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ANTAGONISTIC CHARACTERIZATION OF MARINE MICROALGAE EPIPHYTIC BACTERIUM PSEUDOMONAS MALTOPHILIA SU2 AGAINST SELECTED CLINICAL PATHOGENS

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ABSTRACT

Marine epiphytic bacteria become an important array of study in the exploration for novel microbial products. In recent years pharmaceutical, industrial focus on screening of marine microorganisms for biologically active secondary metabolites. We have studied antagonistic epiphytic bacteria from marine microalgae *Tetraselmis suecica* (Kylin). The antagonistic screening was carried out against selected clinical pathogens by the isolates. The potential epiphytic bacterium was identified as *Pseudomonas maltophilia* SU2 and its antagonistic ability was assessed at different physico-chemical parameters. Among the pathogens tested, highest antibacterial activity was noticed against *Vibrio cholera* (18mm) when the isolate incubated at 35oC at the pH 9. The crude ethyl acetate extract was partially purified by column chromatography. Each fraction was subjected to perform thin layer chromatography to check the purity and to calculate the retention factors (Rf). The Rf value of fraction 1, 2 and 3 were 0.70, 67 and 0.58 respectively. All the fractions were subjected to antibacterial screening against selected human pathogens. A total of six fractions was tested, interestingly fraction 1&2 showed maximum activity (10 ± 0.70 mm) against *Staphylococcus aureus* and *Streptococcus pyogenes*. The crude compounds were identified using GC-MS analysis. The extract from this epiphytic bacteria contain an unusual chemical compound of 2, 6, 10, 14 Tetramethyl heptadecane with the molecular weight of 296.57. This finding indicates that marine epiphytic bacteria have a wide array of biosynthetic capabilities for the production of novel and unique structures for clinical applications.

KEY WORDS: Microalgae, epiphytic bacteria, antibiogram, clinical pathogens

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INTRODUCTION

Marine habitat possesses an unbounded resource of valuable chemical substances for the development of new drugs for agriculture, aquaculture and biomedical applications. Marine natural products (MNP) are a major resource for new drug development for the pre existing as well as newly emerging diseases. A large number of algae, bacteria, fungi, actinomycetes and other marine organisms have been examined for bioactive secondary metabolites[1, 2]. Among these, marine bacteria constitute a prominent resource of untapped novel and unique metabolites[3]. Bacteria associated with marine organisms have been reported to demonstrate significant bioactivity including antibacterial, antifungal, antiviral, antitumor, anticoagulant and anti-cardio active potentials[4, 5]. A few of these substances have unique chemical structures that are unlike any other compounds, may serve as leads to the discovery of new drugs[6]. Bacteria are often found on the surface of many species of marine algae collected from natural habitats. The surface associated bacteria have been demonstrated to possess potential bioactive substances[7]. Most of the algal species need specific vitamins for their growth and associated bacteria may perhaps partially responsible for the production of these substances. Moreover, certain marine algae is capable of synthesizing antiviral, antibacterial, and antifungal compounds against several pathogenic microorganisms. It is believed that associated bacteria found in the algae are the producers of these bioactive compounds[8, 9]. Development of new natural products from extreme habitat recommends a hopeful alternative to the synthetic chemotherapeutic drugs for current clinical practices. Continuous use of chemotherapeutic drugs exhibit some undesirable side effects and the infectious microorganisms become resistant. The infections caused by resistant microorganisms do not answer to the ordinary treatment, which result in a long illness and the risk of death[10]. Hence the natural product researchers have been initiated to focus on natural resources for the development of new drugs to treat both acute and chronic infectious diseases caused by drug resistant microorganisms. Marine resources specifically microalgae associated bacterial natural products (MABNP) have a great impact on human health and are still scanty to be discovered and developed for biomedical applications. In this context an attempt has been mooted out to identify the marine microalgae epiphytic bacteria (MMEB) for the extraction of bioactive secondary metabolites. The main objective of the present investigation was to evaluate the antibacterial activities of the organic solvent extracts of epiphytic bacteria isolated marine microalgae and chemical constituent responsible for bioactivity using gas chromatography and mass spectrometry (GC-MS) analysis.

MATERIALS AND METHODS

Microalgae

Marine microalgae *Tetraselmis suecica* (Kylin) Butcher **(**Kingdom: Viridiplantae; Phylum: Chlorophyta; Class: Prasinophyceae; Order: Chlorodendrales; Family: Chlorodendraceae; Genus: Tetraselmis; Species: *Tetraselmis suecica***)** was collected from Centre for Marine Fisheries Research Institute (CMFRI) Tuticorin, Tamilnadu, India in a sterile screw cap tube which was kept in a ice chest box and brought to our laboratory. The microalgae were sub-cultured and maintained as a pure culture was chosen for the present microalgae associated bacterial metabolites investigation.

Isolation of epiphytic bacteria

One milliliter of exponential phase microalgae culture sample was taken and subjected to serial dilution up to 10-5 dilution and the aliquots are plated in Zobell marine agar (ZMA) plates by pour plate method. The plates were incubated at 37°C for 24 hours.Morphologically dissimilar well isolated colonies were randomly selected and stored at 4oC for further identification and antagonistic studies.

Identification of epiphytic bacteria

Algal associated bacteria showed prominent and broad spectrum activity was taken and purified by a streak plate technique. The isolates were identified based on the morphological, physiological and biochemical characteristics and compared with Bergey's Manual of Determinative Bacteriology[11].

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Chemicals and culture media
All chemicals and culture media components were procured from Hi media Laboratories Private Limited, (Mumbai, India) used to carry out the present investigation.

Bacterial human pathogens
Gram negative bacterial pathogens such as *Vibrio cholerae, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella* sp.*, Proteus* sp. and gram positive pathogens, namely *Streptococcus pyogenes, Staphylococcus aureus, Bacillus megaterium* and *Bacillus subtilis,* were collected from Kanyakumari Medical College and Hospital (KMCH), Kanyakumari District, Tamilnadu, India was selected for the present antibacterial susceptibility study.

Primary screening of antibiogram
The antibiogram activity (ABA) was tested by following cross streak assay method[12]. Single streak (4 – 6 mm diameter) of the isolated stains were streaked on the surface of nutrient agar plates and incubated at 37°C for 48 h. On obtaining a ribbon-like growth, the overnight culture of pathogenic bacteria was streaked at perpendicular to the original streak of epiphytic bacteria and incubated at 37^{0}C for 24h. Triplicates were maintained for each isolates to assess the antibiogram activity. The zone of inhibition was measured and recorded in mm in diameter. A control plate was also maintained without inoculating epiphytic bacteria to evaluate the normal growth of bacteria. All the bacterial isolates were assayed for the antibiogram activity and those isolates which showed prominent and broad spectrum activity were taken for further species level identification and mass scale production.

Mass cultivation of potential strain
Out of twenty epiphytic bacteria screened, *Pseudomonas maltophilia* SU2 strain which showed higher promising antagonistic activity was selected for mass cultivation for the extraction of required quantity of antimicrobial metabolites. A loopful of chosen bacterial strains were inoculated into 100ml of Zobel Marine broth (ZMB) and incubated in orbital shaking incubator (NEOLAB) for 24h. Ten percent of inoculum was transferred to 2000 ml conical flask containing 1000 ml of Zobel Marine broth with different growth parameter including pH (3, 5, 7, 9 and 11), and temperature (25, 30 and 35°C) were optimized independently. The fermentation flasks were incubated for 48h with continuous shaking.

Extraction of secondary metabolites
Mass cultivated broth (MCB) was mixed with 500 ml of ethyl acetate (1:0.5 v/v) in a separating funnel and vigorously shaken to extract the bioactive compound[13]. The mixture was kept in a stand in an undisturbed condition for 15min. The lower aqueous phase was collected in a beaker, the upper solvent phase was collected in a separate beaker to get crude extract. This process was repeated thrice to obtain complete extraction of active principles. The crude extract was concentrated in a vacuum evaporator at 40°C for 24h to obtain dry powder. This crude extract was used for further secondary screening studies against clinical pathogens.

Antibacterial assay
Antibacterial activity was determined against the chosen clinical pathogens using paper disc assay (PDA) method[14]. Whatman No.1 filter paper discs of 6mm diameter were cut and sterilized by autoclaving. The sterile discs were impregnated with different bacterial extracts (50µl/per discs). Control discs also maintained for each bacterial extract by impregnating ethyl acetate alone. Muller Hinton Agar (MHA) plates were prepared and overnight broth culture (1.2×10^{8} cfu/ml) of test pathogens were inoculated uniformly using sterile cotton swab. The impregnated discs were placed on the plates using sterile forceps suitably spaced at equal distance. Triplicates were maintained for each test pathogen. The plates were incubated at 37°C for 24h. The zone of inhibition appearing around the discs were measured and expressed in mm in diameter.

Characterization of bacterial secondary metabolites
Column Chromatography (CC) of bioactive metabolites
The crude extract was dissolved in an appropriate volume of ethanol and used for further purification by the standard methods[15]. The extract was purified by silica gel column chromatography (SGCC) using silica gel (60 –

120 mesh) as stationary phase and ethanol: water (1:1 ratio v/v) as solvent phase. The column (35 X10 mm) was cleaned and packed with silica gel with sterile distilled water. The secondary metabolites were partially purified by elution with (10% stepwise) 0–100% by volume of ethanol in water through a silica gel column. The fractions were collected based on the elution at a particular period of interval.

Thin Layer Chromatography (TLC) of bioactive metabolites

Each fraction was analyzed by thin layer chromatography (TLC) using pre-coated silica gel plates of 0.25 mm thickness (Merck, India) to identify the fraction that contained bioactive metabolites. To develop a chromatogram, Ethanol, water and chloroform were used in a volume ratio of 90:25:4. The eluted spot in the plate was visualized in the iodine vapour chamber and the Rf value of partially purified bioactive metabolite was calculated by the following formula.

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

GC-MS analysis of bioactive metabolites

The gas chromatography combined with mass spectrometry (GC-MS) detection technique is a qualitative and quantitative analysis. By this technique traces of active constituents can be detected with high sensitivity even from crude extracts. The GC-MS analysis was done by the standard method with standard specification of dissolving 10mg of crude extracts in 1 ml of ethyl acetate[16]. The aliquot of 0.1 µl was injected automatically into 0.25 mm x 25 m column of GC-MS model (GC 17A, Japan) 5% phenyl poly siloxane as stationary phase. Helium was used as a carrier gas at 17.69 psi pressure with the flow of 3ml/min at the flow rate of 0.4m/min. The temperature gradient program was implemented for the evaporation of organic solvent to identify the chemical moiety. The initial temperature was 70°C and acceleration at a rate of 10°C per minute to reach 250°C gradually. The sample was injected after 18 minutes at the final temperature of 250°C. The maximum peaks representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds[17]. The concentration of such compound was calculated by the following formula:

Compound concentration percentage = [P1/P2] x 100

Where, P1 is the peak area of the compound and P2 is whole peak areas in the fractionated extracts.

Data analysis

The data were statistically analyzed through TWO way ANOVA using MINITAB software and means of different parameters were separated by applying least significant difference (LSD) test at 0.05 % level of probability to know their significance status by the method of Steel *et al*[18].

RESULTS

Screening of antagonistic isolates

The primary screening of antagonistic isolate was performed by cross streak assay method. Antagonistic activity of isolates were measured, recorded and is portrayed in Table 1. A total of 20 epiphytic bacteria has been isolated and subjected to screening for the antagonistic activity against selected human pathogens. In the present investigation 80 % of isolates showed antibiogram activity against at least one of the tested human pathogens. From the isolates, a strain which had broad spectrum of antibiogram activity against all the human pathogens has been chosen for further studies. The potential isolate was subjected to further strain identification and mass production of secondary metabolites. The microalgae associated bacterial strain which showed prominent and broad spectrum activity were subjected to species level identification (Table 2). The epiphytic bacteria were identified and named as *Pseudomonas maltophilia* SU2.

Table 1
Screening of antibiogram activity of epiphytic bacteria by cross streak assay method

Epiphytic bacterial isolates	Test pathogens									
	Vibrio cholerae	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Salmonella sp*	*Proteus sp*	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Bacillus megaterium*	*Bacillus subtilis*
SU1	++	+++	++	++	+++	++	+	++	+++	+++
SU2	++	++	++	+	++	++	++	+++	+++	++
SU3	+	++	+++	+	+	+	+	++	++	++
SU4	++	+	++	++	+	+	+	+	++	++
SU5	++	+	+	-	+	+	++	++	++	++
SU 6	-	-	-	-	-	-	-		+	+
SU 7	+	+	+	+	+	+	+	+	-	-
SU 8	-	-	-	-	-	-	-	-	-	-
SU 9	-	+	+	+	+	+	++	++	+	+
SU 10	+	-	-	-	+	+	++	++	+	+
SU 11	-	-	-	-	-	-	+	+	+	+
SU 12	+	+	+	+	+	+	-	-	-	-
SU 13	-	-	-	-	-	-	-	-	-	
SU 14	+	+	+	+	++	++	-	-	-	-
SU 15	-	-	-	-	-	-	-	-	-	
SU 16	-	-	-	-	-	-	++	++	+++	+++
SU 17	+	+	+	+	+	+	++	++	++	++
SU 18	-	+	+	+	+	+	++	++	++	++
SU 19	+	+	+	+	+	+	-	-	-	-
SU 20	-	-	-	-	-	-	-	-	-	-

Reference: +++ 10 - 15 mm; ++ 5-10 mm; +>5mm;- No activity

Table 2
Biochemical characteristics of epiphytic bacteria Pseudomonas maltophilia SU2

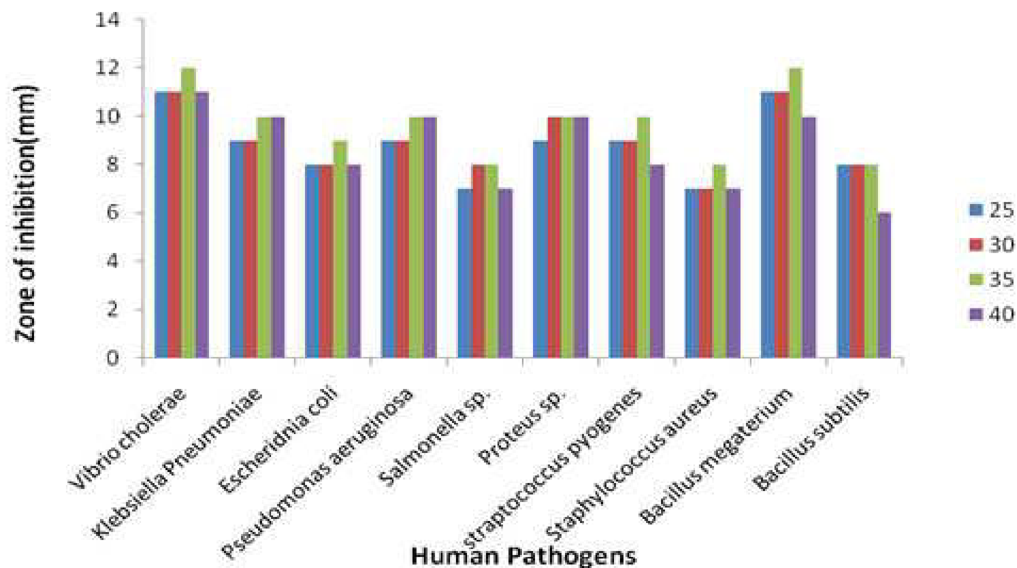
Biochemical characteristics	Test results
Gram's staining	G –ve rod
Motility test	Motile
Indole test	-
MR test	-
VP test	-
Citrate test	+
Urease test	-
Oxidase test	+
Nitrate test	+
H2S production	-
Glucose	-

"+"- positive reactions; "-"- negative reactions

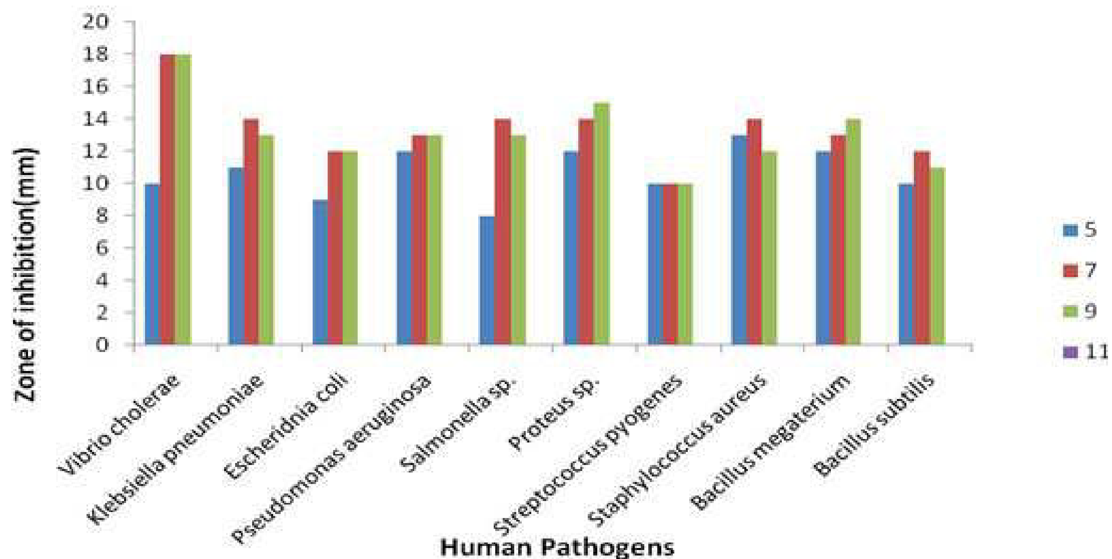
Antibacterial activity

The most promising potential candidate strain of *P. maltophilia* SU2 was adopted for further mass scale production of secondary metabolites in different parameters under experimental conditions separately. Antibacterial activity of compounds extracted from epiphytic bacteria was studied at different temperature and pH. The antibacterial susceptibility of crude extract of *P. maltophilia* SU2 at various temperatures and pH is depicted in graph1 & 2 respectively. Maximum antibacterial susceptibility (12mm) was noticed against *V. cholera* and *B. megaterium*, when the isolate incubated at 35oC. Highest antibacterial activity (18mm) was noticed against *V. cholera*, when the media adjusted at the pH 9. On analyzing the antimicrobial activity data obtained using supernatant of epiphytic bacterial extract against human pathogens, the values were significant ($P < 0.05$) between organisms at pH 7 and highly significant at pH 7 and 9.

Graph 1
Antibacterial activity of P.maltophiila SU2 against human pathogens at different temperature



Graph 2
Antibacterial activity of P.maltophiila SU2 against human pathogens at different pH



Purification of bacterial secondary metabolites

The crude ethyl acetate extract of *P. maltophilia* SU2 metabolites was partially purified by column chromatography. In the present investigation column chromatography fractions were separately collected based on the band separation at specified time intervals. Each fraction was subjected to perform thin layer chromatography to check the purity and to calculate the retention factors (Rf) value of the metabolite. A single separate band of the antimicrobial compound was observed by TLC. The Rf value of fraction 1, fraction 2 and fraction 3 was 0.70, 67 and 0.58 respectively. All the fractions were subjected to antibacterial propensity against selected human pathogens is depicted in Table 3. A total of six fractions tested, fraction 1 exhibited antimicrobial activity against all the tested pathogens. Fraction 2 & 3 showed antimicrobial activity against all the tested pathogens except *K.*

pneumoniae and *B.megaterium* respectively. Interestingly fraction 1&2 showed maximum activity (10 ± 0.70 mm) against *Staphylococcus aureus* and *Streptococcus pyogenes*.

Table 3
Antibacterial susceptibility of partially purified metabolites of P. maltophilia SU2

Antibacterial metabolites	Zone of inhibition in mm diameter										
	Control	*Vibrio cholerae*	*Klebsiella pneumonia*	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus megaterium*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Salmonella* sp.	*Proteus* sp.	*Streptococcus pyogenes*
Fraction 1	-	10.4 ± 1.14	9.6 ± 0.54	9.4 ± 0.54	10 ± 0.70	9.6 ± 0.89	10 ± 1.22	8.6 ± 1.34	9.8 ± 0.83	10 ± 0.70	10 ± 0.70
Fraction 2	-	9.4 ± 1.14	-	8.6 ± 0.54	8.6 ± 0.54	8.2 ± 0.83	8.4 ± 1.34	7.8 ± 0.83	8.8 ± 0.83	9.4 ± 1.14	10 ± 0.70
Fraction 3	-	9 ± 0.70	8.4 ± 0.54	8.8 ± 0.83	10 ± 1	-	10.2 ± 0.83	9.6 ± 1.14	9.4 ± 1.14	9.2 ± 0.44	9.6 ± 1.34
Fraction 4	-	-	-	-	-	-	-	-	-	-	-
Fraction 5	-	-	-	-	-	-	-	-	-	-	-
Fraction 6	-	-	-	-	-	-	-	-	-	-	-

"-" No activity ; Each value is the mean ± SD of three individual estimates

Identification of bioactive principle

The bioactive compounds extracted from algal epiphytic bacteria *P. maltophilia* SU2 was dissolved in ethyl acetate and 0.1 µl of sample was injected into GC-MS (GC17A, Japan) with standard specification. The different compounds identified using GC-MS analysis of microalgae associated bacteria *P. maltophilia* SU2 extract is illustrated in Fig. 1a. The number of compounds (peaks) reported in the crude extract of epiphytic bacteria is depicted in Table 4. The peak separation of main chemical constituents present in the crude extract of *P. maltophilia* SU2 at the retention time of 18.625 with base peak 56.90 is portrayed in Fig. 1b. The extract from this epiphytic bacteria contain an unusual chemical compound of 2, 6, 10, 14 Tetramethyl heptadecane with the molecular weight of 296.57 is presented in Fig. 1c. This chemical marker superimposes way for future research to pinpoint the chemical constituent that possesses the antimicrobial activity.

Table 4
Number of compounds (peaks) reported in the crude extract of P. maltophilia SU2

PK. NO	R.Time	I.Time	F.time	Area	Height	A/H(sec)	MK % Total Name
1.	11.256	11.117	11.483	4018814	544580	7.380	31.16
2.	12.847	12.725	12.975	1747883	207883	8.408	13.55
3.	13.054	12.975	13.250	1216767	153931	7.905	9.43
4.	16.013	15.892	16.175	2640250	459677	5.744	20.47
5	18.616	18.500	18.767	1748784	299455	5.840	13.56
6.	20.195	20.075	20.350	1525424	230649	6.614	11.83

Figure. 1a
Detection of mixed secondary metabolites produced by P.maltophilia SU2 using GC-MS analysis

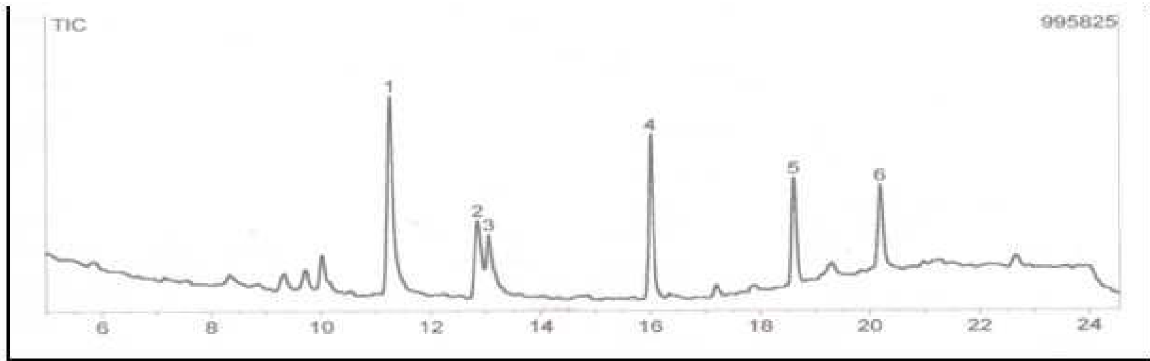


Figure. 1b
Peak separation at the retention time of 18.625; base peak 56.90

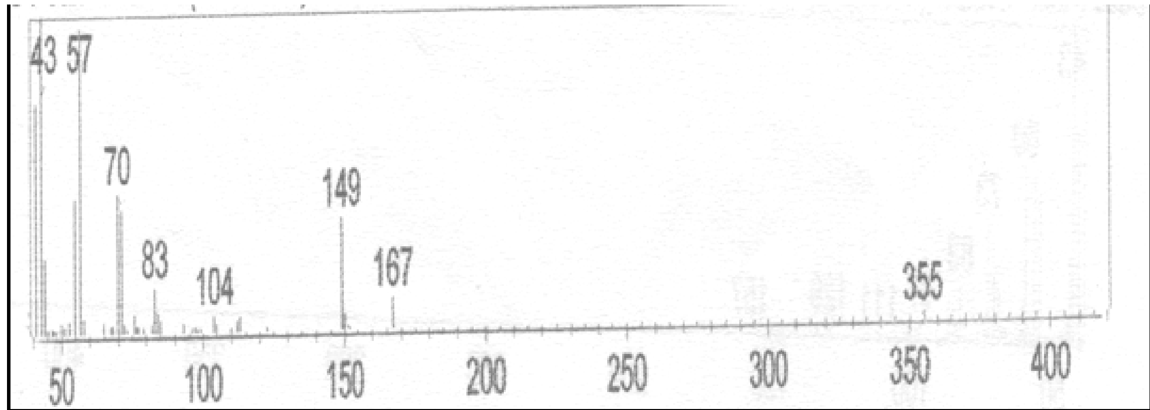
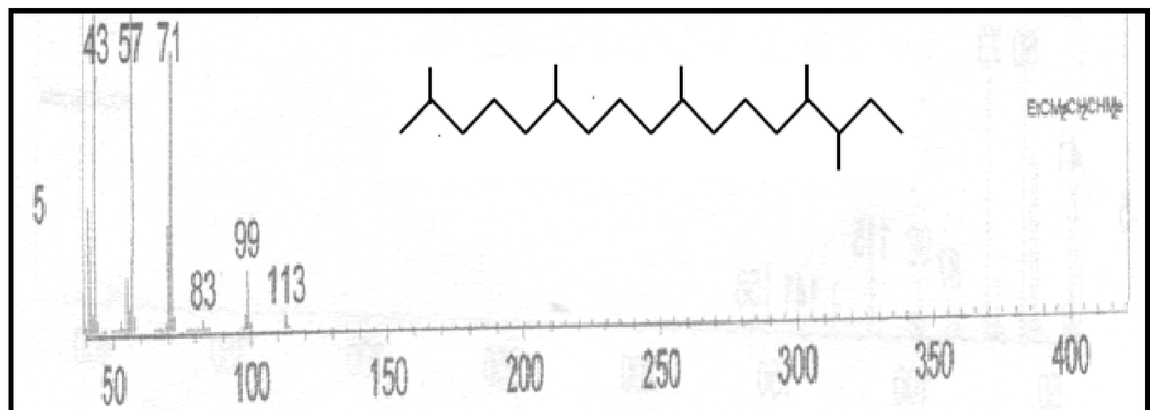


Figure. 1c
2, 6, 10, 14 Tetramethylheptadecane (M.W: 296.57)



DISCUSSION

Microalgae and bacteria association is commonly observed in both freshwater and marine ecosystems. Microscopy studies have documented a number of algal – bacterial interactions[19]. Epiphytic bacteria growing on the surface of marine algae and other organisms live in a highly competitive environment to access nutrients and space. These bacteria can produce secondary metabolites which inhibit the settlement of potential competitors. Epiphytic bacteria are therefore attracting considerable attention as a source of new natural products[20]. Many studies are under taken in this direction to isolate the bacteria from marine algal surface. The present study portrays the potential of marine epiphytic bacteria to produce bioactive compounds active against drug-resistant clinical pathogens. In this investigation the antimicrobial secondary metabolites were isolated from marine microalgae symbiotic bacteria *P. maltophilia* SU2. The isolate had antimicrobial activity against selected human pathogens. Longeon *et al*[21] reported that the marine bacterium *Pseudoalteromonas* sp. strain X153 had antimicrobial activity against human pathogens. In the present study with epiphytic bacteria for antimicrobial substance production was maximum at pH of 7 compared to other pH values studied. Similar research was carried out by Marwick *et al*[22] indicated that the pH level of the growth medium has a marked effect on secondary metabolic production. Marine bacterium *Alteromonas leuteoviolacea* produced violacin at an optimum pH of 7. Similarly *P. fluorescence* produced phaenazine at an optimum pH of 7. The pH used as a stressor to indicate methylomycin synthesis by *Streptomyces* sp. a technique that merits further investigation as a bioprocess intensification tool[23]. The identified epiphytic bacteria *P. maltophilia* SU2 had a wide range of significant antagonistic activity against selected bacterial pathogens. Similar results was observed in *Pseudoaltromonas* sp isolated from the marine habitat[24, 25]. Radjasa *et al*[26] revealed that green algae *Halimeda* sp associated bacterium *Pseudoalteromonas* sp. H17 showed strong growth inhibition against *Staphylococcus aureus*. Marine bacteria have recently emerged as an entirely new source of structurally novel natural product for the development of new drug candidates. The present study also made an attempt to find out the chemical structure of the antimicrobial substance isolated from epiphytic bacteria *P. maltophilia* SU2. In the present study, we have made an attempt in a notable way to the structural elucidation of inhibiting compounds. Until recently, the final purification and establishment of structures have been achieved only in few cases. The first documented identification of a bioactive marine bacterial metabolite was the highly brominated pyrrole antibiotic from *Pseudomonas bromoutilis* from the surface of the Caribbean Sea grass *Thalassia*[27]. In the present investigation three active fractions were identified at the Rf value of 0.70, 67 and 0.58 respectively. Fraction 1&2 showed significant antibacterial activity against selected clinical pathogens. Madhava Charyulu *et al*[28] reported that partially purified marine bacterial secondary metabolites demonstrated antimicrobial activity against both gram positive and gram negative organisms. These results indicate that partially purified secondary metabolites have selective antibacterial response mechanisms against pathogens rather than the crude extract. Therefore it is possible that the microalgae epiphytic bacteria used in this study, share similar defense mechanisms, creating an ecological and biotechnological interest in marine natural product.

CONCLUSION

The present investigation showed that the epiphytic bacteria *Pseudomonas maltophilia* SU2 isolated from marine microalgae *Tetraselmis suecica* had broad spectrum of antagonistic effect against gram positive and gram negative clinical pathogens such as *V. cholera, B. megaterium, S. aureus* and *Streptococus pyogenes*. The bioactive compounds produced by the epiphytic marine bacterium have to be tested in clinical trials in order to make it as a wide range of antibiotics for clinical applications. It is obvious that the epiphytic bacterial strain produces a novel compound which may be the substance responsible for actual antimicrobial activity.

Thus, the epiphytic bacteria associated to this microalga may perhaps yield an immense array of new compounds with unique activities. This study will open a new avenue to provide a novel drug to fight back against a number of clinical pathogens which are currently resistant to conventional chemotherapies.

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