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ISOLATION, PRODUCTION AND OPTIMIZATION OF ANTIMICROBIAL COMPOUND PRODUCED FROM SELECTED MARINE SOURCES

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ABSTRACT
Currently, multiple antibiotic resistances in pathogenic bacteria is increasing, compromising the clinical treatment of growing number of infectious disease. There is an urgent need for new drugs effective against those antibiotic resistant pathogens and opportunistic pathogens. In the present work, samples like seawater, seashore sand and sediments from four different sampling sites, Vishakapatnam, Machilipatnam, Chennai and Kanyakumari. The marine bacterial species were isolated on Zobell Marine agar medium using spread plate technique. Among those isolates MB39 isolate showed maximum degree of inhibition against 6 strains of human pathogens like *Staphylococcus aureus, Bacillus substilis, Escherichia coli, Klebsiella pneumonia, Salmonella typhi* and *Candida albicans* compared to other isolates. The isolate was obtained from Vishakapatnam Sea Sediment sample (SS1), belonged to *betaproteobacteria* and phylogenetically related to *Alcaligenes faecalis sp.* was identified by according to the Bergey's manual. For optimization the selected isolate MB39, grown by using Glucose as a carbon source showed good antimicrobial activity against the test pathogens. The degree of production was increased with increasing culture time with a maximum zone of inhibition obtained after 48 hours, after which decline in growth was observed at 72 hrs. Maximum zone of inhibition was observed at pH 9. For purification of the active compound solvent extraction was performed with five different organic solvents Chloroform, Methanol, Diethyl ether, Acetone and Ethyl acetate. Maximum activity was observed with ethyl acetate extract.

KEYWORDS: Marine bacteria, Sea sediment, Antimicrobial activity, MB39, Betaproteobacteria.

INTRODUCTION
The discovery of novel antibiotic and non-antibiotic compounds lead molecules of pharmaceutical interest through microbial secondary metabolite screening is becoming increasingly fruitful. There is wide acceptance that microorganisms are virtually unlimited sources of novel substances with many therapeutic applications. In the past decades, the increasing needs for drugs to control new illness or resistant strains of microorganisms stimulated to look for unconventional new sources of bioactive metabolites. The oceans found to be an attractive field and great efforts have been accomplished worldwide aiming the isolation of new novel products from marine microorganisms (Kelecom, 2002). Culturing the culture would represent a unique and promising source for the discovery of novel secondary metabolites. As marine microorganisms have evolved great genomic and metabolic diversity, efforts should be directed towards exploring marine microorganisms as a source of novel secondary metabolites.

This study aims at exploring the antibiotics production potentials of some indigenous marine bacterial species isolated from the coast of southern India.

MATERIALS AND METHODS
Collection of samples
Four different locations such as Vishakapatnam, Machilipatnam, Chennai and Kanyakumari were selected so as to cover maximum variation in latitude and longitude along the southern coast of India. Sampling was carried out from July to December 2017. Among the sample types seashore sand, water and sediment (of not more than 15 meters depth) were collected aseptically and transferred into sterilized glass bottles and fresh polythene bags.

Isolation and enumeration of marine bacteria

A total of 12 samples have been used. Marine agar media (high media laboratory, Mumbai, India) was used for isolation. In brief stock was prepared by adding 1 ml of sea water samples in 9 ml of sterile sea water 5 ml of sediment slurry was suspended in 45ml of sterile sea water and 5gm of sand in 45ml sterile sea water. Stock was further serially diluted up to dilution of 10^{-10}. Aliquote of 100 micro liters of each dilution was plated triplicate on marine agar media supplemented with nystatin (30mg /ml, antifungal). The plates were incubated at room temperature (28°C) for 24-48 hours, after which total direct counts of bacteria were determined and colony morphology was noted. Further individual colonies were picked and streaked repeatedly until pure cultures were obtained, and stored in Zobell marine agar slants at 4°C for further use.

Antimicrobial assay against human pathogenic bacteria

Antimicrobial susceptibility testing was done using the agar well diffusion method (Perez et al.,1990) against both gram positive (*Staphylococcus aureus* and Bacillus substilis) gram negative (*Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi*) bacteria and fungi (*Candida albicans*) all of which are opportunistic human pathogens.

For antimicrobial assay, pure culture of each isolate was inoculated in 100 ml of Zobell Marine broth and incubates at room temperature (28°C) in a rotary shaker 200 rpm. After 48 hours, 10 ml of culture was taken and centrifuge at 1200 rpm for 15min. Supernatant thus obtained was further filtered through membrane filter (Millipore filter; 0.45millimeter) to obtain a cell free supernatant. In parallel, freshly grown colonies of the bacterial pathogens were used to inoculate 100ml of Mueller –Hinton broth (Himedia laboratories, Mumbai, India) and incubated in shaking incubator for 4-6 hours until turbidity was reached (Baker et al., 1983). A sterile swab was used to evenly distribute the culture over Mueller –Hinton agar (Himedia laboratories, Mumbai, India) plates. The plates were allowed to dry for 15 minutes before use in the test tubes. Wells were then created using a sterile cork borer of 6 mm diameter and a pipette was used to plays 50 µl of the cell–free crude extract into each well. The plates were incubated at 37°C for 24 hours after which they were examined for inhibition zones against different pathogens. A caliper was used to measure the inhibition zones. Each experiment was repeated twice to ensure reliability.

Identification of Isolates

Isolated colonies were identified through the Gram staining and biochemical tests such as oxidase test, catalase test, urease test, lipase test, amylase test, gelatinase test, citrase test, nitrate reductase test and H_2S production test etc. (Cappuccino and Shearman, 2006; Sleigh and Duguid, 2005).

Optimization conditions for the production of the antimicrobial compound

Effect of carbon sources on the production of antimicrobial metabolite

Conical flasks containing 100ml sterile marine broth containing carbon sources like 1% Sucrose, 1% Mannitol, 1% Lactose and 1% Glucose was separately prepared, they were inoculated with test culture and incubated at 28 $\pm$ 2^0c for up to 48 hrs. The contents of the flasks were harvested by centrifugation at 10,000 rpm for 10 minutes. The culture filtrates were extracted using ethyl acetate in separation funnel. The concentrated ethyl acetate extracts were tested for antibacterial activity by agar well diffusion method (Ripe et al., 2009).

Effect of time on the production of antimicrobial metabolite

Conical flasks containing 100ml sterile marine broth were inoculated with test culture and incubated at 28 $\pm$ 2^0c for up to 4 days. On the day, the flasks were harvested by centrifugation at 10,000 rpm for 10 minutes. The culture filtrates were extracted using ethyl acetate in separation funnel. The concentrated ethyl acetate extracts were tested for antibacterial activity by agar well diffusion method (Ripe et al., 2009).

Effect of p^H on the production of antimicrobial metabolite

The effect of P^H on the antimicrobial metabolite production was studied by inoculating test cultures into flasks containing 100ml sterile marine broth adjusted with various P^H viz., 3,5,7,9 and 11(Using 0.1N HCL or 0.1N NaOH). The flasks were incubated at 28$\pm$ 2° C for 24 hrs. The contents of the flasks were centrifuged at 10,000 rpm for 10minutes. The culture filtrates thus obtained were extracted using ethyl acetate in separation funnel. The concentrated ethyl acetate extracts were tested for antibacterial activity by agar well diffusion method (Ripe et al., 2009).

Effect of temperature on the antimicrobial metabolite

1 ml of culture filtrate was dispensed in sterile eppendrof tubes and was incubated as room temperature (28° C), 40°C, 50°C, 60°C, 70°C and 80°C for 30 minutes. From each eppendrof, fraction of 100 μl was tested against the test pathogen for inhibitory activity.

Purification of the antibiotic substance

Solvent extraction

The supernatant was extracted with five different organic solvents, chloroform, dichloromethane, ethanol, hexane and ethyl acetate. The organic layer was concentrated at room temperature and the concentrate was assayed for antimicrobial activity employing disc diffusion method. The concentrated organic layer thus obtained was termed as crude inhibitor.

Column Chromatography

The crude inhibitor was subjected to column chromatography using a 2cm by 30cm silica gel column (60-120mesh, E.Mesh) and a linear solvent gradient of n-hexane, Dichloromethane, chloroform, ethyl acetate and Methanol (Sigma-Aldrich). A total solvent volume of 0.5 L was applied, and 40, 10 mL fractions were collected. All fractions were assayed for activity against test Pathogen Staphylococcus aureus on Mueller Hinton agar using disc diffusion assay.

Thin Layer Chromatography

Active fraction obtained after column chromatography was further purified by preparative TLC (Zeeshan et al., 2012). The purity of the compound was checked by TLC using various solvent systems. The ethyl acetate extract obtained after column chromatography was re dissolved in 1 mL ethyl acetate. For the TLC analysis, 25µl of the extract was loaded on TLC (Silica gel 60) plates (Merck, Germany). The solvent ratio used for the separation of the compounds was chloroform: ethyl acetate (4:1,v:v). UV transillumination of the plates at 365 nm revealed one band. This band was eluted separately with a minimum amount of ethyl acetate. Active band was scrapped off from the silica gel and eluted in ethyl acetate (HPLC grade, Merck, India) followed by centrifugation at 10,000 rpm. Clear supernatant was then dried aseptically and subjected to antimicrobial assay.

RESULTS AND DISCUSSION

Total count of bacteria at different sites

Marine bacterial species were isolated on Zobell Marine agar medium using spread plate technique. Diversity in pigmentation, colony shape, size and texture were observed on marine agar plates. Colonies with yellow, orange, brown and red pigments, ranging from 1 to 10 mm, with rough or smooth appearance having irregular, entire or rhizoidal margins were observed. Most of the colonies were opaque with few translucent colonies. Colony forming units (CFUs) were counted for all the samples from sample type and sites. Weighted mean of CFU from each sample type, sea sediment, sea sand and sea water, from all sampling sites show that the marine heterotrophic bacterial diversity at all the sites from east coast i.e. Vishakapatnam, Machilipatnam and Chennai and southernmost site i.e. Kanyakumari. CFU values were also found higher in sediment samples from all the sites. Total 120 heterotrophic marine bacterial species were isolated.

Diversity in colony morphology and pigmentation was observed. Isolate ***MB7*** showed the presence of yellow diffusible pigments and isolate ***MB 39*** was pale yellow colored, while remaining isolates showed no pigmentation.

Anti-microbial activity

A total of 120 heterotrophic marine bacteria were isolated on the basis of diversity of colony morphology. Out of which, 9 isolates showed some degree of inhibition against 6 strains of human pathogens. Among those isolates MB39 isolate showed maximum degree of inhibition against 6 strains of human pathogens compared to other isolates. The isolate MB39 obtained from Vishakapatnam Sea Sediment sample (SS1).

Table 1: Colony forming units (CFU) of sea sediments, sea shore sand sea water samples Collected from four different sites of southern India.

S.No	Site	Colony forming unit (CFU)		
		Sea sediments	Sea shore sand	Sea water
1.	Vishakapatnam	15	9.0	2.0
2.	Machilipatnam	18	11	3,0
3.	Chennai	10	4.0	1.0
4.	Kanyakumari	13	9.0	2.0

Table 2: Anti-microbial activity of bacteria isolated from Sea Shore Sand (SSS), Sea Sediment (SS), Sea Water (SW) against *Staphylococcus aureus* (SA), *Bacillus Subtilis*(BS), *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP) *Salmonella typhi* (ST) and Candida Albicans (CA) of human bacterial pathogens. Zone of Inhibitions (in mm) of active isolates from Sea Shore Sand (SSS), Sea Sediment (SS), Sea Water (SW) of 4 sampling sites.

Site	Strain	Sample code	Zone of inhibition (mm)					
			SA	BS	EC	KP	ST	CA
Machilipatnam	MB2	SSS2	11	9.0	13	8.0	16	10
	MB7	SS2	16	12	18	22	19	12
Vishakapatnam	MB24	SSS1	10	8.0	14	7.0	15	13
	MB39	**SS1**	**22**	**18**	**24**	**28**	**25**	**22**
	MB40	SW1	8.0	6.0	9.0	6.0	7.0	8.0
Chennai	MB68	SSS3	9.0	7.0	11	6.0	12	11
	MB74	SS3	14	10	16	18	15	12
Kanyakumari	MB88	SSS4	12	11	14	10	17	14
	MB118	SS4	17	13	19	23	20	16

Morphological and Biochemical characteristics

Colonies of MB39 were observed as small, circular, smooth and opaque on marine agar plate. Gram staining of the cells show gram positive short nods. The biochemical features of MB39 are presented in table-3. Isolate MB39 belonged to *betaproteobacteria* and phylogenetically related to *Alcaligenes faecalis sp.* was identified by their morphological and biochemical characters according to the Bergey's manual. Scanning electron micrographs of MB39 shows very short rods with coccobacilli like appearance.

Table 3: Morphological and Biochemical characteristics of strain *MB39.*

S. No	Tests	Result
1	Diffusible pigment test	Negative
2	Production of melanin pigment	Negative
3	Gram staining	Posittive
4	Oxidase test	Posittive
5	Catalase test	Negative
6	Urease test	Negative
7	Lipase test	Negative
8	Amylase test	Negative
9	Gelatinase test	Negative
10	Citrase test	Positive
11	Nitrate reduction test	Positive
12	H_2S production test	Negative

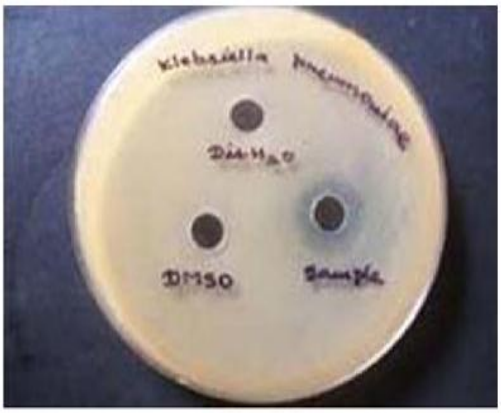
Antimicrobial activity of MB39

MB39 was found to be the most active of all the isolates. It showed activity against all the pathogens tested. It inhibited the growth of *Staphylococcus aureus, Bacillus substilis, Escherichia coli, Klebsiella pneumonia, Salmonella typhi* and *Candida albicans* (figure.1).

Staphylococcus aureus

Bacillus subtilis

Escherichia coli



Klebsiella pneumoniae

Salmonella typhi *Candida albicans*

Fig. 1: Anti-microbial activity of strain MB39 against selected pathogenic microorganisms.

Control: Dimethyl sulphaxide (DMSO) and Sterile Distilled Water Sample

Supernatant of Strain MB39

Optimization conditions for the production of the antimicrobial compound

The selected isolate MB39, grown by using Glucose as a carbon source showed good antimicrobial activity against the test pathogens among the 4 carbon sources tested (fig.2). Effect of time on the production of antimicrobial metabolite was studied. It was observed that degree of production of antimicrobial metabolite increased with increasing culture time with a maximum zone of inhibition obtained after 48 hours of growth after which decline in growth was observed at 72 hours (fig.3). Maximum zone of inhibition was observed in cultures grown at alkaline pH with a maximum activity zone at pH 9 (fig.4). The antimicrobial activity remained unaltered after being held at 50°C for 30 min, started to decrease after being held at 60°C for 30 min, and totally lost when held at 80°C for 30 min (fig.5).

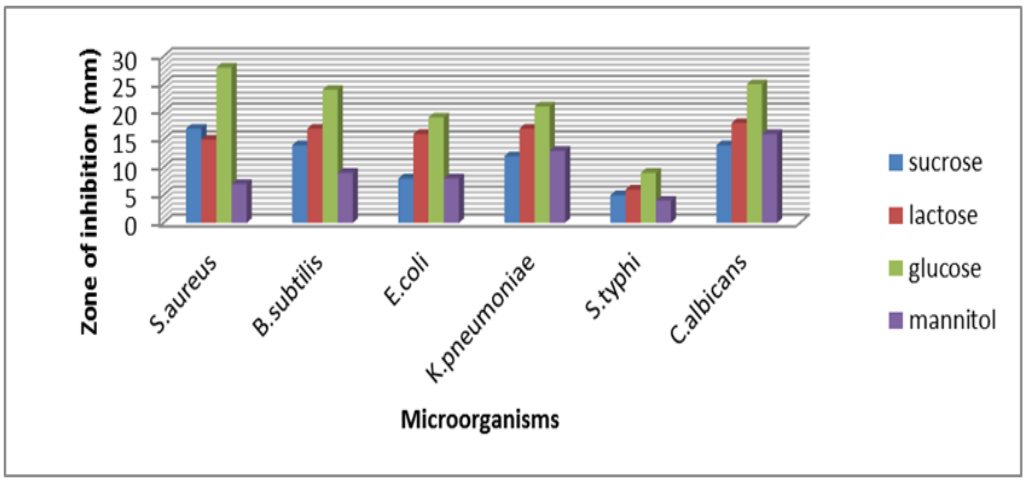


Figure 2: Effect of carbon sources on the production of antimicrobial metabolite.

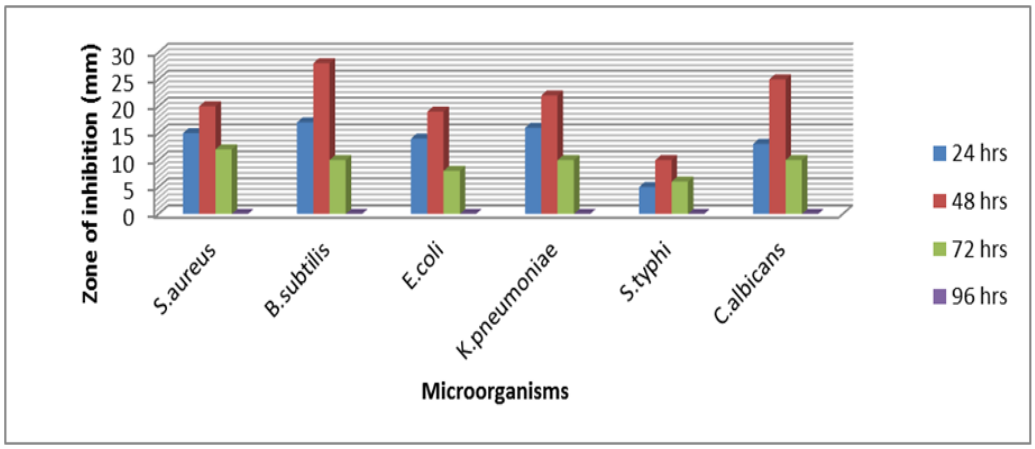


Figure 3: Effect of time on the production of antimicrobial metabolite.

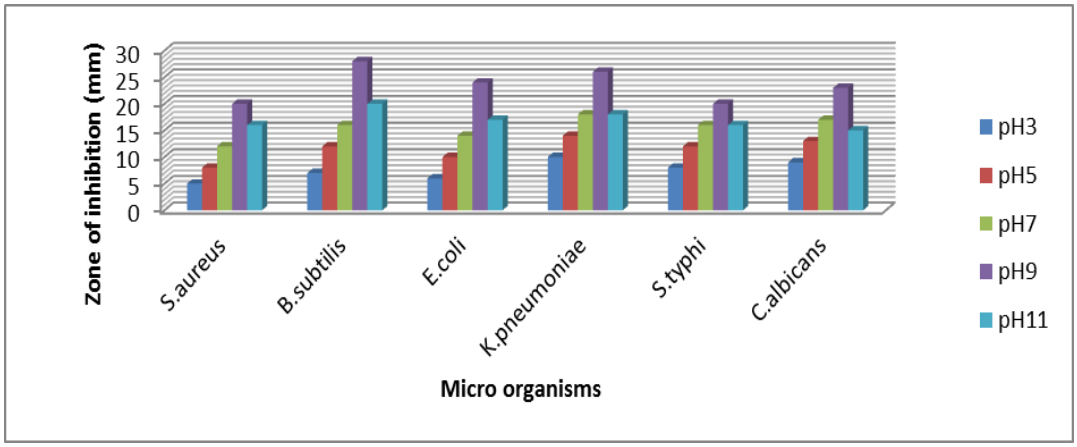


Figure 4: Effect of pH on the production of antimicrobial metabolite.

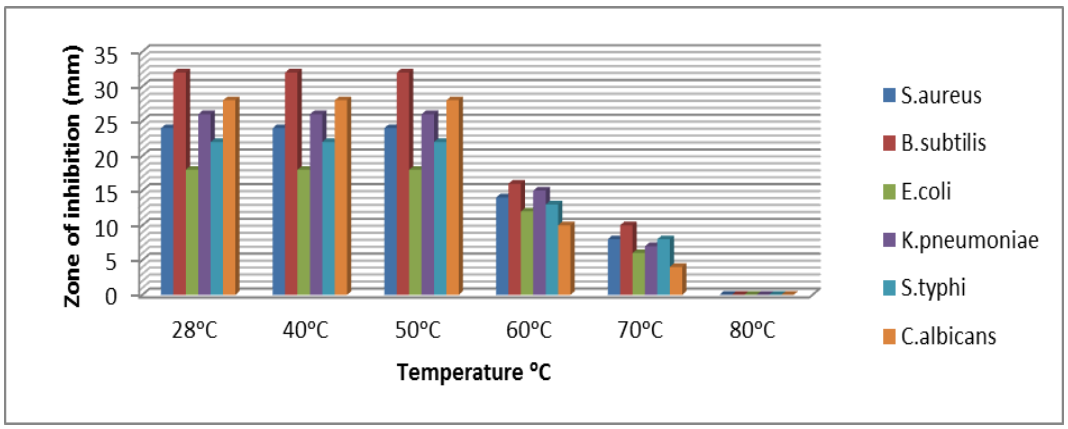


Figure 5: Effect of temperature on the production of antimicrobial metabolite.

Partial purification and characterization of the antimicrobial metabolite

Solvent extraction

For purification of the active compound solvent extraction was performed with five different organic solvents Chloroform, Methanol, Diethyl ether, Acetone and Ethyl acetate. Maximum activity was observed with ethyl acetate extract. Ethyl acetate extract was used for purification using column chromatography.

Column chromatography

The fractions were collected from each column. The fractions were observed under ultraviolet column ultraviolet radiations for presence of different compounds. Under UV the fractions absorbing UV radiation in the range of 254 nm were observed for isolate MB39.

Thin layer chromatography

Based on observation of TLC, amides and alcohol derivatives of aromatic compounds in the active fraction of MB39 isolate.

CONCLUSION

Four different locations such as Vishakapatnam, Machilipatnam, Chennai and Kanyakumari were selected; the samples like seashore sand, water and sediment were collected. A total of 120 heterotrophic marine bacteria were isolated on Zobell Marine agar medium using spread plate technique, out of which, 9 isolates showed some degree of inhibition against 6 strains of human pathogens. Among those isolates, MB39 isolate showed maximum degree of inhibition against 6 strains of human pathogens like *Staphylococcus aureus*, *Bacillus substilis*, *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi* and *Candida albicans* compared to other isolates. The isolate MB39 obtained from Vishakapatnam Sea Sediment sample (SS1), belonged to *betaproteobacteria* and phylogenetically related to *Alcaligenes faecalis sp.* was identified by the Bergey's manual. Scanning electron micrographs of MB39 shows very short rods with coccobacilli like appearance. For optimization the selected isolate MB39, grown by using Glucose as a carbon source showed good antimicrobial activity, the degree of production of antimicrobial metabolite increased with increasing culture time with a maximum zone of inhibition obtained after 48 hours, pH 9 showed maximum activity. The antimicrobial activity remained unaltered after being held at 50°C for 30 min, started to decrease after being held at 60°C for 30 min, and totally lost when held at 80°C for 30 min. For purification of the active compound solvent extraction was performed with five different organic solvents maximum activity was observed with ethyl acetate extract. The fractions were observed under ultraviolet column. Under UV, the fractions absorbing

UV radiation in the range of 254 nm were observed for isolate MB39. Based on observation of TLC, amides and alcohol derivatives of aromatic compounds in the active fraction of MB39 isolate.

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