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ISOLATION AND SCREENING OF POTENT FUNGI FOR THE PRODUCTION OF BIOACTIVE METABOLITES FROM MARINE HABITATS OF SURYALANKA BEACH, ANDHRA PRADESH

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ABSTRACT

Searching for novel antibiotics is the need of the hour to combat multidrug resistant pathogens. Hence, an attempt was made to isolate and identify promising antimicrobial metabolite producing fungi from marine soils of Suryalanka beach, Andhra Pradesh, India along with the study of their antimicrobial potential. The samples collected from mangrove habitats were pretreated with calcium carbonate, diluted and plated on Soybean casein digest agar (SCDA) and Malt extract agar (MEA) media to isolate marine fungi. Over all 10 fungal strains were isolated and screened for their antimicrobial activity. Among them designated as NRI-16 and NRI-21 were exhibited high antimicrobial potential was identified as *Penicillium* NRI-16 and *Aspergillus* NRI-21 based on their micro-morphological studies. The culture broth extracted with ethyl acetate was tested for antimicrobial activity and found to be active against pathogenic Gram-positive and Gram-negative bacteria as well as fungi. It was evident from the present study that marine habitats of Suryalanka serve as a good source for potent fungi possessing broad spectrum antimicrobial activity.

KEYWORDS: Marine fungi, *Penicillium* NRI-16 and *Aspergillus* NRI-21, Antimicrobial activity.

INTRODUCTION

Infectious diseases are leading health problems with high morbidity and mortality in the developing countries. The development of resistance to multiple drugs is a major problem in the treatment of these infectious diseases caused by pathogenic microorganisms. This multidrug resistance is presently an urgent focus of research and new bioactive resistance pathogens. On this view point, attempts have been made to develop novel drugs against infectious diseases for the mitigation of suffering of the vast masses of humanity.[3] The marine realm represents the largest inhabitable biosphere where living organisms flourish, especially microbes. Marine microorganisms thrive everywhere in the sea, from the surface water to the deep sea and from coastal to offshore regions, in both general areas and in specialized niches.[1] Marine life is broadly classified into six main groups: animals, plants, chromists, fungi, protozoa, and bacteria. All groups are heterogeneous in nature and are interdependent for survival. Marine-derived fungi have been widely studied for their bioactive metabolites and have proven to be a rich and promising source of novel anticancer, antibacterial, antiplasmodial, anti-inflammatory and antiviral agents. Considering the number of novel bioactive compounds that have been isolated from marine-derived fungi and the fact that the number of the compounds is increasing rapidly.[2-4] In optimizing the search for new bioactive secondary metabolites a fungus synthesizes may correspond with its respective ecological niche, e.g. the mycotoxins of plant pathogens that metabolic interactions may enhance the synthesis of secondary metabolites. Thus, the fungi screened should originate from biotopes from which fungi have not been previously isolated for biochemical purposes and they should have metabolic interactions with their environment. This is an example of intelligent screening and is a strategy for exploiting the untapped potential for secondary metabolites that fungi offer.[6,7]

Bioactive natural compounds produced by microorganisms have been promising potential usefulness in safety and human health concerns, although there is still a significant demand of drug industry for synthetic products due to economic and time-consuming reasons. Recent review by Newman and Cragg, provides us a list of all approved agents from 1981 to 2006, from which a significant number of natural drugs are produced by microbes. Marine microorganisms have been an important source of pharmacologically active metabolites.[15] Considering that only a small amount of mangrove fungi have been studied, recently, several research groups have been motivated to evaluate and elucidate the potential of these microorganisms applied on biotechnological processes focusing on the production of anti-cancer compounds.[8,9] It is fascinating to discover how simple fungi could hold the key to powerful new approaches to treat cancer. Mangrove associated fungi provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others.[10,11] There are some evidences that bioactive compounds produced by fungi associated with plants could be alternative approaches for discovery of novel drugs, since many natural products from plants, microorganisms, and marine sources were identified as anticancer agents. Cancer is a group of diseases characterized by unregulated growth and spread of abnormal cells, which can result in death if not controlled. It has been considered one of the major causes of death worldwide: 7.4 million (about 13% of all deaths) in 2004.[13,14] The anticancer drugs show nonspecific toxicity to proliferating normal cells, possess enormous side effects, and are not effective against many forms of cancer. Thus, the cure of cancer has been enhanced mainly due to diagnosis improvements, which allow earlier and more precise treatments.[12]

MATERIALS AND METHODS

Sample Collection

The samples were collected from the Suryalanka beach, Guntur South coast of Andhra Pradesh, India (fig.1). The samples were collected randomly at a depth of 10-20 cm and shade dried at room temperature.

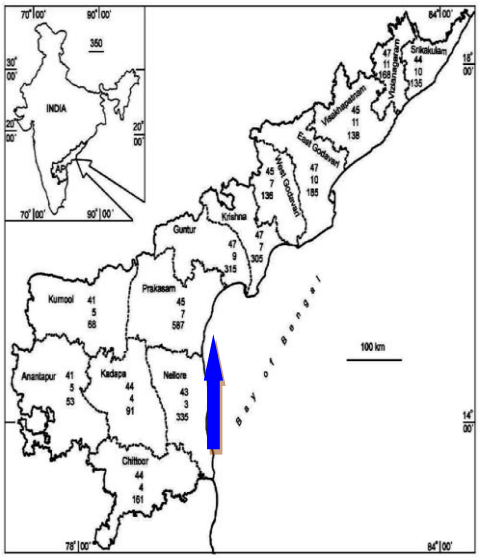


Fig. 1: Sample collection location sites of Guntur.

Pretreatment of soil samples: The collected soil samples were air-dried at room temperature for 3 days. The soil sample was ground and sieved in 2 mm screen.1 g of the sieved soil was suspended in 100 ml of sterile water and heated for 1 hour. The sample was vigorously agitated and then supernatant was diluted. This dilution series were cultured on SCDA, MEA media and incubated for 5-7 days at room temperature. Single colonies from the plates were picked and purified by restreaking. The pure strains were maintained in agar culture in test tube. Indupalli *et al.* (2016) reported, air-dried samples were pretreated with calcium carbonate (10:1) and incubated at 37C for 4 days. Plated on selective media such as humic acid vitamin (HV) agr medium supplemented with 3% NaCl, nystatin and streprtomycin (25 ug/mL) to reduce the bacterial and fungal contaminants.

Physico-chemical characteristics of soil samples

Moisture content

To determine the moisture content, 10g of soil sample was dried in a hot air oven (105ºC) until a constant weight is obtained. The difference between the weights of pre-drying and post-drying was taken as the moisture content of the initial soil sample.

Soil pH

To determine the pH of the soil sample, 20g of sample was mixed with 40ml of distilled water in a 100 ml beaker. The soil suspension was mixed thoroughly and the clay particles were allowed to settle down. The pH of sample was recorded after 1h.

Isolation of marine fungi

The shade dried soil samples were used for the isolation of bacteria by serial dilution plate technique. Soybean casein digest agar (SCDA) and Malt extract agar (MEA) media amended with sodium chloride (2%) are used for the isolation

of fungi. Streptomycin (50µg/ml) was added to retard the growth of bacteria. Aliquots of samples were prepared through serial dilution plate technique. An aliquot of 0.1ml of each dilution (10^{-2} to 10^{-4}) of samples was spread evenly over the surfaces of SCDA and MEA media. The plates were incubated at 37°C and observed for bacterial growth. After 2 days of incubation at 27°C, the inoculated agar plates were examined daily for the presence of developing bacterial colonies, using a dissecting microscope at 20× magnification. Distinct fungal colonies on the agar plates were then transferred to new agar plates for further isolation and purification.[8]

Primary screening of isolated fungi

All the fungal cultures were inoculated in to fermentation broth supplemented with Filtered seawater and incubated at 28ºC for 3 weeks. After incubation, the mycelial growth was removed by using centrifugation. After collecting the supernatant used for primary screening of antibacterial activity against test organisms. The supernatant was load in a sterilized disc placed over the Muller-Hinton Agar plates inoculates with 5 test organisms as described above. All the plates were incubated at 37ºC for 24 hours. The zone of inhibition was measured and expressed in diameter in mm. Based on the results of preliminary screening best three fungal isolates were selected as potential strains for further investigations.[11]

Screening of fungi for the Production of Antimicrobial Metabolites

All the fungal strains isolated were screened for antimicrobial activity against *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (ATCC 6633), *Xanthomonas campestris* (MTCC 2286), and *Escherichia coli* (ATCC 9027) and antifungal activity against *Candida albicans* (MTCC 183).

Screening of fungal strains for bioactive metabolites

Over all 10 fungal strains were isolated and all of them were selected to screening for their production of bioactive metabolites. Seed cultures were prepared by transfer the pure cultures into seed medium broth (Nutrient broth). From the seed medium, 10% of seed cultures were inoculated into the Sabourad dextrose broth as production medium and incubated at room temperature for nine days at 120 rpm. Flasks were harvested after incubation period and biomass was separated from the broth. The crude metabolites produced by the strains were extracted by solvent-extraction method (Elliah *et al.*, 2005). The obtained culture filtrates were extracted with the solvent ethyl acetate (1:1) and this solvent extracts were evaporated in water bath and the obtained residues were used to determine antimicrobial potential by agar well-diffusion method.

Agar well diffusion technique

Antimicrobial potential was evaluated by agar-well diffusion method (Cappuccino and Sherman, 2004). The Czapek-Dox (CD) and Nutrient agar (NA) media were used for culturing test fungi and bacteria. For antibacterial assay, the NA medium was prepared (121ºC/15lbs) and inoculated with 0.1 ml of test bacterial suspensions. After mixing thoroughly, the inoculated medium was poured into sterile petriplates. For antifungal assay, the Czapek-Dox (CD) agar was prepared and inoculated with the test fungi (10^5cells/ml) and the medium was poured into sterile petriplates. After media get solidified, 6 mm diameter wells were punched with metal borer. 60µl of crude ethyl acetate extracts were added to each and every well while only ethyl acetate maintained as control. The plates were incubated 24h for bacteria, 24-72h for fungi and the effectiveness of antagonistic activity was determined by zone of inhibition (mm) (Kapur *et al.*, 2018).

Trial microbial pathogens

Bacteria: *Escherichia coli* (ATCC 9027), *Staphylococcus aureus* (MTCC 3160), *Klebsiella pneumoniae* (ATCC 10031), *Staphylococcus aureus, Bacillus subtilis* (ATCC 6633), *Xanthomonas campestris* (MTCC 2286) and *Pseudomonas aeruginosa* (ATCC 9027). **Fungi:** *Candida albicans* (MTCC 183).

Identification of the fungal Isolates:

Micro-morphology was studied using lacto phenol cotton blue staining. All isolates were identified at genus level, based on mycelial morphology.[9] The effects of salt concentration on the growth of isolated fungi were determined Joshi et al, 2008.[10] The selected fungi were cultured with Nutrient Broth medium with varying salt concentration at 1, 2.5, 5, 7.5, 10.0% (w/v) respectively. After cultured at 28^0C for 4-5 days, the relative levels of bacterial growth at certain conditions were determined.

RESULTS

Physico-chemical characteristics of soil samples

Moisture content

To determine moisture content, 10g of soil sample was dried in a hot air oven (105ºC) until a constant weight is obtained. The difference between the weights of pre-drying and post-drying was taken as the moisture content of sample.

Soil pH

To determine the pH of soil, 20g of sample was mixed with 40ml of distilled water in 100 ml beaker. The soil suspension was mixed thoroughly and clay particles were allowed to settle down. The pH of sample was recorded after 1h.

Isolation and characterization of marine fungi

Totally 10 fungal isolates were recovered and morphologically three different strains were sub cultured and maintained for further analysis. The Microscopic and Cultural characteristics of the isolates were observed. The isolates were belonged to the genera *Penicillium* and *Aspergillus.* Effects of sodium chloride on the growth of fungal isolates were tested. All the isolates were takes growth up 1% to 10% sodium chloride concentration. All the colonies were well growing in NaCl contain medium.

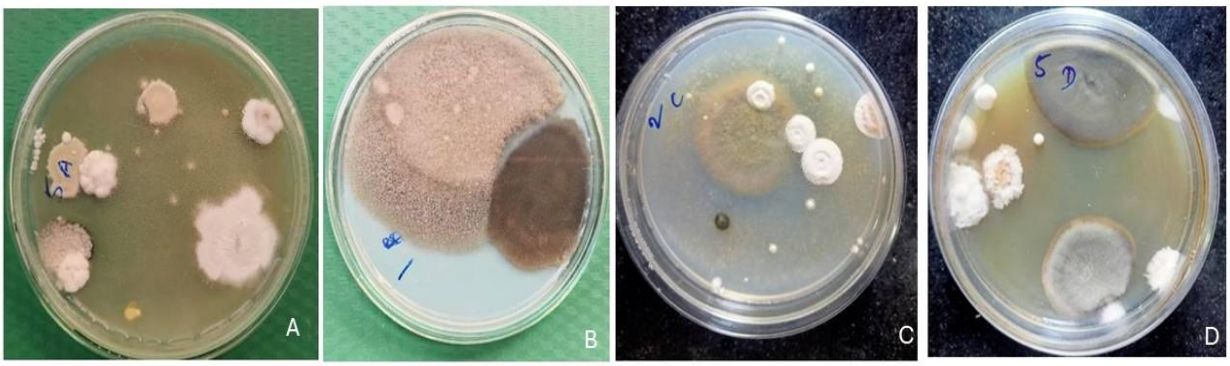


Fig. 1: Isolation of marine fungi on Soybean casein digest agar (SCDA) and Malt extract agar (MEA) media.

Morphological Characteristics of the fungal strains

The fungal isolates were identified initially by morphological characterization through plating the bacteria on SDA and incubated for seven days. The growth and presence/absence of pigmentation were noted. The fungi were identified by studying their cultural characteristics, spore formation, and mycelium arrangement. Slides were prepared by the tease mount method using Lacto-phenol Cotton Blue (LPCB) reagent.

Microscopic characteristics of Penicillium include septate, hyaline hyphae, which are branched and multicellular. From these hyphae arise conidiophores, specialized stalks that branch and end in flask-shaped cells called phialides. These phialides produce chains of conidia (asexual spores) that appear as a brush-like structure, often referred to as a "penicillus". Under the microscope, Aspergillus is characterized by its branching septate hyphae and distinctive conidial heads, which are formed by a conidiophore that expands into a vesicle. This vesicle supports phialides (spore-producing cells) in either a single row (uniseriate) or double row (biseriate) from which chains of conidia are produced. Key characteristics like conidia color and the arrangement of phialides help differentiate species, for example, A. niger has black conidia and A. flavus has yellow-green ones.

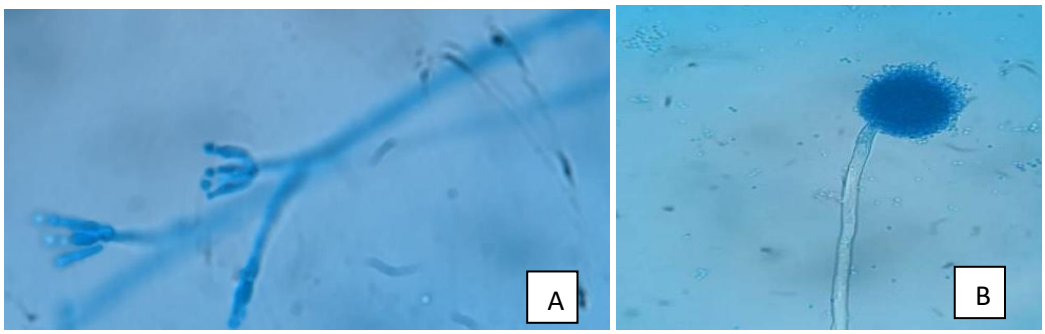


Fig. 2: Microscopic observation of the fungal isolates A: *Penicillium* NRI-16 and B: *Aspergillus* NRI-21.

Extraction of Metabolites and Antimicrobial Assay

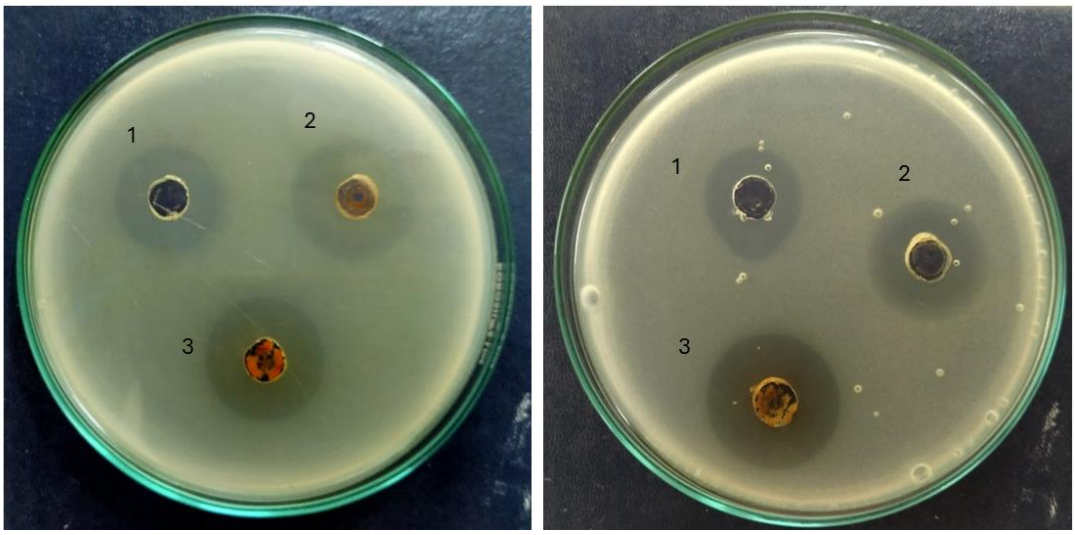
The antimicrobial activity of the strain was determined by agar well diffusion assay. The homogenous culture suspension prepared by suspending 7-day-old culture in sterile saline was used to inoculate SD broth (seed medium) and incubated at 30 °C for seven days on a rotator shaker at 180 rpm. Seed culture at the rate of 10% was transferred to SD broth (fermentation medium). The fermentation was carried out at 30 ± 2 °C for twenty days under agitation at 180 rpm. The antimicrobial compound was recovered from the filtrate by the solvent extraction method. Ethyl acetate was added to the filtrate (1:1) and shaken vigorously (Fig.3). The ethyl acetate extract was evaporated to dryness on a water bath and the residue thus obtained was used to determine antimicrobial activity. Ethyl acetate itself was used as a negative control. About 80 µl of the crude extract and 80 µl of negative control were poured into separate wells. The standard antibiotic disc was placed on the agar surface as a positive control. Plates were incubated at 37 °C for 48 h, and inhibition zones (mm) were measured.

Fig. 3: Crude metabolites from fungi (NRI-16 and NRI-21) after solvent extraction with ethyl acetate.

Antimicrobial assay

Table 1: Antimicrobial assay of secondary metabolites produced by fungi.

Test organism name	Zone of Inhibition (mm)	
	NRI-16	NRI-21
S. a	18	22
E. c	06	18
X. c	14	14
P. a	10	10
K. p	8	08
C. a	16	22



1: Standard Antibiotic Postive control Streptomycin; 2: Metabolites produced by the strain NRI-16; 3: Metabolites produced by the strain NRI-21

Fig. 4: Antimicrobial activity of against *Staphylococcus aureus* and *Candida albicans.*

CONCLUSION

The present investigation highlights the antimicrobial potential of *Penicillium* NRI-16 and *Aspergillus* NRI-21. It is evident from the present study that marine habitats of Suryalanka, serve as a good source for the isolation of potent fungi with broad spectrum antimicrobial activity. Further study on chemical characterization of bioactive compounds of the isolate is in progress.

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Conflict of Interests: Declared none.

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