Available online at www.elixirpublishers.com (Elixir International Journal)

Bio Technology

Elixir Bio. Tech. 40 (2011) 5618-5622

ISSN: 2229-712X

Isolation of heavy metal resistant marine fungi and Bacteria and their antibacterial activity

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ARTICLE INFO

Article history:

Received: 25 October 2011;

Received in revised form:

15 November 2011;

Accepted: 28 November 2011;

Keywords

Marine fungal species *Aspergillus, Mucor and Fusarium,*

Zinc ,

Antimicrobial activity.

ABSTRACT

The marine micro organisms are the store house of a wide variety of biologically active products. Among the vast population of microorganisms which include algae, bacteria, actinomycetes etc., marine fungi are considered as the most useful microorganisms in biotechnology field. The marine environment conditions the micro flora to generate metabolites that have antimicrobial qualities as a self- defending mechanism. The objective of this paper was to isolate the Zinc resistant fungal strains from marine samples like wood , seashore sand , sea water and wooden boat and identify the isolated cultures morphologically till the genus level. The antimicrobial compounds were extracted using different organic solvents and their antibacterial activity was cheked on the selected pathogenic bacteria at different varying concentration. A confirmatory test was performed for each test organism in their selective media. The fungal cultures were found to belong to the genus Aspergillus, Mucor and Fusarium. The extracts of hexane and chloroform on mucor showed very good activity on E.coli while average activity on Pseudomonas aeruginosa and Klebsiella pneumonia and least activity in S. aureus cultures was found.

Introduction

The marine environment is a rich source of metabolites but the marine micro organisms are mostly very hard to culture as they require different cultural conditions for their growth. Marine source has vast bioprospecting aspects and exploration in this field has started giving proofs regarding this with the discoveries of new drugs and various useful products such as antibiotics, organic acids, flavouring agents, chemical compounds and biofertilizers. Among the vast population of microorganisms which include algae, bacteria, actinomycetes etc., marine fungi are considered as the most useful microorganisms in biotechnology field. The fungi are mostly multicellular microorganisms which exist in either of the two forms- yeasts and molds. The filamentous molds, with high biomass, have lot of advantages over the slimy yeast. Marine fungi are commercially important and are mostly used in industries. Products from marine fungus which includes acids such as citric acid, fumaric acid, lactic acid and itaconic acid and gibberellins are used in printing, leather and tanning industries. The other industrial products include soft drinks, medicines, effervescent salts, fibres, paints, adhesives and thickening agents. Marine fungus takes part in bioremediation of pollutants from the soil and environment.

The heavy metals which are known to pollute the aquatic environment and cause severe hazards to the ecosystem are absorbed by the fungal biomass .The absorption may be intracellular or extracellular depending on the nature of the metal. These heavy metals include zinc, lead, calcium and copper, which cause many serious consequences. The microbial secondary metabolites include a number of antimicrobials, antiviral and antitumor agents. They also act as agricultural and pharmacological agents. The secondary metabolites which are produced by the fungi affect the cell growth as well as affect the cell metabolism by interaction with certain target sites of the bacteria.

The antibacterial property of fungi was first described by Tyndall in 1876, when he showed the effect of Penicillin on bacteria. After penicillin was a success in the year 1945 Giuseppe Brotzu carried out a study on sea water samples in Sardinia and analyzed them for any antibiotic producing microbes. Brotzu then discovered Cephalosporin C which exhibited antibacterial property. Marine fungi can be divided into two categories i.e. obligate and facultative fungi. The obligate species of fungi are those that are found only in the marine environments whereas the facultative ones are found in fresh water as well as terrestrial environment (Kohlmeyer, 1974).

Marine fungi have always been the subject of vigorous chemical investigation for new marine natural products (Libberra et.al, 1995;Faulkner, 2001; Blunt et.al ,2003). Biologically active compounds are expected to be produced by marine microorganisms as they live under such extreme environmental conditions (Braueurs et.al ,2000). The fungal species of Genus Phoma, which grows well in both terrestrial as well as marine environment, were isolated from a marine microbial mat collected in Bahamas . two new polyketides phonoxin and phomoxide were isolated and their structures were studied. They were produced from liquid broth culture of the isolates of Phoma spp. Earlier different chemical studies were carried on several strains of Phoma spp & resulted in the production of some diterpenoid platelet actiating factor (PAF) antagonists, the phomactins (Sugano et. Al. ,1991) which gave the idea that these marine species, like their terrestrial counterparts, show a unique structure and biologically active secondary metabolites. In 2000,Fusetani isolated more than 10,000 metabolites from marine microorganisms including

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marine fungi which showed many pharmaceutical properties. They showed antibiotic ,antifungal, cytotoxic, nuerotoxic and antiviral activities.

Here, the isolation of antibacterial components was done on the basis that the marine based compounds may have different form of activity than the usual. The fungal culture were isolated by giving a selective pressure of some heavy metals so that the fungus isolated has a resistance towards it, as they may have some novel antibiotic components.

Materials and Methods

SDA media, GYP media, Bacteriological Agar Powder, Nutrient Agar, Nutrient Broth, Gram's Crystal Violet, Gram's Iodine, Gram's Decolorizer, Saffranin, Malachite Green, 3% H2O2, HiMedia Oxidase Discs, Peptone, Kovac's Reagent, MR-VP Media, Methyl Red, Creatine, Simmon Citrate Agar, TSI Agar Media, Glucose, Mannitol, Sorbitol, Ethanol(99.95% & 70%) are the media used. Sample was collected from Marine water sample from Marina beach and the Ennore beach ,sand samples from Marina beach, Chennai Wood sample from marina and Ennore beach .

The marine fungi were isolated using SDA & GPY media. The media was prepared using 50% sea water which allows the isolation of marine species only. The marine bacteria were isolated using Nutrient Agar media.The Screening tests conducted were aimed at selecting the pure culture of the marine bacteria, were performed. These tests included the preliminary tests like Gram Staining, Spore Staining(Schaffer-Fulton Staining), Catalase Test followed by the confirmatory tests like for Indole Production Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilization Test. Other biochemical tests done were Triple Sugar Iron Agar Test, CO2 gas Production Test and Mobility test to identify the specific marine bacteria. Disc diffusion test was also carried out for the lawn culture of: E.coli on EMB agar , Pseudomonas aeruginosa on King's B agar and Staphylococcus aureus on Mueller Hinton agar.four antibiotics namely: ampicillin, tetracycline, bacitracin, and chloramphenicol, were tested and the Zone of inhibition was measured which tells about the efficacy of the extracts.

The prelimnary test carried for fungi was Lacto Phenol Cotton Blue staining technique,after which the extracts were prepared with different solvents . The bacterial colonies purified were then incubated in Nutrient broth. The inoculated broth was then incubated at room temperature in the beaker at 120 rpm for 24 hours.

Antibacterial activity was tested by well diffusion method.(Madhumita Rakshit 2010).The fungal metabolite was added in the well and the 24 hours bacterial culture was swabbed in the Muler Hintor Agar plate. The plate was incubated for 24 hours and the zone of inhibition was measured.

Results and discussions

Preliminary test:

Gram Staining: Gram Staining is one of the preliminary tests performed to characterize bacteria and divide them into the classes of Gram positive and Gram negative. After Staining the Gram positive bacteria were observed as purple-blue color while the Gram negative ones were red colored. The results of Gram Staining and the morphology of the isolates are Gram negative and bacillus shaped.The bacterial morphology as well as their classification in the Gram positive and Gram negative classes were established. The isolate was Gram negative and their morphology are bacillus.

Spore Staining: Spore staining was performed on pure cultures. The cultures retained red color of saffranin while none showed green color of malachite green.It was established that the test culture was Endospore negative.The bacteria doesn't show Endospores at any stage of their lifecycle. So they show negative result with Spore Staining. Further tests were performed on the culture.

Catalase Test: The test was performed on pure selected culture. Effervescence was observed forming after the test. The presence and absence of effervescence indicates that the cultures were catalase positive and negative respectively. The basis of Catalase test is the presence of catalase enzyme in the bacteria. The enzyme prevents the H2O2 accumulation which is toxic. Catalase breaks the H2O2 to O2 which results in the bubble formation. The bacteria is catalase positive. Since the test cultures tested to be positive for catalase, it was tested further.

Confirmatory Tests:

After the preliminary tests, the test cultures were tested with the confirmatory tests. These mainly include the biochemical tests – the IMViC test along with TSI and CO2 gas production Test.

Indole Test: The Indole test was performed on the same selected culture in 4 sets. No change in color was observed in media for the test organisms. This indicated that the test cultures tested negative for the Indole Test.

Vogues Proskauer Test: Culture showing negative result for the indole test was further subjected to Vogues Proskauer test in two sets. Here also the culture showed no color change. Thus the test proved to be negative.

The basis for VP test is the production of acetyl methyl carbinol that can be detected by oxidation reaction. The glucose phosphate broth when supplied with NaOH produces the carbinol due to the fermentation of glucose. Now the acetyl methyl carbinol formed is oxidized in the presence of air to form diacetyl. The latter gives pink color with creatine. The absence of pink color indicated the inability of the bacteria to form carbinol. Hence, the negative result for the cultures

Citrate Utilization Test: After Vogues Proskauer (VP) test this test was done to further establish the nature of the bacteria. . Here pure culture showed color change from green to blue. Thus the test proved to be positive.

H2S Production Test: This test was done along with TSI test. H2S was not produced as black color was not observed in the slant of TSI agar test. This resulted in negative test for selected pure culture.H2S is produced by the bacteria utilizing the sugars present in the media (glucose, sucrose and fructose). H2S reacts with the ferrous sulfate present in the media to produce black color. In theslants, H2S was not produced so no black color was evident. Hence, the test turned out to be negative for the selected culture.

Methyl Red Test: When the isolate was tested for the methyl red test, showed color formate on fermentation of Glucose. Methyl red is the indicator which changes the color to pink in the acidic media.

Triple Sugar Iron Agar Test: The test organism did not show any change in colour (that is it remained red in colour). The color change was not observed in whole regions of the slant and butt.

CO2 Production Test: The test was done for the selected cultures. The test was done using the Durham tube. Gas bubble formation was observed in test tube having glucose. While in mannitol and sorbitol no

bubble was formed.For CO2 test the presence of bubble in glucose indicated positive test(that is, the production of CO2). No bubble in mannitol and sorbitol indicated negative test. The CO2 Production Test is based on the fermentation of the the sugar, mainly glucose, and the formation of CO2 in the media. This is indicated by the formation of bubble in the Durham tube.The formation of CO2 means that the bacteria are Hetero-fermentative and no CO2 means that the culture is of Homo-fermentative.

Biochemical Test Results

Based on the above results of biochemical tests, the bacterial strain isolated from the sample was concluded to be *Proteus mirabilis.*

Lacto Phenol Cotton Blue Staining of fungal culture:-

It shows that spore like structures which may belong to ascomycetes and further biochemical testing which is to be done for identification for specific fungus. Delicate blue hyphae and fruiting structures were visible with a
pale background.

Results Obtained On Fungi Showing Antibacterial Property

With the selective pressure of the heavy metals in the medium we were able to get three well growing heavy metal resistant fungal strains. These strains showed good growth in the sea water medium only. These failed to grow well in any low salinity grade medium. These limitations have helped in avoiding the common contaminants and the cultures were maintained safe.

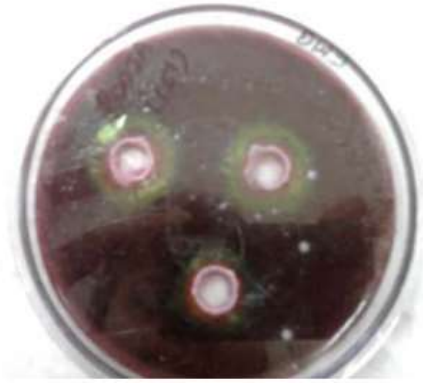

Figure 1: fungal culture in wells showing zone of inhibition against E.coli culture grown on EMB agar showing the antibacterial property of marine fungi. NOTE: EMB agar is the selective media for the growth of E.coli which can be detected by a green metallic sheen

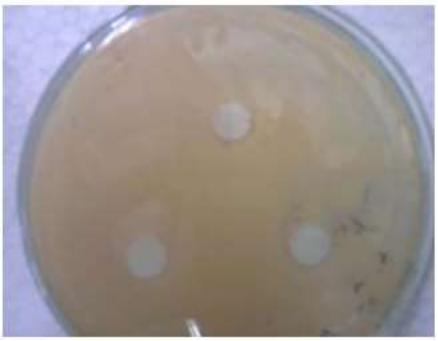

Figure 2: fungal culture in wells showing zone of inhibition against the bacteria Pseudomonas aeruginosa swabbed on Mueller hinton agar. Diameter of zone of inhibition is less compared to E.coli

Figure 3 : showing marine marine fungi do not grow in low salinity grade medium thus preventing contamination. Here marine fungal cultures are in wells showing no zone of inhibition against Staphylococcus aureus in nutrient agar media

Result of Disc Diffusion Test:

Fig 4: showing zones of inhibition (in cm) for S.Aureus in Mueller Hinton Agar

Fig 5 : Showing zones of inhibition(in cm) for E.coli in EMB Agar

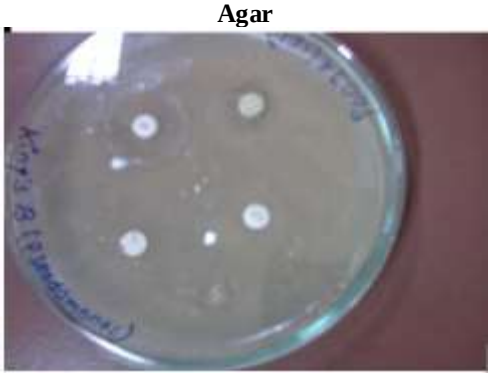

Fig 6 : Showing zones of inhibition(in cm) for P. aeruginosa in King's B agar

Conclusion

The fungus which were selected for antibacterial study belonged to the genus genus Aspergillus, Mucor and Fusarium. The one against *E.coli* in EMB agar was found to be very active

than the other bacterial strains. The other bacterial strain which showed good activity but less than E.coli were *Pseudomonas aeruginosa* in Mueller Hinton agar. Three solvents were used for extracting the antibacterial compound from the fungal broth. These solvents included ethyl acetate, hexane and chloroform. The hexane and chloroform extracts showed good activity whereas ethyl acetate showed the least activity of them all. The ethyl acetate extract showed some activity against *Escherichia* coli only. *Staphylococcus aureus* showed most resistance towards the fungal extracts. The extracts showed moderate activity on *Pseudomonas aeruginosa*. The extracts showed their best activity on Escherichia coli than the other pathogenic strains of bacteria. In the present study three marine fungal species were collected from marine environment and they were isolated in media containing 50% natural sea water which ensured their marine origin. The fungal extracts have been checked for their antibacterial activity against an array of pathogenic bacteria. This work showed that the isolated fungal species showed antibacterial activity towards the test organisms. The fungus produces biologically active substances and with more advanced techniques and more research work in this field many novel components can be isolated from marine fungus.

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Table 1: results of all the tests performed on bacteria

BIOCHEMICAL TEST	RESULT
1) Simmon's Citrate Agar	Positive
2) INDOLE	Negative
3) TRIPLE SUGAR TEST	Positive
4) METHYL RED	Positive
5) VOGUES PR0SKAUER	Positive/Negative
6) MOTILITY	Positive
7) Gas From MANNITOL	Negative
8) Gas From SORBITOL	Negative
9) Gas From GLUCOSE	Positive

Table 2: bacteria showing zone of inhibition (cm) against fungal extracts at three different Volumes

Bacterial Strain	100 (microlitre)	200 (microlitre)	300 (microlitre)
E.coli	2.5 cm	2.7 cm	1.1 cm
P. aeruginosa	0.7 cm	1.9 cm	2.2 cm
S. aureus	-	-	-

.

Table 3: showing zones of inhibition (in cm) for S.aureus in Mueller Hinton Agar

Antibiotics	**Diameter (cm)**
Tetracyclin	2.6
Chloramphenicol	1.6
Ampicillin	1.1
Bacitracin	1

Table 4 : showing zones of inhibition(in cm) for E.coli in EMB Agar

Antibiotics	**Diameter (cm)**
Tetracyclin	2.1
Chloramphenicol	3.5
Ampicillin	1.9
Bacitracin	0.7

Table 5 :showing zones of inhibition(in cm) for P. aeruginosa in King's B agar

Antibiotics	Diameter (cm)
Tetracycline	1.3
Chloramphenicol	3.2
Ampicillin	1.2
Bacitracin	0.8