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One-pot green synthesis of eumelanin: Process optimization and its characterization.

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Abstract

Advances in biocatalysis are determinant for lowering the environmental footprint of chemical processes. In this context, the present study reports for the first time the use of Taguchi design of experiment (DOE) methodology for optimizing the one-pot synthesis of eumelanin, using tyrosinase enzyme from *Amorphophallus campanulatus* and L-Dopa as substrate. Using the Taguchi approach, an L₂₇ orthogonal array (OA) was applied and five critical parameters / factors *i.e.* pH, temperature, oxygen level, reaction time and enzyme units were selected for the optimization. In order to maximize the response, signal to noise (S/N) ratio related to larger-the-better was used. The main effect and interaction plot of various factors were analyzed. Response table and ANOVA revealed that pH was the most significant factor for melanin synthesis ($P < 0.05$). The yield of melanin and the rate of production under the best conditions were 1176 ± 3.2 mg L⁻¹ and 24.5 mg L⁻¹ h⁻¹ respectively. Further, the biosynthesized melanin was characterized using various analytical techniques. The empirical formula of synthesized melanin was found to be C₈H₈O₄N. Based on the elemental analysis and A₆₅₀/A₅₀₀ ratio, the biosynthesized melanin was classified as indole-type eumelanin. Thus, the present study describes the means to increase the yield of melanin, considering the wide applications it has in various fields.

Keywords: *A. campanulatus*, Green technology, Optimization, Taguchi, Melanin.

1. Introduction

Melanin is a negatively charged, hydrophobic, macromolecular dark brown or black pigment. Broadly it is categorized into two major classes: eumelanin and pheomelanin. Both are found in the skin, hair and eyes of many animal species, including humans, where they act as photo-protectants. Eumelanin is known to be a macromolecule composed of indole units and is black to brown in color. Pheomelanin, on the other hand, is a sulfur containing macromolecule composed of benzothiazine units and is red to yellow in color.¹ Among the various biopolymers, melanin has received wide attention in recent years. The reason for this is rooted in the fact that melanin displays diverse functions in the biosystem that include metal biosorption,² radioprotection,³ and free radical quenching.⁴ Melanin covered nanoparticles have shown a protective action during radiation therapy for cancer.⁵ Melanin also confers resistance to UV light by absorbing a broad range of electromagnetic waves due to which it is widely used in cosmetics, photo-protective creams and eyeglasses.⁶ The immuno-pharmacological properties of melanin are of great interest as it holds promise for AIDS treatment.⁷ Melanin sensitized piezoelectric sensor has also been reported for the picogram detection of metal ions.⁸ Thus, considering the beneficial properties of melanin there is an increased demand for the same.

Generally plants are considered to be a good source of pigments such as melanin. The biological properties of melanin obtained from different plant sources have been extensively studied.^{9,10} However, purification of this pigment involves harsh procedures that modify the native structure of melanin. Hence, there is a need for its in-vitro synthesis. Melanin can be synthesized by different methods.^{11,12} However, these methods involve the use of chemicals that create a load on the environment. In contrast to this, in-vitro biological approaches for melanin synthesis are eco-friendly and consequently more favorable. These mainly include the use of

microorganisms,¹³⁻¹⁵ callus culture¹⁶ and enzymes.² Enzymes are generally more specific and overcome the cumbersome procedure of inoculating and maintaining the microorganisms on specific media and also cause less pollution when compared to chemical catalysts. Enzymatic synthesis of melanin can be performed using the enzyme tyrosinase (EC 1.14.18.1) which utilizes molecular oxygen to carry out two different reactions. (i) ortho hydroxylation of monophenols to o-diphenols (monophenolase activity) (ii) oxidation of o-diphenols to o-quinones (diphenolase activity). These reactive quinones further polymerize to yield macromolecular melanin which is a high value compound.

Various process parameters such as pH, temperature, reaction time etc. influence the yield of melanin. Thus, optimizing the process parameters is an essential step to obtain maximum production. In the conventional approach for optimization, one parameter is altered at a time keeping the other parameters constant, to understand the impact of one particular parameter.^{17,18} Although several processes have been optimized using this method, this optimization procedure consumes a lot of time and is labour intensive. Also, it is costly and cannot provide the information on mutual interactions of the parameters on the desired outcome.

Optimization techniques such as Response Surface Method (RSM), Genetic Algorithm (GA), Artificial Neural Network (ANN), and Taguchi approach are being popularly followed for parameter optimization in DOE.¹⁹ Various groups have successfully used these approaches in biological sciences.^{20,21} These methods not only help to save considerable time and cost but also result in a process which is efficient and reproducible. The core purpose in using statistical experimental design is to provide maximum and reliable information. For this purpose, Taguchi approach is preferred as it allows simultaneous examination of many factors and extracts quantitative information by conducting only a few experiments. This method differs from the

other design of experiment techniques in: [i] Taguchi method strongly relies on researcher's experience and knowledge to choose the right parameters and their level, [ii] it also uses OA's to investigate the main and interaction effects of parameters through an optimum number of experiments and [iii] in Taguchi's design, researchers have to determine a target for their response variables such as "smaller is better", "larger is better" or "nominal is best". Therefore, depending on the target being smaller, larger or nominal, every response data is read as S/N ratio. Then the maximum values of S/N ratios are used for selecting optimum levels for each factor.²²

Thus, in the present study, optimization of parameters for melanin synthesis was done using Taguchi approach, involving a system of OA's that allow a maximum number of main effects to be estimated. For this purpose, tyrosinase enzyme from *A. campanulatus* was used and experiments were designed with five different factors (pH, temperature, oxygen level, reaction time and enzyme units) each at three different levels with an orthogonal layout of L₂₇. Minitab 16.0 software was used for analysis of the experimental results. The main effect and interactions between different factors was studied. Validation of the Taguchi model was proved by running the confirmatory experiment. Biosynthesized melanin was further characterized by physicochemical and analytical techniques such as ESEM-EDS, UV-visible spectroscopy, FT-IR, ¹H NMR and thermogravimetric analysis.

2. Experimental

2.1. Materials

3,4-dihydroxyphenylalanine (L-Dopa, ≥ 98%) was purchased from Sigma-Aldrich (USA, product number: D9628-25G), tyrosinase was extracted from the corm of *A. campanulatus* (a tropical tuber crop widely spread throughout the Asian region). Soluene-350 was procured from

PerkinElmer (USA, product number: 6003038). Ultra pure water with a specific resistance of 18.2 M Ω cm was used for all experimental purpose (Elix[®] Millipore). All the other reagents used were of analytical grade.

2.2. Extraction of tyrosinase enzyme

Tyrosinase enzyme was extracted from *A. campanulatus* as described by Saini *et al.*²³ Briefly, *A. campanulatus* (2 kg) was homogenized in phosphate buffer (pH 6.0, 0.1 M) and filtered using cheese cloth. The filtrate was then subjected to centrifugation at 2500 rpm for 7 min at 4 °C. The supernatant obtained was precipitated with ammonium sulphate (60%) under cold conditions. Fresh extraction buffer was used to re-dissolve the pellet obtained after precipitation and it was further subjected to dialysis against ultrapure water. This gave the partially purified tyrosinase enzyme that was stored at 4 °C. The enzyme activity was calculated by measuring the formation of dopachrome ($\lambda_{\max} = 475$ nm, $\varepsilon = 3700$ M⁻¹ cm⁻¹). One unit of enzyme activity was defined as an increase in absorbance by 0.001 per minute at 475 nm at pH 6.0 and temperature of 30 °C.

2.3. Taguchi DOE methodology

The Taguchi DOE methodology used in the present study was divided into four distinct phases, *i.e.* designing the experiment, conducting the experiment, data analysis and validation as shown in Fig. 1. Each phase has its own significance in the overall optimization process as discussed below.

2.3.1. Design of experiment

In order to reduce experimental errors and to evaluate the efficiency and reproducibility of experiments, Taguchi established the concept of OA's. This is a systematic and statistical way of testing pair wise interactions. The symbolic designation of these arrays indicates the main information on the size of the experimentation, *e.g.* L₉ has 9 trials. The total degree of freedom

available in an OA is equal to the number of trials minus one. In an array, column represents the factors which influence the results and row represents the combination of various levels assigned to each factor. In the present study, Minitab16.0 software was used for automatic design and analysis of Taguchi's experiments. All five columns are assigned with different factors as indicated in Table 1 and each factor is assigned with three different levels. Table 2 shows the layout of the L_{27} OA used in the present study.

2.3.2. Conducting the experiment

In this study, five different factors each at three different levels *i.e.* pH (5, 6, 7), temperature (25, 37, 45 °C), oxygen (0.5, 1, 1.5 litres per minute [LPM]), reaction time (8, 24, 48 h), enzyme (5000, 10000, 15000 units) were considered for optimizing the production of melanin. The experiments were performed in a 500 mL Erlenmeyer flask as the reaction vessel, by employing the selected 27 experimental trials (Table 2). Melanin synthesis was carried out using 40 mL of L -Dopa (30 mM) as substrate and 160 mL of phosphate buffer (0.1 M). The final concentration of L -Dopa in the reaction system was 6 mM. Rest of the conditions was as specified in the design matrix. The yield of melanin was reported as mean value with standard deviation for three individual determinations.

2.3.3. Analysis

S/N ratio is used as a statistical measure of performance to analyze the results obtained from the experimental trials. The term signal represents desirable value (mean) and noise represents any undesirable value (standard deviation from mean). In each experimental run, yield of melanin was considered as the response and correspondingly their S/N ratios were calculated. Different types of S/N ratios such as larger the better, nominal is best and smaller is better are used to indicate the quality of optimization process. However, in the present study S/N ratio of larger is

better was chosen, as the goal was to maximize the response (melanin production). Here, the S/N ratio is calculated using the following mathematical formula.

$$S/N = -10 \log \left(\frac{\sum_{i=1}^n [1/y_i^2]}{n} \right) \quad (1)$$

where y is the number of experimental value obtained at each trial and n is the number of samples.

2.3.4. Validating the results

Validation was confirmed by carrying out the confirmatory experiment under the best selected conditions. The best conditions were selected from the main effect plot for S/N. The yield of melanin obtained from the confirmatory experiment was compared with the expected yield value predicted by the software.

2.4. Estimation of melanin

Melanin was estimated spectrophotometrically according to Ozeki *et al.*²⁴ In brief, a small aliquot (1 mL) was taken from the reaction system and precipitated using 6 N HCl (by adjusting the solution pH to 2). It was then subjected to centrifugation at 10,000 rpm (Spinwin, Tarsons) for 10 min. The pellet obtained was extensively washed with ultrapure water and centrifuged. It was then dissolved in 1 mL of solouene-350:water mixture in the ratio of 9:1(v/v), by heating in a boiling water bath for 45 min. The sample was then cooled to room temperature and total melanin was estimated by measuring the absorbance of the solution at 500 nm. Background correction was done using solouene-350:water (9:1) mixture as blank. The ratio of A_{650}/A_{500} was calculated and used to estimate the amount of eumelanin in relation to total melanin.

2.5. Characterization of the biosynthesized melanin

After completion of the reaction, pH of the solution was adjusted to 2 using HCl (6 N). This resulted in the precipitation of the black pigment. Following washing with ultrapure water it was further subjected to lyophilization and stored at -20°C . Characterization of melanin pigment was performed as follows:

2.5.1. Solubility of melanin

For solubility studies, melanin powder (20 mg) was added to 10 mL each of ultrapure water, HCl solution (0.5 M) and common organic solvents such as benzene, methanol, acetic acid, n-hexane, ethanol, chloroform, acetone, ethyl acetate, DMSO, toluene: water mixture and allowed to stir for 1 h. The solutions were then allowed to stand for 10 min and then centrifuged at 10,000 rpm for 10 min. The insolubility of melanin in different solvents was determined based on the observation of a black pellet after centrifugation.

2.5.2. Elemental analysis

The percent composition of C, H, O, N and S in synthesized melanin was determined with the help of an elemental analyzer (FLASH EA 1112 series, Thermo finnigan, Italy). The procedure involves the complete and instantaneous oxidation of the sample by flash combustion. In this set up, the analyzed products *i.e* carbon, hydrogen, nitrogen and sulphur were oxidized to form CO_2 , H_2O , NO_2 and SO_2 respectively. The combustion products were then separated with the help of a chromatographic column and detected using a thermal conductivity detector. The weight percent value was converted to moles, by dividing the percentage of each element in the sample by their respective atomic weight. The empirical formula was then calculated to the relative N value, by taking N as the lowest integral number.

2.5.3. UV-Visible Spectroscopy

For studies on UV-visible absorption, melanin powder (1 mg) was dissolved in solouene350:water mixture (9:1) described as mentioned above. The spectrum was recorded using Jasco V-530 UV-visible double beam spectrometer in the wavelength range of 285-700 nm at 25 °C in a quartz cuvette with a 1 cm optical path.

2.5.4. ESEM-EDS of biosynthesized melanin

ESEM (Quanta 200 ESEM, FEI, USA) was employed to observe the nature of the precipitated melanin. The procedure involved sonicating the suspension of melanin powder (1 mg) in ultrapure water (10 mL) using a sonicator (DP-120, Dakshin, India). Sonication was carried out for 10 cycles with an 'on' cycle for 2 min and 'off' cycle of 1 min. A double coated carbon tape was placed on the surface of the stub and a small aliquot of the sample was placed on the tape and dried. The parameters for ESEM-EDS were as follows: voltage:20 kV, working distance:10.5 mm, magnification: 50,000 X.

2.5.5. FT-IR spectroscopy

FT-IR spectrum of melanin was recorded with Jasco FT-IR-660 plus infrared spectrometer. For the purpose of measurement, melanin powder at 4% (w/w) composition was mixed with dried KBr powder (IR grade). The mixture was then ground using a mortar and pestle till it achieved uniform color. The spectrum was recorded over a range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1} .

2.5.6. Thermo-gravimetric (TG) analysis of biosynthesized melanin

TG data of synthesized melanin sample (10 mg) was recorded using NETZSCH Thermal analyzer (STA 409 pc Luxx) under an inert atmosphere of nitrogen gas. The measurements were performed in an alumina crucible and the heating rate was 10 $^{\circ}\text{C min}^{-1}$. The data was recorded over the temperature range from 35 $^{\circ}\text{C}$ to 1000 $^{\circ}\text{C}$.

2.5.7. ^1H NMR characterization

For the ^1H NMR measurements, biosynthesized melanin (10 mg) was suspended in deuterium oxide (99.9 atom %, Aldrich, 0.7 mL). The solution was adjusted to pH 10 with the help of aqueous ammonia (33 %), which resulted in a dark black colour solution. The ^1H NMR spectra for synthesized melanin was recorded at 25 °C on Mercury Plus 300 MHz NMR spectrometer (Varian, USA) in a 5 mm NMR tube using the following parameters: internal reference: D_2O ; spectral width: 4800 Hz; number of data points: 19192; acquisition time: 2 s; recycle delay: 0.05 s and number of scans: 3200. The water suppression experiment was performed using a 2S homo-nuclear decoupling before the observation pulse.

3. Results and discussion

In our previous study, we have discussed the use of tyrosinase from *A.campanulatus* for melanin production.² The synthesis of melanin was carried out under un-optimized conditions using 10,000 units of tyrosinase enzyme at pH 6.0, temperature of 37 °C and time period of 8 h. Under these un-optimized conditions, a maximum of 93 mg L⁻¹ of melanin was synthesized. The reaction for melanin synthesis proceeds via the formation of an orange colored intermediate called dopachrome. Fig. 2; represents the different stages during the reaction of melanin synthesis. As seen from the figure, a broad peak is observed at 475 nm at 30 min, which is attributed to dopachrome formation.

Considering the increasing applications of melanin, it was decided to optimize the yield of melanin using Taguchi DOE methodology. From our previous experience and literature, we selected those parameters that can affect the synthesis of melanin. It is reported that oxygen plays an important role in the efficient functioning of tyrosinase enzyme and so oxygen was selected as one of the important parameter for melanin synthesis.²⁵ Hence, the parameters chosen for

optimization of melanin synthesis included pH, temperature, oxygen level, reaction time and enzyme units. For optimization, each parameter was then divided into 3 different levels as listed in Table 1.

3.1. Effect of different factors on melanin yield

Taguchi DOE methodology has proven to be a powerful tool for optimizing the process parameters for various biological experiments.²² It allows the calculation of maximum melanin yield based on experiments in which all the factors are varied within the selected range. Also, the interaction effect between various process parameters can be studied. Taguchi utilizes the S/N ratio to measure the deviation of the quality characteristic from the desired value. Table 2 shows the L_{27} array that was used for the present study. Each row in the table indicates the combination of different levels for each factor and the columns represent the different factors chosen. The values for the yield of melanin are reported in Table 2 as mean \pm S.D for three different experiments. The last column in the table shows the corresponding S/N ratio calculated by the software for each of the experiment.

3.2. Main effect plot

The impact of selected factors and their assigned levels on melanin synthesis is shown with the help of main effect plot (Fig. S1). A main effect is present when any level for the factors chosen, affects the response in a different manner. The average for each factor level is calculated and then used to plot the main effect. There is a line that connects the points for each factor. When the line is parallel to the x-axis, then there is no main effect present. This indicates that each level for the factor affects the characteristics in the same manner and the average is same along all factor levels. However, when the line is not horizontal, there is a main effect present. Greater the difference in the vertical position of the point, greater is the magnitude of main effect.²⁶ From the

main effect plot it is observed that increasing the oxygen level had a negative effect on the synthesis of melanin, whereas, increasing pH, temperature, reaction time and enzyme units had a positive effect. The S/N ratio as well as the melanin synthesis increased with rise in pH and were found to be highest at pH 7. Similar results were also observed for melanin produced by *Klebsiella* sp. GSK¹⁵ and recombinant *E.coli*²⁷ Also, a rise in temperature from 25 °C to 45 °C had a positive impact on melanin synthesis. pH as well as temperature are important parameters that affect enzyme activity and hence, melanin synthesis. Optimum pH for tyrosinase enzyme has been reported in the range of 6-7,²⁸ whereas the temperature optima is 37 °C as reported by Marsden²⁹. Although oxygen is required by the enzyme for its action, increase in oxygen level leads to inactivation of the enzyme.³⁰ Fig. S1, shows the negative effect of increasing oxygen levels on the synthesis of melanin pigment. Increasing the reaction time helps to maximize conversion of the substrate to product. Thus, melanin synthesis increased as the reaction time was increased.

3.3. Response table for various factors and ANOVA

Table 3 represents the response table for S/N ratio (larger is better) obtained from L₂₇ orthogonal array. The last two rows in the table *i.e.* delta values and the assigned ranks, helps us to understand which factor has a major impact on the outcome of interest. Delta value represents the difference between the highest and the lowest characteristic average value of a particular factor. Higher the difference, greater is the impact of that factor on the outcome. Based on these delta values, rank arranges the factors from the greatest effect to the least effect on the response characteristics. The order in which each individual factor affects the synthesis of melanin can be ranked as pH > temperature > reaction time > enzyme units > oxygen. This suggests that pH of the system had a major impact on melanin synthesis, whereas oxygen level had the least effect.

Individually, oxygen at level 1, temperature at level 3 and pH at level 3 had a major impact on melanin synthesis. In addition to the S/N ratio analysis, ANOVA was also carried out and the results are tabulated in Table S1. ANOVA table indicates the significance of selected parameter on melanin production, which varied from factor to factor. pH with the least P-value and highest F-value (at 95% confidence level) was found to be the most significant factor for melanin production.

3.4. Effect of interactions between parameters

3.4.1. Interaction plot

Understanding the interaction between two factors gives a better insight into the overall process analysis. Any individual factor may interact with any one or all of the other factors creating the possibility for a large number of interactions. The interaction effect plot is shown in Fig. 3 and it represents the mean response at all possible combinations of any two factors. If two lines intersect each other, then there is a possible interaction between the two factors. As seen from Fig. 3, it is clear that there is a strong interaction between each of the two factors, except for enzyme, where only minor interactions were observed with respect to all the other factors. When pH of the reaction system was set to 7, it resulted in maximum response at all levels for the other factors, describing the importance of high pH for increasing melanin synthesis. It is possible that the conversion of substrate (L-Dopa) to melanin occurs at a higher extent at pH 7 as compared to pH 5 and 6. A temperature of 37 °C was observed to give maximum response at lower oxygen level (0.5 LPM) and higher reaction time (48 h). A higher response at 37 °C was seen because tyrosinase enzyme which catalyzes the synthesis of melanin has temperature optima of 37 °C.^{28,29} Interaction between oxygen at low levels and reaction time at high level had a positive impact on melanin synthesis. Also, at low oxygen level, increase in enzyme units did

not make a major difference in melanin production. Besides enzyme units displayed only a minor interaction with respect to temperature.

3.4.2. Contour plot

From Table 3, it was found that pH showed the maximum delta value and hence was assigned 1st rank. Also, from Table S1, it was observed that pH was the most significant parameter affecting the process. Considering the importance of pH, contour plots for pH versus all other parameters were generated and analyzed. It was observed that maximum response was obtained at pH 7 and temperature of 37 °C (Fig. S2 A). Further increase in temperature probably leads to a decrease in enzyme activity, which resulted in a lower response at higher temperature. Also, as the pH of the system was increased, a decrease in oxygen levels and an increase in both reaction time and enzyme units were found to have a positive impact on the synthesis of melanin (Fig. S2 B-D).

3.5. Confirmatory experiment and validation of the model

The confirmatory experiment is an important aspect to verify the experimental conclusions drawn, based on Taguchi approach. It is necessary to compare the experimental yield value under optimized conditions with the expected yield value predicted by the software. The confirmatory experiment is performed by choosing combination of optimum level for each factor. In this study three independent confirmatory experiments were performed under the best selected conditions. The mean melanin yield predicted by the software under best selected conditions (pH = 7, temperature = 45 °C, time period = 48 h, oxygen = 0.5 LPM and enzyme unit = 15,000 units) was $1169 \pm 4.6 \text{ mg L}^{-1}$ and that from the confirmatory experiment was determined as $1176 \pm 3.2 \text{ mg L}^{-1}$, which is because of the combined effect of enzyme and auto-oxidation. Before optimization melanin yield was 93 mg L^{-1} as reported earlier by our group.² Thus, due to optimization the yield of melanin increased 12 times as compared to un-optimized conditions. Also, the yield of

melanin was better when compared to Manivasagan *et al.*³¹ and Inamdar *et al.*¹⁶ who have reported the biosynthesis of melanin using statistical optimization procedures. From Table 4, it is seen that the production rate of melanin achieved by other authors was in the range from 2.4-23.9 mg L⁻¹ h⁻¹ respectively. However, in the present study, production rate of melanin was 24.5 mg L⁻¹ h⁻¹. Additionally, our method for optimizing melanin synthesis is simple and does not involve the cumbersome aseptic procedure for inoculation and growing the micro-organisms on special growth medium. Also, melanin produced by the present study has less contamination unlike when produced by the bacterial or fungal cultures which require further purification steps. The experimental value was very close to the predicted value which confirmed the validity of Taguchi DOE methodology for optimizing parameters for melanin synthesis. Thus, it was possible to increase melanin production using the proposed statistical approach. Considering that the reaction consists of homogenous components, the process can be scaled up. Further, the system was scaled up to 5 L using the optimized conditions and it was possible to obtain a yield of 5630 ± 10.4 mg L⁻¹.

3.6. Characterization of biosynthesized melanin

3.6.1. Solubility of melanin

The physicochemical characteristics of biosynthesized melanin are listed in Table S2. It was observed that, synthesized melanin was insoluble in distilled water and in most of the common organic solvents studied. In weakly basic solution, aggregation of melanin pigment takes place. However, the solubility of melanin was increased by increasing the solution pH. It was observed that melanin was completely soluble in hot soluene-350:water mixture (9:1). Soluene-350 is a strong organic base, formulated with toluene that has an excellent capacity for solubilization of various biomolecules and hard tissues. Solubilization under basic conditions is most probably

due to the deprotonation of the phenolic and carboxylic moieties present in the structure of melanin. Thus, biosynthesized melanin showed a similar solubility characteristic as reported in general for melanin pigment.

3.6.2. Elemental analysis

The general classification distinguishes melanin into two major types *i.e.* eumelanin and pheomelanin. A major difference between the two is the content of sulphur. Eumelanin does not contain sulphur whereas, pheomelanin contains a variable percentage of sulphur.³³ As shown in Table 5, biosynthesized melanin showed the presence of 50.5% C, 4.3% H, 7.8% N and 33.3% O. The C/H ratio in biosynthesized melanin was high, indicating the presence of many aromatic structures in melanin. The N content between 6-9% and the absence of S indicates the presence of an indole-type melanin, which is in agreement with the literature.³⁶ Due to the presence of large amount of C, H, N and absence of S, melanin synthesized in the present study can be classified as eumelanin. Similar observations have also been made by other researchers (Table 5). Also, from the elemental composition the empirical formula for melanin was calculated and was found to be $C_8H_8O_4N$. Similar results were also reported for melanin obtained after purification by different protocols.³⁷ Also, eumelanin synthesized enzymatically from dihydroxyindole (DHI) and dihydroxyindole-2-carboxylic indole (DHICA) was found to have the same empirical formula.³⁸

3.6.3. UV-visible study

Synthesized melanin exhibited very poor solubility in acidic solution and most of the organic solvents. Therefore, to scan the absorption spectra, the tyrosinase synthesized melanin was suspended in toluene:350:water mixture (9:1) and heated in boiling water bath. As shown in Fig. 4A, UV- visible spectra of biosynthesized melanin showed a broad, strong absorbance in the UV

region, which is a characteristic feature of melanin. The strong absorption in the UV range can be attributed to the n to π^* and π to π^* transition of the carboxylic and aromatic moieties present in the structure of melanin.³⁷ The absorption was highest in the UV region and it went on increasing as the wavelength decreased. This phenomenon is due to the presence of complex conjugated structures in melanin.¹⁵ The ratio of A_{650}/A_{500} helps to estimate the amount of eumelanin in relation to total melanin. A ratio above 0.25 indicates the presence of eumelanin-rich pigment whereas, a ratio below 0.15 is consistent with pheomelanin-rich pigment.²⁴ In the present study, A_{650}/A_{500} ratio was calculated to be in the range of 0.5-0.7 for all the 27 experimental trials that were conducted. This value is well above the value of 0.25 and indicates that the melanin synthesized using tyrosinase enzyme is rich in eumelanin content.

3.6.4. ESEM-EDS study of biosynthesized melanin

ESEM micrographs of the synthesized melanin show aggregates of tightly packed small spherical granules (Fig. 4B). Aggregation of melanin is well known due to their hydrophobic nature.³⁹ The fundamental units are planar and form a layered structure that undergoes lateral aggregation and stacking. This results in the formation of a long chain and branched structure. The internal diameter of these spherical molecules ranges between 150-200nm. Also the EDS spectrum reveals that the synthesized melanin has carbon [C] as the major component, along with nitrogen [N] and oxygen [O] present in lower amounts (Fig. 4C). The peaks for sodium [Na] and phosphorus [P] are because of the buffer used in the preparation. The trace of chlorine [Cl] is due to HCl that was used to precipitate the pigment.

3.6.5. FT-IR spectroscopy

FT-IR spectroscopy helps to investigate the vibrational characteristics of various functional groups. Fig. 4D shows the FT-IR spectrum for the synthesized melanin. The bands near 1700 cm^{-1}

¹ are ascribed to free carboxylic group (-COOH) and those at 1400 cm^{-1} are because of the ionized (-COO⁻) carboxylic moiety. The spectral region from $1100\text{-}1050\text{ cm}^{-1}$ is ascribed to the hydroxyl/ alcoholic groups and peaks in the region $1240\text{-}1200\text{ cm}^{-1}$ indicate the presence of phenolic groups.³ Peaks in the region of 1600 cm^{-1} are due to the vibrations occurring in the plane of HC=CH bonds and due to aromatic -C=C- stretching vibrations, pointing towards the presence of an aromatic structure in the compound.¹⁵ This is in agreement with the elemental analysis and UV visible spectroscopy data which together reveal that biosynthesized melanin contains large number of aromatic moieties. The presence of -OH functional group and the aromatic moieties indicate that the -OH functionality might exist as phenolic OH group in the system. Also, the elemental analysis indicated the presence of an indole-type melanin, which signifies the presence of hydroxy indole moieties, which is the basic constituent of melanin. The region of $1460\text{-}1450\text{ cm}^{-1}$ belongs to the vibrations of aliphatic -C-H bonds; whereas the stretching vibrations of the -C=O bond gives rise to peaks in the region of $1720\text{-}1710\text{ cm}^{-1}$. Also, the region of 3400 cm^{-1} showed stretching vibrations of carboxylic/phenolic -O-H and -N-H-groups.³⁷

3.6.6. TG analysis

As seen from Fig. 4E, high thermal stability is one of the characteristics of biosynthesized melanin. Overall, the thermogram shows that there is a decrease in the weight of melanin as the temperature was increased. From the graph, it was observed that an initial weight loss is caused by the water removal, indicating that nearly 20% of the initial weight of melanin was strongly bound water. Similar trend was observed by Sajjan *et al.*,³³ wherein they studied the properties of melanin pigment extracted from *Klebsiella* sp. GSK. Also, in the present study biosynthesized melanin retained 50% of the initial weight even after exposing it to temperature of $1000\text{ }^{\circ}\text{C}$. P.

Schaeffer suggested a relation between the origin of melanin molecule and its resistance to temperature.⁴⁰ Hence, melanin synthesized in the present study, is stable towards high temperature treatment.

3.6.7. ¹H NMR characterization

The ¹H NMR spectrum of biosynthesized melanin shows signal for both aliphatic as well as aromatic region (Fig. 5). In the aliphatic region of the spectrum, signals in the range from 0.6-1.0 ppm can be assigned to the -CH₃ group of various alkyl fragments. The signal (doublet) at 1.3 ppm can be ascribed to -CH₂- group. A sharp peak at around 1.8 belong to the protons from the -CH₃ group attached to an ethylene carbon atom. The signals at around 2.2 and 2.8 ppm are characteristic of methylene groups. The peaks in the absorption region from 3.7-4.2 ppm can be due to the protons on carbon attached to nitrogen and/or oxygen atoms. In the aromatic region, a broad peak centered at around 7 ppm can be assigned to the indole units present in the structure of melanin.⁴¹ A singlet at 8.4 ppm could be assigned to a -N=CH-Ar group (Ar - Aromatic).

Thus, from all the above studies it can be concluded that the biosynthesized melanin showed similar characteristics to eumelanin.

4. Conclusion

Melanin pigment is gaining much attention because of its numerous applications. Thus, there is a need for devising ways to increase the yield of melanin in order to meet its demand. The present study describes the use of Taguchi's DOE methodology to optimize the one-pot, green technology protocol for *in vitro* synthesis of melanin. Our experimental results indicate that Taguchi approach is an efficient method for optimizing the process parameters so as to increase the yield of melanin. A pH of 7, temperature of 45 °C, oxygen level of 0.5 LPM, reaction time of 48 h and 15,000 units of tyrosinase enzyme were found to be the best conditions for melanin synthesis based on the main effect plot of S/N ratio. After optimization, the yield of melanin increased to 12 times and a better rate of melanin production was achieved as compared to the un-optimized conditions. This highlights the significance of the present study. Also, from the rank obtained in the response table and ANOVA (95% confidence), pH was the most significant factor affecting melanin synthesis. A better understanding of the interactions between different factors at different levels was obtained from the interaction effect plot. Solubility studies revealed the insolubility of synthesized melanin in most solvents. ESEM-EDS and elemental analysis showed that the major component present in the synthesized melanin was carbon. The empirical formula was derived as C₈H₈O₄N based on the percentage composition. The N content (6-9 %), absence of S and the ¹H NMR spectrum indicate the presence of an indole-type melanin. The A₆₅₀/A₅₀₀ ratio was in the range of 0.5-0.7, which proves the presence of a high content of eumelanin. The process optimized in the present study is an alternative to the other methods of melanin production. Also, the use of enzyme for *in vitro* synthesis provides an economic advantage over the cumbersome extraction of this pigment from animal, plant or microbial

source. Hence, we anticipate that melanin synthesized through green technology may find numerous applications at the industrial as well as research scale.

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Figure captions:

Fig. 1 Schematic representation of the steps involved in Taguchi DOE methodology for optimization.

Fig. 2 Graph showing the different stages of melanin synthesis.

Fig. 3 Interaction plot of selected factors and their levels.

Fig. 4 [A] UV-visible spectrum [B] ESEM micrograph at 50,000 X [C] EDS spectrum [D] FT-IR spectrum [E] TG of biosynthesized melanin.

Fig. 5 ^1H NMR spectrum in $\text{D}_2\text{O}/\text{aq. ammonia}$ (pH 10) of biosynthesized melanin.

List of tables:

Table 1 Factors selected for optimizing melanin synthesis along with their respective levels

Sr. No.	Factors	Level 1	Level 2	Level 3
A	pH	5	6	7
B	Temperature(°C)	25	37	45
C	Oxygen (LPM)	0.5	1	1.5
D	Time (h)	8	24	48
E	Enzyme units	5000	10000	15000

Table 2 Experimental table showing the conditions used and the corresponding yield of melanin

Sr. no	pH	Temp. (°C)	Oxygen (LPM)	Time (h)	Enzyme units	Mean melanin yield (mg L ⁻¹)	S/N ratio (dB)
1	5	25	0.5	8	5000	18.17 ± 0.3	25.2
2	5	25	0.5	8	10000	27.38 ± 1.3	28.7
3	5	25	0.5	8	15000	33.76 ± 1.5	30.6
4	5	37	1	24	5000	23.22 ± 1.8	27.3
5	5	37	1	24	10000	50.80 ± 1.5	34.1
6	5	37	1	24	15000	121.38 ± 1.8	41.7
7	5	45	1.5	48	5000	43.24 ± 2.1	32.7
8	5	45	1.5	48	10000	84.49 ± 2.1	38.5
9	5	45	1.5	48	15000	100.76 ± 2.5	40.1
10	6	25	1	48	5000	105.26 ± 2.3	40.4
11	6	25	1	48	10000	93.36 ± 2	39.4
12	6	25	1	48	15000	100.48 ± 1.7	40.0
13	6	37	1.5	8	5000	39.22 ± 1.6	31.9
14	6	37	1.5	8	10000	93.42 ± 2	39.4
15	6	37	1.5	8	15000	118.01 ± 1.9	41.4
16	6	45	0.5	24	5000	224.56 ± 2.6	47.0
17	6	45	0.5	24	10000	207.85 ± 2.7	46.4
18	6	45	0.5	24	15000	215.44 ± 2.5	46.7
19	7	25	1.5	24	5000	233.93 ± 3	47.4

20	7	25	1.5	24	10000	277.78 ± 3.3	48.9
21	7	25	1.5	24	15000	275.50 ± 2.1	48.8
22	7	37	0.5	48	5000	1499.00 ± 3.6	63.5
23	7	37	0.5	48	10000	1043.00 ± 5.1	60.4
24	7	37	0.5	48	15000	1516.01 ± 5.8	63.6
25	7	45	1	8	5000	315.83 ± 1.8	49.9
26	7	45	1	8	10000	674.00 ± 1.4	56.6
27	7	45	1	8	15000	609.45 ± 3.3	55.7

Table 3 Response table based on S/N ratio

Level	pH	Temp (°C)	Oxygen (LPM)	Time (h)	Enzyme units
1	33.19	38.82	45.78	39.94	40.59
2	41.40	44.80	42.79	43.13	43.59
3	54.98	45.95	41.00	46.52	45.39
Delta	21.78	7.13	4.77	6.58	4.80
Rank	1	2	5	3	4

Table 4 Comparison of the batch scale yield of melanin and conditions used

Source	Melanin yield (mg L ⁻¹)	Production rate (mg L ⁻¹ h ⁻¹)	pH	Temp. (°C)	Time (h)
<i>Actinoalloteichus</i> sp. MA-32 ³¹	85.4	0.5	7	28	168
<i>Yarrowia</i> <i>lipolytica</i> ¹⁴	160.2	8.9	-	20	18
<i>Aspergillus</i> <i>nidulans</i> ¹³	172.8	2.4	-	37	72
<i>Klebsiella</i> sp. GSK ¹⁵	537.6	6.4	7.2	37	84
<i>Mucuna</i> <i>monosperma</i> callus ¹⁶	888	18.5	5.85	-	48
Biosynthesized melanin ^{Present study}	1176	24.5	7	45	48
<i>Aeromonas media</i> ³²	1720.8	23.9	7.2	30	72

Table 5 Elemental composition of biosynthesized melanin compared with other melanins

Source of melanin	C%	H%	N%	O%	S%
<i>Klebsiella</i> sp. GSK melanin ³³	47.9	6.9	11.9	-	0.9
<i>Sepia</i> melanin ³⁴	43.3	3.3	7.6	-	-
Taihe black-bone silky fowl melanin ⁴	59.5	4.2	6.6	29.2	0.6
Synthetic Dopa melanin ³⁵	56.5	3.1	8.5	31.8	0.09
Biosynthesized melanin ^{Present study}	50.5	4.3	7.8	33.3	-

Fig. 1

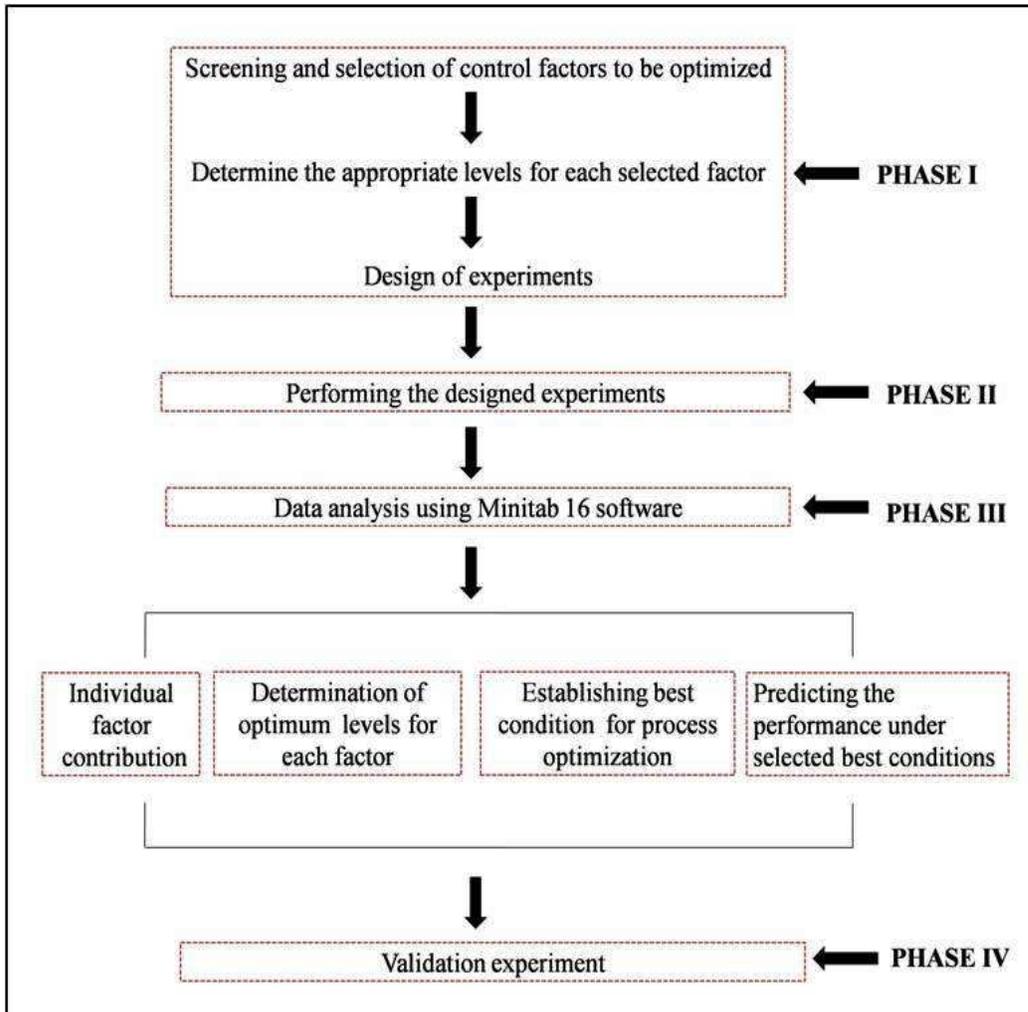


Fig. 2

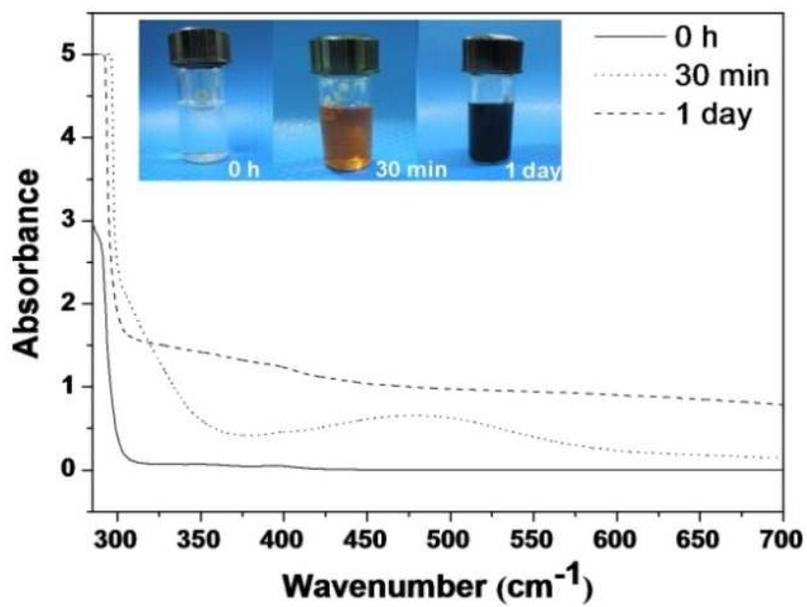


Fig. 3

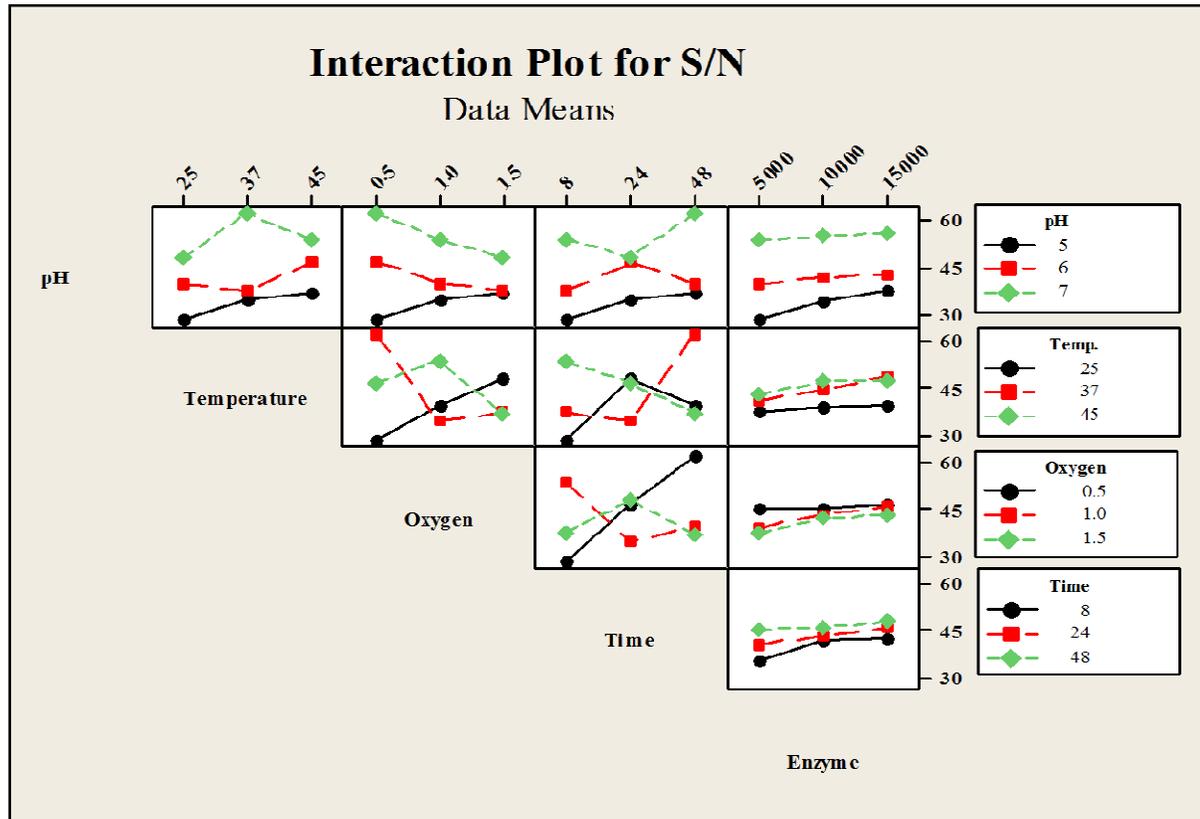


Fig. 4

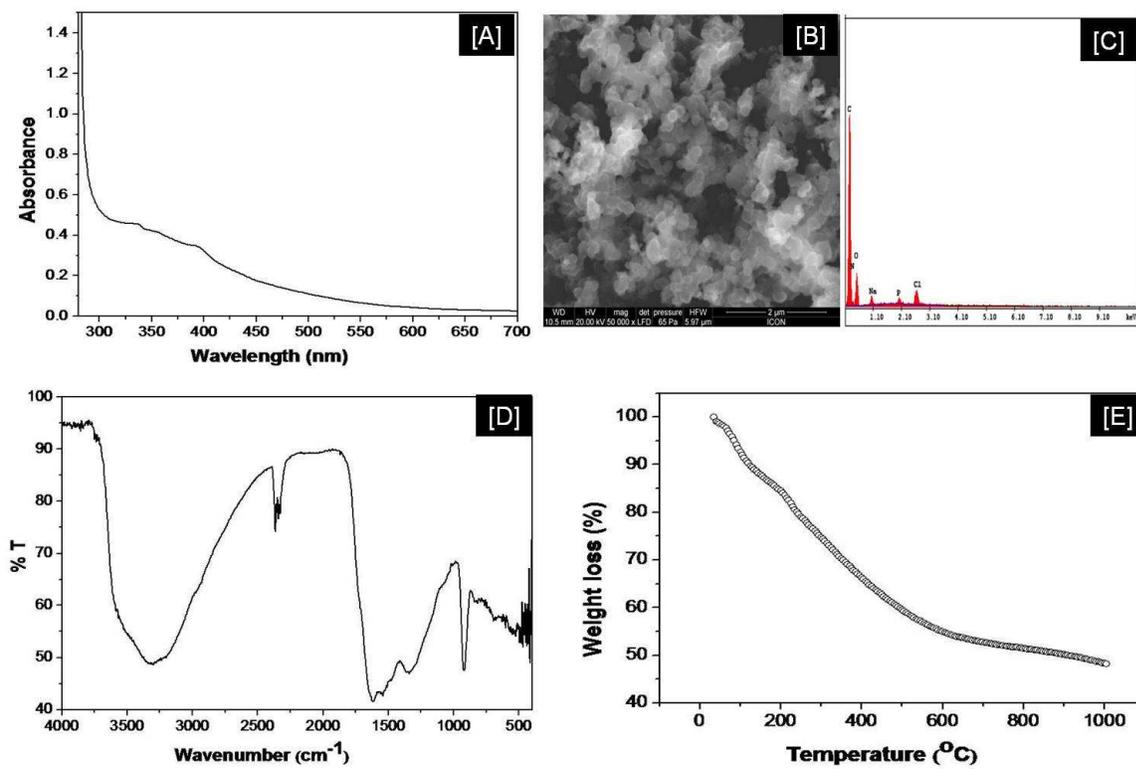


Fig. 5

