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Original Research Article

Extraction and characterization of antimicrobial pigment from marine bacteria and testing of antimicrobial activity against penicillin resistant *Staphylococcus aureus*

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ABSTRACT

The current study shows the extraction of pigment from pigment producing bacteria isolated from marine sediment. The isolation of pigment producing bacteria was done by spread plate method. The marine nutrient medium was used for the isolation procedure. Further, subculture was followed to get desired pure culture. From the isolated culture, various preliminary test, biochemical test, molecular characterization and antimicrobial activity were performed. To extract the pigment a solvent centrifugation process was carried out. The extracted pigment was tested against drug resistant Staphylococcus aureus. Based on the FTIR spectrum respective functional group were identified from extracted pigment. After GC-MS spectral analysis specific Neoisolangifolene (32.73%) compound was identified from the total pigment extract. UV-Visible spectral studies of the pigment extract have confirmed with the variations in absorption peak of total pigments. The isolated organisms were identified based on 16s rRNA partial gene sequence analysis identified as Micrococcus flavus.

1. Introduction

Interest in synthetic pigments has decreased as they are toxic, carcinogenic, and teratogenic in nature. Pigments produced from natural sources are gaining significance worldwide in biomedical and other food industries.[1] Microorganisms are considered as potential source of bio pigment production over plants because of their availability, less cost, easy cultivation, rapid growth, considerable yield independent from weather conditions and colors of different shades. Microbial pigments also possess antimicrobial activity against pathogens, anticancer, anti-inflammatory activities.[2,3]

The marine environment is a productive resource for the isolation of novel microbial products exhibiting antimicrobial activity.[4] Penicillin resistant pathogens like Staphylococcus aureus and Vancomycin resistant S. aureus has become a serious global public health issue now a days.[5] According to World Health Organization (WHO), the European Centre for Disease Prevention and Control (EDDC) and the Centre for Disease Control and Prevention in the US (CDC) the multidrug resistant (MDR) pathogens attribute to increasing mortality rates. It is reported that the antibiotic resistance would become the major cause of mortality by 2050 (WHO 2019). Hence, powerful antimicrobials are essential to overcome antibiotic resistant pathogens. There is few research reported on the discovery of bio active compounds from marine bacteria.[6] Owing to this, the present study has been carried out to isolate potential pigment producing bacteria from marine sediment soil and to extract and characterize antimicrobial pigments and study its effect on the control penicillin resistant *Staphylococcus aureus* along with conventional antibiotics.

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2. Materials and Methods

2.1. Sample collection

Marine water and sediment sample were collected from Pulicat Lake, Tamilnadu, India at the depth of 2-10 meters. The collected sample was brought to the laboratory using sterile poly propylene covers and PET bottle (Figure 1). The sample was stored under 4°C in a ice bag and samples were processed on the collected day itself.

Fig. 1: Samples collected from marine water

2.2. Isolation of bacteria

The serial dilution spread plate method was followed to isolate bacteria from the water and sediment sample. The plates were incubated at 25°C for 24-48 hours to obtain colonies. Individual colonies were picked upon the basis of their macroscopic characters such as size, shape, surface, appearance, texture and color. The putative colored bacterial colonies were selected and streaked in a marine nutrient agar plate.

2.3. Isolation and screening of pigmented organisms

1. Composition Grams/litre
2. Peptone 10.0g
3. Yeast extract 10.0g
4. Agar 15.0g
5. Distilled water 50L
6. Sea water 50L

0.1 ml of the diluted sample was spread on marine nutrient agar. The agar plates were incubated at 37°C for 5-6 days. After incubation, yellow pigmented colonies were selected from the mixed plate culture and sub cultured to get pure form of colonies.

2.4. Extraction of pigment from isolated bacteria

The pigment producing isolates were inoculated in sterile marine nutrient broth and incubated at room temperature for one week. The culture suspension was centrifuged at 2500 rpm for 15 minutes. To the pellet 5ml acetone was added and kept in boiling water bath at 60°C for 20minutes. Until all visible pigment got extracted. The supernatant was collected by centrifugation at 2500rpm and filtered through what man's no.1 filter paper. The acetone was allowed to evaporate and resulted pigment was collected.

2.5. Antimicrobial activity

The antimicrobial activity was determined by well diffusion method. About 25ml of molten Muller Hinton agar was poured into sterile Petri plates. The plates were allowed to solidify, after which 18 hours grown 100μl of pathogenic bacteria was transferred onto plate and made culture lawn by using sterile L-rod spreader. The test samples were loaded into wells with various concentrations such as 50μg/well, 75μg/well, 150μg/well and 200μg/well. The plates were incubated at 37°C in a bacteriological incubator for 24 hours. The Solvent saline loaded well served as negative control and Streptomycin (30μg/ml) well served as positive control. The plates were incubated at 37°C in a bacteriological incubator for 24 hrs. The antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale. Synergy was tested by various concentrations of the pigment with standard drug penicillin by well diffusion method since zone can be readily visualized.

2.6. Identification of pigmented bacteria

The pigmented bacteria were identified by standard biochemical tests.

2.7. Characterization of pigment

2.7.1. FTIR analysis

IR spectra for the purified compounds were recorded on a Bruker Alpha II series FTIR spectrometer using KBr pellets. For the identification of functional group FTIR analysis was carried out.

3. Gas Chromatography

3.1. Shimdzu GC-2010 Plus gas chromatography was used

3.1.1. Mass spectrum

A Direct connection with capillary column metal quadupole mass filter period rod mass spectrometer operating in electron ionization (EI) mode with software GCMS solution ver. 2.6 was used for all analyses. Identification of the components of the compound was matching their recorded spectra of NIST library V 11 provided by the instruments software. GCMS metabolomics Database was used for the metabolomics. Database was used for the similarity search with retention index.

3.1.2. UV–Vis spectra

UV–Vis spectra were recorded using a UV–Vis spectrophotometer (UV 1800, Shimadzu, Japan) between

200 and 800 nm. Methanol was used as blank.

3.2. Molecular studies

3.2.1. Extraction of DNA from isolates

The microbial culture was centrifuged at 5000 rpm for 5 mins. The pellet was suspended into 200 μl of TE buffer (10 mm TrisHcl, 1 mm EDTA, pH — 8) and add 50 mg of RNase to digest the contaminating RNA. 400 μl of solution 1 {1% W/V Sarkosyl, 0.5M NaCl (1% W/V) SDS} was added and mixed well and the tubes were incubated for 10 mins at 37ºC with intermittent shaking at every 5 minutes. Immediately equal volume of PCI (PhenolCholoroformisoamyl alcohol) (25:24:1) was added and mixed by inversion. The suspension was centrifuged at 10,000 rpm for 5minutes at 37ºC and carefully transfer the supernatant into a Eppendorf tubes. 100 μl of sodium acetate (3M, pH — 5.2) and 600 μl of isopropanol were added and mixed gently by inverting the tubes for 6 minutes. The suspension was centrifuged for 5 minutes at 10,000 rpm and supernatant was discarded. The pellet DNA was washed with 1 ml of 70% ethanol and centrifuged at 10,000 rpm for 3 minutes. The supernatant was discarded and air dried the pellet. The DNA pellet was resuspended in 30 μl of sterile TE buffer and the suspension was stored at -20ºC until for use. The DNA was mixed with tracking dye and was run in agarose gel electrophoresis.

3.3. Identification of bacterial isolates using 16s rRNA gene

PCR amplification was performed using a 50ml reaction mixture containing 100ng of template DNA, 20 mmol of 16s rRNA primers, 200l of dNTPs.,1.5 mM of., 1U of Taq DNA polymerase (MBI Fermentas) 10 l of 1 Tap polymerase buffer. The sequences of 16S rRNA primers used as follows. 27f: (5'-AGAGTTTGATCCTGGCTCAG-3') 1522r: (5'-AAGGAGGTGATCCANCCRCA-3').

Amplification was carried out with an initial denaturation at 95 C for min followed by 35 cycles of denaturation at 94 C for 45sec, annealing at 56 C for 45 sec, extension at 72 C 1 min and final extension at 72 C for 5 min using a thermocycler (iCycler; Bio-Rad Laboratories, CA). PCR products were analyzed on 1% agarose gel 16s rRNA amplicons in 1x TBE buffer at 100 V.

3.4. Sequence analysis of PCR products

The 16S rRNA and ITS region fragments were purified using the QIA quick gel extraction kit (Qiagen, Valencia, CA) from the agarose gel and sequencing using automated DNA sequence (Model 3790, Applied Biosystems, USA). The sequences were analyzed using the option Basic Local Alignment Search Tool (BLAST) software available in NCBI.

4. Result

4.1. Isolation of microbial (Micrococcus flavus) pigment

Isolation of micro-organism was carried out using appropriate media. The culturing of organisms was done by using Marine nutrient agar medium. Screening was done for pigmented yellow colonies. The growth of the isolated organism was observed. Identification of the isolated organism was done by standard biochemical tests

4.2. Extraction of pigments from pigment producing bacteria

The pigments extracted were yellow in color (Figure 2). The optical density of the pigment was measured and further processed for antimicrobial activity. The pigments produced in this study have shown different optical density. The peak optical density was at 450nm and the pigment which showed maximum optical density was yellow pigment.

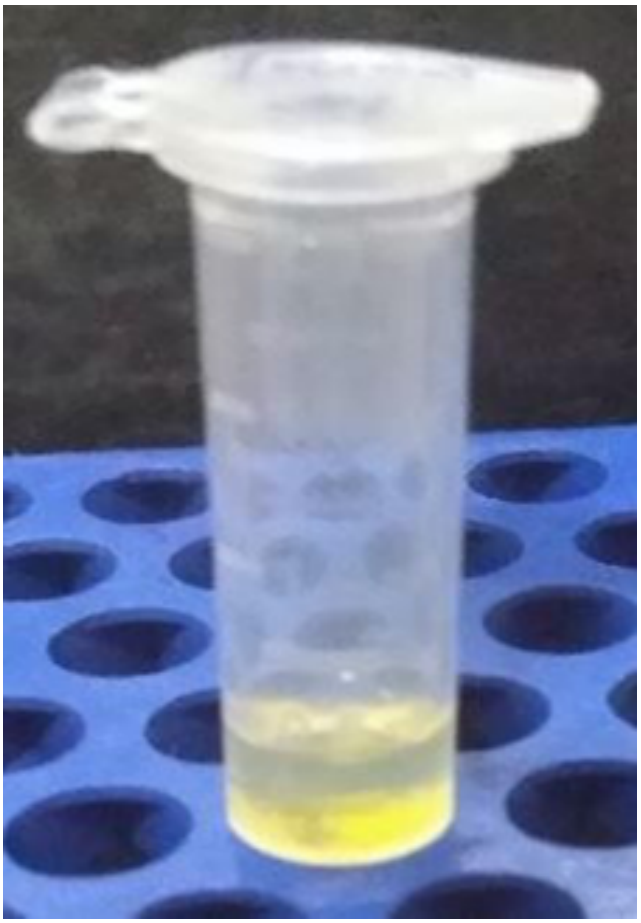


Fig. 2: Extracted pigment

4.3. Antimicrobial activity

The extracted pigments were dissolved with solvent acetone to evaluate the antimicrobial activity against human pathogen by well diffusion method. The bacterial pathogen was *Staphylococcus aureus.* The zone of inhibition (ZOI) of the pigment against this pathogen was measured to evaluate antimicrobial activity. This study clearly indicated that the inhibitory metabolites produced by isolated organism were intracellular. The pigments extracted from the isolates; yellow pigment showed better anti- bacterial activity in terms of zone of inhibition.

The antibiotic resistance spectrum of *S. aureus* was tested with standard antibiotic penicillin at different concentration (10 to 40 units/ml). It was noted that, the standard penicillin did not show any of the inhibition at any concentration tested. It was clearly indicated that, the test strain was definitely a methicillin resistant as phenotypic characterization.

4.4. Antibacterial effect of extracted pigment

Antimicrobial activity of the selected culture pigment extracts was tested against the resistant *S.aureus* isolate. The tested yellow-colored pigment showed a good spectrum of antibacterial effect (Figure 3). The antibacterial activity of the yellow pigment and penicillin combination showed a very good spectrum of antimicrobial activity (Figure 4)

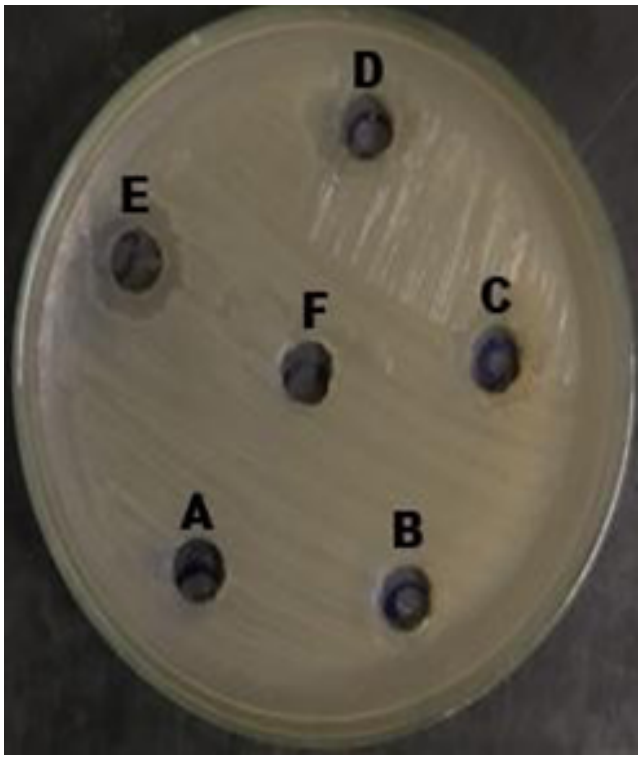


Fig. 3: Antibacterial activity of yellow pigment

A:0 μg/ml (Sterile water); B: 50 μg/ml;YP C: 100 μg/ml; YP D: 150 μg/ml; YP E: 200 μg/ml; YP F: 40 μg/ml YP (Penicillin)

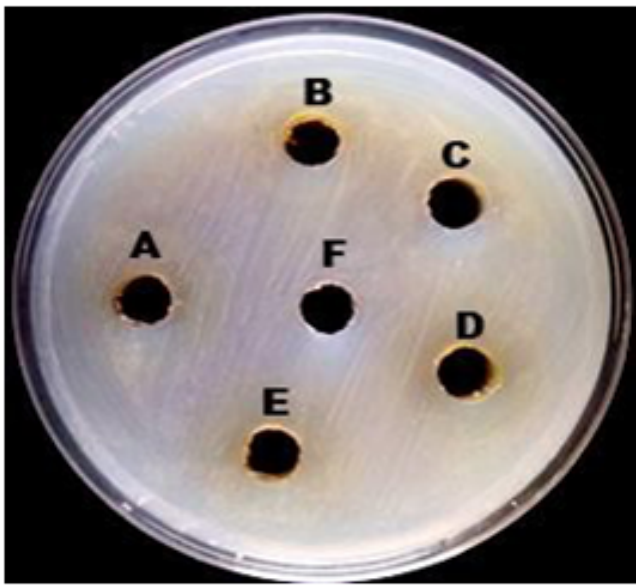
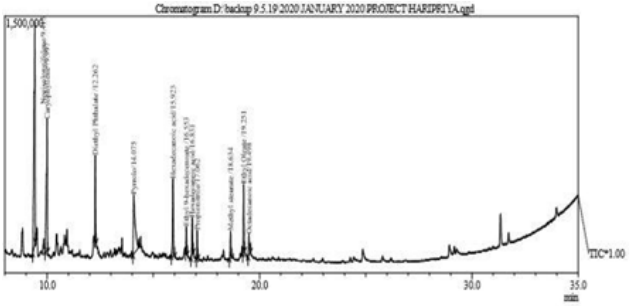


Fig. 4: Antibacterial activity of yellow pigment +penicillin against

A:0 μg/ml (Sterile water); B: 50+50 (YP+P) μg/ml; C:100+100(YP+P) μg/ml; D: 150+150 (YP+P) μg/ml; E: 200+200 (YP+P) μg/ml; F: 40 μg/ml (Penicillin) (YP: Yellow pigment; P: Penicillin)



Peak Report TIC

Peak#	R.Time	Name	Area	Area%	Height	Height%
1	9.415	Neoisolongifolene	4692526	32.73	1314077	28.53
2	9.997	Caryophyllene	2611369	18.22	776685	16.86
3	12.262	Diethyl Phthalate	857151	5.98	497939	10.81
4	14.075	Pyrrolo	2201051	15.35	347249	7.54
5	15.923	Hexadecanoic acid	1245220	8.69	454188	9.86
6	16.553	Ethyl 9-hexadecenoate	318847	2.22	154331	3.35
7	16.831	Hexadecanoic acid	480455	3.35	217459	4.72
8	17.062	Propionitrile	304533	2.12	163034	3.54
9	18.634	Methyl stearate	358386	2.50	158540	3.44
10	19.251	Ethyl Oleate	842929	5.88	400346	8.69
11	19.498	Octadecanoic acid	423651	2.96	122074	2.65
			14336118	100.00	4605922	100.00

Fig. 5: Gas chromatography mass spectrography

GC-MS results showed the different compounds presents in the extracted yellow pigment crude extract (Figure 5).

Among the compounds found in the GC-MS spectrum, the compound neoisolangifolene having the presence of 32.73% area coverage. The compound nature was evaluated in the Pub chem database showed the physical properties of the compound is slightly yellowish color indicative the abundance and color matched with this compound may be the extracted yellow pigment.

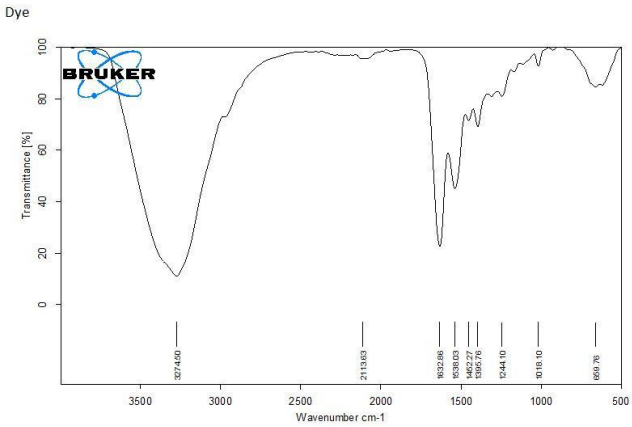


Fig. 6: FTIR analysis

In yellow pigment the finger print region was observed with predominant peak at 1632 86 bond denotes stretching vibration of carbonyl group, denotes the presence of secondary amide. The peak at 1538.03 denotes the N-O stretching nitro group. The peak at 1452.27 denotes the C-H bending alkane. Also, a prominent peak at 1395.76 denotes the C-H bending aldehyde. The peak at 1244.10 denotes the C-H stretching bond and peak at 1018.10 corresponded to the peak at aromatic C-H in bend. The FTIR spectrum showed the peak at 3274 cm^{-1} indicative of hydroxyl group in the compound. Carbonyl group showed at the peak at 2113 cm^{-1}. The H-O-H bending stretch of the compound showed a broad peak at 1632 cm^{-1}.

4.5. UV-VIS spectrum

The UV- visible spectrum showed three peaks at 236, 251 and 379 nm of UV absorbable peaks (Figure 7)

4.6. Phylogeny tree analysis

The sequence of these 16S rRNA genes were compared against the sequence available from GenBank using the BLASTIN program.

5. Discussion

Staphylococcus aureus has been reported resistance towards penicillin and its derivatives and has put a demand to search for novel antimicrobial compounds to meet the requirement. Marine sediment remains a source of providing immense possibility to identify microorganisms capable of producing

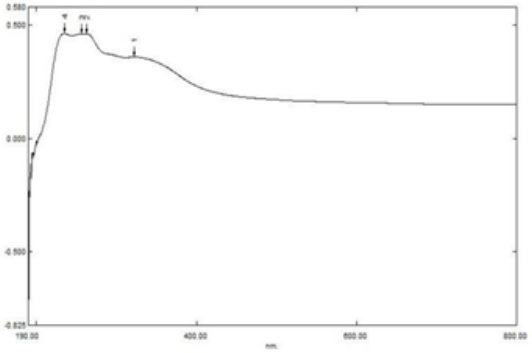


Fig. 7: UV-VIS Spectrum

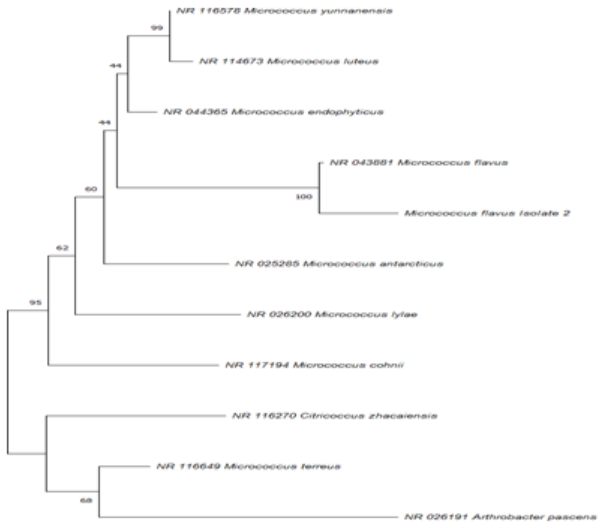


Fig. 8: Phylogenetic tree

many drugs and pharmacologically active substances. So in the present study potential pigment producing bacteria were isolated from marine sediment soil and antimicrobial activity of the pigment was evaluated against drug resistant S aureus.

From the sample obtained from Pulicat lake variety of pigmented isolates were observed. Among these yellow pigments were abundant and showed maximum optical density. Morphological, cultural and biochemical test of isolated pigment producing bacteria were carried out and was identified by comparing the results with Bergey's manual of Determinative Bacteriology. The results revealed that the strains were oxidase positive and catalase positive Gram-positive cocci in clusters producing yellow pigment as *Micrococcus sp.* Oxidase test differentiates *Micrococcus sp*. from *Staphylococcus sp* (which is oxidase negative). The pigment was extracted using acetone as a solvent.

The antimicrobial susceptibility pattern studied showed that *S. aureus* was resistant to penicillin. The extracted

pigment was tested for antimicrobial activity against *S. aureus* in varying concentrations of 50,100,150 and 200μg/ml. The pigment at 200μg/ml gave a significant zone of inhibition against S. aureus with a 15 mm zone of inhibition. Penicillin was used in determining synergism with the isolated pigment against the resistant *S. aureus.* Synergy is defined as a decrease in the viable organism as a result of the combination when compared with the antibiotic when tested alone. Synergy was tested by various concentrations of the pigment with standard drug penicillin by well diffusion method since zone can be readily visualized. Concentrations of 200μg of the pigment and 200 μg of penicillin resulted in synergy at. 37°C overnight incubations resulting in a 21mm zone of inhibition. The antibacterial activity of the yellow pigment and penicillin combination showed a very good spectrum of antimicrobial activity indicative of the yellow pigment having role on the beta lactamase inhibition. It was due to the inhibition of beta lactamase by yellow pigment the antibiotic penicillin showed activity.

The strain was subjected to molecular identification. Total DNA was extracted from culture and the amplicons were analyzed by agarose gel electrophoresis. PCR amplification of the 16s rRNA gene was performed. The sequence was compared with available gene sequence using BLASTn. To know the relevance taxonomy neighbor-joining method was applied to minimize the total branch length at each stage of clustering based on clustal W, clustering of operational taxonomic units (OTUS) was carried out. For phylogenetic analysis, based on ancestral similar sequence through Blast to avail 16srRNA similarity was obtained. Further neighbor joining method was applied for phylogenetic tree construction. Among clades of species of interest, differences were arrived based on closest proximity algorithm multiple lineages of branch point to extended branches and clades further shown 100% similarity of unknown 16s rRNA sequence with Micrococcus flavus NR 043881 strain type. They both share similar proximity among their clades. Therefore, this unidentified strain had been reported as Micrococcus flavus isolate 2.

The GC-MS results showed the compound neoisolangifolene was the major compound with 32.73% area coverage. The compound nature evaluated in pub chem database showed the physical property of the compound to be yellowish matching with the extracted yellow pigment. Various functional group denoted alkane, aldehyde, nitro amide and aromatic groups were identified through FTIR analysis of the crude extract. The results obtained with the pigments against pathogenic organisms might provide some illuminating data in the control of these disease. The current effort for isolation and characterization of pigment from marine sediment bacteria isolated can be a basic step for discovery of novel antibiotics effective against multi drug resistant pathogens.

6. Conclusion

The study reported on the production of antimicrobial substance by pigmented bacteria *Micrococcus flavus* confirmed by 16s rRNA studies to be active against drug resistant *Staphylococcus aureus.* The study contributes marine bacterial pigments as valuable bioactive compounds having potential activity against drug resistant pathogens.

7. Source of Funding

None.

8. Conflicts of Interest

None.

9. Acknowledgement

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