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ISOLATION AND SCREENING OF MARINE BACTERIA FOR THEIR POTENTIAL ANTIMICROBIAL PROPERTIES

Short title: Antimicrobial Properties of Marine Bacteria

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Abstract: Marine microbiology is gaining popularity in various parts of the world, with a distinct concentration on bioactive compounds. The marine biological miscellany has immense potential for the production of new natural products, many of which are promising prospects for the biomedical sector's development. Antimicrobials are derived in major part from terrestrial microorganisms, although these species are still being investigated extensively, and the number of novel compounds discovered by terrestrial microorganisms is decreasing. The ecology of marine microorganisms, on the other hand, is remain unclear, which is likely why scientists and ecologists have paid little attention to these organisms for many years. As they flourish in a variety of ecological stresses, temperatures, and food sources, these marine species acquire specific adaption mechanisms. This can encompass the struggle for habitat, predator avoidance, the ability to breed effectively, and a variety of other defense processes that aren't well understood. The evolution/production of exceptional and normal bioactive metabolites, which can be useful to humans in a variety of ways, is one such result of these adaptations. As a result, the goal of this study was to identify and screen the antimicrobial metabolite producing marine bacteria from the Krishnapatnam area of Andhra Pradesh, India. Five marine bacteria with significant antibacterial and antifungal activities have been successfully isolated.

Index Terms **– Marine Bacteria, Metabolites, Antimicrobial properties, Multidrug resistance.**

I. INTRODUCTION

The marine habitat is rich in biological and chemical diversity, with almost 300,000 species discovered and described in the oceans, which cover around 71 % of the earth's surface area (361 million square kilometres) [1,2]. This number, however, is believed to be a small percentage of the total number of species yet to be discovered and described [3]. Moreover, microbial secondary metabolites have received considerable attention due to their useful biological activities, particularly in the positive effects of human health [4]. The emergence of novel antibiotic resistance has prompted in-depth discussions on the next source of new chemical entities that can face the issue of continuously evolving drug resistance [5]. Despite significant development in the disciplines of chemical synthesis and engineered biosynthesis of antimicrobial chemicals, nature remains the most abundant and adaptable source of novel antibiotics. The marine environment, which represents approximately half of the global biodiversity, contains a rich source of structurally diverse and biologically active metabolites [3-5]. Researchers studying marine natural products have shifted their focus from macro-organisms including algae, sponges, ascidians, and soft corals to marine microorganisms over the last 2 decades. Marine microorganisms have recently garnered considerable attention as potential sources of physiologically active secondary metabolites for the creation of novel pharmaceuticals. Antimicrobial, cytotoxic, anticancer, anti-diabetic, anti-fungal, anti-coagulant, anti-inflammatory, and other pharmacological actions have been demonstrated in products derived from marine organisms [6-10]. In terms of antimicrobial capabilities, it is thought that the marine environment can provide new leads against pathogenic bacteria that are evolving and developing resistance to present medications [11]. To combat and reverse the spread of antibiotic resistant bacteria, innovative anti-biotics are urgently needed. Therefore, this study was focused on the bacterial isolates of marine sediment soils collected from the Coastal areas of Krishnapatnam, Nellore District, Andhra Pradesh, India.

Sediment is a term that refers to any solid piece of inorganic or organic material. Ocean sediments include those found along the coast, such as pebbles and cobbles on the beach, seashell fragments, and sand and mud at the sea's bottom [12].

Continental and oceanic crust, volcanoes, bacteria, plants and animals, chemical processes, and outer space all contribute to the formation of marine sediments [13]. Granular sediments, which emerge from the fragmentation of inorganic or biological parent materials, are the first form of sediment (mud, silt and sand) [14]. Chemical sediment, on the other hand, is formed directly from dissolved components in sea water (fragments of lime stone and lime-stone like rocks) [15]. Despite the fact that microorganisms have been isolated from a range of marine sources, sediments continue to garner the most interest, possibly due to their resemblance to terrestrial soils and their ability to provide therapeutically beneficial metabolites. The largest supply of structurally distinctive, physiologically relevant non-ribosomal peptides, especially cyclopeptides derivatives, is marine microorganisms from granular sediments [16,17]. As a result, in this investigation, we used granular sediments to isolate marine bacteria and screen their metabolites for antibacterial properties.

II. METHODOLOGY

Collection mangrove soil sediment samples

Mangrove soil sediments were collected from Krishnapatnam coastal regions of region, Andhra Pradesh, India. Samples were collected at a depth of 5–10 cm and placed in sterile plastic bags. All samples were transported to the laboratory at Department of Zoology, Vikrama Simhapuri University Post-Graduation Centre, Kavali, India for further study.

Isolation of marine bacteria

Sediment samples were collected and prepared in ten-fold dilution series to 10^{-4} in sterile 70% seawater. Then, 0.1 (100 microliters) mL from each dilution was spread onto two different selective isolation media like Zobell marine agar (ZMA) media, and Luria bertin agar (LB) media. The plates were incubated at 37°C for 1- 5 days. Colonies of various marine bacteria were picked up and purified by streaking onto a ZMA and incubated at 37°C for 5 days. Isolated bacterial pure cultures were stored on Zobell agar slants for working stock and in 20% glycerol at -80°C for long-term storage.

Cultural Characteristics

Morphological features of the selected marine bacterial strains were characterized on ZMA after incubation at 37 °C for 1-5 days. The culture characters like pigmentation (color), appearance, size and microscopic characters like Grams staining, spore morphology and motility were observed. The color of substrate, and soluble pigment production were determined using the NBS/ISCC color system. The taxonomic identification like Utilization of carbon and nitrogen sources of isolated bacterial species were based on Nonomura's key and Bergey's Manual [18,19].

Metabolic extract preparation and screening for antimicrobial activity

The isolates were cultured in 50 mL of liquid media (Zobell media). Cultures were incubated for 1-5 days depending on their growth rate at 37 ^{0}C while shaking at 130 rpm. The isolates were left in the incubator shaker 3–4 days after their growth to release their metabolites in the culture media. An equal volume of methanol was added to the liquid cultures for cell lysis and shaking was continued (130 rpm, 1 h at room temperature). The broth was centrifuged (5000 rpm, 15 min at 37 ^{0}C), the supernatant was evaporated and the extract was dissolved in acetone. ZMA was used for testing antimicrobial properties of methanolic extract against bacteria (*Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*) and fungi (*Aspergillus niger* and *Candida albicans*) by disc method. Cycloheximide was used as positive control and the acetone was used as negative control. The antimicrobial activity was recorded by measuring the inhibition zone diameter (mm) after incubation at 37 ^{0}C for 24 h for bacteria and 48 h for fungi.

III. RESULTS AND DISCUSSION.

A total of 5 bacterial isolates (KPN 1 to KPN 5) were obtained in this study and the highest proportion of the strains were recovered by using ZMA followed by LB. The data about the sampling site of isolates and their colony characteristics were illustrated in Fig. 1. Table 1 summarizes the colony and biochemical characteristics of isolated marine bacteria. All the 5 bacterial isolates were grown in ZMA whereas isolate KPN 2 and 3 were not grown in LB media. This indicates that the ZMA is suitable for isolating marine bacterial. Colony appearance of the all isolates was circular and organisms are rod shaped with unipolar nature. All the isolates are Gram-^{Ve} and possess similar biochemical properties. Mucoid colonial characteristics are present in all five isolates, and these characteristics serve as selection criteria for bacteria that produce exopolysachharide [20]. When compared to other tested strains, KPN-5's secondary metabolite possesses the highest antibacterial properties (Table 2 & Fig. 2). KPN-5 has antibacterial characteristics that are almost identical to those of the positive control, Cycloheximide. The highest zone of inhibition with the KPN-5 metabolite was observed in Staphylococcus aureus (9.1 mm), followed by *Pseudimonas aeruginosa* (5.2 mm), *Bacillus subtilis* (3.2 mm), and *Escherichia coli* (2.3 mm). However, when it comes to antifungal characteristics, all of the isolates exceeded the positive control, cycloheximide (Table 3 & Fig. 3). Isolate, KPN-5 had the best antifungal efficacy against both Aspergillus niger (7.3 mm) and Candida sp (4.3 mm). Whereas in positive control, Cycloheximide inhibited the growth of *Aspergillus niger* with 2.3 mm and in case of *Candida* sp., the inhibition was 4.3 mm. The five isolated bacteria's morphophysio-biochemical features, including colony characters, were not identical, indicating that the bacteria's structure and functions were distinct. As a result, the findings of this study show that marine microorganisms found in coastal environments are a rich source of new antibiotics. Isolation, characterization, and study of marine bacteria are expected to aid in the discovery of novel bacterial species.

The exploration of the marine environment to provide numerous, often extremely complex chemical compounds is a current trend in drug development from natural sources [21]. Antimicrobial drug development continues to rely heavily on marine natural product research. This research can continue to provide new medicines for unmet medical needs given recent breakthroughs in selective organic synthesis, ribosome crystallography, chemical biology techniques for target elucidation, and novel methods for discovering new natural products. Many studies have shown that marine microorganisms develop unique secondary metabolites as a pathogenic invasion resistance mechanism [22-25]. Thus far, a large number of anti-microbial compounds have been discovered in only a few of the one million different microbial species; however, it is thought that searching for natural products produced by microorganisms could be a promising way to solve the problem of microbial resistance to some commonly used drugs and meet the immediate demand for highly effective, low toxicity, and anti-microbial compounds [26].

Although a large number of antimicrobial compounds have been extracted from marine sources, non-specific toxicity in humans, poorly understood biosynthetic processes, and low antimicrobial compound yields hinder research in this area [27]. However, future research should focus on optimizing fermentation conditions that have been found to show bioactivity in order to increase the yield of active substances synthesized by microbes, as well as searching for the regulatory gene in the biosynthesis pathway of antimicrobial compounds and using genetic engineering technology to increase the production of anti-infective substances. The urgent need for newer drugs to treat cancer, HIV, and other infectious diseases, as well as other disorders, necessitates a thorough investigation of all means of extracting fungal and bacterial metabolites from marine sources.

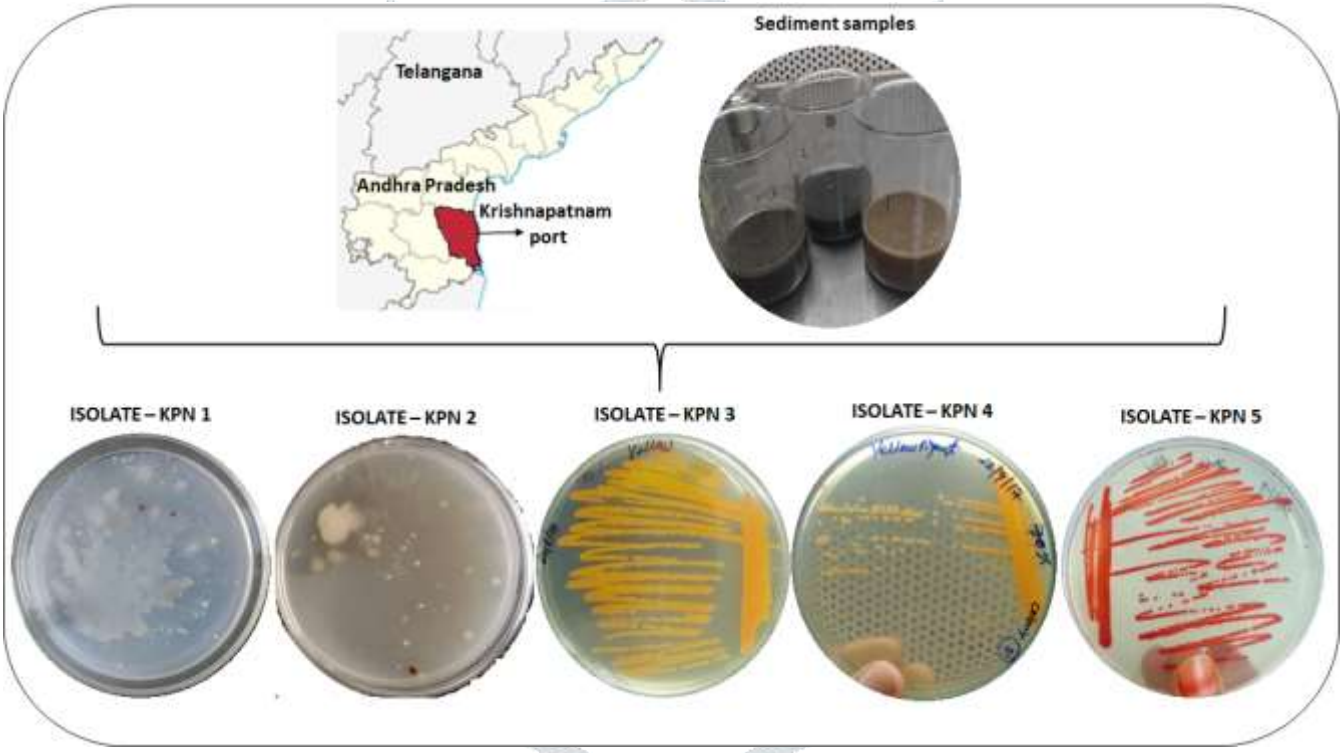

Fig. 1. Sampling site, collected sediments and the isolated marine bacteria

Table 1. Colony and biochemical characteristics of isolated marine bacteria

Character	KPN-1	KPN-2	KPN-3	KPN-4	KPN-5
Appearance	Circular, mucoid	Circular, mucoid	Circular, mucoid	Circular, mucoid	Circular, mucoid
Shape	Long rods	Short rods	Long rods	Long rods	Long rods
Motility	Unipolar	Unipolar	Unipolar	Unipolar	Unipolar
Colony Colour	White	Creamy	Yellow	Light Yellow	Red
Flagella	Single	Single	Single	Single	Single
Gram stain	-ve	-ve	-ve	-ve	-ve
Starch Hydrolysis	+	+	+	+	+
Gelatine Hydrolysis	+	+	+	+	+
Casein Hydrolysis	+	+	+	+	+
	Carbohydrate utilization				

Glucose	+	+	+	+	+
Mannitol	-	-	-	-	-
Sucrose	+	+	+	+	+
Growth media	ZMA and LB	ZMA	ZMA	ZMA and LB	ZMA and LB

Table 2. Antibacterial properties of marine bacterial isolates (KPN-1 to KPN-5 and C- Positive control)

S. No.	Name of Isolate	*Bacillus subtilis*	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
		Zone of inhibition (mm)			
1.	KPN-1	2.4 ± 0.12	2.7 ± 0.05	5.4 ± 0.04	3.5 ± 0.38
2.	KPN-2	2.1 ± 0.03	1.8 ± 0.02	4.3 ± 0.25	2.6 ± 0.27
3.	KPN-3	2.3 ± 0.12	3.7 ± 0.25	6.3 ± 0.24	4.5 ± 0.04
4.	KPN-4	1.7 ± 0.14	2.7 ± 0.14	7.5 ± 0.16	3.7 ± 0.24
5.	KPN-5	3.2 ± 0.11	2.3 ± 0.13	9.1 ± 0.78	5.2 ± 0.53
6.	Cycloheximide	**3.4 ± 0.21**	**2.4 ± 0.21**	**8.9 ± 0.33**	**7..1 ± 0.41**

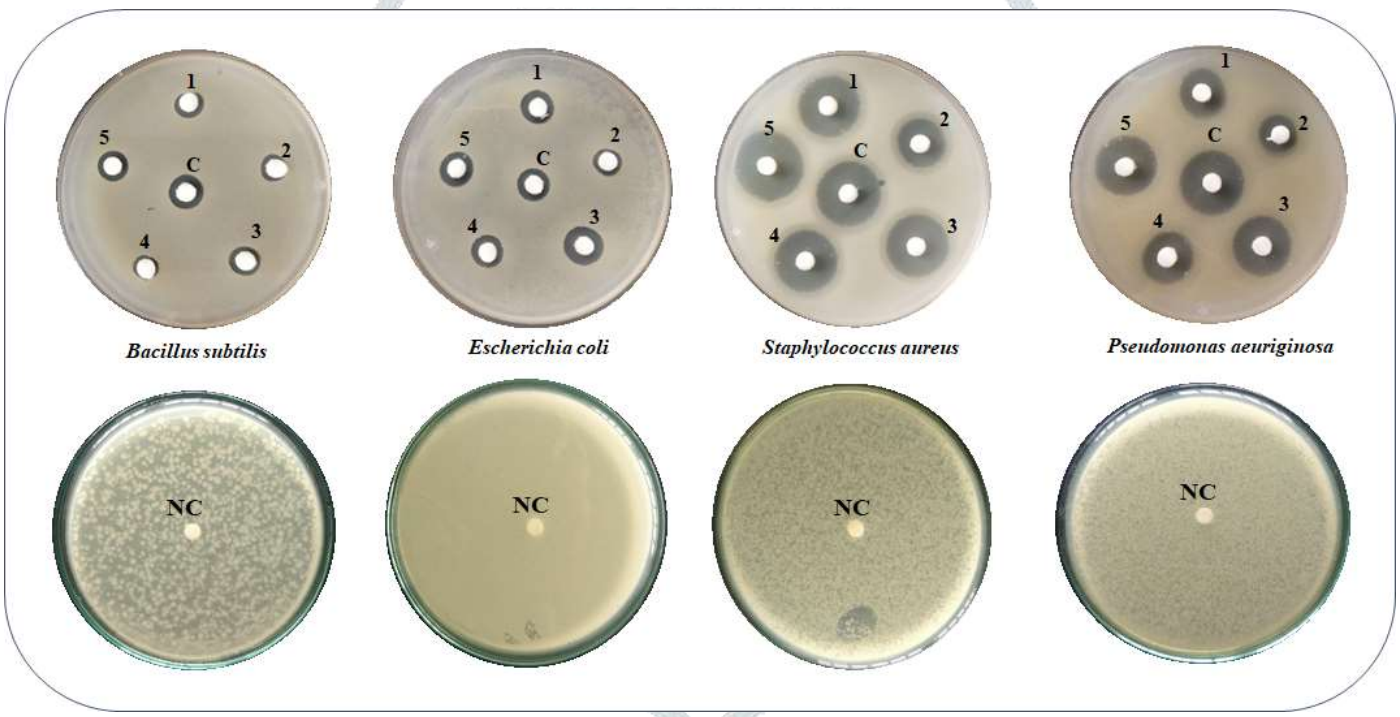

Fig. 2. Antibacterial properties of five marine isolates (KPN-1 to KPN-5 and C- Positive control (Cycloheximide); NC-Negative Control)

Table 3. Antifungal properties of Krishnapatnam isolates (KPN-1 to KPN-5 and C- Positive control)

S.No.	Name of Isolate	*Aspergillus niger*	Candida
		Zone of inhibition (nm)	
1.	KPN-1	2.7 ± 0.32	2.3 ± 0.38
2.	KPN-2	2.5 ± 0.51	2.3 ± 0.69
3.	KPN-3	4.5 ± 0.41	3.2 ± 0.38
4.	KPN-4	2.5 ± 0.69	2.1 ± 0.55
5.	KPN-5	7.3 ± 0.65	4.3 ± 0.49
6.	Cycloheximide	2.3 ± 0.98	**2.5 ± 0.38**

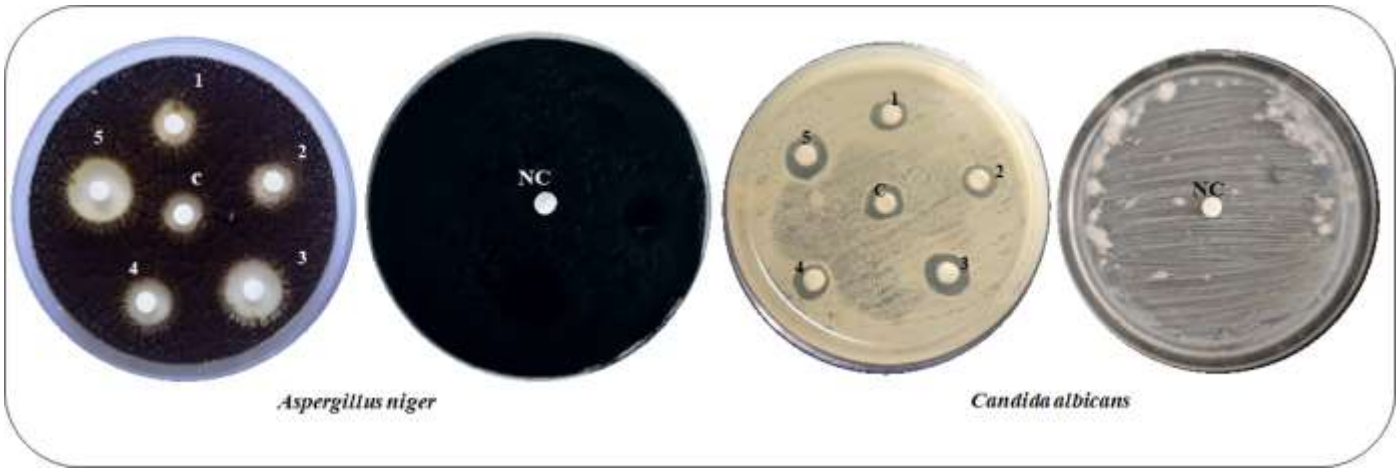

Fig. 3. Antifungal properties of five marine isolates (KPN-1 to KPN-5 and C- Positive control)

IV. SUMMARY AND CONCLUSION

Marine microbiology is gaining popularity in several parts of the world, with an emphasis on bioactive metabolites and the development of new natural products from marine biological miscellany. Many of these products have great potential for the biomedical sector's future. Antimicrobials are primarily produced from terrestrial microorganisms, albeit these species are currently under investigation and the number of unique compounds discovered by terrestrial microorganisms is decreasing. The ecology of marine microorganisms, on the other hand, is still unknown, which is likely why scientists and ecologists have ignored these species for so long. These marine species develop particular adaptation mechanisms as they thrive under a variety of ecological pressures, temperatures, and food sources. This can include the fight for habitat, predator avoidance, the ability to procreate effectively, and a range of other poorly understood defense mechanisms. One of these adaptations is the evolution/production of exceptional and normal bioactive metabolites, which can be valuable to humans in a variety of ways. We were successful in isolating 5 marine bacterial species in our investigation, and when their metabolites were analyzed, they showed antibacterial and antifungal activities. KPN-5 has the ability to demonstrate both antibacterial and antifungal characteristics when compared to all isolated organisms. As a result, additional work is being done to identify the KPN-5 at the species level using molecular methods. Furthermore, work is underway to purify the metabolite and describe it in order to determine its structure.

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