ANTIMICROBIAL ACTIVITIES OF "MARINE DERIVED FUNGI"

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BY

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UNDER THE GUIDANCE OF

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DECLARATION

I hereby declare that work incorporated in this dissertation, which is in partial fulfilment of the M.Sc. degree course, is original and carried out in the School of Earth, Ocean and Atmospheric Sciences, Goa University, Goa and it has not been submitted in any part or in full for any other degree or diploma of any other university.

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This is to certify that the dissertation report **"Antimicrobial activities of Marine derived Fungi"** is a bonafide work carried out by **Ms Aarti Ashok Satuse** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the **Marine Microbiology** at the School of Earth, Ocean and Atmospheric Sciences Goa University.

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This is to certify that the dissertation entitled **as "Antimicrobial activities of Marine derived fungi"** is a bonafide and an authentic record of the research carried out by Ms. Aarti Satuse, Student of M.Sc. Marine Microbiology, under my supervision and guidance at the School of Earth, Ocean and Atmospheric Sciences, Goa University, Goa, in partial fulfilment of the requirement of M.Sc. Marine Microbiology Degree of the University and that no part has been submitted before for any other degree or diploma in any other University.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Fungi are pervasive creatures that are found throughout all ecosystems, even marine ones. The final remaining, less explored territory for biodiversity is the ocean (Samuel et al, 2011). Even though 70% of the planet's surface is made up of maritime habitats, little is known about their biology. A significant fraction of the microbial diversity on Earth is represented by fungi, which are important players in both terrestrial and marine settings (Gonçalves et al, 2022). Marine fungi have been extensively defined as "marine-derived fungi," especially in the field of natural product chemistry (Samuel et al, 2011). In contrast to the traditional definition, "marine-derived fungus" refers to fungi that have been "separated" from habitats and substrates that are marineassociated and are not always considered to be ecological occupants of marine settings. Due to their capacity to create bioactive metabolites marine fungus has drawn interest from researchers and industry since the first investigations on them which was conducted in the early 1900s (Kwon et al, 2021). Several definitions of marine fungi have been employed over time. Physiological traits of marine fungi, such as the need for more than 30% salinity to thrive, were initially used to define them (Gonçalves et al, 2022). Marine fungi are a complex group of microorganisms, that are diverse both chemically and biologically. The classical definition classifies marine fungus as obligatory or facultative (Kwon et al, 2021). Facultative marine fungi are those from freshwater or terrestrial environments that can grow and sporulate in marine environments. Obligate marine fungi are those that grow and sporulate solely in a marine or estuarine habitat. A possible source of novel bioactive natural chemicals is represented by marine fungus, which are a biochemically varied collection of species. Terpenes, steroids, polyketides, peptides, alkaloids, and polysaccharides are a few of the secondary metabolites that marine fungi produce. Most of these metabolites have

antibacterial, antiviral, anticancer, antioxidant, and anti-inflammatory properties (Gonçalves et al, 2022).

Marine fungi can grow on a broad range of substrates, including wood, sediments, muds, soils, sand, algae, corals, calcareous tubes of mollusks, decomposing mangrove leaves, intertidal grasses, and even living creatures (Hyde et al, 1998). In coastal waters, marine fungi are well-known to serve as the major degraders (Zhou et al, 2016).

In comparison to the terrestrial compartment, marine fungi encounter significant difficulties. Osmotic and ionic stress result from salt levels that are too high. However, the high-osmolarity-glycerol (HOG) signalling system, a conserved route, allows fungi to regulate their turgor. This route controls the salt efflux pumps and generates osmolytes that are appropriate for cellular functioning (Gonçalves et al, 2022). A significant source of novel biologically natural compounds has been found in marine fungi. In contrast to terrestrial fungi, they may have evolved particularly in secondary metabolic pathways due to their unique living environment, salinity, nutrition, increased pressure, temperature fluctuations, and competition with bacteria, viruses, and other fungi (Samuel et al, 2001).

1.1.1 “ANTIBACTERIAL ACTIVITIES”

Among the most widely used medications are antibacterial. There has been a recent increase in demand for novel antibacterial and antifungal chemicals due to the seriousness of bacterial and fungal infection resistance. Because of the wide variety of fungal species, the abundance of secondary metabolites, and advancements in genetic breeding and fermentation techniques, naturally occurring fungi are regarded as a significant source of new antibacterial and antifungal chemicals. One of the most

common genera of marine fungi is *Aspergillus*, and this genus has created more novel antibacterial and antifungal chemicals than any other (Christophersen et al, 1998).

1.1 LITERATURE REVIEW:

The scientific community has been challenged to investigate novel environmental contexts and the related microbial diversity due to the impact of bioactive substances from natural sources on human existence, particularly in pharmacology and biotechnology (Nicoletti et al, 2018).

The term "antimicrobial" refers to any agent, whether natural, semi-natural, or synthetic, that kills or slows the growth of microorganisms while posing minimal or no risks to the host (Steeve et al, 2013). Antimicrobial agents, which can be categorised as bactericidal or bacteriostatic, employ several techniques against bacteria to thwart their pathogenesis. Antimicrobial agents come in a variety of forms, including antibiotics, disinfectant, and food preservatives, and they can be applied to microorganisms to lower their growth potential, obstruct their multiplication, or even kill them (Abushaheen et al, 2020). Penicillin, which was discovered by Fleming in 1929 and was derived from the fungus *Penicillium notatum*, is unquestionably one of the most well-known natural product discoveries (Dias et al, 2012).

Following the "Golden Age of Antibiotics" and the widespread motivation to find new antibiotics, many large pharmaceutical corporations at the time started natural product discovery (NPD) programmes that concentrated on infectious disorders in addition to antibacterial and antifungal targets. For the treatment of cancer, microbial infections, hypercholesteremia, and tissue rejection in organ transplants, these programmes delivered lead compounds (Dias et al, 2012).

According to Schumacher et al (2015) the marine environment is home to a variety of organisms that are beneficial for human health. These organisms include those that abolish self-sufficiency in growth signals, restore sensitivity to growth-inhibitory signals, cause apoptosis, act as antiangiogenic compounds, lower the replicative potential, and prevent tissue invasion and metastasis.

Because resident harmful microbes, such as bacteria, provide a constant threat of infection to marine creatures, they have developed complex chemical compounds with antibacterial action from a variety of biological antecedents (Abad et al, 2015).

1.2.1 HISTORY OF ANTIMICROBIAL MOLECULES:

It is well known that throughout history, people have fought a long-running fight with microbes, particularly bacteria, which has resulted in enormous morbidity and mortality in various human populations all over the world (Abushaheen et al, 2020). In 1940, Selman Waksman coined the term "antibiotic" to refer to any tiny chemical produced by a bacterium that inhibits the growth of other microorganisms (Clardy et al, 2006). Penicillin was a powerful antibacterial antibiotic for bacteria in the early 1940s. As a result, it was widely utilised by individuals to combat numerous infectious disorders. However, due to overuse of penicillin, its effectiveness reduced as bacteria began to develop a variety of resistance mechanisms (Abushaheen et al, 2020).

1.2.2 MECHANISM OF ANTIMICROBIAL:

It is essential to understand how antimicrobial drugs function in comprehend the processes of resistance. Specific important bacterial functions are the focus of antimicrobial agents. The method how each class of antimicrobial drugs kills or inhibits the bacterium differs (Abushaheen et al, 2020). Antimicrobial agents are frequently categorised based on their main mode of operation. Among the mechanisms are

disruption of bacterial membrane structure (polymyxins and daptomycin), interference with protein synthesis (macrolides and tetracyclines), interference with nucleic acid synthesis (fluoroquinolones and rifampicin), inhibition of a metabolic pathway

(trimethoprim-sulfamethoxazole), and interference with cell wall synthesis (Tenover, 2006). Antimicrobials can be secreted outside of cells or to the periplasm via ubiquitous transmembrane transporters, which are overexpressed efflux pumps, among other processes that cause antimicrobial resistance. Reports on bacterial efflux pumps are scarce, despite some studies showing marine natural products to be effective in overcoming multidrug resistance in the mammalian efflux pump Pglycoprotein (Durães et al, 2021). Finding novel compounds with the potential to act as antimicrobial adjuvants and the benefit of not being susceptible to resistance is therefore crucial (Durães et al, 2021).

1.2.3 MARINE MICROORGANISM AS SOURCE OF ANTIMICROBIAL METABOLITES:

Majority of the world's marine life, which makes up marine animals, provides a rich source of structurally and functionally diverse bio-functional elements. Over 22,000 substances have so far been extracted from marine species as sponges, coelenterates, microbes, algae, echinoderms, mollusks, and bryozoans as well as from other sources like marine processing wastes (Santos et al, 2014).

More attention has been paid to marine microorganisms such bacteria, actinomycetes, fungus, and cyanobacteria as potential lead compound makers. They are a more sustainable and reproducible source than marine invertebrates since they can be cultured and even imagined as fantastic microbial factories for natural products (Jin et al, 2016). Technology and methodology advancements have made it possible to effectively explore the marine environment and identify the chemical and biological

profiles of substances obtained from marine sources. New scaffolds have been found as a result, and research has focused on these areas; also, drugs developed from marine macro-organisms have already received regulatory approval for use as medicines. But lately, attention has switched to marine microorganisms like bacteria and fungi, which have the advantage of being simple to grow in a lab setting and having the capacity to produce more secondary metabolites (Durães et al, 2021).

Bergmann reported the initial identification of a bioactive marine natural substance in the late 1950s. The first proof that naturally occurring nucleosides might contain sugars other than ribose and desoxyribose came from the arabino and ribo-pentosyl nucleosides that were recovered from marine sponges. Two derivatives of nucleosides having antiviral action, vidarabine and cytarabine, were created through chemical synthesis. For many years, these two nucleosides have been used in medical therapies for ages now (Santos et al, 2014).

1.2.4 SECONDARY METABOLITES OF MARINE DERIVED:

Fungi have drawn more attention in recent years due to their ubiquitous presence in many ecosystems and their propensity to form symbiotic relationships with higher animals. There have been numerous reports of fungal strains producing medications that were previously attributed to marine plants and animals (Nicoletti et al, 2018).

"Secondary metabolism" refers to the process by which an organism produces substances (natural products), which are frequently discovered to be specific to an organism or a manifestation of the individuality of a species. Secondary metabolites are typically not necessary for an organism's growth, development, or reproduction and are either produced as result of the organism adapting to its environment or as a potential defence mechanism against predators to help the organism survive (Dias et

al, 2012). Some of the most significant natural products for the pharmaceutical sector have been produced by bioactive secondary metabolites obtained from fungal sources (Dias et al, 2012). The identification of various structurally varied secondary metabolism with a variety of activities, including antibiotic, anticancer, antiinflammatory, antiviral, and antioxidant action was made possible by the natural products of marine derived fungi (Chen et al, 2015). Alkaloids, terpenes, polyketones, peptides, sterols, and lactones are chemicals derived from marine fungi that may exhibit beneficial properties such as antibacterial, antioxidant, anticancer, anticoagulant, and enzyme inhibitory effects. Polyphenols, which are phenolic chemicals, are secondary metabolites important for maintaining human health (Adeoyo et al, 2019). As a result, marine fungi are a valuable source for the creation of new, highly effective medications with low toxicity (Wang et al, 2021). Particularly, the *Aspergillus* and *Neosartorya* genera have undergone substantial research and are well known for the bioactive chemicals that they may produce (Durães et al, 2021). For instance, gastrointestinal issues and tumours have been treated with basidiomycetes such *Piptoporus betulinus, Inonotus obliquus, Boletus edulis*, and *Fome fomentarius* (Adeoyo et al, 2019).

Despite this fact more and more novel marine natural compounds originating from fungi have been discovered over the past three decades, only the broad-spectrum cephalosporin C can be traced back to marine fungi. The early 1940s saw the isolation of cephalosporins C. from a strain of *Acremonium chrysogenum* found in a sample taken from sewage water along the Sardinian coast. This led to the discovery of cephalosporin C (Gomes et al, 2021). Since then, bioprospecting of marine fungi has made it possible to identify number of metabolites with antibacterial effects, many of which are proving to be active against multi-drug resistant strains. Available data also

suggests that some of these metabolites might boost the pharmaceutical firepower towards some of the bacterial pathogens listed as a priority by the World Health Organisation (Gomes et al, 2021).

1.2.5 ANTI- INFECTIVE COMPOUNDS FOUND IN FUNGI:

Polyketides, terpenes, peptides, alkaloids, shikimates, and sugars were the six main chemical classes that divided the structures of marine natural products into (Santos et al, 2014)

1) Polyketides

Compounds of this class are frequently highly oxygenated; they include macrolides, poly-ethers, polyols, and aromatics. Polyketides constitute a highly diverse class of compounds both in terms of structure and biological activities; their biological activities are diverse and include antibiotic, anticancer, antifungal, antiparasitic, immunosuppressive, and neurotoxic activities (Santos et al, 2014). Due to their great commercial value, polyketides are a significant class of secondary metabolites that have a significant impact on the pharmaceutical business. These naturally occurring substances are used in human therapy as immunosuppressants, antibiotics, and antifungals. Famous examples of this class include the macrolide antibiotics amphotericin, nystatin, and rapamycin (Abad et al, 2015). Example: a polyketide that was discovered in the fungus *Penicillium chrysogenum* and has a mild cytotoxic effect on a cell line derived from human hepatocellular liver cancer (Santos et al, 2014).

2) Terpenes

The terpenoids are one of the more often reported and identified marine natural compounds to date. The isoprene units are typically linked head-to-tail during the formation of terpenoids, and the number of units incorporated into a specific

unsaturated hydrocarbon terpenoid determines how these compounds are classified: monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25), and meroterpenoids (C26) (Abad et al, 2015). To maintain their existence, marine species create terpenes as primary and secondary metabolites. These substances originate from a five-carbon isoprene structure and can be further separated into biogenetic classes such as monoterpenes, sesquiterpenes, diterpenes, sesterpenes, triterpenes (steroids), and tetra-terpenes (carotenoids) depending on the arrangement of units (Santos et al, 2014).

3) Peptides

Bioactive peptides are certain protein fragments that, although inactive inside their parent proteins, when released have beneficial effects on human health. In addition to serving as sources of nitrogen and amino acids, they may also have a variety of physiological effects on the body, including opioid and immunomodulatory action as well as antimicrobial, antithrombotic, and antihypertensive effects (Santos et al, 2014). Antimicrobial peptides and proteins are abundant in the marine environment, which attests to their general significance in the development of most species' defensive mechanisms. They are also known as "natural antibiotics" since they are thought to be a component of the humoral natural defence of invertebrates against infections (Abad et al, 2015).

4) Alkaloids

"Cyclic organic compounds containing nitrogen in a negative oxidation state that are of limited distribution among living organisms" (Santos et al, 2014). Alkaloids with distinctive chemical properties and strong chemical activity are known to be abundant in marine animals and microbes, which raises the possibility that they could be useful

as lead structures for the creation of novel medications. Several of these substances have antibacterial and antiviral characteristics that could have therapeutic effects (Abad et al, 2015).

5) Shikimates

During the production of aromatic amino acids some bacteria, fungi, plants produce shikimate (Santos et al, 2014).

6) Sugars

Polysaccharides, which make up majority of carbohydrates, can be separated from marine organisms. In the biotechnology, pharmaceutical, and chemical sectors, polysaccharides can be used in several ways., including the creation of gels, drug delivery mechanisms, tissue engineering, wound healing, and blood dialysis membranes (Santos et al, 2014). The field of marine polysaccharides is continually changing due to the large diversity of polysaccharides that can be taken from marine plants and animal life or synthesised by marine fungi. Interesting antibacterial and antiviral action was demonstrated by some of these marine polysaccharides (Abad et al, 2015).

1.2.6 METABOLITES GENERATED FROM MARINE DERIVED FUNGI ISOLATED FROM VARIOUS SOURCES:

SEA ANIMALS

1) SPONGE

The fungus *Arthrinium arundinis* ZSDS1-F3, which was collected from sponge (Xisha Islands, China), produced two novel 4-hydroxy-2-pyridone alkaloids called

arthpyrones (1-2). The K562, A549, Huh-7, H1975, MCF-7, U937, BGC823, HL60, Hela, and MOLT-4 cell lines were significantly cytotoxic to compounds 1 and 2 in vitro, with IC50 values ranging from 0.24 to 45 M. Additionally, compound 2 significantly inhibited AchE (IC50 = 0.81 M), whereas compound 1 only slightly did so (IC50 = 47 M) (Jin et al, 2016)

2) CORAL

The South China Sea's *Echinogorgia aurantiaca* gorgonian corals are where the fungus *Aspergillus terreus* SCSGAF0162 was discovered. Under solid-state fermentation of rice, three lactones, including three territrem derivatives (15–17) and a derivative of butyrolactone (18), were recovered from the fungus. Among them, compounds 15 and 16 had potent acetylcholinesterase inhibitory action, with IC50 values of 4.2 0.6 and 4.5 0.6 M, respectively. Compounds 17 and 18 were initially reported to have antiviral activity against HSV-1 with IC50 values of 16.4 0.6 and 21.8 0.8 g mL-1, respectively. Additionally, compound 15 demonstrated clear antifouling efficacy, with EC50 values of 12.9 0.5 g mL-1 towards the barnacle. *Balanus Amphitrite* larvae (Jin et al, 2016).

SEA WATER

Trichoderma sp. strain MF106, a marine fungus isolated from the Greenland Seas, yielded the unique pyridone trichodin A (143). With an IC50 value of 24 M, compound 143 demonstrated antibacterial activity against *Staphylococcus epidermidis* (Jin et al, 2016). Seawater from the Zhanjiang Mangrove National Nature Reserve in Guangdong Province, China, was used to isolate the fungus *Penicillium* 303#. Compounds 144–146, three novel metabolites, were recovered from the fungal fermentation medium. Those substances had modest to moderate cytotoxic effects on MDA.-MB-435 (Jin et al, 2016).

ALGA

Iscocyanthisterol (121) was produced by the *Aspergillus ustus* cf-42 strain, which was isolated from the fresh tissue of the sea green alga *C.fragile*. At 30 mg/disc, compound 121 only mildly inhibited the growth of S. aureus and E. coli (inhibitory diameters, 5.7 and 6.7 mm, respectively) (Jin et al, 2016).

1.2.7 ANTIMICROBIAL RESISTANCE

Health services must deal with the issue of existing dangerous bacteria and fungi becoming resistant to commercial medications, which has gained global attention. This scenario has been favoured by a variety of variables, including the broad and frequently improper use of antibiotics, poor hygienic conditions, constant movement of travellers, a growth in the number of immunocompromised patients, and a delay in infection detection. As a result, a thorough search for new, efficient antimicrobial drugs is required, which is made easier by investigating novel niches and ecosystems (Santos et al, 2015). Some antibiotics are also referred to as narrow spectrum because they exclusively function against Gram-positive or Gram-negative bacteria, as opposed to broad-spectrum antibiotics, which simultaneously act against Grampositive and Gram-negative bacteria. Although some substances may be predominantly effective against Gram-positive bacteria, they may also inhibit some Gram-negatives, therefore this distinction is not always absolute (Giguère et al, 2013).

The search for novel chemicals that not only have antimicrobial activity but also have ability to take up that activity and reverse antibiotic resistance is one of the key quests going on right now (Durães et al, 2021).

Antimicrobial resistance is a serious problem that prevents diseases from being treated effectively. Researchers are currently looking for powerful new medications from

unusual natural sources, which will significantly support and enhance human health. Some strains of bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*, have been reported to be resistant to antibiotics. This report revealed that antibacterial resistance jeopardises the effective use of medications in the prevention and treatment of various infections brought on by microorganisms (Adeoyo et al, 2019).

The search for new antimicrobials as well as substances that can stop resistance mechanisms and be combined with effective antimicrobial medications has been continuing. There is research done by Durães et al (2021) which aimed on capacity to block the bacterial efflux pumps as well as the antimicrobial-related processes, like preventing the growth of biofilms and quorum sensing. By using marine derived fungi. The NIH/3T3 mouse fibroblast cell line was used to assess the cytotoxicity of these substances. The findings point to the possibility of marine-derived fungal metabolites as a source of chemicals that could reverse antimicrobial resistance and act as an inspiration for the synthesis of new antimicrobial medications.

Study done by Bhimba et al (2012) isolated a foliar fungi from mangroves namely *Rhizophora mucronata, Avicenna officialis* and *Avicenna marina.* The ethyl acetate extract had the strongest antibacterial activity, which was followed by tests of various concentrations against bacterial pathogens and anticancer activities for Hep2 and MCF7 cell lines in vitro.

Recently work by Ifatul et al (2016) focused on testing the 4 different marine fungal isolates from Pulau Redang and Pulau Payar Marine Parks, Malaysia for antibacterial activity against *Salmonella typhi, Listeria monocytogenes, Staphylococcus aureus,*

and Escherichia coli. Fungi were inoculated in broth to create crude extracts, which were then tested for antibacterial activity.

1.2.8 AIMS AND OBJECTIVES

The need for new drugs is essential in recent times as pathogens have been developing resistance mechanism to protect themselves from antimicrobial activities. Marine-derived fungi are looked upon as a good source for antimicrobial activity. Hence to know more about them, the present study was planned with the aim to find a fungal isolate that will have the potential of antimicrobial activity against majority of pathogenic bacteria. keeping this in mind, the following objectives were put forth:

1) To isolate different fungi from sea water.
2) To identify the fungi based on cellular morphology.
3) To test the fungi for antimicrobial properties against 5 different species of marinederived human pathogenic bacteria.

CHAPTER 2

MATERIASL AND METHODS

2.1 SITES OF COLLECTION

Two estuaries i.e., Mandovi and Zuari, were chosen as collection site. Mandovi river is located at 15º 15' to 15º 40" N and "73º 15' to 73º 45" E. Zuari river is located at 15° 24' 59.99" N and 73° 53' 59.99" E. Seawater was collected in October 2022.

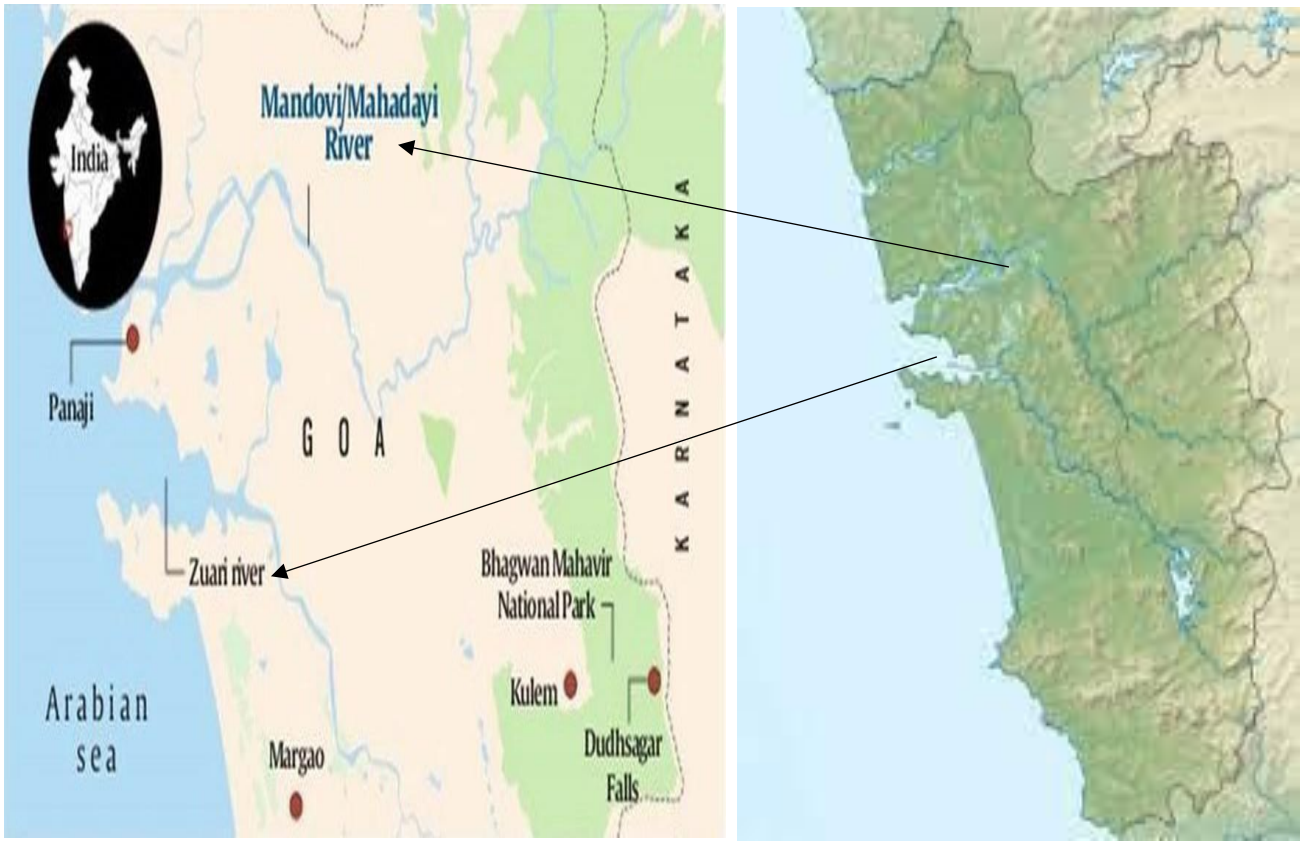

Fig 2.1: Maps showing Mandovi and Zuari River.

2.2 COLLECTION AND PREPARATION

The collection and preparation were done using standard protocol. Care was taken that each sample was handled in a proper manner to minimize handling error.

Water samples were collected in the month of October and November. One day prior to collection of water different types of media for the isolation of fungi were prepared.

For isolation of fungi 2 different media were used, they were Potato dextrose agar (PDA, HiMedia) and Malt extract agar (MEA, HiMedia).

All the apparatus to be used were autoclaved at 121°C 15 PSI for 20 min. Media were also autoclaved and then plates were poured in laminar air flow to maintain sterilized zone. Water sample from the selected site were brought one day prior to autoclave it and use it as a diluent while making dilutions.

2.3 ISOLATION OF CULTURE

To isolate the fungal cultures dilutions were made using sea water as the diluent. Dilutions was made up-to 10^{-5} using sterile seawater.

Before spread plating on MEA and PDA for fungus, antibiotics were spread plated on these media to inhibit the growth of bacteria (ampicillin 5µg/ml and chloramphenicol 5µg/ml). Once this antibiotic dried than 0.1ml of sample was spread plated in laminar air flow. Plates were incubated at room temperature for 3-4 days for fungal growth.

Plates were monitored every day for growth. After the incubation period of 3-4 days fungal growth was obtained on the plates and each colony was sub-cultured on separate MEA plates.

2.4 TESTING FOR ANTIMICROBIAL ACTIVITIES

Fungal isolates were sub-cultured on MEA or MEB and incubated at RT till visible growth. These were sub-cultured again in broth for 4-5 days. Antimicrobial activity was carried out after that by 2 different methods.

1) Agar plug diffusion assay (Marcellano et al, 2017). 2) Agar well diffusion assay (Guven et al, 2005)

2.5 TEST MICROORGANISM

Five different types of bacteria were selected namely *E. coli, Salmonella, Shigella, Vibrio cholerae* and *Vibrio parahaemolyticus* which were maintained on Zobell Marine Agar (ZMA) slants. These were previously isolated from the estuarine waters by plating on selective media. Test bacteria were also sub cultured one day prior to the experiment on ZMA plates and ZMA plates were prepared one day in advance to use it for experiment.

5 ml suspension of 24 hours old bacterial culture was prepared by adding autoclaved seawater and was vortexed for 1 min.

1) AGAR PLUG DIFFUSION ASSAY

Hundred µl of each bacterial suspension was spread plated on ZMA and was kept for drying. Once it was dried than, agar plugs with diameter approximately 8mm were plugged from the MEA plate having actively growing fungal culture and were, transferred to the ZMA plate having bacterial test culture. Plates were than properly sealed with paraffin tape and were incubated for 24 hours at room temperature. After incubation period plates were checked for zone of inhibition (ZOI) and that was measured using a ruler.

2) AGAR WELL DIFFUSION ASSAY

Fungal cultures were sub-cultured in Malt extract broth (MEB) approximately 5 days in advance to get growing culture. Suspension of 24 hours old bacterial culture in sterile sea water was prepared and hundred µl of suspension was spread plated on ZMA plates.

Allowing them to dry. after drying wells was plugged from those ZMA plates of approximately 8mm in diameter.

Broth having growing fungal culture was filtered and hundred µl of that broth was put in that wells.

Plates were systematically sealed with paraffin tape and was incubated for 24 hours at room temperature.

After 24 hours plates were checked for zone of inhibition and it was measured.

2.6 IDENTIFICATION OF FUNGAL CULTURE

Fungal cultures were sub-cultured in Malt extract broth and was incubated for 4-5 days. Small part of fungal colony was plucked with the help of sterile forceps and was placed on clean slide. Fixing it with Lugols iodine. Slide was covered with cover slip making sure no air bubbles were trapped inside and was mounted under microscope at 10x and 40x observation was recorded.

Macroscopic observation was also done by noting down its morphological characteristics.

2.7 CONFIRMATION OF BACTERIAL CULTURE

Bacteria was first Gram stained and was observed under microscope at 100x oil immersion lens. Several biochemical tests were performed to confirm the bacterial culture. By referring to Bergey's manual.

a) Motility test (*Salmonella, shigella*)

100ml Nutrient agar (NA 31g in 1000ml) was prepared by adding 0.4g agar-agar autoclaved at 121°C. 24 hours old bacterial culture was stabbed inside the agar with the help of sterile pointed nichrome loop. Incubated for 24 hours at room temperature and was checked if agar was diffused which indicated positive result.

b) Indole test (*Salmonella, Shigella, E. coli*)

1% L- Tryptophan was added to Nutrient Broth and was autoclaved at 121°C. bacteria was inoculated in broth and was incubated for 24 hours at room temperature. Next day 2-3 drops of kovac's reagent were added. Pink ring formation indicated the positive result.

c) Citrate test (*E. coli*)

Slants was prepared of Simmons citrate agar and bacteria was streaked on it. If colour of media changes from green to blue would indicate positive result.

d) Urease test (*Salmonella, Shigella*)

Slants was prepared of Urease agar. Bacteria was streaked on it. After incubating for 24 hours at room temperature if colour of media changes to pink it would indicate positive result.

e) Glucose fermentation test (*Vibrio cholera, Vibrio parahaemolyticus*)

Bacteria was grown on different agars like SS, TCBS, MacConkey's and morphological characteristics was noted down.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 ISOLATION OF FUNGAL CULTURES

In total 26 fungal cultures were isolated from Mandovi and Zuari estuaries. These were named as MnPz or ZnPz for Mandovi and Zuari estuary respectively. ‘n’ stands for the sampling site and ‘z’ stands for the plate number in which the isolate was obtained.

These were tentatively identified based on colony and cellular morphology as observed under microscope (Table R1 and R2).

Table R1: Colony characteristics of fungal isolates from Mandovi estuary.

ISOLATE NO	COLOUR	SPORU-LATING YES/NO	TEXTURE	SHAPE	ELEVATION	ISOLATE NAME
M3P5	White border with grey in middle	yes	filamentous	circular	raised	*Aspergillus sp.*
M3P4	Dull white	no	fuzzy	circular	raised	*Fusarium sp.*
M4P17	Dark green border light green middle	yes	powdery	irregular	flat	*Aspergillus sp.*
M1P1	Green in middle with white border	yes	dusky	irregular	raised	*Trichoderma sp.*
M4P7	Grey	yes	filamentous	circular	raised	*Chaetomium sp.*
M4P6	Dark Green	yes	powdery	circular	raised	*Cladosporium sp.*
M3P3	Yellow with white below	yes	powdery	irregular	raised	*Aspergillus sp.*
M5P5	Blackish	yes	filamentous	circular	raised	*Chaetomium sp.*

M1P2	Greenish	yes	dusky	irregular	raised	*Aspergillus sp.*

Table R2: Colony characteristics of fungal isolates from Zuari estuary.

ISOLATE NO	COLOUR	SPORULATING YES/NO	TEXTURE	SHAPE	ELEVATION	ISOLATE NAME
Z4P17 (a)	Green middle, yellow border	Yes	powdery	irregular	raised	*Penicillium sp.*
Z4P18	Green with white border	Yes	powdery	circular	raised	*Aspergillus sp.*
Z1P1	White	no	fluffy	circular	raised	*Fusarium sp.*
Z4P17 (b)	Dimorphic offwhite	no	Milky	irregular	flat	*Phytophthora sp.*
Z4P15	Green in between, yellow border	yes	dusky	irregular	raised	*Aspergillus sp.*
Z2P9	red	no	Filamentous, fluffy	circular	raised	*Fusarium sp.*
Z5P21	Grey with whiteish border	yes	fluffy	circular	raised	*Aspergillus sp.*
Z4P1	Blackish grey	yes	powdery	irregular	raised	*Fusarium sp.*
Z3P11	Dark green	yes	dusky	circular	raised	*Cladosporium sp.*
Z3P13	Green with dark green border	yes	powdery	irregular	raised	*Aspergillus sp.*
Z5P22	Pink spores with white border	yes	powdery	irregular	raised	*Aspergillus sp.*
Z1 P18	Off-white	no	filamentous	irregular	flat	Non sporulating, unidentified
Z3P12	Dark dull green	yes	fluffy	circular	raised	*Cladosporium sp.*
Z2P8	yellow	yes	powdery	irregular	raised	*Aspergillus sp.*
Z5P18	Yellow with green border	yes	powdery	irregular	raised	*Penicillium sp.*
Z1P25	Orange, white	no	filamentous	circular	raised	*Trichophyton sp.*

Z2P10	Pink sporulating, white border	yes	powdery	irregular	raised	*Aspergillus sp.*
Z3P14	Pink, white	no	filamentous	circular	raised	*Fusarium sp.*

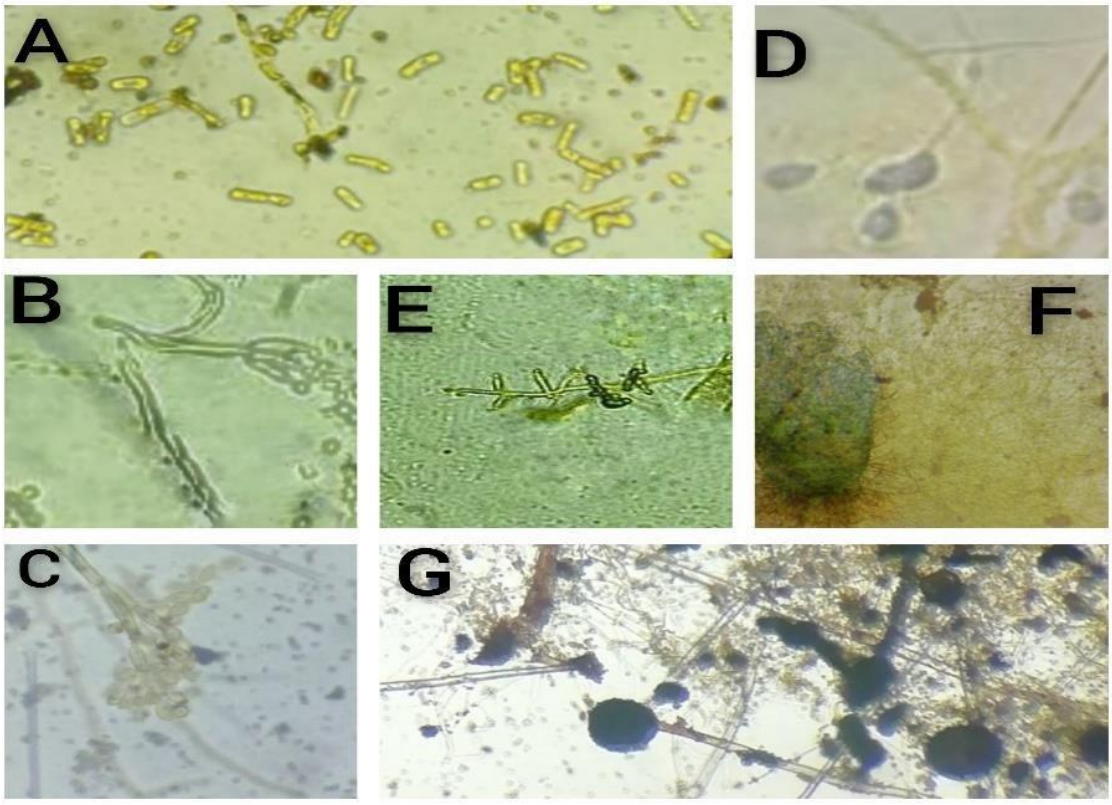

Fig 3.1 Cellular morphology under 10x objective of microscope. *Fusarium sp.* (Z1 P1) (A) *Penicillium sp.* (Z5 P18) (B) *Cladosporium sp.* (Z3 P11) (C) *Phytophthora sp.* (Z4P17 b) (D) *Trichoderma sp.* (M1P1) (E) *Chaetomium sp.* (M5P5) (F) *Aspergillus sp.* (M4P17) (G)

Among the total of 26 isolates, 2 isolates showed promising results against two bacteria (Table R3). These two isolates were M4P6 (Fig. 3.2) and Z1P18 (Fig. 3.3), one each from Mandovi and Zuari estuary. Isolate M4P6 showed average zone of inhibition of diameter 0.1 cm around the well of 1.2 cm against *Vibrio parahaemolyticus* and isolate Z1P18 showed average zone of inhibition of diameter 0.25 cm around the well of 1.2 cm against *Vibrio cholerae*. In addition to the above, some fungal isolates showed antifungal activities against other fungal isolates (Fig. 3.4).

Table R3: Fungal cultures test results against all 5 human pathogenic bacteria.

Plate name	*E. coli* Isolate 1	*V. parahaemolyticus* Isolate 2	*V. cholerae* Isolate 3	*Salmonella* Isolate 4	*Shigella* Isolate 5
M4P6	-	+	-	-	-
Z1P18	-	-	+	-	-

3.2 ZONE OF INHIBITION

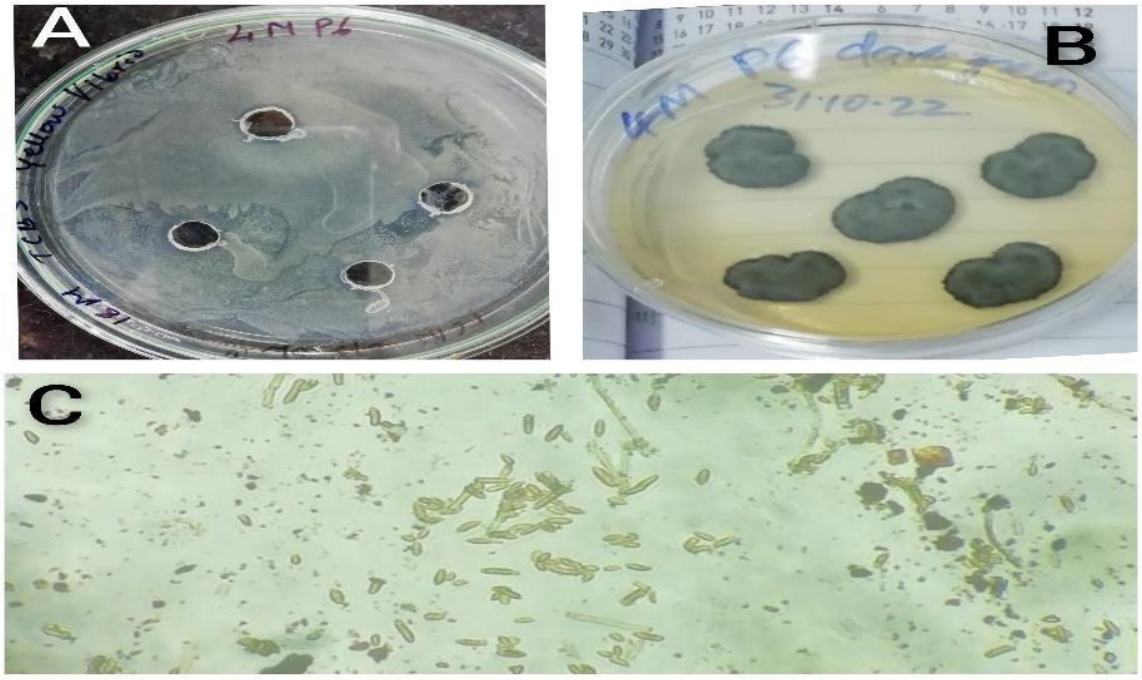

Fig. 3.2. Fungal isolate M4P6 from Mandovi estuary showing positive result against *V. parahaemolyticus* (A), M4P6 colony on MEA plate (B) and cellular morphology of the same under 10X objective of microscope (C).

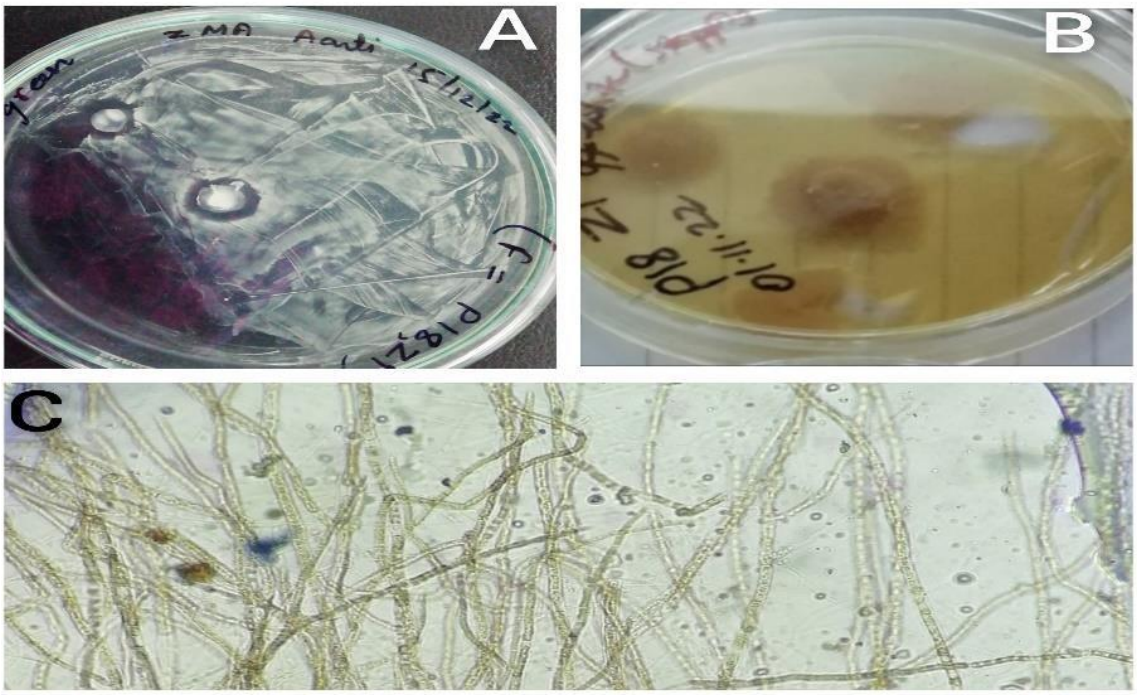

Fig. 3.3. Fungal isolate Z1P18 from Zuari estuary showing positive result against *V. cholerae* (A), Z1P18 colony on MEA plate (B) and cellular morphology of the same under 10x objective of microscope (C).

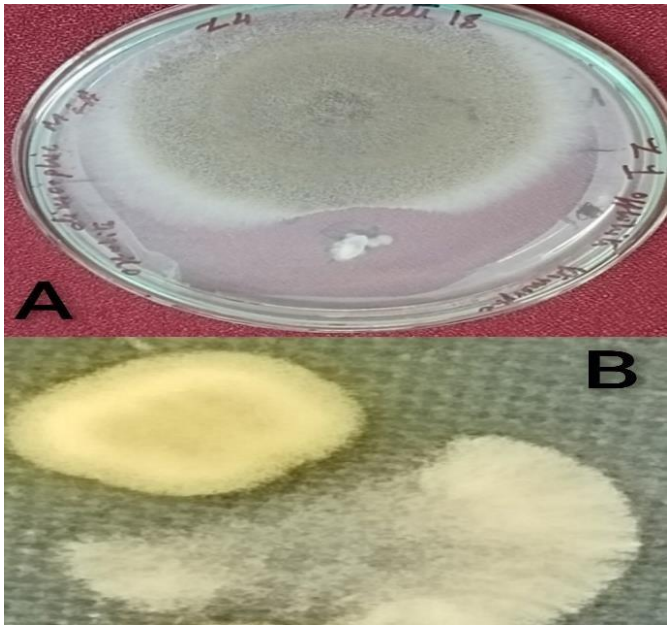

Fig 3.4. Isolates Z4P18 *Aspergillus sp.* showing antifungal activity against dimorphic fungal colony (A) and *Fusarium sp.* showing antifungal activity against *Aspergillus sp.* (B).

3.3 CONFIRMATORY TEST FOR BACTERIA

The bacterial isolates used in the study gave the following results according to Bergey's manual (Table R4, Fig. 3.5 and 3.6). Positive indole test indicated presence of *E. coli* (isolate 1). Negative urease production and negative citrate test with positive glucose fermentation indicated *Salmonella* (isolate 4) and negative glucose fermentation indicated Shigella (isolate5). Yellow colony on TCBS Agar indicated *V. cholerae* (isolate

3) and green colony on TCBS Agar indicated *V. parahaemolyticus* (isolate 2)

Table R4: Selected biochemical tests performed on the pathogenic bacteria used in this study.

Biochemical test	*E. coli* isolate 1	*V. parahaemolyticus* isolate 2	*V. cholerae* isolate 3	*Salmonella* isolate 4	*Shigella* isolate 5
Motility	+	-	-	+	-
Indole	+	-	-	-	-
Citrate	-	-	-	-	-
Urea	-	-	-	-	-
Glucose fermentation	+	+	+	+	-

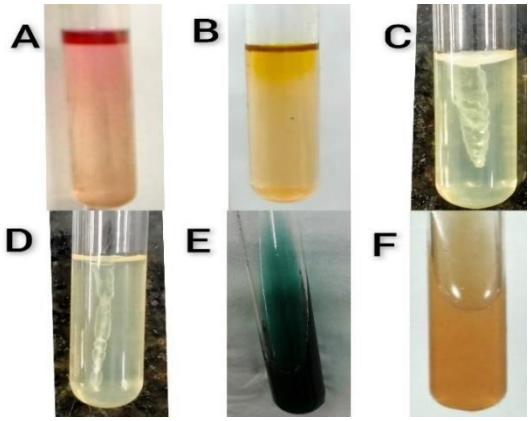

Fig 3.5 Photographs of a few biochemical tests such Indole test positive (a) and negative (b) as seen in isolates 1 and 4 respectively, Motility test positive (c) and negative (d) as seen in isolates 4 and 5 respectively,

Citrate test negative (e) in isolate 1, and Urease test negative (f) in isolate 4.

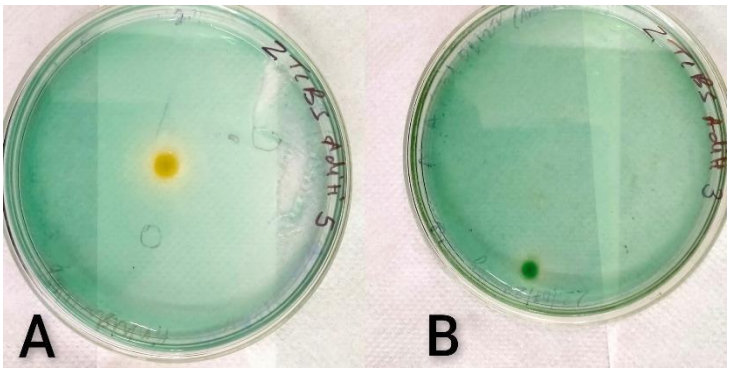

Fig 3.6 Showing bacterial colony on selective media (TCBS Agar) Yellow colony of *Vibrio Cholera* (A), Green colony of *Vibrio parahaemolyticus* (B).

3.4 DISCUSSION

Marine organisms and especially fungi have come to the attention of natural product chemists, despite this fact that terrestrial fungi have long been the primary source of the most significant antibiotics in human history (Mohseni et al, 2013; Priya et al, 2015; Rajabalaya & David 2020; Zhou et al. 2016). Due to the development of microbial resistance to the available antimicrobial drugs, microbial infections have now emerged as a substantial clinical hazard with significant associated morbidity and mortality. As a result, techniques for determining novel antimicrobial drugs and testing for antimicrobial susceptibility have been widely employed and are still being developed (Balouiri et al, 2016). Antimicrobial resistance is on the rise globally, necessitating a variety of strategies in the quest for new antibacterial drugs (Masota et al, 2021). To this, more focus should be driven to studies that involve identifying potential antimicrobial activity possessing fungal cultures. Numerous aquatic fungal isolates were tested against bacterial cultures in the present study. These numerous marine derived fungi were belonging to the species *Aspergillus sp. Chaetomium sp.*, *Penicillium sp.*, *Fusarium sp.*, *Cladosporium sp*. and *Trichoderma sp.* and were tested against the human pathogenic bacteria isolated from

estuarine waters namely *E.coli, Salmonella, Shigella, Vibrio parahaemolyticus,* and *Vibrio cholerae*. These bacteria were specifically isolated on selective media for the same. A few biochemical tests were carried out to confirm identity to some extent. However, molecular identification by 16S rDNA amplification and sequencing, which could have proved their identity further could not be carried out. Among the fungal isolates only 2 showed promising results so this study supports the theory that if we take more interest in finding antimicrobial activities of specifically the marine-derived fungi then there may be high possibilities that will find positive results. Fungi belonging to *Fusarium* sp., *Penicillium* sp., *Aspergillus* sp. etc. were found to possess antibacterial activity against many pathogenic bacteria in a study by Paria & Chakraborty (2019).

Most common methods to check the antibacterial activities are those which are used in these studies but according to Balouiri et al (2016) Time-kill test and flow cytofluorometric methods are suggested for more thorough investigation of an agent's antimicrobial effect. The latter two reveal the type of inhibitory effect (bactericidal or bacteriostatic), as well as whether it is time-dependent or concentration-dependent and how much cell damage the test microorganism sustains. Since these were not done in the present study, the type of inhibitory effect could not be deduced.

Although it has been said that the discovery of antibiotics is one of the greatest medical discoveries of the 20th century, our failure to develop a sustainable platform for antibiotic discovery has left us with one of the greatest liabilities, especially now in the 21st century. Number of targets for broad-spectrum medications restricts the number of antibiotic types (Hoffman 2020). As a result, the number of antibiotic classes will generally be matched by the number of resistance mechanisms. It is therefore unlikely to result in the production of new chemical classes of antibacterial by altering the genes linked to secondary

metabolism, notwithstanding the possibility of enhancing an existing scaffold which could be challenging in this 21st century.

CHAPTER 4

SUMMARY

4 SUMMARY

This research was done to identify potential marine-specific antimicrobial fungi. Different fungal cultures were isolated from the Mandovi and Zuari estuaries during October 2022, and their ability to suppress potential human pathogenic bacteria was evaluated. Malt extract agar and potato dextrose agar, which are used to cultivate fungi especially in the presence antibiotics to prevent bacterial development, were the medium utilised in this investigation to establish fungal cultures. Human pathogenic bacteria were maintained on Zobell Marine Agar after being isolated from selective

media such as Salmonella Shigella Agar, Thiosulfate-Citrate-Bile salts-Sucrose Agar, and MacConkey's Agar.

Antibacterial activity of the fungal isolates was examined by two separate techniques viz., the agar plug diffusion assay and the agar well diffusion assay. The 4-5 days old fungi were tested against 24 h old bacteria. Two fungal isolates viz., Cladosporium sp. M4P6 isolated from Mandovi and Unidentified non-sporulating Z1P18 isolated from Zuari estuary showed positive antibacterial activity against *V. parahaemolyticus* and *V. cholerae*, respectively.

It can be concluded that *Cladosporium sp.* and non-sporulating fungi, particularly of marine origin, may have good antimicrobial activities. Subsequent research in this area may be conducted, and it may be used as a significant antimicrobial agent in novel therapeutic drugs.

CHAPTER 5

APPENDIX

MEDIA COMPOSITION

1) Potato Dextrose Agar (PDA)

7.8g of PDA (HiMedia) in 100ml sea water.

2) Malt Extract Agar (MEA)

3g of MEA (HiMedia) in 100ml distilled water.

3) Zobell Marine Agar (ZMA)

5.25g of ZMA (HiMedia) in 100ml distilled water.

4) Nutrient Agar (NA)

3.1g NA (HiMedia) in 100ml of distilled water.

Add 1.5g of tryptophan for Indole test.

5) Urease Agar

2.52g of Urease agar (HiMedia) in 100ml distilled water.

6) Simmons Citrate Agar

2.24g of Simmons Citrate Agar (HiMedia) in 100ml of distilled water.

7) TCBS Agar

8.9g of TCBS Agar (HiMedia) in 100ml of distilled water.

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DISTRIBUTION OF BACTERIA AND FUNGI IN THE COASTAL WATERS OF GOA.

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Abstract: Goa being a tourist destination, the health of its water bodies and beaches is important for recreational activities. The presence of bacteria that indicate sewage contamination such as faecal coliforms have been reported at a few instances in the monsoonal estuaries of Goa. The present study is carried out to examine if the presence of different bacteria would fall in support of the previous studies or not. Different locations in the North Goa district were chosen for this study. Seawater samples were plated on various nutrient media such as Zobell Marine Agar, Thiosulphate Citrate Bile salts Sucrose Agar (TCBS), Salmonella-Shigella Agar and Mac Conkey's Agar to check for the growth of bacteria, and on Malt Extract Agar and Potato Dextrose Agar for the growth of fungi. Vibrio-like colonies were found to grow after plating most of the samples. Coliforms were found only in a few samples and their numbers were within the permissible limits. Surface waters of the Mandovi and Zuari estuary comprised more number of human pathogenic bacteria than the beach seawater samples. Different types of fungi were obtained from the beach seawater than the estuarine seawater. The fungi could be responsible for restricting the human pathogenic bacterial load.

INTRODUCTION

- Goa: popularly known as 'Pearl of the orient' and tourist paradise.
- Water sports for tourists include surfing, water-scooter, scuba diving, etc.
- Rapid and uncontrolled tourism development in coastal areas has exposed their fragile ecosystem to an ever-increasing risk of environmental degradation.
- Negative impacts of tourism that lead to environmental degradation include: crowding, poor sewage disposal, boat/trawler-generated waste, over-fishing, etc.
- Waste disposal in waters shoot up the nutrient levels, and consequently the autochthonous microbial populations, and expose them to allochthonous microflora.
- Aquatic microbiota: an important factor in the sustainability of the natural water ecosystems.
- Hence it is significant to study the distribution of microorganisms in these waters.

METHOD

Collection of surface seawater

Making dilutions up to 10-5 using sterile seawater (collected in advance from far-away location) as diluent

Spread plating 0.1 mL sample of
10^-3, 10^-4 and 10^-5 dilutions on Zobell Marine Agar (ZMA),
10^-4, 10^-5 and 10^-6 on thiosulphate Citrate Bile salts Sucrose Agar (TCBS), Salmonella Shigella Agar (SS), Mac Conkey's agar,
Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA). MEA and PDA supplemented with antibiotics (ampicillin 3µg/ml and chloramphenicol 3µg/ml).

Counting of colonies and subculturing for identification

To carry out biochemical assays.

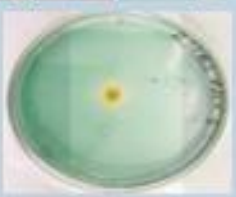
PLATE PHOTOS

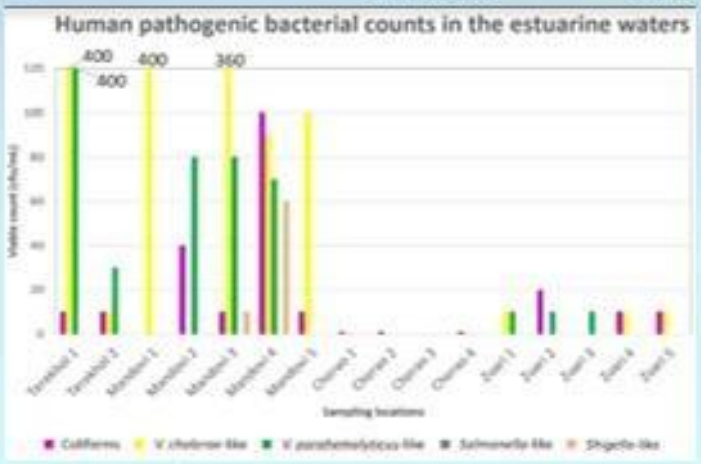

MEA Plate


MEA Plate


ZMA Plate


ZMA Plate


TCBS Plate



HARMFULL EFFECTS

Exposure to microbially polluted recreational waters

- Human health risk
- recreational waterborne illness (RWI)
- gastrointestinal illnesses, neurological infections, skin infections, earaches, eye infections, and respiratory infections

SIGNIFICANCE OF ANTIBACTERIAL ACTIVITY

- Most important characteristics of medical textiles
- Search for new, effective bactericidal materials is significant for combatting drug resistance.
- Fungi naturally produce antibiotics to kill or inhibit the growth of bacteria.
- Fungi are well known for their production of substances with antimicrobial activities, several which have formed the basis for development of new clinically important antimicrobial agents.

ANTIBACTERIAL ACTIVITY

Experiment is still in progress, antibacterial activities are yet to be checked.

ANTIFUNGAL ACTIVITY

SUMMARY

Sea water sampling from different locations in the estuaries of Goa showed the presence of human pathogenic bacteria and various fungi. Numbers of the former were within permissible limits during the sampling time studied. More work in these locations will shed more light on the prevalence of these bacteria in the waters. Different fungi obtained seem to be promising for antifungal activity, hence this activity and their antibacterial profile will be further tested.

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This is to certify that Prof/ Dr / Shri / Smt ...Aarti Satuse.....of
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entitled Distribution of bacteria & fungi in the Coastal waters of Goa.
during the ASM seminar on Microbial Diversity & Ecology - Tropics to the Polar Regions, held at National Centre for Polar and Ocean Research, Goa in association with American Society for Microbiology, CUSAT - NCPOR Centre for Polar Science and Goa University on 09th November 2022.

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