

Isolation of Melanin Pigment Producing Marine Actinobacterium of Streptomyces Isolated From Marine Sediment Samples and Their Antibacterial Activity

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ABSTRACT

Introduction: Melanin is a pigment produced by organisms throughout all domains of life. In the vast majority of studies, melanin has been either chemically synthesized or isolated from animals. Marine organisms are the source of thousands of substances, which also have antibacterial and antifungal effects. Microbial mediated production is the best viable alternative to obtain melanin. This method has the advantage of being scalable and providing a good yield of melanin. *Streptomyces* is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae.

Materials and method: The sediment samples were collected from parangipettai, Tamilnadu. The sediments were sundried for 24-48 hrs and turned into a fine powder with mortar and pestle. Isolation of Actinobacteria, Identification of marine actinobacteria, Production and purification of melanin, Chemical analysis of Melanin. **Antibacterial activity:** The antibacterial activity of melanin was performed with disc diffusion method. Minimum Inhibitory Concentration was also observed.

Results: Presence of actinobacteria from the isolated sediment sample is confirmed by chemotaxis characteristics. The antibacterial activity of the melanin pigment isolated from *Streptomyces* sample was analysed and found that the minimum inhibitory concentration was more compared to the standard antibacterial agent that is tetracycline.

Conclusion: In the present study, produced melanin pigment was found to have potent antibacterial potential activity. Further characterization and bio active properties should be done in future studies, and more articles in future are yet to come in various properties of melanin pigment.

Key words: Melanin, Pigment, Marine, Antibacterial activity, Streptomyces, Sediments

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INTRODUCTION

Melanin is a pigment produced by organisms throughout all domains of life. In the vast majority of studies, melanin has been either chemically synthesized or isolated from animals [1]. In previous researches DOPA melanin showed a promising activity as an antibacterial natural product against 12 pathogenic bacteria from hospital isolations [2]. Currently, the increasing resistance of microorganisms to antibiotics is a serious problem. Actinomycetes, one among the most colourful microbes, are characterized by the production of various pigments on different natural and synthetic media [3]. Marine organisms are the source of thousands of substances, which also have antibacterial

and antifungal effects. Microbial mediated production is the best viable alternative to obtain melanin. This method has the advantage of being scalable and providing a good yield of melanin [4]. Color of melanin ranges from yellow to black and depends on the metabolic pathway which synthesises them. Melanins are used in a wide variety of applications in day-to-day life including cosmetics, optical lenses, pharmaceuticals, and batteries to name some [5-10].

In previous studies three strains among 21 Actinomycetes sp isolates produced a diffusible dark pigment on starch casein an agar medium which was water soluble. That study proved that sugarcane waste can be used for the production of melanin and it (melanin) has potential antibacterial activity [11]. A study suggested that, on the basis of anti-bacterial activity and MIC test of melanin pigment from *Streptomyces* sp., MVCS13 can be selected as an effective antibacterial agent for ornamental fish culture. (Melanin from marine *Streptomyces* sp. (MVCS13) with

potential effect against ornamental fish pathogens of *Carassius auratus*. In an article published it proved that melanin has an antibacterial, antifungal, antioxidant activity and also it can be used for industrial biotechnological application because microbial melanin can be produced in large scale with low production cost [12]. Also few articles proved that actinobacteria isolated from marine environments have been dominated by *Micromonospora*, *Rhodococcus* and *Streptomyces* species. Recent culture-independent studies have shown that marine environments contain a high diversity of actinobacterial species that are rarely, if at all, recovered by cultivation-based methods [13]. Our team has extensive knowledge and research experience that has translated into high quality publications. The present study proves to be unique as not many articles have been published on the antibacterial effect of melanin isolated from marine sediments. The aim of this study is to extract melanin from marine actinobacterium of *streptomyces* from marine sediments and to analyse the antibacterial activity [14-17].

MATERIALS AND METHODS

Isolation of actinobacteria

Isolation and enumeration of actinobacteria were carried out in Kuster's Agar Medium (KUA) supplemented with 0.5% (W/v) NaCl. To minimize the fungal and bacterial contamination, KUA medium was supplemented with cycloheximide (10 µg/ml) and nalidixic acid (10 µg/ml) respectively. Collected sediment samples were serially diluted and inoculated on KUA medium and incubated at 36°C for 7 days. The colonies were counted and the population density has been expressed as colony forming units per gram (CFU/g) of sediments. Morphologically distinct colonies were selected and pure cultures were obtained.

Identification of marine actinobacteria

Aerial mass colour: The colour of the mature sporulation aerial mycelium was recorded in naked eye. When the aerial mass colour fell between two colours series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also, both the colour series were noted. The media used were yeast extract malt extract agar and inorganic salt starch agar [17,18].

Melanoid pigments: The grouping was made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium [19]. The strains were grouped as melanoid pigment produced (+) and not produced (-). In a few cases, the production of melanoid pigments was delayed or weak, and therefore, it was not distinguishable. This is indicated as variable (V). This test was carried out on the media ISP-1 and ISP-7 (Appendix I), as recommended by the International Streptomyces Project [20].

Reverse side pigments: Reverse side pigment production of the isolate was determined on ISP7 medium [21]. The pigment production was noted as distinctive (+) and not distinctive or none (-). In case, a colour with low chroma such as pale yellow, olive or yellowish brown occurred, it was included in the latter group (-).

Soluble pigments: Soluble pigment production of isolate was observed on ISP7 medium. The diffusible pigment production other than melanin was considered positive (+) and not produced (-). The colour was recorded (red, orange, green, yellow, blue and violet).

Spore chain morphology: Spore morphological characters of the strains were studied by inoculating a loopful of one week old cultures into solidified agar medium containing sterile glass slides. The cultures were incubated at 28±2°C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology [22].

Chemotaxonomical characteristics

Hydrolysis: Hydrolysis was done for releasing amino acids. Harvested cells of each strain weighing 20 mg (fresh) were placed in an ampo bottle and 1 ml of 6N HCl was added and sealed with an alcohol blast burner [23]. The samples were kept at 121°C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of 28±2°C. Hydrolysis was also done for releasing sugars. Harvested cells of each strain weighing 50 mg (fresh) were placed in an ampo bottle and 1 ml of 0.5 N HCl was added and sealed with an alcohol blast burner. The samples were kept at 110°C for 2 h. The bottles were then cooled by keeping them at a room temperature of 28±2°C.

Thin Layer Chromatography (TLC): Spotting of the whole cell hydrolysates was made carefully on TLC plate using a microliter pipette. Spots were 5-10 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by a hand dryer [24-28].

Amino acids: Each sample (3 µl) was applied on the baselines of the TLC plate (20 cm x 20 cm). Adjacent to this, 1 µl of DL-diaminopimelic acid (an authentic material mixture of DAP isomers) and 1 µl of amino acetic acid (glycine) were spotted as standards [29]. The TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H₂O (5: 0.5: 0.125: 2.5 v/v). It took approximately more than 4 h for development. The spots were visualized by spraying with 0.4% ninhydrin solution in water-saturated n-butanol, followed by heating at 100°C for 5 min. Spots of amino acids ran faster than DAP. The sample spots were immediately compared with the spots of the standards since spots gradually disappeared in a few hours [30].

Whole-Cell sugars: On a cellulose TLC plate (20 cm x 20 cm), 5 µl of samples was spotted along with 3 µl of sugar solutions as standards on the same plates. Galactose, arabinose, xylose and madurose were the sugars, which

were used as standards. The TLC plate was developed with the solvent mixture containing ethyl acetate: pyridine: acetic acid: distilled water (8: 5: 1: 1.5 v/v). The development time was more than 4 h. Spots were visualized by spraying with aniline phthalate reagent (3.25 g of phthalic acid dissolved in 2 ml of aniline and made upto 100 ml with water saturated n-butanol). The sprayed plate was heated at 100°C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots [31-34].

Assimilation of carbon source: The ability of the actinobacterial strain in utilizing various carbon compounds as source of energy was studied, following the method recommended by International Streptomyces Project [35]. Chemically pure carbon source certified to be free of admixture with other carbohydrates and contaminating materials were used for this purpose. Carbon sources for this test were Arabinose, Xylose, Inositol, Mannitol, Fructose, Rhamnose, Sucrose and Raffinose. These carbon sources were sterilized by ether sterilization without heating. The media and plates were prepared and inoculated according to the convention of ISP project. For each of the carbon sources, utilization is expressed as positive (+), negative (-), or doubtful (\pm). In the 'doubtful' strains, only a trace of growth slightly greater than that of the control was noticed [36-38].

Production and purification of melanin

The ISP-2 medium prepared in sea water was used for the development of inoculum. The 2 ml of spore suspension from inoculums medium was inoculated in the fermentation medium containing 0.65 ml of glycerol, 0.63 gm of Yeast extract, 0.55 gm of glucose, .08 gm of $MgSO_4$.

The 2 ml of spore suspension was inoculated into fermentation medium (ISP7) for 12 days under the agitation for 200 rpm at ambient temperature. Then cell free supernatant was collected by centrifugation at 10,000 rpm for 15 min. The harvested cell free supernatant containing melanin was adjusted to pH 2 with con. HCl and kept at room temperature for 3 hrs. After incubation the suspension was centrifuged at 10000 rpm at 28°C for 20 mins to palletize the melanin pigment. The pellet was washed 3 times with distilled water and dissolved in a phosphate buffer (PH8) [39,40].

Chemical analysis of Melanin

The chemical test was carried out with a little modification. The solubility test for the black pigment was tested by adding 100 μ l of melanin pigment in 1 ml of distilled deionized water, 1N HCl, 1M NaOH, absolute ethanol, acetone (warm), chloroform (warm), Phenol and

Benzene. The reaction with the following oxidizing agents was also determined by adding 100 μ l of melanin to the 1 ml of 30% Hydrogen peroxide (H_2O_2) solution. The precipitation test was carried out by adding 100 μ l of purified melanin to 1 ml of 1% $FeCl_3$ solution and 1 ml of Con. HCl.

Bacterial Suspension: The pathogenic bacterial strains *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Vibrio cholerae* were collected from the Department of Microbiology, Saveetha medical college and hospital, Tamilnadu. The bacterial pathogens were cultured in Muller -Hinton Broth for 24 hr at room temperature. From this bacterial suspension was prepared with saline and the optical density was measured at 600 nm. The concentration of microbial suspension was fixed as 106 CFU/ml. 1 ml of suspension was spread over on Muller Hinton agar plate and incubated for 24 hrs at ambient temperature [41,42].

Antibacterial activity: The antibacterial activity of melanin was performed with a disc diffusion method. Whatman filter paper discs (5 mm) were impregnated with various concentrations (0.5, 1, 1.5, 2, 2.5 and 3 mg/ml) of leaf extract using ethanol and methanol solvent. The inoculated plates were incubated for 24 hr at room temperature and the inhibition zones around the discs were measured. All the results were expressed from an average of three with standard deviation [43].

Minimum Inhibitory Concentration

Minimal Inhibition Concentration of melanin was determined in 5 concentrations (10-5 0 μ g/ml/0.001 to 0.1 mg/ml) with blank (extract in Muller Hinton broth). The inoculated bacteria in test tubes are incubated for 24 hr in ambient temperature then the optical density was observed [44-46].

RESULTS AND DISCUSSION



Streptomyces

Reticulum apertum chain

Figure 1: The figure shows the isolation and melanin pigment separation.

Table 1: The table shows the cultural characteristics of Actinobacteria. The colour was found to be grey with morphology of the reticulum apertum chain.

Positive control - Tetracycline	
Color of aerial mycelium	Grey
Melanoid pigment	+

Reverse side pigment	-
Soluble pigment	-
Spore chain	RA
Arabinose	+
Xylose	+
Inositol	-
Mannitol	±
Fructose	+
Rhamnose	+
Sucrose	+
Raffinose	-

Melanoid pigment was found and the other pigments like soluble pigment and reverse side pigment were absent. Assimilation of carbon source has shown the presence of xylose and rhamnose among all the carbon compounds mentioned above. These results helped in the isolation of *streptomyces* species.

The isolated bacteria produced melanoid pigment, but reverse side pigment and soluble pigment was not produced. The reverse side pigment was not distinctive. The streptomyces were grey in colour. It had reticulum apertum chain morphology (Table 1). The cell wall of the bacteria does contain LL-DAP, Glycine but does not contain Arabinose and Galactose. MesoDAP is absent in the bacterium's cell wall. The type of cell wall is type I (Table 2). The pigment produced by actinobacteria of the species *Streptomyces* proved to be melanin by few characteristics [47,48]. It was black in colour as dark pigments produced by marine organisms are considered to be melanin, if they are dark, insoluble in organic).

Table 2: The table shows the cell wall characteristics of Actinobacteria. Only LL-DAP and glycine were present and the other cell wall amino acids mentioned above were absent and also both the cell wall cell wall sugars i.e., arabinose and galactose were absent. The cell wall belongs to the first type. This shows an index for *Streptomyces* species.

Cellwall amino acids			Cell wall sugar			Index
LL-DAP	MesoDAP	Glycine	Arabinose	Galactose		
+	-	+	-	-		<i>Streptomyces</i>

Table 3: The table shows the zone of inhibition of the melanin pigment isolated from the *streptomyces* sample against particular bacteria.

Melanin pigment µg/ml	<i>Staphylococcus</i>	<i>Pseudomonas</i>	<i>Vibrio. Sp.</i>
0	0	0	
50	6 ± 1.07	7 ± 2.4	9 ± 1.6
100	10 ± 1.9	12 ± 1.7	13 ± 2.5
150	14 ± 2.3	15 ± 2.4	17 ± 2.3

200	20 ± 3.2	19 ± 2.1	21 ± 2.8
250	23 ± 2.4	22 ± 2.8	24 ± 2.2
300	27 ± 2.6	26 ± 2.4	28 ± 2.5

Table 4: Minimum inhibitory concentration of the melanin pigment isolated from *streptomyces* in the 3bacterias was compared with the standard antibacterial agent-tetracycline.)

	0	10	20	30	50	MIC
<i>Staphylococcus</i>	+	+	+	-	-	30 µg/ml
<i>Tetracycline</i>	+	+	-	-	-	20 µg/ml
<i>Pseudomonas</i>	+	+	+	-	-	30 µg/ml
<i>Tetracycline</i>	+	-	-	-	-	10 µg/ml
<i>Vibrio.sp</i>	+	+	+	-	-	30 µg/ml
<i>Tetracycline</i>	+	-	-	-	-	10 µg/ml

Table 5: The table shows the characteristics of pigment obtained from *streptomyces* species.

The Colour of the pigment was black. The pigment derived showed solubility in phenol which is an organic solvent and insoluble in the other organicsolvents and all the inorganic solvents. It showed precipitation in the presence of FeCl₃ and Conc. HCl. The pigment was found to be oxidised with peroxide (H₂O₂).

Melanin chemical analysis	
Color	Black
Solubility in inorganic solvents	
H ₂ O (pH 7)	Insoluble
1N HCl	Insoluble
1M NaOH	Insoluble
Solubility in organic solvents	
Ethanol	Insoluble
Chloroform	Insoluble
Acetone	Insoluble
Benzene	Insoluble
Phenol	soluble
Precipitation reaction	
FeCl ₃	Precipitated
Conc.HCL	Precipitated
Oxidation process	
H ₂ O ₂	Oxidized

In previous research, Melanin pigments were extracted from a wide variety of microorganisms including bacteria and fungi. In that study isolation, identification and characterization of melanin from marine actinobacterium (*Streptomyces sp. MVCS13*) and its potential activities against fish pathogens were investigated [49,50]. Culture conditions and medium composition for the melanin production were optimized. Further, pigment was characterized by UV-vis absorption spectroscopy and FT-

IR infrared spectrometry. It has potential antibacterial activity against ornamental fish pathogens *Vibrio sp. FPO5* (15 ± 0.01 mm) which is similar to that of the present studies. The Minimum Inhibitory Concentration (MIC) ranges were observed between 18 ± 0.01 and 27 ± 0.03 µg/ml which is lesser than the results obtained in the present study [51]. The previous study suggested that, on the basis of anti-bacterial activity and MIC test of melanin pigment from *Streptomyces sp. MVCS13* can be

selected as an effective anti-bacterial agent for ornamental fish culture ('Melanin from marine *Streptomyces sp.* (MVCS13) with potential effect against ornamental fish pathogens of *Carassius auratus*. Various articles have been done on isolation of different types of pigments from marine sediments. Mostly extracellular polysaccharides were extracted from actinobacteria in many studies done previously. The marine actinobacteria isolated in this study is *streptomyces* whereas other studies mostly *nocardiosis sp.* were extracted. Similar to the present study, the antimicrobial activities were done in many studies (First Report on Marine Actinobacterial Diversity around Madras Atomic Power Station (MAPS), India, no date) (Physicochemical Profile of Acacia Catechu Bark Extract-An *in vitro* Stud - International Journal of Pharmaceutical and Phytopharmacological Research, no date) (Awareness of Drug Abuse among Teenagers - International Journal of Pharmaceutical and Phytopharmacological Research, no date) (COX2 Inhibitory Activity of *Abutilon indicum* - Pharmaceutical Research and Allied Sciences, no date).

CONCLUSION

In the present study, *Streptomyces* produced melanin pigment was found to have potent antibacterial potential activity. Further characterization and bio active properties should be done in future studies, and more articles in future are yet to come in various properties of melanin pigment.

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CONFLICTS

The author declared there is no conflict of interest in the present study.

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