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Bioactivity Assay of an Isolated Marine *Fusarium sps*

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Abstract

Marine fungi are the potential and promising sources for biologically active secondary metabolite productions. Secondary metabolites are the chemical compounds that are produced during the stationary phase of the organism. Many years of study revealed that fungi are excellent sources for novel bioactive secondary metabolites. In the present study fungi were isolated from marine water samples and serial dilution method was performed to isolate single colonies. Later microscopic and macroscopic observations were done to identify the organism. Organisms are inoculated into medium for production of secondary metabolites. One week later ethyl acetate is added to the broth in 1:1 ratio and kept for incubation in shaker. Solvent is separated by separating funnel and solvent is extracted by rotavapour to collect the crude extract. Crude extract is dissolved in DMSO and further tested for biological activities. The Fungi was identified as Fusarium species and its secondary metabolites showed potential antimicrobial activity. The present work was attempted to isolate organisms which are capable of producing efficient antibacterial and antifungal compounds. Further we identified the organism as Fusarium sp. The future scope of this work is to isolate these biologically active compounds to use in pharmaceutical applications.

Key words*: Marine fungi, Crude extract, Bioassays, TLC purification*

1. Introduction

Since the discovery of penicillin, a potent antibiotic produced by *Penicillium notatum*, Fleming, 1929, a new area in natural product research has started. Fungi were noticed as a source of chemically new compounds with various biological activities. The marine environment offers a wide array of potential fungal sources including sediment, sand, driftwood, mangrove wood, sea water, algae, sponges and other invertebrates [4]. Fungi obtained either from sponges, algae, or wooden substrates account for about 70 % of chemistry described from marine-derived fungi. Sponge-derived fungi comprise about 33 % of the total compounds in the literature and have the highest number of novel metabolites.

Marine fungi have proved to be a rich source of new biologically natural products [1]. Because of their particular living conditions, salinity, nutrition, higher pressure, temperature variations, competition with bacteria, viruses and other fungi, they may have developed specific secondary metabolic pathways compared with terrestrial fungi [5]. Marine microorganisms have proven to be a promising source for the production of novel antibiotic, anti tumor, and anti inflammatory agents. The marine fungi particularly those associated with marine alga, sponge, invertebrates, and sediments appear to be a rich source for secondary metabolites [2]. A number of antibiotics have been obtained from the culture broths of filamentous fungi to date. Recent investigations on marine filamentous fungi looking for biologically active secondary metabolites indicate the tremendous potential of them as a source of new medicines [6].

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The genera *Fusarium sps*, contains a large number of species which occur in all climate zones and are pathogenic to agriculturally important crops. The majority of the species produce secondary metabolites, many of which are toxic to plants, animals and humans. The metabolites which are produced from these species are Fumonisins, Zearalenone and Trichothecenes and they show their bioactivity on mycotoxins. The diversity of biochemical properties has been demonstrated by the continued discovery of novel compounds that have pharmacological properties [8]. From the literature it suggested that various useful products like Fusaric Acid was produced from the *Fusarium* Species [7]. The antibacterial activity was also reported from endophytic *F. oxysporum* isolated from *Dioscorea zingiberensis* rhizomes. In many instances metabolites extracted from endophytic *F. oxysporum* has displayed only antibacterial activity. But effective inhibition of both Gram positive and Gram negative bacteria and moderate antifungal activity suggest the current metabolite to be broad spectrum in nature.

2. Materials and Methods

2.1. Collection of Sample

Soil sample was collected from Vishakhapatnam in Vishaka district. Andhra Pradesh, India. The water samples were collected in sterile tight bottles and transferred to the laboratory in 24 hrs of duration.

2.2. Isolation of Fungi from Soil using Soil Dilution Method

A wide variety of microorganisms can be isolated from the soil environment and cultivated (grown) on media in the laboratory. Different media will encourage the growth of different types of microbes through the use of inhibitors and specialized growth substrates. We will use several different media to isolate microbes from soil. For fungi Rose Bengal media is used as its composition encourages growth of fungi only.

2.2.1 Rose Bengal Chloramphenicol Agar:

Composition:

Mycological peptone	5.0 g
Dextrose	10.0 g
Mono potassium Phosphate	1.0 g
Magnesium Sulfate	0.5 g
Chloramphenicol	0.10g
Rose Bengal	0.05 g
Agar	15.5 g

Dissolve 32.15 gm of Rose Bengal Chloramphenicol Agar in 1000 ml of Distilled water.

2.2.2. Inoculation:

After shaking, 100 µl from the soil solution is transferred using micropipette on the rose Bengal medium plates. Uniformly spread the soil solution onto the surface of the plate using “L’ shaped glass rod (spreader) and incubate for 2-3 days at 27°C. After incubation, isolated colonies formed are transferred to freshly prepared potato dextrose agar plates for identification.

2.3 Microscopic Observation:

Identification and microscopic observation are done by slide culture technique or war cup method. After incubation of slide culture, slides are stained with Lactophenol blue for

microscopic examination. First the slides are observed under light microscope at 10x resolution and then at 45x and 100x (oil immersion) for morphology of fungi. Based on the mycelium, spore morphology, fungi was identified by using the reference book on "**Illustrated Genera of Imperfect Fungi" by H. L. Barnett and Barry B. Hunter.**

Hyaline septate hyphae, conidiophores, phialides, macroconidia, and microconidia are observed microscopically. In addition to these basic elements, chlamydospores are also produced by *Fusarium chlamydosporum*, *Fusarium napiforme*, *Fusarium oxysporum*, *Fusarium semitectum*, *Fusarium solani*, and *Fusarium sporotrichoides* [8]. Phialides are cylindrical, with a small collarette, solitary or produced as a component of a complex branching system. Monophialides and polyphialides (in heads or in chains) may be observed.

Fusarium sps.
Kingdom: Fungi
Phylum: Dikarya
Subphylum: Ascomycotina
Class: Sordariomycetes
Order*:* *Hypocreales*
Family: Nectriaceae
Genus: *Fusarium*

The genus *Fusarium* currently contains over 20 species. The most common of these are *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium chlamydosporum*. Please refer to the table of synonyms for a much more complete list of the currently recognized *Fusarium spp.*

Macroscopic and microscopic features, such as, color of the colony, length and shape of the macro conidia, the number, shape and arrangement of micro conidia, and presence or absence of chlamydospores are key features for the differentiation of *Fusarium* species [3]. Molecular methods, such as 18S rRNA gene sequencing, may be used for rapid identification of *Fusarium* strains to species level.

2.4. Culturing of the Isolated Fungi

Inoculate the pure culture of ***Fusarium sps*** in the sterilized media and the culture flasks were incubated at 27°C for 15 days.

2.4.1 Media preparation:

Dissolve 24 grams of Potato Dextrose broth in 1 liter of water. This prepared media was sterilized in an autoclave at 121^0C 15 lbs pressure for 15-20 min.

2.5. Extraction of Secondary Metabolites

2.5.1. Preparation of Potato Dextrose Broth Media:

Media is prepared using composition with suitable conditions. A little amount of culture is inoculated into required broth and kept for incubation for a period of 6- 7 days. Change in the media color indicates the production of secondary metabolites. After the production, ethyl acetate as solvent is added to production media in 1:1 proportion. Media along with solvent is kept for incubation in shaker to dissolve the produced compounds into solvent. Later we observed the formation of two layers (broth: solvent).

2.5.2. Separation of Metabolites:

The metabolites which are now dissolved in ethyl acetate solvent are separated by using separating funnels. In the separating funnel add the media with ethyl acetate. To

that add some amount of ethyl acetate, shake well and allow it to settle for a few minutes. Two layers were observed. The bottom layer is discarded and the upper layers of ethyl acetate with metabolites are collected which is called the organic layer. The washes were repeated for three times to extract the complete metabolites. This separated extract was Rota vapored for the collection of crude extract. Crude extract is weighed and stored for further purpose.

2.6. Anti-Microbial Activity of the Crude Extract

The concentrated metabolite collected from Rota vapor is used for anti-microbial activity.

2.6.1. Antibacterial Activity:

The in *vitro* antibacterial activity of the newly isolated secondary metabolites was studied against the bacterial strains, viz., Gram-positive organism's viz. *Bacillus subtilis, Staphylococcus aureus, Streptococcus. epidermidis* and Gram-negative organisms viz *Escherichia coli, Pseudomonas aurogenosa, klebsiella pneumonia* by agar cup diffusion method and the strains were obtained from the Institute of Microbial Technology, Chandigarh. The ready-made Nutrient agar (NA) medium (Himedia, 39 g) was suspended in distilled water (1000 ml) and heated to boiling until it is dissolved completely, the medium and Petri dishes were autoclaved. Agar cup bioassay was employed for testing antibacterial activity **.**The medium was poured into sterile Petri dishes under aseptic conditions in a laminar flow chamber. When the medium in the plates was solidified, 0.5 mL of (week old) culture of test organism was inoculated and uniformly spread over the agar surface with a sterile L-shaped rod. Solutions were prepared by dissolving in DMSO and different concentrations were made. After inoculation, cups were scooped out with 6 mm sterile cork borer and the lids of the dishes were replaced. To each cup, different concentrations of test solutions were added. Controls were maintained with DMSO. The plates were kept at 37^0 C for 48 h. Inhibition zones were measured and the diameter was calculated in millimeter. Three to four replicates were maintained for each treatment.

2.6.2. Antifungal Activity:

The in *vitro* antifungal activity of the newly isolated secondary metabolites was studied against the fungal strains, viz., *Candida albicans, Candida rugosa, Saccharomyces cereviseae, Aspergillus niger* and by agar cup diffusion method and the strains were obtained from the Institute of Microbial Technology, Chandigarh. Controls were maintained with DMSO. The treated and the controls were kept at 27^0 C for 48 h. Inhibition zones were measured and the diameter was calculated in millimeter. Three to four replicates were maintained for each treatment.

2.7. Purification by TLC

Thin layer chromatography is performed to purify the crude extracts. Silica gel (60*120) is taken as stationary phase, silica slurry is prepared and evenly spreaded over silica plates and made it dry in oven at required temperature. Solvents are taken as mobile phase. Silica plate after drying is placed in jar at vertical position by placing a spot at the end of plate marking at 0.5 mm from the margin. Solvents are prepared in different proportions, till the compounds are perfectly separated. After separation of fractions, TLC plate is labeled with dye to observe the fractions in crude extract.

3. Results

3.1. Amount of Secondary Metabolite Extracted per Batch

Secondary metabolites are produced in two different batches. When the organism was inoculated into 1 lit as the first batch 120 mg amount of compound was collected and again for second batch from 500 ml media 80 mg of compound is collected.

3.2. Table 1. Antibacterial Activity of the Crude Extract of *Fusarium sps..:*

Zone of Inhibition in mm						
Conc	Gram Positive Organisms			Gram Negative Organisms		
	Bacillussubtilis	*S. mutant*	*Staphylococccus epidermidis*	*Escherchia coli*	*Pseudomona s.aeroginosa*	*Klebsiella pneumoniae*
50 µg	-	11	-	11	11	0
100 µg	-	11	-	14	12	10
150 µg	14	12	-	14	12	10
200 µg	14	12	-	18	14	12

The above tables show the activity of crude extract against different bacteria. The best activity was observed against *Escherichia coli at* a concentration of 200µg (diameter is 18cm)

Table 2. Antifungal Activity of the Crude Extract

Zone of Inhibition in mm					
Concentration	*candida. rugosa*	*Fusarium oxysporium*	*saccharomyces. cerevisiae*	*Rhizopus. oryzae*	*Aspergillus flavus*
50 µg	10	-	-	12	10
100 µg	10	-	-	15	11
150 µg	12	11	10	17	14
200 µg	14	12	10	20	14

Represents the anti fungal activity of the crude extract against *Rhizopus.oryzae* , *Aspergillus flavus*, *7* , *candida. rugosa*, and *saccharomyces. cerevisiae*. The extract shows activity against all the five fungus.

Measurement of Zones for Anti fungal Activity:

The above tables show the activity of crude extract against different fungi. The best activity was observed against ***Rhizopus oryza*** at a concentration of 200µg (zone diameter is 20mm)

3.3. Separation of Compounds, Chromatography (TLC) by Thin Layer

Thin layer chromatography (TLC) is a chromatography technique used to separate chemical compounds. Crude extract after running TLC, it is known to identify the presence of 3-4 sub compound when separated using Hexane and Ethyl acetate solvents as stationary phase.

R_f value=Distance travelled by the solute / Distance travelled by the solvents

4. Discussion

The ocean is a unique living environment of high salinity, high pressure, low temperature and limited nutrition .To adapt to the environment, marine micro organisms

have evolved distinct metabolism from the terrestrial microorganisms. Marine derived fungi have been recognized as a potential source of structurally novel and biologically potent metabolites, and a growing number of marine fungi have been reported to produce novel bioactive compounds.Marine microorganisms became an important source of pharmacologically active metabolites. These organisms produced various types of metabolites which are capable to inhibit other organisms for competing to the same ecological niche. Secondary metabolites are produced after active growth of the organism with structurally diversified compounds. *Fusarium sp* is one of the marine fungi from which the secondary metabolites are produced. In this present work the *Fusarium sps* is tested against various bacteria and fungi and it showed good antibacterial and antifungal activities. From the TLC it is known that the fungi had 2-3 compounds which have to be purified and identified in future work.

5. Conclusion.

The present work was attempted to isolate organisms which are capable of producing efficient antibacterial and antifungal compounds. Further we identified the organism as *Fusarium sp*. and isolated its secondary metabolites and tested against bacterial and fungal strains. The *Fusarium sps* showed good antibacterial and antifungal activities. The future scope of this work is to isolate these biologically active compounds to use in pharmaceutical applications.

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