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Isolaton of Soft Corals Associated Fungi from Andaman and Nicobar Marine Water and Screening for Antimicrobial and Protease Activity

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In search of bioactive compounds, 3 fungal isolates associated with soft corals were isolated from Andaman and Nicobar marine water. The pure cultures were obtained after a series of sub culturing done and were maintained on slants of 3 different media, Potato Dextrose Agar (PDA), Sabouraud's Dextrose Agar (SDA) and Czapek's Solution Agar (CZSA) for the further experiments. The spores were subjected to Lacto Phenol Cotton Blue staining procedure for the morphological characterization. The 3 isolates FS1, FS2, FS3 were assayed for anti-microbial activity against 5 pathogenic bacteria and 5 clinical fungal strains of which 2 are dermatophytes. It was observed that they potently inhibited *Streptococcus* and *E.coli* among bacteria and *Candida* and one of the dermatophytes. The isolates were further screened for their Protease activity which exhibited positive result. The secondary metabolites produced by fungi will have similar characteristics as those of soft corals in which they live in symbiotic association. So fungi associated with the soft corals are studied for their bio-active compounds as it is easy to culture.

Keywords: Anti-microbial assay, Bio-active compounds, Lacto Phenol Cotton Blue Stain, Protease activity, Soft corals.

Corals are solitary or colonial polypoid coelenterates living in a secreted skeleton of their own. Some grow as massive solid structures, others as large branched colonies. Corals serve as breeding grounds for many marine micro organisms. Marine sponges harbor abundant diverse microbes.

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Up to 40% of the biomass of sponges can be constituted by micro organisms. Most works on corals concentrate on relationships between coral animals, zooxanthellae and calcification[1]. Less attention is paid to other co-existing organisms, such as phototrophic endolithic algae and heterotrophic fungi that may interact with coral in interrelationships other than symbiosis. Endolithic algae and fungi that penetrate coral skeleton are of particular interest. Low light intensities[2] and low fluctuating oxygen pressures[3] within coral skeleton render this environment an ecological extreme, which only a few specialized organisms can endure.

The Andaman and Nicobar (A&N) Islands have a considerable stock of marine resources and are richly fringed by mangroves, coral reefs, sea grasses and seaweed ecosystems[4].

It is well known that coral reefs are economically important for the livelihoods and social welfare of coastal communities, providing up to 25% of the total catch of fish[5]. Further, about 135 species of coral in 59 genera have been reported from the A&N Islands[6]. The Andaman reefs contain about 80% of the maximum coral diversity found anywhere in the world, making them the richest coral reefs in the Indian Ocean and an area of global significance[7,8]. The total area of coral reefs in India is estimated to be 2374.9 km^2 and out of that, about 959.3 km^2 is in the A&N Islands[9].

The presence of endolithic fungi in mollusk shells has been known for more than a century[12] and a number of papers discuss their taxonomic identity and ecological significance[13,14,15]. Less information is available on identity, diversity and ecological roles of fungi in corals. Most reports on coral diseases[16,17,18,19] do not include fungi among coral pathogens. It is assumed that their effect on the host is negligible, or that the fungi in corals are saprophytes that exploit dead organic matter incorporated in coral skeletons by the coral or produced by endolithic algae and cyano-bacteria[20].

Proteases are robust enzymes having wide industrial applications in detergents, leather processing, healthcare, food processing etc[21,22]. Fungi are well known source of extra cellular enzymes and protease from the genus *Aspergillus* has been extensively studied[23]. Fungi from marine environment and endolithic fungi have been screened for protease production. Fungi associated with marine organisms, due to their unusual niche, may be new source to isolate protease. Proteases constitute one of the largest groups of industrially important enzymes.

The present study includes the isolation of fungi from soft corals and obtaining the pure cultures by repeating a series of sub cultures. The isolates were subjected to spore staining and their antimicrobial activity was found against pathogenic fungi as well against bacteria. In the present study marine fungi associated with soft corals were screened for protease activity. Optimization of enzyme production and characterization are future avenues of research which would reveal relevance of these proteases in biotechnology.

MATERIALS AND METHODS

Sampling Site & Collection of Sample

Two field stations Station-I (Pongibalu), 11°30' 0" –11°34' 0" N latitude and 92°38' 0" –92°40' 0" E longitude and Station-II (Chidiya Tapu), Bay of Bengal, 92° to 94° East. Longitude 6° to 14° North Latitude, 25km from Port Blair within the coastal zone of Andaman and Nicobar were sampled during January 2010 (Fig. 1). This coast is known to be flanged by coral reefs with rich coral bio-diversity.

Sediment samples were collected by SCUBA diving, from a depth of 8 – 10m from the coastal waters or with a gravity corer (66 cm length and 7 cm diameter) from these two locations. The overlying water was siphoned out and the cores were cut at 2 cm intervals down to 8 cm and extruded into alcohol sterilized clean plastic containers. They were processed in the laboratory for isolation of fungi and fixed in formalin for direct detection of fungi and bacteria. The remaining sediments were stored at -20°C.

Isolation of fungi

Isolation by particle plating technique

A portion of the sediment from the middle of each sub-section was removed with a flame sterilized spatula and placed in sterile vials for isolation of fungi[25]. The media used for isolations were Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA) and Czapeks Solution agar (CZSA). All the media were used at 1/5 strength to discourage the growth of fast growing fungi. They were prepared in seawater and fortified with streptomycin (0.1 g in 100 ml medium) and penicillin (40,000 Units in 100 ml medium) to inhibit bacterial growth. Fungi were isolated by modified particle plating technique[26]. For this approximately 1 g of sediment slurry was sieved successively through a mesh size of 200 µm and 100 µm screens. The particles that passed through 200 µm mesh but were retained on the 100 µm mesh were spread-plated[27]. Fungi isolated from the sediments were sub cultured and maintained on PDA slants at 5°C. Sporulating cultures were the preserved for further experiment.

Spore Staining

The cultures were subjected to spore staining using Lacto phenol Cotton Blue Stain is recommended for mounting and staining fungi.

Lacto phenol Cotton Blue Stain is formulated with lacto phenol, which serves as a mounting fluid, and cotton blue. Organisms suspended in the stain are killed due to the presence of phenol. The high concentration of the phenol deactivates lytic cellular enzymes thus the cells do not lyse. Cotton blue is an acid dye that stains the chitin present in the cell walls of fungi.

Method of Use

Place a drop of Lacto phenol Cotton Blue Stain in the center of a clean slide. Remove a fragment of the fungus colony 2-3mm from the colony edge using an inoculating loop or teasing needle. Place the fragment in the drop of stain and tease gently. Apply a cover slip. Do not push down or tap the cover slip as this may dislodge the conidia from the conidiophores. Examine the preparation under low and high, dry magnification for the presence of characteristic mycelia and fruiting structures.

Preparation of liquid culture media for the growth of fungal strains and accumulation of antifungal substances

The liquid culture media with different nutritional compositions were used in this experiment for extraction of antifungal substances. The media composition is Glucose (20g/lit), Soy meal (18g/lit), Calcium Carbonate (1g/lit), Magnesium sulphate (0.2g/lit), Di potassium Hydrogen Phosphate (2g/lit), Potassium Chloride (0.5g/lit), Peptone (2g/lit), Casein (3g/lit). The initial pH of each medium was adjusted to 6.0. The experiment was carried out in 500mL Erlenmeyer flasks containing 200mL of medium. Each of the medium was inoculated with 2mL of a 24 hours preculture of all the 3 fungal isolates. Inoculated flasks were incubated at 28 ± 2°C on a rotary shaker at 110rpm for 5 days.

Extraction of antifungal compound into the broth

After completion of the incubation period, each of the liquid culture was centrifuged at 10,000 rpm for 20 min. The cellular pellet was discarded and the supernatant was acidified to pH 2.5 with 6M HCl. The solution was autoclaved at 0.75atm for 10min and then centrifuged again at 8,000rpm for another 10min and the precipitate was again discarded. The supernatant was adjusted to a final pH of 7.0 with phosphate buffer (1M, pH8). This solution was designated as crude supernatant and was used for the antimicrobial activity.

Table 1. Antibacterial activity of 3 isolates

Test	Standard Antibiotic		Fungal isolates		
Organism	Tetracyclin	Erythromycin	FS 1	FS 2	FS 3
Streptococcus	25	16	29	26	35
S.aureus	23	10	23	28	30
B.subtilis	16	18	27	30	25
E.coli	18	13	28	26	31
P. vulgaris	21	13	26	29	31

Table 2. Antifungal activity of 3 isolates

Test	Standard Antibiotic		Fungal isolates		
Organism	Clotrimazole	Flucanozole	FS1	FS2	FS3
Pencillium	10	12	30	25	22
T. mentagrophytes	20	11	42	17	23
Candida	12	7	20	25	20
Rhizopus	8	20	20	21	20
T. rubrum	12	8	32	-	32

Table 3. Protease Activity

Fungal Isolates	Growth on basal medium with 1%w/v skimmed milk	Activity Range
FS 1	Very Good	++
FS 2	Good	++
FS 3	Very Good	+++

Antimicrobial assay by Agar Well Diffusion Method

The 3 cultures were tested for antimicrobial activity against 5 pathogenic fungal strains collected from Dept. of Microbiology, Andhra Medical College, King Goerge Hospital (KGH), Visakhapatnam and 5 standard bacterial strains collected from Dept. of Microbiology, Andhra University, Visakhapatnam.

The Petri plates were spread with SDA and a sterile borer is used to prepare wells in the media with a diameter of 6mm. The test fungal strains were innoculated on the surface of the media using a sterile spreader cleaned with ethanol. Each of the 3 wells of a plate is filled with 25µl crude supernatant, 25µl of standard antibiotics like Tetracycline and Erythromycin (100µg/ml) for antibacterial activity and Fluconazole and Clotrimazole Candid (250µg/ml) for antifungal activity. The plates were inoculated and wells were filled with crude sample as well standard antibiotics and were placed in refrigerator for 1hr so that the antibiotics diffuse into the media. Later, they were placed in the incubator at 28±2°C for 3-5 days. After the incubation period plates were observed for the inhibition zones and measured in mm.

Screening for Protease activity

The innoculum for the spot inoculation was prepared by growing the fungi for 48hrs on PDA. Screening for protease activity was done using modified Gonzalez et al method. A basal medium of composition Ammonium sulphate 0.1%w/v, Potassium dihydrogen phosphate 0.1%w/v, Disodium hydrogen phosphate 0.09%w/v, Magnesium sulphate 0.02%w/v, Yeast extract 0.1%w/v and Agar 3% w/v prepared in 50% v/v filtered seawater was used with 1%w/v of skimmed milk. Cultures were spot inoculated on the plate and incubated at 28°C for 3-5 days. The appearance of clearance zone in the medium around the colony indicated protease activity. The zone was measured from the edge of the colony to the edge of the clearance zone and recorded in mm.

RESULTS

Isolation of fungi

A total of 3 strains of fungi were isolated from the deep sea sediment. Three media PDA, SDA, CZSA were used for the isolation of fungi where all the three media has shown the similar growth characteristics and released their secondary metabolites into the media during their incubation time. The incubation period ranges from 3-5 days for all the 3 media and the temperature maintained during the incubation is 28±2°C. The isolates were named as FS1, FS2, and FS3.

The FS1 (Fig. 1) isolate has a characteristic feature of changing the color of agar into black noticed on the reverse side of the plate since day 2 of incubation irrespective of the composition of media.

The FS2 (Fig. 2) isolate also shows this peculiar characteristic of imparting red color to the agar noticed on the reverse side of the plate in all the 3 media.

The FS3 (Fig. 3) isolate did not show this feature but has a wide spread of black spores throught out the plate.

Spore staining

The spores were obtained from the cultures of all the three media and were stained with Lacto phenol Cotton Blue. The spore structure was observed under the light microscope with 10X and 40X magnifications. All the three fungal strains grown on the 3 different culture media has shown the different spore structures.

The FS1 spores (Fig. 4) appear to be elliptical in shape and spores have taken up the stain Lacto phenol cotton blue.

The FS2 spores (Fig. 5) appear to be present in chains associated with the hyphae and are distorted spherical in shape.

The FS3 spores (Fig. 6) appear to be absolutely spherical in shape.

Antibacterial assay of fungal isolates

The antibacterial activity of these fungal isolates was identified after the specified incubation time. The 5 test organisms used against the 3 fungal isolates were *Streptococcus*,

Isolation of Pure Cultures

Fig. 1. FS 1, FS 2 and FS 3 isolated on CZSA

Fig. 2. FS 1, FS 2 and FS 3 isolated on SDA

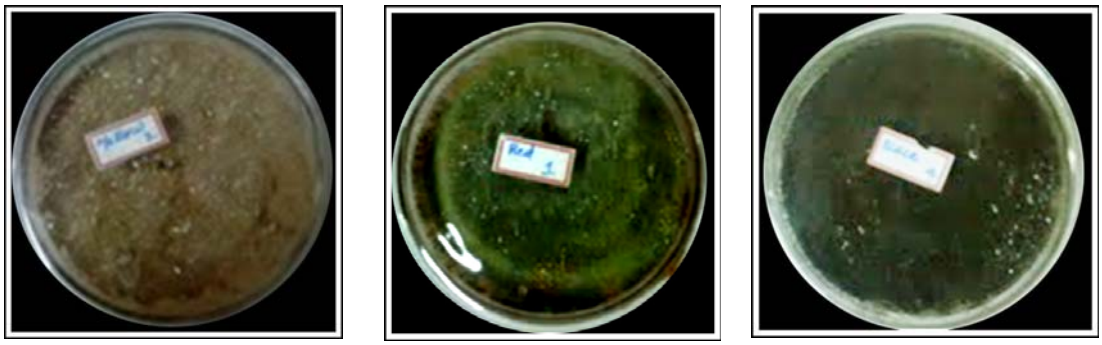


Fig. 3. FS 1, FS 2 and FS 3 isolated on PDA

Spore Staining

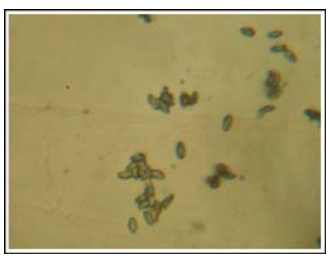


Fig. 4. FS 1

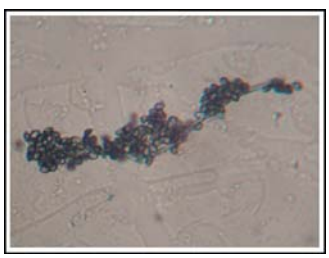


Fig. 5. FS 2

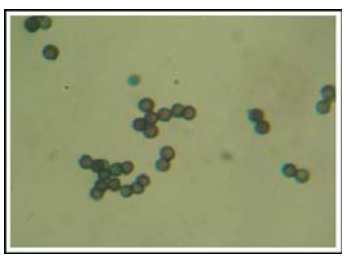


Fig. 6. FS 3

Antimicrobial Activity

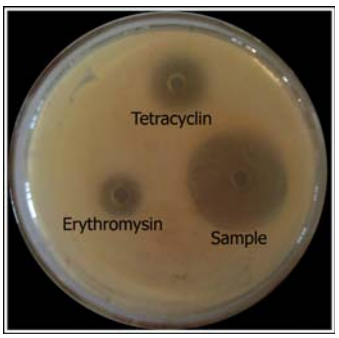


Fig. 7. Antibacterial Activity

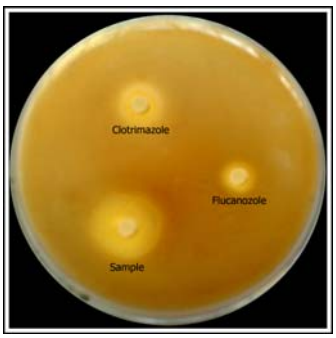


Fig. 8. Antifungal Activity

Protease Activity

Fig. 9. FS1 isolate

Fig. 10. FS2 isolate

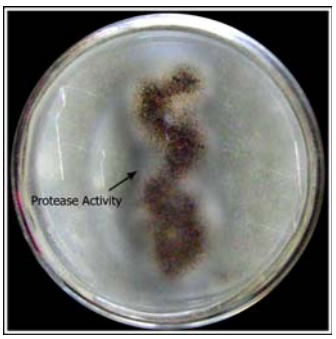


Fig. 11. FS3 isolate

Staphylococcus aureus, *Bacillus subtilis*, *Escherichia coli*, and *Proteus vulgaris.*

The FS1 isolate (Figure 7) has shown the maximum inhibition against *Streptococcus sps.* and minimum against *S. aureus*. The FS2 isolate has shown the maximum inhibition zone against *B. subtilis* and minimum against organisms *Streptococcus* and *E. coli.* The FS3 isolate has shown the maximum inhibition zone against *Streptococcus sps.* and minimum against *B. subtilis.*

The inhibition zones were measured in mm and were tabulated in Table 1 along with Graph 1.

Antifungal activity of fungal isolates

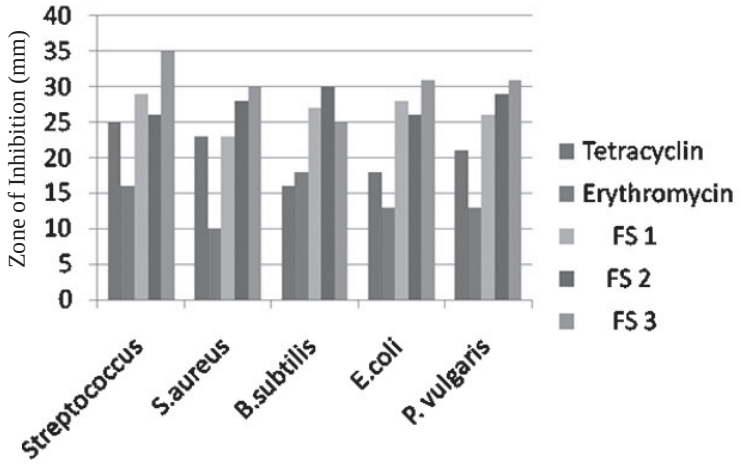
The antifungal activity was found against the test organisms *Pencillium*, *Trichophyton mentagrophytes*, *Candida*, *Rhizopus*, *Trichophyton rubrum*. Of these strains *T. mentagrophytes* and *T. rubrum* are pathogenic dermatophytes.

The FS1 (Figure 8) isolate has shown the maximum inhibition zone against *T. mentagrophytes* and minimum against *Candida* and *Rhizopus sps.* The FS2 isolate has shown the maximum inhibition zone against *Candida* and *Pencillium sps.* and minimum is that there is no inhibition zone against *T. rubrum.* The FS3 isolate has shown maximum inhibition zone against *T. rubrum* and minimum against *Candida* and *Rhizopus sps.*

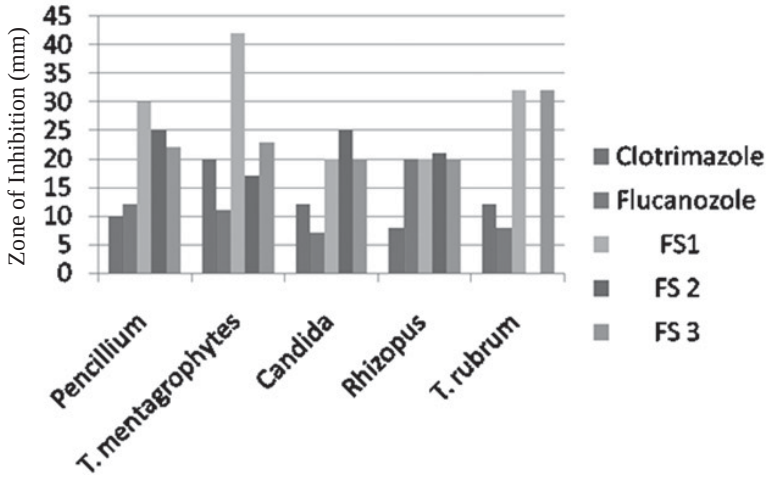
The inhibition zones were tabulated in mm and were tabulated in table 2 along with graph 2.

Protease activity

Almost all the three isolates (Figure 9, 10, 11) have shown protease activity which was observed on the plate with clear growth zones (Table 3) around which the digestion of proteins present in the skimmed milk by the protease enzyme produced by the fungi.



Graph 1. Antibacterial activity



Graph 2. Antifungal activity

DISCUSSION

The three fungal strains have shown the same growth patterns on the 3 media. The spore staining of these isolates has been carefully observed, of which 2 isolates share similar characteristics having round spores freely dispersed as well in chains and the 3rd isolate has spores which are elliptical in shape.

The antibacterial activity infers that the isolates are potent enough in inhibiting the pathogenic bacterial strains. The antifungal activity also infers the same and the strains could inhibit the pathogenic dermatophytes along with *Candida* species which is mainly responsible for dangerous vaginal fungal infections. So the 3 fungal isolates have antibiotic producing capacity which can be exploited for preparing drug against dermatophytes as well *Candida* causing infections.

The protease activity with 1% skimmed milk was found to be good in the case of all the three isolates. The zone of clearance can be easily identified which infers that the skimmed milk present in the medium was subjected to protease enzyme released by these three isolates. Fungal enzymes are gaining importance in agriculture, industry and human health, as they are often more stable (at high temperature and extreme pH) than the enzymes derived from plants and animals

Fungi have proven themselves invaluable sources of natural products for agriculture as well

as biomedical development for over a half century[28,29,30]. As fungi thrive in competitive environments, it is hypothesized that their metabolic compatibility has been strongly influenced by natural selection[31]. Bioactive product discovery depends on the knowledge of habitats where fungi are abundant and the strength of culture collection[32]. Screening this fungal resource for novel metabolites and enzymes and their application are major goals of current research to accomplish environment-friendly technological development.

CONCLUSION

The work clearly infers that the fungal isolates are potent enough in producing antibiotic compound which can be incredibly used for the welfare of mankind against pathogenic fungal and bacterial infections. The isolates are also capable enough for protease production.

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