Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

IN VITRO ANTIMICROBIAL AND ANTIOXIDANT PROFILE OF STREPTOMYCES SP. ISOLATED FROM COROMANDEL COAST REGION, INDIA

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ABSTRACT

Marine actinomycetes were isolated from the sediments collected from Coromandel Coast region, India. The strain designated as BI244 was found to be an efficient pigment (dark brown-black) producer and has been selected for further studies. The organism was identified as Streptomyces sp. based on morphological, biochemical and physiological characterization. Phylogenetic tree constructed for 16s rRNA gene sequence by weighted-neighbor joining method showed 98% similarity to Streptomyces sp. NBRC 13020. The pigment extracted with methanol showed broad spectrum antimicrobial activity towards gram positive and gram negative bacteria and yeast. The extract also possessed DPPH free radical scavenging activity and the IC_{50} value was found to be 3.5mg/mL. The results of antimicrobial and antioxidant activity produced by the pigment extract clearly indicated that the marine environment is a untrapped source for isolation of bioactive compounds from actinomycetes.

KEYWORDS: Actinomycetes, Pigment, Phylogenetic tree, 16s rRNA, antimicrobial, DPPH free radical, Streptomyces sp.

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1. INTRODUCTION

Natural products have been regarded as important sources that could produce potential chemotherapeutic agents. Research into pharmacological properties of marine natural products has led to the discovery of many compounds considered worthy of clinical applications. There are great potential in bioprospecting from the sea and marine natural products research has just started to bloom. Today, marine sources have the highest probability of yielding natural products with unprecedented carbon skeletons and interesting biological activities. The marine environment supports a great biodiversity with a correspondingly great potential for the discovery of unique and pharmaceutically active secondary metabolites. There are several organisms that can produce pigments, which is one of the important class of secondary metabolites and are often referred as biopigments. These biopigments can be obtained from two major sources, plants[1,2] and microorganisms[3-8]. The accessible authorized biopigments from plants have numerous drawbacks such as instability against light, heat or adverse pH, low water solubility and often non-availability throughout the year. The latter are of great interest owing to the stability of the pigments produced[9] and the availability of cultivation technology[10,11]. Biopigments, not only play an important role in bacterial life, but also possess diverse biological properties such as antibiotic, antioxidant and anticancer activities. The latter is of special interest due to the consistent requirement for chemotherapeutic drugs with high selectivity towards malignant cells.

2. MATERIALS AND METHODS

2.1. Sample collection and processing

Marine sediment samples were collected as part of an expedition to the Marina Beach, Chennai (Latitude (N) 13.0542°; Longitude (E) 80.2837°) at a depth of 30-60cm in the Coromandel coast region of the Bay of Bengal, India. Samples were collected in sterile plastic containers and were transferred immediately to the laboratory and stored at 4°C. The air dried sediment samples were ground aseptically with Mortar and Pestle, mixed thoroughly and passed through 2 mm sieve filter to remove gravel and debris. Samples were kept at 55 °C for 10 minutes for pretreatment[12].

2.2. Isolation of actinomycetes from collected samples

The sediment samples collected from the station mentioned above were pooled. Actinomycetes were isolated from the soil sample by serial dilution method using Sea water Yeast Extract Peptone agar medium (SYEP) after an incubation period of 5 days at 30ºC[13]. Three replicates were maintained and the pigmented colonies were isolated. Based on pigmentation one strain has been chosen for further studies.

2.3. Characterization of selected actinomycete

2.3.1. Morphological characterization

The strain was identified upto generic level by macroscopic and morphological methods as per Bergey's manual[47] (2000).The macroscopic method was done by colony characterization on yeast malt extract agar medium and SYEP agar medium. Colour of the colony and presence of pigmentation were noted. The microscopic characterization was done by standard cover slip culture method and observed after three days. Further characterization of the selected strain was done by using recommended procedures given by the International Streptomyces Project (ISP)[14].

2.3.2. Biochemical characterization

The ability of actinomycete strains to utilize various carbon compounds as energy source was studied by the following method as recommended in International Streptomyces Project[15]. Carbon utilization medium was used in this carbon assimilation test. Growth of actinomycete strain was checked by taking 1% different carbon source viz. D-Glucose, L-Arabinose, D- Sucrose, D-Maltose, D-Xylose, D-Raffinose, D-Mannitol, Sorbitol, Rhamnose and

Inositol in SYEP media. Strain was inoculated and incubated at 30°C for 5 days. Growth of the strain was observed by comparing them with positive and negative control.

2.3.3. Molecular characterization

The strain was grown on SYEP liquid medium under constant shaking at 30 ± 1°C for 3 days. Spore was obtained by centrifugation and filtration. Extraction of genomic DNA of the strain was performed according to the method described by Lee[16]. PCR amplification of 16S rDNA was performed using both universal and specific primers: forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-CGGTTACCTTGTTAC GACT-3'). The reaction mixture was prepared in a total volume of 50 µL containing 10XPCR reaction buffer (5µL), $MgCl_2$ (2 mM), Taq DNA polymerase (1.5 U), dNTP (200 µM), forward primer (2 µM), reverse primer (2 µM) and template DNA (2 µL). The amplification was performed using a thermal cycler (ABI2720). The PCR programme started with an initial denaturation at 94°C for 5 min. Then followed by 35 cycles of PCR programmed by denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min, and a final extension at 72°C for 5 min and cooled to 4°C. The PCR product was detected by agarose gel electrophoresis and visualized by UV fluorescence after ethidium bromide (EB) staining. Then, the PCR product was sequenced using an ABI 3500 XL Genetic Analyzer. A distance matrix was constructed using the Jukes-Cantor corrected distance model[17] and the tree is generated using Weighted Neighbor Joining method[18].

2.4 Extraction of the pigment

The pigmented strain BI244 was inoculated to Erlenmeyer flask containing 50 mL of seed medium (SYEP broth), followed by incubation at 30°C for 2 days on a rotary shaker (160 rpm). The culture was transferred as 5% (v/v) inoculum into 100 mL of the production medium (SYEP) contained in 20×250 mL Erlenmeyer flasks. The flasks were then incubated on an incubator shaker (160 rpm) at 30°C for 5 days. After incubation the broth was filtered through Whatmann No.1 filter paper to remove the mycelial mat. The filtrate was further centrifuged at 8,000 rpm for 30 min at 4°C; the crude bioactive compound was recovered from the supernatant by solvent extraction with thrice the volume of methanol. The mixture was shaken overnight and then allowed to stand for 60 min for complete separation of the aqueous phase and organic phase. The organic phase was evaporated to dryness in room temperature and powdered pigment residues were collected and weighed. The crude extract was used further for analysis of antimicrobial and antioxidant activity.

2.5 Bioactivity of the crude pigment

2.5.1 Determination of Antimicrobial activity

Antimicrobial activity of crude pigment was determined by using disc diffusion method[19]. Ten different pathogens namely Staphylococcus aureus , Bacillus Subtilis , Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella sp., Proteus vulgaris, Proteus mirabilis and Candida albicans were inoculated on Trypticase Soy Broth (Himedia) and incubated for 24h at 37˚C. The counts of pathogenic cultures were adjusted to yield of 10^6-10^8 CFU/mL using the McFarland Standard. Then the test pathogens were inoculated with a sterile swab on the surface of prepared, sterilized Muller Hinton Agar (MHA) plates separately and left at room temperature for 3-5min to allow for any moisture to be absorbed before applying the extract. Ten mg/mL of crude extract was impregnated with filter paper disc (6mm diameter) and placed on to the surface of MHA medium, then left for 15mins to allow the extract to diffuse. The plates were incubated at 37° C for 24h and zone of inhibition was measured to evaluate the antimicrobial activity of the crude pigment. All the experiments were done in triplicates and the zone of inhibition expressed as Mean ± SD.

2.5.2 Estimation of DPPH radical scavenging activity

Free radical scavenging effect was estimated according to the method of Blois[20] as modified by Zhu et al.,[21]. Briefly, a 1mM solution of DPPH (1,1, Diphenyl-2-Picryl hydrazyl) radical solution

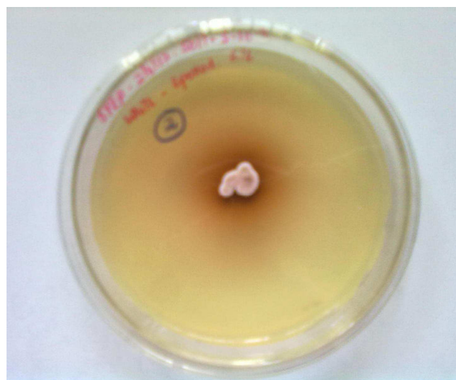
was prepared in methanol, and then 1mL of this solution was mixed with different concentrations of methanolic extract (sample); the mixture was then vortexed vigorously and left for 30 min at room temperature in the dark and the absorbance (OD) was measured at 517 nm with a spectrophotometer and is calculated as DPPH Scavenging activity % = [(Control OD – sample OD)/Control OD] x 100. The control was prepared without any extract containing 1.0mL of methanol and 1mL of 1mM solution of DPPH radical solution and the rest of the procedures remaining the same.

3. RESULTS AND DISCUSSION

3.1 Isolation and selection of the actinomycete strain

The Indian subcontinent has an immense biological diversity and it is increasingly recognized that a large number of chemical entities exists as metabolites in the micro flora. Actinomycetes have evolved as a group with greatest genomic and metabolic diversity[22]. Actinomycetes are indeed well adapted and are functional members of the aquatic microbial community. Distribution and diversity of actinobacteria have been reported from marine habitats such as marine sediments by Jensen et al.[23]. Actinomycetes comprise 10% of the total bacterial colonizing marine aggregates[24]. Actinomycetes represent only a small component of the total bacterial population in marine sediments[25]. Actinomycetes account 70% of the earth's surface and represent an attractive source for isolation of novel microorganisms and production of potent bioactive secondary metabolites. In our present study a total of 10 marine actinomycetes were isolated from the sea sediment samples on SYEP medium using dilution plate technique and pour plate method. The strains BI244 and BI245 produced dark brown-black pigment and brown pigment respectively. Based on the pigment colour (Fig.1) and amount of pigment production the strain BI244 was used for further studies.

Figure 1
Plate showing the culture BI244 and the pigment production



The soluble dark brown-black pigment probably refers to melanin[26]. Melanin is a common substance produced by animals, plants and microorganisms. Melanins are pigments of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds and usually are dark brown or black[27-29]. Actinomycetes are able to synthesize and excrete dark pigments, melanin or melanoid, which are considered to be a useful criterion for taxonomical studies[30].

3.2 Identification and characterization of BI244 strain

The cultural, morphological, physiological and biochemical characteristics of the isolate has been shown in Table1. On the SYEP medium, the color of the aerial mycelium was white, while the color of substrate mycelium appeared dark

brown for the strain. Diffusible dark brown-black pigmentation was observed in the SYEP medium. Microscopic observation of the spore chain morphology of the strain grown on SYEP medium for 7 days showed rectiflexibile mature spore chain. The strain had the ability to secrete enzymes such as gelatinase, amylase and proteinase, but did not produce cellulose and nitrate redutase. Carbohydrate utilization test play a prominent role in the taxonomic characterization of actinomycete strains[15]. Strain BI244 efficiently utilized the carbon sources such as glucose, arabinose, xylose, fructose, maltose and mannitol. Sorbitol, sucrose and rhamnose were not assimilated by the strain which exhibited positive response to melanoid pigmentation on Yeast extract iron agar and Sea water yeast extract peptone agar. Temperature is one of the physical factor that governs the distribution and activities of actinomycetes in natural habitats. The optimum temperature and pH for growth of the strain was 30ºC and 7 (data not shown) respectively.

Table 1

Morphological, biochemical and physiological characteristics of the actinomycete strain.

Characteristics	Results
Spore chain morphology	Rectiflexible
Spore surface	Smooth
Color of aerial mycelium	Dull White
Color of substrate mycelium	Dark Brown
Spore mass	White
Gram staining	Positive
Decomposition	
Nitrate	+
Starch	+
Pectin	-
Gelatin liquefactions	+
Xylan	-
Milk coagulation	+
Melanoid pigment	+
Carbon source utilization (1% w/v)	
D-glucose	+
L-arabinose	+
Sucrose	-
D-xylose	+
Fructose	+
Maltose	+
Rhamnose	-
Mannitol	+
Sorbitol	-
Physiology	
Optimum temperature for growth (°C)	***30ºC***
Optimum pH for growth	***7***

The 16S rDNA gene of the strain BI244 was PCR amplified, sequenced. A total of 1057bp has been obtained (Fig. 2a). A BLAST search of the Gen Bank database results showed that the new isolate had the highest similarity (98%) with identity with Streptomyces sp;NBRC 13020. (GenBank entry: AB184261) and 82% identity with Streptomyces Sp; 80866 (GenBank entry: AY996821). The phylogenetic tree generated by a weighted neighbor-joining (Fig. 2b) method clearly revealed the evolutionary relationship of the strain BI 244 to a group of Streptomyces sp. Thus, this strain was designated Streptomyces sp BI244. Actinomycetes especially Streptomyces, have been reported from the marine subhabitats such as marine sediments[31,32]; marine soils[32,33] and also from almost all parts of the world. Streptomyces sp. was the most frequently documented genus among others in marine sediment soils[34]. Also the dominance of Streptomyces among the actinomycetes especially in soils has been reported by many workers[23,33]. Hence they have worldwide distribution, which indicate their

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plasticity and adaptability to extremely varied environment[35].

Figure 2a

PCR amplification of ~1.5kb 16srDNA fragment from genomic DNA of BI244

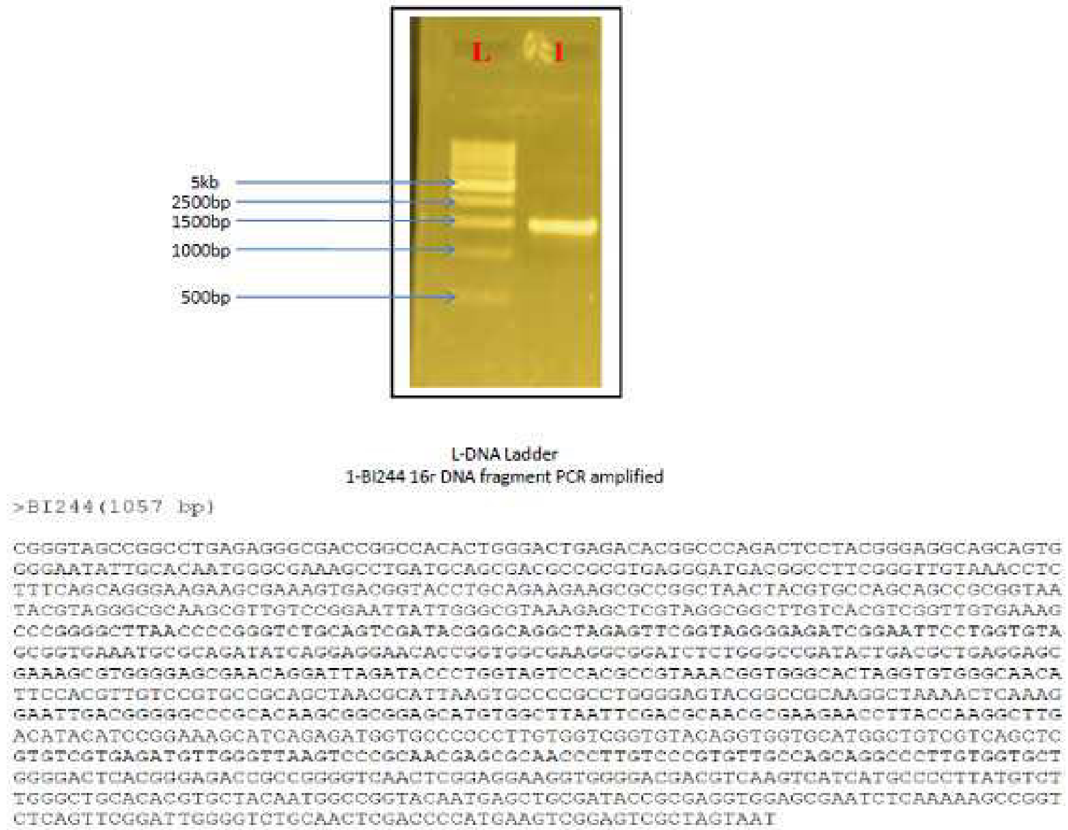
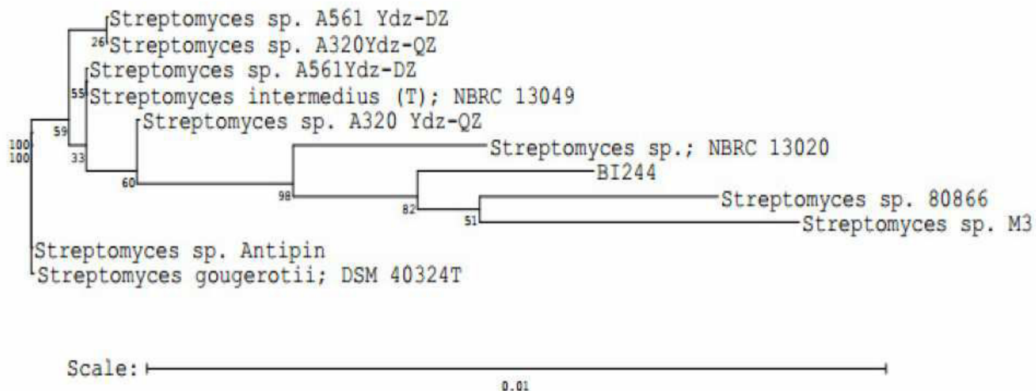


Figure 2b

Phylogenetic tree based on weighted neighbor-joining method for the selected strain



3.3 Extraction of pigment

Fermentation of the strain was carried out at 30°C for 5 days with shaking conditions at 160 rpm/min. Pigment production was observed on day 3 after incubation and pigments continued to accumulate throughout the fermentation period. However, the pigment yield reached its highest level on day 5. The fermented broth was

centrifuged and methanol was added to cell free supernatant and mixed well. The solvents were then evaporated and the powdered pigment residues were collected. This procedure yielded 2.5g of crude pigment per L of fermented broth.

3.4 Bioactivity of the crude pigment

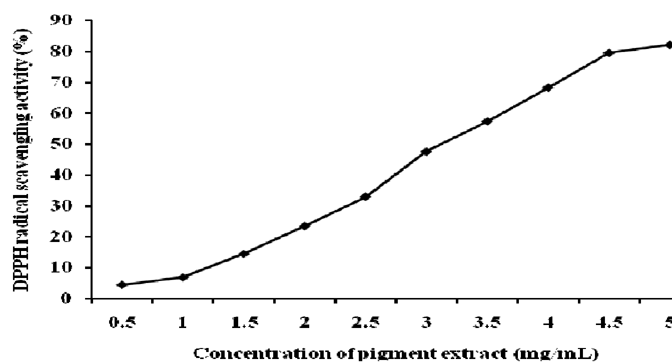
3.4.1 Determination of antimicrobial activity

The antimicrobial activity of crude extract of the pigment of marine Streptomyces sp. against test pathogens was determined using disc diffusion method. This study indicated that the pigment extract have shown good antimicrobial activity against Staphylococcus aureus (17±0.3mm), Enterococcus faecalis (16±0.041mm) and Bacillus Subtilis (13±0.2mm) and moderate activity towards Escherichia coli (10±0.3mm), Klebsiella pneumoniae (7±0.1mm), Salmonella typhi (10±0.1mm), Shigella sp. (9±0.4mm) and Candida albicans (10±0.2mm). The crude extract of the pigment showed no zone of inhibition against Proteus vulgaris and Proteus mirabilis. The pigment showed more inhibitory activity against Gram positive organisms than Gram negative organisms. This is in accordance with the report of Kokare et al.[36] who stated that during the screening for novel secondary metabolites, Actinomycetes showed more active antimicrobial activity against gram positive bacteria than gram negative bacteria. Streptomyces species showed significant antibacterial activity against S. aureus and Pseudomonas aeruginosa[37]. Marine actinomycetes are efficient producers of new secondary metabolites that show a range of biological activities. Several species of actinomycetes were found to produce pigments, most of which are having antibiotic properties. The pigments of actinomycetes are found to posses antimicrobial activity[38], antitumor activity[39] and other pharmacologically significant activities. Melanin extract showed strong antibacterial activity against both E. coli and P. vulgaris at a concentration of 1µL pigment extract and the range of the zone of inhibition was between 17 to 20 mm[40]. The extract also showed strong activity against S. aureus, P. mirabilis, V. cholerae, S. typhi, S. paratyphi and K. oxytoca. This is in accordance with our finding but the crude extract did not show any activity against Proteus sp. About 95 microorganisms mainly Streptomycetes, were examined for prodiginine pigments. Streptoverticillium sp. 26-1 furnished good yields of butylcycloheptlyprodiginine (I) and its antimicrobial activity determined. Pigmented antibiotic, found to be a novel peptide antibiotic and was extracted from soil Streptomycetes showing wide antibacterial activity[41]. Compared to other Actinomycetes, Streptomyces species showed efficient antagonistic activity[42]. Eighty three percent of actinomycetes isolated from Sagamy Bay were found to posses antifungal activity. Many marine microorganisms showed antifungal activity against Aspergillus niger but not against C. albicans. But our pigment showed moderate antagonistic activity against C.albicans. Actinomycetes isolated from mangrove sediments of Pichavaram southeast coast of India exhibiting prominent antibiotic activity against C. albicans[33].

3.4.2 Determination of DPPH activity

Free radicals are chemical species containing one or more unpaired electrons that make them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. In recent years much attention has been devoted to natural antioxidants and their association with health benefits[43]. In the present study the DPPH free radical scavenging activity of the pigment extract was found to increase in concentration dependent manner (Fig.3). The addition of an antioxidant resulted in decrease of absorbance proportional to the concentration and free radical scavenging activity of the compound and it indicated an increase of the DPPH radical scavenging activity[44]. The IC_{50} value of the pigment extract was found to be 3.5mg/mL.

Figure 3
DPPH radical scavenging activity of pigment extract



Recently researchers have focused the usage of pigments from coloring agents as antioxidants in pharmaceutical and food industries. Diraviyam[45] and co-workers reported the antioxidant activity of melanin pigment from Streptomyces species D5. The antioxidant activity of crude carotenoid extracted from Halorubrum sp. was tested and recorded activity was equivalent to the antioxidant activity of standard leutin and astaxanthin at their 4mM concentration[46].

5. CONCLUSION

Dark brown-black pigment producing actinomycetes isolated from marine ecosystem have been chosen for our present study. The organism has been identified as Streptomyces sp. based on morphological, biochemical and 16s rRNA gene sequencing. The pigment was extracted and its bioactivity has been analyzed. The results obtained highlighted the effectiveness of the pigment extract as a potent antimicrobial and antioxidant compound. Further studies have to be focused on elucidating the chemical nature of the pigmented compound and their antitumor activity.

6. CONFLICT OF INTEREST

Conflict of interest declared none.

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