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Research Article

SCREENING AND IDENTIFICATION OF MARINE SPONGE ASSOCIATED FUNGUS PRODUCING NOVEL BIOACTIVE MOLECULES

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ABSTRACT

The marine environment is a rich source of both biological and chemical diversity, which has been the source of unique chemical compounds with the potential for industrial development. In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms. Several robust new compounds derived from marine natural products are now in clinical pipeline, with more clinical development. In our present study, we had collected the sponge samples from the Bay of Bengal. From that we had isolated microorganisms, screened for activity, extracted the bioactive compound from the selected isolate and identified the isolate. Samples of different sponges are collected by removing a piece of the animal (sponge) and kept in sterile YEME broth in a screw capped tube and stored at low temperatures. We isolated microorganisms from various sponge samples by following screening procedures of which one sample was primarily selected for further studies. From the selected sample namely KSP 03 09 2010, morphological studies of the isolates 2F, 3F, 5F, 6F and 7F were performed and identified as fungal isolates. Phenotypic characterization of the fungal isolate 5F by IMTECH, Chandigarh revealed that it belongs to the genus *Penicillium*. Bioprocess parameters like production medium, sea water composition and incubation time for growth and production of secondary metabolites were optimized to get high yield of the bioactive. Bioautography of the crude extract of 5F revealed that our compound was a mixture of 2- 3 compounds.

Keywords: marine sponges, screening and isolation of fungi, bioactivity, optimization.

INTRODUCTION

The marine environment is a rich source of both biological and chemical diversity, which has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals and agrochemicals. In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms. The marine world offers an extremely rich resource for novel compounds; it also represents a great challenge that requires inputs from various scientific areas to bring the marine chemical diversity up to its therapeutic potential.

Unlike the long-standing historical medical uses of terrestrial plants, marine organisms have a shorter history of utilization in the treatment and/or prevention of human disease. Among the first bioactive compounds from marine sources, spongouridine and spongothymidine from the Caribbean sponge (*Tethya crypta* Fig. 1.1), were isolated serendipitously in the early 1950's. They were approved as an anticancer drug (cytosine arabinoside, Ara-C) and an antiviral drug (adenine arabinoside, Ara-A), respectively, 15 years later. The secondary metabolites of marine organisms have been studied extensively over the past 30 years, since a small number of academic chemists began to isolate and elucidate novel compounds from marine sources in the 1970's. Drug discovery

research from marine organisms has been accelerating and now involves interdisciplinary research including biochemistry, biology, ecology, organic chemistry, and pharmacology. Recently, much attention has been given to marine organisms due to their considerable biodiversity that has been found in the widespread oceans that cover over 70% of the world. Structurally unique secondary metabolites have been isolated and identified from marine organisms and consequently, a compound based on new chemical template has been developed and launched in 2004, while numerous other candidates are in clinical trials.

Marine fungi comprise of an estimated 1500 species, excluding those that form lichens. This number is low compared to the number of described and estimated terrestrial fungi (over 250,000). So far, less than 500 filamentous higher marine fungi have been described and only 79 are associated with algae as parasites or symbionts and 18 with animal hosts. A number of interesting compounds, such as cytoglobosins and halovirs, had been isolated from marine fungi. Hence, we consider that there are numerous marine fungi containing further remarkable structures as well as bioactive compounds.

Marine-derived fungi have been shown in recent years to produce a plethora of new bioactive secondary metabolites, some of them featuring new carbon frameworks hitherto unprecedented in nature. These compounds are of interest as new lead structures for medicine as well as for plant protection. 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. Some marine fungi have unique new carbon frameworks which are exceptional in nature. Compounds produced by such fungi are of interest as new lead structures for medicine as well as for plant protection.

Kjer *et al.,* (2010) have defined a detailed protocol for their isolation and cultivation from various marine organisms (sponges, algae and mangrove plants) in order to characterize and elucidate the structure of secondary metabolites produced by these fungi .

Du *et al.,* (2007) isolated a novel anthraquinone derivative with naphtha [1, 2, 3-de]chromene-2, 7-dione skeleton, and named it aspergiolide A. It was isolated from a marine filamentous fungus, *Aspergillus glaucus* in the Fujian province of China, and was found to exhibit cytotoxicity against K562 and P388 cell lines. The same group has recently worked on the antitumor activities of alkaloids isolated from a *Penicillium sp.* derived from deep ocean sediment. They isolated two new meleagrin analogs, meleagrin D and E, and two new diketopiperazines, roquefortine H and I which showed weak cytotoxicity as compared to the previously reported meleagrin B and meleagrin that induced HL-60 cell apoptosis or arrested the cell cycle through G2/M phase, respectively. They proposed that the distinct substitutions on the imidazole ring could have a significant influence on the cytotoxicity of these alkaloids.

A number of novel compounds and metabolites with bioactive potential continue to be isolated and characterized from marine derived fungi, which are capable of producing not only antimicrobial but also antifouling compounds. In 2006, a bioassay-guided isolation and purification procedure was used to obtain a novel antifouling and antimicrobial compound from a marine-derived fungus *Ampelomyces sp*. The isolate, 3-chloro-2, 5-dihydroxybenzyl alcohol effectively inhibited larval settlement of the tubeworm *Hydroides elegans* and of cyprids of the barnacle *Balanus amphitrite* and was non-toxic; suggestive of a potent antifoulant and/or antibiotic activity. Another study from the same group concerned the antibiotic and antifouling compound production by the marine derived fungus *Cladosporium* sp. F14. They reported that in nutrient enriched cultivation media, this strain produced antibiotic and antifouling compounds in the presence of glucose or xylose. In the search for novel antimitotic and antifungal substances from marine-derived fungi, Gai and coworkers (2007) reported that low concentration of the EtOH extracts of the culture broth of a *Fusarium* sp. (strain 05JANF165) were bioactive. Their search for the basis of this bioactivity led to the identification and purification of a new antifungal antibiotic and the chemical structure was elucidated as Fusarielin E.

A Korea based study resulted in the isolation of a novel antibacterial dioxopiperazine, dehydroxy bis dethio bis-methyl thio-gliotoxin and the previously reported bis dethio bis-methylthio-gliotoxin and gliotoxin from the broth of a marine derived fungus of the genus *Pseudallescheria* and its structure was assigned through NMR. All three compounds exhibited potent antibacterial activity against the methicillin resistant and multidrug resistant *Staphylococcus aureus*, whereas Gliotoxin showed a significant radical scavenging activity against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) with IC_{50} value of 5.2 µM.

Another study from the same source, reported two novel antibacterial aspyrone derivatives *viz*. Chlorohydroaspyrones A and B and the

previously described aspyrone, asperlactone and penicillic acid from the broth of a marine isolate of the fungus *Exophiala* and were found to have mild antibacterial activity against *Staphylococcus aureus.*

Marine fungi have also been reported to have a nematicidal effect, for example, nematicidal and antimicrobial metabolites were reported previously from marine ascomycete, *Lachnum papyraceum* (Karst.) Karst III and the production of novel isocoumarin derivatives were achieved under halogenated conditions.

Nenkep *et al.,* (2010) recently reported the isolation of halogenated benzoquinones (bromochlorogentisylquinones A and B), with significant radical scavenging activity against DPPH, from a marine derived *Phoma herbarum* strain (Ira Bhatnagar and Se-Kwon Kim, 2010).

The aim of this protocol is to give a detailed description of methods useful for the isolation and cultivation of fungi associated with various marine organisms (sponges, algae and mangrove plants) for the extraction, characterization and structure elucidation of biologically active secondary metabolites produced by these marine-derived endophytic fungi, and for the preliminary evaluation of their pharmacological properties based on rapid 'in house' screening systems.

MATERIALS AND METHODS

Isolation of fungi from marine samples

1. Collection of samples

In our present study, we had collected the sponge samples from the Bay of Bengal. From that we had isolated microorganisms, screened for activity, extracted the bioactive compound from the selected isolate and identified the isolate.

Samples of different sponges namely Axinella, Clathria, Haliclona, Callyspongia and Ircinia species are collected from Kulasekharapatnam, Thoothkudi district, Tamil Nadu, India. The samples are collected by removing a piece of the animal (sponge) and kept in sterile YEME broth in a screw capped tube and stored at low temperatures.

Among these, sample from Clathria procera is selected for screening procedure and was coded as KSP 03 09 2010.

2. Isolation of fungi

After collection, the sample, KSP 03 09 2010 was screened for isolation of microorganisms. The sample was taken and macerated to homogenize the sponge sample. From this, 1mL of the broth was taken after proper mixing with the help of micropipette. This 1mL was transferred to 50mL of sterile sea water, mixed well and kept on a rotary shaker at 120 rpm for 30 min. After 30 min, serial dilutions were made from this solution upto the order of 10-10 dilutions by taking 1mL and diluting to 10mL with sterile sea water.

1mL of each of the solution was added to 50mL of sterile molten YEME medium and plated in sterile Petri plates of 30cm size. Antimicrobial agents were also added to the agar medium to prevent the contamination from the surrounding atmosphere. These agents include Rifampicin and Cycloheximide. These were used in combination to prevent the growth of both fungi and bacteria and aid the growth of actinomycetes and Rifampicin only to prevent the growth of bacteria (fungi and actinomycetes both can grow). 1mL of each of the antimicrobial agent was added to 50mL of the agar medium. The plates were then incubated for the growth of the colonies at 27ºC for 10 to 21 days.

3. Isolation and maintenance of cultures

After observation of growth on the plates, the colonies were isolated. This was done by taking/removing a small portion of the colony with the help of an inoculating loop and streaked it on agar slants. YEME medium prepared by using 100% sea water was used for maintenance of the isolated cultures.

The composition of YEME medium was as follows

Yeast Extract	0.4 gm
Malt Extract	1.0 gm
Dextrose	0.4 gm
Agar agar	2.0 gm
Sea water (100%)	100 mL
pH	7.3

4. Preparation of suspension of test organisms

i) Preparation of the McFarland standard

0.5 mL of 0.048M $BaCl_2$ (1.17%w/v $BaCl_2.2H_2O$) was added to 99.5 mL of 0.18M H_2SO_4 (1% w/v) with constant stirring. The standard was distributed into a screw-cap tube of the same size and the volume as those used to prepare the test inoculum. Tubes were tightly sealed to prevent loss by evaporation. Standard was protected from light and stored at room temperature. Before use, turbidity standard was vigorously agitated using a vortex mixer. Standard may be stored up to six months.

ii) Inoculum preparation

Bacterial inoculum preparation: A pure colony of the test organism was taken using a sterile loop and transferred into tubes having a sterile nutrient broth and incubated with shaking at

35ºC -37ºC until the visible turbidity was equal to that of the 0.5 McFarland standard.
Fungal inoculum preparation: Three to seven days slant of fungal culture on potato dextrose agar was taken and scrapped to form a suspension in sterile water. The mixture was vortexed and heavy particles were allowed to settle. The homogenous suspension was adjusted to 0.5 McFarland standards.

iii) Adjustment of the test organism suspension to McFarland standard

Density of the test organism suspension was adjusted to be equal to that of the 0.5McFarland standard. If the bacterial suspension is too turbid, it can be diluted by adding more sterile water. If the suspension is not turbid enough, more bacterial culture can be added. To aid comparison, the suspension and standard against a white background with a contrasting black line can be taken. The suspension should be used within 15 minutes.

iv) Inoculation procedure

Within 15 minutes after adjusting the turbidity of the inoculum suspension, sterile cotton swab was dipped into the suspension. The swab was pressed firmly against the inside wall of the tube above the fluid level, the swab was rotated to remove excess liquid. The swab was streaked over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of inoculum. Finally all round the edge of the agar surface was swabbed.

5 Determination of antimicrobial activity

The antimicrobial activity of the isolates were determined by performing two methods

1. Cross streak method
2. Cup plate method

1. Cross streak method

This method is followed to screen the isolates initially.

Procedure

In this method, molten sterile YEME agar medium was poured in sterile Petri plates and allowed to solidify for about 30min. After solidification of the agar medium, the isolates were streaked in a single straight line in the center of the Petri plates and incubated for growth upto stationary phase (4-5days) where the secondary metabolites are produced in general.

After 4-5 days of growth, test organisms were streaked in perpendicular to that of the isolate. The line of streaking was made from the near end of the isolate and continued to the rear end. The test organisms used include bacterial (G+ve and G-ve) and fungal species respectively. The plates cross streaked with bacteria were incubated at 37ºC in an incubator and those with fungi at 28ºC at room temperature. Results were observed after 24 hrs for bacteria and 3-4 days for fungi respectively.

2. Cup plate method

After observation of results, the extent of the activity of the isolates was determined by observation of zones of inhibition. This was done by cup plate method. In this method submerged fermentation was followed for the isolates to obtain the bioactive principle which was then used to determine the activity of the isolate.

Procedure

In this method submerged fermentation was done by selecting a medium where the isolate produces secondary metabolites in the medium. P.M-2 was selected for submerged fermentation procedure for the detection.
Initially inoculum medium was prepared to increase the cell mass of the isolate. This was then added to fresh sterile production medium to obtain the secondary metabolite. Both inoculum and production media used were same.
Inoculum medium was prepared by adding 5 mL suspension of the microorganism to 45 mL of sterile inoculum medium. Sea water at a concentration of 50% was used for the preparation of production medium. Then it was kept on rotary shaker at 120 rpm for 2 days. After 2 days, 5-10% of inoculum was transferred to sterile production medium, kept on shaker for 5 days.
At the end of 7^{th} day of the total production, the mycelium and the supernatant were separated by centrifugation in a rotary centrifuge at 7000 rpm for 10 min. After separation, the supernatant was extracted with ethyl acetate and the ethyl acetate fractions were concentrated in a rotary evaporator.
The activity was determined for this concentrate by cup plate method by measuring the zone of inhibition. This was done by adding 50µL of the concentrate to the solidified agar medium which was previously inoculated with test organism against which activity was to be determined. The plates were kept in a refrigerator for about 15min for diffusion of the concentrate into the surroundings and then kept for incubation at 27ºC for fungi and 37ºC for bacteria.

6. Selection of isolates

After observing the results of both cross streak and cup plate techniques, the isolates 2F, 3F, 5F, 6F and 7F were selected for further studies for obtaining the bioactive molecule by optimization studies.

Taxonomy of the selected isolates

Morphological studies

Characteristics of spores and spore bearing structures (hyphae) and spore chains are determined by direct microscopic examination of the spore surface with adequate magnification 40X and 100X.

Morphological characterization of the isolates 2F, 3F, 5F, 6F and 7F were done for preliminary identification. This was done by observing the thin smear on Petri plate that consists of the microorganism in its early stages of growth under Trinocular microscope with different magnifications. Features observed under the microscope were structure of hyphae, aerial mycelium, presence of spores, fragmentation etc.,

Smear of the isolate was prepared by adding 0.5mL of suspension of the isolate to dried thin smear of YEME medium on a sterile Petri plate and spreading the suspension by gently rotating the Petri plate.

7. Phenotypic characterization

Phenotypic characterization of the promising isolate (Identification of the isolate 5F to species level) was further done by IMTECH, Chandigarh, India.

8. Fermentation and extraction of bioactive molecules from the selected isolate:

The selected isolate 5F was kept for submerged fermentation process to extract the bioactive molecules.

Fermentation procedure

Inoculum medium

Soy bean meal	1.0 gm
Dextrose	1.0 gm
Calcium Carbonate	0.1 gm
Sodium Chloride	0.5 gm
Sea Water (50%)	100 mL

Production medium

Soy bean meal	1.0 gm
Dextrose	1 .0gm
Calcium Carbonate	0.1 gm
Sodium Chloride	0.5 gm
Sea Water (50%)	100 mL

4-5 days slants of the isolate were selected for inoculum preparation. To that slants 5 mL of sterile distilled water was added, made to a suspension and then added to 45mL of production medium sterilized in 250 mL conical flasks. After transfer of the spore suspension, the flasks were kept on a rotary shaker at 130 rpm for 3 days. At the end of 3rd day, 10% of the inoculum medium was added to freshly prepared sterile production medium, kept on rotary shaker for 5 days.

Extraction

After 7 days of production, the mycelium and the supernatant were separated by centrifugation in a rotary centrifuge at 7000 rpm for 10 min. After separation, the supernatant was extracted with ethyl acetate and the ethyl acetate fractions were concentrated in a rotary evaporator.

The concentrated extract was used to determine the activity and purification of the bioactive molecule.

9. Biological characterization of active compounds

The biological characterization was done by bioautographic technique. It is used to determine the biological activity of the crude extract. Paper chromatography followed by bioautography was used for the first time in1946 by Goodall and Levi to estimate the purity of penicillin. This was called contact bioautography (i.e., the developed plate/ paper chromatogram was placed onto the inoculated agar layer enabling the diffusion of antibiotics from paper to agar).

Procedure:

About 0.02mL of crude extract was loaded onto the precoated TLC plate using capillary tube using ethyl acetate as mobile phase. The developed chromatograms were dried in air completely until the smell of solvent escapes and then these plates were aseptically transferred on to the inoculated agar plate. The plates with the chromatograms were refrigerated for about 3 hrs for complete diffusion of the crude extract into the agar. After 3hrs, the TLC plates were removed aseptically and the sterile petri plates were then incubated at 37ºC for 24 hrs for growth of test organisms.

10. Optimization studies

In our study, we optimized some bio process parameters for obtaining the maximum yield of the bioactive molecule. The bio process parameters include percentage of sea water, pH, production medium, incubation time.

1. Optimization of production medium

Five different media were used for the optimization of production medium. The production was carried for 7 days initially to

determine the suitable medium. The composition of the media were

Production medium- 1	
Soy bean meal	1.0 gm
Corn steep liquor	0.5 gm
Soluble starch	1.0 gm
Dextrose	0.5 gm
Calcium Carbonate	0.7 gm
Sea Water (50%)	100 mL
pH	7.2

Production medium- 2	
Soy bean meal	1.0 gm
Dextrose	1.0 gm
Calcium Carbonate	0.1 gm
Sodium Chloride	0.5 gm
Sea Water (50%)	100 mL

Production medium-3	
Glycerol	2.0 gm
Peptone	0.5 gm
Yeast Extract	0.3 gm
Malt Extract	0.3 gm
Calcium Carbonate	0.25 gm
Sea Water (50%)	100 mL
pH	6.8

Production medium -4	
Corn steep liquor	1.0 gm
Soluble starch	2.5 gm
Ammonium Sulphate	0.5 gm
Calcium Carbonate	0.5 gm
Sea Water (50%)	100 mL
pH	6.8

Production medium -5	
Glucose	1.0 gm
Starch	1.0 gm
Peptone	0.75 gm
Meat Extract	0.75 gm
Sodium Chloride	0.3 gm
Sea Water (50%)	100 mL
pH	6.5

Procedure

4-5 days slants of the isolate were selected for production. To that slants 5 mL of sterile distilled water was added, made to a suspension and then added to 45mL of production medium sterilized in 250 mL conical flasks. The production was kept for 7 days and at the end of 7th day, the production was stopped, centrifuged, separated the mycelium and supernatant, extracted the supernatant with ethyl acetate, concentrated the extracts and tested for activity against test organisms using cup plate method.

2. Optimization of sea water concentration

Water is essential for the growth of microorganisms, particularly in submerged fermentation. The composition of sea water used in production influences the growth and production of secondary metabolites.

Six different compositions of sea water are used for optimizing P.M-2 medium for obtaining the maximum yield of the bioactive molecule. The different concentrations used were

- 100% sea water + 0% distilled water
- 80% sea water + 20% distilled water
- 65% sea water + 35% distilled water
- 50% sea water + 50% distilled water
- 20% sea water + 80% distilled water
- 0% sea water + 100% distilled water

Procedure

Production medium P.M-2 was made with the above concentrations and 4-5 days slants of the isolate were selected for production. To that slants 5 mL of sterile distilled water was added, made to a suspension and then added to 45mL of production medium sterilized in 250 mL conical flasks. The production was kept for 7 days and at the end of 7th day, the production was stopped, centrifuged, separated the mycelium and supernatant, extracted the supernatant with ethyl acetate, concentrated the extracts and tested for activity against test organisms using cup plate method.

3. Optimization of incubation time

Time plays a crucial role in the production of secondary metabolites in microorganisms. The production of secondary metabolites by microorganisms differs from one another. Hence in our study, optimization of incubation time is done to produce high amount of secondary metabolite by the isolate.

The time required for the isolate to produce the bioactive molecule in high amounts is called the optimum incubation period. P.M-2 medium prepared by using 50% sea water

was kept for production upto 8 days in different flasks. Each of the flasks was removed at the end of 5th. 6th, 7th and 8th day of production, centrifuged, extracted with ethyl acetate, concentrated and the extent of the bioactive molecule production was determined by following cup plate technique.

Results

Sample collection

A total of 40 fungal species were isolated from 8 different sponges (Fig.1&2) collected from Kulasekharapatnam, Thoothukudi district, Tamil Nadu in sterile screw capped bottles containing YEME medium(Fig.3).

Screening

The number of isolates obtained from each sample through screening (Fig.4 &5) was shown in table 1.

Determination of antimicrobial activity

1. Cross streak method

Results (Table: 2 and Fig. 6 to10) were observed after the incubation time of the test organisms. All the isolates were found to inhibit the growth of the test organisms and were considered as bioactive isolates.

2. Cup plate method

The results of cup plate method were shown in Table: 3 as observed

From the results of zones of inhibition (Fig. 11, 12 & 13), it was evident that the fungal isolates 2F, 3F, 5F, 6F and 7F were found to possess higher antimicrobial activity than the remaining fungal isolates. Also these fungal isolates didn't inhibit the growth of the pathogenic fungus *Candida albicans* but able to inhibit the other test organisms.

Taxonomy of the selected isolates

Morphological studies of the isolates 2F, 3F, 5F, 6F and 7F revealed the spore structures, their characteristics and were preliminarily identified it to be fungal isolates under the Trinocular microscope (Fig. 14 to 20).

Phenotypic characterization of the fungal isolate 5F performed by IMTECH, Chandigarh, India reported the fungal isolate to be *Penicillium citrinum* with reference number MTCC 10984.

Colonies with restricted growth, blue-green, reverse bright with the same pigmentation diffusing into the agar, conidiophores smooth-walled stipes, bearing divergent whorls of metulae, conidia globose to subglobose, smooth walled, 1-celled were found to be some of the unique characteristics of the fungal isolate 5F.

Fermentation procedure

Fermentation was carried out for the selected fungal isolate 5F with P.M-2 made with 50% sea water.

Biological characterization of active metabolite

TLC of the compound was run with 100% Ethyl acetate as mobile phase. The bioactive molecule moved along with the solvent and at the end, the spot was found at the top of the chromatogram (Fig.21).

After incubation for growth of the test organism, the crude extract was found to inhibit the growth of the test organism indicated by the zone of inhibition around the diffused extract (Fig.22 & 23).

Optimization studies

1. Optimization of production medium

Five different media were used for optimization of production medium. The extracts were determined for antimicrobial activity and amount of bioactive metabolite production by measuring the inhibition zone diameters of the crude extracts (Fig. 24 to 27) on test organisms *Bacillus pumilis* and *Proteus vulgaris* respectively.

The observations were shown in table 4 and the inhibition zone diameters showed that our fungal isolate produced more amount of bioactive compound in P.M-2 followed by P.M-4.

2. Optimization of sea water concentration

P.M-2 was selected and optimized with 6 different compositions of sea water for high production of bioactive metabolite.

The results were shown in table 5

P.M-2 medium with 50% sea water concentration showed good zone of inhibition on test organisms *Bacillus pumilis* and *Proteus vulgaris* respectively (Fig. 28 & 29).

3. Optimization of incubation time

The results were tabulated in table 6 and indicated the production of bioactive metabolite was more after 7th day and decreased on 8th day (Fig. 30 & 31).

DISCUSSION

In our study, we isolated microorganisms from various sponge samples by following screening procedures of which one sample was primarily selected for further studies. From the selected sample namely KSP 03 09 2010, the bioactivity of the isolates were determined by performing cross streak and cup plate techniques. Among them isolates showing good antimicrobial properties were selected and phenotypic characterization of a

fungal isolate 5F was carried out to identify it. Bioprocess parameters like production medium, sea water composition and incubation time for growth and production of secondary metabolites were optimized for the fungal isolate 5F to get high yield of the bioactive molecule. Bioautography was also performed to determine the presence of mixtures in the bioactive compound of the fungal isolate 5F.

CONCLUSIONS

A total of 40 fungi were obtained through screening from 8 different sponge samples. From the selected sponge sample KSP 03 09 2010, 10 fungi were isolated and screened for antimicrobial activity where only 9 fungi were found to be active against test organisms. From the sample KSP 03 09 2010, the isolates 2F, 3F, 5F, 6F and 7F were selected from the inhibition zone diameters for further studies. Results from antimicrobial activity revealed that our isolate KSP 03 09 2010 5F was found to have broad spectrum of antimicrobial activity on both types of bacteria (G+ve and G-ve) and fungi. Morphological studies of the isolates 2F, 3F, 5F, 6F and 7F were performed and identified as fungal isolates. Phenotypic characterization of the fungal isolate 5F by IMTECH, Chandigarh revealed that it belongs to the genus *Penicillium* and identified it to be *Penicillium citrinum*. Bioprocess parameters like production medium, sea water composition and incubation time for growth and production of secondary metabolites were optimized to get high yield of the bioactive molecule and observed that P.M-2 with 50% sea water concentration for 7 days showed good yield of the bioactive molecule. Bioautography of the crude extract of 5F revealed that our compound was a mixture of 2- 3 compounds and have to be purified. Finally, the purification and characterization of the bioactive molecule from the crude extract have to be done.

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Fig.1: Callyspongia diffusa

Fig. 2: Clathria procera

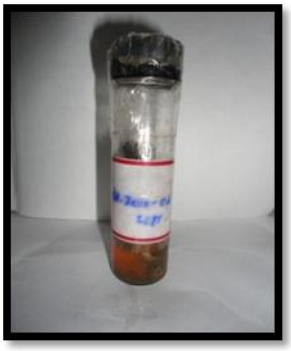

Fig. 3: Sample Collected

Table 1:

Sl no	Sample No.	Name of the sponge	No of fungal isolates
1.	KSP 03 09 2010	*Clathria procera*	10
2.	KSP 07 10 2010	*Haliclona sps*	10
3.	KSP 12 10 2010	*Callyspongia diffusa*	6
4.	KSP 02 10 2010	Unidentified sponge	2
5.	KSP 05 10 2010	*Ircinia sps*	3
6.	KSP 06 10 2010	Unidentified sponge	-
7.	KSP 01 10 2010	*Axinella doneni*	8
8.	TCH 08 10 2010	*Ircinia sps*	2

Screening plates: (Fig..4 & 5)

Fig. 4:

Fig. 5:

Table2:

Sl no	Isolate No	Test organism						
		P.c	***A.n***	***A.o***	***B.p***	***S.a***	***E.coli***	***P.a***
1.	1F	+	-	-	+	+	+	+
2.	2F	+	+	-	+	+	+	+
3.	3F	+	+	+	+	+	+	+
4.	4F	+	+	+	+	+	+	-
5.	5F	+	+	+	+	+	+	+
6.	6F	+	+	+	+	+	+	+
7.	7F	+	+	+	+	+	+	-
8.	8F	-	-	-	-	-	-	-
9.	9F	+	+	+	+	+	+	+
10.	10F	+	+	+	+	+	+	+

'+'- indicates inhibition of growth;
'-'- indicates no growth inhibition
P.c-Penicillium chrysogenum; A.n- Aspergillus niger; A.o- Aspergillus oryzae; B.p- Bacillus pumilis; S.a- Staphylococcus aureus; E.coli- Escherichia coli; P.a- Pseudomonas aerogenosa.

Results of cross streak technique of different fungal isolates

Fig. 6: KSP 03 4F

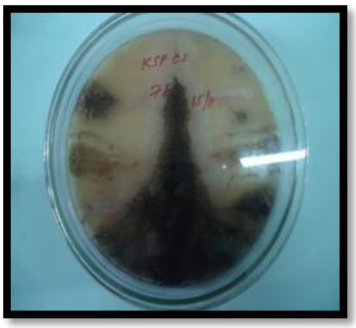

Fig. 7: KSP 03

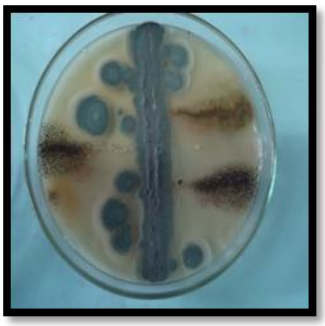

Fig. 8: KSP 03 5F

Fig. 9: KSP 03 2F

Fig. 10: Agar overlay of KSP 03 5F

Table 3:

Sl. No	Isolate	Inhibition zone diameters in mm						
		Test organism						
		P.c	***A.n***	***A.o***	***C.a***	***S.a***	***E.coli***	***P.a***
1.	1F	18	20	15	-	13	14	15
2.	2F	22.5	22	20	-	20	20	17
3.	3F	20	30	18	-	19	18	11
4.	4F	16.5	20	20	-	10	9	-
5.	5F	22	20	20	-	22	20	-
6.	6F	23	20	20	-	30	16	17
7.	7F	25	22.5	20	-	13	11	-
8.	8F	-	-	-	-	-	-	-
9.	9F	10	12	10	-	18	17	-
10.	10F	12	15	13	-	22	-	20
11.	Ethyl acetate (blank)	-	-	-	-	-	-	-

P.c-Penicillium chrysogenum; A.n- Aspergillus niger; A.o- Aspergillus oryzae;
C.a- Candida albicans; S.a- Staphylococcus aureus;
E.coli- Escherichia coli; P.a- Pseudomonas aerogenosa.

Fig. 11:

Fig. 12:

Fig.13:

Inhibition zones of the crude extracts on fungi and bacteria

Morphological observation of fungal isolates under 40X magnification

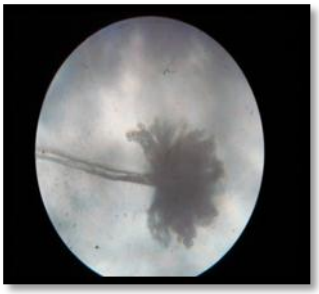

Fig. 14 2F:

Fig. 15 3F:

Fig. 16 5F:

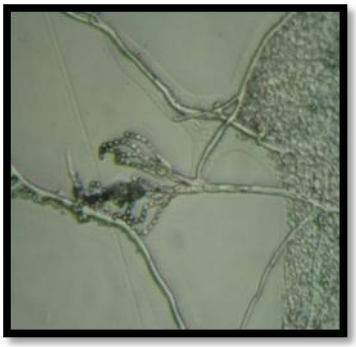

Fig. 17 5F:

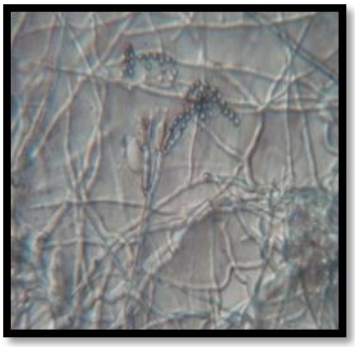

Fig. 18 6F:

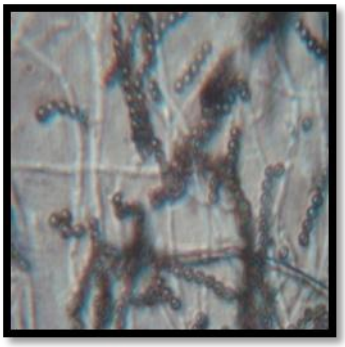

Fig. 19 6F:

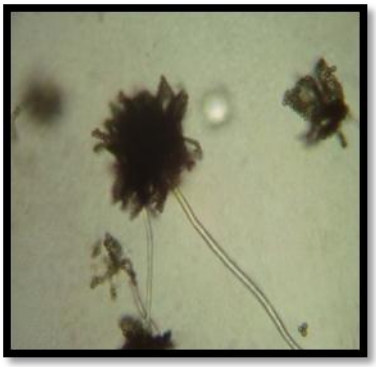

Fig. 20 7F:

TLC of the crude extract 5F

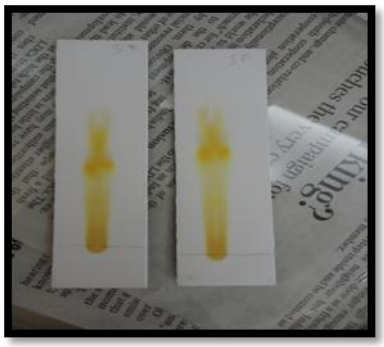

Fig. 21:

Bioautography of the extract 5F

Fig. 22:

Fig. 23:

Table 4: optimisation of production medium

Sl.no	Production medium(P.M)	Inhibition zone diameter in mm	
		Test organism	
		B.p	P.v
1.	P.M-1	5	5
2.	P.M-2	19	18
3.	P.M-3	7	5
4.	P.M-4	17	17
5.	P.M-5	-	-

B.p- Bacillus pumilis; P.v- Proteus vulgaris

Fig. 24:

Fig. 25:

Fig. 26 :

Fig. 27:

Inhibition zone diameters for production media P.M-1, P.M-2, P.M.-3 P.M-4 and P.M.-5

Table 5: optmization of sea water concentration

Sl.no	Composition of sea water	Inhibition zone diameter in mm	
		Test organisms	
		B.p	***P.v***
1.	100%	2	-
2.	80%	-	-
3.	65%	-	-
4.	50%	22	20
5	20%	-	-
6.	0%	4	2

B.p- Bacillus pumilis; P.v- Proteus vulgaris

Fig. 28:

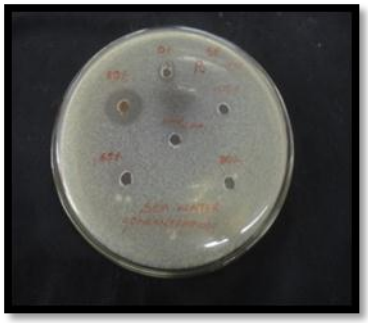

Fig. 29:

Inhibition zone diameters of the crude extract of different compositions of sea water

Table 6: optimisation of incubation time

Sl.no	Incubation time	Inhibition zone diameter in mm	
		Test organisms	
		B.p	***P.v***
1.	5th day	6	4
2.	6th day	12	10
3.	7th day	19	18
4.	8th day	10	8

B.p- Bacillus pumilis; P.v- Proteus vulgaris

Fig. 30:

Fig. 31:

Inhibition zone diameters of the extract 5F on *B.pumilis* and *P. vulgaris* on 7th day

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