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FULL LENGTH ARTICLE

Optimizing the Biosynthesis of Melanin Nanoparticles Used for Heavy Metal Removal

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ABSTRACT

Melanins are nitrogenous polymeric compounds with indole ring as their monomeric unit.They have immense applications in the field of agriculture, cosmetics and pharmaceutical industries due to their photo-protective, UV protective and anti- oxidant activities. The metal ion chelation property of natural melanin can be exploited for removal of heavy metals. The objective of this study was to investigate the effects of different growth media and process parameters on melanin production. The marine bacterial strain Pseudomonas stutzeri HMGM-7(MTCC 11712) was selected and the effect of different media and various medium components were investigated with a one factor at a time approach. Parameters like shaking frequency, inoculum age, inoculum size and pH were also investigated in order to obtain the optimum conditions for maximum melanin production. The highest yield of melanin concentration, 0.27 g/L, was obtained in nutrient broth at 32 hours. The yield was 1.53 times higher than the melanin obtained before optimization, 0.177 g/L at 48 hours. The increase in the productivity of melanin after selection of suitable medium and optimization of process parameters was 128.73%. Particle size analysis of the biosynthesized melanin shows that particles are nanoparticles with a size of 32 ± 0.98 nm

Keywords— *melanin, Pseudomonas stutzeri HMGM-7, one factor at a time, nanoparticle.*

INTRODUCTION

Pigments are colorful chemical compounds which absorb light in the visible spectrum. The produced color is due to the absorption of energy by a group of molecules known as chromophore which leads to the excitation of an electron. The non-absorbed energy which is reflected or refracted is captured by the eye to generate neural impulses which are then carried to the brain where they could be decoded as a color [1]. Pigments are of two types, synthetic pigments and natural pigments. They are widely used in clothes, cosmetics, furniture, foods, medicines, and in other products. Based on their structural characteristics, the natural pigments are classified as Tetrapyrrole derivatives, Isoprenoid derivatives, Benzopyran derivatives, Quinones and Melanins [1]. Melanins are nitrogenous polymeric compounds with indole ring as their monomeric unit but they are not homopolymers. Generally, they are present as a mixture of macromolecules and are responsible for most of the black, brown and gray colorations of plants, animals, and microorganisms. Melanins are classified into three groups, they are; Eumelanins which are black or brown pigments and are widely distributed in vertebrates and invertebrates. It is the most common type of melanin. Pheomelanins which are yellow to red pigments and are found in mammals and birds. And finally allomelanins that are present in fungi, seeds and spores. The very first report of melanin production was reported in *Pseudomonas aeruginosa* producing pyomelanin [2] followed by *Shewanella. colwelliana, Vibrio cholera*, and *Hyphomonas* strain [3, 4].The melanin synthesis using homogentisic acid as a precursor was first reported in *Vibrio cholerae, Hyphomonas* species and *Shewanella colwelliana* [3]. The synthesis of melanin and its characterization such as solubility, free radical nature was initially studied in *Proteus mirabilis* [5]. A novel marine bacterium *Alteromonas* strain MMB-1, was isolated from the Mediterranean Sea and its melanin synthesis ability was studied using L-tyrosine as a precursor [6]. Melanin production was studied by a UV-resistant mutant of *Bacillus thuringiensis* subsp. *Kurstaki* and its UV-protection ability for insecticidal crystals were tested [7]. The thermo tolerant strains of *Bacillus thuringiensis* were also reported for melanin production [8]. Another important melanin producing bacterium reported was *Marinomonas mediterranea*, which produces black eumelanin from L-tyrosine [9]. A marine bacterium, *Pseudomonas stutzeri* HMGM-7 producing considerable amount of melanin in sea water medium without the addition of L-tyrosine was also reported [10]. The different properties and functions of melanins are being explored for various applications. The high reactivity of melanin due to the presence of =O, -OH, -NH, and –COOH groups is the

reason behind growing interest in melanin research. Natural melanin has considerable affinity towards metal ions. Possible functional groups responsible for metal binding are carboxyl (COOH), Amine groups (NH) and phenolic hydroxyl (OH). Melanins also have a broad spectrum of biological applications. This includes inhibition of Human Immunodeficiency Virus (HIV) replication, antivenin activity, antimicrobial activity and antioxidant activity. Melanins also have physical and chemical applications. This includes their use in nano particle synthesis, cosmetics and in lenses.

In spite of so many potential applications, melanins are not used due to non-availability of a sustainable and cost effective method of melanin production. The major sources of melanin are cephalopods, plants and microorganisms. Melanin obtained from microbes has great advantages over melanin from animals and plants. Microorganisms do not cause the problems of seasonal variations and are fast in growth. They also modify themselves according to the medium and conditions provided. The bacterial sources have been used as a main source of melanin with immense applications in the field of agriculture, cosmetics and pharmaceutical industries [11], hence its optimization is important for large scale production. The high production cost and high commercial value of melanin has given rise to the need of a demanding research for cheaper production methods. The present study aims to increase the productivity of melanin from an epiphytic bacterium known as *Pseudomonas stutzeri* HMGM-7 (MTCC 11712). This bacterium was isolated from the branches of sea weed *Hypnea musciformis*, which released a black extracellular pigment into the medium. The physical parameters and nutritional requirements which play an important role in the production of melanin were optimized in the present study.

MATERIALS AND METHODS

Microorganisms and culture conditions

Nutrient agar slants and plates were prepared for maintenance of the organism (*Pseudomonas stutzeri* HMGM-7) obtained from MTCC Chandigarh. Periodical subcultures were done for maintenance of the viability of the strain. Media volume used throughout the experiments was 50ml in 250 ml Erlenmeyer flasks which were maintained at 37^0C at 150 rpm. Nutrient broth prepared in distilled water was used for shake flask studies.

Growth Studies

2% inoculum volume was added to each flask and incubated at 37^0C and observed at two different rpm: 150 and 250. During the incubation period of 72 h, the OD of the samples was measured at 660 nm after every 4 h using appropriate blank. Biomass dry weight for a volume of 28ml of the culture was accounted for by centrifuging (8000 rpm, 8mins, and 40C) and drying the pellet for 8 h at 60^0C in a hot air oven. The supernatant obtained after centrifugation is filter sterilized with 0.45µm syringe filters and their absorbance is measured at 400 nm to quantify the melanin.

Optimization of nutritional parameters for melanin production

All the experiments conducted used a constant media volume of 50 ml in 250ml Erlenmeyer flasks. NB was used as the basal medium which was incubated at 37^oC and 150 rpm unless otherwise stated. The effect of different parameters on the production of melanin such as pH, temperature, carbon sources, nitrogen sources, trace elements was evaluated by keeping Nutrient broth as the basal medium. The factorial design of experiments known as 'one factor at a time' method was applied to improve the reproducibility of the experimental results and to optimize the entire biosynthesis process. The experiments were conducted by varying one factor at a time and keeping the remaining factors constant.

Effect of Inoculum age

The effect of age of the inoculum on the melanin production was studied using Nutrient Broth medium by using 6, 12, 18 and 32 hrs old cultures maintained at 37^0C and 150 rpm.

Effect of Inoculum size

0.5%, 5%, 10%, 15% and 20 % inoculum volume were evaluated for melanin production using Nutrient Broth medium at 37^0C and 150 rpm.

Effect of shaking frequency

The shaking frequency was optimized by incubating the Erlenmeyer flasks in an incubator shaker at 100,150, 200 and 250 rpm at 37^0C with a shaking diameter of 25mm.

Effect of pH

The optimum pH for the production of melanin was determined by setting initial pH of the medium to 4, 5, 6, 7, 8 and 9 by using 0.1 N HCl and 0.1 N NaOH.

Effect of different growth media

Four different growth media, Nutrient Broth (NB), Luria Bertini (LB) Broth, Bushnell- Haas Broth (BHB) and Trypticase Soy Broth (TSB) were studied for melanin production by culturing the microorganism in each of the media at 37^0C and150 rpm.

Effect of Carbon sources

The effect of various carbon sources was studied by adding each carbon source in the medium at the concentration (2.5 g/l). The carbon sources evaluated were glucose, sucrose, lactose, fructose, starch, xylose, maltose, glycerol and dextrose.

Effect of Organic nitrogen sources

To evaluate the various nitrogen sources for maximum melanin, the production medium was supplemented with each organic nitrogen source at the concentration (1.5 g/l). The organic nitrogen sources tested were peptone, beef extract, yeast extract, and tryptone.

Extraction and purification of melanin.

The extraction of melanin was done in accordance with the procedure described for the purification of melanin from the culture of *Aspergillus bridgeri* with some minor modifications. In short, the medium was centrifuged at 5000 g for 10 minutes to remove the biomass. The supernatant collected was then treated with 1 M NaOH and then autoclaved at 120°C for 15-20 minutes. After autoclaving, the solution was cooled and centrifuged at 5,000*g* for 10 min to collect the alkylated supernatant which was then acidified to pH 2 by using 1 N HCl, in order to precipitate the melanin. The precipitated melanin was collected by centrifuging at 12,000 *g* for 20 min and washed with distilled water and evaporated to dryness at room temperature and was stored for further use.

Characterization studies.

Purified melanin was dissolved in 0.1 N NaOH for UV-visible spectrophotometric analysis. The solution was scanned from 200 to 900 nm. The absorbance was measured by using a double beam UV-visible spectrophotometer (Hitachi, Labomed Inc). The absorption spectrum of the melanin pigment from the *Pseudomonas stutzeri* HMGM-7 strain was compared with that of standard melanin. For FT-IR analysis, the pigment and standard melanin were scanned between the wavenumber range of 4,000–400 cm-1 by using KBr discs with an FT-IR spectrophotometer (IR Prestige, Shimadzu). Particle size analysis of the biosynthesized melanin was done using 'Nanopartica' Nanoparticle analyzer SZ–100, HORIBA Scientific. Pure samples were dispersed in distilled water using an ultrasonic bath before introducing into the instrument. Scanning Electron Microscopy was used to investigate the nature and surface morphology of Biosynthesized and Synthetic melanin.

RESULTS AND DISCUSSIONS

Effect of inoculum age

After evaluating different inoculum age (6, 12, 18, and 32 hrs) for their melanin production, the 12 h old inoculum gave the highest melanin concentration (197 mg/l) at the 48th hr. When 6 h old culture was used, the highest melanin concentration (195.2 mg/l) was attained in the 40th h itself. The maximum biomass yield was obtained for the 32 h old culture (1.434 g/l 12th h) whereas the 6 h old culture managed to attain its maximum biomass in the 22nd h (1.69 g/l). 1.344 g/l and 1.410 g/l were the highest biomass concentrations for cultures that were 12 and 18 hrs old respectively. The highest melanin obtained in the control medium was 177 mg/l and biomass attained was 1.107 g/. The increase in inoculum age thus results in an increase in biomass whereas reduction in inoculum age resulted in an increase in melanin production.

Effect of inoculum size

Different inoculum volumes (0.5%, 5%, 10%, 15% and 20%) were investigated to observe their effect on melanin production. The highest melanin production of 270.9 mg/l (32nd h) was achieved when 10% inoculum was added to 50ml of Nutrient Broth medium. Change in inoculum size did not alter the biomass yields, 1.425 g/l, 1.4 g/l, 1.46 g/l, 1.418 g/l and 1.385 g/l being the maximum biomass concentrations obtained at 0.5%, 5%, 10%, 15% and 20% inoculum sizes respectively. As a result of which, 10% inoculum volume was selected to be the optimum inoculum volume to be used for further investigations. The melanin production was observed after 10th h. The highest yield of melanin was achieved (270.9 mg/l) at the 32nd h. Thus there was a 53% increase in melanin productivity.

Effect of shaking frequency on biomass production

Nutrient broth medium was used as the growth medium which was maintained at 37^{0}C and the shaking frequency was varied to study its effect on the melanin production. The maximum biomass production obtained for *Pseudomonas stutzeri* HMGM-7 was 1.107g/l at the 8th and 12th h when the organism was allowed to grow for a period of 72 hrs at 150 rpm. In the medium prepared in sea water without adding L-tyrosine, Ganesh Kumar et al. (2013) obtained maximum biomass production of 2.5g/l. There was a substantial increase in the biomass at the increased shaking frequency of 250 rpm as compared to 150 rpm.

Effect of shaking frequency on melanin production

There was a steady increase in the melanin production till the 48th h when the culture flasks were maintained at 37^{0}C and 150 rpm, where maximum production of 0.177 g/l was obtained followed by a decline in its productivity by the end of the incubation period .The onset of melanin production was significant only after the 8th h. Ganesh Kumar et al.[10] obtained the maximum melanin production of 6.7g/l at the 60th hr in the sea-water medium without L-tyrosine supplementation. The melanin yield obtained in this study is comparatively lesser since Nutrient broth medium prepared in distilled water was used instead of the sea- water medium which is known to be conducive for marine species like *Pseudomonas*. Melanin production at 37^{0}C and 250 rpm increased till the 32nd h, where maximum production of 0.164 g/l was obtained followed by a decline in its productivity by the end of the incubation period. When L-tyrosine was used as a sole carbon and nitrogen source into the melanin production media containing KH_2PO_4, NaCl and $MgSO_4.7H_2O$ made in Distilled water by Noble K Kurian et al. [12],*Pseudomonas stutzeri* Strain BTCZ10 produced 47.47±0.2µg/mL of melanin. Thus, in the present study it was found that increase in shaking frequency from 150 to 250 rpm caused a decrease in melanin productivity.

Effect of growth media

Nutrient Broth (NB), Luria-Bertani (LB) broth, Bushnell- Haas broth (BHB) and Tryptic Soy broth (TSB) were the four different growth media that were utilized in this study to screen for the medium that produced more amount of melanin. The highest melanin yield, 167.38 mg/l was obtained at the 32nd h in NB, followed by TSB and LB, whereas BHB had very low melanin production (Fig. 1.1). None of the additional nutrients could affect a significant rise in melanin production when compared to NB alone.

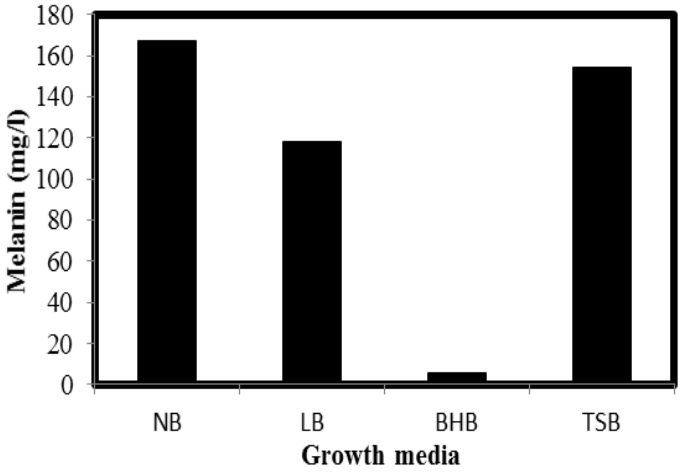

Fig. 1.1. Melanin productions for different growth media.

Characterization studies.

The spectral property of the pigment was analyzed to confirm the nature of the pigment. Its UV spectrum was found to be similar to that of synthetic melanin which exhibited absorption peak of maxima between 200nm and 300nm (Fig. 1.2).

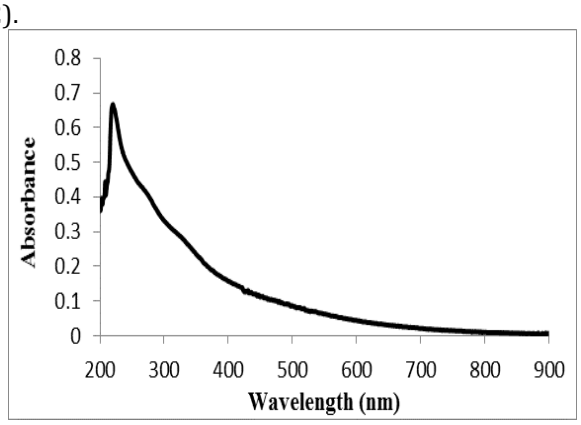

Fig. 1.2. The UV absorption spectrum of melanin produced by *Pseudomonas stutzeri* HMGM-7.

It showed a high degree of similarity when the main absorption peaks in the FT-IR spectra of the synthetic melanin (Sigma-Aldrich) and melanin obtained from *Pseudomonas stutzeri* HMGM-7 were compared (Fig. 1.3).

FT-IR spectrum showed a broad absorption peak at 1,622 cm-1 , which is due to the vibrations of the aromatic groups (C=O or C=C). The peak at 3,512 cm-1 was attributed to stretching vibrations of the –OH and–NH2 groups. While the peak observed at 2,916 cm-1 suggested the presence of –CH groups.

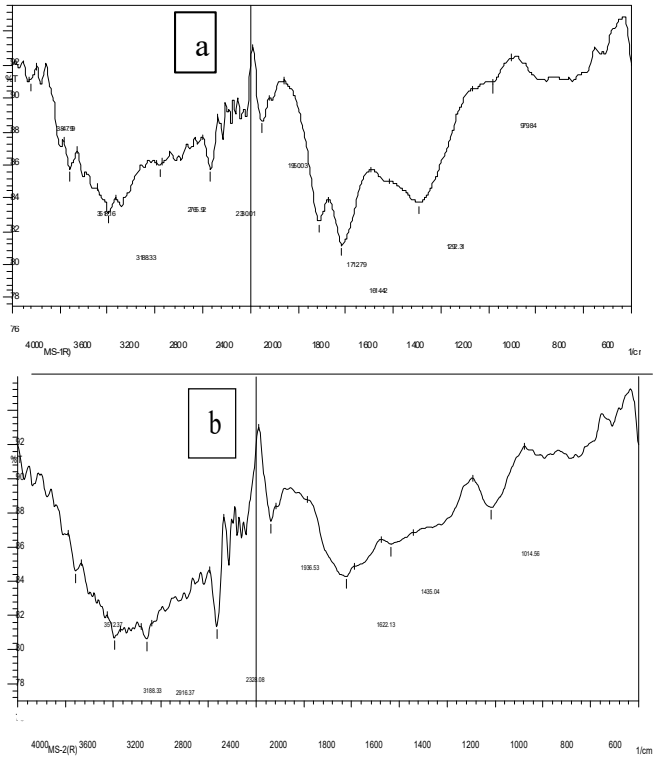

Fig. 1.3. a) FTIR spectra of synthetic melanin, b) FTIR spectra of melanin obtained from *Pseudomonas stutzeri* HMGM-7.

Particle size analysis of the biosynthesized melanin shows that particles are nanoparticles with a size of 32 ± 0.98 nm (Fig: 1.4).

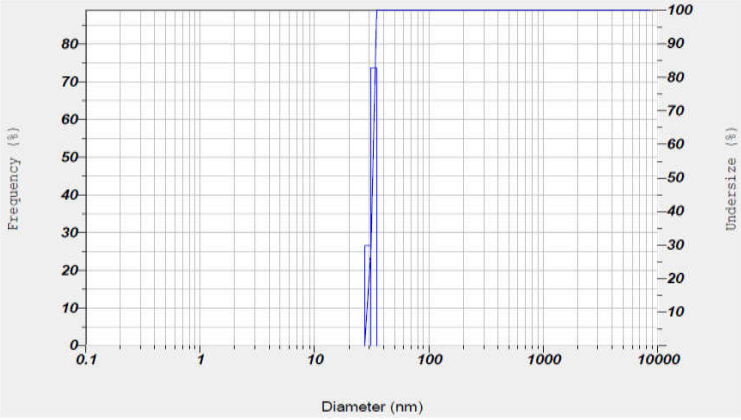

Fig. 1.4. Particle size analysis of the biosynthesized melanin.

SEM results showed similarities between Biosynthesized melanin and natural melanin (Fig: 1.5).

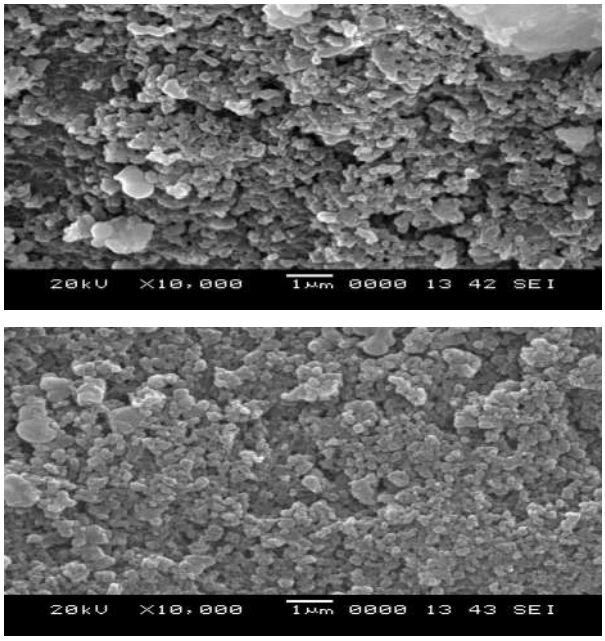

Fig. 1.5. a) SEM image of Synthetic Melanin b) SEM image of Biosynthesized melanin

CONCLUSION

Many bacterial sources have been used widely as a major source of melanin in recent years and hence its optimization is important for large scale production. *Pseudomonas stutzeri* HMGM-7 used in this investigation has a competence to produce melanin under various process conditions and in different growth medium and can prove to be of commercial use for the large-scale industrial production. Physical parameters and nutritional requirements often determine the melanin productivity that can be obtained from bacterial sources, and hence these parameters were evaluated in the current study. *Pseudomonas stutzeri* HMGM-7 was able to produce its highest melanin yield within shorter incubation period (32hrs) for most of the studies that were conducted by varying different nutritional and process parameters. The optimum inoculum age and size that produced higher melanin yield was found to be 6 h and 10% respectively. Nutrient broth along with three different media (TSB, BHB , LB) were evaluated for its melanin production, from which Nutrient broth proved to be the best, 0.27g/L being the highest melanin yield produced across all the experiments conducted. There was no significant increase in the melanin production when the media was supplemented with additional nutrients.

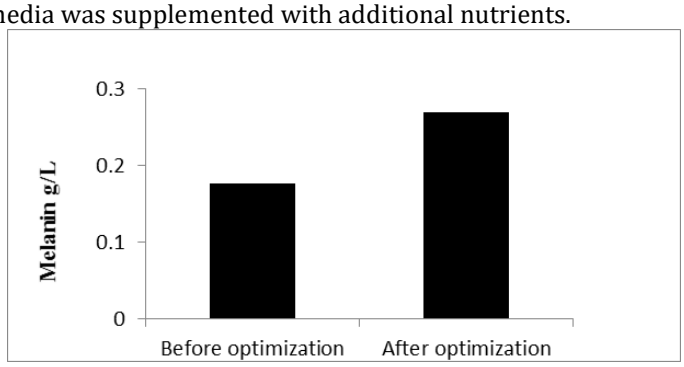

Fig. 1.6. Melanin yield before and after optimization.

The increase in the productivity of melanin after selection of suitable medium and optimization of process parameters was 128.73%. The melanin yield obtained can be further enhanced by statistical optimization and evaluating the effect of different combinations of nutrients like carbon and nitrogen sources and trace elements and further scale-up of the process can be done.

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