Antioxidant potential from melanin pigment produced by Marine Actinobacterium of *Nocardiopsis* species

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ABSTRACT:

Introduction: Melanin refers to the group of natural pigments found in most organisms. It is produced through a multistage chemical process known as melanogenesis, where the oxidation of the amino acid tyrosine is followed by polymerisation. Special skin cells called melanocytes make melanin. *Nocardiopsis* species are an ecologically versatile and biotechnologically important group of Actinomycetes. The members of this genus have established a place for themselves and are of considerable value in producing compounds with profound bio medical applications

Materials and methods: The sediment sample was collected from the Pichavaram mangrove forest area, Tamil Nadu. The collected sample was sun dried for 48 hrs and turned into fine powder by mortar and pestle. Isolation and enumeration of actinobacteria were carried out in Kuster's agar medium. identification of marine actinobacterium is by looking at aerial mass colour, grouping of melanin pigments, determination of reverse side pigments and soluble pigments on ISP7 medium, spore chain morphology by inoculation. Chemotaxonomical characteristics like hydrolysis, thin layer chromatography, amino acid application, spottered whole cell sugars and assimilation of carbon source. Finally the derived pigment was sent for antioxidant activity by 3 methods: Total antioxidant activity, DPPH assay, and scavenging of nitrous oxide.

Results: The isolation and culture of actinobacteria colour was found to be grey with long spore chain morphology. Melanoid pigment was found and the other pigments like soluble pigment and reverse side pigment were absent. Assimilation of carbon source has shown the absence of xylose and rhamnose among all the carbon compounds mentioned above. These results helped in the isolation of *Nocardiopsis* species. The cell wall belongs to the third type. This shows an index for *Nocardiopsis* species. The pigment derived showed solubility in phenol which is an organic solvent and insoluble in the other organic solvents 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 (H_2O_2). All the three activities for antioxidant potential i.e., total antioxidant activity, DPPH assay and NO scavenging assay showed a potential activity of melanin pigment.

Conclusion: The produced melanin pigment from the Actinobacteria of *Nocardiopsis* species was found to have potent antioxidant potential activity. Further characterisation and bio active properties should be done in the further studies, and more articles in future are yet to come in various properties of melanin pigment.

Key words: Actinobacteria, *Nocardiopsis* species, Antioxidant activity, Melanin, characterization

INTRODUCTION:

Melanin refers to the group of natural pigments found in most organisms. It is produced through a multistage chemical process known as melanogenesis, where the oxidation of the amino acid tyrosine is followed by polymerisation. Special skin cells called melanocytes make melanin. Everyone has the same number of melanocytes, but some people make more melanin than others. Melanin is a natural pigment produced through the oligomerization of 5,6-DHI and 5,6-DHICA catalysed by tyrosinase further forming 3 to 9 monomer units. Organisms produce these pigments for protection in the biological systems (1). Protective activity of melanin like pigment derived from tea was studied using benzidine as a DNA reactive chemical. MLP prevented the production of free radicals due to metabolic transformation of BZ (2). The most significant categories affecting melanin production are incubation period, protease-peptone and ferric ammonium citrate. Maximum melanin production was achieved by high tyrosinase activity (3).

Nocardiopsis species are an ecologically versatile and biotechnologically important group of Actinomycetes. The members of this genus have established a place for themselves and are of considerable value in producing compounds with profound bio medical applications. Melanin production was done using submerged cultures of mushroom *Auricularia auricula,* methanol, peanut oil and palmitic acid increased the melanin production from these species. Seven kinds of amino acids were used to change the non-water-soluble extracellular melanin from *lachanum* YM-36. Through this species a new water soluble arginine-melanin pigment was prepared for the first time (4); (5); (6).

Melanin protects melanocytes and keratinocytes from the induction of DNA strand breaks by hydrogen peroxide, indicating that this pigment has an important antioxidant role in the skin. Chestnut shell melanin is used as a colorant and antioxidant which was further fractionated into three fractions with various physicochemical properties. The addition of an antioxidant prolongs the shelf life of food products and based on these reasons there are many investigations going on antioxidant activities of different (7); (8). Fungal melanin pigments were found to have a potent antioxidant activity. Melanin was found to have Gene protective properties, increase in number of methyl substitutes is related to decrease in inhibitory efficiency of peroxidase mediated oxidation (9).

The present study focuses on the antioxidant property of melanin pigment which is derived from the marine Actinobacteria *Nocardiopsis* species. Previous studies on antioxidants and the other properties had shown that melanin pigment has a potent antioxidant and cytotoxic effects. These properties of melanin had increased the use of pigment over industries and all over the country. Our team has extensive knowledge and research experience that has translate into high quality publications(10–14),(15),(16),(17),(18),(19),(20)

MATERIALS AND METHODS:

Sample collection and preparation

The sediment sample was collected from the Pichavaram mangrove forest area, Tamilnadu. The collected sample was sun dried for 48 hrs and turned into fine powder by mortar and pestle.

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 (Kathiresan et al., 2005). 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 sporulating 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.

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. 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 (Shirling and Gottlieb, 1966).

Reverse side pigments: Reverse side pigment production of the isolate was determined on ISP7 medium. 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 occured, 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 $28+2^0$ C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology.

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 6 N HCl was added and sealed with an alcohol blast burner. The samples were kept at 121^0 C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of $28+2^0$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 0.5N HCl was added and sealed with an alcohol blast burner. The samples were kept at 110^0 C for 2 h. The bottles were then cooled by keeping them at a room temperature of $28+2^0$ 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.

Amino acids: Each sample (3 µl) was applied on the baselines of the TLC plate (20 cm x 20 cm). Adjacent to this, 1µl of DL-diaminopimelic acid (an authentic material mixture of DAP isomers) and 1µl of amino acetic acid (glycine) were spotted as standards. TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H_2O (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 100^0 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.

Whole-Cell sugars: On a cellulose TLC plate (20 cm x 20 cm), 5µl 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. 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 100^0 C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots.

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 (Shirling and Gottlieb, 1966). 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 (Shirling and Gottlieb, 1966). For each of the carbon sources, utilization is expressed as positive (+), negative (-), or doubtful (±). In the 'doubtful ' strains, only a trace of growth slightly greater than that of the control was noticed.

Production and purification of melanin

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

The 2ml of spore suspension was inoculated into fermentation medium (ISP7) for 12days under the agitation for 200rpm at ambient temperature. Then cell free supernatant was collected by centrifugation at 10,000 rpm for 15min. The harvested cell free supernatant containing melanin was adjusted to pH 2 with con. HCl and kept at room temperature for 3hrs. After incubation the

suspension was centrifuged at 10000rpm 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).

Chemical analysis of the pigment

The chemical test was carried out with a little modification (Fava et al., 1993). The solubility test for the black pigment was tested by adding 100 µl of melanin pigment in 1ml 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 100µl of melanin to the 1ml of 30% Hydrogen peroxide (H_2O_2) solution. The precipitation test was carried out by adding 100 µl of purified melanin to 1ml of 1% $Fecl_3$ solution and 1ml of Con. HCl.

Total antioxidant activity: Total antioxidant activity of the melanin was determined by following method: 0.3 ml of sample was prepared in different concentrations (0.5 – 3mg/ml) with 3ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). Reaction mixture was incubated at 95ºc for 90 minutes in a water bath. Absorbance of all sample mixtures was measured at 695 nm. Total antioxidant activity has been expressed as the number of equivalents of ascorbic acid.

DPPH Assay: The antioxidant potential activity of melanin was determined on the basis of their scavenging activity of the stable 1,1- diphenyl-2-picryl hydrazyl (DPPH) free radical. Different concentrations (0.5-3mg/ml) of samples were mixed with 2.9ml diphenylpicrylhydrazyl (DPPH) solution (120µM) in methanol and incubated in darkness at 37°c for 30 minutes. The absorbance was recorded at 517 nm. Inhibition of free radical by DPPH in percentage (I %) was calculated with the following equation:

$$\text{Percentage of Inhibition (I \%)} = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

Where, A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of the sample.Ascorbic acid was used as positive control (Kamala et al., 2015) and all the tests were carried out in triplicate.

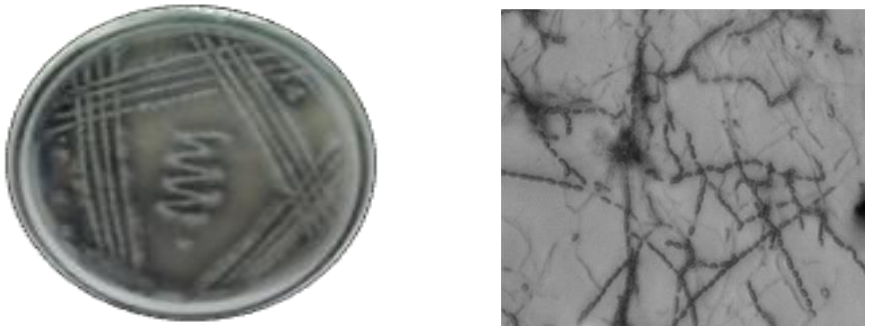
Scavenging of nitric oxide: The reaction mixture (3ml) containing 10mM sodium nitroprusside and the sample (0.5-3mg/ml) in benzene: chloroform was incubated at 25ºc for 150 min. After incubation, 0.5ml of the reaction mixture was mixed with 1ml of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) and allowed to stand for 5min for complete diazotization.

Then, 1ml of naphthyl ethylenediamine dihydrochloride (0.1%) was added and allowed to stand for 30 min at 25ºC. A pink coloured chromophore was formed and the absorbance was measured at 540 nm against the blank solution. Percentage of scavenging of NOwas calculated as stated above.

Scavenging effect (%) = $(A_{cont} - A_{test})\ A_{cont} \times 100$

RESULTS:

The colour was found to be grey with long spore chain morphology (Fig.1B). Melanoid pigment was found and the other pigments like soluble pigment and reverse side pigment were absent. Assimilation of carbon source has shown the absence of xylose and rhamnose among all the carbon compounds mentioned in the table-1. These results helped in the identification of *Nocardiopsis* species.



(A) (B)

Figure - 1 The figure shows the (A) isolated Actinobacteria and (B) the Morphology of the Actinobacteria

Table 1: The table shows the cultural characteristics of Actinobacteria

Color of aerial mycelium	Grey
Melanoid pigment	+
Reverse side pigment	-
Soluble pigment	-
Spore chain	Long chain

Assimilation of carbon source	
Arabinose	+
Xylose	-
Inositol	+
Mannitol	+
Fructose	+
Rhamnose	-
Sucrose	+
Raffinose	+

Table 2: The table shows the cell wall characteristics of Actinobacteria.

Cell wall amino acids			Cell wall sugar		Cell Wall type	Index
LL-DAP	MesoDAP	Glycine	Arabinose	Galactose		
-	+	-	-	-	III	*Nocardiopsis*

Only MesoDAP was found 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 third type (Table-2). This shows and index for *Nocardiopsis* species

Table 3: The table shows the characteristics of pigment obtained from *Nocardiopsis* species.

Melanin chemical analysis	
Color	Black
Solubility in inorganic solvents	
H2O (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	
FeCl3	
Conc.HCL	
Oxidation process	
H2O2	Oxidized

The colour of the pigment was black. The pigment derived showed solubility in phenol which is an organic solvent and insoluble in the other organic solvents 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 (H_2O_2). In case of antioxidant, DPPH assay and NO scavenging assay showed a potential activity using melanin pigment. The different concentration (25µg/ml to 150 µg/ml) of melanin was used and ascorbic acid act as control. While increasing concentration, the percentage of inhibition also increased for both DPPH and NO scavenging assay (Fig.3&4).

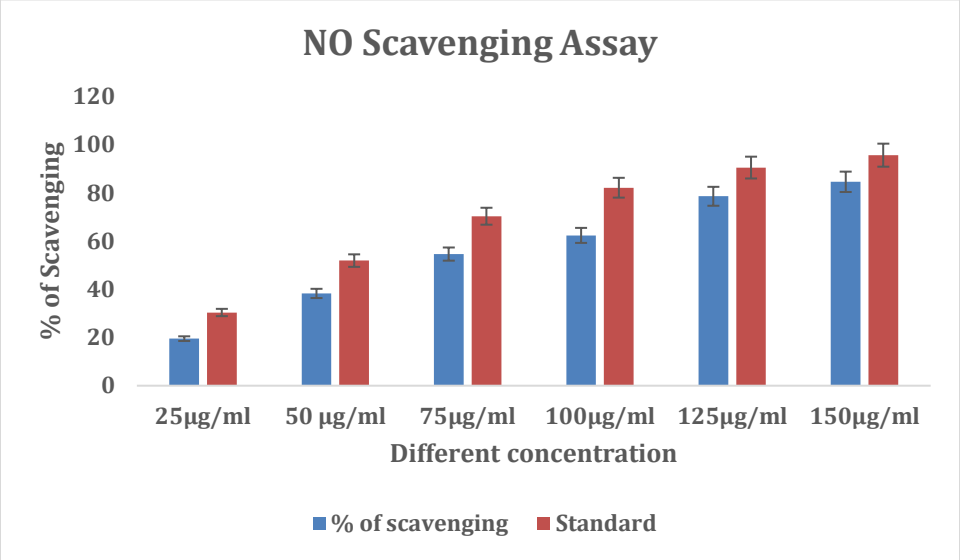


Fig.3 NO activities using melanin pigment

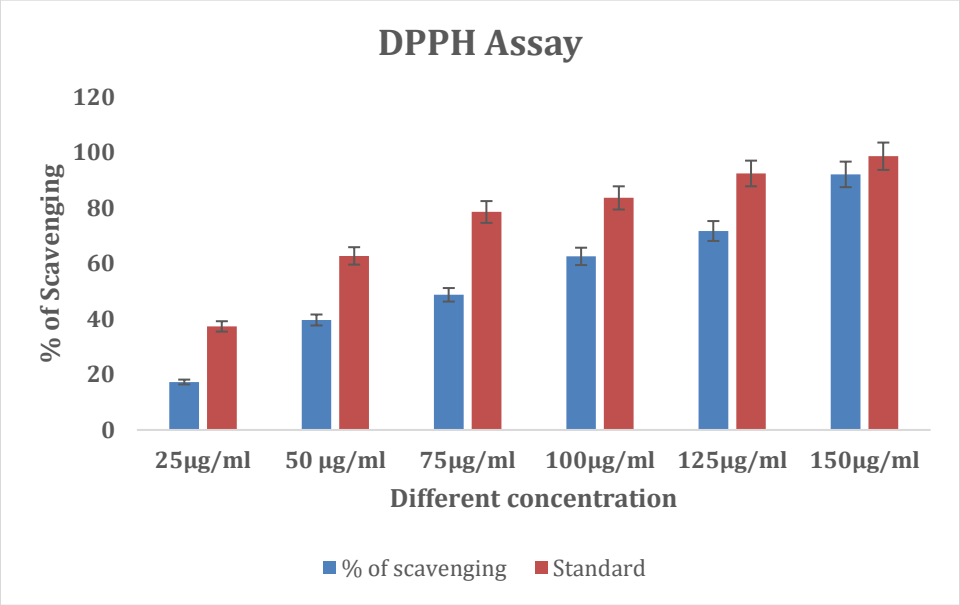


Fig. 4 DPPH activities using melanin pigment

DISCUSSION:

The colour was found to be grey with long spore chain morphology. Melanoid pigment was found and the other pigments like soluble pigment and reverse side pigment were absent. Assimilation of carbon source has shown the absence of xylose and rhamnose among all the carbon compounds mentioned above. These results helped in the isolation of *Nocardiopsis* species. The cell wall belongs to the third type. This shows an index for *Nocardiopsis* species. The pigment derived showed solubility in phenol which is an organic solvent and insoluble in the other organic solvents 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 has an antifungal, antibacterial, antioxidant activity and also plays a vital role in industrial purposes and biotechnological application due to the reason that melanin pigment is ankle to produce large scale goods with low production cost. It was shown that melanin pigment is suitable for dyeing and printing of wool fabrics in place of synthetic dyes (21). (22). The melanin pigment was evaluated as a potent bioactive molecule with antimicrobial, antioxidant, anti-inflammatory, and anticancer activities. It shows the therapeutic nature of melanin pigment has been increased over years. In Spite of all these activities, melanin pigment was found to produce killer toxin activity against the pathogenic yeast *Candida albicans (23)*. This study revealed melanin production could contribute to antibiotic resistance to the bacteria and the mechanism underlying needs to be explored further. As melanin is a pigment with highest pharmacological and cosmetological importance, the melanin produced by the bacteria in the study can be utilized in developing cosmetics and future drugs (7); (24). Cytotoxicity of DOPA melanin against cervical cancer cell line showed a dose-response activity and IC50 value was found to be 300µg/mL. This DOPA melanin has shown a potent activity as an antibacterial natural product against 12 pathogenic bacteria from hospital isolations, particularly against *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* (25).

Antioxidant activities of the fractions from chestnut shell were comparatively evaluated for the first time. The fractions exhibited different antioxidative potentials in different evaluation systems. The fractions included soluble water and insoluble substances which may be organic or inorganic. All fractions were inferior to BHT in peroxidation inhibition and o2- scavenging and reducing

power. Based on all the results the author concluded that the melanin pigment fractions are effective antioxidant colorants (26);(24,25). The author synthesized two types of chemically modified melanin pigments. The results of elemental analysis were found to correlate with the antioxidant activity of each melanin sample. None of the melanins showed a significant cytotoxicity and antioxidant activity, indicating that these melanins have attractive characteristics for potential medicinal applications (27); (28). This study examined antioxidant activity of synthetic melanin extracted from the mycelium and the culture medium after the growth of highly melanized strain from A.nidulans. The results showed that the pigment of the MEL strain competes with TNB for H2O2 and HOCl oxidant which was compared with the inhibition of synthetic melanin having IC50 values close to both the pigments. Thus the author suggested that the melanin obtained from A.nidulans is a potential HOCl scavenger and is able to be used in the cosmetic industry for the formulation of various creams and lotions that protect the skin against possible oxidative damage (29). There was no significant difference between A. auricula melanin control group and waste residue melanin on ABTS, DPPH, and hydroxyl radical scavenging activity. Waste residue melanin significantly inhibited the cell death caused by H_2O_2, and the cell viability was restored to 98.09 ± 5.97% when the melanin concentration was 1.6 mg/ml. (30); (5,28).

CONCLUSION:

The produced melanin pigment from marine actinobacterium was found to have potent antioxidant potential activity. Further characterization and bio active properties should be done in the further studies, and more articles in future are yet to come in various properties of melanin pigment.

CONFLICTS OF INTEREST:

The authors declare that there were no conflicts of interest in the present study.

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