Production of melanin by soil microbial isolate on fruit waste extract: two step optimization of key parameters

Korumilli Tarangini *, Susmita Mishra

Department of Chemical Engineering, National Institute of Technology, Rourkela 769 008, Orissa, India

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A B S T R A C T
In this study, optimization of production parameters influencing melanin production in an economical fruit waste extract was attempted using a garden soil isolate (Bacillus safensis). Taguchi approach was adopted for screening of critical parameters and further optimization was done using a central composite design of response surface methodology (RSM). At optimum conditions (pH 6.84 and Temp 30.7°C), a significant yield of ~6.96 mg/mL was observed. Statistical analysis revealed that the experimental results fitted well to the statistical model with model R² value 0.982. The optimization of process parameters using RSM reported a 15% increase in the pigment yield than average yield obtained from the studied model. The melanin produced was confirmed by UV–visible spectroscopy, FTIR and XRD analysis. Moreover melanin obtained has significant photoprotective, radical scavenging and metal chelating activity. Thus, B. safensis has the potential to be a new source for the production of melanin, which is of industrial interest.

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1. Introduction
Melanins are the natural pigments which have their presence in animals, plants and in most of the microorganisms [1]. They are the dark coloured negatively charged high molecular weight pigments which are formed due to polymerized phenolic and/or indolic compounds. These complex polymers are amorphous in nature and shows solubility in neither aqueous nor organic solvents. They showed resistance to concentrated acids and are susceptible to bleaching by oxidizing agents [2]. They play a vital role in defense and protection mechanisms that improve the survival and competitiveness of the organisms [3]. Melanin is known for its absorption capacity of radiation of all wavelengths with an optimum absorbance at UV range [4] which prevents photo induced damage. Hence it is used in the preparation of photo absorbing optical lenses and in bioplastics. Besides photo protection it has versatile biological activities such as radical scavenging, antioxidant, antitumor, anti-inflammatory [5] and as immune stimulating agent [6].

Melanin obtained from microbes has great advantages over melanin from animals and plants. Microorganisms don’t cause the problems of seasonal variations and are selected arsenals as they modify them according to the medium and conditions provided to them [7]. Targeting melanogenisis in microbes may help to discover antimicrobial drugs. For example, melanins produced by Cryptococcus neoformans and Burkholderia cepacia offer virulence and contribute to the growing resistance of these pathogenic bacteria towards antibiotics [2,8]. The melanin synthesized by microbes shows metal chelating ability too (sorb the radioactive wastes uranium) [9]. There are reports that showed the anti HIV properties of melanin and their role in photo voltage generation and fluorescence studies [10–11]. Therefore, all these properties of melanin make them unique and are widely used in cosmetic, sunscreen protection creams, eye glasses, pharmaceuticals, and food industries.

Considering the potential uses and increasing demand for the melanin pigment there is a need to conduct studies on the production of melanin from microbes. There are reports on melanin production from various microorganisms, including Bacillus species which are well known for their pigment production ability in various stress environments [4,12]. Selection of substrate for melanin production has economic importance. For instance till date expensive substrates like NCM media [4], LB (Luria–Bertani) media [12], minimal media supplemented with L-tyrosine [13], amino acids enriched tryptoph broth agar [14] and so on [15–16] were used for high yield of melanin. Owing to the economy and practicability of the melanin production process; the need to use economically feasible substrates along with optimization of the key parameters is needed. In recent years, considerable interest
has been developed in using agro-industrial wastes as substrates for valuable products like pigments. The abundantly available fruit waste in India used widely as animal feed or disposed to the soil. The effective utilization of this waste which is rich in carbohydrates and other nutrients can address our primary objective of melanin production in a cheaper way. An optimization strategy like Taguchi method [17] is a systematic technique of design and analysis of experiments that has been employed successfully in recent years to design, improve the product quality economically [18], and a central composite design (CCD) approach has been used to fit a polynomial model. The complementary use of both the methodologies provides a great amount of information based on only a small number of experiments and to scheme a process.

In this study, a bacterium capable of producing melanin was isolated from garden soil and subsequently characterized. The strain was cultivated on the fruit waste extract (FWE) as the sole source of energy to produce significant amounts of melanin. The key parameters in melanin production were identified and optimized using simple two steps Taguchi and CCD (central composite design) approach. Upon purification and characterization, the obtained melanin was tested for in vitro sun protection effect, free radical scavenging and metal chelating activities.

2. Materials and methods

2.1. Chemicals and microorganism

DPPH (2,2-diphenyl-1-picrylhydrazyl), purchased from HiMedia chemicals, Mumbai, India. Ascorbic acid was purchased from Merck, India. Ferrozine and melanin (synthetic) were purchased from Sigma–Aldrich, India. Ethanol, NaCl, NaOH, HCl are from Merck, India and all other chemicals used were of analytical reagent grade throughout the study. Ultrapure water was used for the experiments and aseptic conditions were maintained wherever necessary.

The microorganism used in this study was isolated from garden soil in front of Department of Chemical Engineering, National Institute of Technology, Rourkela, India using serial dilution technique on a nutrient agar (NA) [19]. Using 10⁷ dilution of soil sample on NA plates, the melanin producing organism was identified. It was separated by observing a diffusible black pigment on NA plates after 24 h. The isolated culture was preserved on NA slants at 4°C and sub-cultured at monthly intervals.
2.2. Substrate preparation

Fruit waste was obtained from a fruit juice shop of a local market. The material used is from single batch i.e., used in all the experiments to minimize the disturbances in the results due to variations in composition. The waste contains major portions of pineapple and orange waste and minor portions of pomegranate waste. Fruit waste includes extracted carpels of oranges, core of pineapple and orange waste and minor portions of pomegranate. The soluble sugars were extracted from 1 kg of fruit waste by adding 2 l of distilled water and boiled at 100 °C for 30 min. The resultant straw colour FWE was filtered and stored at 4 °C for further experimentation.

2.3. Production and purification of melanin

Nutrient broth (peptone-5 g/L, beef extract-3 g/L, NaCl-5 g/L) was used for inoculum preparation and FWE was used as a production medium for melanin. About 10 μL (10⁶ CFU/mL) culture suspension was added to the FWE medium in 250 mL flasks with a working volume 50 mL. The medium was then incubated at 30 °C on a rotary shaker moving at 200 rpm for 24 h. A dark pigmented and nearly opaque FWE medium was observed (Fig. 1a). After the incubation time, the medium was centrifuged using REMI-RM12C, India centrifuge at 9200 g for 15 min to separate the broth (supernatant) and the cells. The solid pellet of cells was separated and suspended in distilled water. The cells were further centrifuged to collect the supernatant. Melanin was extracted from the overall supernatant by addition 2 l of distilled water and boiled at 100 °C for 5 min to prevent the formation of melanoidins. As a final point, the crude pigment pellet was collected after centrifugation at 4600 g for 15 min.

2.4. Parameters optimization using Taguchi and CCD design

Preliminary idea on growth conditions suggest Taguchi method to be employed for the optimization of culture conditions for high yield melanin pigment production. Optimization of three vital factors like pH, temperature and agitation in 6-3-3 levels respectively was done as a starting point of the study (Table 1). Then Taguchi method was performed by 18 different experiments by using L18 orthogonal array as shown in Table 2. Shown values of melanin (mg/mL) are the average of the results of two replicates. Based on the obtained results, the optimum conditions of the used parameters were identified and an analysis of variance (ANOVA) for the obtained results was investigated.

Once the critical factors were identified, in addition to the above, a CCD for independent variables was used for further optimization. Two variables at two levels were used to fit a polynomial model. A two level full factorial is performed with a model equation designed such that the variance of Y is constant for all points equidistant from the centre of the design. Minitab (14.0) statistical software package was used in the experimental design and data analysis. Response surface graphs were obtained to know the effect of the variables, individually and in combination, and to determine their optimum levels for maximum melanin production. All trials were performed in duplicate, and the average melanin yield was used as response Y.

Table 1

| Factor           | Levels | 1  | 2  | 3  | 4  | 5  | 6  |
|------------------|--------|----|----|----|----|----|----|
| pH               |        | 4.3| 5  | 6  | 7  | 8  | 9  |
| Temperature (°C) |        | 15 | 30 | 45 |    |    |    |
| Agitation (rpm)  |        | 90 | 140| 180|    |    |    |

Table 2

| pH                  | Temperature | Agitation | Melanin (mg/mL) |
|---------------------|-------------|-----------|-----------------|
| 4.3                 | 15          | 90        | 0.001           |
| 4.3                 | 30          | 140       | 0.01            |
| 4.3                 | 45          | 180       | 0.011           |
| 5                   | 15          | 90        | 0.08            |
| 5                   | 30          | 140       | 0.124           |
| 5                   | 45          | 180       | 0.11            |
| 6                   | 15          | 140       | 0.309           |
| 6                   | 30          | 180       | 0.331           |
| 6                   | 45          | 90        | 0.328           |
| 7                   | 15          | 180       | 0.522           |
| 7                   | 30          | 90        | 0.655           |
| 7                   | 45          | 140       | 0.577           |
| 8                   | 15          | 140       | 0.356           |
| 8                   | 30          | 180       | 0.446           |
| 8                   | 45          | 90        | 0.428           |
| 9                   | 15          | 180       | 0.219           |
| 9                   | 30          | 90        | 0.231           |
| 9                   | 45          | 140       | 0.218           |

3. SPF/DPPH/metal chelating assay

3.1. In vitro sun protection factor

As per the adapted method, spectra of melanin samples were collected over the spectral range 400–280 nm with 1 nm data point resolution on a UV-visible UV-3200 double beam spectrophotometer (LABINDIA analytical Instruments Pvt Ltd India.). The SPF values of melanin from the purchased strain and microbial isolate were determined using Mansur mathematical Eq. (1).

\[
SPF = CF \times \frac{\sum_{i=1}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)}{290}
\]

where, SPF=Sunscreen protection factor; EE(λ)=erythremal effect spectrum, I(λ)=solar intensity spectrum; Abs(λ)=absorbance of sunscreen product; CF=correction factor (36.2 for SNOW LOTUS SPF 15 and 30) taken from Huang et al. [20]

3.2. DPPH assay

The radical scavenging activity by melanin pigment was investigated by the modified method of Ju et al. [21]. Primarily, 1 mg/mL of microbial melanin/standard (Ascorbic acid) in different dilutions was added to 2 mL of DPPH in ethanol; so that the total strength of the melanin used was varied from 15–100 μg/mL in each solution. After keeping for 30 min at 37 °C the absorbance at 516 nm was measured using UV-spectrophotometer with reference blank samples. The experiment was performed in duplicate. The absorbance of DPPH as control was measured at 516 nm. The lower absorbance of the reaction mixture indicated higher radical scavenging activity. The scavenging effect was measured using Eq. (2).

\[
\text{DPPHInhibition} (%) = \left( \frac{\text{Controlabsorbance} - \text{testabsorbance}}{\text{Controlabsorbance}} \right) \times 100
\]

3.3. Determination of metal chelating activity

The chelation of ferrous ions by the melanin pigment was estimated by the method of Huang et al. [20]. Different concentrations of melanin were mixed with a solution of 2 mM
4. Characterization/analytical methods

XRD analysis was performed using the CuKα radiation (λ = 1.5406 Å) X-ray diffractometer [Philips PW1830HT], in the range of 20–90° (2θ) at 0.05°/s with an accelerating voltage 35 kV and applied current (30 mA). The absorption spectra of the purified melanin solutions at room temperature were obtained by UV-visible spectrophotometer. Structural functional groups were identified by FTIR, [Bruker, Germany] equipped with attenuated total reflectance (ATR) mode with zinc selenide (ZnSe) crystal.

5. Results and discussion

5.1. Strain selection and Pigment production

The melanin producing soil microbial isolate from NA plates was carefully separated and cultivated on fresh agar plates (Fig. 1b) for 24 h. These colonies were examined microscopically for their morphological characteristics as shown in Fig. 1c. The isolated strain upon 16S rDNA sequencing identified a novel bacterial species B. safensis strain ZJHD1–43 (GenBank Accession Number: KF585035.1). The phylogenetic tree was constructed showing the position of isolate with reference to related strains in Fig. 1d. The evolutionary history was inferred using the Neighbor-Joining method. All ambiguous positions were removed for each sequence pair and the Gene accession numbers are also shown in Fig. 1d. Some taxonomic, morphological and biochemical characteristics of the microbial isolate was given in Table 3.

At usual conditions, FWE appeared to be most suitable medium for melanin production. An intense coloration of the medium from straw colour to brownish black was observed within 24 h at a pH of 7 and with agitation of 100 rpm.

5.2. Taguchi design for screening critical factors

Effect of pH, temperature and agitation were studied employing a Taguchi method, which is a fractional factorial experimental design tool. Experiments performed at experimental conditions (pH 7; temperature 30°C; agitation 90 rpm) produced maximum melanin of 0.655 mg/mL on an average as shown in Table 2. Each of these factors such as pH, temperature and agitation influenced significantly on melanin production represented as “main effect” and illustrated in Fig. 2. Using the ANOVA software tool, significance of two important factors pH and temperature was reflected as per Table 4. The F value represents the relative contribution of estimated variance to residual variance. Large F value is desirable and indicates the significance of that parameter in the optimization method. In addition, further confirmation of the significant effect is understood from P value. Using P-value prob >F test that indicates the probability of F value that will be observed when P<0.05. Thus we found that pH and temperature have significant influence in the optimization of process conditions towards melanin production, whereas agitation has negligible effect.

Furthermore, Table 5 shows the suggested condition as predicted from the optimization tool. Statistical calculations predicted that at these conditions (Table 5) the melanin yield should reach 0.620 mg/mL. However, this value is slightly less than and almost equals the value by trial no: 7 (from the array of experiments given in Table 2). Hence, further investigation of the suggested conditions (Table 5) was discontinued.

6. Optimization by response surface methodology (CCD design)

Optimization of process parameters was carried out using the CCD design with the parameters found to be significant from the Taguchi approach, including pH (X1) and temperature (X2). Table 6 represents the design matrix and the results of the 13 experiments carried out using the CCD design. The data obtained provided the regression model using ANOVA software.

\[
Y = 6.7014 - 0.3367(x_1) + 0.2083(x_2) - 0.6048(x_1 \times x_2) - 0.1175(x_1 \times x_2)
\]

where X1 and X2 represents pH and temperature respectively. The estimated regression coefficients from response surface analysis of the quadratic regression model (Table 7) demonstrate that Eq. (4) is a highly significant model with goodness of fit R² = 0.982 and adjusted R² = 0.969. These values indicate that the model equation...
was adequate for predicting the melanin production under any combination of values of the variables.

### 6.1. Interaction effects of variables

The graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable and the type of interactions between the test variables in order to identify the optimum conditions. The interaction effects and optimal levels of the variables were determined by plotting the three dimensional (3D) response surface curves. The response surface curve in Fig. 3a,b represents the interaction between pH and temperature, which showed that the maximum melanin yield was obtained toward neutral pH, while melanin yield was significantly affected with an alkaline pH.

### 6.2. Validation of the model

Validation was carried out under conditions predicted by the model. The optimum conditions predicted by the model are pH 6.84, Temp \(25^\circ\text{C}\) with yield of \(6.3\ mg/mL\) and the actual yield obtained was \(6.96 \pm 0.6\ mg/mL\). The close correlation between the experimental and predicted values signifies the reliability of the response methodology (CCD design) over traditional optimization approach. The increased yield at the optimum conditions were comparable\(^{10}\) to and better than some microbial sources\(^{13,22}\) reported in the literature. As the reported studies utilized relatively expensive media, our results shows the suitability of significant melanin production on a cheaper substrate FWE and has huge scope for larger scale production.

### 7. Spectroscopy, IR and XRD analysis of melanin

#### 7.1. UV–vis analysis

The absorption spectrum of natural melanin is shown in Fig. 4a. The UV–visible wavelength scan showed that absorption was highest in the UV region (200–300 nm), but diminished towards lower wavelengths.
The inter layer spacing obtained. The signals from 3600 cm$^{-1}$ and 24.32\degree for bacterial and purchased melanins respectively. The value of $d$ is in good agreement with reported value of the inter layer spacing in the stacked sheets model of the melanin 1. An estimate of average grain size of melanins can be calculated from the Dedye - scherrer Eq. (1).

$$D = 0.9 \frac{\lambda}{FWHM \cos \theta}$$

where $FWHM$ is full width at half maximum of diffraction peak. The obtained $D$ values are 0.668 and 0.568 nm for the bacterial and purchased melanins. The closeness of the grain size values indicates the quality of the purified bacterial melanin. Furthermore % crystallinity was also calculated for the stated melanins by considering glass substrate as background. The calculation is as follows:

$$\%\text{crystallinity} = \left(\frac{\text{total area} - \text{background profile area}}{\text{total area}}\right) \times 100$$

Although both melanin samples exhibited the lack of structure in the diffraction pattern corresponding to any significant crystallinity, the % crystallinity values (Fig. 4c, picture indicated by arrow) further indicate bacterial melanin from FWE was far less crystalline when compared to the purchased melanin. Lack of crystallinity is a significant sign of consistent physical property of melanin [25].

### 7.4. Sunscreen protection factor (SPF) values determination

The determination of SPF values for samples (bacterial and purchased melanin) was made through the UV spectrophotometer using the Mansur equation [20]. The SPF value for melanin from FWE was 53.36 $\pm$ 0.009, while it was 59.34 $\pm$ 0.006 for purchased melanin. As melanins are known for their photoprotective role [26], the obtained SPF values state that melanin from FWE might have profound protection effect against dermal damage related to photoaging as that of purchased melanin.

### 7.5. DPPH assay

DPPH accepts an electron to become a stable diamagnetic molecule. The ethanolic solution of DPPH (violet colour) has got a strong absorbance at 516 nm which is in the visible region of the electromagnetic spectrum. The presence of a reducing agent in this ethanolic solution pairs the odd electrons of DPPH radical and further the solution losses colour stoichometrically and also the absorbance of the solution decreases at 516 nm. Reduction of absorbance at 516 nm and colour of DPPH associated with different melanin doses was verified. The % increase in radical scavenging activity from Fig. 5a indicates the diminished behaviour of the radical. The data obtained from Fig. 5a states that scavenging activity of the melanin was higher than the control ascorbic acid at each and every dose studied. This behaviour shows 30% enhanced reductive capability of the obtained bacterial melanin than ascorbic acid for a constant dose of melanin dose of $\sim$ 100 $\mu$g/mL.

### 8. The metal binding capacity of melanin

The metal binding capacities of melanin from FWE was determined by assessing its ability to compete with ferrozine for the ferrous ions. The concentration dependent metal chelating
The reduction in spectrum with an increase in melanin dose indicates that melanin compound was interfering with the formation of ferrous and ferrozine complex. This suggests the chelating effect of melanin and its ability to capture ferrous ions before ferrozine. Maximum effect (~64% chelation) was observed for a dose of 0.2 mg/mL (Fig. 5c). The results suggest that the action of melamins as oxidation protection factors may be predominantly due to their iron binding capacity.

9. Conclusions

From the results of this study, it is concluded that the use of two step statistical approach not only helped in locating the optimum levels of the most significant factors considered with minimum resources and time but also proved to be a useful and satisfactory method in melanin production-optimizing exercise. Thus, the optimization of vital nutritional parameters using response surface methodology significantly enhanced the yield of melanin on fruit.
waste extract has proved its feasibility for large-scale production by a garden soil isolate (Bacillus safensis). The melanin obtained in this study has photoprotective, radical scavenging and metal binding capacity which is of economic importance. So the B. safensis and fruit waste extract can be potential sources for melanin production.

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