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Research Article

Isolation and production of bioactive molecule from marine bacteria associated with sponges and ascidians and its screening for antimicrobial potential

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ABSTRACT

Ethyl acetate extract of strain NIO MU 19.2 showed significant antimicrobial activity against human test pathogens as well as multi-drug resistant pathogens from thirty-eight marine bacteria isolated from three sponges and three ascidians from Mumbai (India). A strain NIO MU 19.2 was subjected to morphological and biochemical characterization. In addition, fermentation media, modified media contents and fermentation conditions were also optimized for maximum production of antimicrobial metabolite. NIO MU 19.2 was mass cultured using optimized conditions and ethyl extract of broth was further fractionated with column chromatography. Through bioassay-guided fractionation, active fraction was identified and subjected for chemical studies such as HPLC, UV, IR, and NMR. Further studies are in progress in order to elucidate the structure and mode of action of bioactive molecule.

Keywords: Marine bacteria, Sponges, Ascidians, Bioactive molecule, Antimicrobial.

1. INTRODUCTION

The marine environment is proving to be a valuable source of novel bioactive compounds with antibacterial, antiviral and anticancer properties. Both free-living bacteria and bacteria that are symbionts of marine invertebrates are likely to be a good source of useful bioactive compounds. It has been recently shown that bacterial species that produce antimicrobial compounds are intimately associated with sponges and ascidians.[5] Marine invertebrates and a growing number of marine bacteria have also been observed as the sources of novel, bioactive secondary metabolites.[6, 1]

Over the past 60 years, between 30,000 to 50,000 natural products have been discovered from microorganisms. More than 10,000 of these compounds are biologically active and more than 8,000 are with antibiotics and antitumor properties. Today, over 100 microbial products continue to be used clinically as antibiotics and antitumor agents.[2] For instance, about 300 patents on bioactive marine natural product have been issued between 1969 and 1999.[3]

The biodiversity of the ocean is underscored by the fact that 34 of the 37 phyla of life occur in the ocean, as opposed to only 17 of 37 on land. So why should we be discouraged about marine natural products when the greatest resource of biological and genetic diversity resides in the ocean? [4, 7]

A strain affiliated with the *Roseobacter* clade and producing a new antibiotic named tropodithietic acid was isolated from the German Wadden Sea. Antibiotic production was found to occur during the complete growth phase.[8]

The analysis reported by sponge derived *Salinispora* strain demonstrates that *Salinispora* isolate does produce compounds of the rifamycin class, including rifamycin B and rifamycin SV. Thus, *Salinispora* group of actinobacteria represents a potential new source of rifamycins outside the genus *Amycolatopsis* and the first recorded source of rifamycins from marine bacteria.[9]

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Antimicrobial activity was found in several isolates, two of which were identified as *Rhodococcus sp.* and *Pseudomonas sp.* by partial 16S rRNA gene sequencing. The recovery of strains with antimicrobial activity suggests that marine sponges represent an ecological niche, which harbors a largely uncharacterized microbial diversity and a yet unexploited potential in the search for new secondary metabolites.[10]

2. MATERIALS AND METHODS

2.1 Sample collection

Sample specimens of the sponges were collected by SCUBA diving from a depth of 5 -15 meters from Mumbai west coast, India and placed into sterile plastic bags, cooled in ice and transported to the laboratory. In laboratory sponges were cut into small pieces and stored at -20^{0}C for the isolation of episymbionts and endosymbionts.

2.2 Isolation of marine bacteria[11]

2.2.1 Episymbionts

A sample was taken in a sterile petri-plate and cut. The piece was taken in a test tube containing sterile seawater and vortexed. The washing was decanted, same process was repeated. Serial dilutions were performed and spread on ZMA plates in which an antifungal agent Nystatin was added. The plates were then incubated at 37^0 C for 48 hours.

2.2.2 Endosymbionts

A piece of sample was taken in a sterile test tube containing sterile seawater and vortexed for 30 seconds and then decanted. The procedure was repeated. The sample was vortexed in 70% ethanol, washed with sterile seawater and triturated. Serial dilutions of the same were made up and spread on ZMA containing an antifungal agent Nystatin. The plates were incubated at 37^0 C for 48 hours till colonies were observed visually.

2.3 Purification of isolates

The cultures grown on the Zobell Marine Agar (ZMA) medium were sub cultured on the respective medium. Then they were purified by continuous streaking on ZMA plates.

2.4 Preservation of strains

The purified isolate cultures were grown in Zobell Marine Broth (ZMB) and incubated for 24 hours at 37^{0}C. After the sufficient growth at the end of 24 hours, culture broth was transferred into sterile vials. The vials were earlier added with sterile 70% glycerol, and these vials were preserved at – 20 ^{0}C in triplicates.

2.5 Preliminary screening

Based on the clear zone around colonies at the time of isolation, 8 out of 38 strains were selected for primary screening as follows.

NIO MU 2.1	NIO MU 16.5	NIO MU 19.1
NIO MU 2.2	NIO MU 17.1	NIO MU 19.2
NIO MU 9.1	NIO MU 18.1	

2.5.1 Cultivation of bacterial strains [12]

Isolated colonies were picked and inoculated separately in ZMB, after incubation at 37^{0}C for 72 hours; each strain was inoculated in 100 ml of following different media.

a) Zobell Marine Broth (ZMB) b) Marine Broth (MB) c) SM-12 media.

Based on growth of bacteria marine broth was decided to use for further cultivation.

2.5.2 Extraction of broth[13, 14]

The broth was centrifuged at 10,000 rpm for 20 mins. The cell pellets were collected and extracted with methanol. The supernatant was extracted with ethyl acetate, remaining extract was considered as aqueous. All of them were assayed for antimicrobial activity.

2.6 Determination of bioactivity of crude extracts [15,16]

2.6.1 Agar diffusion assay

Crude extracts were dissolved in respective solvents at a concentration of 100 µg/ml. 2x10 µl samples were used to saturate antimicrobial assay paper discs with a period of drying between each application. The discs were placed on the Mueller Hinton agar surface containing the test microorganisms, and incubated at 37^{o}C for 24 hours (bacteria) and 48 hours (fungi).

2.6.2 Mass culturing

According to preliminary screening results, NIO MU 19.2 was found to be a potential strain for production of bioactive antimicrobial molecule so the same was seed cultured by

inoculating 50 ml Marine broth in a 100 mL Erlenmeyer flask in a shaker (37^{o}C/200 rpm) for 24hr. Then this seed culture was reinoculated in Erlenmeyer flask containing 1 L marine broth and incubated with shaking (200 rpm) for 3 days at 37^{0} C. Total 20 L of broth was cultured. Cells were separated by centrifugation (10000rpm/20 min). The supernatant was extracted with ethyl acetate.

2.6.3 Identification of bacteria [17,18]

Morphological characterization was performed by a visual observation of bacterial colonies. Gram staining was examined under a light compound microscope. Gram-reaction was done by using Himedia K001 Gram Stain Kit.

Motility test was performed in which semisolid ZMA was used. Catalase test was demonstrated to detect the presence of the enzyme catalase. The capacity of an organism of utilizing citrate as sole carbon source for metabolism was determined by citrate utilization test using Simmon's citrate agar. Oxygen Fermentation medium was used. to perform the oxidative or fermentative metabolism of a carbohydrate.

ZMB of different NaCl concentration was prepared. The flasks were inoculated and incubated. Growth in each flask with different NaCl concentration was observed.

2.7 Optimization parameters

Different concentration range 0.1% - 0.6% of peptone and yeast extract 0.04% - 0.14% were used. Media with different P^{H} ranges from 5 – 7.5 were also used. Fermentation time was also optimized by collecting samples for regular time interval.

2.8 Chemical characterization

Thin layer chromatography was performed using pre-coated layer silica gel G plates. different solvent systems and different visualizing agents were used. Sephadex column was used for further separation, ethyl acetate extract was loaded uniformly on the surface of the column by dropper. Totally around 50 fractions were collected and each fraction was subjected to TLC. Purity was checked by using HPLC technique.

2.9 Spectroscopic studies

UV spectrum for isolated bioactive fraction was recorded in Shimadzu UV spectrophotometer using ethyl acetate as a solvent.

FT-IR spectrum of isolated bioactive fraction was recorded in Shimadzu IR spectrometer using KBr pellets. The purified compound was subjected to analysis by NMR spectroscopy (300 MHz Bruker NMR).

2.11 Antimicrobial screening of fractions [19, 20]

Various fractions which were obtained from separation of ethyl acetate extract by column chromatography were studied further for their antimicrobial potential. Study was carried out by Cup plate method.

3. RESULTS AND DISCUSSION

3.1 Sample collection

In the course of screening for bioactive molecules possessing antimicrobial properties from sponge associated bacterial samples, antibiotic-producing bacterial cultures were recorded from sponge symbionts samples collected from Mumbai by SCUBA diving.

In the present study, about thirty-eight bacteria were isolated from sponge samples as endosymbionts and episymbionts, using ZMA as an isolation media and Nystatin as an antifungal agent in it. Nystatin was used to eliminate growth of fungal culture associated with sponges and only to isolate bacterial colonies. The sponge samples were collected and processed aseptically which helped to prevent contamination of growth of unwanted bacteria and fungi. We had selected eight bacterial colonies having good zone of inhibition around it and were purified by continuous streaking on ZMA plates. Each purified strain was preserved in agar slants at 4° C and also at -20^{0} C in glycerol. The strains which were preserved at 4° C, used for routine serial culturing and that of at -20^{0} C in glycerol were kept for long time preservation as it does not show any growth in those condition. The bacterial samples isolated and selected for further study were named according to their associated sponge samples. The morphological and cultural characteristics

of the isolated strains were studied on ZMA plates by naked eye.

Isolated colonies were inoculated separately in ZMB, MB and SM-12 media. The good growths of all bacterial isolates were observed in MB, the same media was selected for the further study. For preliminary screening, each strain was inoculated and incubated in MB and extracted with ethyl acetate and methanol.. The extracts (Table 1) from isolated strains were tested for antimicrobial test against bacterial, fungal and MDR strains by paper disc method.

Table 1: Agar diffusion assay (paper disc method) against bacterial pathogens.

Extracts	B1	B2	B3	B4	B5	B6	B7	D1	D2	D3	D4	F1	F2	F3	F4	F5
MU2(1)EA	-	-	+	-	-	-	+	+	-	-	-	+	-	+	-	-
MU2(1)MeOH	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	+
MU2(1)Aq	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
MU2(2)EA										-	++	+	-	+	+	-
MU2(2)MeOH	+	-	-	+	-	-	+	-	+	-	-	-	+	-	-	-
MU2(2) Aq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MU9.1EA	++	++	++	-	-	-	++	-	++	++	-	++	-	+	-	-
MU9.1MeOH	++	-	-	-	++	-	++	-	-	-	++	-	++	-	-	-
MU9.1Aq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
MU16.5EA	++	++	-	++	-	-	++	-	++	+	++	+	-	-	++	++
MU16.5 MeOH	++	+	-	-	++	-	++	++	-	-	-	-	-	-	++	-
MU16.5Aq	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-
MU17.1EA	-	+	+	-	-	-	+	-	-	++	-	++	+	+	-	+
MU17.1 MeOH	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	+
MU17.1Aq	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-
MU18.1EA	+	++	-	++	-	+	-	-	-	-	+	-	+	+	+	-
MU18.1 MeOH	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	+
MU18.1Aq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MU19(1).EA	++	+	-	-	-	+	-	+	-	++	++	+	-	-	+	+
MU19(1).MeOH	-	-	+	+	+	+	-	+	-	-	-	-	+	+	-	-
MU19(1).Aq	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-
MU19(2).EA	**++**	**-**	**++**	**++**	**++**	**++**	**+++**	**+**	**++**	**-**	**-**	**++**	**-**	**++**	**++**	**-**
MU19(2).MeOH	-	++	++	-	-	-	-	-	-	++	++	++	++	-	++	-
MU19(2).Aq	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Standard	+++	+++	++	+++	+++	+++	+++	++	++	++	++	++	++	++	++	++

B1: Escherichia coli, B2: Pseudomonas aerugunosa, B3: Staphylococcus aureus , B4: Salmonella typhi B5: Shigella flescineri, B6: Klebsiella, B7: Vibrio cholerae, D1: Streptococcus pyogenes, D2: Acinetobacter, D3: MDR Salmonella typhi, D4: Methicillin resistant Staphylococcus aureus F1: Aspergillus fumigatus, F2: Rhodotorula, F3: Candida albicans, F4: Cryptococcus neoformans, F5: Aspergillus nige. EA:ethyl acetate, MeOH: methanolic, Aq: aqueous, Disc diameter:6 mm,+: 6-10 mm, ++: 11-15 mm, +++: 16-19 mm

Table 2: Biochemical test of NIO MU 19.2

TEST	RESULT
Catalase	Positive
Citrate Utilisation	Positive
Oxygen fermentation	Negative
Growth at 4^0 C	-
Growth at 40^0 C	+

3.2 Optimized conditions

The optimization parameters ranges were taken considering MB media content concentrations and MB contents shows maximum zone of inhibition (Table 3) so the same were used for further study. The optimized fermentation parameters were taken as follows:

Media	: Marine Broth	Temperature	: 37^0 C
pH	: 7.0	Duration	: 72 hours
RPM	: 200		

Table 3: Optimization of media content and fermentation parameters.

Sr. No.	Yeast extract concentration (%)	Zone of inhibition	Peptone concentration (%)	Zone of inhibition	pH	Zone of inhibition	Incubation time	Zone of inhibition
1	0.04%	12	0.1%	12	5	13	24	14
2	0.06%	13	0.2%	12	5.5	14	36	14
3	0.08%	13	0.3%	13	6	14	48	14
4	**0.10%**	**16**	0.4%	13	6.5	14	60	15
5	0.12%	15	**0.5%**	**15**	**7**	**15**	**72**	**16**
6	0.14%	15	0.6%	12	7.5	13	84	13

Disc diameter = 6 mm, Organism used = *Vibrio cholerae*

3.3 Mass culturing

The strain NIO MU 19.2 showing good yields and significant antimicrobial potential was selected for study. It was mass cultured in a large-scale i.e. 20 liters. From the same 800 mg of product was extracted and used further for chemical characterization and antimicrobial study.

3.4 Biochemical test

NIO MU 19.2 was further studied using different test for its identification and it was found to be gram negative and non-motile in nature. Growth in different NaCl concentrations was also observed which shows good growth in range of 0.05% - 6.0%. It was also subjected for various biochemical tests.

3.5 Chemical characterization

TLC of the extracted compound was performed. The spots on the plates were developed by sulphuric acid and also with iodine vapors, which showed four different spots. The column chromatography of ethyl acetate extract yields a major (43-45) fraction having Rf value 0.687.

Table 4: Antimicrobial screening of fractions

Extracts	B1	B3	B4	B5	B6	B7	D1	D2	F3	F4
MU19(2).EA 1-20	+	-	-	-	+	-	-	-	-	-
MU19(2). EA 21-42	-	+	-	-	-	-	-	-	++	+
MU19(2). EA 43-45	**++**	**++**	**++**	**++**	**++**	**+++**	**++**	**++**	**++**	**++**
MU19(2). EA 46-50	-	-	-	-	+	+	+	-	-	-
Standard	+++	++	+++	+++	+++	+++	++	++	++	++

B1: Escherichia coli, B3: Staphylococcus aureus, B4: Salmonella typhi B5: Shigella flescineri, B6: Klebsiella, B7: Vibrio cholerae, D1: Streptococcus pyogenes, D2: Acinetobacter, F3: Candida albicans, F4: Cryptococcus neoformans, EA:ethyl acetate, MeOH: methanolic, Aq: aqueous, Disc diameter:6 mm,+: 6-10 mm, ++: 11-15 mm, +++: 16-19 mm

3.6 Physical properties of purified product

The isolated compound was found to be yellow colored amorphous powder. It showed clear solubility in ethyl acetate, methanol, chloroform and sparingly soluble in DMSO.

3.7 Determination of MIC

The MIC of bioactive molecule was found to be 125 μg/ml against *Staphylococcus aureus, Shigella flescineri, Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans,* 100 μg/ml against *Escherichia coli, Streptococcus pyogenes, Acinetobacter and* 50 μg/ml against *Vibrio cholerae.* While MIC for anti-tubercular activity was found to be significant at the concentration of 75 μg/ml.

3.8 Spectroscopical studies

In attempt to establish chemical structure of bioactive molecule produced by NIO MU 19 (2), spectral studies such as UV, IR (table 5) and NMR (table 6) were performed.

In UV, λmax of the isolated compound was observed as 3.5390 at 216 nm which indicates the presence of chromophoric group while HPLC data showed 99.612 % of purity at retention time 6.715 min.

Table 5: IR Spectroscopical data

Sr. No.	Wave number	Functional groups
1	3224	OH –str
2	2931,2960	Aliphatic C-H str
3	1666	C=O str
4	1514	C-H bending
5	1109	C-O str
6	702,750	Substituted aromatic ring

Table 6: NMR Spectroscopical data

NMR	δ Values	Position of nucleus
^{1}H	1.05 - 1.53	Protons of aliphatic chain
^{13}C	29.70 - 28.83	Carbons of aliphatic chain
DEPT	24.37	$-CH_3$
	29.18	$-CH_2$
	58.66	-CH
	128.06	-CH

4. CONCLUSION

The present study concludes that the marine microorganism have tremendous potential of producing antimicrobial compounds. The study also showed that the seaweed associated microorganisms are capable of producing the potent pharmaceuticals.

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