Study of Sand Dunes Bacteria and Bioprospecting for their Bioactive Molecules

A Dissertation For

Course code and Course Title: MIC-651 Discipline Specific Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

Master of Science in Microbiology

By

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I hereby declare that the data presented in this dissertation report entitled, "Study of Sand Dune Bacteria and Bioprospecting for their Bioactive Molecules" is based on the results of investigations carried out by me in the School of Biological Sciences and Biotechnology at the Microbiology department, Goa University under the supervision of Dr Milind Mohan Naik 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 not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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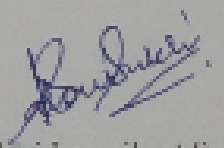

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PREFACE

Due to the improper and extensive use of antibiotics; most of the bacteria are developing resistance towards antibiotics giving rise to multi drug resistant (MDR) bacteria. MDR bacteria are bacteria that show resistance towards two or more types of antibiotics. Hence, immergence of MDR bacteria is one of the major medical issues faced by the doctors and the scientists around the globe. Therefore, there is a need to find novel antibiotics or novel approaches to deal with MDR bacteria. Another major problem is the unavailability of enough natural resources to reach the demands of the increasing population, whilst chemical resources are economically and environmentally unsustainable. Microbes are producers of multiple hydrolytic enzymes which can be utilised in different industries as natural resources. Microbes require less time to grow and can also produce high yield hence they provide an economical and environmentally sustainable alternative for industrial use. Sand dune ecosystem is a dynamic environment, hence microorganism found here have abilities to deal with the dynamic conditions like varying pH, extreme temperatures and salinity and are reported to contain agriculturally, industrially and pharmaceutically diverse microbial diversity. The aim of this project was to study Sand dune Bacteria and bioprospecting for their bioactive molecules. Screening the isolates for antibacterial and quorum quenching capabilities was one of the study's goals. Additionally, they were examined for the presence of hydrolytic enzymes, and a few isolates were tentatively identified.

ACKNOWLEDGMENT

I want to take this opportunity to express my gratitude to everyone that has supported me in my venture and helped me finish my thesis.

Firstly, I would like to thank God in giving me the patience and endurance to finish this project.

I would also like to extent my gratitude to my guide Dr Milind M. Naik, Assistant Professor, Department of Microbiology, School of Biological Sciences and Biotechnology, Goa university for his valuable time, supervision, endless patience, wisdom and support through the course of the dissertation. I would like to thank Dr Lakshangy Charya Programme Director, Department of Microbiology, School of Biological Sciences and Biotechnology for her support and cooperation.

I would also like to thank the teaching staff as well as the PhD scholars for acknowledging my queries and proving necessary help when needed. Special thanks to the nonteaching staff for providing timely support during the entire course of my dissertation.

Lastly, I would like to thank my fellow dissertation mates Rachel D'Souza, Apurva Vaigankar, Arzoo Mulla, Sainil Naik, Sarvasvi Devidas, Nikita Gaonkar and others, as well as my Juniors Harun Sayed and Marlon Cardozo for providing all the necessary help mentally as well as hands on during the entire course of dissertation.

TABLES

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ABBRVIATIONS USED

Entity	Abbreviation
Acylated Homoserine Lactone	AHL
American Type Culture Collection	ATCC
Carboxymethylcellulose	CMC
Cellulase producing Bacteria	CPB
Coastal Sand Dune	CSD
Deoxyribonucleic Acid	DNA
Embryo Dune	ED
Fore Dune	FD
Generally Regarded as Safe	GRAS
Gray Dune	GD
Hugh Leifson	HL
Kilo meter	Km
Maure Dune	MD
Methyl Red	MR
Nutrient Agar	NA
Quorum Quenching	QQ
Quorum Sensing	QS
United States Food and Drug Administration	USFDA
Vogue Proskauer	VP
Yellow Dune	YD
Zobell Marine Agar	ZMA

ABSTRACT

Coastal sand dunes are a hostile environment due to its ecological and physicochemical parameters. The oligotrophic nature of the sand dunes makes them a hot spot for finding novel microbes with novel qualities. Despite this fact, little is known about the composition and diversity of the bacterial community established in the sand dunes. In this study, isolates from Miramar sand dune and Morjim sand dunes samples were screened for antibacterial and quorum quenching activity using cross streak method against standard ATCC cultures *Salmonella typhimurium* and *Streptococcus pyogenes* (for antibacterial activity) and *Chromobacterium violaceum and Serratia marcescens* (for quorum quenching activity). Quorum quenching potential was confirmed by overlay method. Isolates were also screened for production of hydrolytic enzymes like Amylase, Cellulase, Protease, Lipase and Pectinase. Isolates SMI 6, SMI 7, SMJ 8 showed antibacterial activity against the standard ATCC cultures. Quorum quenching potential against standard ATCC cultures were shown by SMI 7 and SMJ 8 isolates. Isolates from both the sampling sites also showed production of multiple hydrolytic enzymes. Morphological and biochemical identification of the isolates was done and one of the isolate was tentatively identified to belong to the genus *Pseudomonas* sp.

Keywords: Coastal sand dunes, Antibacterial Activity, Quorum Quenching Activity, Hydrolytic Enzymes

1. INTRODUCTION

1.1 Background

Microorganisms are microscopic creatures that are found everywhere on living as well as dead surfaces. Microbes can be classified as mesophiles and extremophiles. Extremophiles are microorganisms that survive in harsh conditions. These microorganisms are known to colonise harsh environments like extreme temperature (freezing and 50°C), pH (acidic and alkaline), pressure, salt concentrations, low water activities, etc. Isolation of organisms from extreme conditions can provide mankind with many potential applications in various fields such as agriculture, pharmaceuticals, cosmetics, and food industries (Gupta et al 2014). Therefore; their usage by scientific communities for different applications is been expanding progressively. Halophilic organisms are a potential candidate for the development of novel industrial applications and biotechnological processes due to their unusual properties **(**Moreno et al 2013**)**. Halophilic microorganisms are salt-loving that can regulate the osmotic pressure of their surroundings and live in a variety of habitats. The diversity of halophilic microorganisms is extremely varied, encompassing members of three distinct life domains: Archaea, Bacteria, and Eukarya. They can be found in various ecosystems from hypersaline soils, salt lakes, marine sediments, saltpans, brines and so on **(**Moreno et al 2013).

Sand dunes ecosystem is widely distributed across the globe, covering $6X10^6$ Km^2 of its land surface. The effect of sea makes this ecosystem a unique and different ecosystem compared to the inland dunes that are formed by deposition and transportation of sand due to the action of wind. Sand dunes act as the natural barriers against the wave action of the sea, protecting the natural habitat and the shoreline. Sand dunes is a hostile ecosystem for the establishment of normal life forms due to the constant strong winds and the saline mist that may not support the establishment of normal biodiversity (Godinho et al 2010**)**.

The ecosystem of sand dunes has absence of a lot of greenery hence there is limitation of carbon availability in the soil which affects the microbial biomass. Due to the varying environmental and physicochemical parameters like fluctuating temperatures, low pH, high salinity and lack of water holding capacity of sand the diversity of microorganisms, flora and fauna that can survive in such stressful environment are expected to accommodate properties that are industrially, agriculturally and pharmaceutically important (Nayak et al 2019, Godinho 2010). Despite of the fact that these ecosystems harbour industrially valuable atypical biodiversity; unlike desert dunes, sand dunes in general have been neglected and attracted little attention from investigators for studying their biodiversity (Nayak et al 2019).

The consequences of such environmental stresses have not only affected the pattern of vegetation and fauna diversity of the indigenous organisms but it has also influenced the dynamics and composition of microbial community. It has been seen that such dynamic environmental variations can benefit microorganisms by increasing their competence and possibly increasing their capacity for bioactivity. Microorganism with characteristics that aid in the growth of plants are known as plant growth promoting bacteria. They are extremely important to agriculture and can be used in biofertilizers, among other applications.

Microorganisms isolated from such areas (sand dunes) with their ability to grow at such varying conditions could provide an ecological and ecofriendly alternative to deal with different industrial and environmental problems. Protease, lipase, amylase, cellulase, and xylanase are among the advantageous enzymes derived from halophiles that may be utilised in industries because of their ability to be functional in harsh conditions such as varying pH, temperature, and salt concentrations. **(**Ibrahim et al 2020). The oligotrophic environment of sand dune means limited availability of nutrients for the growth of macro and microorganisms. This results to an evolution of biodiversity for the sequestration of nutrients, involving several strategies employed by microbes. Microbes compete for resources in two ways: directly through

interference competition, in which individual cells harm one another, and indirectly through exploitative competition, which happens through resource consumption. Microorganisms isolated from such harsh conditions (oligotrophic, dry, competition) are expected to be under stress which in turn demands them to produce certain secondary metabolites having antioxidant, antimicrobial, antitumor and many other beneficial properties.

1.1.1 Antimicrobial activity of microorganisms

Numerous strategies used by microorganisms to compete and thrive in their surroundings can be linked to the broad variety of antimicrobial properties they display. Here are a few important details on the antibacterial properties of microbes. **1. Production of Antibiotics:** As secondary metabolites, a wide variety of microorganisms, mostly bacteria and fungi, produce antibiotics. By preventing the growth of other microbes, these antibiotics provide their users a competitive edge. Penicillin, which is synthesized by *Penicillium* fungi, and streptomycin, which is produced by *Streptomyces* (actinomycetes) bacteria, are two examples. **2. Biofilm Inhibition:** Certain microbes have the ability to prevent the development of biofilms, which are communities of microorganisms covered in an extracellular polymeric matrix (EPS) that forms on its own. Biofilms are multiple layers of microbes embedded in Exopolymeric matrix. Since biofilms frequently withstand antibiotics treatment, they provide a serious problem in both industrial and medicinal contexts. **3. Inhibition of Quorum Sensing:** Bacteria utilize quorum sensing as a communication mechanism (talk between bacteria) to regulate gene expression in response to population density. Certain microbes secrete substances that obstruct quorum sensing signals, hindering bacterial from coordinating with each another and possibly preventing the formation of biofilms and virulence factors. **4. Enzyme Production:** microbes produce enzymes like proteases and lysozyme, which can break down the membranes or cell walls of other microbes, causing lysis and death. The growth and colonization of harmful bacteria can be inhibited by microorganisms through competition for resources. **5. Bacteriocin**

Production: Bacteriocins are the antimicrobial peptides produced by bacteria to stop the reproduction of species or strains of bacteria that are phylogenetically related. They are able to attack cell membranes, prevent the production of nucleic acids, or obstruct the formation of cell walls. **6. Antifungal properties** Some bacteria synthesis substances that have antifungal capabilities, which can damage fungal cell membranes or inhibit fungal growth. **7. Antiparasitic Properties:** In addition to their property to directly target parasites, microorganisms can also create chemicals that have the potential to modulate host immune responses in order to directly combat parasites. **8. Synergistic Interactions:** To increase antibacterial activity, microorganisms may cooperate in communities. One microbe might, for instance, produce a substance that boosts the activity of another microbe against a pathogen of interest (Kumar et al 2022).

1.1.2 Quorum sensing ability of microorganisms

Bacteria use a process called quorum sensing (QS) (also called as cell to cell talk) to coordinate behaviour and communicate throughout a population. Autoinducers are the signalling molecules which are synthesised, released, and sensed. As the bacterial population increases, these chemicals (signals) build up and eventually reach a threshold concentration that causes particular patterns of gene expression. Important points regarding quorum sensing consist of: small signalling molecules, such as oligopeptides (cyclic peptide) in Gram-positive bacteria and acyl-homoserine lactones (AHLs) in Gram-negative bacteria, are produced and released by bacteria. (Prabhu et al 2019) Since bacteria possess sensor proteins or receptors, they are able to sense the concentration of signalling molecules in their environment (Dong et al 2007). Gene Regulation: Variations in gene expression within the bacterial population occur when the concentration of signalling molecules crosses a crucial threshold (Boyer et al 2009). Coordinated behaviours resulting from this (Quorum sensing), include expressing genes that grant antibiotic resistance, bioluminescence, sporulation, biofilm formation, pigment

production, and the synthesis of virulence factors. The development and maturation of biofilms: organized bacterial colonies surrounded by an extracellular matrix are significantly influenced by QS. Biofilm generation, adhesion, and dispersal genes are regulated by QS. Virulence: A large number of harmful bacteria rely on QS to control the expression of virulence factors, that are chemicals (biomolecules) that give them the ability to evade the host immune system, enter host tissues, and spread illness. Antibiotic Resistance: Through controlling the expression of resistance genes, QS can also affect antibiotic resistance. When opposed to planktonic cells, bacteria in biofilms show higher antibiotic resistance. Interference and Inhibition: One possible tactic to manage bacterial behaviour and fight bacterial illnesses is to target QS systems for interference or inhibition. Quorum quenching agents (QQ), or compounds that cause QS to be disrupted, are being researched as possible antibacterial treatments (Hemmati et al 2020). Applications: Knowledge of QS mechanism may be utilised in a variety of disciplines, such as biotechnology (to control bacterial behaviours in industrial processes), agriculture (to control plant diseases), and medicine (to develop innovative antibacterial techniques). Quorum sensing, in general, is an amazing system that plays crucial roles in both symbiotic and pathogenic relationships by enabling bacteria to coordinate their behaviours and adapt to changing environments.

1.1.3 Quorum quenching ability of microorganisms

The term "quorum quenching" (QQ) describes the suppression or interruption of cell-to-cell communication bacterial quorum sensing (QS) signalling. This disruption of bacterial communication may contain crucial application for the medical, agricultural, and industrial sectors, among other domains. A variety of molecules (synthetic or natural) can lead to quorum quenching in microbes (Hemmati et al 2020). One popular strategy is to stop bacterial receptors from detecting QS signalling molecules by enzymatically modifying or degrading signals. The production of substances that prevent the generation of QS signals or obstruct QS receptor

binding is another method. Naturally Occurring Quorum Quenchers: A wide range of organisms, such as plants, animals, and other microorganisms, naturally produce quorum quenching chemicals (Dong et al 2007). These substances may be involved in competitive or symbiotic relationships within microbial communities which possess the property to obstruct bacterial communication. Synthetic Quorum Quenchers: Scientists have also synthesised artificial substances that exhibit quorum quenching properties. These compounds have the capacity to interfere with QS signalling pathways, which may provide them a vital role as additives to regulate bacterial behaviour in industrial processes or as therapeutic agents to treat bacterial illnesses. Potential Uses in Medicine: Quorum quenching holds great promise in the creation of innovative antibacterial treatments. Quorum quenching techniques aim to disrupt bacterial communication networks instead of simply killing germs, which could reduce the rate of development of antibiotic resistance and provide an alternate course of treatment for bacterial illnesses. Inhibiting the production of biofilms is a significant challenge in both industry and medicine, and quorum quenching may have some consequences in this regard. Quorum quenchers may increase the effectiveness of antimicrobial therapies by preventing the production of new biofilms or facilitating the dispersal of already-existing biofilms by interfering with the quorum sensing signals involved in biofilm growth. Agricultural and Environmental Applications: By altering bacterial behaviours in the rhizosphere, quorum quenching can also have potential for their use in agriculture to suppress plant diseases or stimulate plant growth. Quorum quenching techniques may also lessen the negative effects of bacterial infections on aquaculture and other environmental contexts (Bzdrenga et al 2017).

In order to determine whether the bacteria from the sand dune ecosystem have any speciality that could give them properties that enable them to be used for commercial or biotechnological uses, that was the aim of this study. The selected organisms were screened for antibacterial activity and inhibition of quorum sensing ability against Gram negative as well as Gram

positive human pathogenic bacteria and also checked for production of hydrolytic enzymes (amylase, lipase, cellulase, protease and pectinase).

1.2 Aims And Objectives

Aim: To study sand dune bacteria for bioprospecting of antibacterial, quorum quenching molecules and hydrolytic enzymes.

Objectives:

- Screening and isolation of bacteria from sand dune ecosystem.
- Screening of isolates for antibacterial and Quorum Quenching biomolecules.
- Screening of isolates for hydrolytic enzymes.
- Morphological and biochemical identification of bacterial isolates.

1.3 Hypothesis

Given that the sand dune environment is dynamic in terms of both biodiversity and physiological characteristics, it is possible to identify novel creatures with unique traits there. It is expected that pigmented bacteria will be present in stressed environments because pigment is a secondary metabolite produced under such circumstances. There is competition amongst microbes in this oligotrophic and stressed environment, which increases the likelihood that they will produce quorum quenching and antimicrobial molecules. The severe circumstances also help bacteria produce hydrolytic enzymes that are stable in a range of salinity, pH, and temperature.

1.4 Scope

Sand dune bacterial isolates can be used as tools for dealing with multi drug resistance by providing novel antibiotic molecules or quorum quenching molecules. They can also be employed in various industries to overcome industrial issues for their property of producing multiple hydrolytic enzymes.

2. LITERATURE REVIEW

2.1 Bioprospecting of microorganisms

The demand for food, energy, and biological resources rises in tandem with the world's population growth, affluence, and global economy. It is estimated that there might be a significant problem in supplying a sustainable source of food to the increasing population in near future. Microorganisms are regarded as a promising alternative to deal with scarcity of food and an essential source of sustenance. The term bioprospecting refers to the process of locating and proposing novel products that are derived from nature. It basically implies searching for organic substances that may be beneficial to humans. Owing to their ecologically and biologically varied ecosystem, microorganisms are able to produce a wide range of important biological substances that help human nutrition, agriculture, health, and remediation. (Ruginescu et al 2020). Microorganisms play a crucial role in the ecosystem hence it is crucial to study about these microbial community structures in order to understand better about these communities. Numerous natural activities, including the recycling of nutrients (nitrogen and phosphorus), decomposition, mineralization, bioremediation, biofertilization, and many others, are carried out by microbes. According to several studies conducted it was revealed that microorganisms are the building blocks and the fundamental element of the environment be it terrestrial or marine. Microorganisms also play an important role in the development of the soil structure especially in the coastal sand dunes (Wasserstrom et al 2017)

2.2 Sand dune environment

Across the world, sand dunes are extensively dispersed, making about 6×10^6 km^2 of the land area. This particular ecosystem is thought to be distinct from inland dunes because of the influence of the sea. The shoreline is shielded from the force of waves by sand dunes, which operate as natural barriers. These structures (sand dunes) support important plant, animal, and

microbial communities, which are essential to the creation of the ecosystem (Nayak et al 2019)**.** Sand dunes are aeolian bedforms that emerge in areas where the wind's ability to carry objects is compromised. The ecology of sand dunes is considered extremely fragile, vulnerable and dynamic regardless of its terrain covered with vegetation or bare due to its tendency to alter in response to even minute environmental changes (Rodrigues et at 2011). Sand dunes are considered natural defence system. They stop sand from blowing, divert wind upward, and keep waves away from the hinterland. In addition to providing beach nourishment, sand dunes also neutralize and disperse wave and current energy in the coastal zone. As a result, they contribute specifically to the maintenance of the biological equilibrium of the coastal zone by replenishing lost material from erosion. Sand conservation is therefore emphasised as it is needed for the protection of the coast from erosion and also to replenish the sand lost due to wave and current energies (Rodrigues et al 2011**).** Sand dunes are considered a very hostile and dynamic ecosystem due to the presence of many limiting factors like scarcity of major nutrients notably of nitrogen, phosphorus and potassium. It is challenging for plants to thrive because of the shifting sands, salty environment, lack of humus, high temperatures, and deep subsurface water. The vegetation found in such areas have adapted to these volatile conditions which makes it possible for them to endure such circumstances. The buildup of humus enhances the developing dune soils' ability to retain moisture and nutrients. (Godinho et al 2010).

2.3 Sand Dune succession

The formation of a coastal sand dune occurs when shore waves carry marine sand onto the beach. Sand dries out in the air and becomes susceptible to aerodynamic processes. Grasses present in the dune environment temporarily stabilise the dunes by holding them in to place with their roots, while their leaves capture sand and encourage dune expansion (Muthukumar and Selvin, 2011). The availability of sediments, speed of wind, size of the grains, sand surface,

topography and surface roughness are the key elements required for the formation of sand dunes (Hesp, 1989). Depending on the grain size, a minimum threshold wind speed needs to be reached in order to move the sand particles. The wind-blown sediments from the sea carry it near to the ground after the threshold speed is crossed. The mechanism that causes each individual grain to move in a succession of tiny jumps is called "saltation" (Bass 2019).

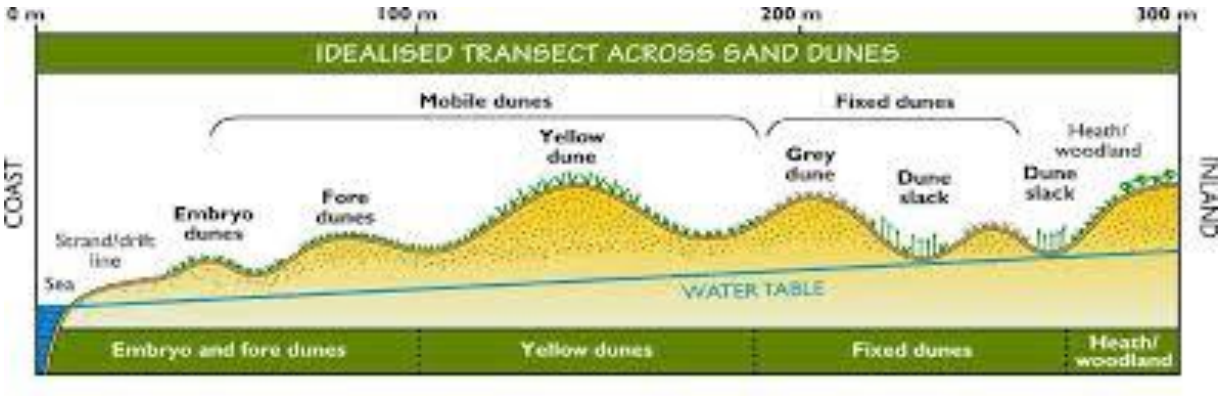


Fig 2.1: Different Coastal Sand dune Zones.

2.4 Different zones of Coastal Sand Dune (CSD)

Depending on the pH and the vegetation found at the coastal sand dunes, these dunes can be further classified into 6 different zones namely Embryo dunes, Fore dunes, yellow dunes, Gray dunes, Dune slack and Mature dunes (Dang et al 2021).

Embryo dunes (ED) are found parallel to the Ocean on the seaward coast along sand ridges. Strong winds and frequent seaside salt spray make for unfavourable weather at ED. This particular dune is home to several pioneer species, including sea wort grasses, *Ipomoea pes-caprae, Spinifex littoreus,* and marram (*Ammophila arenaria*). Due of the calcium carbonate from seashell debris, the pH is alkaline (8.5) (Shet Set al 2021).

Fore dunes (FD) extend parallel to the first beach ridge and are situated somewhat above the high tide line. *Ipomoea pes-caprae, Spinifex littoreus*, *Amaranthus spinosa*, and *Elymus arenarius* are among the plants that may be found in Fore dunes. These fit the FD circumstances perfectly. The water table can be reached by the deep roots of these grasses. The majority of plants have nitrogen-fixing root nodules that draw nitrogen from the atmosphere. FD has a pH between 7.5 and 8 (Shet et al 2021).

The **Yellow Dunes** (YD) are slightly higher than the Fore Dune, but they are parallel to it. Compared to the Fore dune, this dune has more humus. Three plant species were identified in this dune: *Carex arenarium, Eryngium maritimum*, and *Calystegia soldanella*. The plants in this region covers the CSD surface and form a horizontal root network which provide them with the ability of binding the surface sand. "Yellow Dune" refers to the area where a significant amount of immaculate yellow sand may still be seen through the uneven plant cover. The pH of YD is 7.5 (Shet et al 2021).

Gray dunes (GD) are positioned 50-100 m from the margin of the sea towards the terrestrial side of the yellow dune. The dune is heavily colonized by green or grey lichens, which give the sand its distinctive dirty grey colour. Gray dunes are home to mosses including *Tortula ruraliformis* and *Brynum*. The amount of humus in these dunes is substantial. At 6.5, the pH is rather acidic. (Shet et al 2021)

Humus leaches from all dunes and washes away into forming **Dune slacks**. Only marsh plants, such creeping willow, can thrive in these wet slacks. Due to the significant humus concentration, the pH in Dune Slack is acidic (pH 5). (Shet et al 2021)

Mature dunes (MD) are located a few hundred meters from the coast. Climax plants, like oak and birch trees, are found here. Because of the high humus content created by the decomposing

leaves and other organic debris, the mature dune has an acidic pH of 2-3. This area is home to a variety of plants, including *Terminalia* spp., *Acacia* spp., *Banksia integrifolia*, *Casuarina equisetifolia*, and *Pandanus* spp. (Shet et al 2021).

Three primary varieties of dunes are found in Goa's coastal sand dunes: white dunes (Embryo and Fore dune), yellow dunes, and Gray dunes, according to research done by (Desai et al 2002).

2.5 Diversity of plants/ creepers microorganisms in sand dune environment

Ammophilia arenaria, *Elymus arenarium*, and *Spinifex littoreus* are the pioneer grasses that grow on sand first and collect sand that is driven by the wind. These grasses decompose and provide the soil with humus, enabling the growth of other plants (Maun, 1998). The vegetation in the sand dunes has evolved to withstand stress conditions by means of a variety of adaptation mechanisms. For instance, *Spinifex littoreus* has roots long enough to reach quite deep, which aids in sand retention. Thick cuticles covering the leaf's outer surface also aid in the plant's ability to shield its tissues from heat. Water retention is also aided by thick cuticles (Divyashree et al 2019).

Ipomoea pes-caprae creates substantial mats that help keep dunes stable. They make great sand binders. (Barbour et al 1985). Plants such as *Spinifex littoreus, Cyperus arenarius, Spermacoce stricta, Launea pinnatifida, Justicia simplex, Lactuca remotiflora, Ipomea-pes caprea, Sporobolus virginicutes,* and *Clerodentron inerma* can be found growing on the sand dune at the coastal areas of Goa (Desai et al 1995). The predominant plants on the Goan shore are *sphinifex littoreus, ipomea pes-caprea,* in their embryo and foredune. Bacteria from the genera *Brevibacillus, Chryseobacterium, Pantoea, Agrobacterium, Bacillus, Acinetobactor Erwinia,*

Rhizobium, Microbacterium, Rhodanobacter, and *Xanthomonas* are found at the coastal sand dunes, in the rhizospheric region of plants, such *as Lanthyrus japonica, Carex kobomugi, Calystegia soldanella, Vitex rotundifolia, Artemisia fukudo, Glehnia littoralis* and *Messerchmidia sibirica, Elymus mollis* (Lee et al 2006).

There have been reports of the genus *Pseudomonas* in both rhizosphere and non - rhizosphere regions of CSD (Shin et al., 2007). *Pseudomonas fluorescens* was discovered at Tortula and Sphagnum plants rhizosphere. Alkaliphilic pigmented bacteria, *Microbacterium arborescens*, was isolated from *Ipomoea pes-caprea* rhizosphere (Godinho et al 2007). Non rhizosphere region of coastal sand dunes was seen to have *Pseudomonas aeruginosa* as its indigenous microorganism (Gaonkar et al 2012).

The ecology in CSD is unfriendly to life. CSD is a unique habitat because of the several unfavourable environmental conditions that still exist there. Microorganisms that are able to adapt to the harsh conditions of CSD are found in such habitats. A general picture of bacterial adaptations to low nutrition soil environments is provided by *Collimonas fungivorans*, which was isolated from the CSD of Dutch Wadden Island, Terschelling (de Boer et al 2004; Leveau et al 2010*). C. fungivorans* can draw nutrients from minerals, rocks, and live fungus. Two of the main herbaceous CSD plants, *Calystegia soldanella* and *Elymus mollis* are thought to be in charge of maintaining the rhizosphere and balancing the biodiversity in CSD. There was a record of the variety of culturable bacteria linked to these two main sand dune plants. The findings demonstrated the distinctions between the root endophytic and rhizosphere microbial communities. Both the rhizosphere and the endophyte communities are dominated by *Pseudomonas* sp. *Alphaproteobacteria* in the endophytes and members of *Bacteroidetes* and *Firmicutes* in the rhizosphere were also isolated (Lee et al 2006). The CSD plant *C. soldanella* was discovered to be associated with *Pseudomonas, Chryseobacterium,* and *Pantoea*. (Lee et al 2006). Moreover, *Pseudomonas* and *Aeromonas hydrophila* were discovered to be

associated with *E. mollis*, the CSD plant. In coastal areas of Tae-an, Korea, *Elymus mollis*, and *C. taeanense* were isolated from the rhizosphere region of CSD plants, *Calystegia soldanella* and *Chryseobacterium soldanellicola* (Park et al 2005). Few attempts have been made to use culture-dependent and independent methods to investigate the bacterial diversity of rhizosphere, non-rhizosphere, and root endophytic communities of CSD plants.

Pigmented strains of *Pseudomonas* sp. and *Bacillus* sp. were shown to be prominent at CSD in a study that depended on culturing Goa's Miramar sand dunes (Gaonkar et al 2011 and Godinho et al 2010). *Chryseobacterium soldanellicol*a and *C. taeanense*, two yellow pigmented bacteria, were isolated from the rhizosphere of CSD plants, *Calystegia soldanella* and *Elymus mollis* (Park et al., 2005). *Microbacterium arborescenes* AGSB sp. Nov., an orange-coloured bacterium, was isolated from the *Ipomoea pes-caprae* rhizosphere region from Goa, India. (Godinho, 2007). *Streptomyces griseus*, a red-coloured bacterium, was isolated from the non-rhizosphere of sand dunes (Antony-babu et al 2008). *Pseudomonas aeruginosa*, a green-coloured bacterium, was identified from the non-rhizosphere of CSD at Miramar, Goa, India (Gaonkar et al 2012). From Goa's CSD, *Bacillus marisflavi*, a yellow-coloured bacteria, was isolated (Prabhu et al 2018).

2.5 Adaptation mechanism of halophilic and halotolerant microorganisms

Halophiles are an extreme group of organisms that inhabit highly salinized habitats. They are characterized by their ability to flourish and survive in such extreme conditions. These organisms can be found all over the Bacteria, Archaea, and Eukarya domains, which are the three domains of life. According to their optical NaCl concentration for growth, (Kushner and Kamekura 1988) divide them into many categories: i) Not halophilic, needing less than 1% NaCl ii) Mild halophiles, needing between 1% and 3% NaCl iii) Moderate halophiles, needing

between 3% and 15%. According to Moreno et al. (2013), IV) Extreme halophiles grow best in environments containing 15%–30% NaCl. V) Halotolerant can withstand a broad range of salt concentrations, although it grows best at less than 1%. Halophilic bacteria can withstand the osmotic and ionic stressors brought on by high salinity. The ability of halophiles to tolerate the high concentration of NaCl in their niche, which results in osmotic pressure, is what allows them to adapt to these severe conditions. Numerous adaptive methods have been employed by microbes to go past the harsh environment.

i. Salt-in strategy: This approach involves the sequestration of inorganic ions within cells in order to maintain a higher intracellular osmotic potential and equipoise the ionic concentration of the external environment. Ion channels and pumps such as Cl^- pumps work in alliance with arginine and lysine residues to facilitate the uptake of Cl^- ions, or K^+ channels or ATP-dependent transport systems, to transport ions across membranes. Energy is the driving force behind these ion exchange pathways. Light-driven processes utilizing membrane-bound pigments, such as bacteriorhodopsin and halorhodopsin (found, for example, in *Halobacterium salinarium*), can supply the necessary energy (Gunde et al., 2018).

ii. Salt-out strategy: It is sometimes referred to as the “compatible solute strategy.” These tiny, low-molecular-weight organic compounds are known as osmolytes. The organism can either generate the suitable solutes from scratch or absorb them from its immediate surroundings. Osmotic potential is lowered and turgor pressure is maintained by the cells' concurrent extrusion of salt and accumulation of compatible solutes (Iqbal, M.J 2018; Oren, 2002). Compatible solutes play a role in DNA stability, enzyme stabilization, and protection against stressors including freezing, desiccation, and heating in addition to preserving osmotic potential. Sometimes, osmolytes are referred to as chemical chaperones, which support appropriate

polypeptide chain folding (Shivanand & Mugeraya, 2011). Incorporate betaine, ectoins, alcohols, amino acids, and highly water-soluble carbohydrates or their derivatives (Shivanand & Mugeraya, 2011).

2.6 Applications of halotolerant microorganisms

The cytoplasm of halobacteria and certain highly halophilic bacteria is where inorganic ions (K^+, Na^+, Cl^-) are accumulated to maintain the medium's osmotic pressure equilibrium, and they contain certain proteins that remain active and stable when salts are present. On the other hand, moderate halophiles cause large numbers of particular organic osmolytes to collect in the cytoplasm. These osmoprotectants maintain osmotic equilibrium without altering the cell's regular metabolism. (Nieto et al 2002). Studies on halophilic microorganisms have been conducted recently due to their potential in various biotechnological sectors (Mellado et al 2003). Applications include the employment of these microorganisms in environmental bioremediation procedures as well as the utilization of various products, such as suitable solutes, biopolymers, or carotenoids, in an array of businesses. In addition to having inherent stability and activity at high salinities. Furthermore, halophilic enzymes present significant prospects for biotechnological uses, including environmental bioremediation, food processing, and biosynthetic procedures. Accordingly, it is crucial to discover new enzymes with optimal activity across a variety of pH, temperature, and salt concentration (Gomez et al 2004). It is crucial to emphasize that the stability of halophile enzymes at high salt concentrations is not the sole element that determines their usefulness in industrial settings; these extremozymes are typically also stable at high temperature and when organic solvents are present (Oren, A 2010). In addition to activity at low water activity, resistance to denaturation, and the function of salt in preserving structure (Karan et al 2012). Lipase, amylase, cellulase, protease, pectinase and other such enzymes are frequently sought after industrial enzymes (Rathakrishnan & Gopalan 2022).

2.6.1 Amylases

Amylases belong to the class of hydrolase enzymes that is responsible for hydrolysis of starch. The polymers amylopectin, a branching polymer of glucose, and amylose, a linear polymer of glucose, combine to form starch. Endoamylases and exoamylases are the two classes into which amylases fall. Endoamylases randomly hydrolyse the starch molecule to produce branched and linear short chains of varying lengths (Gupta, et al 2003). Amylase breaks down the starch molecule's α-1-4 glycosidic linkages. For instance, α amylase hydrolyses α 1,4 glycosidic linkages in starch to produce maltose, glucose, or limit dextran. Industries prefer bacterial amylase because to its affordability, environmental friendliness, high quality, and plentiful supply. Most of Bacillus species, such as *B. subtilis, B. megaterium, B. vulgaris, B. licheniformis, B. amyloliquefaciens, B. cereus*, and many more, are the producers of bacterial amylase (Gopinath et al 2017). Short-end products are produced by exoamylases hydrolysing the non-reducing ends of starch molecules. One example is β amylase, which cleaves either α-1,4 or both α-1,4 and α-1,6 glycosidic linkages to produce glucose units or maltose and limit dextran (Tiwari, et al 2015). Applications of amylases in the food brewing, distilling, and textile industries for starch saccharification. *Thalassobacillus* sp. LY18 was the source of one identified extracellular halophilic amylase. Excellent stability was shown by this enzyme over a wide range of temperatures (about 30°c to 90°c), pH levels (3-6) and NaCl concentrations (0%–20%) exhibiting outstanding characteristics that are thermotolerant, alkali tolerant and halotolerant (Li & Yu, 2012). Produce an amylase active at 3M NaCl and 45°C, *Haloarcula* sp. may find application in the food, textile, and brewing industries (Siroosi et al. 2021). Amylases are seen to have applications in varied industries such as food, detergent, paper, textile and pharmaceutical industries (Souza, 2010). Amylase activity was detected in *Bacillus megaterium* from the CSD environment in Miramar-Goa (Nayak et al 2013).

2.6.2 Cellulase

These are the enzymes that catalyse the breakdown of cellulase polysaccharides by acting on β 1,4-glycosidic bonds. Tightened by β 1,4-glycosidic bonds, cellulose is a linear polymer of D glucose. Rather than operating as a single enzyme, cellulase is a collection of enzymes which includes β glucosidase, endoglucanase, and exoglucanase. Cellobiose units and products are produced by endoglucanase and exoglucanases respectively. One of the ways that β-glucobiose helps disintegrate cellulose into its monomer components is by converting cellobiose to glucose (Jayasekara & Ratnayake 2019). Bacteria that produce cellulases (CPBs) have been identified from a range of different sources, such as compost, soil, organic matter, decomposing plant matter, and severe environments like hot springs. Cellulases have multiple industrial applications. The textile, paper, and pulp industries employ these enzymes for bio stoning and biopolishing; the detergent and laundry industries use them for pulping and deinking; the food processing industries utilize them, and agriculture in conjunction with other (Jayasekara & Ratnayake 2019). As an illustration, consider the alkali-tolerant, solvent-tolerant cellulase that was recovered from the halotolerant Bacillus subtilis SU40 (Asha & Sakthivel 2014). *Bacillus cereus, B. subtilis, Paenibacillus campinasensis, B. polymyxa, Cellulomonas cellulans, B. licheniformis, Bosea* sp.*, and B. thuringiensis* are a few examples of CPB (Sadhu and Maiti 2013).

2.6.4 Protease

These are the enzymes that catalyse the breakdown of the peptide bonds found in proteins, producing amino acids and shorter polypeptide chains. Proteases are produced by bacteria, fungi, plants, mammals, and archaea and are found all across nature. Proteases are categorised into two groups: exo proteases, that exclusively function at the extremities of polypeptide chains, and endo proteases, which cleave the core peptide bonds of proteins. Protease is mostly

used in the detergent business, and as it must function in an alkaline environment, researchers are focusing their research on alkaline protease. Proteases are vital industrial enzymes that account for 20% of all commercially valuable enzymes. The market for these enzymes is growing quickly on a global scale (Mokshe, et al 2018). These enzymes are used in the leather, textile, and detergent industries they also have function in food business (cheese manufacturing, meat tenderization, dough activation, etc.) (Okpara 2022). Fish meat can be softened with the help of *B. megaterium*'s extracellular proteases. *B. subtilis* uses the waste products from the leather industry to produce high-quality glue that is appropriate for use in carpentry (Anwar and Saleemuddin, 1998). Most halophilic proteases are classified as serine endoproteases, that resist temperatures ranging from 40 to 75 degrees Celsius and function at salt concentrations of approximately 4-5 M NaCl and pH of 5 to 10. *Halobacterium* sp. is the source of these salt-stable serine protease families known as halolysins (Mokshe, et al 2018). Majority of the proteases found in halotolerant species are classified as serine proteases and metalloproteases (Mokshe et al 2018). The alkalohalophilic bacterium *Bacillus luteus* produces an extracellular serine endoprotease that is stable at approximately 5M NaCl and active over a wide pH and temperature range (Kalwasinska et al 2018).

2.6.4 Lipase

These enzymes work on the ester bond of glycerol to catalyse hydrolysis of tri-, di-, and monoglycerides (Casas-Godoy et al 2012). Because the substrates are immiscible in water, these hydrolytic processes occur at the lipid-water interface. Lipases catalyse processes for esterification and transesterification in addition to hydrolysis (Casas-Godoy et al 2012). Owing to their property to withstand high salinity, high temperatures, and organic solvents, halophilic lipases are believed to be ideal candidates for numerous harsh industrial processes (Que et al 2021*). Pseudomonas* sp.*, Pseudomonas aeruginosa, Serratia rubidaea, Staphylococcus caseolyticus, Bacillus stearothermophilus, Acinetobacter radioresistens Burkholderia cepacia,*

Burkholderia multivorans, Bacillus sp., *Bacillus coagulans*, and *Bacillus subtilis* are microorganisms that are reported in the literature to produce lipase (Li et al 2005; Treichel et al 2009). Lipases, such as the alkali-stable, organic-solvent tolerant extracellular lipase identified from *Idiomarina* sp. W33 utilizing jatropha oil (Li et al 2014) and another lipase isolated from *Haloarcula* sp. G41 (Li & Yu 2014), are employed in the manufacturing of biodiesel.

2.6.5 Pectinase

A class of enzymes known as pectinases targets pectin and breaks it down into smaller pieces by hydrolysing the ester link between the methyl and carboxyl groups of the pectin and trans-eliminating it (Ceci et al. 2008). Pectin is a class of intricate polysaccharides that are present in higher plants' cell walls and act as a cellulose network cement (Thakur et al 1997). Pectinases are classes of enzymes that hydrolyse pectin, which is a chain of molecules linked to other polymers and carbohydrates and contains a rhamnogalacturonan backbone (Thakur et at 1997). The fruit juice and wine industries both make extensive use of these pectinases. It is used in the fruit juice business for clarification, which results in a decrease in viscosity and the generation of clear juice. Pectinases assist create pulpy products by liquefying pulps enzymatically and macerating organized tissue into a suspension of intact cells. This increases the output of juices (Sierio et at 2012). The food industry uses *Aspergillus niger, Aspergillus oryzae,* and *Penicillium expansum*, which the United States Food and Drug Administration (USFDA) has classified as generally regarded as safe (GRAS). Additionally employed are some yeasts such as *Saccharomyces, Candida,* and *Actinomycetes* like *Streptomycetes,* as well as some bacteria *(Lactobacillus, Bacillus licheniformis, Aeromonas cavi,* etc.) (Pandey et al 2009).

3.

MATERIALS AND METHODS

3.1 Sampling

The sample was collected from Sand dunes of Miramar beach-Panaji; Morjim beach, Goa India. The samples were collected during the months of September and February 2023-2024. The coordinates of the locations are Lat 15.482462° and Long 73.807508°, Lat 15.618142° and Long 73.732851° respectively. The sand sample was collected in a sterile centrifuge tube and the roots were segmented with the help of sterile scalpel and collected in 50 ml sterile centrifuge tubes.

Figure 3.1: sampling sites (sand dunes): Miramar beach and Morjim beach Goa India.

3.2 Isolation and purification of isolates

3.2.1 Preparation of media:

The isolation media used for marine bacteria was ZMA – Zobell Marine Agar media. (appendix I) ZMA media of quantity required for 300ml was weighed as per instructions by Himedia

chemicals, media pH was adjusted to 7.6 ± 0.2. Media was autoclaved at temperature 121°C for 15 lbs pressure and when temperature of media reach around 45°C the media was poured in autoclaved plates under sterile conditions (laminar air flow).

3.2.2 Isolation of potential halotolerant bacterial cultures

1g sand sample was added to 9 ml sterile 1% saline (Appendix II) and tenfold serial dilution was carried out (up to 10^{-3}). 0.1 ml of all the dilutions was spread plated onto the prepared ZMA plates. The incubation period for the plates was maintained at room temperature (28± 2°C) for 24-48h until diverse colonies were visible on the plates.

Ipomea sp. root hair along with attached sand was added to 9 ml sterile 1% saline (Appendix II) and tenfold serial dilution was carried out (up to 10^{-3}). 0.1 ml of all the dilutions was spread plated onto the prepared ZMA plates. The incubation period for the plates was maintained at room temperature (28± 2°C) for 24-48h until diverse colonies were visible on the plates.

Root hairs are also directly placed on ZMA plates and incubated at room temperature (28± 2°C) for 24-48h until diverse colonies were visible on the plates.

3.2.3 Purification of Isolates

Morphologically different colonies (appeared on the plates) from the spread plated plates were chosen and carefully picked up from the master plates and quadrant streaked onto fresh ZMA plates to get isolated colonies. Repeated quadrant streaking was carried out multiple times until pure isolates were obtained. The colony characteristics of each isolate were carried out.

3.3 Determination of Antibacterial potential of bacterial isolates.

Nutrient agar (Appendix I) plates were prepared. Isolates to be tested for antibacterial potential were streaked vertically on the nutrient agar plates and maintained for 18 hours at room temperature (28±2°C). After 18 hours standard ATCC organisms (*Salmonella typhimurium* 14028 and *Streptococcus pyogenes* 19615*)* were streaked horizontally to the isolate streak. The plates were maintained for 24 hours at 37°C. If the isolate has potential antibacterial activity, growth inhibition of the standard ATCC organisms is observed.

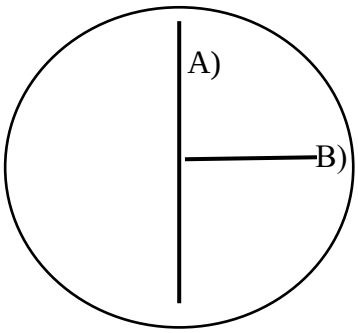


Fig 3.2: Cross streak method to check for antibacterial potential of bacteria. A) test isolate, B) standard ATCC organism.

3.4 Determination of Quorum Quenching potential of bacterial isolates.

Nutrient agar plates were prepared. Isolates to be tested for quorum quenching activity were streaked vertically on the nutrient agar plates and incubated for 18 hours at room temperature (28±2°C). After 18 hours reporter organisms (*Chromobacterium violaceum* and *Serratia marcescens)* were streaked horizontally to the isolate streak. The plates were incubated for 24 hours at room temperature (28±2°C). Quorum quenching activity detected by the potential of the isolate to inhibit quorum sensing mechanism of the reporter organisms which is detected by pigment inhibition of the violacein pigment of *Chromobacterium violaceum* and Prodigiosin pigment of *Serratia marcescens.* Cross streak method is used to check quorum quenching potential. *Chromobacterium violaceum* produce pigment controlled by QS system. If test bacteria (isolates) produce QQ biomolecules than the *Chromobacterium violaceum* streaked

very close to it will not produce violacein pigment and will appear white. *Serratia marcescens* produce pigment controlled by QS system. If test bacteria (isolates) produce QQ biomolecules than the *Serratia marcescens* streaked very close to it will not produce prodigiosin pigment and will appear white.

3.5 Screening for enzyme activity

3.5.1 Amylase activity

Plates were prepared using ZMA medium supplemented with 1% soluble starch. The cultures were spotted on these plates. The plates were than incubated for 24-48 hours at room temperature (28±2°C). After the incubation period, plates were flooded with iodine solution (Appendix II). Amylase production is indicated by the presence of clear zones at the periphery of the spot-inoculated culture to a blue-black background (Delgado et al. 2014).

3.5.2 Cellulase activity

0.5% carboxymethylcellulose (CMC) was added to ZMA media as the substrate for screening cellulase production. The cultures were spotted onto the media plates and incubated for 24-48 hours at room temperature (28±2°C). After incubation, the plates were flooded with 0.2 % Congo Red solution (Appendix II) and washed with 1N NaCl (Appendix II). The presence of clear zones around the spotted culture indicates cellulase production (Delgado et al. 2014).

3.5.3 Protease activity

Skimmed milk was sterilised separately and used as substrate for detection of protease activity. 10 ml of skimmed milk was inoculated into sterilised ZMA media (100ml) (under sterile conditions) before pouring the media onto the plates. The isolates were spotted onto the media

plates and incubated for 24-48 hours at room temperature (28±2°C). The formation of clear zones/halo around the colonies indicates positive protease production (Delgado et al. 2014).

3.5.4 Lipase activity

1% olive oil was used as the substrate for detection of lipase activity. Substrate was inoculated in autoclaved ZMA media under sterile conditions and poured into the plates. The isolates were spot inoculated on the media plates and incubated for 24-48 hours at room temperature (28±2°C). The presence of white precipitate around the vicinity of the culture colony indicates lipase production (Delgado et al. 2014).

3.5.5 Pectinase activity

0.5 % Pectin was inoculated into ZMA media as the substrate for detecting pectinase activity. The culture was spot inoculated onto the media plates and incubated for 24-48 hours at room temperature (28±2°C). Plates were flooded with iodine solution (Appendix II) after the incubation period. Pectinase production is indicated by the presence of clear zone around the periphery of the spot-inoculated culture in contrast to yellowish background (Delgado et al. 2014).

3.6 Morphological characteristics

3.6.1 colony characteristics

The selected isolates were quadrant streaked on sterile ZMA plates and incubated at room temperature for 24 hours. The colony characteristics of the grown isolates were then recorded in term of size, shape, opacity, margin, colour, consistency, and elevation.

3.6.2 Gram staining

The 18 hours old culture isolates were smeared onto a clean, grease-free slide and heat fixed. The slide was then immersed with crystal violet (Appendix II) and held for 1 min. The stain was discarded and followed by flooding with Gram's iodine (Appendix II) for about 30 seconds. The slide was then gently rinsed with water. The slide was then decolourised with ethanol for about 30 seconds. Saffranine (Appendix II) was used as counterstain for 1 min. Slide was then gently rinsed, air-dried and observed under an oil immersion lens (100X) (Moyes et al 2009).

3.6.3 Endospore staining

Endospore staining was carried out by heat fixing the bacterial smear on a clean grease free slide. The slide was than covered with a blotting paper and the paper was immersed with malachite green stain solution (Appendix II) and slide was steamed for 5 minutes. The slide was rinsed with water and counterstained with saffranine for 30 seconds. The slide was then rinsed gently, aired dried and observed under oil immersion lens (100X). Endospores stain green in colour while vegetative cells stain pink (Hussey et al 2007).

3.6.3 Citrate test

The usage of citrate as the carbon source was detected using Simmons Citrate Agar (Appendix 1). Weighing the necessary quantity of media components as per Himedia instructions on media bottle and suspend in distilled water, pH was adjusted to 6.9 ± 0.2. The medium was autoclaved to prepare it. The medium has a vivid green colour. After that, the agar media was inoculated into sterile test tubes, which were then kept tilted/slanted so the agar solidified and formed slants. After streaking the cultures onto the slants, they were left to incubate for 24 hours at room temperature. A positive test result was detected by the medium changing from green to blue in colour.

3.6.4. Methyl red (MR) and Vogues Proskauer (VP) test

The cultures were maintained for 24 hours at room temperature after being inoculated into a 10 ml glucose phosphate broth medium (Appendix II). Two portions of the medium are separated following incubation into two separate test tubes. Three drops of methyl red solution were added to one portion (first test tube). A positive MR test is shown by the colour changing to red. Three drops of O'Meara's reagent (Appendix II) were added to the other portion (second test tube) and thoroughly mixed. After that, the tubes were incubated for an hour. The broth's pink colouring suggested a positive test.

3.6.5 Indole test

The culture isolates were inoculated into 5 ml of sterile tryptone water broth medium (Appendix II). After that, the tubes were incubated for a full day at room temperature. Five drops of Kovac's reagent (Appendix II) were applied to each broth tube after incubation. A positive test result is shown by the reddish-pink colour ring of the broth in the reagent layer situated at the top of the medium broth.

3.6.6 Hugh- Leifson test (HL)

This test is done to detect the oxidative fermentative characteristics of microorganisms. The prepares Hugh-Leifson medium (Appendix II) was digested on water bath for 15 minutes and autoclaved at 121°C for 20 minutes. 5 ml media was dispensed in sterile test tubes. Loop full of culture suspension was stabbed in the media tube. Two tubes of each isolate were made respectively for oxidative and fermentative test. Cover one tube with mineral oil for checking for fermentative property and second tube is not covered with mineral oil (oxidative). The change in the medium from green to yellow indicates acid production due to utilisation of the

carbohydrate (glucose). Oxidative organisms produce an acidic reaction in the tube having aerobic conditions. Organisms that change the media colour throughout both aerobic as well as anaerobic tubes have fermentative characteristic. Alkaline reaction in the open tube and no reaction in the tube covered with oil indicates that the organism is unable to utilise the carbohydrate.

3.6.7 Oxidase test

Oxidase test indicates the existence or absence of cytochrome C oxidase enzyme within the organism. The test is conducted by inoculating culture isolate directly on to the oxidase disc with the help of sterile loop. The reduced colourless reagent N, N-methyl-p-phenylenediamine develops a deep blue-purple colour that indicates positive test.

3.6.8 Motility test

Motility test is used to detect the motility of the microorganism. 0.5% agar is added to Nutrient broth (Appendix II) and autoclaved. The media is poured in sterile test tubes under aseptic conditions and are allowed to solidify. Culture isolate is then stabbed in to the agar and incubated for 24 hours at room temperature. Motility of the organism is observed by cloud of growth away from the line of inoculation.

4. RESULTS AND DISCUSSION

4.1 Isolation of potential halophilic microorganisms

The colonies started appearing after 24 hours on incubation on ZMA. As the incubation period increased, more number of pigmented colonies appeared.

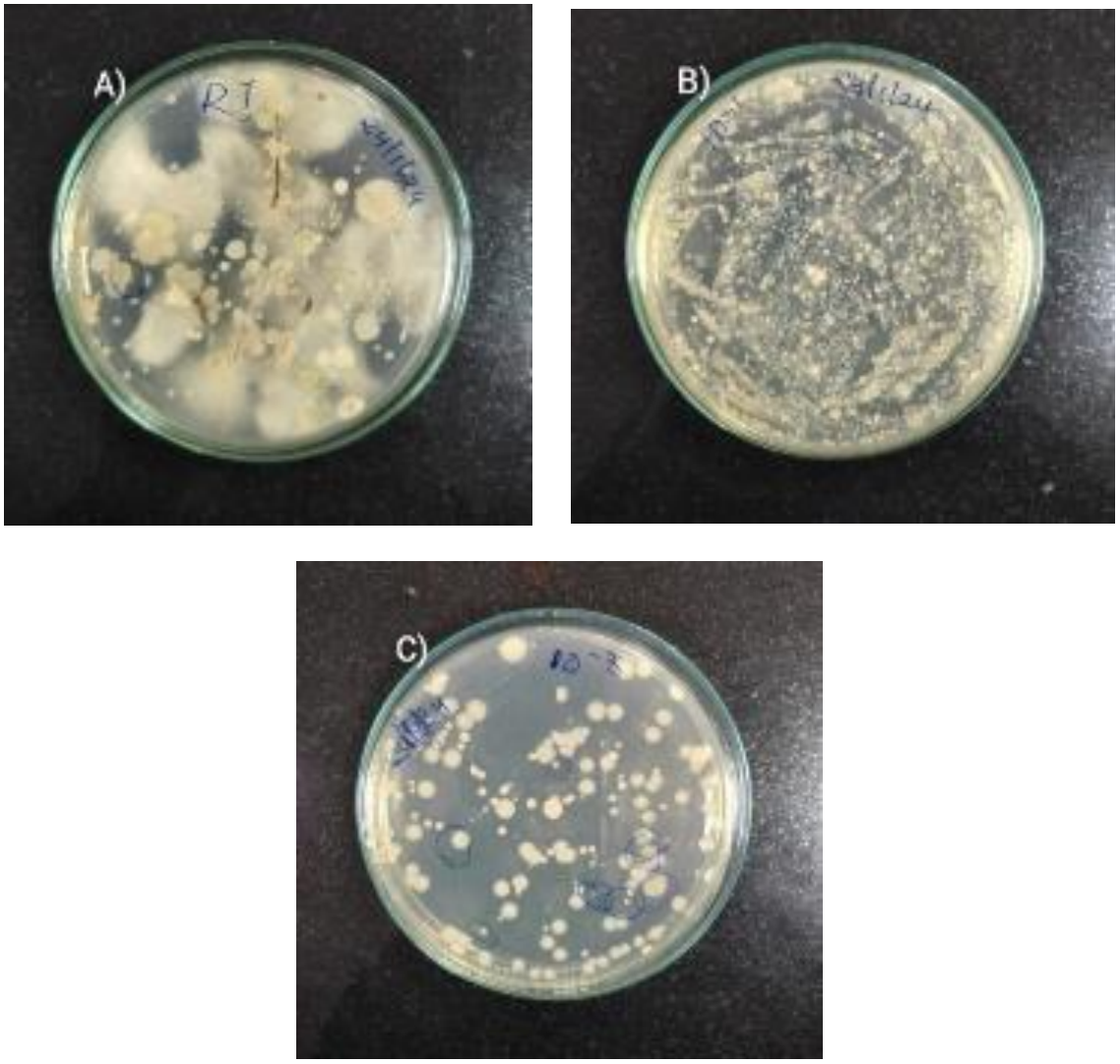


Figure 4.1: A) Master plate containing root hair associated bacterial isolates, B) and C) Dilution 10^{-1} and 10^{-3} of sand sample and root sample respectively.

4.1.1 Purification of bacterial isolates

Morphologically distinct colonies indicating distinct colony characteristics and pigments were selected from each master plate and purification was done by repeated quadrant streaking onto ZMA plates. Isolates obtained from Miramar beach sand dune sample were named with the acronym SMI 1, SMI 2, SMI 3 up to SMI 10 (Total 10 isolates). Isolates obtained from Morjim beach sample were named with the acronym SMJ 1, SMJ 2, SMJ 3 up to SMJ 12 (Total 12 isolates)

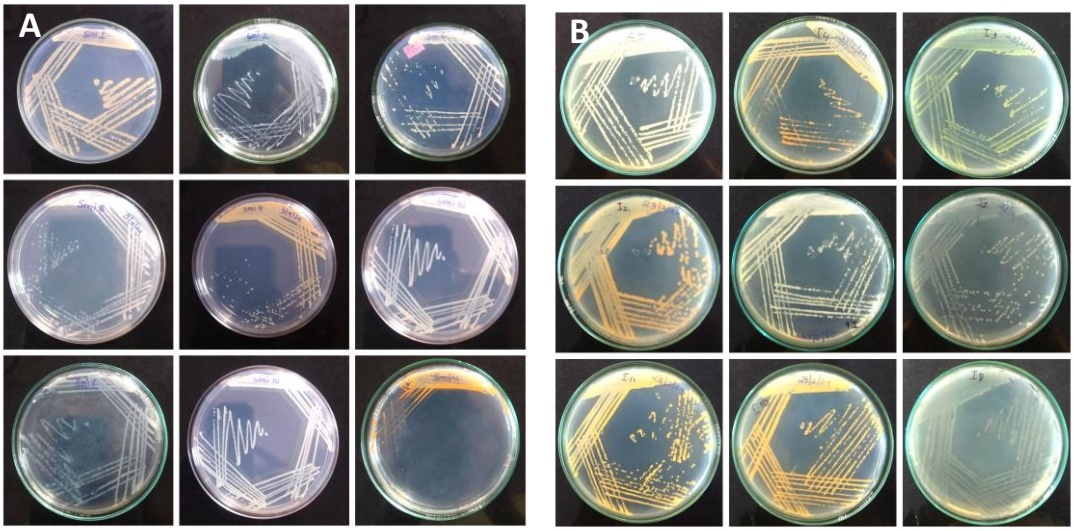


Figure 4.2: Purification of isolates using Quadrant streak method. A) Miramar beach isolates, B) Morjim beach isolates.

4.1.2 Maintenance of bacterial isolates

Culture isolates were streaked on ZMA slants and preserved at 4°C for around a month. Every month these sand dune bacterial isolates were sub cultured onto fresh slants.

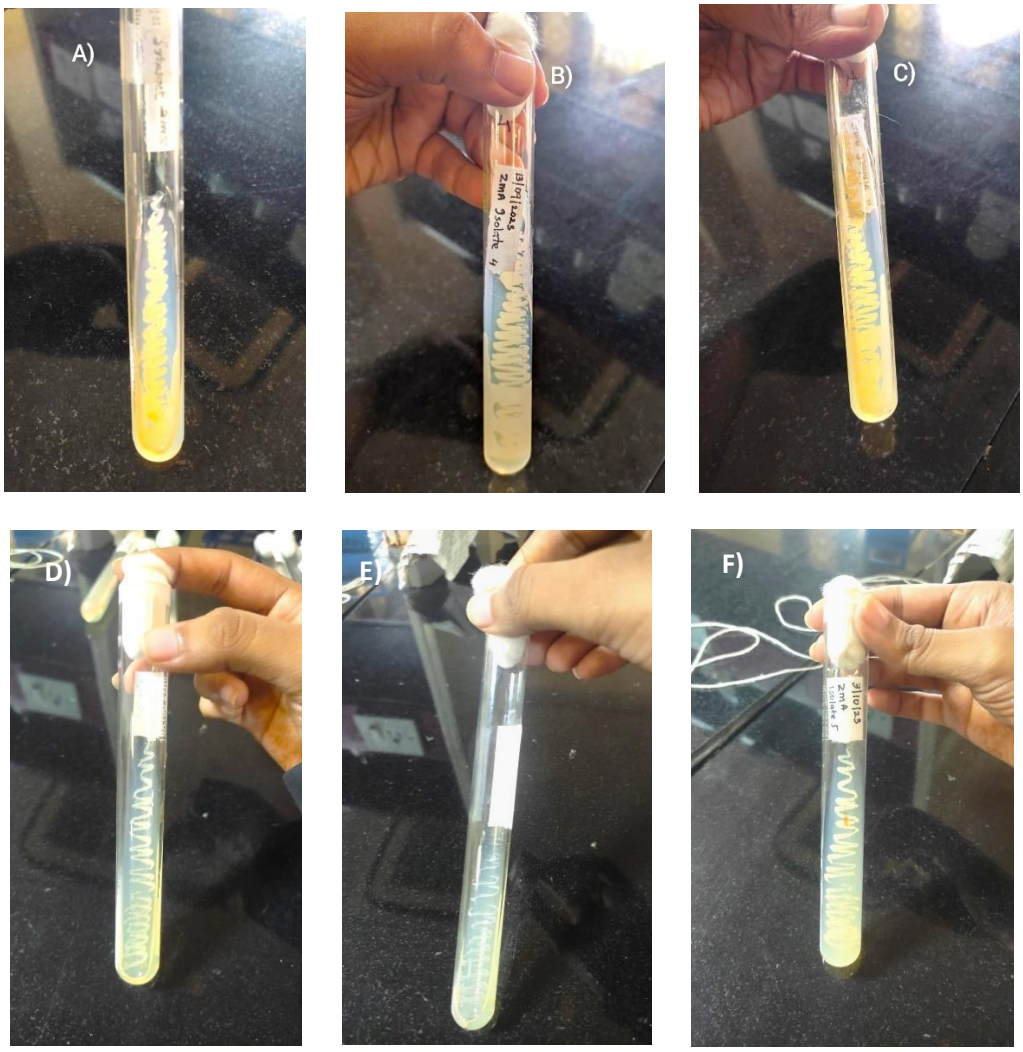


Figure 4.3: Maintenance of isolates on ZMA slants.

4.2 Determination of antibacteria potential of bacterial isolates.

All the 22 isolates were streaked vertically on NA plates and incubated at room temperature for 18 hours. Standard organism *Salmonella typhimurium* ATCC 14028 and *Streptecococcus pyogenes* ATCC 19615 were cross streaked across the test isolate and plates were incubated at 37°C for 24 hours. Isolates SMI 7, SMI 6, SMJ 8 showed antibacterial activity against both *S. pyogenes* and *S.typhymurium* indicated by growth inhibition of the standard ATCC culture organisms in the vicinity of the test isolate.

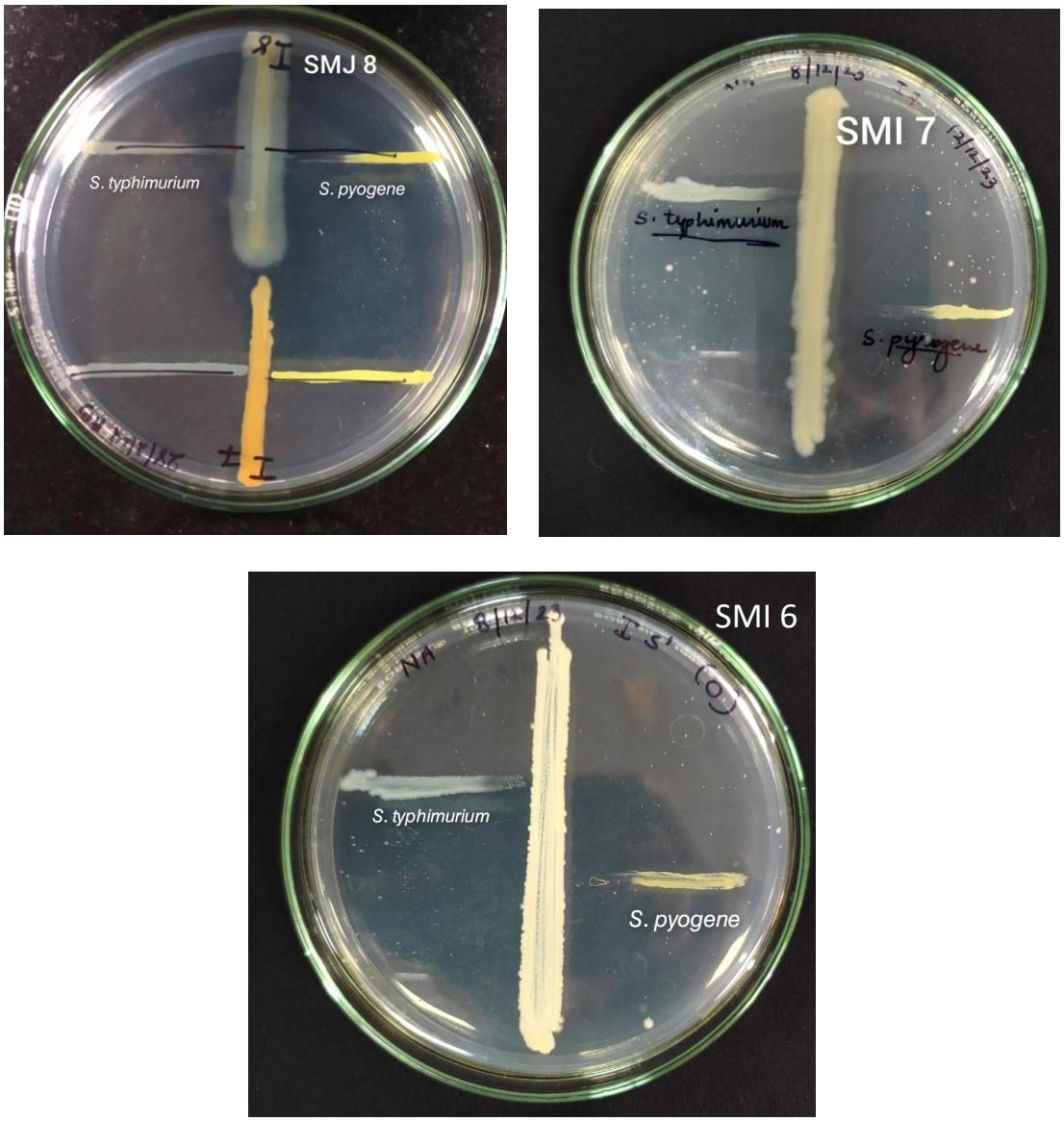
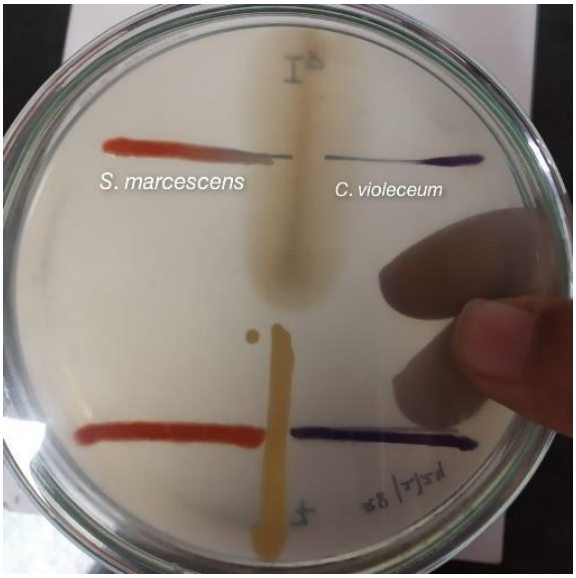


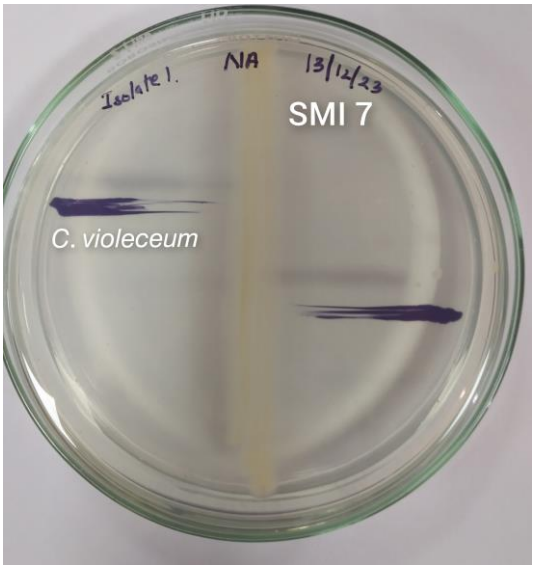
Figure 4.4: Sand dune bacteria isolates showing antibacterial activity/ growth inhibition against reporter (standard ATCC cultures) organisms *S. typhimurium* ATCC 14028 and *S. pyogenes* ATCC 19615

4.3 Determination of Quorum Quenching potential of sand dune bacterial isolates.

All the 22 isolates were streaked vertically on NA plates and incubated at room tempeature for 18 hours. Reporter culture *Chromobacterium violaceum* and *Serratia marcescens* were cross streaked across the test isolate and plates were incubated at room temperature for 24 hours. Isolates SMI 7 and SMJ 8 showed Quorum Quenching activity against the reporter organisms indicated by inhibition of pimentation of the reporter cultures in the vicinity of the test isolate. *Chromobacterium violaceum* produce pigment controlled by QS system. If test bacteria (isolates) produce QQ biomolecules than the *Chromobacterium violaceum* streaked very close to test isolate will not produce violacein pigment and will appear white.

Serratia marcescens produce pigment controlled by QS system. If test bacteria (isolates) produce QQ biomolecules than the *Serratia marcescens* streaked very close to it will not produce prodigiosin pigment and will appear white.





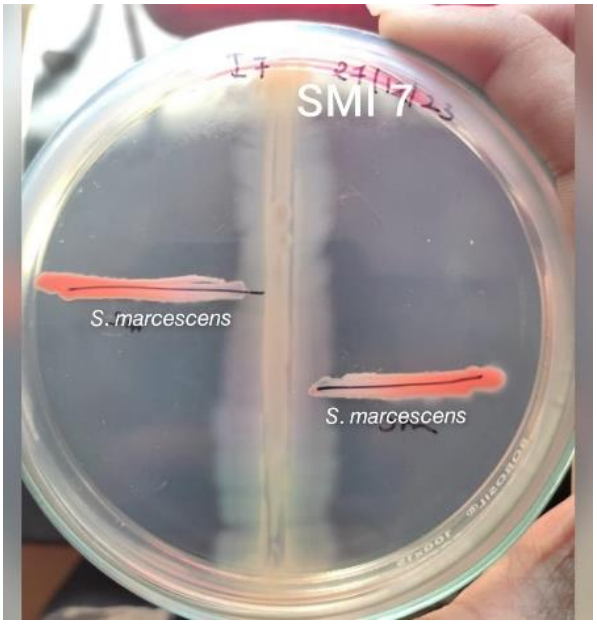


Figure 4.5: isolates showing Quorum Quenching potential by pigment inhibition against *C. violaceum* and *S. marcescens*

In above picture Isolate SMI7 showed pigment inhibition of *S. marcescens* which confirms QQ potential of SMI7. Isolate SMJ 8 also shows pigment inhibition of *S. marcescens* and *C. violaceum* which confirms QQ potential of SMJ 8.

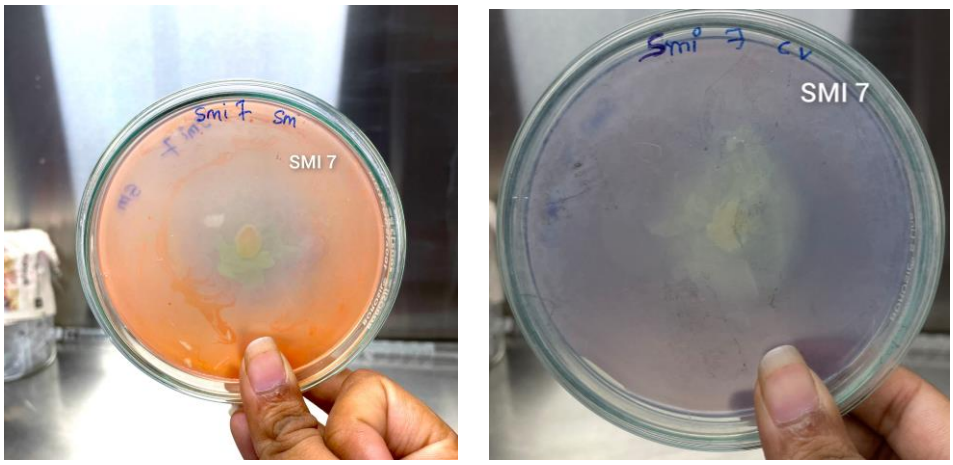


Figure 4.6: Quorum Quenching activity of isolate SMI 7 and SMJ 8 was seen against *C. violaceum* and *S. marcescens*

In above picture it is evident that both isolate SMI 7 and SMJ 8 show Quorum Quenching activity against *C. violaceum* and *S.marcescens* using agar overlay method. Here we can see both isolate SMI 7 and SMJ 8 inhibition of violacein and prodigiosin pigments produced by *C. violeceum* and *S.marcescens* . Their pigments are controled by AHL based QS system.

4.4 Screening for hydrolytic enzymes

All 22 isolates acquired from the sand dune sand samples were screened for the production of hydrolytic enzymes.

4.4.1 Amylase activity

All the 22 isolates were screened for Amylase activity, using 1 % soluble starch. Out the the 22 isolates screened, 4 isolates- SMI 1, SMI 2, SMJ 7 and SMJ 9 showed amylase activity.

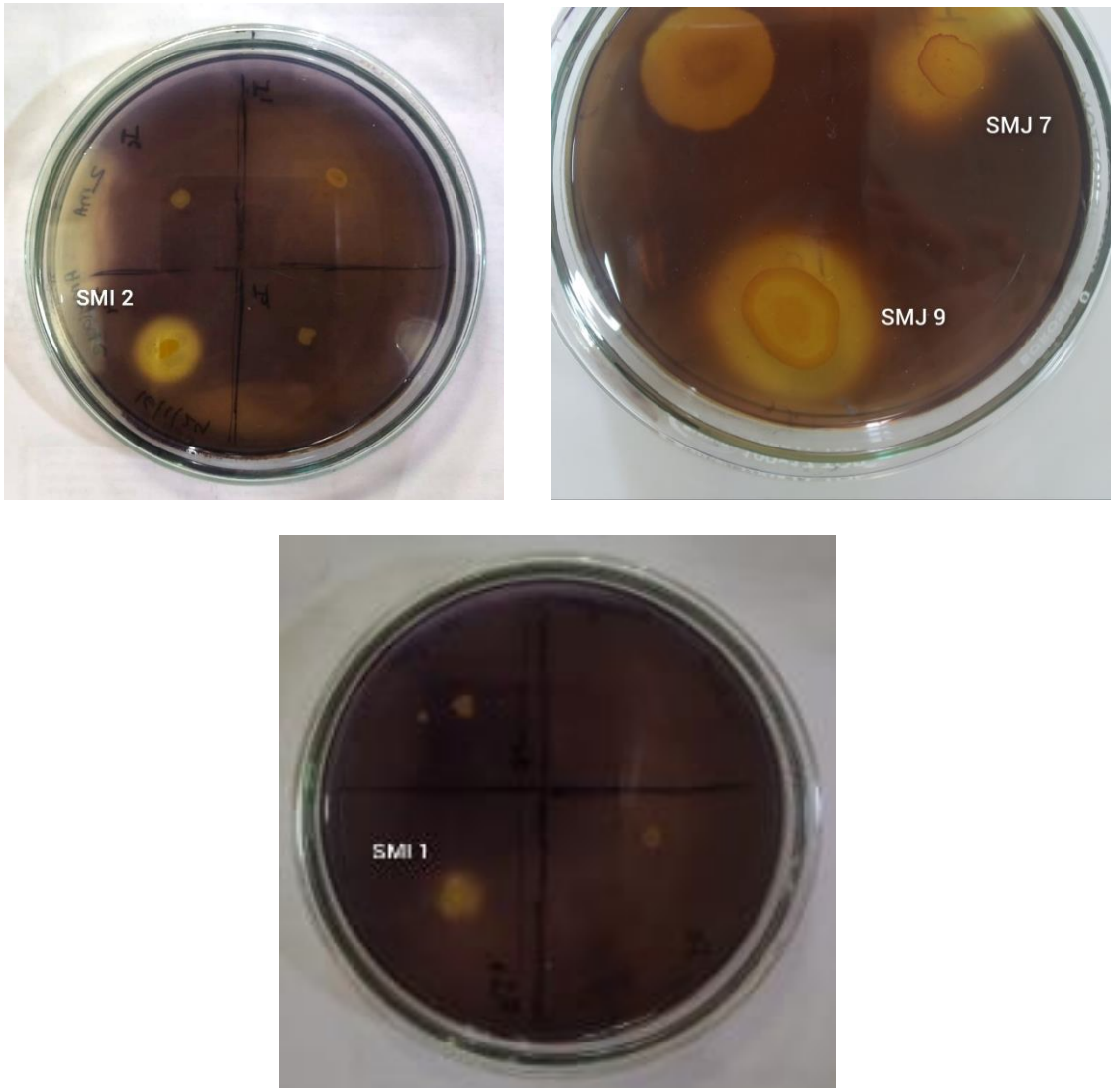


Figure 4.7: Clear Zone around the colonies after flooding with iodine solution indicating Amylase Activity.

4.4.2 Cellulase activity

All the 22 isolates were screened for cellulase activity using 0.5% carboxymethylcellulose (CMC) as the substrate. Out the 22 isolates screened 7 isolates- SMI 2, SMI 3, SMI 5, SMI 6, SMJ 1, SMJ 2 and SMJ 5 showed cellulase activity after flooding with 0.2% Congo Red solution and then washing with 1N NaCl solution.

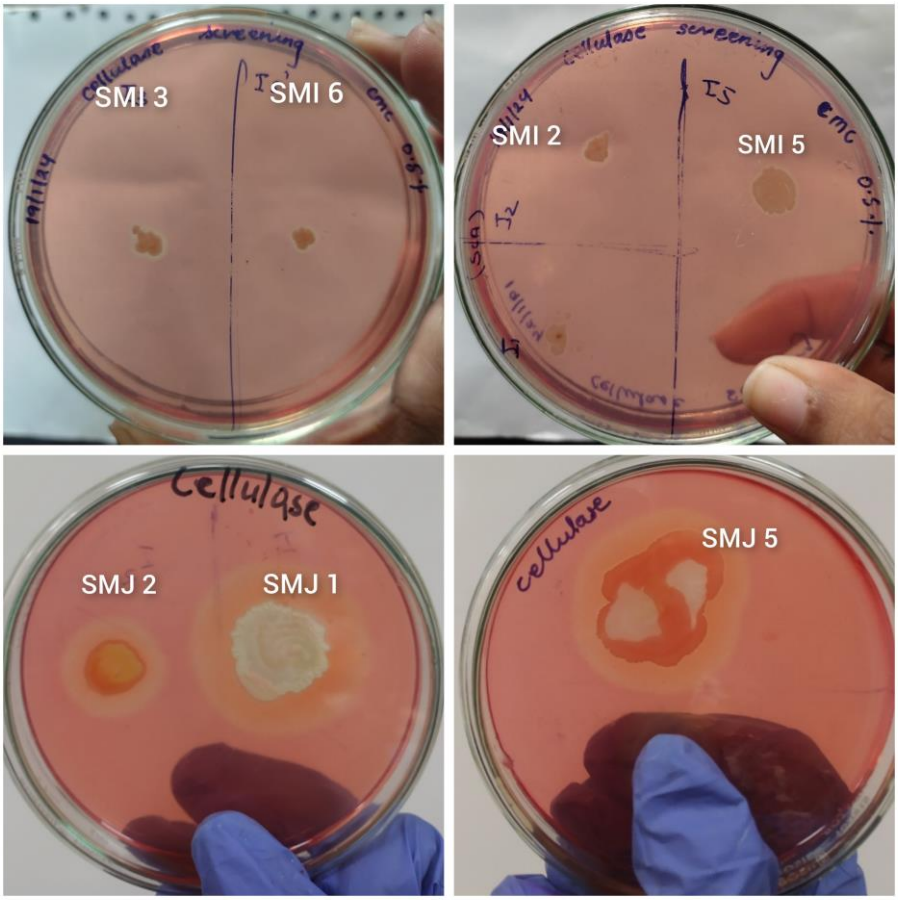


Figure 4.8: Zone of Clearance around the colonies after flooding with Congo red solution indicates cellulase activity.

4.4.3 Lipase activity

All the 22 isolates wee screened for lipase activity using 1% olive oil as the substrate. No white precipitation by any of the isolates was seen even after incubation of the plates for a week. Therefore none of the 22 isolates showed lipase activity.

4.4.4 Protease activity

All the 22 isolates were screened for protease acivity using 10% skimmed milk as the substrate. Out of the 22 isolated screened, 15 isolates- SMI 1, SMI 2, SMI 3, SMI 4, SMI 5, SMI 7, SMJ 1, SMJ 2, SMJ 3, SMJ 5, SMJ 7, SMJ 8, SMJ 9, SMJ 10 and SMJ 11 showed zones of clearance around the vicinity of the colonies after the incubation time, indicating protease production.

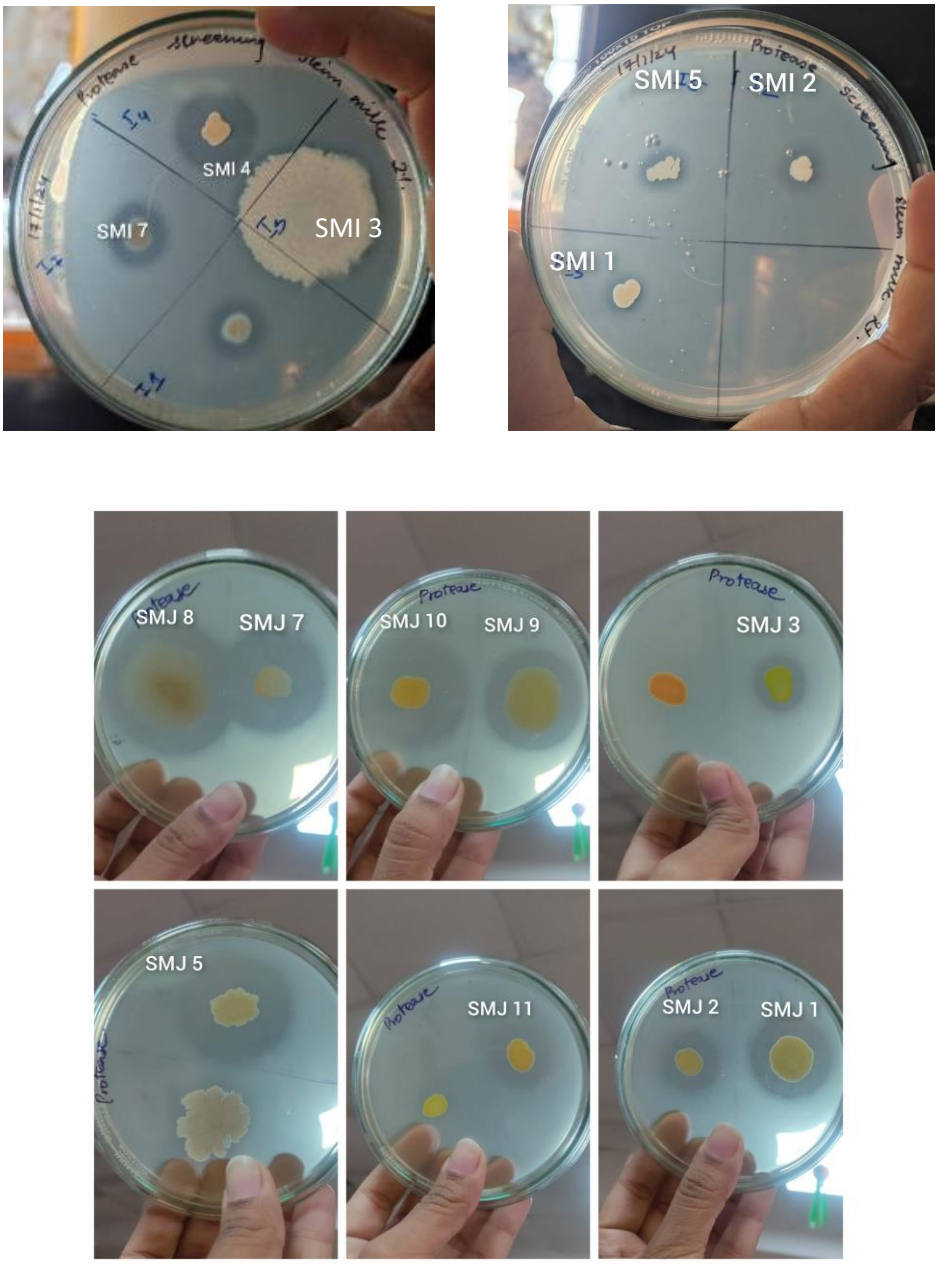


Figure 4.9: Clear zone/ halo formation around the colonies indicates Protease activity

4.4.5 Pectinase activity

All the 22 isolates were screened for pectinase activity using 0.5 % pectin as the substrate. Out of the 22 isolates screened, 4 isolates- SMI 3, SMI 4, SMJ 3 and SMJ 9 showed a zone of clearance around the periphery of the colonies after flooding it with iodine solution indicating positive for pectinase activity.

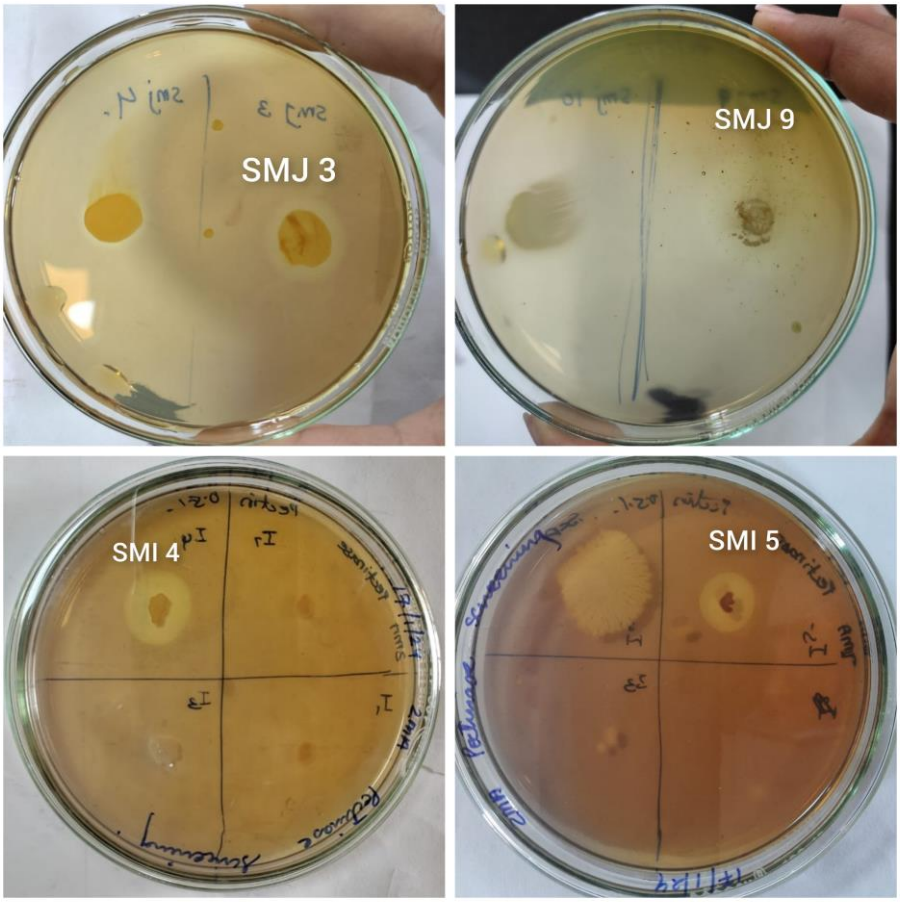


Figure 4.10: Clear zone around the colonies after flooding the plates with iodine solution indicates pectinase activity.

4.5.1 Morphological identification

Isolates	**SMI 1**	**SMI 2**	**SMI 3**	**SMI 4**	**SMI 5**
Media	ZMA	ZMA	ZMA	ZMA	ZMA
Time	24h	24h	24h	24h	24h
Temperature	28±0.2	28±0.2	28±0.2	28±0.2	28±0.2
Size	Small	Small	Small	Small	Small
Shape	Circular	Circular	Circular	Circular	Lobbed
Colour	Cream	White	White	Orange	White
Margin	Even	Lobbed	Lobbed	Even	Lobbed
Elevation	Flat	Raised	Flat	Convex	Flat
Surface texture	Smooth	Matt	Matt	Smooth	Matt
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Gram Character	+ bacilli	+ baciili	+ bacilli	+ cocci	+ short rods

Table no: 4.1: Morphological characteristics of Miramar sand dune isolates (SMI 1-SMI 5)

Isolates	**SMI 6**	**SMI 7**	**SMI 8**	**SMI 9**	**SMI 10**
Media	ZMA	ZMA	ZMA	ZMA	ZMA
Time	24h	24h	24h	24h	24h
Temperature	28±0.2	28±0.2	28±0.2	28±0.2	28±0.2
Size	Small	Small	Small	Small	Small
Shape	Circular	Circular	Circular	Circular	Circular
Colour	White	Grey	Pink	Orange	White
Margin	Even	even	Wavy	Even	Even
Elevation	Raised	Flat	Flat	Raised	Flat
Surface texture	Wrinkled	Smooth	Wrinkled	Smooth	Smooth
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Gram Character	+ Short rods	- Short rods	- bacilli	+ bacilli	+ short rods

Table no: 4.2: Morphological characteristics of Miramar sand dune isolates (SMI 6- SMI 10)

Isolate	**SMJ1**	**SMJ2**	**SMJ3**	**SMJ4**	**SMJ5**	**SMJ6**
Media	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA
Time	24h	24h	24h	24h	24h	24h
Temperaure	28±0.2	28±0.2	28±0.2	28±0.2	28±0.2	28±0.2
Size	Small	Small	Small	Small	Small	Small
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Colour	White	Orange	Lemon yellow	Peach	White	Peach
Margin	Wavy	Even	Even	Even	Wavy	Wavy
Elevation	Flat	Flat	Flat	Convex	Flat	Flat
Surface texture	Smooth	Smooth	Smooth	Smooth	Wrinkle	Wrinkle
Consistency	Butyrous	Mucoid	Mucoid	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Gram character	+ bacilli	- short rods	- short rods	+ cocci	+ bacilli	+ short rods

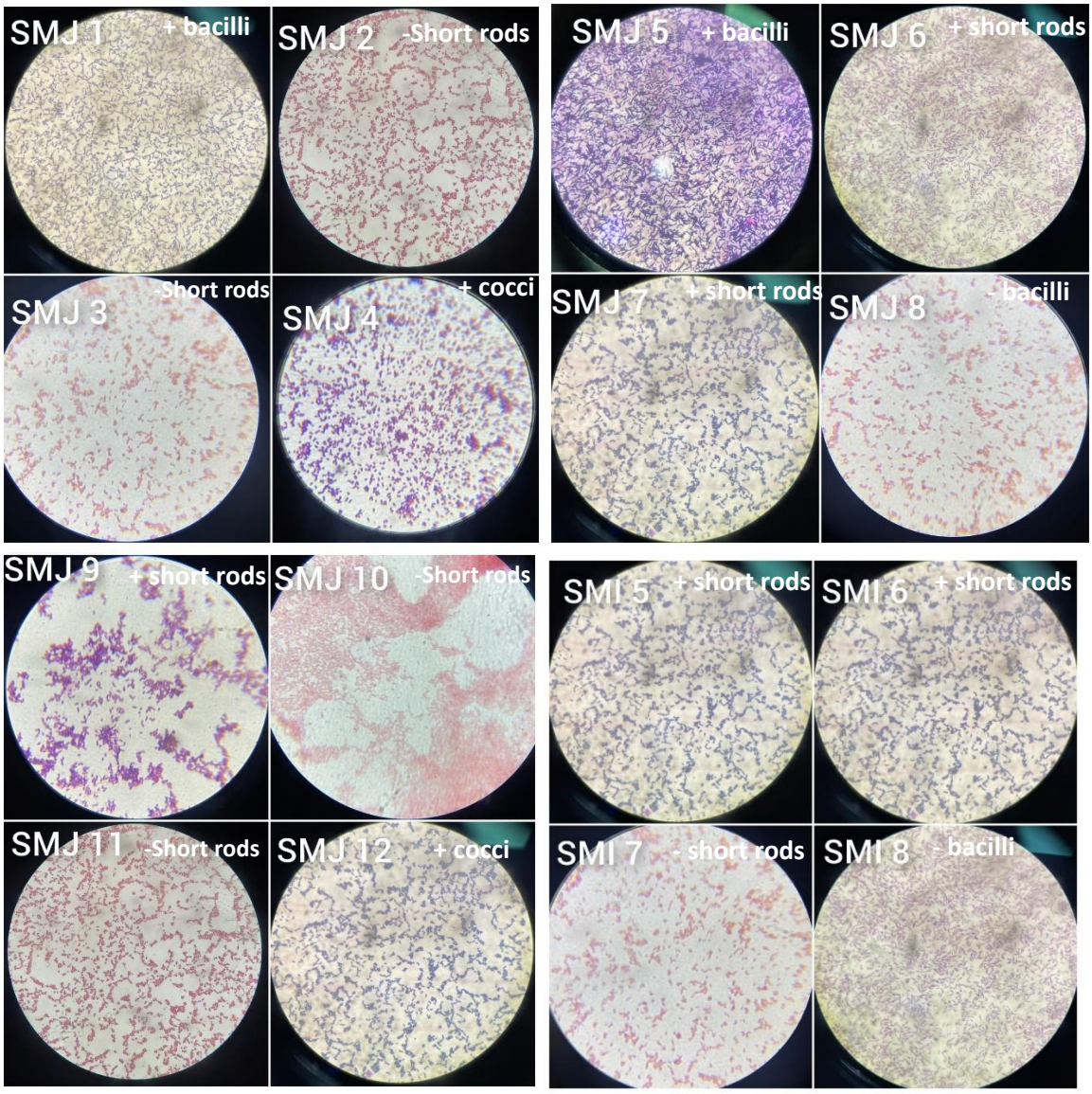
Table no: 4.3: Morphological characteristics of Morjim sand dune isolates (SMJ 1-SMJ 6)

Isolate	**SMJ7**	**SMJ8**	**SMJ9**	**SMJ10**	**SMJ11**	**SMJ12**
Media	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA
Time	24h	24h	24h	24h	24h	24h
Temperature	28±0.2	28±0.2	28±0.2	28±0.2	28±0.2	28±0.2
Size	Puntiform	Small	Small	Puntiform	Small	Small
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Colour	Orange	Brown	Cream	Orange	Orange	Yellow
Margin	Even	Even	Even	Even	Even	Even
Elevation	Flat	Flat	Flat	Convex	Convex	Pulvinate
Surface Texture	Buyrous	Butyrous	Butyrus	Butyrous	Butyrous	Butyrous
Consistency	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Gram Chracter	+ short rods	- bacilli	+ short rods	- short rods	- short rods	+ cocci

Table no: 4.4: Morphological characteristics of Morjim sand dune isolates (SMJ 7-SMJ 12)

4.5.1 Gram Staining

Gram staining was conducted using 18 hours old cultures using appropriate gram staining protocol. Pink colour cells indicate Gram negative organism. Purple colour cells indicate that the organism has Postive Gram Character.



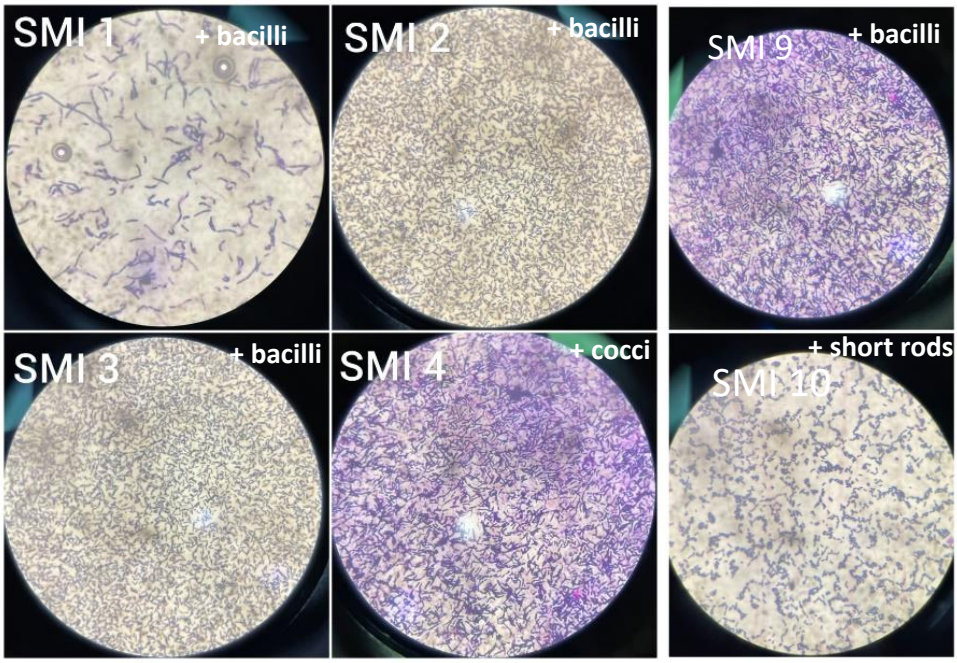


Figure 4.11: Gram staining of Miramar beach and Morjim beach Sand dune Isolates.

4.5.2 Endospore staining

Endospore staining of the culture isolates was conducted using appropriate protocol. None of the isolates that were observed for endospores showed the existence of endopores.

4.5.3 Biochemical tests

Isolate	**SMI 1**	**SMI 2**	**SMI 3**	**SMI 4**	**SMI 5**
Lactose fermentation	-	-	-	-	-
Indole	-	-	-	-	-
Vogues-proskaur	-	-	-	-	-
Methyl red	-	-	-	-	-
Citrate	-	-	-	-	+
Oxidase	-	+	+	+	+
Motility	+	-	-	+	+
Hugh-Leifson	Aerobic	Facultative anaerobe	Facultative anaerobe	Facutative anaerobe	Facultative anaerobe
Gram character	+ bacilli	+ bacilli	+ bacilli	+ cocci	+ short rods

Key :- + positive test, - negative test

Table no:4.5: Biochemical test results of Miramar sand dune isolates (SMI 1-SMI 5)

Isolate	SMI 6	SMI 7	SMI 8	SMI 9	SMI 10
Lactose fermentation	-	-	-	-	-
Indole	-	-	-	-	-
Vogues-proskaur	-	-	-	-	-
Methyl red	-	-	-	-	-
Citrate	-	+	-	-	-
Oxidase	-	+	+	+	-
Motility	+	+	-	+	-
Hugh-Leifson	Faculttive anaerobe	aerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Gram character	+ short rods	- Short rods	- bacilli	+ bacilli	+ short rods

Key : + positive test, - negative test

Table no:4.6: Biochemical test results of Miramar sand dune isolates (SMI 6-SMI 10)

Isolate	SMJ1	SMJ2	SMJ3	SMJ4	SMJ5	SMJ6
Lactose fermention	-	-	-	-	-	-
Indole	-	-	-	-	-	-
Vogues-proskaur	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-
Citrate	+	+	-	-	+	+
Oxidase	+	-	-	+	+	+
Motility	+	-	-	-	+	-
Hugh-Leifson	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anarebe	aerobe	Aerobe
Gram charachter	+ bacilli	- Short rods	- Short rods	+ cocci	+ bacilli	+ short rods

Key:- + positive test, - negative test

Table no: 4.7: Biochemical test results of Morjim sand dune isolates (SMJ 1-SMJ 6)

Isolate	**SMJ7**	**SMJ8**	**SMJ9**	**SMJ10**	**SMJ11**	**SMJ12**
Lactose fermentative	-	-	-	-	-	+
Indole	-	-	-	-	-	-
Vogues-proskauer	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-
Citrate	+	+	-	+	+	-
Oxidase	-	+	-	-	-	-
Motility	+	+	+	-	+	+
Hugh-Leifson	aerobe	Aerobe	Non saccharolytic	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Gram Character	+ short rods	-bacilli	+ bacilli	- Short rods	- Short rods	+ cocci

Key:- + positive test, - negative test

Table no: 4.8: Biochemical test results of Morjim sand dune isolates (SMJ 6-SMJ 12)

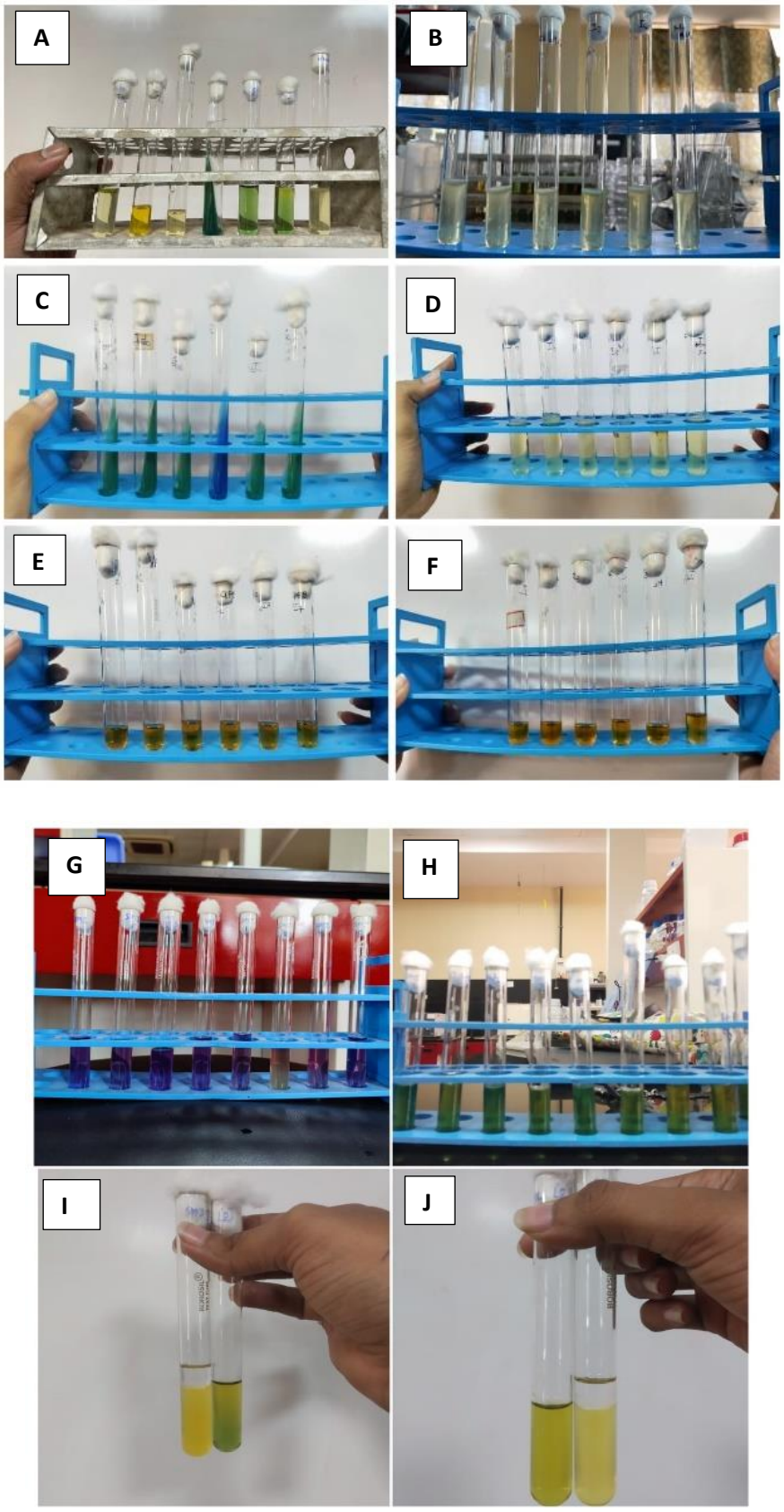


Figure 4.12: A) Biochemical tests of an isolate B) Motility test C) Citrate test D) Indole test E) MR test F) VP test G) Lactose fermentation test H) Hugh Leifson test I) and J) Tubes showing fermentative property of isolates.

Based on the results obtained after performing of the biochemical tests, isolates SMI 7 and SMJ 8 were tentatively determined to belong to the genus *Pseudomonas* sp. Apart from them the results obtained for other isolates were not conclusive enough to draw conclusion for tentative identification. More biochemical tests needed to be done.

ISOLATES		**SMI 1**	**SMI 2**	**SMI 3**	**SMI 4**	**SMI 5**	**SMI 6**	**SMI 7**	**SMI 8**	**SMI 9**	**SMI 10**
ANTIBACTERIAL ACTIVITY	***S. typhimurium***	-	-	-	-	-	+	+	-	-	-
	S. pyogenes	-	-	-	-	-	+	+	-	-	-
QUORUM QUENCHING POTENTIAL	***Chromobacterium violaceum***	-	-	-	-	-	-	+	-	-	-
	Serratia marcescens	-	-	-	-	-	-	+	-	-	-
HYDROLYTIC ENZYME ACTIVITY	**Amylase**	+	+	-	-	-	-	-	-	-	+
	Protease	+	+	+	+	+	-	+	-	-	+
	Cellulase	-	+	+	-	-	+	-	-	-	-
	Lipase	-	-	-	-	-	-	-	-	-	-
	Pectinase	-	+	+	-	-	+	-	-	-	-

Key:- + positive , - negative

Table no:4.9 (a): Table represents antibacterial, quorum quenching and hydrolytic enzyme production by Miramar sand dune isolates.

ISOLATES		**SMJ 1**	**SMJ 2**	**SMJ 3**	**SMJ 4**	**SMJ 5**	**SMJ 6**	**SMJ 7**	**SMJ 8**	**SMJ 9**	**SMJ 10**	**SMJ 11**	**SMJ 12**
ANTIBCTERIAL ACTIVITY	***S. typhimurium***	-	-	-	-	-	-	-	+	-	-	-	-
	S. pyogenes	-	-	-	-	-	-	-	+	-	-	-	-
QUORUM QUENCHING POTENTIAL.	***Chromobacterium violaceum***	-	-	-	-	-	-	-	+	-	-	-	-
	Serratia marcescens	-	-	-	-	-	-	-	+	-	-	-	-
HYDROLYTIC ENZYME ACTIVITY	**Amylase**	-	-	-	-	-	-	+	-	+	+	-	-
	Cellulase	+	+	-	-	+	+	-	-	-	-	-	-
	Lipase	-	-	-	-	-	-	-	-	-	-	-	-
	Protease	+	+	+	-	+	-	+	+	+	+	+	-
	Pectinase	-	-	+	-	-	-	-	-	+	-	-	-

Key :- + positive, - negative

Table no: 4.5.9 (b): Table represents antibacterial, quorum quenching and hydrolytic enzyme production by Morjim sand dune isolates.

In this study, the marine bacterial cultures obtained from the Miramar beach sand dunes and the Morjim beach sand dunes were screened for antibacterial activity, quorum quenching activity and production of hydrolytic enzymes. Total of 22 isolates were purified from the sand dune samples. The isolates from Miramar beach were named with the acronym SMI 1, SMI 2, SMI 3, SMI 4, SMI 5, SMI 6, SMI 7, SMI 8, SMI 9 and SMI 10. Similarly, the isolates from Morjim beach were named with the acronym SMJ 1, SMJ 2, SMJ 3, SMJ 4, SMJ 5, SMJ 6, SMJ 7, SMJ 8, SMJ 9, SMJ 10, SMJ 11 and SMJ 12. The antibacterial activity of the isolates was checked by cross streaking the isolate against two standard organisms; *S. typhimurium* ATCC 14028 and *S. pyogenes* ATCC 19615. Isolates SMI 6, SMI 7 and SMJ 8 showed antibacterial activity against *S. typhimurium* ATCC 14028 as well as *S. pyogenes* ATCC 19615 detected by growth inhibition of these organisms at the vicinity of the isolates by cross streak method. The remaining isolates didn't show antibacterial activity against the two using cross streak method. The isolates were than screened for inhibition of quorum sensing activity also called as quorum quenching activity. Quorum quenching potential of isolates was checked by streaking the isolates against pigmented bacteria *Chromobacterium violaceum* (produce violacein pigment controlled by AHL based QS) and *Serratia marcescens* to check for inhibition of pigment which indicates inhibition of quorum sensing. Isolate SMI 7 from the Miramar beach sample and isolate SMJ 8 from the Morjim beach sample showed quorum quenching activity against both *Chromobacterium violaceum* and *Serratia marcescens.* Here when SMI 7 and SMJ 8 crossed streaked against *Chromobcterium violaceum* and *Serratia marcescens* showed violacein and Prodigiosin inhibition respectively without inhibiting their growth which confirms QQ potential. Quorum Quenching activity was further confirmed by overlay method showing the pigment inhibition of *Chromobacterium violaceum* (violacein

pigment inhibition) and *Serratia marcescens* (Prodigiosin pigment inhibition) without inhibiting their growth. None of the other isolates had Quorum Quenching activity against *Chromobacterium violaceum* and *Serratia marcescens.* All the 22 isolates were screened for the production of hydrolytic enzymes Amylase, Protease, Cellulase, Lipase and Pectinase. Most of the isolates from Morjim as well as Miramar beach showed the production of hydrolytic enzymes. None of the 22 isolates had lipase enzyme production. Protease was the most abundantly produced hydrolytic enzyme compared to other hydrolytic enzymes. Isolate SMI 2 was the best hydrolytic enzyme producer showing almost all hydrolytic enzyme production except lipase enzyme. Followed by SMI 3 having the ability to produce three hydrolytic enzymes. Over all, SMI 7 and SMJ 8 are the best isolates showing antibacterial as well as Quorum quenching activity, but lacks in hydrolytic enzyme production; only showed amylase production. Isolate SMI 6 showed antibacterial activity but lacked Quorum quenching activity; instead showed production of two hydrolytic enzymes – cellulase and pectinase. Isolates SMI 7 and SMJ 8 can be utilised as potential solutions for certain medical issues as they show antibacterial as well as quorum quenching activity. Isolate SMI 6 can play a beneficial role in medical field as it shows antibacterial activity against *S. typhimurium* ATCC 14028 and *S. pyogenes* ATCC 19615. SMI 6 along with SMI 2 and SMI 3 can be used for industrial applications as they have the ability to produce multiple hydrolytic enzymes like Amylase, Cellulase, Protease and Pectinase.

CONCLUSION

In this study we have isolated sand dune bacteria and screened them for antibacterial, Quorum Quenching and hydrolytic enzyme production. Based on the results obtained, isolates have a potential for antibacterial activity as well as Quorum Quenching potential. Isolates also produced positive results for hydrolytic enzymes. The results implies that isolates from such areas have abundant industrial as well as medicinal applications. Isolates SMI 6, SMI 7 and SMJ 8 have potential to be used to deal with medical problems while SMI 2 and SMI 3 along with SMI 6 can be used for industrial applications.

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APPENDIX I

MEDIA COMPOSITION

1) Zobell Marine Agar (ZMA)

Ingredients	**g/litre**
Peptone	5.0
Sodium chloride	19.45
Yeast extract	1
Magnesium chloride	8.8
Ferric citrate	0.1
Sodium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.16
Disodium phosphate	0.008
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Agar	15.0
Distilled water	1L
pH	7.6±0.2

Weigh the appropriate quantity of media and add distilled water. Adjust the pH of the medium to 7.6±0.2. Autoclave the medium at 121°C, 15lbs pressure for 20 minutes.

2) Nutrient Agar (NA)

Ingredients	**g/litre**
Peptone	5
NaCl	5
Beef extract	3
Agar	15
Distilled water	1000 ml
Ph	7.4

Weigh the appropriate quantity of media and add distilled water. Adjust the pH of the medium to 7.4±0.2. Autoclave the medium at 121°C, 15lbs pressure for 20 minutes.

APPENDIX II

BIOCHEMICAL MEDIUM COMPOSITION

1) MacConkey`s Broth (for lactose fermentation)

Ingredients	**g/litres**
Pancreatic digest of gelatin	20
Lactose monohydrate	10
Dehydrated ox-bile	5
Bromo cresol purple	0.01
pH	7.3±0.2

Weigh appropriate quanity of dehydrated medium and add distilled water. Heat to boiling to dissolve the medium completely. Dispense into test tubes with inverted Durham tubes. Sterilise by autoclaving at 15lbs pressure at 121°C for 15 minutes.

2) Glucose phosphate Broth (MR-VP medium)

Ingredients	**g/litre**
Peotone	7.0
Dipotassium Phosphate	5.0
Dextrose	5.0
pH	7.4±0.2

3) Tryptone Broth

Ingredients	**g/litre**
Tryptone	10
NaCl	5.0
Distilled water	1 litre

4) Simmons Citrate Agar

Ingredients	**g/100ml**
NaCl	0.5
$MgS_4.7H_2O$	0.02
NaH_2PO_4	0.1
KH_2PO_4	0.1
Sodium Citrate	0.5
Bromothymol Blue (0.2%)	4.5
Agar	4
Distilled water	100
pH	6.8±0.2

5) Motility agar

Ingredients	**g/litre**
Nutrient broth	1.3
Agar	0.5
Distilled water	100 ml
pH	7.4±0.2

6) Hugh-Leifson medium

Ingredients	**g/litre**
Peptone	2
NaCl	5
Glucose	10
Bromothymol blue	0.03
Agar	3
Distilled water	1000ml
pH	7.1±0.2

REAGENTS AND STAINS

1) 0.2% KI-0.1%I solution

Ingredients	**g/100ml**
Potassium Iodide	0.2
Iodine Crystals	0.1
Distilled water	100 ml

2) 0.2% Congo red solution

Ingredients	**g/litre**
Congo red	0.2
Distilled water	100ml

3) 1M NaCl solution

Ingredients	**g/litre**
NaCl	24.72
Distilled water	100ml

4) 1% saline

Ingredients	**g/litre**
NaCl	1
Distilled water	100ml

5) Methyl red solution (for MR test)

Ingredients	**g/litre**
Methyl red	6.2
Ethyl alcohol	600ml
Distilled water	400ml

6) Kovac`s reagent (Indole test)

Ingredients	**g/litre**
Isoamyl alcohol	150 ml
p-dimethyl amino benzaldehyde	10.0
Concentrated HCL	50 ml

7) Omeara`s reagent (Vogues-Proskauer test)

Ingredients	**g/litre**
Creatine	0.15
KOH	20.0
Distilled water	40 ml

8) Gram`s Crystal Violet

Ingredients	**g/litre**
Crystal violet	2
Ammonium oxalate	0.8
Ethyl alcohol	20ml
Distilled water	80ml

9) Safranine

Ingredients	**g/litre**
Safranine-O	0.5
Ethyl alcohol	50ml

10) Gram`s iodine

Ingredients	**g/litre**
Iodine	1
Potassium Iodide	2
Distilled water	300ml

Store in amber coloured bottle.

11) Malachite Green

Ingredients	**g/litre**
Malachite green	0.5
Distilled water	100ml

Study of Sand dune Bacteria and Bioprospecting for their Bioactive molecules

SUMMARY

Due to the improper and extensive use of antibiotics; most of the bacteria are developing resistance towards antibiotics giving rise to multi drug resistant (MDR) bacteria. MDR bacteria are bacteria that show resistance towards two or more types of antibiotics. Hence, immergence of MDR bacteria is one of the major medical issues faced by the doctors and the scientists around the globe. Therefore, there is a need to find novel antibiotics or novel approaches to deal with MDR bacteria. Another major problem is the unavailability of enough natural resources to reach the demands of the increasing population, whilst chemical resources are economically and environmentally unsustainable. Microbes are producers of multiple hydrolytic enzymes which can be utilised in different industries as natural resources. Microbes require less time to grow and can also produce high yield hence they provide an economical and environmentally sustainable alternative for industries. Sand dune ecosystem is a dynamic environment, hence microorganism found here have abilities to deal with the dynamic conditions like varying pH, extreme temperatures and salinity and are reported to contain agriculturally, industrially and pharmaceutically diverse microbial diversity. The aim of this project was to study Sand dune Bacteria and bioprospecting for their bioactive molecules. Objectives under this study were to screen the isolates for antibacterial and Quorum Quenching potential. They were also screened for production of hydrolytic enzyme and tentative identification was also done for few isolates.

The samples were collected from the sand dunes of Morjim beach and Miramar beach of Goa India. Sand sample along with roots of *Ipomoea* sp. was collected in a sterile 50 ml centrifuge tube with the help of sterile scalpel. Sample were serially diluted and spread plated on ZMA plates. Total of 10 isolates were selected from Miramar sand dune sample and 12 isolates were

selected from Morjim sand dune sample. Isolates from Miramar sand dunes were named with the acronym SMI 1, SMI 2, SMI 3 up till SMI 10. Similarly, isolates from Morjim were named with the acronym SMJ 1, SMJ 2, SMJ 3 up till SMJ 12. This samples were then screened for their antibacterial and quorum quenching potential and were also check for production of hydrolytic enzymes like amylase, cellulase, protease, lipase and pectinase.

ISOLATES		**SMI 1**	**SMI 2**	**SMI 3**	**SMI 4**	**SMI 5**	**SMI 6**	**SMI 7**	**SMI 8**	**SMI 9**	**SMI 10**
ANTIBACTERIAL ACTIVITY	***S. typhimurium***	-	-	-	-	-	+	+	-	-	-
	Strep. pyogenes	-	-	-	-	-	+	+	-	-	-
QUORUM QUENCHING POTENTIAL	***Chromobacterium violaceum***	-	-	-	-	-	-	+	-	-	-
	Serratia marcescens	-	-	-	-	-	-	+	-	-	-
HYDROLYTIC ENZYME ACTIVITY	**Amylase**	+	+	-	-	-	-	-	-	-	+
	Protease	+	+	+	+	+	-	+	-	-	+
	Cellulase	-	+	+	-	-	+	-	-	-	-
	Lipase	-	-	-	-	-	-	-	-	-	-
	Pectinase	-	+	+	-	-	+	-	-	-	-

Table no 1: table represent antibacterial, quorum quenching activity and hydrolytic enzyme production of Miramar beach sand dune bacterial isolates (SMI 1-SMI 10).

ISOLATES		**SMJ 1**	**SMJ 2**	**SMJ 3**	**SMJ 4**	**SMJ 5**	**SMJ 6**	**SMJ 7**	**SMJ 8**	**SMJ 9**	**SMJ 10**	**SMJ 11**	**SMJ 12**
ANTIBCTERIAL ACTIVITY	***Salmonella typhimurium***	-	-	-	-	-	-	-	+	-	-	-	-
	Streptococcus pyogenes	-	-	-	-	-	-	-	+	-	-	-	-
QUORUM QUENCHING POTENTIAL	***Chromobacterium violaceum***	-	-	-	-	-	-	-	+	-	-	-	-
	Serratia marcescens	-	-	-	-	-	-	-	+	-	-	-	-
HYDROLYTIC ENZYME ACTIVITY	**Amylase**	-	-	-	-	-	-	+	-	+	+	-	-
	Cellulase	+	+	-	-	+	+	-	-	-	-	-	-
	Lipase	-	-	-	-	-	-	-	-	-	-	-	-
	Protease	+	+	+	-	+	-	+	+	+	+	+	-
	Pectinase	-	-	+	-	-	-	-	-	+	-	-	-

Table no 2: Table represents Antibacterial, Quorum Quenching activity and Hydrolytic enzyme production of Morjim sand dune bacterial isolates (SMJ 1-SMJ 12).

In this study, the sand dune bacterial cultures obtained from the Miramar beach sand dunes and the Morjim beach sand dunes were screened for antibacterial activity, quorum quenching activity and production of hydrolytic enzymes. Total of 22 isolates were purified from the sand dune samples. The isolates from Miramar beach were named with the acronym SMI 1, SMI 2, SMI 3, SMI 4, SMI 5, SMI 6, SMI 7, SMI 8, SMI 9 and SMI 10. Similarly, the isolates from Morjim beach were named with the acronym SMJ 1, SMJ 2, SMJ 3, SMJ 4, SMJ 5, SMJ 6, SMJ 7, SMJ 8, SMJ 9, SMJ 10, SMJ 11 and SMJ 12. The antibacterial activity of the isolates was checked by cross streaking the isolate against two standard organisms; *S. typhimurium* ATCC 14028 and *S. pyogenes* ATCC 19615. Isolates SMI 6, SMI 7 and SMJ 8 showed antibacterial activity against *S. typhimurium* as well as *S. pyogenes* detected by growth

inhibition of these organisms at the vicinity of the isolates. The remaining isolates didn't show antibacterial activity even against one of the two standard organisms. The isolates were than screened for inhibition of quorum sensing activity also called as quorum quenching activity. Quorum quenching potential of isolates was checked by cross streaking the isolates against pigmented bacteria *Chromobacterium violaceum* and *Serratia marcescens* to check for inhibition of pigment which signals inhibition of quorum sensing. Isolate SMI 7 from the Miramar beach sample and isolate SMJ 8 from the Morjim beach sample showed quorum quenching activity against both *Chromobacterium violaceum* and *Serratia marcescens.* Quorum Quenching activity was further confirmed by agar overlay method showing the pigment inhibition of the pathogenic strains. None of the other isolates had Quorum Quenching activity against *Chromobacterium violaceum* and *Serratia marcescens.* All the 22 isolates were screened for the production of hydrolytic enzymes Amylase, Protease, Cellulase, Lipase and Pectinase. Most of the isolates from Morjim as well as Miramar beach showed the production of hydrolytic enzymes. None of the 22 isolates had lipase enzyme production. Protease was the most abundantly produced hydrolytic enzyme compared to other hydrolytic enzymes. Isolate SMI 2 was the best hydrolytic producer showing almost all hydrolytic enzyme production except lipase enzyme. Followed by SMI 3 having the ability to produce three hydrolytic enzymes. Over all, SMI 7 and SMJ 8 are the best isolates showing antibacterial as well as Quorum quenching activity, but only showed amylase production. Isolate SMI 6 showed antibacterial activity but lacked Quorum quenching activity; instead showed production of two hydrolytic enzymes – cellulase and pectinase. Isolates SMI 7 and SMJ 8 can be utilised as potential solutions for certain medical issues as they show antibacterial as well as quorum quenching activity. Isolate SMI 6 can play a beneficial role in medical field as it shows antibacterial activity against *S. typhimurium* ATCC 14028 and *S. pyogenes* ATCC 19615. SMI 6 along with SMI 2 and SMI 3 can be used to deal with industrial problems as they

have the ability to produce multiple hydrolytic enzymes like Amylase, Cellulase, Protease and Pectinase.

Conclusion

In this study we have isolated sand dune bacteria and screened them for antibacterial, Quorum Quenching and hydrolytic enzyme production. Based on the results obtained, isolates have a potential for antibacterial activity as well as Quorum Quenching potential. Isolates also produced positive results for hydrolytic enzymes. The results implies that isolates from such areas have abundant industrial as well as medicinal applications. Isolates SMI 6, SMI 7 and SMJ 8 have potential to be used to deal with medical problems while SMI 2 and SMI 3 along with SMI 6 can be for industrial applications.