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Isolation and characterization of Marine fungal metabolites against clinical pathogens

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ABSTRACT

Objective: To isolate and identify of marine fungal metabolites against clinical bacterial pathogens. To optimize the production medium for isolated fungus. **Method:** Marine fungus isolated from water and sediment samples from different places of Sundarbans mangrove, Muttukadu (Chennai) and Parangipettai in India. Antimicrobial substance from marine fungi was produced by agar plate method. The potent fungal were inoculated on production medium and extracted was done. The extracted compound was checked for anti bacterial activity. Suitable production medium were optimized. **Result:** Totally 30 fungal isolates were recovered and morphologically 10 different strains were belongs to the fungal genera such as *Fusarium*, *Aspergillus*, *Mucor* and *Penicillium*. Preliminary screening results showed 3 fungal isolates showed promising activity. After production of potent fungal SS2 crude extracts showed highest inhibition against the bacterial pathogens out of 3 fungal isolates. The results showed maximum zone in 20mm against *E.coli* and minimum 10 mm against *Vibrio* sp. **Conclusions:** The present study identified *Fusarium* sp isolated from Sundarbans mangrove water as a potential source for bioactive compounds. Further isolation of active compound from potential fungal isolates will leads to the discovery of effective antimicrobials.

1. Introduction

New trends in drug discovery from natural sources emphasize on investigation of the marine ecosystem to explore numerous complex and novel chemical entities. These entities are the source of new lead for treatment of many diseases such as cancer, AIDS, inflammatory condition, arthritis, malaria and large variety of viral, bacterial, fungal diseases [1]. Because of the highly chemical and physical harsh condition in marine environment, the organisms produce a variety of molecules with unique structural features and exhibit various biological activities [2].

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

compounds are necessary to combat these multidrug 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].

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 [4]. The introduction of penicillin and other antibiotics ushered in an era of effective treatment of microbial infections. However, with the overuse of antibiotics, resistance of pathogens to antimicrobial agents has become an escalating problem [5]. In the past several years, only a few new antibacterial agents have received approval by the US Food and Drug Administration [6]. Therefore, there is an urgent need for effective new antimicrobial agents.

Until recently, the search for new fungal metabolites concentrated mainly on the random screening of isolates. In optimizing the search for new bioactive secondary

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metabolites, it is relevant to consider that the 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 [7].

2. Materials and Methods

2.1. Isolation of marine fungi

The sediment and water sample were collected from the three areas of Sundarbans mangrove, Muttukadu (Chennai) and Parangipettai in India. The samples were diluted at 10 and 100 fold with autoclaved filtered seawater (0.22 μ m). Aliquots of 200 ml from each water sample were spread onto nutrient agar plates (containing 2% glucose, 1% peptone, 2% agar and filtered seawater). The antibiotics streptomycin and penicillin, at final concentrations of 100 and 50 mg^{-1} , respectively, were added to each agar plate to inhibit the growth of bacteria. Two replicate agar plates were used for each sample. After 2 days of incubation at 27 °C, the inoculated agar plates were examined daily for the presence of developing fungal hyphae, using a dissecting microscope at 20 \times magnification. Distinct fungal colonies on the agar plates were then transferred to new agar plates for further isolation and purification [8].

2.2. Identification of the Fungal Isolates

Micromorphology 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 °C for 4–5 days, the relative levels of fungal growth at certain conditions were determined.

2.3. Test Bacteria

Five clinical bacterial pathogens such as *Bacillus* sp., *Staph. Aureus*, *Pseudomonas* sp, *E.coli*, *Klebsiella pneumoniae* and *Vibrio* sp. were obtained from Dept of Microbiology, Sri Sankara Arts and Science College, Kanchipuram, Tamil Nadu.

2.4. Primary screening of antimicrobial substance from marine fungi

All the fungal cultures were inoculated in to fermentation broth supplemented with Filtered seawater and incubated

at 28 °C 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 were load in a sterilized disc placed over the MHA plates inoculates with 5 test organisms as described above. All the plates were incubated at 37 °C for 24 hours. The zone of inhibition was measured and expressed in diameter in millimetre. Based on the results of preliminary screening best three fungal isolates were selected as potential strains for further investigations [11].

2.5. Production of antimicrobial substance from marine fungi

All the potential fungal isolates were inoculated in to each Nutrient Agar plates and incubated at 28°C for 3 weeks. Then the mycelia growth was removes aseptically using sterile spatula. The agar medium was cut in to pieces and extracted using (1:2 ratio) acetone and ethyl acetate respectively, for 24 hours. The acetone and ethyl acetate portion was collected and concentrated by evaporation. Quantity of crude extract was measured and the stock solution was prepared in the concentration of 10mg/ml of acetone and ethyl acetate respectively.

2.6. Antimicrobial activity of fungal extracts

Antimicrobial activity of fungal extracts was tested by using paper disc diffusion method [11]. 10 μ l of crude extract from stock solution was added in to sterile filter paper disc (5mm in diameter) and allowed to dry. Final concentration of crude extract is 100 μ g/disc. The crude extract impregnated discs were placed over MHA plates inoculated with test organisms. All the plates were incubated at 37°C for 24 hours. The zone of inhibition was measured and expressed in diameter in millimetre.

2.7. Medium Optimization

All experiments in this study were carried out in duplicates shaking cultures to determine the physiological and physical conditions that would affect the antimicrobial agent production of the selected strain. Sucrose as a carbon source in Nutrient broth medium (containing Sea water) was replaced by various carbon sources at a concentration of 2% (w/v) to study the effects of different carbon sources on antimicrobial agent production by SS2. The effect of addition of equimolar amounts of different inorganic and organic nitrogen sources to enhance the antimicrobial agent production by SS2 was also studied. Since, Yeast extract might be considered as a growth factor and/or nitrogen source. So, different concentrations of yeast extract were added to test their effect on the antimicrobial agent production. The modified B.S.M was adjusted to appropriate pH value of 4.0 to 9.0 to study the effect of the initial pH values on antimicrobial agent production.

2.8. Time Course of Antimicrobial Agent Production

Time course from one to eight days was followed in shaking incubated flasks containing the modified B.S.M having the optimized culture conditions. The optimum period of incubation for maximum antimicrobial agent production was determined.

3. Results

3.1. Isolation and characterization of marine fungi

Totally 30 fungal isolates were recovered and morphologically 10 different strains were sub cultured and maintained for further analysis. The Microscopic and Cultural characteristics of the isolates were observed. *Aspergillus* species were found in the Sundarbans mangrove, Muttukadu (Chennai) and Parangipettai samples, but *Fusarium* sp only found in the Sundarbans mangrove samples. *Penicillium*, *Mucor* in the Muttukadu (Chennai) sample but not found in the Sundarbans mangrove and Parangipettai samples. The fungal isolates were belongs to the genera *Aspergillus*, *Penicillium*, *Mucor* and *Fusarium*. 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 but PS5 colonies were not show good in the NaCl contain medium.

3.2. Antimicrobial activity of marine fungi

Out of 10 fungal isolates, 3 isolates were showed

antibacterial activity against tested bacterial pathogens maximum zone in 18mm against *E. Coli* and minimum 10mm against *klebsiella pneumonia* (Table 1). The fungal strains SS2, BS8 and PS8 were selected for the secondary screening. Antimicrobial activity of both ethyl acetate extracts showed highest 20mm against *E.coli* and minimum 10mm against *Vibrio* sp. were observed in (Table 2) acetone extract.

3.3. Medium Optimization

This part of work aims at the optimization of some culture conditions to attain maximum antimicrobial agent production. Of all the tested carbon sources (1% w/v) in the basal salt medium (B.S.M) inoculated with SS2 and incubated shaken for 5 days, starch supported the highest level of antimicrobial agent production (Figure 1)

Addition of equimolar amounts of various inorganic and organic nitrogen sources in the B.S.M supplemented with 2.0% (w/v) show that Potassium nitrate was a good inorganic nitrogen source on the antimicrobial agent production, while no obvious effect was detected with organic nitrogen sources (Figure 2). 0.24% (w/v) was the best concentration of potassium nitrate for optimum antimicrobial agent production by SS2. The requirements for specific nitrogen supplement differ from one microorganism to another. In most microorganisms both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. However, it was found that some carbon and nitrogen sources had an inhibitory effect on the antimicrobial agent production and this may be due to organic acid accumulation, oxygen depletion or sugar catabolic repression. No noticeable increase in antimicrobial agent production was detected at

Table 1

Antimicrobial activity of marine fungi (zone of inhibition in mm)

Strain no	<i>Bacillus</i> sp	<i>Staph aureus</i>	<i>Pseudomonas</i> sp	<i>E.coli</i>	<i>Klebsiella pneumoniae</i>	<i>Vibrio</i> sp
SS2	16	–	18	18	17	18
SW3	–	12	–	–	–	14
SS7	–	11	–	–	12	–
BW2	11	–	–	16	–	–
BS8	14	–	15	17	–	12
BS9	–	11	–	11	–	–
PW3	13	–	–	–	–	–
PW4	–	–	12	–	–	–
PS5	–	–	–	12	–	–
PS8	–	12	12	–	10	14

Table 2

Antibacterial activity of fungal extracts

Test organisms	Extracts (zone of inhibition in mm (disc size 3mm))					
	Acetone extract			Ethyl Acetate		
	SS2	BS8	PS8	SS2	BS8	PS8
<i>Bacillus</i> sp.	11	12	–	15	–	14
<i>Staph. aureus</i>	14	17	12	18	20	–
<i>Pseudomonas</i> sp	18	15	–	–	16	–
<i>E.coli</i>	20	–	11	20	–	13
<i>Klebsiella pneumoniae</i>	–	–	–	–	17	–
<i>Vibrio</i> sp	15	10	11	17	–	16

various yeast extract concentrations (Figure 3).

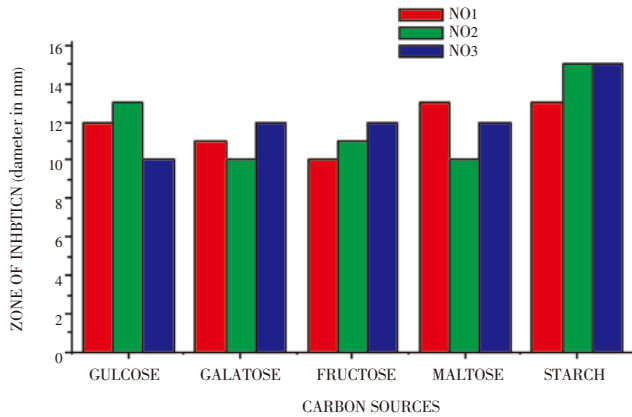


Figure. 1: Effect of various Carbon sources on antimicrobial agent production by *Fusarium* species

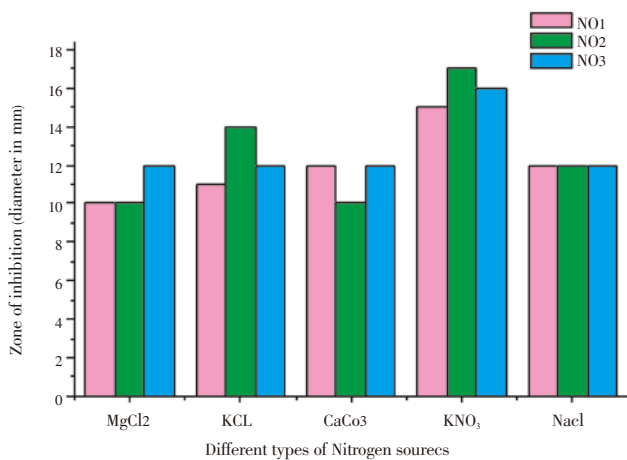


Figure. 2: Effect of different inorganic and organic nitrogen sources for antimicrobial agent *Fusarium* species

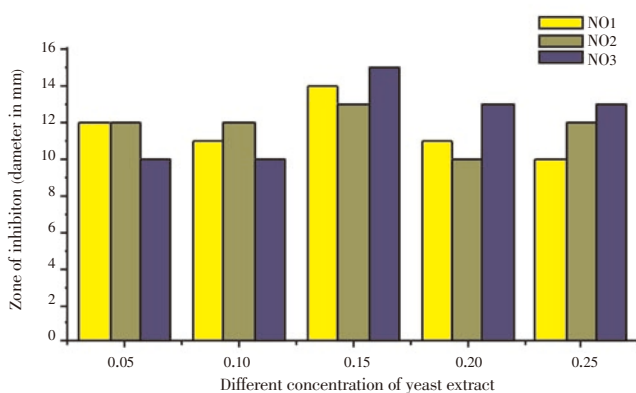


Figure. 3: Effect of addition of the different yeast extract concentrations for antimicrobial agent production by *Fusarium* species

The antimicrobial agent production was affected by the initial pH values of the medium, ranging from pH 5.0 to 9.0. Results indicated that the suitable pH for antimicrobial agent production by SS2 was at pH range from 6.0 to 7.0 while

it was optimum at pH 6.0 (Figure 4).

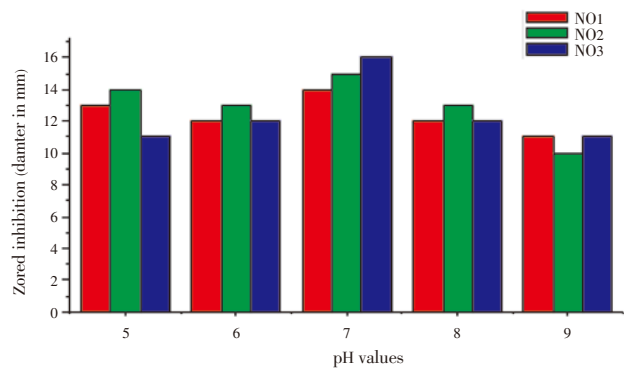


Figure. 4: Effect of initial pH values on antimicrobial agent production by *Fusarium* species

3.4. Time Course of the Antimicrobial Agent Production on the Optimized Medium

Time course from one to eight days was followed in shaking incubated flasks containing the optimized culture conditions inoculated with SS2 (Figure 5). The antimicrobial agent production seems to be stable on the 6th and 7th days of the incubation period, having maximum on the 7th day.

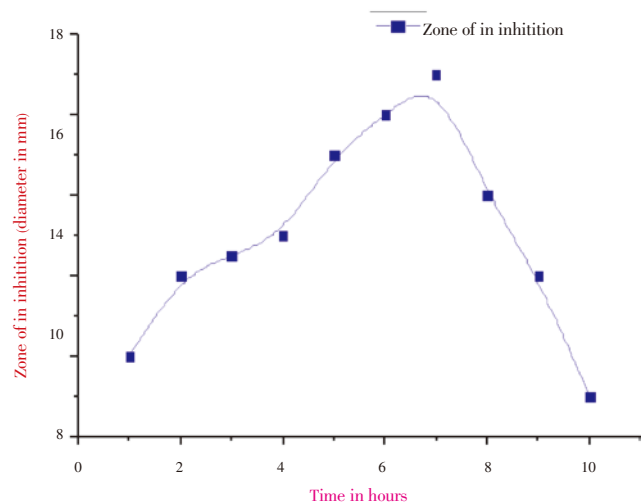


Figure. 5: Time course of the antimicrobial agent production by *Fusarium* species

4. Discussion

Emergence of antimicrobial resistance among clinical pathogens poses serious threat in the treatment of infectious diseases. To overcome the problem of antimicrobial chemotherapy, scientists and academicians in all over the world searching various microbial sources for novel antimicrobials since the continuous searching of terrestrial sources yielded known microorganisms and metabolites in the past. The survey of recent literatures evidenced that marine derived microorganisms are the potential sources of

bioactive metabolites.

In this present study, 30 fungal were isolated from (Sundarbans, Muttukadu and Parangipettai). 10 fungal strains were selected based on the morphological characters. *Aspergillus* species were found in the Sundarbans mangrove, Muttukadu (Chennai) and Parangipettai samples, but *Fusarium* sp only found in the Sundarbans mangrove samples. *Penicillium*, *Mucor* in the Muttukadu (Chennai) sample but not found in the Sundarbans mangrove and Parangipettai samples. The fungal isolates were belongs to the genera *Aspergillus*, *Penicillium*, *Mucor* and *Fusarium*. All the fungal isolates belong to three genera such as *Penicillium*, *Aspergillus*, *Mucor* and *Fusarium*. One of the previous study stated that *Aspergillus* and *Penicillium* was the most abundant among fungal population in the three locations studied at Sundarbans mangroves [12]. Surajit Das [13] reported that totally 90 fungal colonies were isolated, numbered (DSF225.1–DSF225.84, DSF – Deep Sea Fungi) and identified. *Aspergillus* was found to be the dominant genus (33%) followed by *Penicillium* (13%) *Lulworthia* (8%), others (40%) and non-sporulating fungi (6%). Deuteromycotina was dominant in the group contributing 72%, followed by Ascomycotina (20%) and Basidiomycotina (2%). In our present study also supported that *Aspergillus* were found abundant Species in parangipettai area.

Effect of sodium chloride on the growth of fungal isolates was given in table 2. All the isolates were takes growth up 1% to 10% sodium chloride concentration. All the colonies were well growing in NaCl contain medium but PS5 colonies were not show good in the NaCl contain medium. In salt tolerance studies all fungal isolates showed good growth at 1–10 % NaCl which confirm its marine nature. In the present study supported by Bavya et al [14] reported that R1 (Marine Actinomycetes) Showed good growth on ISP1, ISP2, ISP3, ISP4, ISP5, and ISP7 medium and moderate growth on ISP1 medium and No growth on ISP6 medium. Good growth was also observed 20 °C– 40 °C, 0–5% NaCl concentration and ph range from 7–11. The NaCl tolerant of strain R1 confirmed as marine origin.

Though fungal diversity is documented in some extent in Sundarbans mangroves, reports on antimicrobial activity is scanty. In this study, all the fungal isolates were screened for the antimicrobial activity against of clinical bacterial pathogens. Mangrove associated microorganisms are the potential sources for biotechnologically valuable compounds. In this study, 3 out of 10 fungal isolates showed good antimicrobial activity. The results also evidenced that antimicrobial substances produced by all the active fungal isolates are extracellular in nature. Most of the antimicrobial compounds produced from different microorganisms are extracellular in nature [15].

Three fungal isolates which showed antibacterial activity and its selected for further studies. All the isolates showed promising growth during the production of bioactive compounds. In antimicrobial of the ethyl acetate and acetone extracts obtained from fungal isolates SS2 showed a good activity compare than BS8 and PS8. SS2 showed maximum zone of inhibition against the tested pathogens.

Further optimization studies on fermentation, extraction and purification needed to produce the bioactive compounds at maximum level. Saha et al [16] reported the microorganisms isolated from the sunderbans region of the bay of Bengal, India, showed potent antimicrobial activity against gram positive and gram negative bacteria, molds, yeasts, and several multiple drug resistant (MDR) bacteria, including MRSA. Recently in our country, the bacteria isolated from Bhitarkanika mangrove ecosystem showed wide spectrum of antimicrobial activity [17].

In this present the tested carbon sources (2% w/v) in the basal salt medium (B.S.M) inoculated and incubated shaken for 5 days, glucose supported the highest level of antimicrobial agent production by *Fusarium* sp (SS2). In this present study also support the glucose enriched the production antibacterial compound by the fungi of *Fusarium* sp. In contrast, low activity was observed with galactose and starch. The simple carbohydrates like glucose, dextrose through metabolic pathway affect on production of intermediates leading to primary as well as secondary metabolites in addition to CO₂, water and energy. Addition of glucose resulted highest growth of the fungus, but significantly in many fermentation processes higher concentration of glucose has a suppressive effect on production of bioactive metabolites [18].

Among all inorganic and amino acid nitrogen sources, maximum production was obtained with ammonium nitrate indicating that the level of antibiotic production may be greatly influenced by the nature, type and concentration of the nitrogen source supplied in the culture medium. In the contrast in our present study show good activity by using Potassium nitrate as a inorganic sources. While obviously good effect was detected with organic nitrogen sources 0.24% (w/v) was the best concentration of potassium nitrate for optimum antimicrobial agent production by SS2. The requirements for specific nitrogen supplement differ from one microorganism to another.

Amendment of yeast extract enhanced the secondary metabolite production while beef extract and peptone increase biomass production but not bioactive metabolite. Radu et al. [19] also reported the maximum production of antifungal and antitumour compound from endophytic fungi grown in a glycerol (4%) and yeast extract (0.5%) amended media. In this present study *Fusarium* sp (SS2) producing high antibacterial compound in the medium containing Yeast extract was used as an nitrogen source and/or as growth factor in the medium in present study also support the earlier report.

The antimicrobial agent production was affected by the initial pH values of the medium, ranging from pH 3.0 to 7.0. Results indicated that the suitable pH for antimicrobial agent production by SS2 was at pH range from 5.0 to 6.0, while it was optimum at pH 5.0. Gogoi et al [18] reported that initial pH 6 of the medium was found to be the optimal for growth and bioactive metabolites production by *Fusarium* sp. DF2. Neutral pH also supports the growth (3.2 mg/ml) and bioactive metabolite production of the strain. No growth was observed at pH <3 and pH >11.

Time course from one to eight days was followed in shaking incubated flasks containing the optimized culture conditions inoculated with SS2. The antimicrobial agent production seems to be stable on the 5th and 6th days of the incubation period, having maximum on the 5th day. The time course for antimicrobial agent production differs according to the strain and cultivation conditions. For instance, the maximum antimicrobial agent production was achieved after 4 days of incubation of *Cladosporium* sp [20] 5 days of incubation of *Penicillium corylophilum* [19].

The potential fungal isolate SS2 was identified as *Fusarium* sp, BS8 and PS8 were belongs to the *Mucor* sp, *Aspergillus* sp. respectively. Miao et al [21] reported that, *Staphylococcus* strains isolated from Hong Kong waters showed antimicrobial activity especially antifungal activities. The present studies conclude that Sundarbans Soil (SS2) sample as a potential source for bioactive fungi. Further isolation of active compound from potential fungal isolates reported in this study will leads to the discovery of effective antimicrobials.

Conflict of interest statement

We declare that we have no conflict of interest.

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