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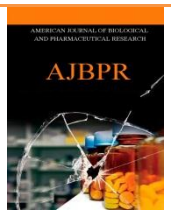

ISOLATION, PURIFICATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE MELANIN FROM MARINE STREPTOMYCES

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ABSTRACT

Melanins are enigmatic pigments and biological macromolecules that are produced by a wide variety of microorganisms including several species of bacteria and fungi. The present study was carried out on isolation and characterization of melanin from marine actinomycetes Streptomyces sp. PA-32 Medium composition and culture conditions for the melanin production by Streptomyces sp. PA-32 were optimized the concentration of the four significant variables: glycerol, l-tyrosine, NaCl and trace salt solution. The melanin was optimally active at pH 7–9 and temperature 45–60◦C and it was most stable up to pH 11 and 4% of NaCl concentration. Melanin was examined by UV–vis absorption spectroscopy and infrared spectrometry. The molecular weight of the purified melanin of PA-32 was 63 kDa. This study suggested that the melanin could potentially be used as a natural antioxidant in the food, cosmetic and pharmaceutical industries.

INTRODUCTION

Melanins are the pigments of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds and are usually dark brown or black in colour [1-3]. They are among the most stable and resistant biochemical materials which enhance the survival and competitive abilities of possessor organisms in certain environs but are not essential for their growth and development [4]. They have several biological functions including photoprotection, thermoregulation, action as free radical sinks, cation chelators and antibiotics. They are frequently used in medicine, pharmacology and cosmetic preparations.

Melanin or melanoid pigments are synthesized and excreted by actinobacteria, which are useful in taxonomical studies [5,6] in addition to their industrial application.

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In the present investigation, production and characterization of melanin pigment of the actinomycetes *Streptomyces* PA-32 isolated from the pitchavaram mangrove sediment which has been studied in detail in order to assess the strain's potential for industrial application.

MATERIALS AND METHODS

Isolation and identification of marine actinomycetes

A marine actinomycetes *Streptomyces* strain, PA-32 was isolated from the mangrove sediment from Pitchavaram (Lat 11°22¢N to 11°30¢N and long 79°45¢E to 79°52¢E), is located in the northern most end of the Cauvery delta and screened for melanin production on Tryptone Yeast Extract agar and Tyro sineagar incubated for 7 days at 28◦C. After incubation, production of melanoid pigments (i.e. greenish brown, brownish black and distinct brown) was noted on the medium.

Development of dark brown to black color around the media was considered positive for melanin pigments.

Melanin production

Composition of the production medium used was Tyrosine agar (Glycerol 15 g; L-tyrosine 0.5 g; L-asparagine 1 g; K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.5 g; NaCl 0.5 g; $FeSO_4.7H_2O$ 0.01 g; Trace salt solution 1 ml; pH 7.0 and 50% aged seawater). All the experiments were carried out in 500 ml Erlenmeyer flasks containing 100 ml of Tyrosine agar medium. Sterile medium was inoculated with 5% of inoculum (3.1-4.7 x 10^4 CFU ml^{-1}), incubated at 28°C and cultivated under agitation at 180 rpm for 7 days. Growth and melanin production were determined from the samples collected at 12 h intervals for which samples (5 ml) were taken from each of three replicate tubes. The cells were harvested by centrifugation at 12,000 rpm for 15 min, at 4°C. The mycelial mass was removed by vacuum filtration and dried in an oven at 80°C to measure dry biomass weight, expressed in mg dry mass per 50 ml^{-1} of culture medium.

Optimization of culture conditions

Out of the 5 actinobacterial strains subjected to the primary screening process only the strain PA-32 showed higher production of melanin pigments. So, this strain was subjected to different culture conditions to derive the optimum conditions for melanin production. Growth and melanin production were estimated at various temperatures (15, 25, 30, 35, 40 and 45°C), pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), different concentrations of sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%), carbon sources (arabinose, xylose, inositol, fructose, rhamnose, sucrose, lactose and raffinose), nitrogen sources (peptone, yeast extract, beef extract, casein and potassium nitrate) and amino acids (L-glutamine, L- asparagine, L-tyrosine, L-lysine, L-dopa and L- histidine).

Temperature

Fermentation was carried out at various temperatures viz. 15, 25, 30, 35, 40 and 45°C to study their effect on melanin production. Experiments were carried out in triplicates and the average values have been reported.

pH

Different pH viz. 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 of the Tyrosine broth were prepared using buffer solution. All the experiments were carried out in triplicates and the average values have been reported.

Sodium chloride

Various concentrations of sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) were used by changing the ratio of volume of sodium chloride solution to the Tyrosine broth and the flasks were incubated in triplicates, keeping the other parameters constant and the average values have been reported.

Carbon compounds

The basal medium of the following composition was used with 1% of carbon source, 2.0 g of $NaNO_3$, 1.0 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of KCl and 0.01 g of $FeSO_4.7H_2O$ in 1000 ml of distilled water (pH 7.2). Basal medium was distributed into various flasks and 1% of each carbon source viz. arabinose, xylose, inositol, fructose, rhaffinose, sucrose, lactose and raffinose was then added before inoculation of the strain and incubated at the optimum pH, temperature and NaCl. The experiments were conducted in triplicates and the average values have been reported.

Nitrogen compounds

Basal medium was used for studying the effect of various nitrogen compounds viz. peptone, yeast extract, beef extract, casein and potassium nitrate. The broth was distributed into various flasks and 1% of each nitrogen source was added before the inoculation of the strain. Cultures in triplicates were incubated at already standardized parameters.

Amino acids

Basal medium was used for studying the influence of amino acids such as L-glutamine, L- asparagine, L-tyrosine, L-lysine, L-dopa and L- histidine on melanin production. The broth was distributed into various flasks and 0.8 ml of each amino acid was then added. Triplicate flasks were incubated, keeping all other conditions at their optimum level.

Melanin formation

Melanin pigment was estimated by taking 2 ml of the culture and 1 ml of 0.4% substrate solution (L-tyrosine or L-dopa) and it was incubated at 37ºC for 30 min for L-tyrosine and 5 min for L-Dopa .Black colouration resulting from dopachrome formation was observed and read spectrophotometrically at 300 nm (UV-1800-Shimadzu scientific instruments, USA). When there was no colouration within these periods, the reaction mixture was further incubated for as long as 2 h. After incubation, melanin was found to be formed within 30 min [7].

UV absorption spectrum of melanin

To determine the UV-visible light absorption spectrum of melanin, 0.1 mg of sample was dissolved in alkaline distilled water at pH 8.0, and the resulting solution was scanned with a spectrophotometer (Shimadzu, UV-1800) to determine its absorption spectrum in the UV-visible light at wavelengths ranging from 200 to 800 nm.

Thin-layer chromatographic analysis

Products liberated by the action of melanin pigment on Tyrosine medium were identified by spotting the melanin pigment and standard (L-tyrosine and L-Dopa) on a silica gel plate activated at 110^{o}C for 2 h. The plates were developed in butanol: ethanol: water solvent (5:3:2) and dried over night at room temperature. The individual melanin pigments were visualized by spraying with aniline-diphenylamine reagent [8].

Purification of melanin

The strain PA-32 culture broth was centrifuged at 12,000 rpm for 10 min at 4^{o}C to remove the cells, and melanin was precipitated by adjusting the pH to 3.0 with 5N HCl. The precipitated melanin was re-dissolved in distilled water at pH 8.0, centrifuged again and dialyzed against distilled water. The dialysis was conducted for at least 24 h and stopped when the dialyzed solution reached the pH 4.5. The dialyzed preparation of melanin was lyophilized.

Further purification was carried out using ion exchange chromatography (Sephadex LH-20). The melanin powder was applied to a Sephadex LH-20 column, pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). After washing the column with 3 vol. of equilibration buffer, bound melanins were eluted stepwise using phosphate buffers of increasing molarity and decreasing pH values at room temperature (approx. 25◦C). The flow rate was adjusted to 24 ml h^{1} and fractions (1 ml each) were collected. The fractions showing high melanin content were pooled. Protein content of the melanin was also estimated [9]at different levels of purification.

RESULTS

Melanin production and mycelial biomass (Plate 5 a and b)

In the strain PA-32, melanin production ranged from 0.051 to 0.595 (Optical Density). The maximum melanin production was observed at 120 hrs (Optical Density 0.595) and minimum was recorded at 12 hrs (O.D 0.051). Biomass varied between 21 to 97 mg 50 ml^{-1}. The maximum biomass was observed at 120 hrs (97 mg 50 ml^{-1}) and minimum at 12 hrs (21 mg 50 ml^{-1}). Melanin (pigments) production and biomass were higher during the stationary phase of growth of the organism at 120 h.

The optimum growth temperature for melanin production by PA-32 was found to be 25^{o}C. Higher amount of melanin production was noticed on temperature at 30^{o}C (OD 0.487) and lower value was recorded at 15^{o}C (0.089) . The optimum pH for melanin production by PA-32 was found to be pH 6. Maximum melanin production (0.561) was observed at pH 7.0 and minimum (0.102) was observed at pH 4.0. The pH ranged from 5 to 13.

The optimum sodium chloride for melanin production by PA-32 was found to be 3% maximum level of melanin production was observed at 4% concentration and the minimum level was observed at 2%.In the different carbon sources tested, melanin production was maximum in sucrose (0.487) and minimum in lactose (0.147). In the different nitrogen sources tested, melanin content was higher (0.445) in casein and lower (0.245) in potassium nitrate.

In the different amino acids tested, melanin production was maximum (0.548) in L-dopa and minimum (0.219) in L- lysine. Optimal levels of the growth parameters, observed in the present study for the production of melanin with respect to the potential actinobacterial strain PA-32 are shown in Table .1

UV absorption spectrum of melanin

The ultraviolet (UV)-visible light absorption spectrum of the purified melanin of the strain PA-32 showed the absorption peak at 300 nm.

Thin layer chromatography

R_f value of the purified melanin was 0.62. It was obtained as reddish crystals with a melting point of 110-120^{o}C. The purified melanin was odourless and appeared as a brown spot on the TLC plate.

Protein content and Molecular weight determination in SDS-PAGE

The protein concentration of the purified melanin was 73 mg/g and Molecular weight of the melanoid pigment was 63 kDa as determined by SDS-PAGE gel electrophoresis.

Table. 1. Optimal levels of growth parameters and sources for melanin production by the strain MA-32.

S. No	Parameters	Optimal Value/Source
1	Temperature (^{o}C)	30
2	pH	7.0
3	NaCl concentration	3%
4	Carbon source	Sucrose
5	Nitrogen source	Casein
6	Amino acid source	L-dopa
7	Incubation time	120 hrs

Figure 1. Optimal levels of growth parameters and sources for melanin production

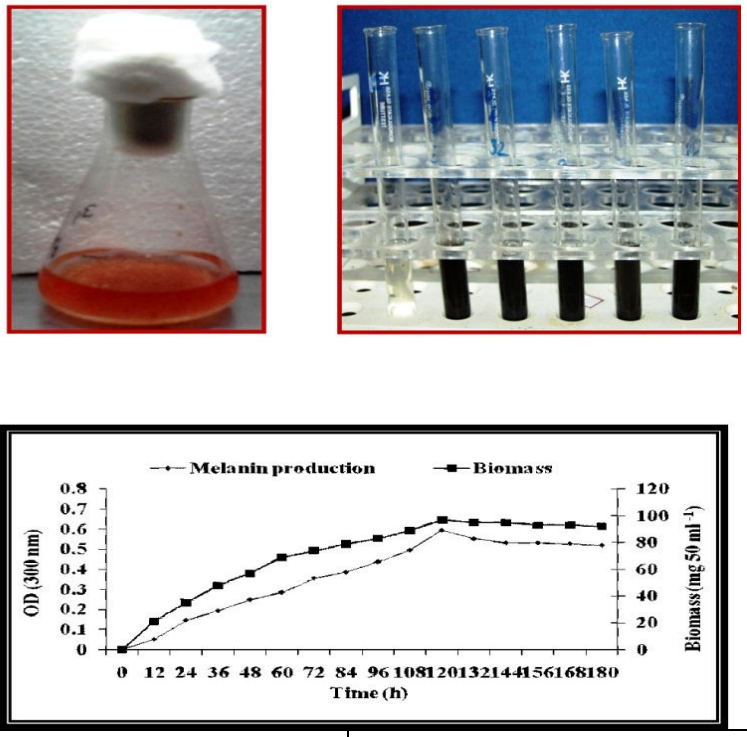


Figure 2. UV-Visible absorption spectrum of the melanin pigment of PA-32.

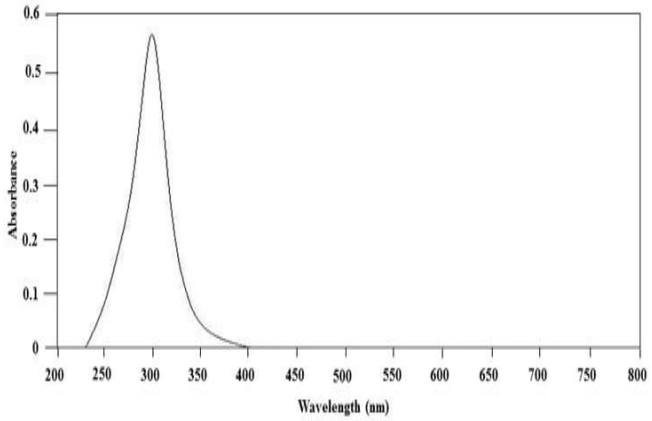
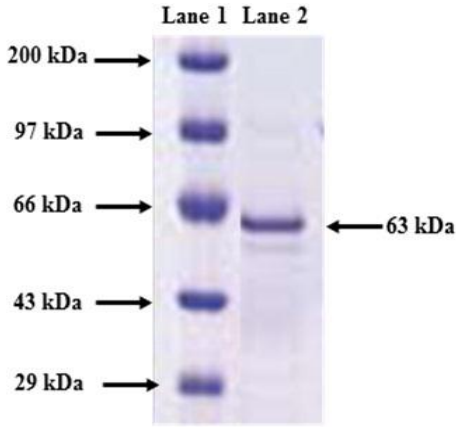


Figure 3. SDS-PAGE of the purified melanin (pigment) (Lane 1- molecular weight markers; and lane 2- melanin).



DISCUSSION

In the development of fermentation technology, determination of growth parameters is an important aspect. Only a few reports are available concerning the optimization of growth parameters for microbes eg. *E. coli* [10]. *Bacillus thuringiensis* [11] *B. cereus*[12]. fungi [13] *Penicillium* [14] and studies are very less in actinobacteria (eg. *Streptomyces* by [15]with melanin production.

In the present study, melanin production by the strain PA-32 was growth independent as maximum melanin production was noticed during the stationary phase of growth of the strain. Among the different parameters, pH of the growth medium is said to play an important role by inducing morphological changes in microbes and their melanin secretion and the pH changes during the microbial growth can also affect product stability in the medium [16].Most of the earlier studies have revealed that the optimum pH range is between 3.0 and 10.0 for the growth of fungal strains and melanin production [14] and in the present study, it was 7.0.

Temperature optimum for melanin production was found to be in the range of 10 and 35°C for the fungi and

the present study has recorded 30°C as optimal, which is in agreement with the earlier findings of [14,17].Incubation period can vary with various melanin productions. In the present study, the melanin production increased steadily and reached the maximum at 120 h of incubation, as against a short duration of 24 h in the case of bacteria [11].

At the sodium chloride concentration of 2%, higher growth of the isolate was observed.[18] have also reported that in *Streptomyces* sp., maximum production of melanin occurred in 2.5% of sodium chloride. It is worth mentioning here that sodium chloride-sensitive actinobacteria can increase their tolerance to higher concentrations during successive cultivations with a significantly different absorbance in the visible spectrum, suggesting the production of new metabolites [19].

With reference to carbon sources, sucrose promoted the growth of the isolate, whereas poor growth was observed in lactose. *Streptomyces plicatus* isolated from the fish, *Gerres filamentoses*, showed more affinity towards glucose[20,21] and *Streptomycetes* obtained from the gut of ornamental fish, *Barbus schwanefeldi*, showed maximum growth in mannitol and xylose[22].

Among the different nitrogen sources used, melanin production was higher in casein and lower in potassium nitrate. In the different amino acids tested, maximum growth was noticed in L-dopa and minimum growth, in L- histidine. [15] have reported that maximum growth occurred in L-citrulline (0.671 O.D) in *Streptomyces* sp. Similar findings have also been made by [17,18].

In the present study, melanin showed absorption peak at 300 nm whereas [23] have found the absorption peak at 250 nm for the melanin pigment extracted from *Phyllosticta capitalensis*. The molecular weight of the purified melanin of PA-32 was 63 kDa whereas [24].obtained purified melanin with 130 kDa in *Bacillus cereus*. Such low molecular pigments would be a asset for further biotechnological application.

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