Study of natural salt from Goan salt pans

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DECLARATION

I hereby declare that the data presented in this dissertation report entitled, "**Study of natural salt from Goan salt pans**" is based on the results of investigations carried out by me in the Biotechnology Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Savita S Kerkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations/ experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Study of natural salts from Goan Salt pans**" is a bonafide work carried out by Mr. Md Adnan under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in Marine Biotechnology in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

This dissertation explores study of five natural salt samples from Goan salt pans. With a focus on the iodine content of the salts their nutritional value and stability in comparison with commercial salts available in the market. Furthermore, this study delves into the ecological aspects of the natural salt pans to isolate important bioactive compounds from microorganisms thriving in the salt pan environment.

Motivated by a fascination for natural salt pans ecology and Goa, this journey of discovery has been guided by the expertise and support of my supervisor, **Prof. Savita S Kerkar.** Additionally, collaboration with colleagues and the unwavering encouragement of family and friends have been instrumental in shaping this research.

As this dissertation contributes to our understanding of Goan salt pan ecology, I am grateful for the opportunity to explore the mysteries of this age old tradition. It is my hope that this work will inspire further inquiry and deepen our appreciation for the intricate processes of natural salt formation its application and the extent of pollution in Goan waters.

MD Adnan

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LIST OF FIGURES

LIST OF TABLES

ABBRIVATIONS USED

µm Micrometer

mm Millimeter

mL Milliliter

nm Nanometer

℃ Degree(s)Celsius

% Percentage

MPs Microplastic

Ppm parts per million

g/L grams per litre

mg/ml milligrams per millilitres

mg milligrams

ABSTRACT

The dominance of salt industries and promotional campaigns advocating for the fortification of natural salt have led to a widespread misconception that all natural salts lack iodine. This false belief has influenced society, including in Goa, to favor refined salt consumption, consequently impacting the decline of traditional, economically viable salt produced by Goan salterns. This reality has motivated the current study to evaluate the quality of natural salt with the primary goal of dispelling the notion that it is inferior and iodine-deficient along with determining its stability and safety of consumption overtime. This study also focuses on the environmental aspects of these salt pans and emphasizes on the search for bioactive compounds extracted from organisms isolated from the salt pan environment. There has been extensive research in micro plastics in sea water and marine organisms but the study of micro plastics from salterns of Goa is still at an early stage. For accurate assessment of the risks and the extent of reach of micro plastics in the salterns of Goa, current information gaps must be filled. Therefore, in this study the prevalence of micro plastics in the salt procured from Goan salt pans were investigated.

CONTENTS

CHAPTER 1: INTRODUCTION & LITERATURE REVIEW

1.1 Introduction:

Salt is a vital component of daily life which balances numerous physiological function of the human body. Since millennia salt has been used as a valuable preservative and seasoning agent and a common food additive (Aquilano *et al*.,2016).The origins of salt production can be traced back thousands of years, with evidence of ancient salt works found in countries like China, Egypt, and Greece. These early civilizations recognized the value of salt not only as a culinary seasoning but also for its preservative properties, which enabled the storage and transportation of food over long distances (Kurlansky, M. (2002). Salt: A world history. Penguin Books.).

Naturally salt is an ionic compound with the matrix of NaCl and its origin is generally derived from the sea. Evaporation of sea water leads to the deposition of salt crystals and other minerals. Solar salt production in natural salt pans represents an ancient and sustainable method of harvesting salt from seawater through evaporation. One of the oldest and most sustainable process of salt production is solar evaporation, a process that relies on the natural forces of the sun and wind to extract salt from seawater or saltwater lakes. In coastal regions, communities constructed shallow ponds or "salt pans" to facilitate the evaporation process. As the seawater evaporated under the sun's heat, salt crystals would form and could be easily harvested. This traditional practice was perfected over generations, with each community developing its own unique techniques and rituals. In many cultures, salt farming became deeply ingrained in the local traditions and way of life, with the knowledge and skills being passed down from elders to younger generations (Mani *et al.*,2012).

Today, while industrial salt production dominates the global market, the ancient art of natural salt farming persists in many parts of the world. Communities in regions like India, Portugal, Indonesia, and Mexico continue to practice traditional salt farming methods, preserving not

only a valuable commodity but also a rich cultural heritage. These salt farmers serve as stewards of a centuries-old tradition, working in harmony with the natural cycles of the earth and the sea. Their intimate knowledge of the local environment, combined with time-honored techniques, allows them to produce salt with unique flavors and properties, often prized by gourmets and health-conscious consumers alike.

Natural salt pans are shallow artificial ponds located in coastal regions, designed to facilitate the evaporation of seawater and the subsequent crystallization of salt. These unique ecosystems have existed for centuries, reflecting the ingenuity of human communities in harnessing the power of nature using solar evaporation to produce this essential mineral. Not only do salt pans serve as a reservoir of natural salt, but they also support a diverse array of halophilic (salt-loving) organisms, creating a delicate yet fascinating ecological balance.

In the recent years the production and popularity of natural salt has seen a decline both in production and marketing which is owed largely to the marketability and branding of refined salts being superior, safe and having more iodine content in them than the natural salts. Refined salt is crystalline white in colour and is produced in factories which is a meticulous process that transforms crude salt into industrial grade salt. The crude salt is first processed after evaporation and then converted into brine along with washing and milting to remove their minerals and any other substances then acid treatment is used to remove the remaining minerals or any other contaminants. Furthermore, additives are added to the refined salt to make it stable.

Iodine is a major element required by the human body for synthesis of two major hormones, namely, thyroxine and tri-iodothyromine that play an important role in the human body metabolism and are produced in the thyroid gland of the human body. Iodine deficiency in humans can lead to goitre and cretinism (Nutrition foundation of India, 1983).

Developed countries commonly address iodine deficiency through the widespread use of potassium iodide (KI) and potassium iodate (KIO3) to fortify refined table salt. However, it's worth noting that while KI is more commonly used, it is less stable and prone to oxidation under various environmental conditions, whereas iodate demonstrates greater resilience. In regions with prevalent iodine deficiency, many individuals rely on unrefined salt, which can be effectively enriched with KIO3 to address iodine insufficiency.

Goa, a coastal state in western India, has a rich tradition of solar salt production that dates back over 1,500 years. This age-old practice has been carried out by several communities, taking advantage of the region's favourable climatic conditions, easy access to seawater, and the presence of estuaries formed by the state's numerous rivers. Salt production was one of the major key factors of Goa economy, even under the Portuguese colonial rule, when it served as a chief export commodity. The salt produced from the salt pans in the region was considered superior in quality and was exchanged and sold to various Asian countries, including Burma and Thailand. While Goa once had major involvement in salt production there has been a decline in natural salt production in Goa This age-old practice not only had a key role in the local economy but also served as a important export commodity during the Portuguese rule (Mani *et al.*,2012). However, in recent times, the natural salt production in Goa has witnessed a sharp decline due to various challenges, putting this unique cultural heritage at risk. Despite its historical significance and ecological importance, the solar salt industry in Goa faces several challenges that have led to its decline. Low income, lack of skilled labor, competition from industrially produced iodized salt, and yearly damage to embankments have caused many salt pan operators to abandon their traditional practices and seek alternative employment opportunities.

Furthermore, beyond the nutritional aspects, there are socio-economic factors at play. The monopolization of salt industries and the promotion of fortified refined salt through

advertisements have perpetuated the misconception that natural salts lack iodine. This misbelieve has significantly influenced consumer behaviour, particularly in regions like Goa and elsewhere, steering individuals towards the consumption of refined salt. Consequently, this shift in consumption patterns has contributed to the gradual decline of traditional, more economically viable salt produced by local salterns. This underscores the importance of education and public awareness campaigns to dispel myths surrounding salt iodization and promote the consumption of iodine-fortified salts, irrespective of their refinement status, to combat iodine deficiency effectively.

Halophiles are microorganisms that thrive in environments with high salt concentrations, such as salt lakes, salt marshes, and solar salterns. These organisms have adapted to survive and grow in these extreme conditions by developing various strategies, including the process of accumulation of essential solutes known as compatible solutes which help in maintaining their osmotic balance and the production of specialized enzymes, known as halophilic enzymes.

Enzymes can be defined as biological catalysts that play crucial roles in various biochemical processes within living organisms. They are essential for the survival and functioning of cells, as they facilitate and accelerate chemical reactions that would otherwise occur at extremely slow rates or not at all under physiological conditions.

Halophilic enzymes are enzymes produced by microorganisms obtained from halophilic condition possess unique properties that make them attractive for various industrial and biotechnological applications. These enzymes are often more functional and active under conditions of high salinity, extreme temperatures, and low water activity, which are typically detrimental to most enzymes derived from non-halophilic sources.

Tyrosinase, a multifunctional copper-containing enzyme, plays a important role in melanin biosynthesis, a crucial process observed in various organisms as an essential process. Melanin, a complex heterogeneous pigment, exhibits diverse biological functions, including protection against harmful ultraviolet radiation, free radical scavenging, and virulence in pathogenic microorganisms. The salt pan environment, a relatively unexplored ecosystem, harbors a wide array of microorganisms, including actinomycetes, which have emerged as a promising source of novel bioactive compounds and enzymes.

Marine actinomycetes, a group of Gram-positive bacteria, have garnered significant attention for their ability to produce a wide range of bioactive compounds with potential applications in various fields, such as pharmaceuticals, agriculture, and biotechnology. These organisms have adapted to thrive in unique and often extreme conditions of the salt pan environment, making them a valuable resource for production of enzymes with unique properties and functionalities.

Tyrosinase enzymes derived from marine actinomycetes have gained considerable interest due to their potent and useful applications in various industries, including food, cosmetics, and bioremediation. These enzymes exhibit remarkable stability and catalytic efficiency under diverse environmental conditions, making them attractive candidates for industrial applications.

Furthermore, tyrosinase enzymes play a significant role in the bioremediation of phenolic compounds and other environmental pollutants, owing to their capability to catalyze the oxidation of these compounds.

In recent years, the concern and impact of plastic pollution has garnered significant attention due to its detrimental effects on the environment, particularly the marine ecosystem. One of the most concerning aspects of this problem is the presence of microplastics, which are small

plastic particles in size measuring less than 5 millimeters. These microscopic fragments are ubiquitous in the oceanic environment and have been deemed a significant threat to aquatic life and the overall health of our oceans. Micro plastics are tiny plastic particles measuring less than 5 millimeters in size, and they can be classified into two main categories: primary and secondary. Primary micro plastics are manufactured as small plastic particles and are commonly used in personal day to day products, such as tooth brushes and toothpaste and in industrial processes like sandblasting and abrasive cleaning. Secondary micro plastics,the second class of micro plastics, are formed through the fragmentation and breakdown of larger plastic items, such as plastic bags, bottles, and fishing nets, due to exposure to environmental factors like UV radiation, mechanical abrasion, and microbial activity.

The ubiquity of MPs in the marine environment is a cause for grave concern. These microscopic fragments have been found in virtually every corner of the world's oceans. Even seemingly pristine beaches and coastal areas are not spared from this pervasive pollution, as these small particles can be transported by wind, rivers, and currents, ultimately ending up in the vast expanse of the seas.

The potential sources of micro plastic pollution in the oceanic environment are diverse and widespread (Rudel *et al*,.2003).. Wastewater treatment plants, which often fail to effectively remove these tiny particles, discharge them into rivers and coastal waters. Storm water runoff from urban areas and agricultural fields carries MPs into waterways, ultimately leading to their accumulation in the ocean. Fishing and shipping industries also contribute significantly to this problem, as abandoned fishing equipments, such as nets and lines, can break down into smaller pieces over time (Rudel *et al*,.2003). Even recreational activities on beaches and coastal areas can introduce MPs directly into the marine environment.

The vulnerability of salt pans to micro plastic contamination lies in their direct exposure to the marine environment. As seawater is drawn using an inland route to the salt pans therefore it carries with it micro plastics present in the ocean, originating from sources such as wastewater discharge, stormwater runoff, and the offbreak of bigger plastic debris.

Once micro plastics enter the salt pans, they precipitate out with the salt crystals during the evaporation process. As the gradual evaporation of sea water takes place, the micro plastics remain trapped within the salt, eventually making their way into the final product consumed by humans (Lee *et al*,.2019).

1.2 Literature review

Salt in Human life and history

Salt, a seemingly simple compound, has played a profound role in shaping human civilization throughout history. From its beginnings as a precious trade commodity and cultural symbol, salt has evolved into a ubiquitous ingredient in modern life, with applications spanning various industries. Solar salt production is one of the most ancient process still performed and known to man even before history started to be recorded. Salt, the humble yet indispensable compound composed of sodium and chloride ions, has played a pivotal role in human civilization for millennia. Beyond its familiar culinary applications, salt has shaped the pathway of history, serving as a valuable trade commodity, a preservative, and even a currency in ancient times (Kurlansky, 2002).

The production of salt dates to the dawn of civilization, with evidence of natural salt production and trade found in various ancient civilizations, including China, Egypt, and the Indus Valley (Kurlansky, 2002). Throughout history, salt has been revered as a precious commodity, often referred to as "white gold" due to its scarcity and significance in preserving food and enhancing flavors (Multhauf, 1978). Salt played a vital role in major trade routes development, such as the famous Salt Roads of the Sahara and the Via Salaria in ancient Rome, facilitating the exchange of goods and cultural practices (Warsh, 2022).Salt has also had a profound impact on cultural and religious practices. In many traditions, salt is considered a symbol of purity, hospitality, and eternity (Willett & Funk, 2014). It is used in sacred rituals, blessings, and purification ceremonies across various cultures and belief systems, such as Hinduism, Judaism, and Christianity (Reddy & Anitha, 2015). The vital role of salt in these practices highlights its deep-rooted connection to human societies and spirituality.

The chemical composition of salt varies depending on its source and production method. Palanichamy *et al*. (2006) provide a comparative analysis of the chemical composition of sea salt and salt obtained from inland brine. Sea salt contains calcium and magnesium, resulting in the formation of calcium sulfate (gypsum) and magnesium salts, such as magnesium sulfate and magnesium chloride. However, inland brine salt is characterized by the absence of significant amounts of calcium and magnesium, leading to the presence of higher levels of sodium sulfate, sodium carbonate, and sodium bicarbonate. On comparison the chemical compositions of different types of salt produced from inland brine, namely Kyar salt, Reshta salt, and Pan salt, highlight variations in their sodium chloride content and concentrations of other compounds (Palanichamy *et al.*,2006).

Trace Metals and Organic Content:

In addition to the major chemical constituents, salt may also contain trace metals and organic compounds. Palanichamy *et al*. (2006) report the presence of trace metals such as vanadium, iron, nickel, copper, aluminum, and titanium in various salt types, while chromium and molybdenum are absent. The presence of certain trace metals and organic compounds can potentially impact the quality and characteristics of the salt produced, highlighting the importance of understanding the brine sources and production processes (Palanichamy *et al.*,2006).

In modern times, salt continues to be a vital component of our daily lives, not only in the culinary realm but also in various industrial applications. It is used in the production of chemicals, pharmaceuticals, and even in the de-icing of roads during winter months (Garrett, 2004). The global salt industry is a multi-billion dollar market, with major producers located in countries like China, United States, Germany, and India (Gevers *et al.*,2018).

Natural salt pans in Goa

The coastal state of Goa, situated on the western coast of India, has a rich history and tradition of solar salt production. This age-old practice, which harnesses the power of the sun and the abundant seawater resources, has played a significant role in the region's economy and cultural heritage (Kerkar S, Fernandes M, 2012).

Salt production in Goa can be traced back to the Portuguese colonial era, with records indicating the establishment of salt pans (known as "agars") along the coastal regions (Nayak, 2002). The traditional methods of salt production have been passed down through generations, with local communities relying on this industry as a source of livelihood (D'Souza, 2018).Salt production in Goa, India, has been a traditional practice for over 1,500 years, deeply rooted in the local communities and their cultural heritage. This age-old industry has played a significant role in the economy of Goa, even during the Portuguese rule, when salt was a chief export commodity (Pinto, 1990; Scammell, 1982; Gracias, 1940). Despite its rich history and cultural significance, the salt production industry in Goa has faced various challenges in recent times, leading to a decline in the number of operational salt pans (Nagvenkar, 1999; Almeida, 1985).

Traditional method of Goan salt production

Solar salt production in Goa follows a well-established process that relies on the principles of evaporation and crystallization. The process typically begins with the construction of shallow, rectangular ponds called "agars" or "salinas" along the coastal regions (Nasnolkar, 2016). These ponds are strategically located to take advantage of the natural tidal flow, allowing for the influx of seawater during high tides. The seawater is then allowed to evaporate gradually in the agars, with the aid of the intense tropical sun and the prevailing coastal winds (Nasnolkar, 2016). As the evaporation process continues, the concentration of

salt in the brine increases, leading to the formation of salt crystals. These crystals are subsequently harvested manually by the salt farmers, a process known as "salt raking" (D'Souza, 2018).

The success of solar salt production in Goa is heavily influenced by various environmental factors, including climate, geography, and tidal patterns. The state's tropical climate, characterized by high temperatures and abundant sunshine, is conducive to efficient evaporation (Nayak, 2002). Additionally, the coastal topography and the presence of estuaries provide an ideal setting for the construction of salt pans (Nasnolkar, 2016).Tidal patterns play a crucial role in the salt production process, as the influx of seawater into the agars is dependent on the tidal cycles. The timing and duration of the tidal cycles, along with the salinity levels of the seawater, can impact the rate of evaporation and the quality of the salt produced (D'Souza, 2018).

Chemical composition of salt

The chemical composition of solar salt produced in Goa is primarily influenced by the composition of the seawater used in the production process. Seawater, being a complex solution, contains various dissolved salts and minerals, including sodium chloride (NaCl), magnesium chloride ($MgCl_2$), calcium sulfate ($CaSO_4$), and trace amounts of other elements (Dabbagh *et al.*,2014).During the evaporation process, the concentration of these salts and minerals increases, leading to the formation of different salt compounds. The primary component of solar salt is sodium chloride, which typically accounts for more than 90% of the total composition (Badrinath *et al.*,2019). However, the salt may also contain varying amounts of other compounds, such as magnesium chloride, calcium sulfate, and trace elements, depending on the specific environmental conditions and the purity of the seawater used (Dabbagh *et al.*,2014).Most of the natural salts produced in the salt pans of Goa have 17

minerals essential for the human body (Kerkar S, Fernandes M, 2012) .The quality of the solar salt produced in Goa is influenced by several factors, including the purity of the seawater, environmental contaminants, and the efficiency of the production process. Contamination from sources such as industrial effluents, agricultural runoff, or even atmospheric pollutants can adversely affect the salt quality (Nasnolkar, 2016). Additionally, the presence of organic matter or sediments in the seawater can introduce impurities into the final salt product (Badrinath *et al.*,2019).

To ensure high-quality salt production, careful monitoring and management of the agars are essential. Proper maintenance, including regular cleaning and repair of the salt pans, can help minimize contamination and improve the overall efficiency of the evaporation process (D'Souza, 2018).

Salt production in Goa has been a community-driven activity, involving five main communities: Mithgaudas, Gauddos, Bhandaris, Agris, and Agers (Singh *et al.*,1993; Sequeira, 2009). The Mithgauda community, a subdivision of the Gauda/Govada community, is believed to have pioneered the art of salt making. These communities either own the salterns (salt pans) or are employed by one another for the production process.

Goan communities involved in salt farming

The communal organization, known as the "comunidade," played a crucial role in governing villages and regulating agricultural activities, including salt production (De Souza, 1990). Each village had its own set of rules and customs, and the comunidade was responsible for reclaiming waterlogged lands (khazans) along the coasts and making them suitable for salt production, aquaculture, pisciculture, and agriculture. The solar salt industry in Goa has significant socio-economic implications for the local communities. It provides employment opportunities and a source of income for many families, particularly in the coastal regions

where alternative economic activities may be limited (Nayak, 2002). The traditional knowledge and skills associated with salt production have been passed down through generations, contributing to the preservation of cultural heritage (D'Souza, 2018). However, the solar salt industry in Goa also faces challenges, such as competition from mechanized salt production methods, fluctuations in market demand, and the impact of climate change on the production processes (Nasnolkar, 2016). Efforts have been made by local authorities and organizations to support and promote the traditional solar salt industry, recognizing its cultural and economic significance (D'Souza, 2018).

Importance of the Iodine content of salt

Iodine deficiency is a major public health concern, affecting both developed and developing countries (Pearce *et al.*,2021). According to the World Health Organization (WHO, 2022), iodine deficiency is the leading cause of preventable mental impairment globally. Severe iodine deficiency during pregnancy can lead to irreversible brain damage in the fetus, resulting in conditions such as cretinism, which is characterized by severe mental and physical retardation (Zimmermann, 2009).

Even mild to moderate iodine deficiency can have adverse effects, including impaired cognitive development in children, reduced productivity, and increased risk of goiter and other thyroid disorders (Bougma *et al.*,2013). The consequences of iodine deficiency are particularly severe for pregnant women, infants, and young children, as their requirements for iodine are higher due to rapid growth and development (Zimmermann & Boelaert, 2015).

The fortification of salt with iodine has been recognized as a safe, cost-effective, and sustainable strategy to address iodine deficiency globally (Diosady *et al.*,2017). Salt is an ideal vehicle for iodine fortification because it is widely consumed by most populations and its production is centralized, making it easier to fortify and monitor (Pearce *et al.*,2013).

Studies have demonstrated the effectiveness of iodine fortification in reducing the prevalence of IDDs. For example, a systematic review and meta-analysis by Aburto *et al*. (2019) found that iodine fortification programs significantly increased urinary iodine concentrations and reduced the risk of goiter in school-age children.

In addition, a study by Pearce *et al*. (2004) reported that iodine fortification of salt in the United States effectively eliminated iodine deficiency and reduced the prevalence of goiter, illustrating the long-term success of such programs.

Recognizing the importance of iodine fortification, various international organizations, such as the WHO, the United Nations Children's Fund (UNICEF), and the International Council for the Control of Iodine Deficiency Disorders (ICCIDD), have advocated for universal salt iodization (USI) programs (WHO/UNICEF/ICCIDD, 2007).

As a result, many countries have implemented mandatory or voluntary salt iodization programs, leading to significant improvements in iodine status and a reduction in the burden of IDDs (Pearce *et al.*,2021). However, challenges remain in achieving optimal iodine intake levels in some regions, emphasizing the need for continued monitoring and program evaluation (Eastman & Zimmermann, 2018). These programs have resulted in the advertisement of refined salt being fortified and sufficient in iodine as a important part of human health and to a no fact biased myth that natural salt is deficient in iodine which has led to a decline in consumption and sale of natural salts which is a myth as Goan natural salts were found to be sufficient in iodine content (Kerkar S, Fernandes M, 2012).

Importance of halophiles from salt pans

The salt pans hypersaline environment harbors a huge community of extremophiles which have the ability to produce bioactive compounds of huge relevance to the biotechnology and can have various industrial applications (Gawas P, Kerkar S, 2023). Salt pan environment

harbors halophiles that have developed the ability, strategy and cellular mechanisms that can ensure their survival in high salt concentrations and make them adaptable to the high salt stress. Salt in and salt out strategy is a prevalent theme in the survival of these halophiles in which in the salt-in strategy the intracellular system is adapted to the high salt concentrations but the consumption of energy in this process is huge whereas in the salt out system organism avoid consumption of energy and use the compatible solutes strategy (Oren, 1999).

The industrial enzyme market is a dynamic sector that serves various industries, including food and beverages, biofuels, textiles, detergents, pharmaceuticals, and more. Enzymes play a vital role in these industries by catalyzing biochemical reactions, accelerating processes, enhancing product quality, and minimizing environmental impact.

In recent years, the industrial enzyme market has experienced significant growth due to factors such as increasing demand for eco-friendly and sustainable products, advancements in enzyme engineering and biotechnology, and heightened awareness of enzyme benefits across diverse applications.

An emerging segment within the industrial enzyme market is halophilic enzymes. Halophiles are organisms that thrive in high-salt environments like salt lakes, saline soils, and salt mines. Halophilic enzymes are enzymes produced by these organisms, adapted to function optimally in high-salt conditions.These enzymes exhibit greater stability in harsh conditions like high temperatures, extreme pH levels, and organic solvents compared to non-halophilic enzymes. This stability makes them valuable for various industrial applications where stability is crucial. Halophilic enzymes have diverse biotechnological applications, including but not limited to food processing, pharmaceuticals, bioremediation, and biofuels production. For instance, they can improve the quality and efficiency of food production processes, enhance drug synthesis, aid in environmental cleanup, and contribute to sustainable energy

production. Halophilic enzymes offer a relatively untapped source of enzymes with unique properties, encouraging innovation and the discovery of novel enzymes with specialized functions. Research into halophilic enzymes expands the enzyme toolbox available to industries, fostering advancements in biotechnology and industrial processes (Das *et al.*,2019).

Phenol and phenolic compounds represent common pollutants found abundantly in the industrial wastewater of various sectors such as metal, steel, coal conversion, petroleum refining, resin, plastic, agrochemicals, pharmaceuticals, and dye industries (Grady, 1990; Ha *et al.*,2000).

These compounds pose significant environmental and health hazards, including cardiac arrhythmias, renal diseases, skin cancer, and even death (Rice and Cohen, 1996; Adeyemi *et al.*,2009).To address the removal of these toxic contaminants from industrial wastewater, various physical, chemical, and biological methods have been employed. Physical methods, such as pulse high-voltage discharge systems and quick sorption on activated sludge, offer effective removal of organic contaminants (Zhao *et al.*,2008; Shi *et al.*,2009). Chemical methods, including the Photo-Fenton reaction and solvent-impregnated resin systems, have been utilized for the removal of phenolic compounds (Gernjak *et al.*,2003; Cuypers *et al.*,2010).

Enzymatic treatment has emerged as a promising alternative for phenol removal from industrial wastewater. Treatment of aqueous phenols using oxidoreductive enzymes, such as peroxidases, has been identified as an efficient and cost-effective method (Klibanov *et al.*,1980). Peroxidases, including laccases and tyrosinases, catalyze the oxidative transformation of a wide range of phenolic and non-phenolic aromatic compounds to their

corresponding quinones (Escribano *et al.*,1997; Dura'n *et al.*,2002; Mayer and Staples, 2002; Saboury *et al.*,2006).

Tyrosinase, a copper-containing enzyme present in various microorganisms, plants, and animals, offers potential for phenol removal due to its ability to catalyze the ortho-hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones (Seo *et al.*,2003). Bacterial tyrosinases, particularly those from Streptomyces species, are considered advantageous over mushroom tyrosinases due to ease of cultivation and scalability (Della-Cioppa *et al.*,1998a, 1998b; Matoba *et al.*,2006; Popa and Bahrim, 2011).

Microplastic pollution in salt pans

Plastic-derived materials have gained widespread use due to their cost-effectiveness, versatility, durability, and other favorable properties (Wang *et al.*,2016). However, a significant portion of manufactured plastics ends up in the environment, contributing to pollution, with plastic waste constituting a substantial proportion of coastal detritus (Moore, 2008; Wang *et al.*,2016). Microplastics, small polymer particles ranging from 1 to 1000 μm, have become pervasive in marine environments, originating from various land- and marine-based sources, such as sewage wastes and recreational activities (Turner and Holmes, 2011). These microplastics, categorized as emerging hazardous pollutants, pose risks to aquatic organisms due to their non-degradable nature and potential to accumulate in ecosystems (Sedlak, 2017; Karami *et al.*,2016; Eriksen *et al.*,2014).

The presence of microplastics in marine organisms, including fish, mollusks, crustaceans, and marine vegetation, has been extensively studied (Pozo *et al.*,2019; Abidli *et al.*,2019; Hossain *et al.*,2020; Naidu *et al.*,2018; Zantis, 2021). Studies have also highlighted the contamination of marine goods like sea salts with microplastics due to their abundance in the marine environment (Karami *et al.*,2017; Blaskovi *et al.*,2017; Lee *et al.*,2019). This contamination

raises concerns as microplastics can enter the human food chain through salt consumption, with commercial salts often derived from sea salts harvested in salt pans or saline evaporation ponds (Selvam *et al.*,2020; Serrano *et al.*,2011).

Salt pans rely on the evaporation of seawater or brine to produce salt, a process that exposes them to potential microplastic contamination. The presence of microplastics in salt pans can result from various sources, including urban runoff, plastic litter, and atmospheric deposition (Turner and Holmes, 2011). Additionally, salt pan infrastructure, such as plastic-lined evaporation ponds and equipment, may contribute to microplastic inputs.

Microplastics pose significant challenges to salt pan ecosystems and salt production processes. These tiny particles can accumulate in sediments, brine, and salt crystals, potentially affecting salt quality and purity. The distribution and fate of microplastics in salt pans are influenced by factors such as hydrodynamics, sedimentation rates, and salt production practices. Transport mechanisms, such as wind and water currents, can disperse microplastics within salt pan ecosystems, leading to widespread contamination.

In India, particularly in states like Tamil Nadu, microplastic contamination in salt pans has emerged as a significant environmental concern. Tamil Nadu, the second-largest salt producer in India after Gujarat, has a prominent salt production industry, with salt pans spread across its coastal regions (Gundogdu and Çevik, 2017; Karthik *et al.*,2018; Krishnakumar *et al.*,2017). These salt pans are crucial for the local economy, providing livelihoods for numerous communities involved in salt harvesting and processing.

Microplastics contamination of salts from salt pans has recently grown to a large number due to anthropogenic activities and is one of the grave threat looming all over the world. Furthermore visual detection and determination of MPs can be performed using μ-Raman

spectroscopy and μ-Fourier transform infrared (FTIR) spectroscopy. (Hidalgo-Ruz, Gutow, Thompson, & Thiel, 2012)

CHAPTER 2: AIMS AND OBJECTIVES

2.1 Aim

To asses and evaluate the potential of natural salt from Goan salt pans for safety of consumption, nutritional value and iodine content and isolation of microorganisms from the salt pans to screen for bioactive compounds of significant use.

2.2 Objectives

The present work was carried out with the following objectives

- Salt sample collection from 5 different salt pans of Goa and titrating their iodine content and checking their stability.
- Morphological differentiation of natural salt using SEM .
- Isolation of culturable bacteria from different salt pan samples and assessing their enzymatic capabilities.
- Assesment of the presence of microplastics in the natural salt and commercial salt present in the market.

2.3 Hypothesis:

The dominance of salt industries and promotional campaigns advocating for the fortification of natural salt have led to a widespread misconception that all natural salts lack iodine. This false belief has influenced society, including in Goa, to favor refined salt consumption, consequently impacting the decline of traditional, economically viable salt produced by Goan salterns. This reality has motivated the current study to evaluate the quality of natural salt with the primary goal of dispelling the notion that it is inferior and iodine-deficient along with determining its stability and safety of consumption overtime. This study also focuses on the environmental aspects of these salt pans and emphasizes on the search for bioactive

compounds extracted from organisms isolated from the salt pan environment. There has been extensive research in micro plastics in sea water and marine organisms but the study of micro plastics from salterns of Goa is still at an early stage. For accurate assessment of the risks and the extent of reach of micro plastics in the salterns of Goa, current information gaps must be filled. Therefore, in this study the prevalence of micro plastics in the salt procured from Goan salt pans were investigated.

CHAPTER 3: MATERIALS AND METHODS

3. **Materials and Methods**

3.1 Site description

Five salt pans of North Goa were selected as sampling site. Samples of salts were collected from Agarwaddo, Curca, Batim, Nerul and Ribandar salt pans during the pre-monsoon season in the month of February 2023.

3.2. Sample collection

Salt, water and soil samples were collected from the 5 sampling sites. The salt samples were collected in sterile autoclaved PVC bags and glass stopper bottles. The water and sediment samples were collected in sterile stopper bottles and PVC bags respectively.

3.3. SEM analysis

Ten grams of each salt sample (Agarwado, Curca, Batim, Nerul and Ribandar) were dissolved in 100 mL of milliQ water individually and were vaccum filtered using a Watman filter paper 1. The respective powdered samples were then placed on nylon stubs with a carbon conductive tape. The samples were then sputter coated (20 nm) with gold. The coated samples were then analysed by SEM (model Carl-Ziess Scanning Electron Microscope) with oxford EDS.

3.4 Estimation of iodine content of salt

The determination of iodine content of the five natural salt sample collected and three refined salt samples were quantified by the process of iodometric titration as described by DeMaeyer *et al*.(1979).

3.5 Measurement of iodine content of salt after boiling

Ten grams of salt sample of both natural and refined salts were weighed and dissolved in 50mL of milliQ water in Erlenmeyer flask and heated till boiling cooled and then titrated against 0.005M sodium thiousulphate by iodometric titration (DeMaeyer *et al*.,1979).

3.6 Periodic testing of stability of iodine

Two samples of refined salt and all five samples of natural salt were selected to test for stability at room temperature .Ten grams of salt samples of each refined and natural salts were weighed and kept exposed in a petri-plate at room temperature. Iodometric titration was carried out using .005 M sodium thiousulphate solution (DeMaeyer *et al*.,1979) after a period of 5, 10,15,20,30 ,40days the titration was carried out at 28 ± 2 ℃ temperature.

3.7 Heterotrophic count on modified Nutrient Agar media

The salts from the five salt pans were respectively serially diluted upto 10^{-8} dilutions and plated on modified nutrient agar (containing 20% w/v natural salt in agar) and were incubated at room temperature (28 ± 2 ℃) for 20 days.

3.8 Heterotrophic count on modified Potato Dextrose Agar media

The salts from the five salt pans were respectively serially diluted upto 10^{-8} and plated on modified Potato Dextrose Agar (containing 20%w/v natural salt in agar) incubated at room temperature (28 ± 2 ℃) for 25 days.

3.9 Enteric pathogens

Salt samples of each natural salt(Agarwado, Curca, Batim, Nerul and Ribandar) (10mg) were dissolved in 10mL of sterile saline solution and a ten fold dilution up to 10^{-8} were prepared. 100µL aliquots of each dilution were plated on MacConkey agar, Eosin methlene blue (EMB) agar, TCBS agar and Salmonella Shigella (SS) agar. All the plates were incubated at 37 °C for 7 days.

3.10 Enteric pathogen test for colonies grown on Nutrient media

Colonies obtained on the modified nutrient agar media were streaked on MacConkey agar, eosin methlene blue (EMB) agar, TCBS agar and Salmonella Shigella (SS) agar and modified agars of the same containing 25% natural salt content. All the plates were incubated at 37 °C for 28 days. All media used were from Hi-Media.

3.11 Blood agar haemolysis test

Salt samples (10mg) from each of the salt pans (Agarwado, Curca, Batim, Nerul and Ribandar) were dissolved in 10ml of sterile milliQ water individually and were plated on Blood agar plates to check for haemolysis.

3.12 Isolation of halophiles from water sample from salt pans.

100 µl of water samples from each salt pan (Agarwado, Curca, Batim, Nerul and Ribandar) was plated on Halopiger medium containing (g/L) natural salt 250, KCl 2, $MgSO_4.7H_2O$ 20, Sodium tri citrate 3, Yeast extract 10, Agar 20 (pH adjusted to 7.2). All the plates were incubated at room temperature (28 ± 2 °C) for 20 days.

3.13 Morphological characterization

For Gram staining, cell suspension of the isolated bacterial culture was prepared and a smear was made on a clean glass slide using 0.8 %NaCl. Smear was heat fixed and staining was performed by following the standard Gram staining protocol. The slides were observed using a phase contrast light microscope (100X objective).

3.14 Screening of isolates for hydrolytic enzymes

The isolates were further screened for production of hydrolytic enzymes using plate assay method. Activity was checked in minimal Norberg Hofstein (NH) media containing (g/l) NaCl 200, MgSO4 7H2O 10, KCl 5, yeast extract 1 (pH adjusted to 7.0 using 1 M KOH). The media was supplemented with the substrate of which the activity was to be checked for. 50µl of each culture was spotted on the respective plates except for checking of esterase and lipase activity on which wells were bored using a sterile cork borer and the respective cultures were added to the wells.

Amylase activity

Plate assay was carried out in Norberg Hofstein (NH) media supplemented with 1 % (w/v) soluble starch. The different cultures were spotted on the plates. The plates were incubated for 20 days at room temperature and were then flooded with 0.3%I_2-0.6%KI solution. Amylase production is indicated by the presence of a clear zone.

Cellulase activity

Plate assay was carried out in Norberg Hofstein (NH) media supplemented with 1 %(w/v) Carboxy Methyl Cellulose (CMC). The different cultures were spotted on the plates. The

plates were incubated for 20 days at room temperature and were then flooded with 2%Congo red solution. Cellulase production is indicated by the presence of a clear zone.

Protease activity

Plate assay was carried out in Norberg Hofstein (NH) media supplemented with 1%(w/v) skimmed milk. The different cultures were spotted on the plates. The plates were incubated for 20 days at room temperature and were then flooded with commasine brilliant blue. Protease production is indicated by the presence of a clear zone.

Lipase activity

Olive oil 1%(v/v) was supplemented in Norberg Hofstein (NH) media. 50µl of culture were added respectively in wells bored on the agar using sterile borer. The presence of lipase activity was determined by formation of white precipitates around the bored wells.

Esterase activity

Tween80 and Tween20 1%(w/v) was supplemented in the above media. 50µl of culture were added respectively in wells bored on the agar using sterile borer. The presence of lipase activity was determined by formation of white sediments or precipitates around the bored wells.

3.15 Screening for L-asparaginase and L-glutaminase activity

Screening for L-asparaginase and L-glutaminase activity was done using rapid plate assay method (Gulati *et al.*,1997). Halopiger medium was supplemented with L-asparagine and L-glutamine (5.0 g/L). 2.5 ml of 1%stock solution of phenol red was added as the pH indicator. Plates were incubated at 37°C for 48 hours. The indicators colour change to pink around the

spotted culture after 24 to 48 hours was the indicator of L-asparaginase and L-glutaminase activity.

3.16 Primary screening for tyrosinase enzyme

Primary screening of tyrosinase producing enzyme was carried on skim milk agar plates. All the isolates were streaked on skim milk agar plates (pH 6) containing natural salt 0.5%, skim milk 10%, yeast extract 0.3%, peptone 1%and agar 2%. All the plates were incubated at 30°C for 48 hrs. After incubation the plates were observed for the zone of clearance around the isolates.

3.17 Isolation of marine actinobacteria from soil samples of salt pans

A 10-fold serial dilution of the soil sample of each salt pan was prepared up to 10^{-6}. 100µL aliquots of each dilution was inoculated into starch casein agar medium (pH 7.2) prepared with 50%seawater to sustain the growth of actinobacteria. To avoid the growth of fungal and bacterial contaminant, potassium dichromate (50 µg/mL) was supplemented to the medium. The plates were incubated at room temperature (28°C) and monitored periodically over 21 days for actinomycetes growth. The pure isolates of the actinobacteria were transferred to ISP2 (Isolation streptomyces project medium No.2) media.

3.18 Secondary screening method

Tyrosinase enzyme producing marine actinobacteria were further screened by following different methods like tyrosine agar plate assay and tyrosine broth.

Tyrosine agar

The isolates were streaked on tyrosine agar (pH 7) containing peptone 0.5%, beef extract 0.3%, agar 2%and L-tyrosine 0.5%and all the plates were incubated at 30°C for 7 days. The occurrence of brown pigmented colonies that gradually changed its color to black (melanin formation) was indication of tyrosinase positive organism (Raval *et al.*,2012) .

Tyrosine broth

The isolates were inoculated into 50 mL of 0.1%tyrosine broth with few drops of chloroform in 100mL Erlenmeyer flasks and incubated at 30°C for 7 days. The deep brown to red colour shows the positive results (Raval *et al.*,2012).

3.19 Ammonium sulphate precipitation

The tyrosine broth was centrifuged at 9500 RPM for 30 min at 4°C in a cooling centrifuge. The supernatant was collected and ammonium sulphate powder was added to the supernatant slowly with continuous stirring in an ice bath, until 60% saturation is achieved. The mixture was kept at 4°C overnight followed by centrifugation at 9500 RPM in a cooling centrifuge. The supernatant was discarded and the precipitate was dissolved in 0.2M phosphate buffer (pH7.0) with continuous mixing using magnetic stirrer.

3.20 Dialysis

The dialysis membrane was cut and pre-treated in boiling water for 60 min and stored in 0.2M phosphate buffer (pH 7.0). The membrane was packed up with the dissolved precipitate and sealed by clamps on both ends. The dialysis bag was suspended overnight at 4°C in a glass beaker containing 0.2 m phosphate buffer (pH 7.0) with continuous mixing using a magnetic stirrer.

3.21 Removal of phenol from waste water.

100µL of the partially purified enzyme was added to 10ml of the waste water sample and was incubated at 35°C. The concentration of phenol remained was estimated by using Folin Ciocalteu reagent (Maurya and Singh, 2010) after every two hours uptill 8 hours.

3.22 Isolation of MPs from salt samples

Ten grams of each natural salt and commercial salt was weighed in a sterile glass petri-plate and were dissolved in 100ml of Hydrogen peroxide in sterile glass stoppered bottles and stored at 65°C in a water bath for digestion for 7 days. After 7 days of incubation the samples were diluted with milliQ water uptil the final volume becomes 1litre and were then filtered through a vaccum filter unit using a filter paper of pore size 0.44µm and 0.22µm.The filter paper was then visualised under light microscope for visual identification and isolation of microplastics.

3.33 Raman spectroscopy analysis of MPs

One sample each of the isolated MP was selected for Raman spectrometry and were designated code names and were analysed using a labRAM Evolution microscope at a wavelength of 785nm. Similarity search for isolated particles was done using KnowItAll Information System 2021 by Wiley Online Raman Database

CHAPTER 4: RESULTS AND DISSCUSIONS

4 Results

The natural salt collected from the five salterns of Goa were assessed for various parameters.

4.1 Iodine content estimation

The iodine content of natural salt and refined salt samples were measured by quantitative titration analysis and were compared as shown in Table 1 and Figure 4.1.1. Among refined salt Saffola salt showed the highest concentration of iodine followed by Tata salt and Tata I shakti salt. Significant amount of iodine was estimated in natural salts from Ribandar, Curca, Batim, Nerul and Agarwado. Among the five natural salts Agarwado had the least amount of iodine and was white in colour as compared to other natural salts as shown in table 1. The stability profile of iodine with exposure to room temperature for a period of 45 days is represented in Figure 4.1.2. A marked decrease in the content of iodine was seen in both natural and refined salts on exposure to room temperature which was in accordance to the study done by Kerkar S *et al*,.2013 as represented in table 2 .

Table 1: Iodine content of Goan natural salt and refined salt before and after boiling.

Salt sample	Without boiling	With boiling
Tata salt	58	46
captain cook	62	50
Tata I shakti	24	10
Curca	42	38
Batim	40	36
Ribandar	44	38
Nerul	40	38

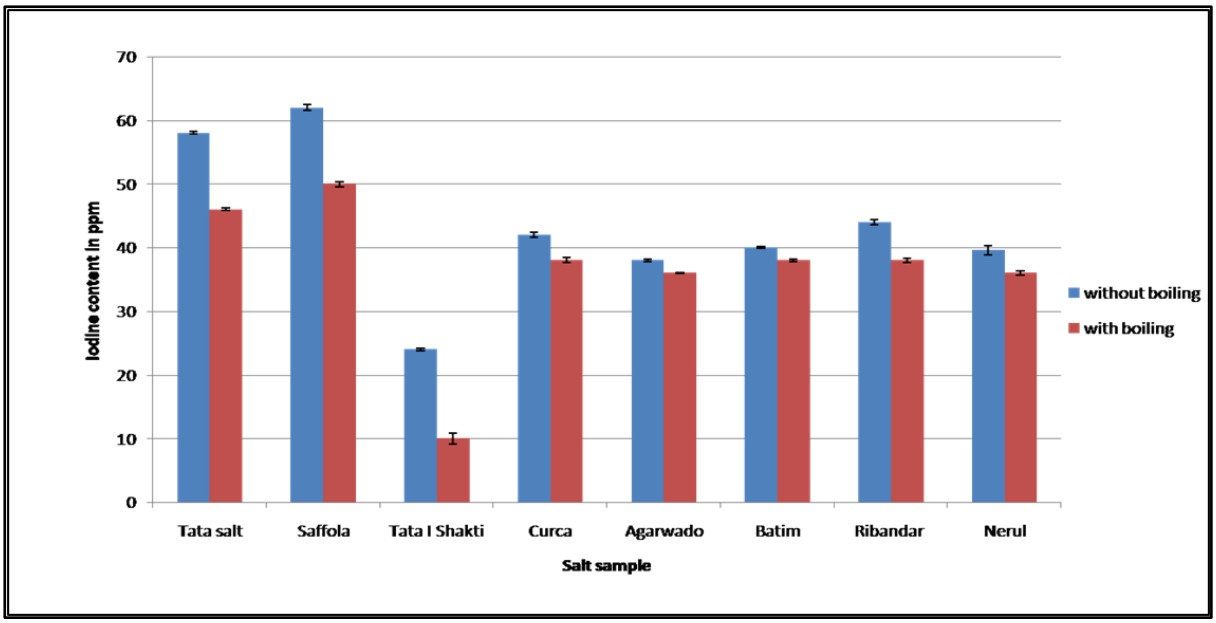

Figure 4.1.1: Iodine content of Goan natural salt and refined salt before and after boiling.

Table 2: Iodine content of salt samples on exposure to Room temperature

Salt sample	Iodine content at day 0	Day 5	Day 10	Day 20	Day 30	Day 45
Tata	58	54	46	32	18	8
Saffola	62	60	48	30	28	8
Tata I Shakti	24	18	12	4	0	0
Curca	42	40	38	30	26	12
Batim	40	37	33	27	20	10
Ribandar	44	37	35	27	22	12
Nerul	40	38.5	32	26	20	10
Agarwado	38	35	33	26	20	8
Tata	58	54	46	32	18	8

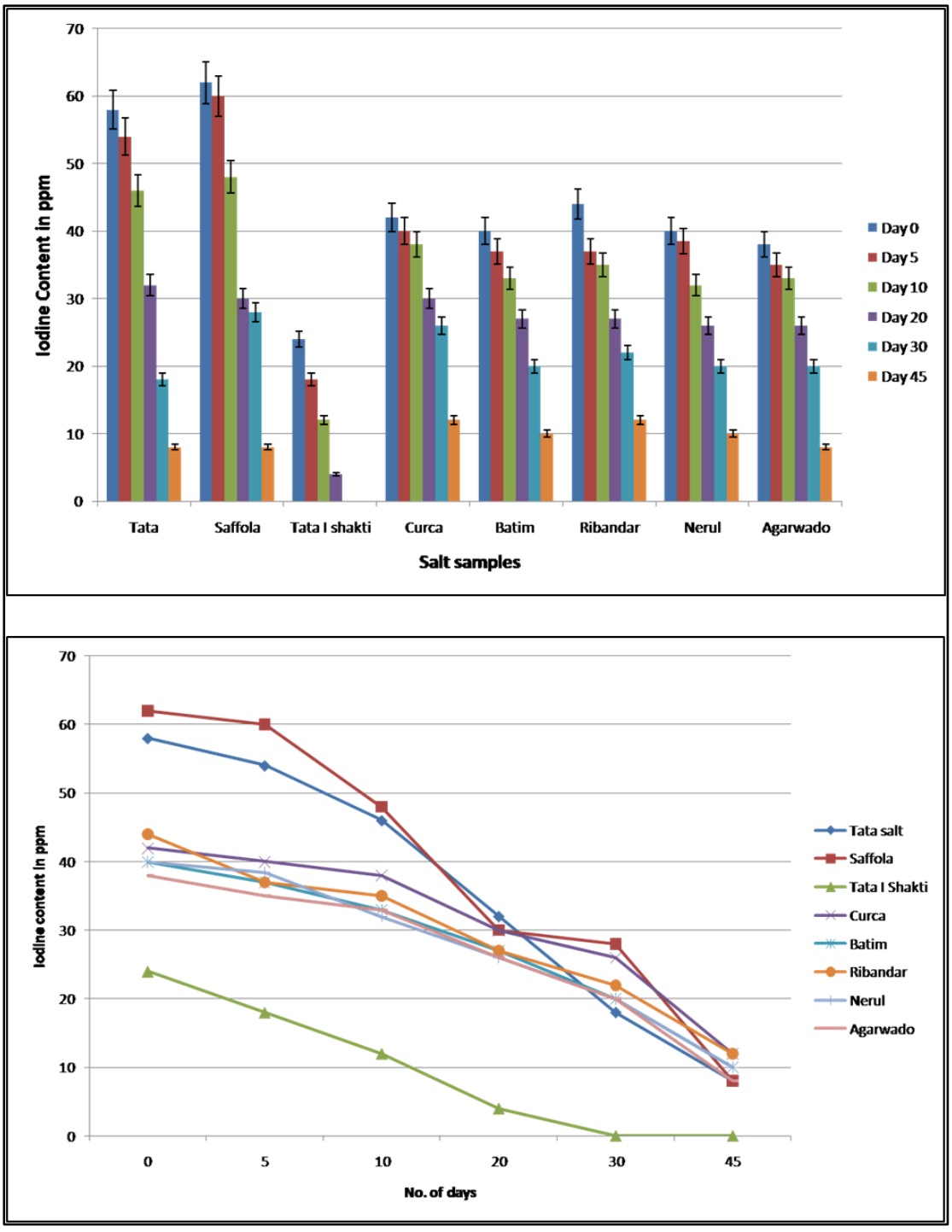

Figure 4.1.2: Comparative study of iodine content of Goan natural salt and refined salt for 45 days of exposure to room temperature.

4.2 SEM analysis

Scanning electron micrographic images revealed the distinct structure of the natural salts from the salterns.

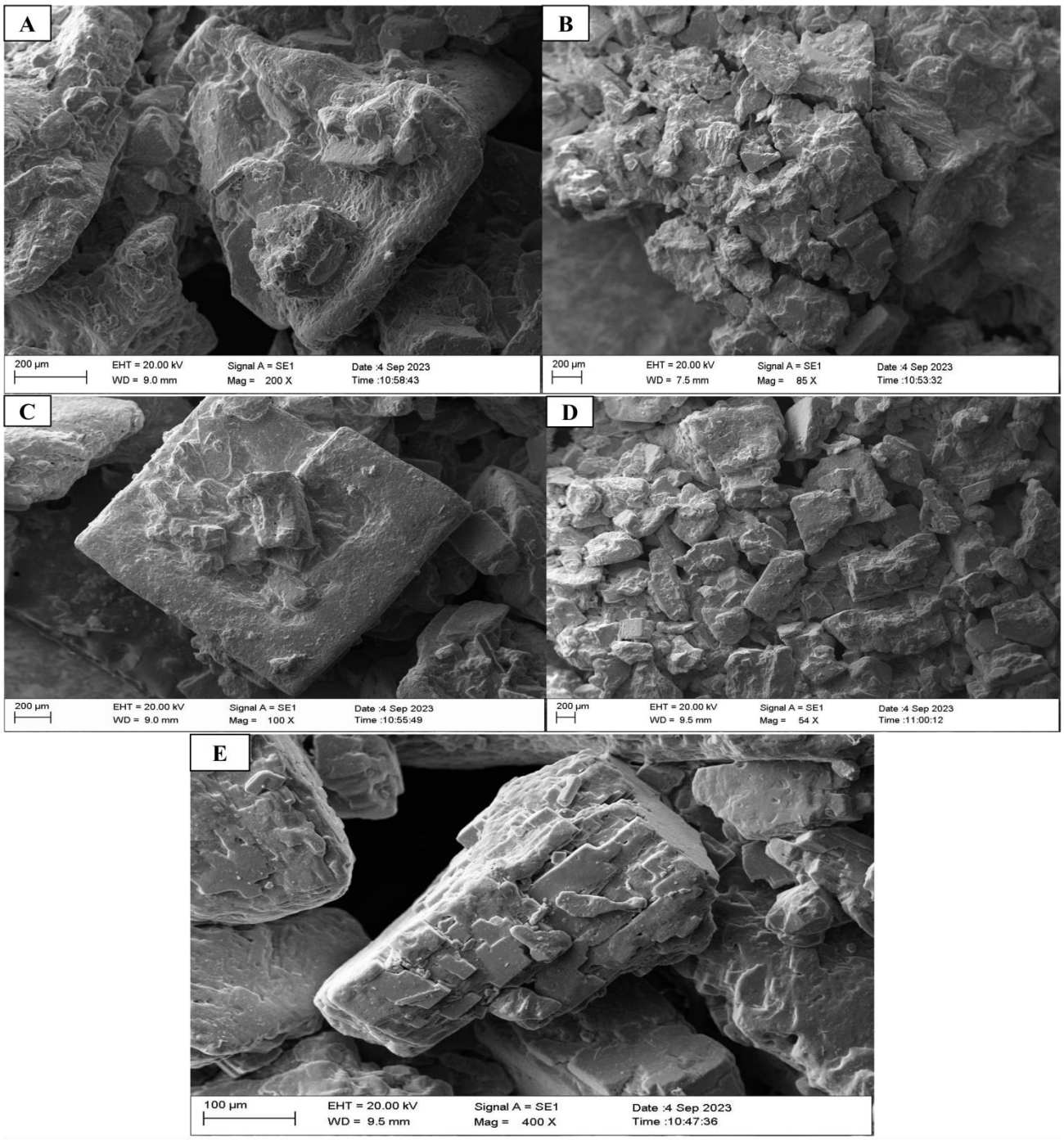

Fig- 4.2.1. Scanning electron micrograph of salt crystals collected from different salt pans of Goa; (A) Salt crystal of Curca salt pan- Bar corresponds to 200 µm; (B) Salt crystal of Agarwado salt pan- Bar corresponds to 200 µm; (C) Salt crystal of Ribandar salt pan- Bar corresponds to 200 µm; Salt crystal of Batim salt pan- Bar corresponds to 200 µm; Salt crystal of Nerul salt pan- Bar corresponds to 100 µm

4.3 Heterotrophic count on modified agar

Heterotrophic bacteria were observed on modified nutrient agar plate on all of the natural salt samples of dilution 10^0 as represented in table 3. No colonies were observed in dilutions of $10^{-1}$to 10^{-8}.

4.4 Heterotrophic count on modified potato dextrose agar(PDA)

No bacterial or fungal colonies were detected on PDA plates of all the natural salts on any dilutions (Table 3).

Table 3: Heterotrophic count of Goan natural salts

Salt pan	Modified nutrient agar (CFU/mL) on 10^0 dilution	Potato dextrose agar (CFU/mL)
Curca	110	Not detected
Agarwado	40	Not detected
Ribandar	330	Not detected
Batim	160	Not detected
Nerul	640	Not detected

4.5 Enteropathogenic test

No enteric pathogens were detected in any of the salt samples plated on the selective media. No growth of the colonies obtained on the modified agar plates were observed on the respective medias.

4.6 Blood agar haemolysis test

Gamma haemolysis (no haemolysis) was observed in all the five natural salt samples.

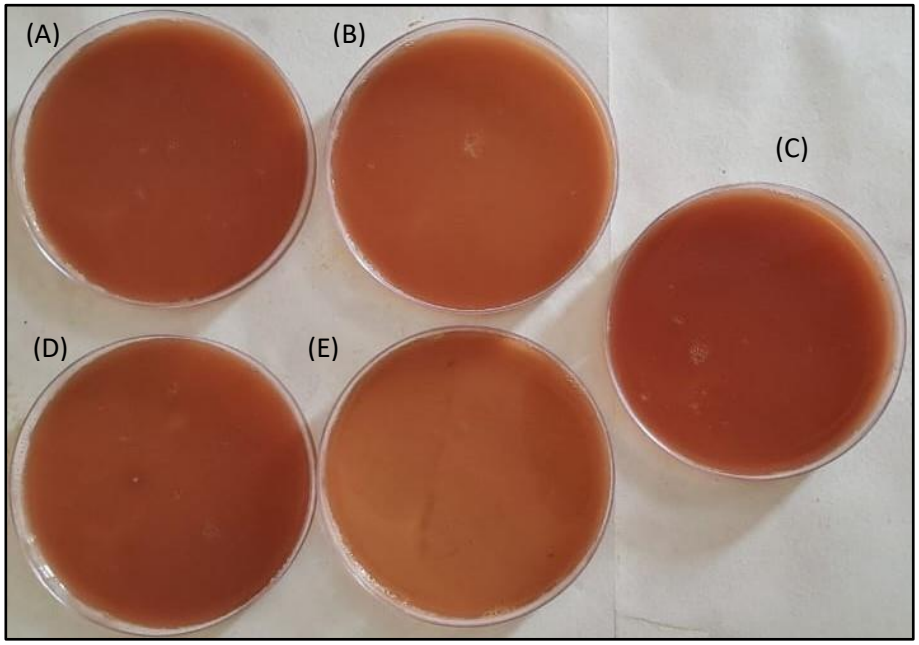

Figure 4.6.1: Blood agar haemolysis test plates of Agarwado (A),Batim (B),Curca (C), Nerul (D), Ribandar (E).

4.7 Isolation, growth and maintenance of halophilic isolates

The samples obtained from various sites were plated out and visually distinct cultures were purified by repeated streaking. Isolates SKA1 and SKA2 were isolated from Halopiger media from Curca soil sample, SKA3 and SKA4 were isolated from Ribandar soil sample and three isolates SKA5 ,SKA6 ,SKA7 were isolated from Nerul soil sample. Table 4 shows details of each sample with respect to their sampling sites and pigmentation. The pigmentation of isolates was in different ranges.

Table 4: Pigmentation of halophilic isolates and their eco-niche.

Isolate	Sampling site	Pigmentation
SKA1	Curca	White
SKA2	Curca	Yellow
SKA3	Ribandar	White
SKA4	Ribandar	Cream
SKA5	Nerul	Yellow
SKA6	Nerul	Orange
SKA7	Nerul	Cream

4.8 Morphological characteristics of the isolates

Gram character of all isolates are shown in Table 5.

Table 5: Gram character of Isolates obtained on Halopiger plates.

Isolates	Gram character	Morphology
SKA1	Gram negative	Rod shaped
SKA2	Gram negative	Rod shaped
SKA3	Gram negative	Rod shaped
SKA4	Gram negative	Rod shaped
SKA5	Gram positive	Cocci
SKA6	Gram negative	Small rods
SKA7	Gram positive	Rod shaped

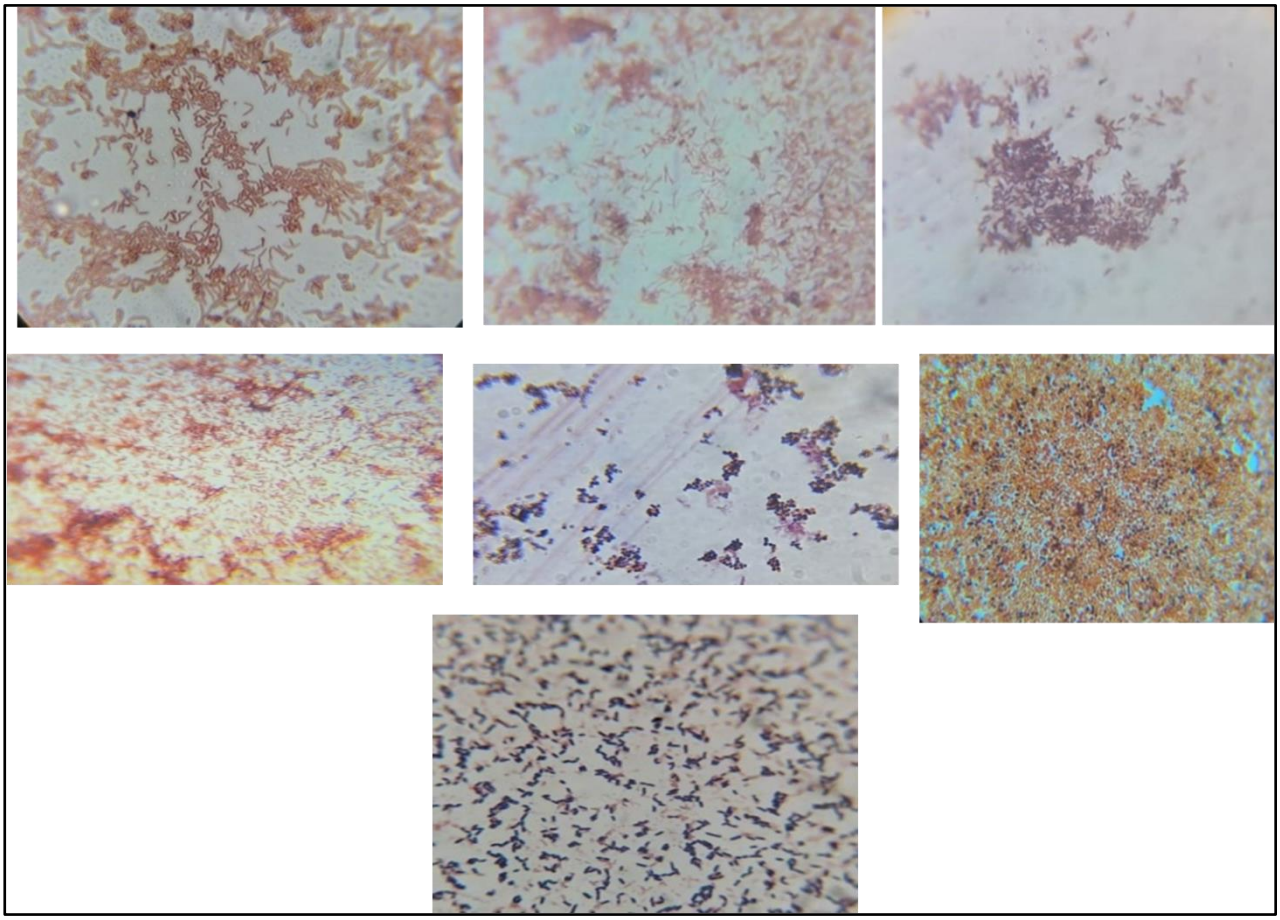

Figure 4.8.1: Isolates (a) SKA1 (b) SKA2 (c) SKA3 (d) SKA4 (e) SKA5 (f) SKA6 (g) SKA7 under 100X objective.

4.9 Screening of isolates for extracellular hydrolytic enzymes

Seven halophilic cultures namely SKA1, SKA2, SKA3, SKA4, SKA5, SKA6, SKA7 were screened for the production of protease, cellulose, amylase, lipase and esterase using plate assay with medias containing more than 25% natural salt(Fig).

Isolates SKA1, SKA2 ,SKA3 ,SKA4, SKA5 exhibited lipase and esterase activity whereas cellulase, protease and amylase were not detected in any of the cultures isolated amylase activity was shown only by SKA5 as shown in table 6.

Table 6: Isolates showing plate assays

Isolates	Amylase	Protease	Cellulase	Esterase	Lipase
SKA1	Negative	Negative	Negative	Positive	Positive
SKA2	Negative	Negative	Negative	Positive	Positive
SKA3	Negative	Negative	Negative	Positive	Positive
SKA4	Negative	Negative	Negative	Positive	Positive
SKA5	Positive	Negative	Negative	Positive	Positive
SKA6	Negative	Negative	Negative	Negative	Negative
SKA7	Negative	Negative	Negative	Negative	Negative

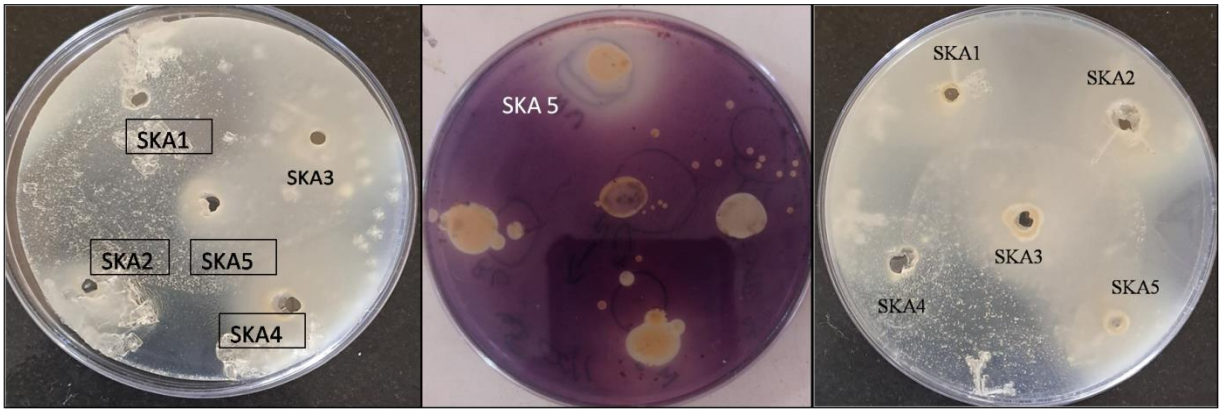

Figure 4.9 plate assay exhibiting esterase (a) amylase (b) and lipase (c) activity.

4.10 Screening for L-asparginase and L-glutaminase activity

All isolates were screened for L-asparginase and L-glutaminase activity. Isolate SKA1 and SKA3 showed both L-asparginase and L-glutaminase activity while L-asparginase activity was only showed by SKA1 and SKA3 whereas L-glutaminase activity was shown by SKA1, SKA2, and SKA3.

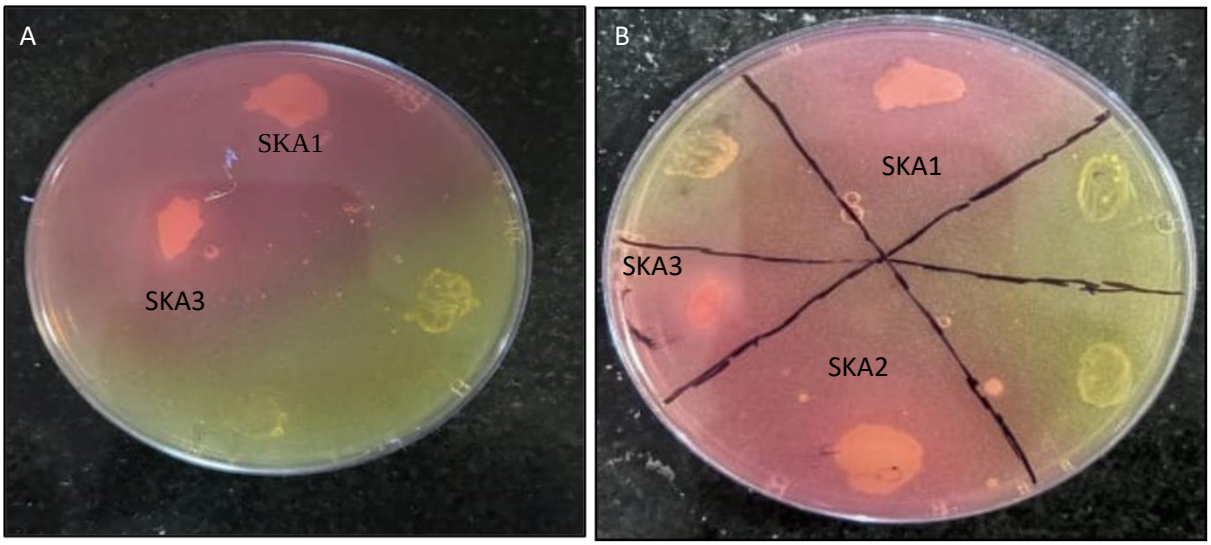

Figure 4.10.1: plate assay exhibiting **(A)** L-asparginase and **(B)** L-glutaminase activity.

4.11 Screening for tyrosinase activity

A total of 11 marine actinobateria were isolated from the soil sample of marine salterns.Out of 11 isolates, isolate SKAC1 showed positive proteolytic activity on Skimmed milk agar and was selected for further studies. In the tyrosine agar plates colonies showed brown colour pigment formation which eventually turned to black giving a positive indication for tyrosinase activity. The colour change of tyrosine broth from yellow to light brown gave positive indication of tyrosinase activity.

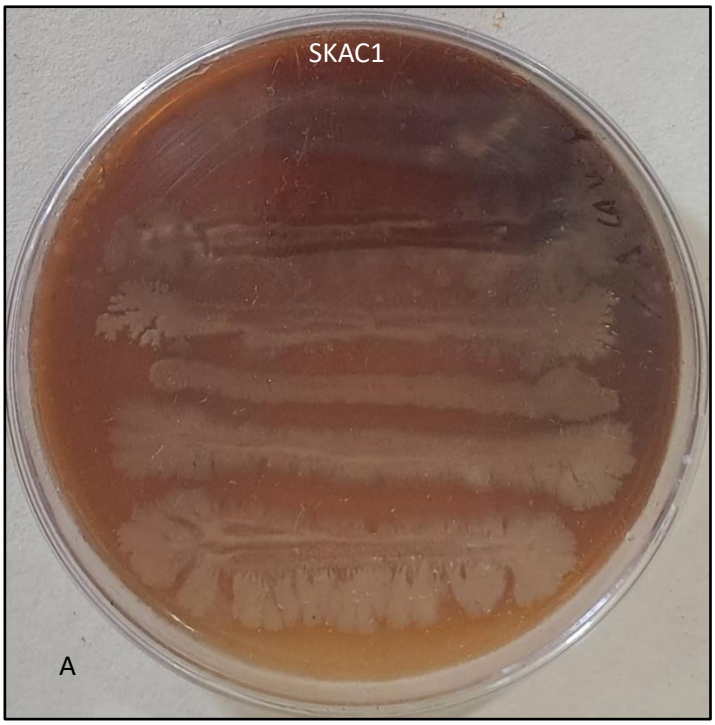

Figure 4.11.1: Tyrosinase plate assay(A) and tyrosine broth (B) exhibiting tyrosinase activity of isolate SQ1

4.12 Purification of tyrosinase enzyme

After incubation the tyrosinase enzyme was further purified using Ammonium sulphate precipitation and dialysis.

4.13 Removal of phenol from wastewater

The addition of the partially purified tyrosinase enzyme (100µl) to 10ml of waste water resulted in the decrease of 60.81% of phenol content from the waste water sample in 8 hours.

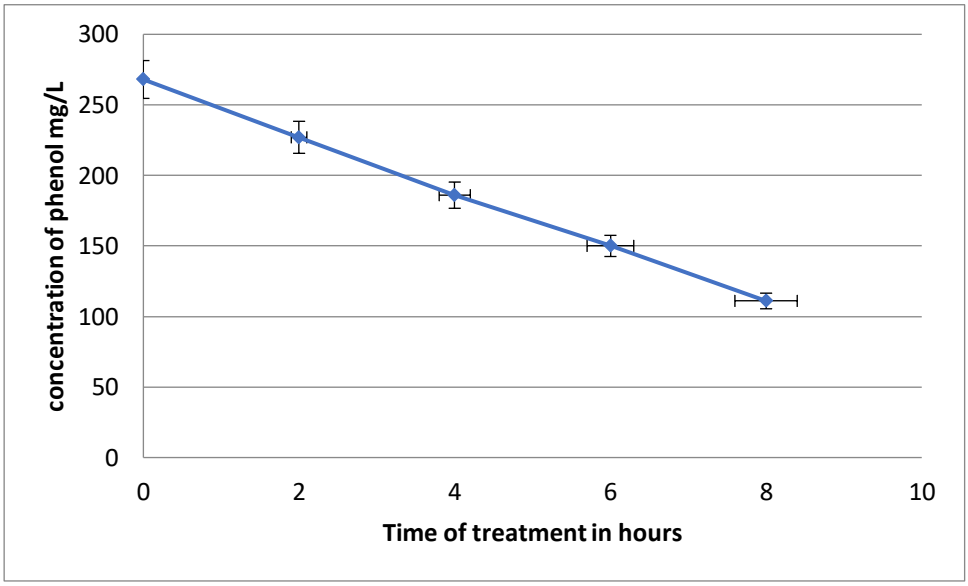

Figure 4.13.1: Removal of phenol from waste water over different period of time.

4.14 Analysis of MPs (0.40µm to 0.22µm)

After the digestion of salt samples with 30% H_2O_2 the salt samples were analysed for the presence of MPs using microscopic and Raman spectroscopy. MPs of size 0.40µm to 0.22µm were collected after passing the samples through nitrocellulose filter paper of the respective sizes. The isolated MPs from salt samples were then visually categorised into groups based on their colour, level of thickness, shape hardness and transparency as shown in Table 7.

4.15 Characterization of MPs

Table 7: Visual characterization of isolated MPs from salt samples.

Name of MP	No.	Colour	Level of thickness	Transparency	Shape of the fragment
TS01	6	Colourless	Thick	Transparent	Fragment
CK02	5	Colourless	Thick	Transparent	Fragment
RB01	4	Colourless	Thick	Transparent	Fragment
CU01	3	Black	Thick	Transparent	Fragment
BA01	4	Colourless	Thick	Transparent	Fragment
AG01	4	Brown	Thick	Transparent	Fragment
NU01	3	Black	Thick	Transparent	Fragment

4.16 Microscopic analysis

As seen in figure 14-20 the microplastics were observed under microscope using 10X objective lens and were in the form of fragments. The colour of MPs ranged from brown, black to colourless.

Figure 14.6.1: Microscopic image of TS01

Figure 14.6.2: Microscopic image of CK01

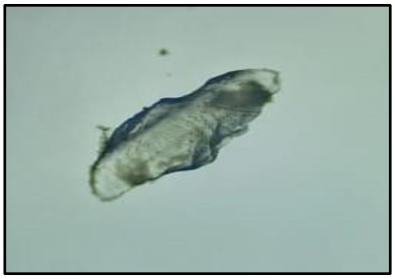

Figure 14.6.3: Microscopic image of RB01

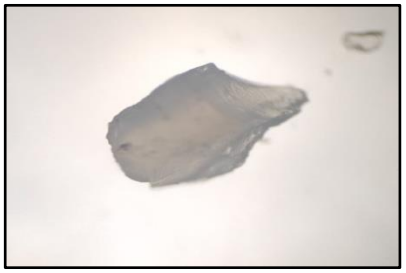

Figure 14.6.4: Microscopic image of CU01

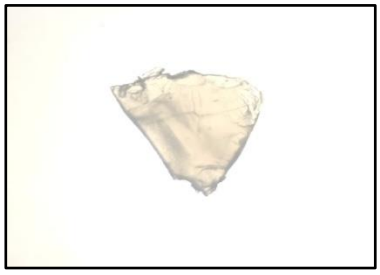

Figure 14.6.5: Microscopic image of BA01

Figure 14.6.6: Microscopic image of AG01

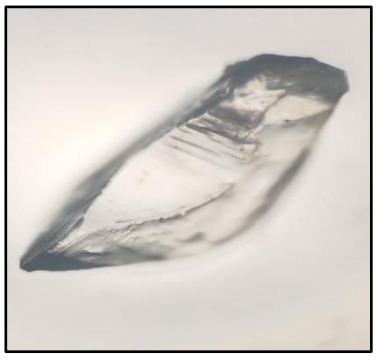

Figure 14.6.7: Microscopic image of NU01

4.17 Raman spectra analysis of the MPs

Raman spectrum were compared to standard plastic polymer spectrum for each of the MP classes using Wiley Science Solutions's KnowItAll Raman Spectral Database Collection. The similarity varied from 60 to 80%. The results for each category are given in table 8.

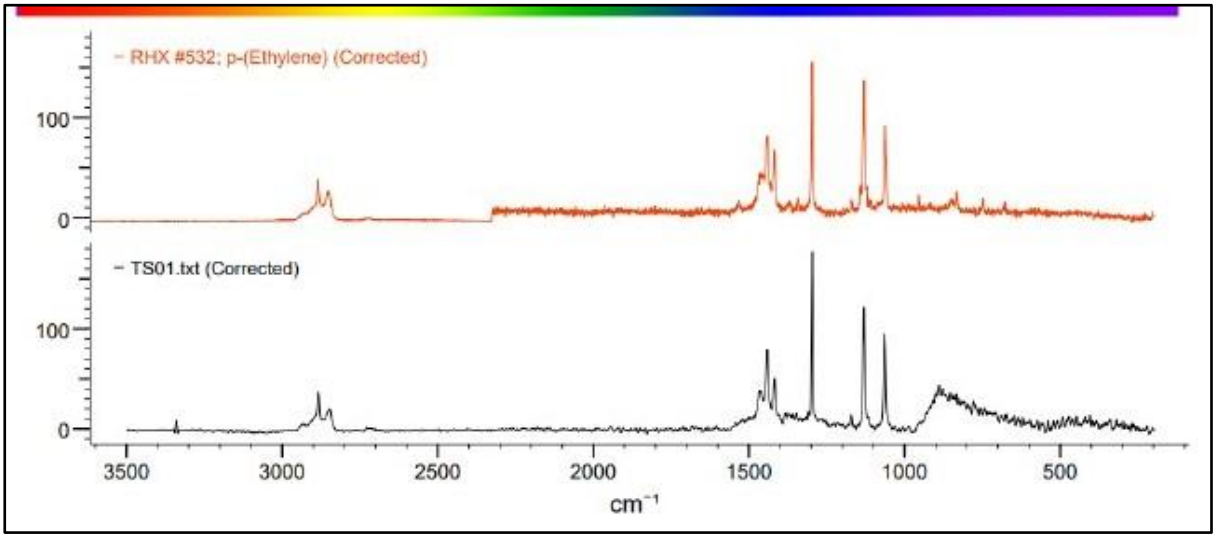

Figure 4.17.1: Raman spectra for TS01

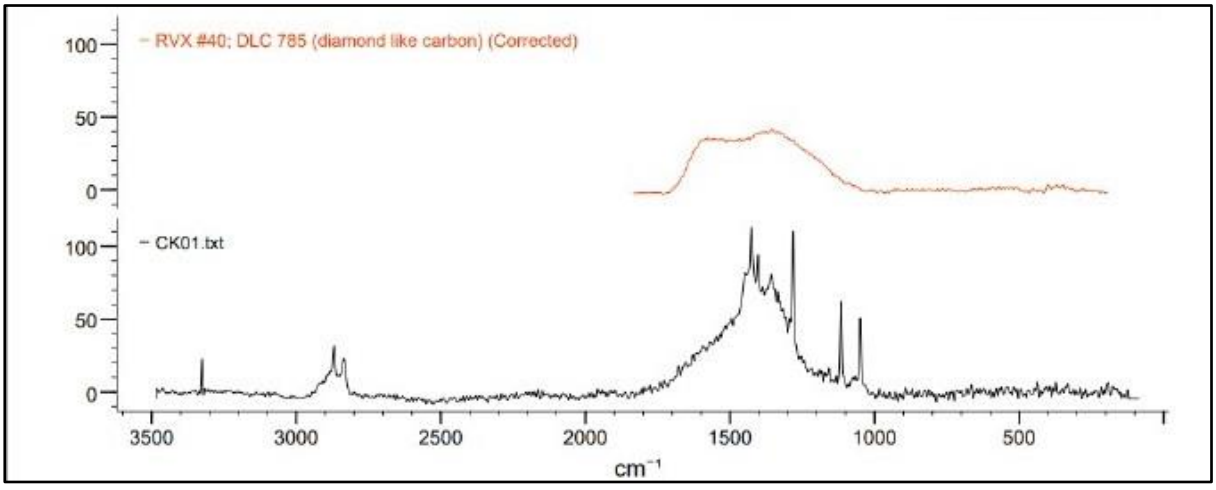

Figure 4.17.2: Raman spectra for CK01

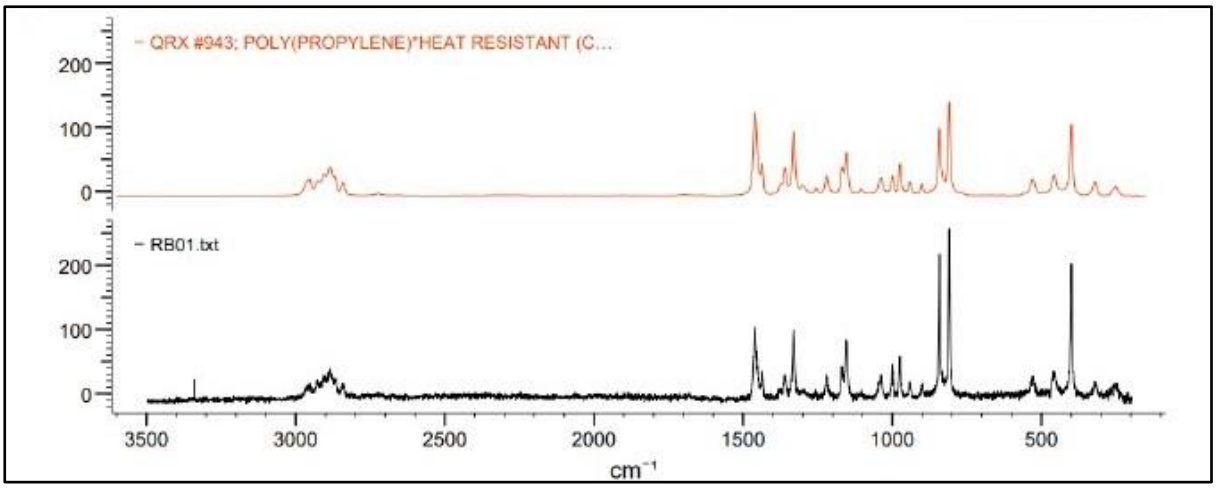

Figure 4.17.3: Raman spectra for RB01

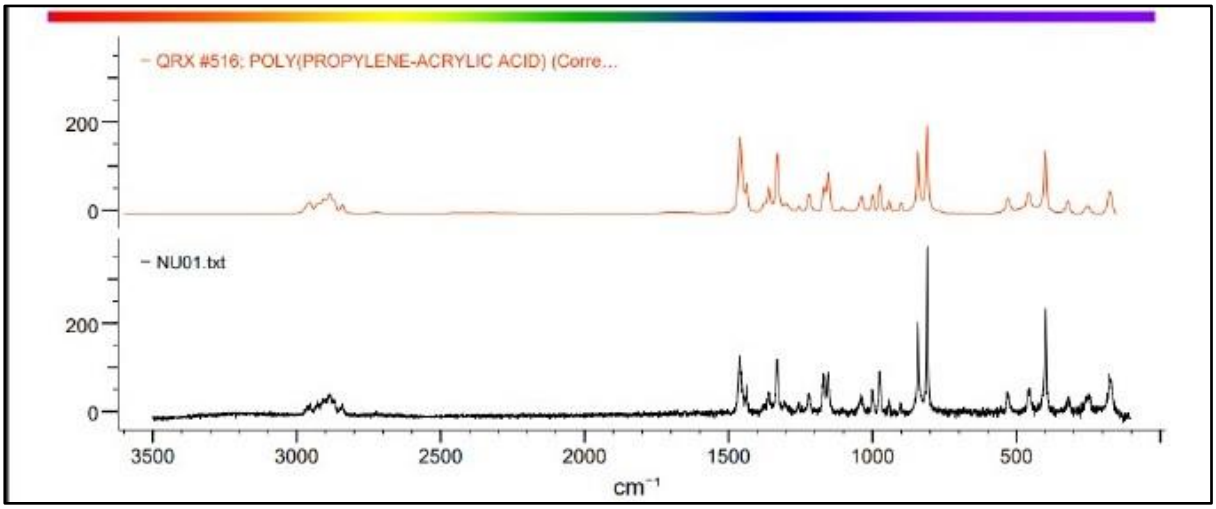

Figure 4.17.4: Raman spectra for NU01

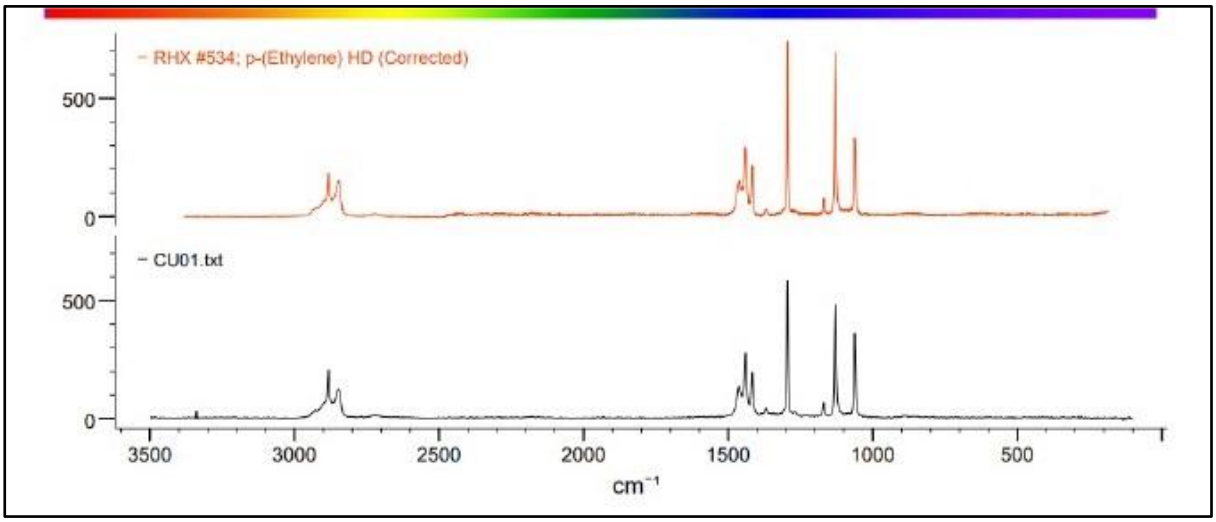

Figure 4.17.5: Raman spectra for CU01

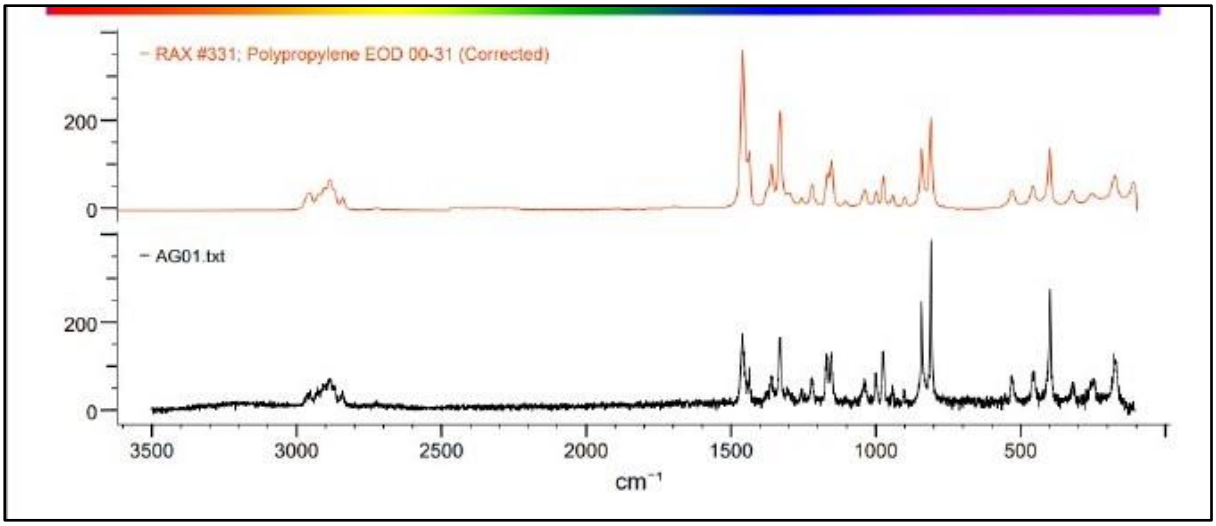

Figure 4.17.6: Raman spectra for AG01

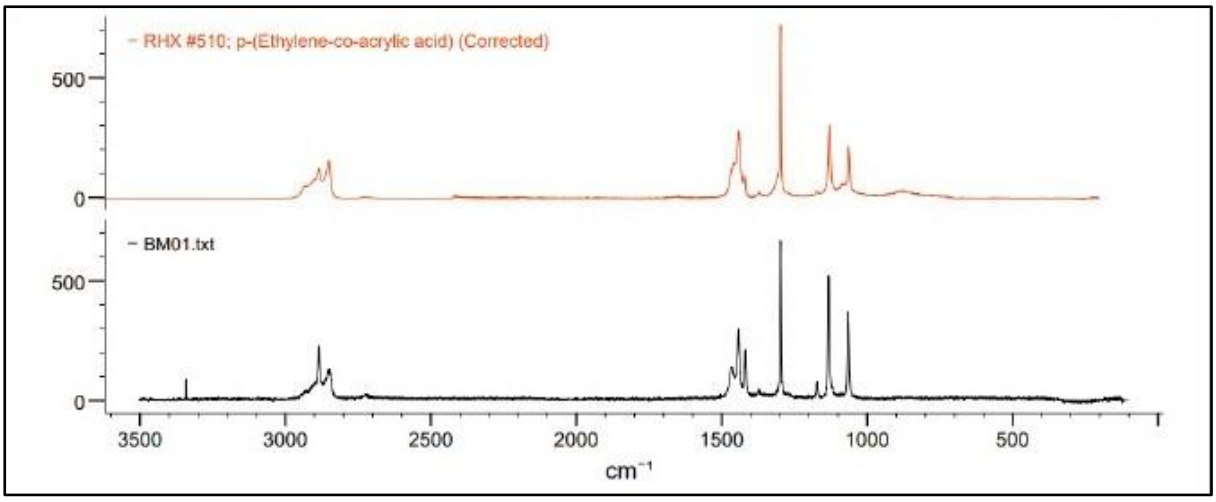

Figure 4.17.7: Raman spectra for BA01

Table 8: Raman analysis for salt samples.

S. no	MP given for Raman analysis	Sample from	Similar to
1	TS01	Commercial salt	Polyethylene
2	CK02	Commercial salt	Diamond like carbon
3	RB01	Ribandar	Poly propylene
4	CU01	Curca	High density polyethylene
5	BA01	Batim	Polyethylene-co-acrylic acid
6	AG01	Agarwado	Polypropylene EOD
7	NU01	Nerul	Polypropylene acrylic acid

Raman spectrum for TS01 showed similarity to polyethylene which is a common material used in the salts packaging material, CK02 showed similarity to diamond like carbon which is used as protective coating of industrial machinery and coatings, RB01 showed similarity to polypropylene which is used to make swim suits, PPE kits ,masks and common day use items, CU1 showed similarity to high density polyethylene, BA01 showed similarity to

Polyethylene-co-acrylic acid which is used in making common packaging materials, AG01 showed similarity to polypropylene and NU01 was similar to polypropylene acrylic acid.

This in accordance with the data provided in Nithin, *et al*,.2021 where polyethylene and polypropylene were found in both natural salts obtained from salt pans of Marakkanam and Parangipettai in Tamil Nadu as well as commercial salt.

CHAPTER 5:SUMMARY

5.1 Summary

In this study the assessment of natural salt produced from five Goan salt pans were done for their estimation of iodine content its stability and nutritional content compared to commercial salt available in the market. The study confirms that iodine content in Goan natural salt is less than commercial salt, but is more than sufficient compared to the standard value set by the World Health organisation and has the necessary elements required for the physiological processes of the human body thus natural salt can provide our requirement of iodine intake sufficiently. Furthermore, natural salts were found to be more stable in retention of iodine content to exposure at room temperature than natural salts and the same was found for after boiling the salts. The salt pan ecosystem supports a variety of microorganisms known as halophiles which produce potent bioactive molecules with important applications in biotechnology and industries. Bacterial isolates were isolated from the water samples of salt pans of Curca, Ribandar and Nerul and were designated as SKA1, SKA2, SKA3, SKA4, SKA5, SKA6, SKA7, out of which SKA1, SKA2, SKA3, SKA4, SKA5 showed the presence of lipase and esterase activity. Isolates SKA1 and SKA3 exhibited L-asparginase and L-glutaminase activity which have shown their use in treatment of lung cancer whereas L-glutaminase activity was exhibited by SKA1, SKA2, SKA3. These bioactive molecules are of great relevancy for various industrial and pharmaceutical application and the presence of these microbes in the salt pan ecosystem makes it more important to save these declining ecosystem which can be a potential source of these bioactive molecules. Marine actinomycetes are a ubiquitous source of potential bioactive compounds that have been used in various fields. In our study 11 marine actinomycetes isolates were isolated from the soil sample of the five salt pans of Goa out of which isolate SQ1 isolated from the soil sample of Curca showed tyrosinase activity. The partially purified tyrosinase enzyme succesfuly removed 60.81% of phenol from waste water in 8 hours.

All the five samples of salt from natural salt pans and three commercial salts were assessed for the presence of micro plastics. The analysis of all the samples was based on the size ranges of 0.44µm to 0.22 µm. The identity of the micro plastics were identified by Raman spectroscopy and the spectrum similarity search was done using KnowItAll Information System 2024 by Wiley Online Raman Database.

Micro plastics were found in both natural and commercial salts and were characterised using Raman analysis. In Tata salt and Captain cook the polymers found showed similarity to Polyethylene and diamond like carbon respectively and from the natural salts the polymers showed similarity to poly propylene in the salts from the salt pan of Ribandar and Agarwado whereas High density polyethylene, Polyethylene-co-acrylic acid and Polypropylene acrylic acid were identified in the salt samples of Curca, Batim and Nerul respectively.

5.2 Future prospects

- Isolation and purification of microbial enzymes and their industrial application.
- Assessment of nano plastics in both commercial and natural salts.
- Study on toxicological effects of micro plastics.

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CHAPTER 7:
APPENDIX

7.1 Chemicals Used

S. No.	Name of the Chemicals and media used	Company
1.	Nutrient Agar	HiMedia
2.	Potato Dextrose Agar	HiMedia
3.	Eosin methlene Blue Agar	HiMedia
4.	MacConkey Agar	HiMedia
5.	TCBS Agar	HiMedia
6.	Sallmonela Shigella Agar	HiMedia
8.	Agar	HiMedia
9.	Ethanol	HiMedia
10.	Hydrogen peroxide	HiMedia
11.	Phenol Red dye	HiMedia
12.	KCl	HiMedia

13.	Agar	HiMedia
14.	$MgSO_4.7H_2O$	HiMedia
15.	Sodium tri citrate	HiMedia
16.	Yeast extract	HiMedia
17.	CMC	HiMedia
18.	Skimmed milk	HiMedia
19.	Starch Soluble	HiMedia
20.	Tween20and Tween80	HiMedia
21.	Olive oil	Fortune
22.	L-aspargine	HiMedia
23.	L-glutamine	HiMedia
24.	Starch casein Agar	HiMedia
25	Ammonium Sulphate	HiMedia
26	Gram staining kit	HiMedia
27	Congo red	HiMedia
28	Hydrochloric Acid	HiMedia
29	Nitric Acid	HiMedia
30	Gallic Acid	HiMedia

7.2 Media composition:

Halopiger media

Natural salt	250 g/L
KCl	2 g/L
$MgSO_4.7H_2O$	20 g/L
Sodium tri citrate	3 g/L
Yeast extract	10 g/L
Agar	20 g/L

(pH adjusted to 7.2).

Norberg Hofstein (NH)

	(g/l)
NaCl	200
MgSO4 7H2O	10
KCl	5
yeast extract	1

(pH adjusted to 7.0 using 1 M KOH).