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Melanin nano-pigments for heavy metal remediation from water

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ABSTRACT

Melanins are water insoluble polyphenol compounds. The metal ion chelating property of natural melanin is exploited for removal of heavy metals from contaminated water. We optimized biosynthesis of melanin from marine bacterium using different growth media, media components, and operating conditions. Optimized medium yielded 513 mg/L melanin at 36 h of incubation, which was 3.15 times higher than the yield before optimization. Particle size analysis of the biosynthesized melanin indicated a size of 32 ± 0.98 nm. Preliminary investigation indicated that melanin nanoparticles could adsorb different heavy metals such as chromium, selenium, and lead from very low initial concentrations.

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KEYWORDS
Heavy metals; adsorption; biosynthesis; melanin nanoparticles; *Pseudomonas stutzeri*

Introduction

Pigments are colorful chemical compounds which absorb light in the region of visible spectrum. Certain molecules known as chromophores which are present in the pigments absorb energy, as a result of which the color is produced.[1] Pigments are mainly of two types, synthetic and natural, and are extensively used in cosmetics, textiles, foodstuffs, furnishings, lenses, drugs, and in other products. The natural pigments are classified into different groups based on their structural characteristics. They are benzopyran derivatives, isoprenoid derivatives, tetrapyrrole derivatives, quinones, and melanins.[1] Melanins are class of polyphenol compounds whose monomeric unit is an indole ring. They are responsible for most of the brown, black, and grey pigmentations in animals, plants, and microbes. Melanins are usually classified into three groups and they are eumelanins which are brown or black in color and are the most common type of melanins. They are distributed widely in vertebrates and invertebrates. Pheomelanins that are yellow or red in color and are found in birds and mammals. And finally the allomelanins which are present in fungi, spores, and seeds.

The melanin has numerous functional groups such as =O, –OH, –NH, and –COOH. These functional groups make melanin an ideal choice for heavy metal remediation. Use of melanin for heavy metal removal is at its nascent stage. Chen *et al.*[2] investigated Pb (II) and Cd (II) remediation from water using squid melanin. Sono *et al.*[3] described the use of hydrophobic polymer PVDF coated with synthetic and hair melanin for the adsorption of Pb (II). Saini and Melo[4] synthesized melanin using tyrosinase enzyme. This melanin was used for the adsorption of uranium. It showed good uptake capacity over a broad range of pH. Sajjan *et al.*[5] have done adsorption of $Cu2^{+2}$ and Pb^{+2} using melanin extracted from *Klebsiella* sp. GSK after immobilizing the melanin with sodium alginate. In all the reports, authors concluded that melanin had excellent heavy metal binding capacity as compared to activated carbon, especially at low metal concentrations.

In spite of melanin's very promising potential for heavy metal remediation, melanins are not widely used due to unavailability of a cost-effective and sustainable method for melanin production. As of now melanin is extracted from hair or from squid. Both the methods are not cost-effective and they will not be able to meet the melanin demand worldwide. Therefore, there is a need to produce melanin at commercial scale. Since they grow faster and adjust themselves with respect to the environment provided, the microorganisms may provide a suitable and cost-effective method for melanin production. At first, the production of melanin was reported in *Pseudomonas aeruginosa* producing pyomelanin[6] followed by *Shewanella colwelliana, Hyphomonas*, and *Vibrio cholera*.[7,8] Later, melanin production in presence of tyrosin precursor was reported in different microorganisms, such as in *Proteus mirabilis*,[9] in *Alteromonas* strain MMB-1,[10] a mutant of *Bacillus thuringiensis* subsp. *Kurstaki* which is UV resistant,[11] the

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strains of *B. thuringiensis* that are thermotolerant,[12] and *Marinomonas mediterranea*.[13] Recently, *Pseudomonas stutzeri* HMGM-7 which is a marine bacteria was reported to produce considerable amount of melanin in the medium prepared in seawater without the addition of tyrosine.[14] Seawater is abundant source (about 96.5%) as compared to potable water (about 2%). Therefore, use of seawater in any bioprocess makes the process more sustainable. Although, melanins are reported to be produced in variety of microorganisms, an optimized bioprocess has not been developed to date for possible large scale production, especially using seawater. Moreover, there are no reports on biosynthesis of melanin nanoparticles.

We report biosynthesis and application of melanin nanoparticles using seawater for the first time. We used microbial strain *P. stutzeri* HMGM-7 to produce melanin nanoparticles. The operating conditions for optimum melanin nanoparticle production are optimized. The nanoparticles of biosynthesized melanin are reported as adsorbents for heavy metal remediation.

Materials and methods

Microorganisms and culture conditions

The bacteria *P. stutzeri* strain HMGM-7 obtained from MTCC Chandigarh was retrieved under laboratory conditions. The pure cultures of bacteria were subcultured routinely on nutrient agar slants. All cultures were subcultured after every 4 weeks and were stored at 4°C. For the shake flask studies, 50 ml of Nutrient broth (NB) prepared in seawater with pH 7 in 250 ml Erlenmeyer flask was used as a medium. The flasks were maintained at 37°C at 150 rpm. Samples were taken at regular time intervals to study the growth and melanin production. The optical density (OD) of the culture was measured at 660 nm after every 6 h using suitable blank. Biomass dry weight was obtained by centrifuging at 5000 rpm for 5 min and 4°C. The pellets were dried for 8 h at 60°C in a hot air oven. The supernatant obtained was filter sterilized with 0.45 µm syringe filters and the OD was measured at 400 nm for the quantification of the melanin.

Optimization of nutritional parameters for the production of melanin

The effects of different nutritional parameters for melanin production such as effect of different growth media, effect of carbon and nitrogen sources, and effect of trace elements were evaluated by keeping NB as the basal medium. Factorial design of experiments known as 'one variable at a time' method was applied to optimize the entire process of biosynthesis.[15]

Effect of media

The effect of growth media on melanin production was studied by inoculating the culture into different growth media such as NB, Bushnell-Haas broth (BHB), Luria Bertani (LB) broth, and Tryptic Soy broth (TSB).

Effect of inoculum age

Inoculum of 6, 12, 18, and 24 h of age was used to study the effect of inoculum age on melanin production.

Effect of inoculum percentage

The effect of inoculum percentage on the production of melanin was studied by changing the inoculum concentrations. Inoculum of different sizes (0.5%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, and 60%) was used to study their effect on melanin production.

Effect of carbon and nitrogen sources

The effect of different carbon sources such as glucose, dextrose, starch, glycerol, and sucrose was investigated by adding different carbon sources to the medium at a concentration of 5 g/L. TSB medium was prepared by mixing all individual components and the carbon source which was present in the media was replaced by other carbon sources.

The effect of different sources of nitrogen such as corn steep liquor, soybean meal, coconut meal, cotton seed meal, and oat meal was studied by adding different nitrogen sources to the medium at a concentration of 5 g/L. TSB medium was used and the nitrogen source which was present in the media was replaced by other nitrogen sources.

Effect of filing volume on production of melanin

Since filling volume inside the flask affects oxygen transfer rate and hence, growth of microorganism, the effect of different filling volumes such as 10, 20, 50, and 100 ml was studied.

Effect of shaking frequency

The optimization of shaking frequency (rpm) was carried out by incubating the 250 ml Erlenmeyer flasks in an incubator shaker at different shaking frequencies such as 100, 150, 200, and 250 rpm at 37°C with a shaking diameter of 25 mm.

Effect of pH

The optimum pH for melanin production was determined by adjusting the initial pH of the medium from 4 to 9 by using 0.1 N NaOH and 0.1 N HCl.

Effect of temperature

The temperature optimization experiments for melanin nanoparticles biosynthesis were done by incubating flasks at 25°C, 30°C, 37°C, 40°C, and 45°C in incubator shaker.

Extraction and purification of melanin

Extraction and purification of melanin was done according to the protocol described for melanin purification from a culture of *Aspergillus bridgeri*[16] with some modifications.

In brief, the culture was centrifuged at 5000 *g* for 10 min to remove the biomass and the supernatant obtained was extracted with 1 M NaOH and then it was autoclaved at 120°C for 20 min. After autoclaving, the solution was then centrifuged at 5000 *g* for 5 min to collect the supernatant. The alkaline pigmented supernatant was acidified to pH 2 by adding 1 N HCl in order to precipitate the melanin which was then collected by centrifuging at 12,000 *g* for 20 min. The melanin collected was then washed with 3 ml of distilled water (×3 times), dried overnight at room temperature and then it was used for further studies.

Quantification of melanin

Quantification of melanin produced in the cell-free culture supernatant was estimated at 400 nm.[17]

Fourier-transform infrared studies

Fourier-transform infrared (FTIR) spectroscopy is considered to be the most informative, well-resolved, and non-destructive method, which will provide information on functional groups and detailed structural analysis of melanin.[5] The purified dark brown powder and standard synthetic melanin were analyzed by FTIR spectroscopy (Thermo Nicolet Avatar) using potassium bromide.

Particle size analysis

Particle size analysis of the biosynthesized melanin was done using 'Nanopartica' Nano particle analyzer SZ–100, HORIBA Scientific. Pure samples were dispersed in distilled water using an ultrasonic bath before introducing into the instrument.

Scanning electron microscopy and transmission electron microscopy analysis

The scanning electron microscopy (SEM) (JSM-6380, JOEL) and transmission electron microscopy (TEM) (JEM-2100, JOEL) were conducted to study the size and morphological characteristics of the biosynthesized melanin.

Adsorption studies

Biosynthesized melanin was used in adsorption studies for the removal of heavy metals such as lead, chromium, and selenium. Heavy metal salts were dissolved to give final concentration of 5 ppm. Batch adsorption was carried out in 100 ml conical shake flasks with 25 ml filing volume and 150 rpm.

Results and discussions

Effect of growth media

TSB media was selected which gave maximum melanin production (238 mg/L) and maximum biomass production (3.96 g/L) at 60th h, followed by LB and NB and BHB gave the least yield. Carbon and nitrogen sources are major components for growth of microorganisms as well as product formation.[18] NB and LB contain carbon and nitrogen sources whereas BHB contains only minerals and salts. That may be the reason why there is less biomass production in BHB and hence the melanin. TSB medium has the highest amount of carbon and nitrogen sources out of all the media selected, which enhanced the cell growth and melanin biosynthesis. Results of effect of different media are shown in supplementary Figure 1(e).

Effect of inoculum age

Age of microorganisms, also called as age of inoculum, plays crucial role in health of any bioprocess. If microorganism age is too young or too old then the biosynthesis of any compound will not occur at optimum level. Usually, optimum inoculum age is different for different bioproduct's synthesis and therefore needs to be optimized separately for each bioproduct.[19] Therefore, biosynthesis of melanin nanoparticles was investigated by using the inoculum grown for 6, 12, 18, and 24 h. Results are shown in supplementary Figure 1(c). After investigating different inoculum age for their melanin production, the 12-h old inoculum gave the maximum melanin yield (262 mg/L) at the 48th h.

Effect of inoculum size

Microbial concentration or inoculum size plays a major role in bioprocess productivity. A low inoculum size may increase lag phase or nonproductive phase of a bioprocess. The large inoculum size may reduce the productive phase of a bioprocess due to relatively fast depletion of nutrients.[19] In most of the microbial production systems, inoculum size plays a crucial role in regulating bioluminescence, antibiotic biosynthesis, catalase activity, virulence determination, and initiation of chromosomal replication.[20] Optimum inoculum size depends on microorganism and bioproduct being formed and therefore, its value differs for different bioprocesses. We obtained the maximum melanin yield of 282 mg/L (36 h) when inoculum size of 10% was added to 50 ml of TSB. Higher inoculum sizes did not give significant increase in melanin yield (supplementary Figure 1(d)). Therefore, inoculum size of 10% was chosen to be the optimum inoculum size to be used for further studies.

Effect of flask volume on production of melanin

Aerobic microorganisms require oxygen for growth and product formation. Oxygen is the least soluble medium component out of all medium components. Therefore, most of the aerobic processes are controlled by the oxygen transfer rate from air to the liquid medium. In shake flasks, oxygen transfer can be increased by lowering the culture volume or filling volume.[21] As oxygen transfer rates are relatively higher, the lower filling volumes produced more biomass and melanin that too in a short time, which was due to higher aeration and the availability of more surface area, which increased overall oxygen transfer rate. Oxygen is required to oxidize tyrosine which is the melanin biosynthesis initiation reaction. The melanin production was higher when the medium filling volume of 10 ml was used and it decreased with increasing filling volume (supplementary Figure 1(b)).

Effect of shaking frequency

As explained above, oxygen transfer rate controls the overall growth rate of any microorganism. Shaking frequency also influences the oxygen transfer rate from air to the liquid medium.[21] As shown in supplementary Figure 1 (a), the highest production of melanin was obtained at 200 rpm (248 mg/L at 48th h). From this experiment, it has been found that the melanin production and biomass production tends to decrease in higher and lower shaking frequencies (250 and 100 rpm). In lower shaking frequencies, there was a decrease in biomass production which may be due to insufficient aeration. Whereas at higher shaking frequency (250 rpm), excessive foam was generated which reduced oxygen transfer rate from air to liquid and hence affected the growth and melanin nanoparticles biosynthesis.

Effect of carbon sources

Microorganisms react differently to different carbon sources. Large carbohydrate molecules are broken down to the simplest six-carbon sugar, glucose, which is used by microorganisms in different metabolic pathways for growth.[19] Although glucose is preferred carbon source, it may inhibit the growth through catabolite repression and therefore, it is necessary to identify a suitable carbon source for the growth of a given microorganism. Figure 1 shows the effect of different carbon sources such as glucose, dextrose, starch, glycerol, and sucrose at a concentration of 5 g/L concentration. From the results, glucose proved to be the best carbon source for melanin nanoparticles biosynthesis, yielding 386.66 mg/L melanin, while starch was found to be poor carbon source. When glucose and dextrose gave higher biomass yield, starch and glycerol resulted in comparatively lower biomass production. Starch and glycerol increased medium viscosity from about 2 to about 50 mPa·s, which reduced oxygen transfer rate and hence low growth was observed. Moreover, microorganisms prefer glucose and the carbon source to get energy for growth and product formation. Large sugars are broken down to glucose by microorganisms. Since, *Pseudomonas* species is not inhibited by glucose, it was the most preferred carbon source for melanin production. Rani *et al.* also reported higher melanin production in presence of glucose.[22]

Effect of nitrogen sources

Figure 2 shows the effect of different sources of nitrogen such as corn steep liquor, soybean meal, coconut meal, cotton seed meal, and oat meal was studied in this experiment. Maximum yield of melanin (300 mg/L) was produced by the medium supplemented with coconut meal in the 44th h, though, melanin biosynthesis did not vary much across all nitrogen sources. Melanin contains only about 6–7% of its weight as nitrogen and therefore we did not observe marked differences in melanin yield with respect to different nitrogen sources. Nearby regions have coconut processing industries nearby which produce coconut meal as by-product, therefore, we used coconut meal as the nitrogen source. These are low cost by-products of food processing industries, which are not used for human consumption. Thus, using them for melanin biosynthesis can impart value addition to the by-products.

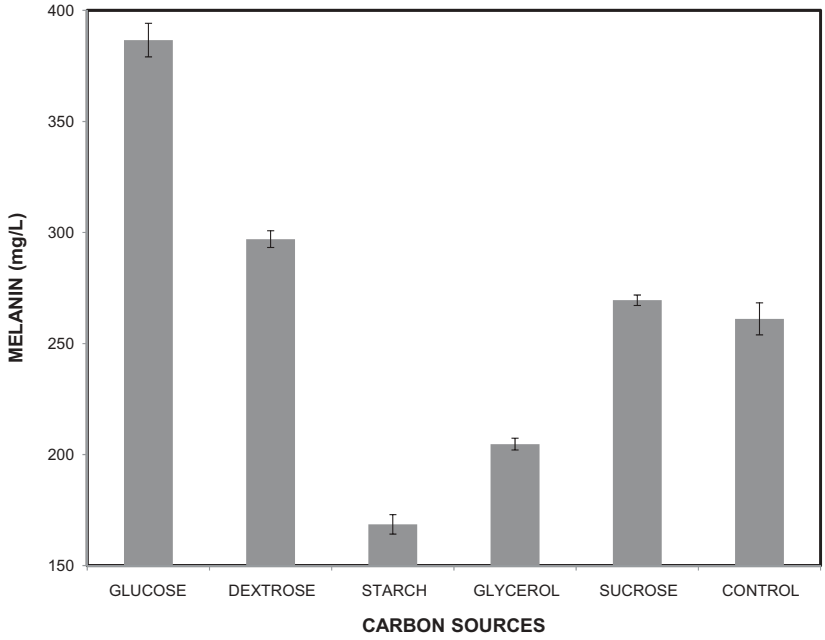

Figure 1. Effect of different carbon sources on the production of melanin. Kindly refer to materials and methods section for operating conditions.

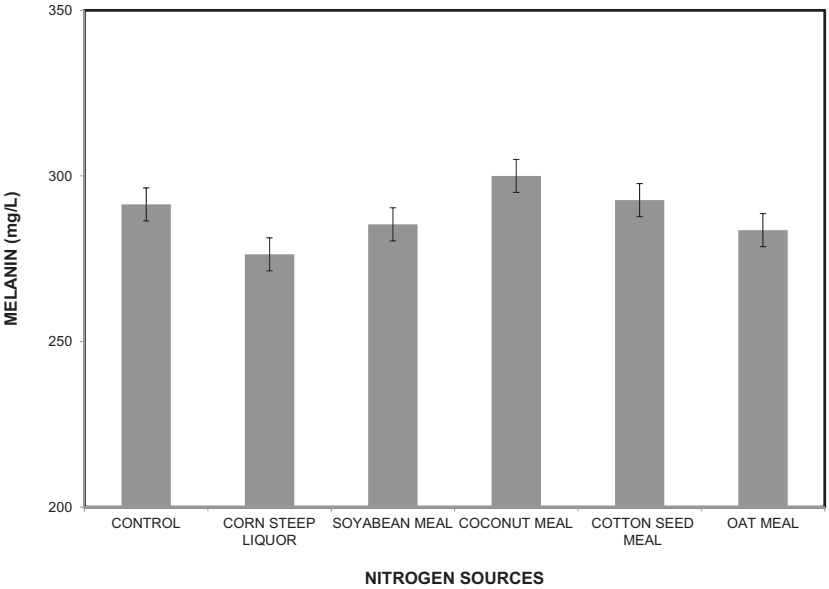

Figure 2. Effect of different nitrogen sources on the production of melanin. Kindly refer to materials and methods section for operating conditions.

Reports are available in which wheat flour and yeast flour were used for melanin production using *B. thuringiensis* subsp. *Galleriae* strain K1,[23] while maize flour and yeast extract for *B. thuringiensis* L-7601,[24] whereas bactotyptone and casein were used for *Bacillus cereus* 58.[25] Rani *et al.* reported higher melanin production when peptone was used as nitrogen source.[22]

Under optimized nutritional conditions and process parameters, the melanin yield was increased from 163 to 513 mg/L. Supplementary Figure 2 shows the photograph of how melanin was biosynthesized over the time in the bioprocess.

Characterization studies

UV-visible spectrophotometric analysis

The spectral property of the melanin was studied to confirm its nature. The UV-visible absorption spectrum

of the biosynthesized melanin and synthetic melanin was found to be similar with their absorption maxima between 200 and 300 nm (Fig. 3).

Particle size analysis and zeta potential

Figures 4 and 5 show particle size distribution and zeta potential of melanin. The mean particle size of melanin particles produced by *P. stutzeri* was 32 ± 0.98 nm and the zeta potential of melanin particles was about −54.9 mV. Zeta potential changed after metal adsorption on the melanin. The zeta potential of chromium- and selenium-bound melanin decreased further to −58.5 and −59.3 mV, respectively. This effect was due to anionic nature of chromium and selenium ions. On the other hand, lead ions are cationic in solution and therefore, the zeta potential of lead-bound melanin increased to −46.2 mV. However, we did not find drastic change in particle size of heavy metal bound-melanin. A plausible reason could be the zeta potential of heavy metal-bound melanin, which was still in the range of −40 to −60 mV due to which nanoparticles, did not agglomerate. This proved that the melanin nanoparticles produced by the *P. stutzeri* is an efficient heavy metal adsorbent due to its size and the high negative charge it possess on its surface.

Scanning electron microscopy and transmission electron microscopy analysis

SEM and TEM images imaging revealed nano-size of melanin pigment. The results revealed that shape of melanin nanoparticles does not change after adsorption of heavy metals (Figs 6 and 7).

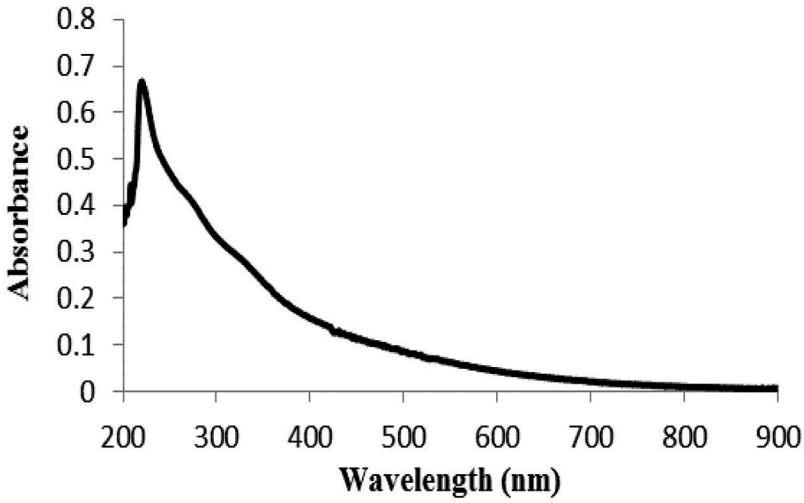

Figure 3. The UV-visible absorption spectrum of melanin produced by the bacteria *Pseudomonas stutzeri* HMGM 7.

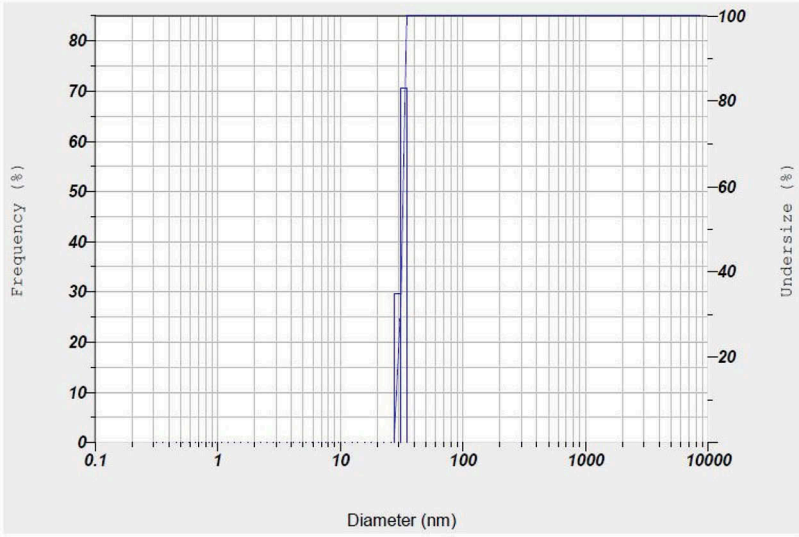

Figure 4. The particles size distribution profile where frequency is plotted against the diameter of the particles.

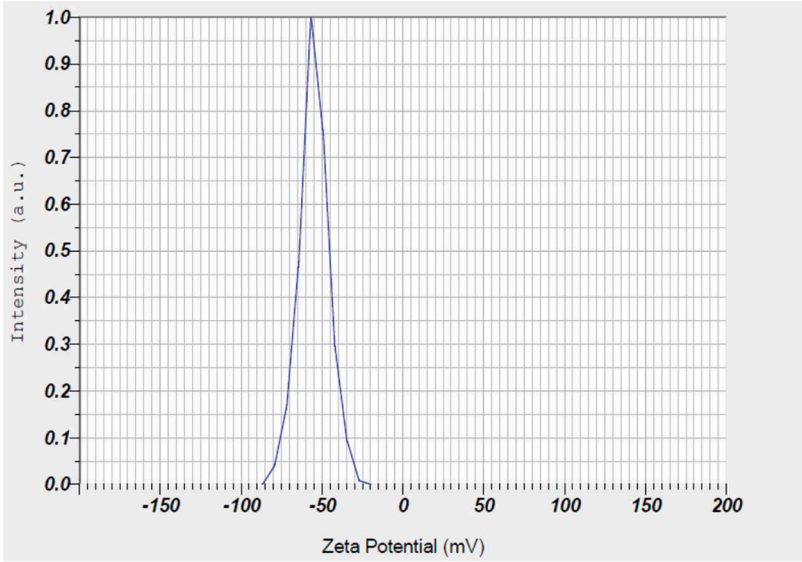

Figure 5. Intensity versus zeta potential distribution of the melanin nanoparticles.

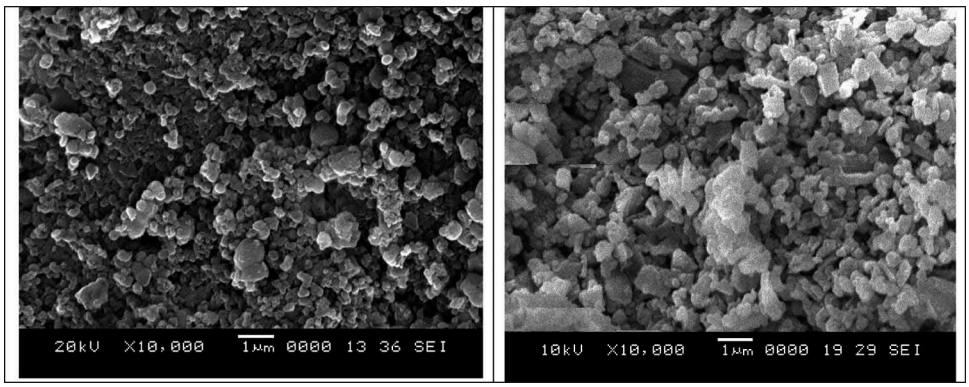

Figure 6. Scanning electron image of bacterial melanin. The left and right side images are biosynthesized melanin and chromium-bound melanin, respectively.

Adsorption studies

Different heavy metals adsorb at different quantities on to melanin. Different parameters like temperature, pH, time, mass loading, rpm, etc. affect the adsorption rate and efficiency. We optimized shaking frequency and pH for adsorption of heavy metals onto the melanin. Supplementary Figure 3 demonstrates the results of optimization. As Fig. 3(a) indicates that heavy metal adsorption increased as shaking frequency increased. This is due to better mixing and hence better contact between melanin and heavy metals. Figure 3(b) shows effect of pH on heavy metal adsorption. Above pH 4, the melanin has negatively charged functional groups such as COO^{-}, which attract positively charged cations such as lead and selenium. Below pH 4, melanin is positively charged due to presence of excess hydronium ions which attracts negatively charged chromate and dichromate ions. Therefore, under optimum conditions, lead showed 97% adsorption efficiency at pH 5, while chromium showed 88.8% at pH 3 and selenium showed 82% adsorption efficiency at pH 5 (Fig. 8). Lead precipitates between pH 5.5 and 8[26] and therefore, we were not able to detect lead in this pH range. However, most of the lead was adsorbed onto melanin at pH 5 itself. Our results demonstrate that melanin proved to be an efficient adsorbent for the removal of heavy metals from synthetic drinking water especially, at lower metal ion concentrations.

Fourier-transform infrared spectroscopy

The FTIR analysis shows bond vibrations in the peaks corresponding to the functional groups –COOH, –CO, –NH, and –OH present in melanin. The bond vibrations represent a change in functional groups which is caused by the bonding of the heavy metals to it (Fig. 9). All the three heavy metal bindings onto melanin

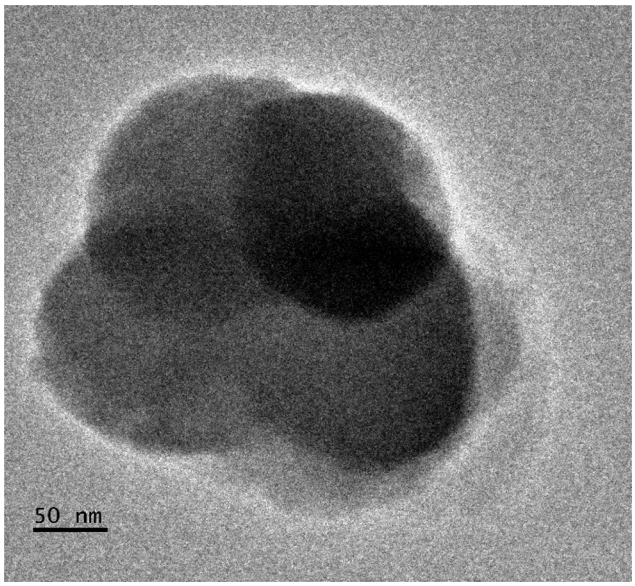

Figure 7. TEM image of an aggregate of melanin particles.

changed FTIR spectrum (solid line). The comparative spectrum of chromium, lead, and selenium is shown in figure. The spectrum of melanin (dotted line) indicates different characteristic stretching and vibration peaks corresponding to functional groups present. In melanin, C-N was identified at wave number 1244.69 cm^{-1}, N-H of the amino group at 1612.73 cm^{-1}, C=O stretching at 1704.18 cm^{-1}, –OH group at 3222.62 and 3342.22 cm^{-1}. Bond shifting vibrations were observed for the chromium-, lead-, and selenium-bound melanin. The heavy metals chemically bonded to the functional groups and there by bond vibrations and stretching changed. The peaks showed bond shifting and difference in intensity confirming that the heavy metals have adsorbed to the functional groups in melanin. For selenium adsorption, wave number corresponding to 639.21 and 676.15 cm^{-1} shows an increase in intensity, which represents –CH bond vibrations. It also shows a shift in that wave number after selenium adsorption indicating a strong chemical bonding between –CH group and selenium. Increase in peak intensity at 837.64 cm^{-1} indicates –NH wag due to the binding of selenium to the –NH group. 1062.31 cm^{-1} also indicates the binding of selenium to –NH group of melanin. The peak corresponding to 1103.10 cm^{-1} shows an intensity increase as well as shift representing a strong chemical bonding of selenium to carboxylic and carbonyl groups in melanin. The peak intensity at 1597.35 cm^{-1} also suggests the adsorption to –NH group.

Conclusion

The microorganism *P. stutzeri*, which was used in this study, has a competency to produce melanin under various process conditions and in different growth medium. From the experimental results, the operating conditions for melanin production from *P. stutzeri* were optimized along with inoculum size and inoculum age.

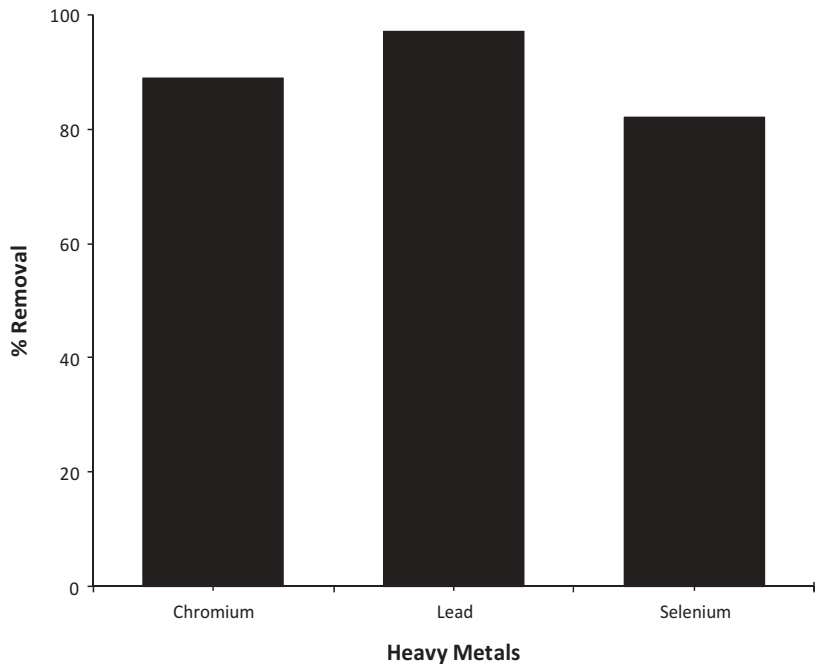

Figure 8. The adsorption efficiency of different heavy metals on melanin. Kindly refer to materials and methods for operating conditions.

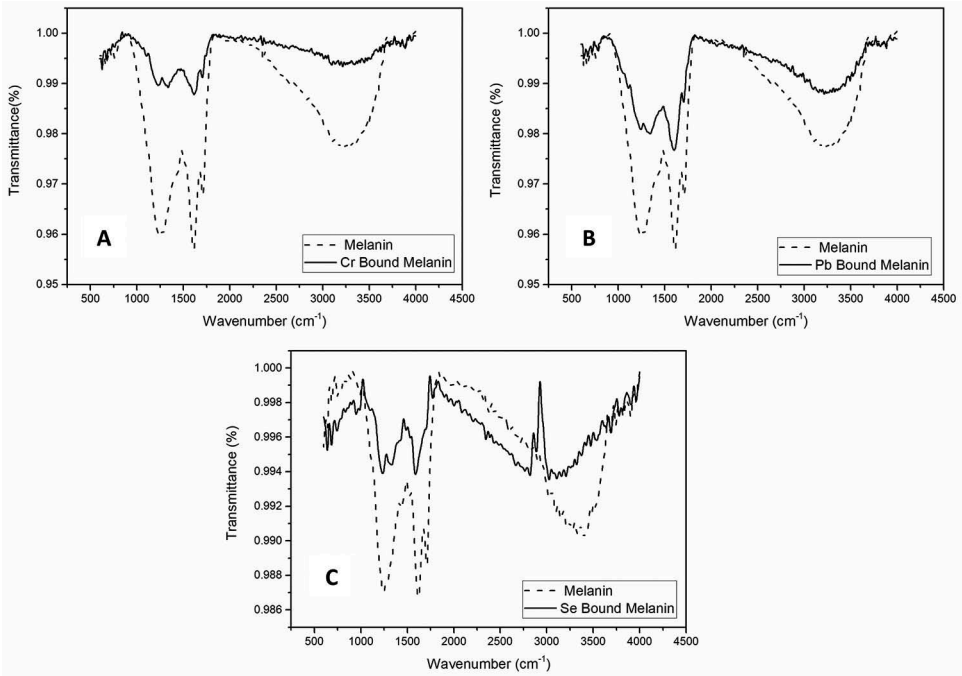

Figure 9. Comparison of FTIR spectra of (a) chromium-bound melanin, (b) lead-bound melanin, and (c) selenium-bound melanin with natural melanin (dotted line). Solid line indicates FTIR spectra of heavy metal-bound melanin.

Two percent inoculum of a 12-h old culture at 37°C temperature, pH 7 at 200 rpm produced maximum melanin. Different media such as NB, LB, TSB, and BHB were evaluated for their melanin production, among which TSB proved to be the best, producing 238 mg/L of melanin. With a view of further enhancing the melanin yield, TSB was modified with different carbon and nitrogen sources. Glucose and coconut meal were selected as good sources of carbon and nitrogen, respectively. After optimization, the melanin yield was increased from 163 to 513 mg/L. The melanin produced by the bacteria *P. stutzeri* is of nano-size of 32 ± 0.98 nm and have −54.9 mV zeta potential. The biosynthesized melanin is an excellent heavy metal scavenger with promising level of removal of lead, chromium, and selenium from drinking water.

Acknowledegments

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