

Molecular Identification of Potent Antimicrobial Marine Fungi from Manginapudi Beach (Machilipatnam, India)

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Abstract: The primary objective of this study is to isolate and characterize marine fungi exhibiting antimicrobial properties, collected from Manginapudi Beach located in Machilipatnam along the Indian coast of the Bay of Bengal. The present investigation involved the isolation of marine fungi from seawater samples, with a 0.1 ml sample residue being introduced onto various fungal growth media. These isolated fungi were identified using the Internal Transcribed Spacer (ITS) region. Subsequently, phylogenetic analysis was conducted using MEGA-X to elucidate the relationships between the isolates and closely related species available in the NCBI-GenBank database. The crude extracts of these isolated marine fungi were then prepared and evaluated for their antimicrobial efficacy against various test organisms. Four fungal isolates, namely SS7, SS9, SS10, and SS11, exhibited a 100% similarity match and were conclusively identified as *Fusarium incarnatum* (SS7), *Hamigera insecticola* (SS9), *Talaromyces tratensis* (SS10), and *Neurospora crassa* (SS11). Notably, this study reports the first-time presence of *Acremonium persicinum*, *Fusarium equiseti*, *F. incarnatum*, *F. pernambutanum*, *Hamigera avellanea*, *H. insecticola*, and *Talaromyces tratensis* in the Manginapudi Beach region. Among the findings, the ethyl acetate extract of *T. tratensis* (SS10) demonstrated a significant zone of inhibition, measuring 22.1 ± 0.28 mm against *Candida albicans*. Similarly, *Acremonium persicinum* (SS6) exhibited the highest zone of inhibition (29.8 ± 0.26 mm) against *Escherichia coli*. The outcomes of this study strongly indicate that Manginapudi Beach serves as a valuable repository of marine fungi displaying significant antimicrobial potential. To fully substantiate and utilize this potential, additional investigation is required to isolate and thoroughly characterize the active components within these crude extracts. This in-depth analysis will offer conclusive evidence regarding the bioactive properties of marine fungi.

Keywords: Manginapudi Beach, Marine fungi, Internal Transcriber Spacer, Phylogenetic Analysis, Antimicrobial activity.

1. INTRODUCTION

Fungi are found everywhere and thrive in a diverse range of environments. At present, the world of fungi is a vast and diverse one, with an estimated range of 1.5 to 7.1 million known species [1]. These remarkable organisms occupy many habitats, from aquatic environments to terrestrial ecosystems. They can be found in water bodies, soils, and the atmosphere, and inhabit both the decaying remnants and the living tissues of plants and animals. Fungi display a remarkable adaptability, thriving across a spectrum of conditions, including those characterized by moderate temperatures ranging from 20 to 35°C.

One of the most intriguing aspects of fungi is their ability to prosper without the presence of chlorophyll, the green pigment found in plants that facilitates photosynthesis. This attribute allows fungi to thrive in conditions where sunlight is either abundant or virtually absent. However, despite the widespread presence and ecological importance of fungi, our understanding of their diversity, particularly in marine ecosystems, remains rather limited. In recent times, fungi have emerged as a compelling frontier in the exploration of biodiversity, posing a significant challenge to the field of microbial ecology. Understanding the vast array of fungal species and their ecological roles has gained paramount importance. To this end, traditional methods of visual identification have been complemented by cutting-edge molecular phylogenetic techniques [2]. These advanced methodologies offer a more precise and comprehensive

approach to the study of fungi, facilitating the exploration of their genetic relationships and the untapped potential they hold in various scientific, medical, and environmental applications.

In recent years, numerous molecular techniques have emerged for the identification of fungal species. One such approach is DNA barcoding, which involves the use of a standardized and highly variable DNA region consisting of 700 nucleotides. This specific DNA segment serves as a unique identifier for discerning different living organisms [3]. Cytochrome c oxidase I (COI), a 648-base pair (bp) mitochondrial gene, has been introduced and established as a standard barcode for animal species [4]. COI has proven to be highly effective in the identification of animal species over the years. Until recently, the Internal Transcribed Spacer (ITS) region of nuclear DNA (rDNA) was the most commonly sequenced region for both species and intra-species identification within the field of fungal taxonomy [5]. The ITS region is more variable than the other rDNA regions (small and large subunits). Consequently, the ITS region is currently widely recognized as the DNA barcode for fungi [6-7].

In recent years, there has been a growing focus on microbes inhabiting marine environments. These microorganisms have been compelled to develop biochemical and biophysical mechanisms, including symbiotic relationships and the synthesis of exceptionally potent bioactive compounds, in response to the substantial chemical and physical transformations in their surroundings. This adaptation enables them to thrive in the face of diverse environmental challenges [8]. For instance, the filamentous fungus *Aspergillus* stands out as a remarkable chemical producer of various secondary metabolites, carrying significant medical and commercial significance [9]. Notably, it is renowned for its synthesis of a range of compounds, including ochratoxin, aspergamides, asperloxins, viomellein, vioxanthin, circumdatins A-G, ochratoxins A and B, xanthomegnins, and 4-hydroxymellein [10]. The growing incidence of multiple drug resistance has hampered the development of new synthetic antimicrobial medicines, necessitating a search for alternate sources. Therefore, the present study aims to identify marine fungi at the morphological and molecular levels (ITS region) and determine whether the detected isolates have any antimicrobial potential that makes them useful as antimicrobial agents.

2. MATERIALS AND METHODS

2.1 Collection of marine water samples

In October 2019, seawater samples were taken from Manginapudi Beach, located on the Bay of Bengal shore in Machilipatnam, India (latitude: 16.24443, longitude: 81.24078) (Figure 1). By immersing the one-liter bottles in the seawater, the samples were obtained. The collected samples are transported to the Department of Biotechnology, Acharya Nagarjuna University, and processed immediately.

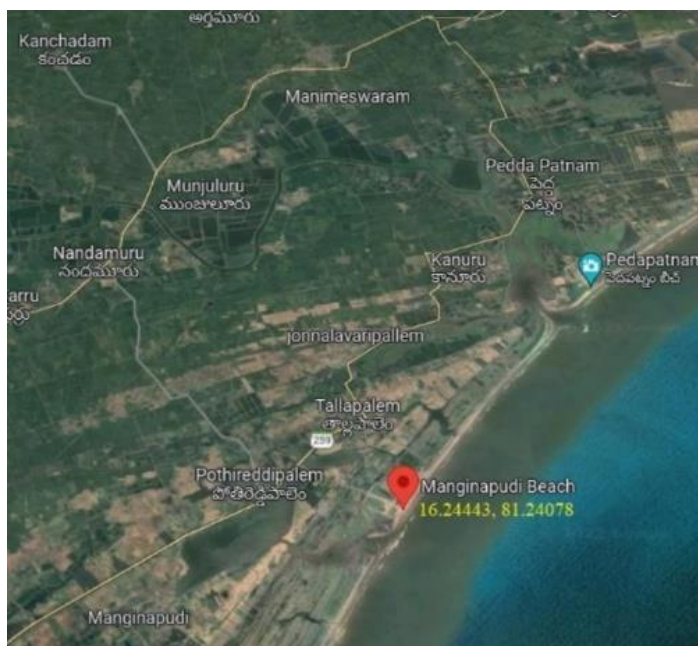


Figure 1: Location of the study area

2.2 Isolation of marine fungi from the water sample

The seawater samples were placed on a clean laboratory workbench, and 10 ml of each sample, in triplicate, were aseptically transferred into sterile centrifuge tubes. These samples were subsequently centrifuged for 10 minutes at a speed of 250 rpm to concentrate them. A small volume (0.1 ml) of the residual material from each sample was then evenly spread onto sterile Potato Dextrose Agar (PDA), Czapek Dox Agar (CZA), Sabouraud Dextrose Agar (SDA), and Rose Bengal Agar (RBA). These agar media were supplemented with 2% NaCl and 50 µg/L of chloramphenicol, as detailed in Table 1. The media were supplemented with chloramphenicol to inhibit the growth of bacteria, and to encourage the growth of fungi. The plates were incubated at room temperature (28°C) for 7 days [11].

2.3 Microscopic Identification

Colony characteristics, including texture, color, zonation, sporulation, and diameter, were employed to discern and identify the growth of fungi on each type of media. Purified isolates were produced by sub-culturing the distinct cultures on RBA slant for seven days at room temperature (28 °C). Microscopic features were observed using an electron microscope equipped with a 40X objective lens. To do this, a small portion of the fungal growth area was mounted on a clean, grease-free slide with a drop of lactophenol cotton blue, covered with a cover slip, and examined as per the standard procedures [12-13]. The characterization and identification of the isolates were conducted with reference to established literature sources [14-22]. The pure isolates were then preserved in Rose Bengal Agar (RBA) slants and refrigerated for subsequent verification.

Table 1: Formulation of various culture media utilized for fungi isolation

Ingredients (g/L)	PDA	CZA	SDA	RBA
Potatoes, infusion from	200.0	-	-	-
Dextrose	20.0	-	20.0	10.0
Sucrose	-	30.00	-	-
Sodium Nitrate	-	2.00	-	-
Dipotassium Phosphate	-	1.00	-	-
Magnesium Sulfate	-	0.50	-	-
Potassium Chloride	-	0.50	-	-
Ferrous Sulfate	-	0.01	-	-
Peptone	-	-	10.0	-
Papaic Digest of Soyabean Meal	-	-	-	5.0
Monopotassium Phosphate	-	-	-	1.0
Magnesium Sulphate	-	-	-	0.5
Rose Bengal	-	-	-	0.05
Chloramphenicol	50 ug/L	50 ug/L	50 ug/L	50 ug/L
NaCl	2%	2%	2%	2%
Agar	30.0	15.0	17.0	15.0
Final pH (at 25°C)	5.6 ± 0.2	7.3 ± 0.2	7.0 ± 0.2	7.2 ± 0.2

PDA-Potato dextrose agar; CZA- Czapek Dox Agar; SDA- Sabouraud Dextrose Agar; RBA-Rose Bengal Agar

2.4 Molecular characterization

2.4.1 Genomic DNA Extraction

Each individual isolate was initially cultivated on Rose Bengal Agar (RBA) at room temperature for a period of 5 days. Following this, each isolate was subsequently transferred to a 250 mL Erlenmeyer flask containing 100 mL of Rose Bengal broth and incubated for an additional 5 days. The mycelial mass of each isolate was then separated from the

broth by filtration, employing clean Whatman No. 5 filter paper. The mycelial mass was crushed with a mortar and pestle and subsequently transferred to Eppendorf tubes in preparation for DNA extraction. Genomic DNA extraction was carried out using the ZR Fungal DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions [23-25]. The quality and quantity of isolated DNA samples were evaluated on 0.8% agarose gel electrophoresis and Double beam UV-visible spectrophotometer at 260 and 280 nm.

2.4.2 PCR amplification of the ITS

In this study, specific primers for the internal transcribed spacer region (ITS) were employed, namely, Forward ITS 5 (5'GGAAGTAAAAGTCGTAACAAGG 3') and Reverse ITS 4 (5'TCCTCCGCTTATTGATATGC3'). These primers were obtained from Macrogen (South Korea) and used to amplify the ITS loci through PCR. The PCR amplification of the extracted DNA was carried out in a 20 μ L reaction mixture, consisting of 1 μ L of genomic DNA template, 0.2 μ L of DNA polymerase, 0.5 μ L of each forward and reverse primers, 1 μ L of dNTPs, and sterile double distilled water to reach a final volume of 20 μ L [26].

For the amplification of ITS loci, PCR reactions were performed in a thermal cycler (BIO-RAD) with the following program: an initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 1 minute. The reaction was completed with a final extension step at 72 °C for 10 minutes. Subsequently, the PCR products were separated by electrophoresis on 1% TAE agarose gels containing Ethidium Bromide (0.5 μ g/mL) and visualized under a UV transilluminator [27].

2.4.3 Sequencing and Phylogenetic Analysis

Fungal isolates were identified through DNA sequencing. The PCR products were sent to Macrogen in South Korea for sequencing. All single-band amplicons obtained were subjected to cleaning using the Exo-SAP method. Bands with molecular weights closely matching the targeted region were further purified using a Multiscreen filter plate from Millipore Corp. Following purification, the final PCR products were subjected to bi-directional Sanger's di-deoxy sequencing [28]. This sequencing process utilized the BigDye (R) Terminator v3.1 sequencing kit on an ABI PRISM 3730XL DNA analyzer, which featured 96 capillary types and was manufactured by Applied Biosystems Inc. in the USA. Sequencing was conducted in a 28 μ L reaction mixture, consisting of 4 μ L of each primer, 8 μ L of purified DNA, and 16 μ L of PCR water. The samples were sequenced using the Di-Deoxy Terminator sequencer.

2.4.4 Analysis of DNA Sequence Data

The chromatograms obtained from the sequencer were base-called using Phred. Base-calling is the process of assigning nucleobases to chromatograms. The sequences with a Phred quality score of more than 20 (Base call accuracy > 99%) were used for further analysis. Both the forward and reverse sequences underwent trimming and assembly, employing Sequencher, a software provided by Gene Codes Corporation based in Ann Arbor, Michigan, USA. Each sequencer project file (.spf) contained all the sequences for a particular species, and its consensus sequences were considered the representative sequences for that species. The contigs, formed by aligning forward and reverse sequences, were analyzed using BioEdit 7.2.5 and aligned using Clustal W in MEGA X.

The resulting sequences were compared with other related sequences through a MEGA BLAST search in GenBank, managed by the National Center for Biotechnology Information (NCBI). Following this comparison, the fungal isolates were assigned species names based on their similarity to representative sequences available in NCBI. The obtained sequences were deposited in GenBank, and each was assigned a unique accession number.

2.5 Fermentation and secondary metabolite extraction

The identified marine fungi were cultured in Rose Bengal Broth (RBB) by separately inoculating them into 250 mL Erlenmeyer flasks, each containing 100 mL of the medium. These flasks were then placed in an incubator at 27-28 °C for a duration of 14 days, with periodic shaking at 200 rpm. After the incubation period, the fermentation broths of the fungi were homogenized by the addition of 10% methanol. Metabolite extraction was carried out through a solvent extraction procedure utilizing ethyl acetate and methanol as the organic solvents.

To the filtrate, an equal volume of solvents was added, mixed thoroughly for 10 minutes, and left to stand for an additional five minutes, allowing the formation of two distinct, immiscible layers. The upper layer, containing the extracted compounds, was separated using a separating funnel. The solvent was subsequently evaporated, and the

resulting compound was dried in a rotary vacuum evaporator to obtain the crude metabolite. This crude extract was then dissolved in Dimethyl sulphoxide (DMSO) at a concentration of 1 mg/mL and stored at 4 °C [29-30].

2.6 Assessment of the antimicrobial potential of crude extracts from identified marine fungi

The antimicrobial activity of crude extracts obtained from identified marine fungi was assessed by testing their effect on a range of microorganisms, including Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*), Gram-negative bacteria (*Escherichia coli* and *Vibrio cholerae*), and fungi (*Aspergillus flavus* and *Candida albicans*). This screening was conducted using the agar well diffusion method.

To perform the screening, bacterial pathogens were spread onto Nutrient Agar Medium (NAM) plates, while fungi were cultured on Potato Dextrose Agar (PDA). Wells were created on the agar plates using a cork borer, and three different concentrations of crude extracts (100 µg/ml, 200 µg/ml, and 300 µg/ml) were placed into separate wells. The antibacterial and antifungal activities were observed after an incubation period of 1-2 days at temperatures ranging from 35 to 37°C for bacteria and 4-6 days at 28°C for fungi. The presence of a clear zone around the wells indicated the bioactive properties of the extracted compounds. Streptomycin and fluconazole were used as positive controls for bacteria and fungi, respectively, while DMSO served as the negative control. The experiments were conducted in triplicate, and the data were presented as the mean value with standard deviation.

3. RESULTS AND DISCUSSION

3.1 Cultural and microscopic characteristics of fungal isolates

In the present study, a total of eight fungal isolates were obtained from the marine habitats of Manginapudi Beach, Bay of Bengal, India. The 8 fungal isolates (SS1, SS2, SS4, SS6, SS7, SS9, SS10 & SS11) belong to 5 genera such as *Acremonium*, *Fusarium*, *Hamigera*, *Neurospora*, and *Talaromyces*. All the isolates showed variation in color, surface characteristics, edge, pigmentation, and diameters after 10 days of incubation at 28 ± 2 °C (Table 2). Isolates such as SS1 (Figure 2A), SS2, SS6 (Figure 2B), and SS7 appeared white on different media. Isolate SS4 (Figure 2C), SS9, SS10 (Figure 2D), and SS11 appeared pale golden, yellowish to dark brown, yellow, and orange (Table 2). During the initial growth phase, across all media, the colonies exhibited characteristics of being smooth, wrinkled, and flat. However, it's important to note that the specific nature of these colonies varied depending on the composition of the respective media (Table 2). Similar observations were reported by Anteneh *et al.* [31] in the characterization of *Magnuscella marinae* a halotolerant fungus from a marine sponge. Isolates SS1, SS2, and SS7 showed no pigmentation on different media used. Isolates SS4, SS6, SS9, SS10, and SS11 showed pale yellow, light pink, golden yellow, yellow, and orange pigmentation. More colony growth was recorded in RBA (Average colony diameter = 72.61 mm) when compared to other media. Fungal isolates on PDA also showed good growth. The poor growth of fungal colonies was observed on SDA and CZA. In this investigation, the fungal isolates were initially identified to a genus level using a morphological evaluation based on the colors of the colony generated.

Further identification was done using a microscopic examination of the spore-producing structures' shapes. As per the findings by Wang *et al.* [32], morphological analysis and fungal identification provide valuable insights, particularly for identifying isolates up to the family or genus level. To achieve a species-level identification of the isolated fungi, however, additional information becomes imperative [33-34]. Scientific names play a vital role in the transmission of knowledge about fungi, serving as indispensable tools for researchers. They enable the identification of closely related species, facilitating a deeper understanding of the evolution of chemical gene clusters. Additionally, these names aid in prioritizing taxonomically related strains, which is particularly valuable when a productive strain may inadvertently reduce the production of crucial bioactive compounds [35]. In the present study, the fungal isolates are arranged according to the classification of Ascomycota (Table 3) given by Wijayawardene *et al.* [36]. The isolated fungi belong to two classes as the following: Eurotiomycetes [*Hamigera avellanea* (SS4), *H. insecticola* (SS9), and *Talaromyces tratensis* (SS10)] and Sordariomycetes [*Acremonium persicinum* (SS6), *Fusarium equiseti* (SS1), *F. pernambutanum* (SS2), *F. incarnatum* (SS7), and *Neurospora crassa* (SS11)].

Table 2: Cultural, morphological, and microscopic traits of fungal isolates on diverse media

Isolate	Media	Color of the isolates	Surface Characteristics	Edge	Pigmentation	Diameter of the colony (in mm) Mean \pm S.D.
SS1	PDA	White	Smooth, branched, cylindrical and septate.	White, circular	White	60 \pm 0.87
	CZA	White	Smooth, branched, cylindrical and septate.	White, circular	White	54.6 \pm 0.54
	SDA	White	Smooth, branched, cylindrical and septate.	White, circular	White	82.6 \pm 0.97
	RBA	White	Smooth, branched, cylindrical and septate.	White, circular	White	85 \pm 0.38
SS2	PDA	White	The basal cell is distinctly foot-shaped.	White, circular	White	59.33 \pm 1.15
	CZA	White	The basal cell is distinctly foot-shaped.	White, circular	White	52 \pm 0.98
	SDA	White	The basal cell is distinctly foot-shaped.	White, circular	White	62.66 \pm 0.52
	RBA	White	The basal cell is distinctly foot-shaped.	White, circular	White	89.33 \pm 0.15
SS4	PDA	Pale golden	Smooth	White, irregular	Pale yellow	87.66 \pm 1.12
	CZA	Pale golden	Smooth	White, irregular	Pale yellow	65 \pm 1.00
	SDA	Pale golden	Smooth	White, irregular	Pale yellow	81 \pm 0.95
	RBA	Pale golden	Smooth	White, irregular	Pale yellow	88 \pm 0.27
SS6	PDA	White	Wrinkled	White, irregular	Light pink	41 \pm 0.34
	CZA	White	Wrinkled	White, irregular	Light pink	20.6 \pm 0.16
	SDA	White	Wrinkled	White, irregular	Light pink	310.86
	RBA	White	Wrinkled	White, irregular	Light pink	49.3 \pm 1.15
SS7	PDA	White	Smooth	White, circular	White	49.6 \pm 0.27
	CZA	White	Smooth	White, circular	White	40.6 \pm 0.35
	SDA	White	Smooth	White, circular	White	59 \pm 0.85
	RBA	White	Smooth	White, circular	White	71 \pm 1.00
SS9	PDA	Yellowish to dark brown	Develop a thin, flat layer of mycelium that is coated in white aerial hyphae.	light brown, circular	Golden yellow	87.6 \pm 0.57
	CZA	Yellowish to dark brown	Develop a thin, flat layer of mycelium that is coated in white aerial hyphae.	light brown, circular	Golden yellow	65 \pm 0.96
	SDA	yellowish to dark brown	Develop a thin, flat layer of mycelium that is coated in white aerial hyphae.	light brown, circular	Golden yellow	83.3 \pm 0.82

Isolate	Media	Color of the isolates	Surface Characteristics	Edge	Pigmentation	Diameter of the colony (in mm) Mean \pm S.D.
	RBA	Yellowish to dark brown	Develop a thin, flat layer of mycelium that is coated in white aerial hyphae.	light brown, circular	Golden yellow	88.33 \pm 0.72
SS10	PDA	Yellow	Smooth and lateral branching.	Yellow, circular	Yellow	20.33 \pm 0.76
	CZA	Yellow	Smooth and lateral branching.	Yellow, circular	Yellow	19 \pm 0.28
	SDA	Yellow	Smooth and lateral branching.	Yellow, circular	Yellow	23 \pm 0.36
	RBA	Yellow	Smooth and lateral branching.	Yellow, circular	Yellow	39.6 \pm 0.57
SS11	PDA	Orange	The colonies grow like slimy yeast cultures instead of as a mycelial mat-like growth.	Light orange, Irregular	Orange	63 \pm 0.12
	CZA	Orange	The colonies grow like slimy yeast cultures instead of as a mycelial mat-like growth.	Light orange, Irregular	Orange	59.6 \pm 0.17
	SDA	Orange	The colonies grow like slimy yeast cultures instead of as a mycelial mat-like growth.	Light orange, Irregular	Orange	69.6 \pm 0.14
	RBA	Orange	The colonies grow like slimy yeast cultures instead of as a mycelial mat-like growth.	Light orange, Irregular	Orange	70.3 \pm 0.68

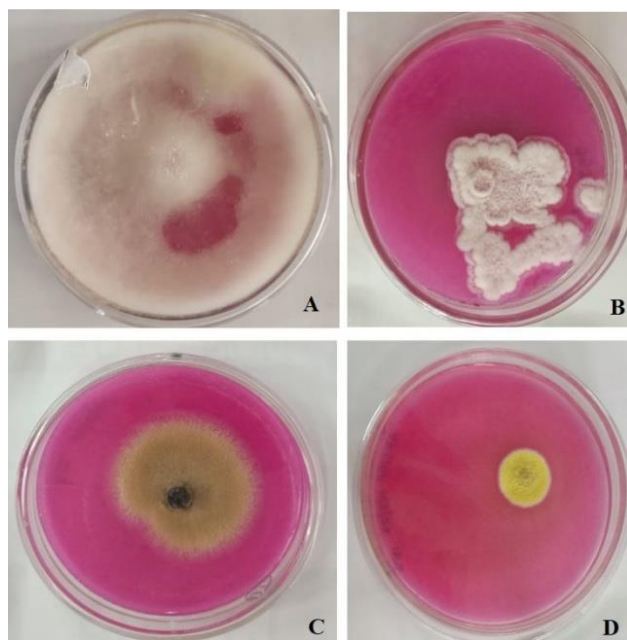


Figure 2: Morphocultural characteristics of fungal isolates on Rose Bengal Agar medium. **A.** *Fusarium equiseti* (SS1); **B.** *Acremonium persicinum* (SS6); **C.** *Hamigera avellanea* (SS4); **D.** *Talaromyces tratensis* (SS10)

Table 3: Fungi isolated and their systematic position*

Isolate	Name of the fungi isolated and its systematic position	Characteristic features observed
Superkingdom: Eukaryota Kingdom: Fungi Subkingdom: Dikarya Phylum: Ascomycota Subphylum: Pezizomycotina		
Class: Eurotiomycetes Sub Class: Eurotiomycetidae Order: Eurotiales		
Family: Aspergillaceae		
SS4	<i>Hamigera avellanea</i>	Colonies of <i>Hamigera avellanea</i> looked pale golden to pinkish, with denser radial aggregates. After 7 days at 25 °C , colonies grew to a diameter of 60 millimeters. Conidiophores exhibit minimal branching and originate from the colony surface. They possess surfaces that can range from smooth to finely roughened, and as they age, conidiophores may develop a vesiculate appearance. Hyphae may occasionally exhibit swelling and are typically septate. At times, an apical cluster of metulae carrying ampulliform phialides can be observed. These structures produce prolate to spheroidal conidia with smooth walls, and metulae and sessile phialides are typically found in subapical positions.
SS9	<i>Hamigera insecticola</i>	<i>H. insecticola</i> initially appeared as a velvety, flat, thin covering of white mycelium that later turned yellowish to dark brown in color. After 7 days of incubation at 28°C, it expanded to a diameter of 90 mm. A few conidial heads are formed by it. A contortion capsule is not formed. Teliocysts develop on a thin, flat layer of mycelium that is coated in white aerial hyphae.
Family: Trichocomaceae		
SS10	<i>Talaromyces tratensis</i>	<i>T. tratensis</i> displayed a distinct yellow coloration and exhibited a relatively slow growth rate. Over a span of 10 days of incubation at 28±1 °C, it managed to achieve a growth of 30mm. The conidia produced by this fungus are aseptate and are associated with phialides and ascospores. Notably, the conidiophores of <i>T. tratensis</i> feature smooth surfaces and exhibit lateral branching.
Class: Sordariomycetes Sub Class: Hypocreomycetidae Order: Hypocreales		
Family: Hypocreales incertae sedis		
SS6	<i>Acremonium persicinum</i>	<i>A. persicinum</i> Colonies were initially white and gradually turned light pinkish as they grew older, eventually reaching 50 mm in diameter after 2 weeks of culture at 28 °C. The center of the colony was originally dark white, but as the colony grew toward the edge, it became pale and crumpled. Erect, simple, or branched conidiophores are observed. The phialides are lateral and acicular. Conidia were oval in outline.

Family: Nectriaceae		
SS1	<i>Fusarium equiseti</i>	The pure culture of <i>F. equiseti</i> initially presented as white colonies, which subsequently transformed into a peach-orange hue. Remarkably, these colonies exhibited vigorous growth, extending to a remarkable 90 mm within just 7 days of incubation at 28±1 °C. The mycelium displayed distinctive characteristics, being smooth, branched, cylindrical, septate, and measuring between 3.05 and 4.60 µm in width. Furthermore, the conidiophores of this fungus appeared cylindrical, relatively short, featured a simple structure, and exhibited septation. Microconidia were oval in outline, hyaline, and septate. Macroconidia displayed a prominent foot cell and an apical cell that had been tapered and elongated. Chlamydospores are spherical and produced singly, in chains, or intercalarily.
SS7	<i>Fusarium incarnatum</i>	The <i>F. incarnatum</i> colonies vary in color from white to orange-red and develop quickly as dense aerial mycelium that grows 90 mm after 7 days of incubation at 25 °C. The aerial mycelium was white, plentiful, thick, floccose, partially tufted, and slightly powdery in older cultures. Aerial conidiophores had flexible, irregular, or verticillate branches, and their apices were primarily covered in polyblastic conidiogenous cells that grew sympodially. Aerial conidia are blastic, primarily spindle-shaped and straight, but occasionally slightly curved, and taper towards both extremities.
SS2	<i>Fusarium pernambutanum</i>	In pure cultures of <i>F. pernambutanum</i> cultivated on RBA medium, distinctive characteristics emerged. These included the presence of thick aerial mycelium, a fully developed colony edge, and a colony that exhibited a predominantly white appearance (with a central brown reverse) after 7 days at 25°C. The conidiophores of this fungus displayed branching and were equipped with apical monophialides, lateral monophialides, and cylindrical sporodochial phialides.
Sub Class: Sordariomycetidae		
Order: Sordariales		
Family: Sordariaceae		
SS11	<i>Neurospora crassa</i>	In a pure culture of <i>N. crassa</i> , notable features included a fluffy aerial growth and the development of an orange-colored appearance on the agar medium. This fungus displayed robust growth, reaching a size of 90 mm within just 4-5 days of incubation at 28±1 °C. The colonies grow like slimy yeast cultures instead of as a mycelial mat-like growth. This filamentous fungus grew quickly as evidenced by the lengthening of the hyphal tips and the development of new tips as branches just behind the main tip.

*Arranged according to Wijayawardene et al, [36]

3.2 Molecular identification

DNA barcoding, employing sequencing of the Internal Transcribed Spacer (ITS) region, served as the molecular identification method of choice. The ITS region is particularly prized for species-level identification because it undergoes rapid evolution. Notably, a consortium of mycologists has officially designated the ITS as the preferred barcode for fungi. This selection is attributed to its ease of amplification, widespread application, and ability to establish a clear and accurate distinction between interspecific and infraspecific variations, commonly referred to as

the "barcode gap." According to Anderson and Parkin [37], the ITS rDNA region sequence is also one of the most crucial tools for identifying fungal species that have been isolated from environmental sources. As a result, it has been widely used to identify the marine fungal community and to improve on traditional identifications. ITS rDNA genes are suitable candidates for phylogenetic analysis because they are widely dispersed, functionally constant, adequately conserved, and long enough to provide an in-depth understanding of evolutionary relationships [38]. The NCBI-BLAST program was used to match the ITS rDNA sequences to those in the databases. Using DNA barcoding, eight species were identified with an identification range of 99.31 to 100% with already existing GenBank sequences (Table 4). Four fungal isolates (SS7, SS9, SS10, and SS11) showed 100% similarity and exactly identified as *Fusarium incarnatum*, *Hamigera insecticola*, *Talaromyces tratensis*, and *Neurospora crassa*. There is a significant amount of similarity between the fungal isolates and those in the NCBI-GenBank, as shown by comparison. This shows that there are known terrestrial isolates that are comparable to this one, and it also shows that the species underwent some little evolution to help ensure their survival.

The degree of commonality between the current isolate and earlier isolates also suggests that the fungus species have not been subjected to environmental or other variables that might encourage increased genetic diversity, or what has been known as concerted evolution [39]. The isolates such as SS1, SS2, SS4, and SS6 displayed 99.64%, 99.82%, 99.31%, and 99.64%, similarity respectively due to 2-4 nucleotide base differences (Table 4).

In the present study, *Talaromyces tratensis* was reported for the first time in seawater. Earlier *T. tratensis* was recorded from the soil, indoors, food, waste, lichens, and marine sponges [40-41]. *T. tratensis* possesses strong antagonistic action against rice disease, as well as plant growth promotion properties that are important in rice production [42]. The results of our study are consistent with the research conducted by Kumar *et al.* [43]. Their studies reported the presence of *Neurospora crassa* in seawater of the Machilipatnam coast, which is identified in the present study area. This filamentous fungus (*N. crassa*) is widely used as a model organism for different branches of biology especially to study host-virus interactions [44]. The present study confirms the findings of Palmero *et al.* [45] by recording *F. equiseti* for the first time on the Machilipatnam coast, confirming previous findings of *F. exquisite* and *F. incarnatum* from Mediterranean coastal regions at depths greater than 1.5 m. These filamentous fungi were reported from seawater elsewhere [46]. The current study confirms observations reported in Dead Sea water by Kis-Papo *et al.* [47] and Buchalo *et al.* [48] that *Acremonium persicinum* is present in seawater off the Machilipatnam coast).

3.3. Maximum Parsimony Analysis of Taxa

Evolutionary analyses were conducted in MEGA X [49]. The evolutionary history was deduced using the Maximum Parsimony method. The bootstrap consensus tree, derived from 1000 replicates, serves as a representation of the evolutionary history of the analyzed taxa, following the methodology proposed by Felsenstein [50]. Branches corresponding to partitions replicated in less than 50% of bootstrap replicates have been condensed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is indicated adjacent to the respective branches (Figure 3). The Maximum Parsimony (MP) tree was constructed using the Subtree-Pruning-Regrafting (SPR) algorithm [51]. The analysis encompassed 26 nucleotide sequences, with codon positions considered including 1st, 2nd, 3rd, and noncoding regions. The final dataset encompassed a total of 708 positions. Within this dataset, there were 398 conserved sites, 263 variable sites, 248 sites that provided parsimony-informative information, 15 singleton sites, as well as 432 zero-fold sites, 62 two-fold sites, and 108 four-fold sites. The phylogenetic tree constructed for the fungal isolates revealed distinct clustering patterns, indicating their close resemblance to one another. Notably, the study's isolates are distinctly highlighted within a designated box, while sequences obtained from NCBI-GenBank are positioned outside this box (Figure 3). This analysis further revealed that the clustered isolates in this study shared a substantial identity of over 90% with those sourced from GenBank. *Talaromyces tratensis* (SS10) exhibited a robust bootstrap value and formed a well-supported cluster with *T. tratensis* isolates retrieved from NCBI-GenBank. Similarly, isolates such as *Fusarium equiseti* (SS1), *F. pernamucanum* (SS2), *F. incarnatum* (SS7), *Hamigera avellanea* (SS4), and *H. insecticola* (SS9) also demonstrated strong bootstrap values, signifying their close relatedness to the isolates acquired from NCBI-GenBank (Figure 3).

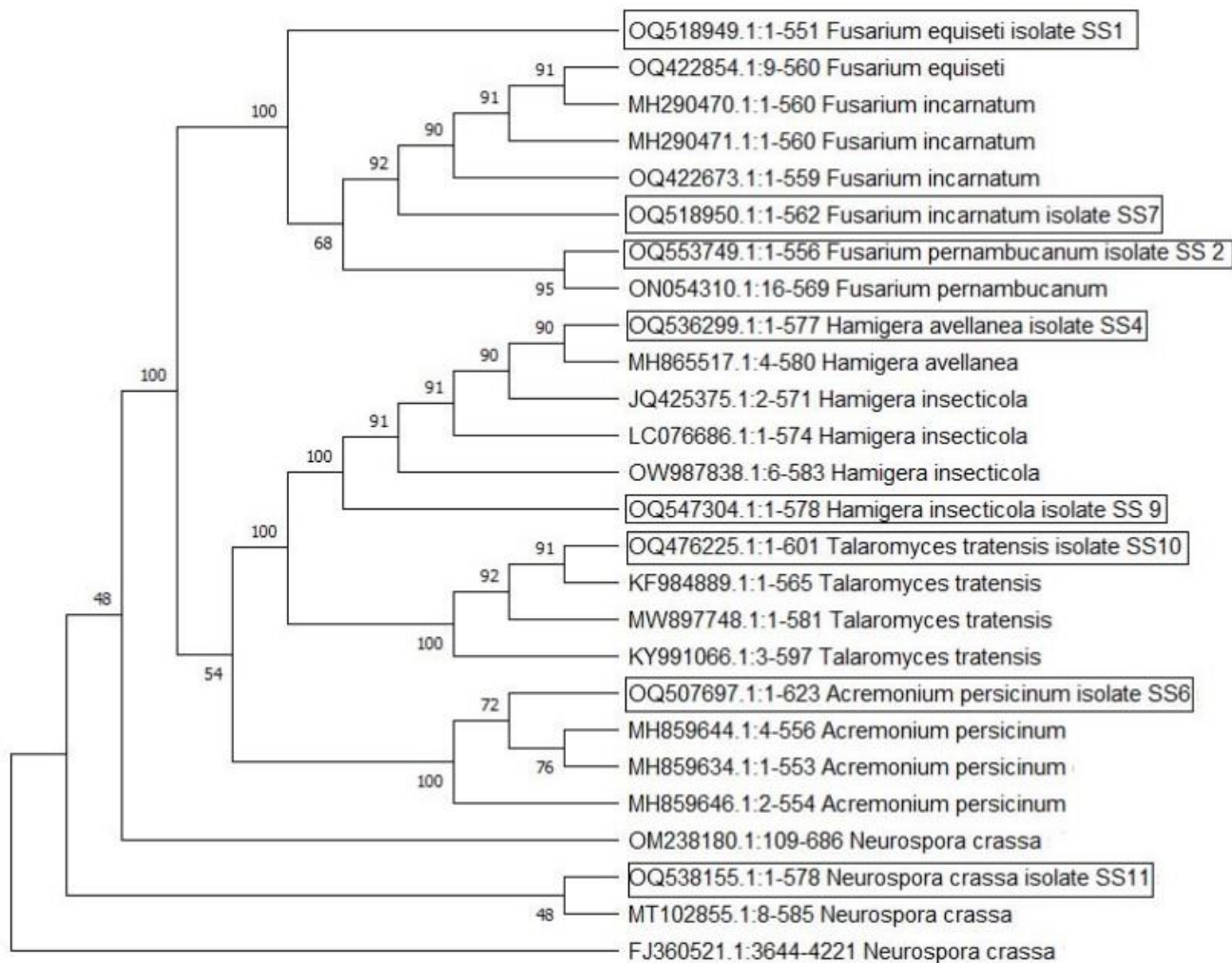


Figure 3: A phylogenetic tree constructed from partial ITS gene sequences, illustrating the interconnections among the fungal isolates examined in this study and their counterparts officially archived in NCBI-GenBank (Isolates described in this study are enclosed within the highlighted box).

Table 4: Nucleotide Sequence homology search results using MEGA BLAST based on ITS rDNA sequences

Fungal Isolate	Name of Organism identified through BLAST	Query cover	Identity (%)	Nucleotide base difference	Nucleotide sequence submitted to GenBank and its Accession Number
SS1	<i>Fusarium equiseti</i>	100%	99.64%	551/553	OQ518949.1
SS2	<i>Fusarium pernambucanum</i>	99%	99.82%	554/555	OQ553749.1
SS4	<i>Hamigera avellanea</i>	100%	99.31%	574/578	OQ536299.1
SS6	<i>Acremonium persicinum</i>	88%	99.64%	551/553	OQ507697.1
SS7	<i>Fusarium incarnatum</i>	99%	100%	559/559	OQ518950.1
SS9	<i>Hamigera insecticola</i>	100%	100%	578/578	OQ547304.1
SS10	<i>Talaromyces tratensis</i>	99%	100%	595/595	OQ476225.1
SS11	<i>Neurospora crassa</i>	100%	100%	578/578	OQ538155.1

3.4 Antimicrobial Efficacy of Crude Extracts of Marine Fungi

In this investigation, the antimicrobial potential of crude extracts from marine fungi was evaluated against a range of microorganisms, including Gram-positive strains (*Staphylococcus aureus* and *Enterococcus faecalis*), Gram-negative bacteria (*Escherichia coli* and *Vibrio cholerae*), and fungi (*Aspergillus flavus* and *Candida albicans*). Various concentrations of the crude extracts were employed for testing. Notably, the ethyl acetate extract derived from *Acremonium persicinum* (SS6) exhibited a remarkable maximum zone of inhibition, measuring 29.8 ± 0.26 mm, when tested at a concentration of $300 \mu\text{g/ml}$ against *Escherichia coli* (Figure 4A). It is worth mentioning that the standard reference antibiotic, streptomycin (at $10 \mu\text{g/ml}$), yielded a zone of inhibition measuring 15 ± 0.82 mm.

Furthermore, when the same extract was assessed at a concentration of $300 \mu\text{g/ml}$, it also displayed a substantial maximum zone of inhibition (18.5 ± 0.86 mm) against the Gram-positive bacterium *Staphylococcus aureus* (Table 5). Notably, the study observed an increase in the antimicrobial activity of the crude extracts with the rise in concentration (ranging from 100 to $300 \mu\text{g/ml}$).

The ethyl acetate extract obtained from *Talaromyces tratensis* (SS10) exhibited substantial inhibitory effects when tested at $300 \mu\text{g/ml}$ against all the examined microorganisms, with the most prominent zone of inhibition measuring 22.1 ± 0.28 mm, notably evident against *Candida albicans* (Table 5; Figure 4B). It is worth noting that the standard antibiotic, Amphotericin B (at $10 \mu\text{g/ml}$), resulted in a zone of inhibition measuring 15 mm when employed against the same fungi. In contrast, the methanol extract of *T. tratensis* at the same concentration displayed moderate inhibitory activity against the fungi tested (Table 5). The variation in activity could be attributed to the distinct secondary metabolites present in the respective crude extracts. Remarkably, the outcomes of this study align with the studies of Dethoup *et al.* [52], where they highlighted the antifungal activity of the crude ethyl acetate extract of *T. tratensis* against *Bipolaris oryzae*, a rice pathogen. In the current investigation, the synergy of all bioactive substances within the crude extracts may have contributed to the enhancement of their antimicrobial properties. This heightened reactivity and increased bioavailability of active molecules likely resulted in a cumulative impact of these active compounds [53].

Furthermore, the ethyl acetate extract derived from *Neurospora crassa* (SS11) demonstrated significant inhibitory effects against both *E. coli* and *C. albicans* (Table 5). These findings are consistent with the research conducted by Kumar *et al.* [43] which also emphasized the antimicrobial capabilities of marine-derived *N. crassa*.

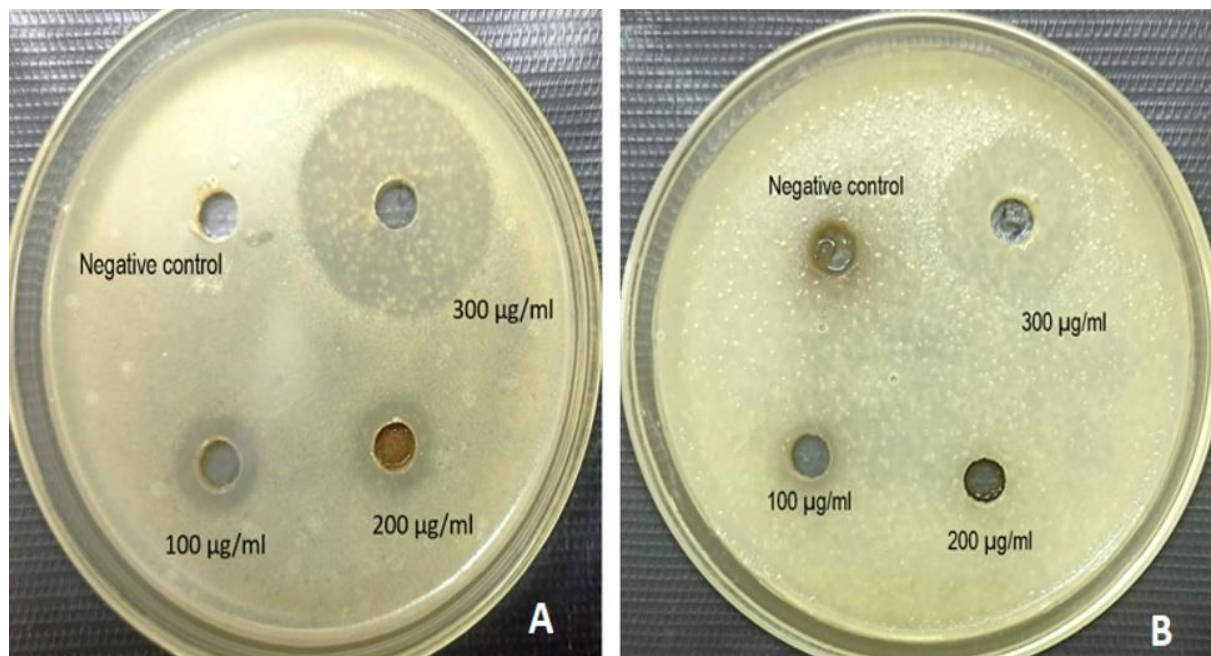


Figure 4: Antimicrobial activity. A. Antibacterial potential of ethyl acetate extract of *Acremonium persicinum* (SS6) ($300 \mu\text{g/ml}$) against *Escherichia coli*; B. Antifungal potential of ethyl acetate extract of *Talaromyces tratensis* (SS10) ($300 \mu\text{g/ml}$) against *Candida albicans*

Table 5: Antimicrobial potential of marine fungi

Marine fungi	Crude extract	Zone of inhibition (in mm) at 300 µg/ml (Mean±S.D.) for test organisms					
		Gram-positive bacteria		Gram-negative bacteria		Fungi	
		<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	<i>Escherichia coli</i>	<i>Vibrio cholerae</i>	<i>Aspergillus flavus</i>	<i>Candida albicans</i>
<i>Fusarium equiseti</i> (SS1)	E.A.E.	-	2.2±0.40	-	-	-	-
	M.E.	-	2.1±0.23	-	-	-	-
<i>Fusarium perambucanum</i> (SS2)	E.A.E.	3.3±0.41	-	-	2.3±0.47	-	-
	M.E.	-	-	-	-	-	-
<i>Hamigera avellanea</i> (SS4)	E.A.E.	-	4.1±0.28	-	-	-	-
	M.E.	-	-	-	-	-	-
<i>Acremonium persicinum</i> (SS6)	E.A.E.	18.5±0.86	12.4±0.80	29.8±0.26	8.2±0.25	8.0±0.36	9.3±0.57
	M.E.	7.0±0.25	6.2±0.25	7.7±0.64	7.2±0.30	5.5±0.60	6.53±0.47
<i>Fusarium incarnatum</i> (SS7)	E.A.E.	-	4.2±0.34	-	-	-	-
	M.E.	-	-	-	-	-	-
<i>Hamigera insecticola</i> (SS9)	E.A.E.	7.6±0.12	-	-	6.6±0.68	13.6±0.42	12.6±0.11
	M.E.	6.3±0.32	-	-	5.8±0.31	11.3±0.87	10.5±0.69
<i>Talaromyces tratensis</i> (SS10)	E.A.E.	12.6±0.65	12.1±1.00	13.6±0.65	6.3±0.32	19.3±0.81	22.1±0.28
	M.E.	8.1±0.15	7.3±0.20	11.6±0.70	10.1±0.28	12.1±0.17	15.3±0.41
<i>Neurospora crassa</i> (SS11)	E.A.E.	5.1±0.28	7.5±0.50	14.8±0.98	3.0±0.20	3.1±0.80	15.6±0.70
	M.E.	6.2±0.37	5.3±0.10	10.4±0.43	7.5±0.43	6.2±0.37	12.7±0.64

E.A.E. – Ethyl acetate extract; M.E. – Methanol extract

4. CONCLUSION

Marine fungi represent a diverse group of organisms with significant chemical and biological variability [54]. However, systematic research into the molecular identification of marine fungi at Manginapudi Beach (Machilipatnam coast), India, has been scarce or virtually non-existent. This study aimed to enhance the identification and molecular characterization of fungal isolates down to the species level through the utilization of PCR amplification and ITS region sequencing. The molecular techniques employed in this research have notably advanced the precision of fungal species identification when compared to conventional methods.

The findings of this investigation suggest that Manginapudi Beach harbors a rich diversity of marine fungi, many of which possess antimicrobial properties. Particularly, the ethyl acetate extracts of *Acremonium persicinum* (SS6) and *Talaromyces tratensis* (SS10) exhibited promising antimicrobial activity against selected clinical pathogens. These extracts hold the potential to serve as sources for the development of novel drugs that could address currently unmet therapeutic needs. To gain a comprehensive understanding of their mechanisms of action at the cellular and molecular levels, further research is required, including the extensive exploration of their antimicrobial potential.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest to disclose.

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