

Molecular Characterization of Pigment Producing Microbes Associated with Marine Sponges and their Microbial Assay

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ABSTRACT

Marine sponges are animals grows under the sea level, contain abundant microbial communities, such as bacteria, archaea, microalgae, and fungi. Sponge associated microorganisms are an untapped source for pigments that can have wide range of applications in industries including food industry and textile industry. In the current study, Four species of sponges (*Echinodictyum sp.*, *Spongia sp.*, *Halichondria panicea* and *Hippospongia lachne*) from a depth of 5–10m were collected from Thiruchendur, South East coast of India and screened for pigment producing microbes. The Isolated microbes were characterized by phenotypic and molecular characterization. The pigments were extracted from the isolated microbes and are characterized by which they found to be carotenoid and chlorophyll. The antimicrobial activity of the pigment was analyzed by antibiotic sensitivity test, in which green pigment showed a broad spectrum of activity against *Staphylococcus sp.*, viz., 22.5 mm, 25mm, 27mm, 30mm, and 32 mm respectively.

Keywords: Marine sponges, pigment producing microbes, 16s rRNA sequencing, antimicrobial activity

Sponges are the marine organism which grows under the sea levels, often found near 5-50 meters deep and grow in wide coastal areas around the world. There are different types of marine sponges under the phylum poriferan. The metabolites of the marine sponges have shown wide range of biological and pharmacological activities such as antimicrobial, antioxidant, anti-inflammatory, antitumor activity, etc., They contain abundant microbial communities (bacteria, fungi, etc.), i.e. 40% of its body volume are composed of microbes, which has a striking similar metabolite of sponges^[29]. In a general survey, several thousands of microbial colonies isolated from the marine sources were found to be coloured (31.3% were yellow, 15.2% orange, 9.9% brown and 5.4% red or pink^[32]). Hence, the marine sponges are potential sources for culturing pigment producing microorganism.

There are large number of natural pigments extracted from natural sources usually from plants, but only few of them are in sufficient quantity used for industries^[17]. Hence, the microbial pigments forms an important source, because of their natural

character and safety to use, and their production are not depended on seasons and environmental conditions, and their yield can be predicted and controlled^[12]. The fast growth of microbes reduces the production time and compared to plant or animal sources for production, microbial sources are adaptable and can be easily controlled^[29]. Hence, nowadays microbial pigment production is one of the arising fields in research to establish its uses for various industrial applications.

The pigments producing microbes from marine sponges have a similar metabolite due to association with them. Hence, the pigments derived from the microbes associated with marine sponges have similar metabolites which have wide range of biological and pharmacological activities. Therefore, these pigments form a potential source for food and pharmaceutical industries^[13]. Hence, in this study the pigment producing microbes are isolated and screened based on their phenotypic and molecular characterization from marine sponges and the pigments are extracted and characterized for identifying its activity.

MATERIALS AND METHODS

Collection of Samples

Four species of sponges (*Echinodictyum sp.*, *Spongia sp.*, *Halichondria panicea* and *Hippospongia lachne*) from a depth of 5–10m were collected from Thiruchendur, South East coast of India. The sponge samples were placed inside sterile ethyl polythene bags underwater and transferred to the lab aseptically in ice boxes^[21].

Preparation of the Sample

The Sponge samples were prepared by washing with jets of filtered and autoclaved seawater until they were visibly free of debris. Then the sponge surface was sterilized by a rapid wash of 70% ethanol and immediately immersed in autoclaved and filtered seawater and then aspirated. One gram of sponge tissue was removed with a sterile scalpel and the tissue was immediately transferred to 99 ml sponge dissociation medium (2.7% NaCl, 0.008% KCl, 0.01% Na₂SO₄, pH 8). The samples were soaked for 20 min and then the tissue and diluents were macerated and the homogenate was used^[23].

Isolation of Pigment Producing Microbes

The homogenate sample was inoculated into the Nutrient agar and potato dextrose media which was prepared using sea water and distilled water in 1:1 ratio and incubated for 2-7 days at 37°C in incubator. They were examined daily for growth and colony Morphology. The isolated colonies were purified from primary culture and stored at -20°C.

Phenotypic Characterization

The isolates were identified based on the colony morphology and phenotypic characterization by Gram staining and Biochemical identification^[19].

Molecular Characterization

16s rRNA Sequencing

Bacterial genomic DNA was isolated by Insta Gene TM Matrix Genomic DNA isolation kit. Using 16S rRNA Universal primers 27f(5' AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTGTTACGACTT-3') was amplified using MJ Research Peltier Thermal

Cyclerz^[9]. Amplified 16S rRNA gene fragments were purified by removing unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean upkit (Millipore). The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Extraction of Pigments

The pigments were extracted through submerged fermentation, the pigment producing bacterial isolates were inoculated into LB broth and incubated for three days at 28±2°C in rotatory shaker. After incubation, cells were centrifuged at 6000rpm for 20min. The supernatant was discarded and methanol(5ml) solvent was added to the pellet. Then it was incubated in water bath (60°C) until all coloured pigments were extracted and then centrifuged at 2000rpm for 15 min. The coloured supernatant was filtered using Whatman no.1 filter paper^[5].

Characterization and Antimicrobial Assay of Extracted Pigments

The extracted pigments can be further characterized for physical and chemical characteristics using various instrumental-based analytical techniques such as TLC (Thinlayer chromatography), UV–vis Spectroscopy, FTIR (Fourier-Transform Infrared Spectroscopy), and antimicrobial assay by disc diffusion method^[26].

RESULTS

Isolation and Identification of Pigment Producing Microbes

In the present study, two isolates were screened and identified to be pigment producers, producing green and orange colonies on the nutrient media.

Phenotypic Characterization

The Gram staining results indicated that both the isolates were gram negative rod (Table 1). This result comparable with Venil *et al.* (2013)^[13]. The isolates were found rod shaped, non-spore producing, motile bacteria. Results on various biochemical tests revealed that the orange, green pigment producing

Table 1: Phenotypic characterization of isolates (Colony Morphology and Gram staining)

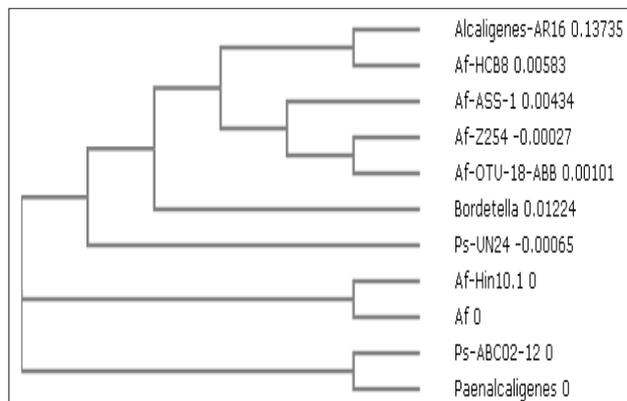
Sl. No.	Organism	Colony morphology		Gram staining
		Colour	Shape	
1	Strain 1	Orange	Round Scattered	(-ve) rod
2	Strain 2	Green	Round Scattered	(-ve)rod

Table 2: Biochemical identification

Sl. No.	Biochemical Tests	Isolate 1	Isolate 2
1	Indole Test	-	-
2	Methyl Red	+	-
3	Voges-Proskauer	+	-
4	Citrate utilization	-	+
5	Urease	+	-
6	Catalase	+	+
7	Oxidase	-	+
8	Triple sugar ion	Acid butt	Alkaline butt
9	Gelatin Test	+	-
10	Carbohydrate Fermentation	Glucose	-
		Lactose	-
		Sucrose	+

“+” = Positive “-” = Negative

bacterium expressed a positive result against catalase reaction, isolate 1 (orange) expressed positive reaction for methyl red, voges-proskauer, urease, catalase, gelatin and sucrose test and the isolate 2 (green) showed positive expression towards citrate, catalase, and Oxidase Tests (Table 2).

**Fig. 1:** Phylogenetic tree of strain 1

Molecular Characterization of Isolated Microorganisms

The identity of the strain was further confirmed by using partial gene sequence of 16S rRNA which was amplified and sequenced. Molecular identification

results showed that isolate 1 has the highest percentage of similarity with *Alcaligenes faecalis* a 98% level value. The partial sequence of the 16S rRNA gene obtained for Isolate 1 was submitted to Gene bank (Accession number KU973626) through Bank. It programme, at NCBI site. Phylogenetic tree and percentage matrix (Fig. 2 and Table 3) shows the phylogenetic affiliation of bacterial isolate with other microorganisms.

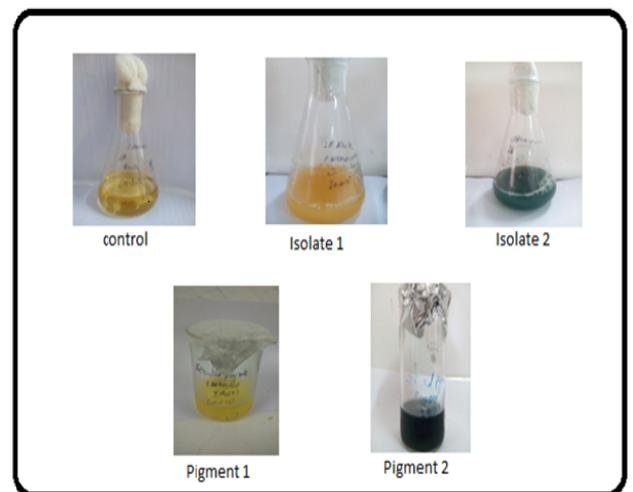
**Fig. 2:** Extraction of Pigments

Table 3: Percentage Identity Matrix of Phylogenetic Tree

	Aleali genes - AR16	Bordete IIa	Af-Hin10.1	Af	Ps-ABC02-12	Paenalcaligenes	Ps-UN24	Af-HCB 8	Af-ASS-1	Af-Z254	Af-OTU-18-ABB
Alcaligenes-AR16	100.00	83.90	85.33	85.06	85.54	85.54	86.44	85.68	84.48	85.34	84.82
Bordelelia	83.90	100.00	98.55	98.56	98.45	98.45	98.56	97.46	98.06	98.45	98.21
Af-Hin10.1	85.33	98.55	100.00	100.00	99.93	99.93	100.00	98.21	98.77	99.19	99.13
Af	85.06	98.56	100.00	100.00	99.93	99.93	100.00	98.22	98.78	99.19	99.14
Ps-ABC02-12	85.54	98.45	99.93	99.93	100.00	100.00	99.92	98.07	98.76	99.11	98.98
Parnalcaligenes	85.54	98.45	99.93	99.93	100.00	100.00	99.92	98.07	98.76	99.11	98.98
Ps-UN24	86.44	98.56	100.00	100.00	99.92	99.92	100.00	98.19	98.72	99.16	99.10
Af-HCB8	85.68	97.46	98.21	98.22	98.07	98.07	98.19	100.00	98.71	98.87	99.00
Af-ASS-1	84.48	98.06	98.77	98.78	98.76	98.76	98.72	98.71	100.00	99.56	99.40
Af-Z254	85.34	98.45	99.19	99.19	99.11	99.11	99.16	98.87	99.56	100.00	99.93
Af-OTU-18-ABB	84.82	98.21	99.13	99.14	98.98	98.98	99.10	99.00	99.40	99.93	100.00

Table 4: Antimicrobial activity of isolated pigments

Sl. No.	Organism	Orange pigment					Green pigment				
		20µl	40µl	60µl	80µl	100µl	20µl	40µl	60µl	80µl	100µl
1	<i>E. coli</i>	-	-	-	-	-	-	-	-	-	-
2	<i>Pseudomonas sp.</i> ,	-	-	-	-	-	22.5 mm	25 mm	27 mm	30 mm	32 mm
3	<i>Staphylococcus sp.</i> ,	-	-	-	-	-	-	-	-	-	-

“µl”= microlitre “mm”= millimeter

Pigment Extraction

Extraction of coloured pigments from the isolated strains was carried out by methanol as a solvent (Fig. 4).

Characterization of Extracted Pigments

UV Spectrophotometric Analysis

The absorption maximum for the extracted orange pigment was found to be 300nm and the absorption maximum for the extracted green pigment was found to be 1070 nm. In the Previous research work, the methanolic extract of *Salinicoccu ssp*, analyzed spectrophotometrically within the region of 400-690nm which was a typical pattern of absorption spectrum of carotenoid^[20].

Thin layer chromatography

Thin layer chromatographic technique was performed and the Rf value of the orange and green pigments were found to be 0.4 and 0.66. The

pigments were separated as pink-yellow and green-blue respectively. In the previous research work, the Rf value was found to be 0.629 and they found as carotenoid^[20].

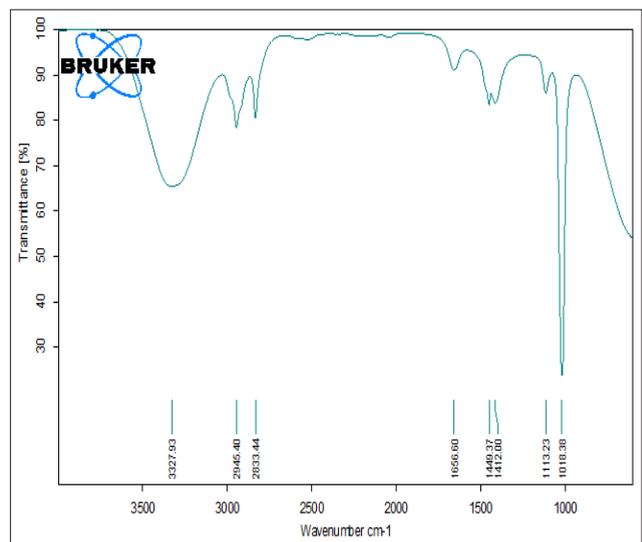


Fig. 3: FT-IR of Orange Pigment

Fourier-Transform Infrared Spectroscopy (FT-IR Spectroscopy)

Fourier-Transform Infrared Spectroscopy (FT-IR Spectroscopy) was performed for the extracted bacterial pigments and the obtained characterization results were related with Nugraheni *et al.* (2010)^[27] that the orange pigment was found to be carotenoid and the green pigment was found to be pyocyanin.

Orange Pigment

FT-IR absorption in for the Orange pigment was dominated by strong bands at 1018.38 cm^{-1} (C=N), 3327.93 cm^{-1} (-C (triple bond) C-H: C-H stretch), 2945.40 cm^{-1} and 2833.44 cm^{-1} (C-H), 1449.37 cm^{-1} and 1412.00 cm^{-1} (C-C), 1113.23 cm^{-1} (C-H wag) and 1656.60 cm^{-1} (-C=C- stretch).

Green Pigment

FT-IR absorption in for the Green pigment was dominated by strong bands at 3341.93 cm^{-1} (N-H stretch), 1637.22 cm^{-1} (-C=C-) and 1695.44 cm^{-1} (C=O), 1238.35 cm^{-1} (C-N) and 1370.59 cm^{-1} (C-H).

Antimicrobial Assay

The extracted pigment was analyzed by antibiotic sensitivity test in this, orange pigment showed no activity this result linked with Kim *et al.* (2006)^[17] that, *Monascus* pigments that were orange are reported that has little or no activity, while the green pigment showed a broad spectrum of activity against *Staphylococcus* sp., and the maximum inhibition zone is 32mm and minimum inhibition zone is 22.5 mm as shown in Table 4. Chen and Tseng (1989)^[6] reported that *Monascus purpureus* of green pigment products have been showed antibacterial assay against *S.aureus*.

DISCUSSION

In the present study samples of four species of sponges (*Echinodictyum* sp., *Spongia* sp., *Halichondria panicea* and *Hippospongia lachne*) are collected from Thiruchendur, South East coast of India. Marine sponges are reported as rich sources of natural compounds, which exhibit wide variety of biological activity^[8]. Hence, many novel microorganisms with potential biological activity were isolated from marine sponges^[16]. Pigments have been a very important taxonomic tool to identify bacteria species,

but identification it's complicated because there are many physical conditions that can make a bacterium change its pigmentation. Some bacteria may lose the pigments when they are in stress^[29]. The two of the pigment producing organisms which were selected are identified primarily using biochemical tests. Based on the results green pigment producing microorganism was identified/closely relate to as *Pseudomonas* sp., Molecular identification was done and the sequence were blasted in NCBI, it reveals similar organism was *Alcaligene faecalis* (98%). The partial sequence of the 16S rRNA gene obtained for Isolate 1 was submitted to Genbank (Accession number KU973626) through BankIt programme, at NCBI site (<http://www.ncbi.nlm.nih.gov/BankIt>). According to Hagstrom *et al.* (2000)^[14], organism which has 16srDNA sequence similarity more than 97 % can represent same species. Therefore, we can conclude that isolate bacterial *Alcaligene faecalis* AR.

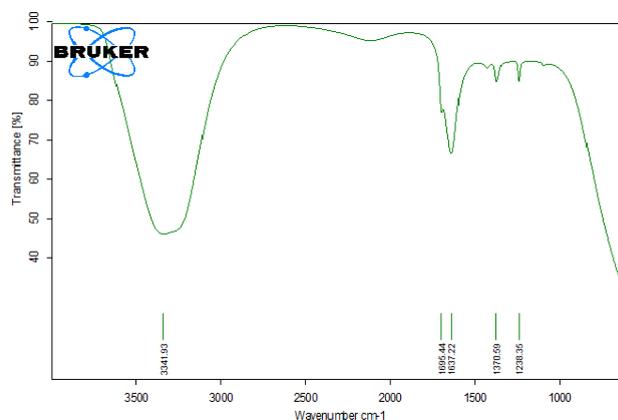


Fig. 4: FT-IR of Green Pigment

While extracting pigments, Methanol showed to be better than acetone, which is not very efficient in the extraction and quantification of pigments from autotrophic cell cultures^[15] so that in the present study we used methanol as a solvent for extraction. Therefore, it is concluded that, bacterial isolates obtained from marine sponges produced as carotenoids and pyocyanin and they showed λ max absorbance at 300nm which is the indication for carotenoids. The negative results in antimicrobial activity of orange pigment showed that they may be fit for human consumption as they do not interfere with the human biota and can have a potential application as food color additive and positive result in antimicrobial activity of green pigment showed that they can be used in pharmacological

industries. Thus, the present study deals with an approach of developing new sources of biocolors from easily cultivated bacterial species that can be further exploited at larger scale.

ACKNOWLEDGEMENTS

Thanks, are due to Shri Nehru Maha Vidhayalaya College for the technical support during this studies.

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