immobilized enzymes are more resistant to environmental changes and are easier to recover or recycle. The main advantage of immobilization is that it shields the enzymes from adverse environmental factors like high temperatures and acidic pH levels. In addition to being used in water treatment plants, the immobilized enzymes can be used in a variety of large-scale industries, including the food, pharmaceutical, water, detergent, and textile sectors. Appropriate carrier materials and an appropriate enzyme immobilization approach are selected based on the needed application.

Techniques for immobilizing enzymes include covalent binding, trapping, encapsulation, adsorption, and so forth (Kuddus, 2018).

Global scientific community's attention was drawn to cold-active enzymes and their potential use in a variety of industries, including the food business. Enzymes that are cold-active, sometimes referred to as psychrophilic enzymes, have significant catalytic activity at low to moderate temperatures. These enzymes use less energy in biological reactions because of their low temperature activity, which also helps to stabilize delicate molecules in the reaction media. Enzymes that are active in cold temperatures are primarily derived from psychrophilic and psychrotrophic bacteria that inhabit cold environments. There are currently very few cold-active enzymes known, and their use in the food business has received very little research compared to mesophilic and thermophilic enzymes (Rabbani *et al.*, 2023).

Microbial lipases are widely used biocatalysts due to their broad range of catalytic activities in both aqueous and non-aqueous conditions. The microbial lipases originating from three different sources: thermophiles, mesophiles, and psychrophiles. The structural variety of mesophilic, thermophilic, and psychrophilic lipases in temperature adaptation and industrial applications. Lipases have microbial origins that are conceptually active, proficient, and have a wide range of commercial applications with the production of modified molecules. Therefore, it is interesting to learn about the molecular mechanisms by which lipases naturally adapt to temperature. Nevertheless, lipases derived from extremophiles, such as thermophiles and psychrophiles, are frequently employed in the design of biotransformation reactions that have been predicted to catalyze reactions that are otherwise not achievable with mesophilic lipases and have higher yields, fewer byproducts, or useful side products. As a versatile biological catalyst, lipases have opened up new possibilities for addressing the needs of a number of industries, including biodiesel, beverages, food, detergents, leather, textiles, and medicines (Hamid *et al.*, 2022).

Compared to their related proteins, psychrozymes are expected to have a more flexible structure. These proteins' structural flexibility allows them to change conformation during catalysis, increasing catalytic efficiency at low temperatures

(Vaseekaran *et al.*,2011). The most common inhabitants of cold environments are psychrophilic microbes, which have the ability to produce a variety of cold-active enzymes with use in both scientific and industrial settings. Psychrophiles have evolved to tolerate cold temperatures while still thriving, proliferating, and engaging in active metabolic processes (Bose *et al.*, 2013).

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To study the enzymatic properties of Southern Ocean bacteria.

3.2 OBJECTIVE

- Collection and screening of enzyme producing bacteria
- Quantitative and qualitative screening of enzymatic bacteria
- Extraction and purification of enzymes
- Characterization and identification of potential strain

CHAPTER 4

MATERIALS AND METHODS

4.1 DESCRIPTION OF PSYCHROPHILIC BACTERIA

For this study, about 30 bacterial cultures were obtained from the Cryosphere Microbial Research Lab, Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu. All the cultures were previously isolated from Sea ice and vertical water samples collected from less explored ecosystems of Southern Ocean region in Antarctica and maintained in respective agar slants as well as in 30% glycerol broth (Table 4.1).

Table 4.1: List of Psychrophilic strains used for enzyme study

S. No	Strain name	Media	Source
1	SOSIST-3	ISP2 50% Sea Water	Vertical water samples
2	SOE-11-09-PP9	ISP2 50% Sea Water	Vertical water samples
3	SOE-11-21-PP9	ISP2 50% Sea Water	Vertical water samples
4	SOSIST-13	NA	Vertical water samples
5	SOSIST-6	ISP2	Vertical water samples
6	SOE-11-01-04	AIA 50% Sea Water	Vertical water samples
7	SOE-11-27-09	ISP2 50% Sea Water	Vertical water samples
8	SOE-11-27-PP3	AIA 50% Sea Water	Vertical water samples
9	SOE-11-09-PP65	ISP2	Vertical water samples
10	SOE-11-09-PP13	ISP2 50% Sea Water	Vertical water samples
11	SOE-11-9-PP15	ISP2 50% Sea Water	Vertical water samples
12	SOE-11-9-PP69	ISP2 50% Sea Water	Vertical water samples

13	SEA ICE-5	AIA 50% Sea Water	Sea ice sample
14	SS-M-11-5	AIA 50% Sea Water	Surface water sample
15	SS-M-7-32	SCA 50% Sea Water	Surface water sample
16	GC-Surface-2	AIA 50% Sea Water	Sediment sample
17	GC-Surface-1	AIA 50% Sea Water	Sediment sample
18	SOE-11-15-57	SCA 50% Sea Water	Vertical water samples
19	SEA ICE-31	SCA 50% Sea Water	Sea ice sample
20	GC-4	AIA 50% Sea Water	Sediment sample
21	SS-7-4	AIA 50% Sea Water	Surface water sample
22	SS-7-20	AIA 50% Sea Water	Surface water sample
23	SEA ICE-9	AIA 50% Sea Water	Sea ice sample
24	SS-7-4	AIA 50% Sea Water	Surface water sample
25	SOE-11-21-PP15	NA 50% Sea Water	Vertical water samples
26	SEA ICE-16	AIA 50% Sea Water	Sea ice sample
27	SEA ICE-14	AIA 50% Sea Water	Sea ice sample
28	SOE-11-9-PP68	ISP2 50% Sea Water	Vertical water samples
29	SEA ICE-1	AIA 50% Sea Water	Sea ice sample
30	SEA ICE-4	AIA 50% Sea Water	Sea ice sample

4.2 PRIMARY SCREENING FOR ENZYMATIC ACTIVITY (QUALITATIVE ASSAY)

Primary Enzymatic screening was studied on enzyme incorporated media plates by spot assay, which includes Amylase, Lipase, Protease, Cellulase, Xylanase, Glutaminase, and Urease. The Enzyme agar plates were prepared accordingly to the substrate and autoclaved at 121 °C for 15 minutes, 15 lbs pressure (Table 4.2). The well grown cultures were inoculated as spot on the respective agar plates. The plates were incubated for 20 – 30 days at 4 °C and 7 – 10 days at 27 °C. After incubation period, the visualizing reagents were used to observe results.

Table 4.2: Media used for enzymatic activity

S. No	Enzyme Agar	Media Composition for 100 mL	Reagents for visualization	Reference
1	Starch Agar	Nutrient Agar -1.3 g, Starch – 1 g	Iodine	(Vaseekaran <i>et al.</i> , 2011)
2	Tributylin Agar	Tributylin agar Powder – 2.3 g, Agar – 2 g and Tributyrin oil – 1 ml	-	(Sirisha <i>et al.</i> , 2010)
3	Skim Milk Agar	Nutrient Agar – 1.3 g, Skim Milk – 1 g	-	(Nurkasanah <i>et al.</i> , 2015)
4	Cellulase Minimal Agar	CMC-1g, KH ₂ PO ₄ – 0.1 g, K ₂ HPO ₄ -0.1g, MgSO ₄ - 0.04 g, NaCl -0.005 g, FeSO ₄ -0.000125 g, Agar- 2	Congo Red + NaCl	(Maki <i>et al.</i> , 2009)
5	Xylanase	Peptone -0.5 g, Xylene – 0.1 g, Agar – 2 g	Congo Red + NaCl	(Kumar <i>et al.</i> , 2018)
6	Glutaminase	KCl-0.05 g, MgSO ₄ - 0.05 g, KH ₂ PO ₄ -0.01g, FeSO ₄ - 0.01 g, ZnSO ₄ - 0.01 g , NaCl – 0.25 g, L - Glutamine -1 g , Agar - 2 g	-	(Balagurunathan <i>et al.</i> , 2010)

7	Urease	Peptone – 0.1 g, Dextrose – 0.1 g, NaCl – 0.5 g, Na ₂ HPO ₄ – 0.12 g, KH ₂ PO ₄ – 0.08 g, Phenol Red – 0.0012, Agar - 2 g, 40 % Urea Solution – 50 µl, pH- 6.8	-	(Senthil Balan <i>et al.</i> ,2012)
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4.3 SEMI QUANTITATIVE ASSAY BY WELL DIFFUSION METHOD

The selected strains which showed enzymatic activity were inoculated into the 100 mL respective broth media and incubated for 20-30 days at 4 °C and 7-10 days at 27 °C. After incubation, the culture broth was centrifuged at 5,000 rpm for 20 minutes and supernatant was collected for this study. The respective enzyme agar media were prepared and a well with a diameter of 7 mm was punched using a well puncher. 100 µL of crude enzyme was loaded into the well, then the plates were incubated for 20-30 days at 4 °C and 7-10 days at 27 °C (Balagurunathan *et al.*, 2010).

4.4 QUANTITATIVE ASSAY

4.4.1 Di-nitro salicylic acid (DNS) for Protease

1000 µl of casein / skim milk, 1000 µl of 0.1 M Phosphate buffer, and 200 µl of diluted supernatant (crude enzyme) was added in the test tube. The pH 8 was maintained and incubated at 40 °C for 15 minutes, then 2 mL of DNS and 4 mL of distilled water were added. The test tubes were boiled for 5 minutes at 100 °C, then quickly cooled in ice water and read the UV Visible Spectroscopy absorbance at 540 nm (Muriithi *et al.*, 2021).

4.4.2 p-nitrophenyl palmitate (p-NPP) for Lipase

2.4 mL of p-NPP solution and 0.1 ml of the cultural supernatant (crude enzyme) was added. Read the UV Visible Spectroscopy absorbance at 540 nm (Bose *et al.*, 2013).

4.5 PARTIAL PURIFICATION OF ENZYME BY AMMONIUM SULPHATE PRECIPITATION TEST

The crude enzyme was partial purified by Ammonium sulphate precipitation method. Add 10 ml of the crude enzyme with 40% of ammonium sulphate (2.26 g). Similarly, for 60% and 80% of ammonium sulphate (3.61 g and 5.16 g). The samples were kept for overnight in shaking condition at 4 °C and 27 °C. The samples were centrifuged at 10,000 rpm for 30 minutes. The suspension was centrifuged at 10,000 rpm for 15 mins at 4 °C. The supernatant was collected and stored for further studies. The partially purified enzymes at different concentrations of Ammonium sulphate were selected and were reconfirmed for its activity by well diffusion method (Duong-Ly *et al.*, 2014).

4.6 ESTIMATION OF PROTEIN BY LOWERY'S METHOD

0.1 mL of 2N NaOH was added to the 0.1 mL of sample and boiled at 100 °C for 10 mins. The mixture was cool down to room temperature and 1 mL of freshly prepared complex reagent was added and left undisturbed at room temperature. Then, 0.1 mL of Folin reagent was added and mix well and let stand at room temperature for 30-60 mins. The absorbance was recorded at 750 nm.

4.7 SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

4.7.1 Acrylamide (30 % stock)

3 g of acrylamide and 0.8 g of methylene bisacrylamide were dissolved in 70 ml of distilled water and made up to 100 ml. The stock solution was stored in amber colour bottles at 4 °C (stored up to 3 months).

4.7.2 Resolving /separating gel buffer (1.5 M Tris-HCl, pH 8.8)

18.17 g tris base was dissolved in 70 ml distilled water. The pH was adjusted to 8.8 with concentrated HCl and made up to 100 ml. This buffer was stored at 4 °C.

4.7.3 Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

6.05 g tris base was dissolved in 70 ml of distilled water. The pH was adjusted to 6.8 with concentrated HCl and made up to 100 ml. This buffer was stored at 4 °C.

4.7.4 Electrophoresis (tank) buffer, pH 8.3

3 g tris base, 14.4 g glycine and 1 g SDS were dissolved in 1000 ml of double distilled water. The pH without adjustment was 8.3.

4.7.5 10 % Ammonium persulphate

100 mg of ammonium persulphate was dissolved in 1 ml of double distilled water. This solution was prepared freshly.

4.7.6 TEMED (N, N, N', N'-tetramethylethylenediamine)

It was used as a catalyst.

4.7.7 SDS (10 %)

2.5 g SDS was dissolved in 25 ml of double distilled water.

4.7.8 SDS-sample buffer

5 mg bromophenol blue, 15 mg SDS, 1 ml glycerol, 1.25 ml stacking gel buffer and 0.5 ml 2-mercaptoethanol were taken in a glass beaker, mixed well and made up to 10 ml.

4.7.9 Staining solution

The CBB staining solution containing 0.25 % coomassie brilliant blue R-250 in 50 % methanol and 7 % acetic acid were prepared. This solution was made up to 100 ml with distilled water.

4.7.10 Destaining solution

Destaining solution containing 30 % methanol and 7 % acetic acid were prepared. Briefly, 30 methanol and 7 ml acetic acid were mixed and made up to 100 ml with double distilled water.

4.7.11 Preparation of gel for SDS-PAGE

The gel plates were assembled according to the manufacturer's instruction and the volume of the gel mould was determined. In a beaker, 12 % resolving gel (2 ml of 30 % acrylamide stock + 1.85 ml of resolving gel buffer (pH 8.8) + 1 ml of distilled water + 50 µl of 10 % SDS + 50 µl of 10 % ammonium persulphate + 5 µl of TEMED)

was prepared. The components were mixed thoroughly and without delay the acrylamide mixture was poured into the glass mould and overlaid with butanol. After polymerization (approximately 20 min), the overlay was removed. 4 % stacking gel was prepared by mixing 0.4 ml of 30 % acrylamide stock, 0.375 ml of stacking gel buffer (pH 6.8), 2.163 ml of distilled water, 30 μ l of 10 % SDS, 30 μ l of 10 % ammonium persulphate and 3 μ l of TEMED. These components were mixed and poured over the resolving gel. Immediately a comb was inserted and the gel was allowed to polymerize. The comb was removed from the mould and the wells were washed with distilled water. The gel was mounted in the electrophoretic apparatus.

4.7.12 Analysis of partially purified enzyme in SDS-PAGE

In order to analyze enzyme in SDS-PAGE, 40 μ l of enzyme containing 32 μ L of sample was mixed separately with 8 μ L of SDS-sample loading buffer. The samples were boiled in water bath for 1 min at 90 °C to denature the proteins. They were kept on ice to retain the denatured stage. The samples were loaded in the wells of gel fixed in the electrophoresis apparatus. The SDS-tank buffer (pH 8.3) was added to the upper and the lower tank of the electrophoretic apparatus. The apparatus was attached to power supply unit and 50 V for stacking gel and 100 V for resolving gel was applied. The power supply was turned off, once the bromophenol blue reached the bottom of the resolving gel. The gel mould was removed from the apparatus and the plates were removed apart using a spatula. The orientation of the gel was marked. The separated proteins in the gel were stained with Coomassie brilliant blue (CBB) G250 for overnight. The gel stained with CBB was destained with the destaining solution. Every 2 hrs the destaining solution was changed. After destaining, the gel was washed with distilled water and was stored in 7 % acetic acid. The bands were visualized using an illuminator and then photographed.

4.8 CHARACTERIZATION & IDENTIFICATION OF POTENTIAL STRAIN

4.8.1 Micromorphology

Gram staining was performed for potential strains and viewed under 100x objective of oil immersion.

4.8.2 Cultural Characterization

The culture morphology was studied by inoculating in AIA media. The colony size, consistency, colony edge, soluble pigment was observed.

4.8.3 Molecular Charecterization

The strain Sea Ice-9 and Sea Ice -5 was subjected to 16s rRNA sequencing. The 16s rRNA gene was amplified by PCR using universal primers 27F (5'AGAGTTTGATCCTGGCTCAG 3') and 1492R (5'TACGGNTACCTTGTTACGACTT 3').

4.8.4 Phylogenetic Analysis

16s rRNA gene sequences of the isolates were compared with 16s rRNA sequencing available in the BLAST search in NCBI. The sequenced strains were deposited in GenBank database.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Description and Morphological Characterization of Selected Psychrophilic Bacterial Strain

For this study, about 30 bacterial cultures were obtained from the Cryosphere Microbial Research Lab, Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu. All the cultures were previously isolated from Sea ice and vertical water samples collected from less explored ecosystems of Southern Ocean region in Antarctica and maintained in respective agar slants as well as in 30 % glycerol broth (Figure 5.1).



Fig 5.1 Psychrophilic bacteria used for enzyme studies where on respective media plates

5.1.1 Cultural Morphology

The growth of bacterial strains and cultural characteristics were listed in the table 5.2. Most of the cultural colony were rough and leathery which seems to be *Streptomyces*. Pigmented colonies were smooth, creamy and few were mucoid in consistency were also identified.

Table 5.1 Cultural morphology of Psychrophilic Bacteria

S. No	Strain No	Texture	Aerial Mycelium	Aerial Mycelium Colour	Substrate Mycelium	Reverse Pigment	Soluble Pigment
1	SOSIST-3	Powdery	+	Grey	—	Brown	—
2	SOE-11-09-PP67	Powdery + Leathery	+	Off White	—	Golden Brown	—
3	SOE-11-21-PP9	Creamy	—	Yellow	—	Yellow	—
4	SOSIST-13	Smooth+ Mucoid	—	Off White	—	Off White	—
5	SOSIST-6	Mucoid	—	Off White	—	Off White	—
6	SOE-11-01-04	Creamy	—	Pale Yellow	—	Pale Yellow	—
7	SOE-11-27-09	Mucoid	—	Pale Yellow	—	Pale Yellow	—
8	SOE-11-27-PP3	Smooth+ Mucoid	—	Off White	—	Off White	—
9	SOE-11-09-PP65	Powdery+ Leathery	+	Off White	—	Off White	—
10	SOE-11-09-PP13	Powdery +	+	Off White	—	Off White	—

		Leathery					
11	SOE-11-9-PP15	Powdery + Leathery	+	Off White	–	Off White	–
12	SOE-11-9-PP15	Powdery + Leathery	+	Off White	–	Off White	–
13	SOE-11-9-PP69	Powdery + Leathery	+	Off White	–	Off White	–
14	SEA ICE-5	Smooth	–	Off White	–	Off White	–
15	SS-M-11-5	Smooth, Mucoid	–	Off White	–	Off White	–
16	SS-M-7-32	Smooth	–	Pale Yellow	–	Pale Yellow	–
17	GC-Surface-2	Smooth, Mucoid	–	White	–	White	–
18	GC-Surface-1	Smooth, Mucoid	–	White	–	White	–
19	SOE-11-15-57	Powdery	+	Greenish Black	–	Black	+
20	SEA ICE-31	Smooth, Mucoid	–	Pale Pink	–	Pale Pink	–
21	GC-4	Mucoid	–	Pale Orange	–	Pale Orange	–
22	SS-M-7-20	Smooth, Mucoid	–	Pink	–	Pink	–

23	SEA ICE-9	Smooth, Mucoid	—	Pink	—	Pink	—
24	SS-M-7- 4	Mucoid	—	White	—	White	—
25	SOE-11- 21-PP15	Creamy	—	Pale Yellow	—	Pale Yellow	—
26	SEA ICE-16	Smooth, Mucoid	—	Pink	—	Pink	—
27	SEA ICE-14	Smooth, Mucoid	—	Off White	—	Off White	—
28	SOE-11- 9-PP68	Powdery	+	White	—	White	—
29	SEA ICE-1	Smooth, Mucoid	—	White	—	White	—
30	SEA ICE-4	Smooth, Mucoid	—	Off White	—	Off White	—

5.2 Primary Screening of Psychrophilic Bacteria by spot assay

In this study, all the 30 psychrophilic strains were screened for enzymatic activity. Around 20 strains showed at least one enzyme activity at 27 °C and 12 strains showed activity at 4 °C in spot assay (Figure 5.3 and Table 5.3).

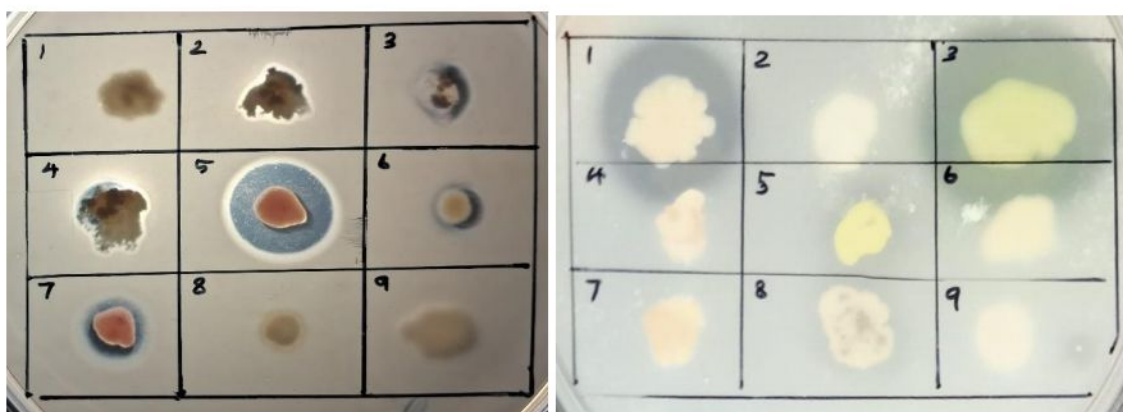


Fig 5.2 Lipase and protease activity by spot assay

Table 5.2 Enzyme Screening at 27 °C by spot method

S. No	Strain No	Amylase	Lipase	Protease	Cellulase	Xylanase	Glutaminase	Urease
1	SOSIST-3	++	++	+	–	–	–	–
2	SOE-11-09-PP67	–	++	–	+	–	–	–
3	SOSIST-13	–	–	+	–	–	–	–
4	SOSIST-6	–	–	+	–	–	–	–
5	SOE-11-09-PP65	–	++	–	+	–	–	–
6	SOE-11-09-PP13	–	+	–	+	–	–	–
7	SOE-11-9-PP15	–	–	–	+	–	–	–
8	SOE-11-9-PP15	+	–	–	–	–	–	–
9	SOE-11-9-PP69	+/-	+	–	–	–	–	–
10	SEA ICE-5	–	+/-	+	–	–	–	–
11	SS-M-11-5	+	+	+	–	–	–	–
12	SS-M-7-32	–	+	–	–	–	–	–

13	GC-Surface-2	–	+/-	–	–	–	–	–
14	GC-Surface-1	–	+	–	–	–	–	–
15	SOE-11-15-57	–	+	+	+/-	–	–	–
16	SEA ICE-31	–	+/-	–	–	–	–	–
17	GC-4	–	++	–	–	–	–	–
18	SS-M-7-20	–	++	–	–	–	–	–
19	SEA ICE-9	–	+++	–	–	–	–	–
20	SOE-11-21-PP15	+++	–	–	–	–	–	–

Table 5.2 Enzyme Screening at 4 °C by spot method

S. No	Strain No	Amylase	Lipase	Protease	Cellulase	Xylanase	Glutaminase	Urease
1	SOSIST-3	–	–	+++	–	–	–	–
2	SOE-11-09-PP67	–	++	–	–	–	–	–
3	SOSIST-13	+		++	–	–	–	–
4	SOSIST-6	–	–	++	–	–	–	–
5	SOE-11-27-PP3	–	++	++	–	–	–	–
6	SOE-11-09-PP65	–	+	–	–	–	–	–
7	SOE-11-09-PP13	–	+	–	–	–	–	–
8	SOE-11-9-PP15	–	+	–	–	–	–	–
9	SOE-11-9-PP69	–	++	–	–	–	–	–
10	SEA ICE-5	++	+	+++	–	–	–	–
11	SS-M-11-5	+	–	–	–	–	–	–

12	SEA ICE-9	—	+++	—	—	—	—	—
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5.3 Semi Quantitative Assay for Psychrophilic Enzymes

By semi quantitative assay, the highest activity for lipase was 43.5 mm and 21.2 mm in diameter for Sea Ice-9 and highest protease activity was 20 mm and 16 mm in diameter in 4° C Sea Ice-5 at both the temperature.

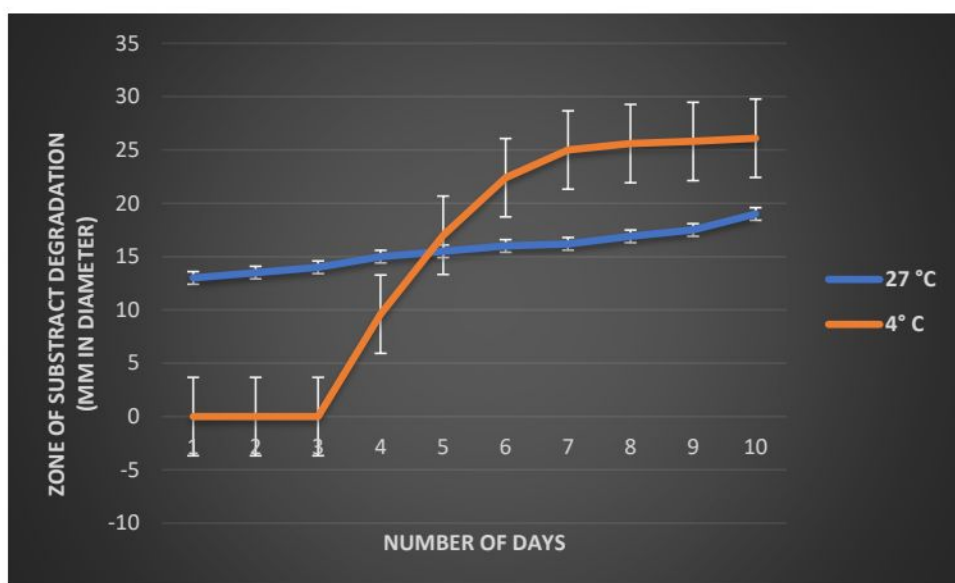


Fig 5.3 Time point assay for Protease enzyme production of Sea Ice – 5

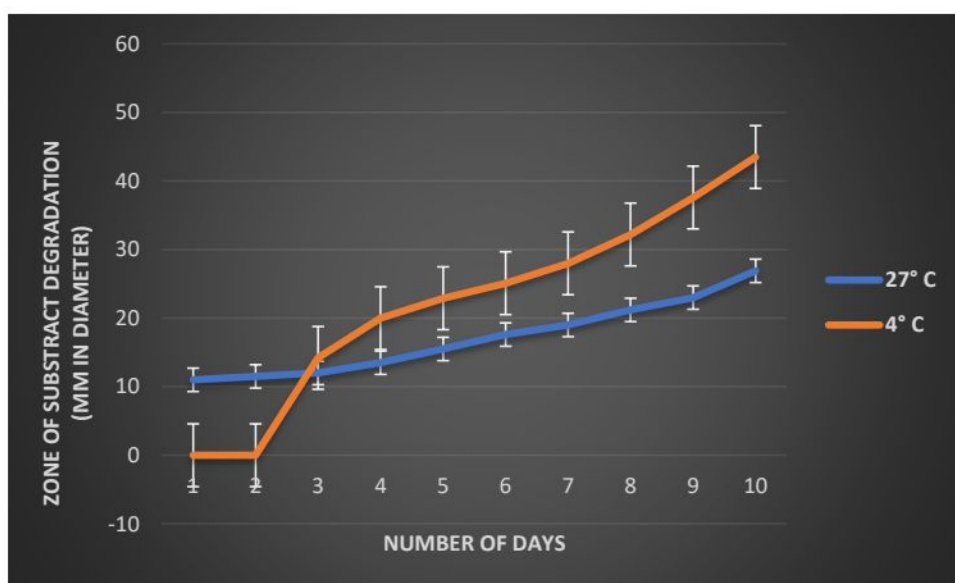


Fig 5.3 Time point assay for Lipase enzyme production of Sea Ice – 9

5.4 Quantitative assay

5.4.1 Quantitative Assay by Di Nitro salicylic acid (DNS) for Protease

In di-Nitro salicylic acid (DNS) assay, the highest enzyme production for Protease was 43.45 (U/mL) for Sea Ice-5 in 20th day of incubation (Table 5.6).

Table 5.4.1 Quantitative assay for Sea Ice-5 by DNS

Days	Enzyme Production (U/mL)
3	6.35
7	14.2
12	24.4
15	28.6
20	43.45

5.4.2 Quantitative Assay by p- NitroPhenile Palmitate (p-NPP) Test for Lipase

In p-Nitrophenyl Palmitate (p-NPP) assay, the highest enzyme production for lipase was 63.70 (U/mL) for Sea Ice-9 in 20th day of incubation (Table 5.7).

Table 5.4.2 Quantitative assay for SI-9 by p-NPP

Days	Enzyme Production (U/mL)
3	8.55
7	16.7
12	4.93
15	53.45
20	63.70

5.5 Partial Purification of Enzyme by Ammonium Sulphate Precipitation Test

The crude enzyme was partially purified and the highest enzymatic activity was showed at 60% and 80% of the Ammonium Sulphate concentration for both Lipase and Protease (Table 5.5).

Table: 5.5 Enzymatic activity of partially purified enzyme at different concentration of Ammonium sulphate

S. No	Ammonium Sulphate Concentration	Skim milk degradation by Sea Ice - 5 (mm in diameter)		Tributyrin oil degradation by Sea Ice-9 (mm in diameter)	
		4° C	27° C	4° C	27° C
1	40%	11	-	11	12
2	60%	12	-	14.3	14
3	80%	14	-	12	13

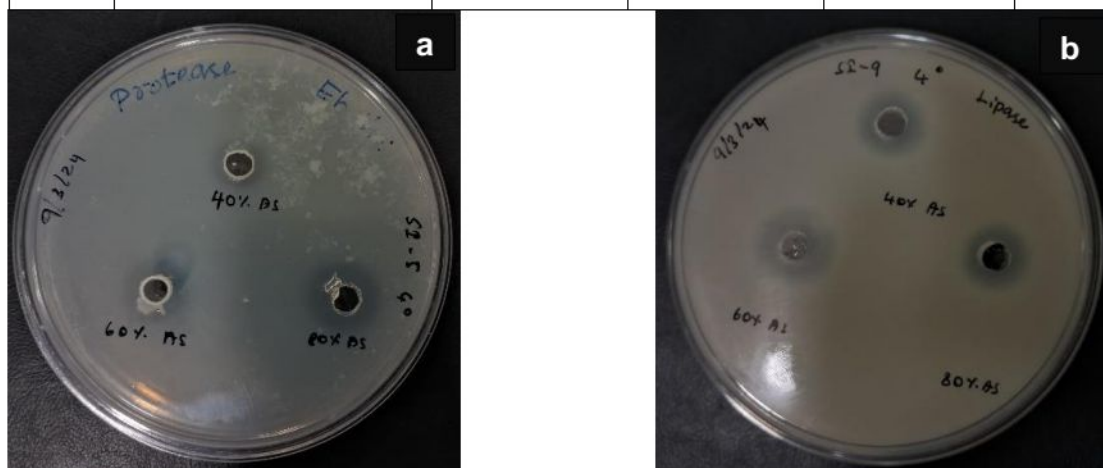


Fig 5.5 Partially purified enzyme activity at various concentration of Ammonium sulphate for Protease (5.5a) and lipase (5.5b)

5.6 Estimation of Protein by Lowery's method

Table 5.6 Estimation of Protein content by Lowry's Method

Bacterial Strain	Estimated Protein Content
SI-5	1.37
SI-9	1.24

5.7 SDS-PAGE

From SDS-PAGE analysis, molecular weight of the lipase and protease had different range from 13-55 kDa (Figure 5.7). It suggested that each bacterial strain has different molecular weight of both the enzyme and also different amount of protein content.

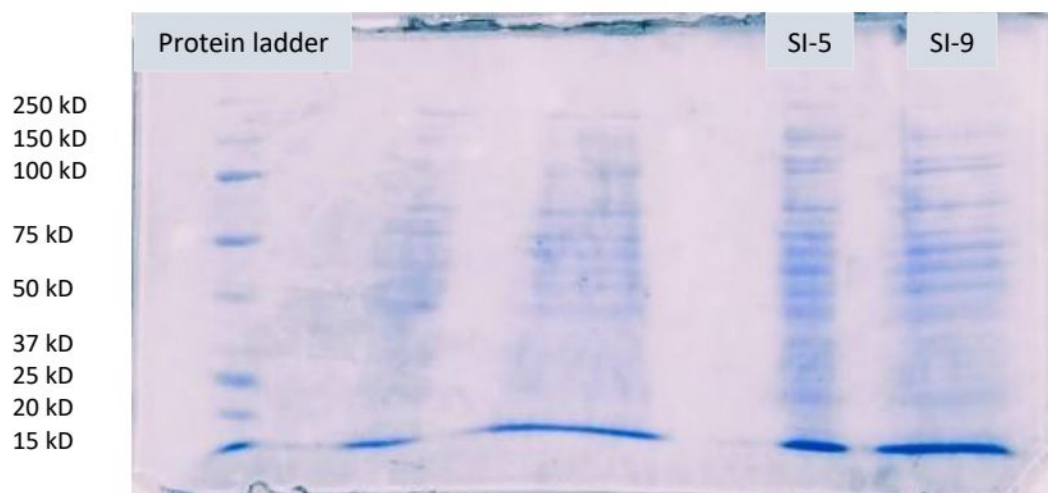


Fig 5.7 SDS-PAGE gel with separated bands for the isolated enzyme based on molecular weight

5.8 Identification and characterization of potential strain

5.8.1 Micromorphology

Under bright field microscope observation, the strain Sea Ice – 9 showed gram positive cocci under 100X objective.

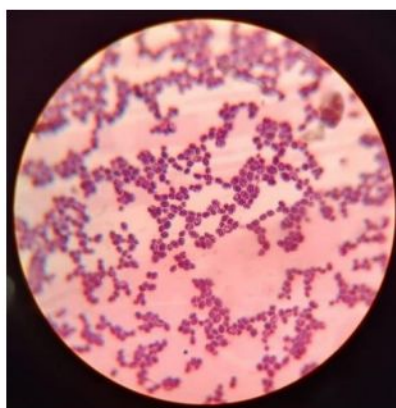


Fig 5.8 Gram staining under 100X objective

5.8.2 Cultural characteristics

The cultural morphology of the strain Sea Ice-5 and Sea Ice-9 were given in figure 5.8. The strain showed good growth on AIA media with smooth, creamy and pink coloured colony of Sea Ice-9 and smooth, mucoid and white coloured colony of Sea Ice-5.

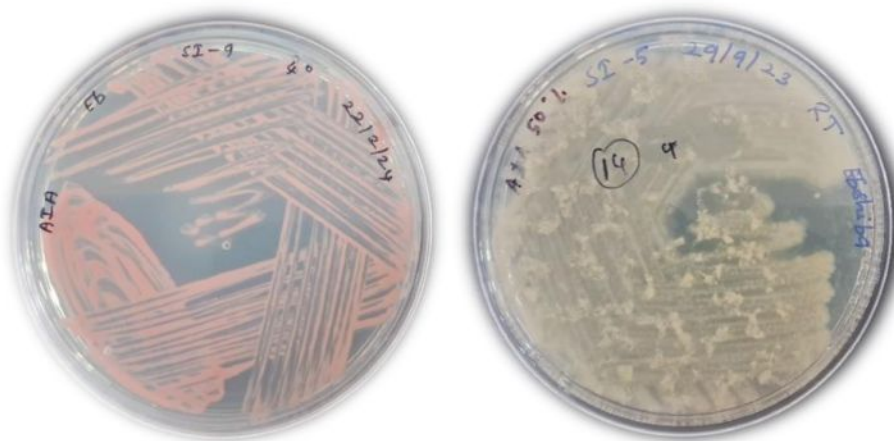


Fig 5.8 Cultural colony appearance on AIA media

5.8.3 Molecular characterization

The PCR amplification of the strain Sea Ice-5 and Sea Ice-9 16s rRNA gene yields 681 and 782 base pairs. NCBI BLAST analysis shows 100% similarity to sequence of *Stenotrophomonas rhizophila* MT373663.1 for Sea Ice-5. For Sea Ice-9, NCBI BLAST analysis shows 99.74% similarity to the sequence of *Burkholderia* sp. MT626032.1.

5.8.4 Phylogenetic Analysis

The evolutionary history was inferred by using the maximum likelihood based on the Tamura-Nei model which confirms distinct Phylogenetic tree construction with Sea Ice-5 designated as *Stenotrophomonas rhizophila* (Figure 5.8). The evolutionary history was inferred by using the maximum likelihood based on the Tamura-Nei model which confirms distinct Phylogenetic tree construction with Sea Ice-9 designated as *Burkholderia* sp.

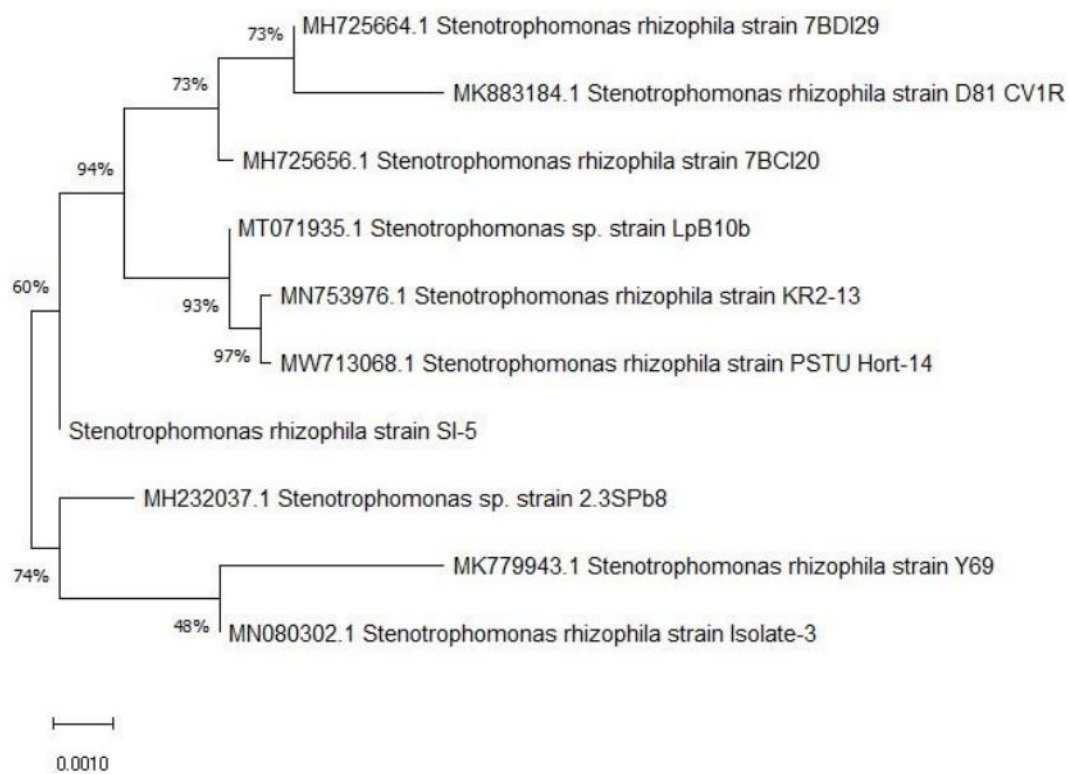


Fig 5.8 Phylogenetic tree for Sea Ice - 5

CHAPTER 6

SUMMARY AND CONCLUSION

The present study attempted to explore psychrophilic bacteria for enzymatic activity against different substrates like starch, tributyrin oil, skim milk, beechwood xylene, cellulose etc. totally 30 bacterial strains were procured from Cryosphere microbial research lab of Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology. The enzymatic activity for all the strains were determined by agar-based spot assay. The strains Sea Ice – 5 and 9 showed promising protease and lipase enzyme production at two different temperatures (4 °C and 27 °C) for about 12 to 20 mm of zone. The active enzymes from the strains were produced by adopting submerged fermentation using AIA broth. The enzyme production was reconfirmed by well diffusion method on respective substrate incorporated media plates for every 24 hours up to 10 days of incubation in both the conditions. The crude enzymes were partially purified by ammonium precipitate method and ran SDS-PAGE electrophoresis to determine the weight of the enzyme which showed around 13-55 kDa. The microscopic and cultural characteristics of the potential bacterial strains were studied by adopting standard procedures. Based on the molecular characterization (i.e.,) BLAST and Phylogenetic tree analysis of Sea Ice – 5 shows 100% showed similarity to the sequence of *Stenotrophomonas rhizophila*. Further studies like protein digestion for mass spectrometry In-Gel will be done in future. The findings of the present study concluded that the strain Sea Ice – 5 and 9 will be a promising source for cold active enzyme productions which can be used biotechnologically.

CHAPTER 7

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ANNEXURE

1. ACTINOMYCETES ISOLATION AGAR (AIA)

REQUIREMENTS	AMOUNT (g/L)
AIA	22.00
GLYCEROL	5.00
AGAR	15.00

2. INTERNATIONAL STREPTOMYCES PROJECT-2 (ISP-2)

REQUIREMENTS	AMOUNT (g/L)
Yeast extract	4
Malt extract	10
D-Glucose	4
NaCl	10
Agar	20

3. NUTRIENT AGAR

REQUIREMENTS	AMOUNT (g/L)
Beef extract	1.00
Yeast extract	2.00
Peptone	5.00
Sodium Chloride	5.00
Agar	15.00

4. STARCH CASEIN AGAR (SCA)

REQUIREMENTS	AMOUNT (g/L)
Soluble Starch	10
Casein (vitamin free)	0.3
Pottasium nitrate	2
Magnesium sulphate	0.05
Ferrous sulphate	0.01
Dipotassium phosphate	2
Calcium carbonate	0.02
Sodium chloride	2
Agar Agar	20
pH	7

5. 5M NaCl

REQUIREMENTS	AMOUNT (g/L)
NaCl	58

6. 0.5% CONGO RED

REQUIREMENTS	AMOUNT (g/L)
Congo Red	5