"A theory is something nobody believes it, except the person who made it. An experiment is something everybody believes it, except the person who made it”

– Albert Einstein
3. Materials and methods

3.1 Chemicals

All chemicals used were of highest purity and of an analytical grade. Synthetic melanin and DEAE-cellulose were purchased from Sigma Aldrich, USA. NH₄Cl, NH₄NO₃, (NH₄)₂SO₄, KNO₃, NaNO₃, CH₄N₂O, NaCl, CoCl₂, CaCl₂, MgSO₄, MnSO₄, FeSO₄, CuSO₄, ZnSO₄, Na₂HPO₄, NaH₂PO₄, KCl, K₂HPO₄, KH₂PO₄, FeCl₃, Na₂MoO₄, MnCl₂, H₃BO₄, ZnCl₂, thiamine, pyridoxine, folic acid, nicotinic acid, cynocobalamine, biotin, riboflavin and Coomassie Brilliant blue R-250 were obtained from Sisco Research Laboratories (SRL), India. Methanol, chloroform, n-butanol and acetic acid were obtained from Merck, India. The HPLC grade water was obtained from S. d. fine chem. Ltd., India. L-tyrosine, L-DOPA, L-tryptophan, L-phenylalanine, L-glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-lysine, L-aspartic acid, L-glutamic acid, L-histidine, L-arginine, L-serine, L-cysteine, L-methionine, L-threonine, L-asparagine, L-proline, and L-glutamine, glucose, sucrose, lactose, fructose, cellulose, starch, xylose, galactose, maltose, manitol, sorbitol, arabinose, mannose, rhamnose, L-ascorbic acid, peptone, yeast extract, tryptone, beef extract, meat extract, soya peptone, gelatin, casein and agar powder were obtained from Hi-Media laboratory, India. Protein markers were obtained from Bangalore Genei Pvt. Ltd. The N, N’-methylene bisacrylamide- 2X and TEMED (N,N,N,N’-Tetramethyl-ethylenediamine) were obtained from Fluka Chemicals, India.

3.2 Isolation and screening of microorganism

L-DOPA and melanin producing bacteria were isolated by serial dilution technique and soil samples were collected from Shivaji University, Kolhapur, India. A modified nutrient agar used for the isolation was composed of (g l⁻¹) peptone 1, yeast extract 1, beef extract 1, NaCl 1, agar 20 and supplemented with L-tyrosine 5 with pH 6.5 were taken in 250 ml of Erlenmeyer flask and autoclaved at 15 psi (121°C) for 20 min. 0.1 ml aliquots of diluted soil sample were spread over agar plates and incubated at 30°C for 48 h. The bacterial isolates showing the dark brown pigment around the colonies were screened for both melanin and L-DOPA producing abilities.
3.3 **Identification of bacteria and phylogenic analysis**

The isolated bacterial strains were identified by 16S rRNA sequencing, carried out at Genombio Technologies Pvt. Ltd., Pune, India and Chromus Biotech Pvt. Ltd. Bangalore, India.

The partial nucleotide sequence (16S rRNA) was obtained and submitted to Genbank, and the strains were identified as a novel bacteria *Bacillus* sp. JPJ (accession no. FJ545652.1.) and *Brevundimonas* sp. SGJ (accession no. HM998899). After release of the sequence by database of NCBI, it was aligned with non-redundant database present in NCBI using BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the homologous sequences of species were used for phylogenetic analysis. The phylogenetic tree was constructed with MEGA4 software (AZ, USA). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. The clock calibration to convert distance to time was 1 (time/node height). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 software (Tamura et al. 2007). This type of phylogenetic analysis was reported earlier (Dhanve et al., 2009).

3.4 **Microorganisms and cultural conditions**

The pure cultures of *Bacillus* sp. JPJ and *Brevundimonas* sp. SGJ were maintained routinely on modified nutrient agar slants having composition stated above for isolation of bacteria. All cultures were sub cultured after every 3 weeks and stored at 4°C.
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3.5 Inoculum development

The inoculum was developed on the basis of dry cell weight. The crude tyrosinase activity was determined with 6 h time interval. 1 ml cell suspension of 6 h of grown Bacillus sp. JPJ having absorbance 0.51 at 660 nm was inoculated in the same medium stated above for the melanin and L-DOPA production.

3.6 Optimization of nutritional parameters for melanin and L-DOPA production

Although L-DOPA synthesized initially in melanin synthesis pathway, the present study primarily focused on melanin production, hence melanin production by bacteria was studied first and L-DOPA production was studied later. The optimization of nutritional parameters was carried out for both melanin and L-DOPA by using similar parameters; hence details of the methods employed were given in under one title.

3.6.1 Melanin and L-DOPA production

The nutrient broth having the composition (g 1⁻¹) peptone 1, yeast extract 1, beef extract 1, NaCl 1, agar 20 and supplemented with L-tyrosine 5 with pH 6.5 was used as production medium and the 250 ml Erlenmeyer flasks were incubated at 30°C and kept at 120 rpm in orbital shaking incubator and after 48 h of incubation period melanin was assayed. The produced L-DOPA was assayed after 18 h in the medium and the utilized L-tyrosine was estimated during L-DOPA production to study efficiency of the bacterial strain to produce L-DOPA at various physical and nutritional conditions. The effect of various parameters on the production of melanin and L-DOPA such as pH, temperature, carbon sources, nitrogen sources, mineral salts, trace elements and vitamins were evaluated.

To enhance the reproducibility of the experimental results and to optimize the biosynthesis process as a whole, the factorial design of experiments, the ‘‘one factor at a time’’ method, was employed in this study. Here, the experimental factors are varied one at a time with the remaining factors held constant. Furthermore the optimized conditions were used in all experiments in sequential order. All the experiments were carried out in triplicates with control unless otherwise stated.
3.6.2 Optimization of agitation rate

As tyrosinase is an oxidizing enzyme (Claus and Decker, 2006), specific agitation rate is required for melanin and L-DOPA production. The agitation rate was optimized by incubating the Erlenmeyer flasks in static and in incubator shaker at 40, 80, 120, 160 and 200 rpm.

3.6.3 Optimization of pH

The pH optima for the production of melanin and L-DOPA were determined by setting initial pH of medium to 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 by using 0.2 N HCl and 0.2 N NaOH.

3.6.4 Optimization of temperature

The optimization of temperature for melanin and L-DOPA production was carried out by incubating flasks at 5, 10, 20, 25, 30, 35, 40, 45 and 50°C in incubator shaker.

3.6.5 Evaluation of carbon sources

The effect of various carbon sources was studied by adding each carbon source in the medium at the concentration (1 g l⁻¹). The carbon sources evaluated were glucose, sucrose, lactose, fructose, cellulose, starch, xylose, galactose, maltose, manitol, sorbitol, arabinose, mannose and rhamnose. A carbon source which allowed highest melanin and L-DOPA production was further optimized.

3.6.6 Evaluation of organic nitrogen sources

To evaluate the various nitrogen sources for maximum melanin and L-DOPA, the production medium containing best carbon source was supplemented with each organic nitrogen source at the concentration (1 g l⁻¹). The organic nitrogen sources tested were peptone, yeast extract, beef extract, meat extract, tryptone, soya peptone, gelatin and casein. As nutrient broth used for the production of melanin composed of three organic nitrogen sources in combination such as peptone and yeast extract and
beef extract, so to study the effect of organic nitrogen sources combination, various combinations were used at the concentration (1 g l⁻¹).

3.6.7 Evaluation of inorganic nitrogen sources

The effect of inorganic nitrogen sources with and without organic nitrogen source was evaluated for maximum melanin and L-DOPA production by using (1 g l⁻¹) ammonium chloride (NH₄Cl), ammonium sulphate [(NH₄)₂SO₄], ammonium nitrate (NH₄NO₃), sodium nitrate (NaNO₃), potassium nitrate (KNO₃) and urea (CH₄N₂O).

3.6.8 Influence of amino acids

The effect of amino acids on melanin and L-DOPA production was deliberated by using (1 g l⁻¹) L- tryptophan, L- phenyl alanine, L-glycine, L-alanine, L-valine, L- leucine, L-isoleucine, L-lysine, L- aspartic acid, L- glutamic acid, L-histidine, L-arginine, L-serine, L-cysteine, L-methionine, L-threonine, L-asparagine, L-proline, and L-glutamine in presence of L-tyrosine in the medium.

3.6.9 Effect of L-DOPA

The L-DOPA was reported earlier for enhancing the melanin production (Shrishailnath et al., 2010). Hence its effect on melanin yield was evaluated by adding L-DOPA in the medium with various concentrations of (g l⁻¹) 0.001, 0.002, 0.003, 0.004 and 0.005.

3.6.10 Influence of mineral salts

To study the effect of various mineral salts on the melanin and L-DOPA yield, mineral salts were added in the medium at the concentrations described earlier for *Bacillus* species (Atlas, 2005) (g l⁻¹) NaCl 1, CoCl₂ 0.1, CaCl₂ 0.05, MgSO₄ 0.1, MnSO₄ 0.001, FeSO₄ 0.1, CuSO₄, ZnSO₄ 0.1, Na₂HPO₄ 1, FeCl₃ 0.01, Na₂MoO₄ 0.01, MnCl₂ 0.1, H₃BO₃ 0.1, ZnCl₂ 0.01.
3.6.11 Effect of CuSO₄

The effect of inducer was studied by using CuSO₄, which was reported earlier for enhancing the melanin production (Shrishailnath et al., 2010). The CuSO₄ added in the medium with various concentrations of (g l⁻¹) 0.001, 0.002, 0.003, 0.004 and 0.005.

3.6.12 Influence of vitamins

The effect of vitamins on the melanin production was studied by supplementing the medium with (0.001 g l⁻¹) thiamine, pyridoxine, folic acid, nicotinic acid, cynocobalamine, biotin, riboflavin and ascorbic acid. In case of L-DOPA to observe the effect, vitamins were added at the 12th h of incubation.

3.5.13 Optimization of precursor (L-tyrosine) concentration

The optimization of L-tyrosine concentrations for melanin and L-DOPA production was studied by supplementing various L-tyrosine concentrations (g l⁻¹) 0.5, 1, 1.5, 2, 2.5.

3.6.14 Effect of incubation period

The effect of incubation period on the melanin and L-DOPA production was studied using medium before optimization and after optimization. The effect of incubation period was studied by estimating melanin and L-DOPA with 6 h of time interval.

3.6.15 Evaluation of raw materials

Evaluations of various raw materials for melanin and L-DOPA were carried out by using sugarcane molasses 0.05 % w/v in the medium containing L-tyrosine 1 g l⁻¹, tryptone 2 g l⁻¹ and beef extract 0.5 g l⁻¹. The dairy whey 20% v/v was used in the medium containing L-tyrosine 1 g l⁻¹. The 1 % of wheat bran, rice husk, and maiz husk were boiled for 15 min. and filtered and the 20 % v/v filtrate was added in the medium containing L-tyrosine 1 g l⁻¹.
3.6.16 Effect of inhibitors

The inhibitors of melanin synthesis were valuable in cosmetic products to increase the fairness of skin. Thus effect of inhibitors on melanin synthesis was assessed by using L-ascorbic acid and kojic acid which were reported earlier as strong inhibitors of tyrosinase (Chang, 2009). The concentrations of L-ascorbic acid and kojic acid added in the medium were (g l⁻¹) 0.02, 0.04, 0.06, 0.08, 0.1.

The effect of standard tyrosinase inhibitors were studied on the production of L-DOPA, which includes pyridoxine (Yokochi et al. 2003), L-ascorbic acid (Kim and Uyama 2005; Chang, 2009), cystine (Kim and Uyama 2005), salicylic acid (Zhang et al. 2006), aniline (Mahmoud and Bendary 2010). These inhibitors were reported earlier for enhancing the yield of L-DOPA by inhibiting diphenolase activity of tyrosinase. This diphenolase activity was involved in conversion of L-DOPA to DOPAquinone. These inhibitors were added in the medium at the 12th of incubation for L-DOPA production.

3.6.17 Effect of medicinal plant extracts on melanin production

Several medicinal plant extracts were reported in ‘Ayurveda’ for the skin fairness hence their effects on melanin production were studied. For this study 1 % v/v juice of Citrus fruit (Citrus medica) was added in the optimized medium. The 1 % aqueous extract with concentration (1 g l⁻¹) of saffron (Crocos sativus) turmeric powder (Curcuma longa), almond (Prunus dulcis) powder, amala powder (Phyllanthus emblica), and sandalwood powder (Santalum paniculatum) were added in the medium.

These natural inhibitors although inhibits the melanin synthesis, but they are useful to enhance the L-DOPA production by blocking the conversion of L-DOPA to DOPAquinone. Hence the effect of these inhibitors was also evaluated on L-DOPA production. These inhibitors were added in the medium at the 12th of incubation for L-DOPA production.

3.6.18 Scale up studies

The scale studies for melanin and L-DOPA production were performed by using lab scale bioreactor (Fermenter) of 5 L capacity (Sartorius Bisostat B plus,
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GmbH, Germany). The inoculum size, Agitation rate and dissolved oxygen are the most affecting parameters for scale up, which were studied in bioreactor using medium optimized during flask studies. The inoculum size, for scale up studied was optimized by varying the 6 h grown inoculum 2, 4, 6, 8, 10 %. The dissolved oxygen (DO) concentration was optimized by supplying it 20, 40, 60, 80, and 100 % while optimum agitation rate was obtained by adjusting the stirrer speed 100, 200, 300, 400 and 500 rpm. The optimum parameters obtained from flask studies were used for scale up studies.

To enhance the yield of melanin and L-DOPA, the intermittent addition of L-tyrosine was done in a bioreactor as described previously (Yoshida et al. 1974), by supplementing the 2 g l⁻¹ of L-tyrosine after 6 h of time interval from 12th h to 30th h for melanin production and from 6th h to 30th h for L-DOPA production.

3.7 Optimization of melanin and L-DOPA production by using cell mass of Bacillus sp. JPJ

The optimization of various factors for both melanin and L-DOPA production in buffer was carried out by using similar factors hence details of the methods employed for this study were given under one title.

3.7.1 Melanin and L-DOPA production in buffer

Initially the biochemical reaction of L-tyrosine to melanin and L-tyrosine to L-DOPA was carried out in the reaction mixture containing, 50 ml of potassium phosphate buffer (0.1M, pH 7) and 1 g l⁻¹ of L-tyrosine in 250 ml of Erlenmeyer flask, autoclaved at 15 psi (121°C). The 18 h grown cells having absorbance 0.71 at 530 nm were harvested by centrifugation at 10000 rpm and the supernatant was decanted and cells were resuspended in reaction buffer stated above.

The cell mass of 0.5 g l⁻¹ was resuspended in the sterilized reaction mixture and incubated at 30°C and kept at 120 rpm in an incubator shaker. The melanin production in the reaction mixture was assayed after 4 h of incubation period. The reaction mixture was assayed after 50 min. of incubation period for the produced L-DOPA and residual L-tyrosine. These reaction conditions were considered as an
initial reaction conditions which were maintained further in optimization studies of the cell mass, pH, temperature and L-tyrosine.

3.7.2 Determination of pH optima

The pH necessary for maximum melanin and L-DOPA bioconversion was determined by using the buffer of pH 3, 4, 5, 6, 7, 8, 9, and 10 and incubated at 30°C for 4 h (melanin) and 50 min. (L-DOPA) with 0.5 g l⁻¹ of cell mass and 1 g l⁻¹ of L-tyrosine.

3.7.3 Determination of temperature optima

To determine the temperature optima, it was varied 5, 10, 15, 20, 25, 30, 35, 40, 45, 50°C and incubated for 4 h (melanin) and 50 min (L-DOPA) with pH 7, 0.5 g l⁻¹ of cell mass and 1 g l⁻¹ of L-tyrosine.

3.7.4 Optimization of cell mass

To study the effect of cell mass on melanin and L-DOPA production, the reaction mixture was added with cell mass (g l⁻¹) 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 in a reaction mixture. This reaction mixture contained 1 g l⁻¹ L-tyrosine incubated with optimum pH and temperature for 4 h (melanin) and 50 min (L-DOPA).

3.7.5 Effect of CuSO₄

The effect of CuSO₄ on the production of melanin and L-DOPA was studied with optimum pH, temperature and cell mass with reaction mixture containing 1 g l⁻¹ L-tyrosine was incubated for 4 h (melanin) and 50 min. (L-DOPA). The CuSO₄ concentrations varied was (g l⁻¹) 0.02, 0.04, 0.06, 0.08, 0.1.

3.7.6 Effect of L-ascorbic acid

The influence of L-ascorbic acid was studied on the production of melanin and L-DOPA with reaction mixture containing 1 g l⁻¹ of L-tyrosine was incubated for 4 h (melanin) and 50 min. (L-DOPA). The L-ascorbic acid concentrations varied was (g l⁻¹) 0.02, 0.04, 0.06, 0.08, 0.1.
3.7.7 Effect of activated charcoal

The effect of activated charcoal was studied by varying the concentrations (g l\(^{-1}\)) 1, 2, 3, 4 and 5 in the buffer for maximum production of L-DOPA.

3.7.8 Effect of L-tyrosine

The effect of various L-tyrosine concentrations on melanin and L-DOPA production was studied by using L-tyrosine (g l\(^{-1}\)) 0.25, 0.5, 1, 1.5, 2, 2.5 incubated for 4 h (melanin) and 50 min. (L-DOPA) with optimum pH, temperature, cell mass, CuSO\(_4\), L-ascorbic acid and activated charcoal.

3.7.9 Effect of incubation period

For studying the effect of incubation period on melanin production, the reaction mixture was incubated for 1, 2, 3, 4 and 5 h., while for L-DOPA production it was incubated for 30, 60, 90, 120, and 150 min. with optimum cell mass, pH, temperature, L-tyrosine, CuSO\(_4\), L-ascorbic acid and activated charcoal concentrations.

3.7.10 Recycling of the cell mass

The recycling of the cell mass for melanin and L-DOPA production was carried out by centrifuging the reaction mixture and resuspending the cells again in another reaction mixture. Total 5 reaction cycles were carried out with optimum conditions.

3.8 Melanin characterization

3.8.1 Melanin assay

The melanin concentration was determined spectrophotometrically at 475 nm and 540 nm by using calibration curve of standard melanin (Sigma St. Louis USA) (Hoti and Balaraman, 1993; Aghajanyan et al., 2005; Kubo et al., 2007).
### 3.8.2 Isolation and purification melanin

To isolate and purify melanin, cell free broth was centrifuged at room temperature at 10000 rpm for 15 min to remove cells and debris. The supernatant was filtered through a 0.45-µm membrane and adjusted the pH 11 with 10 M NaOH and kept overnight to ensure complex polymerization. As solubility of melanin decreased within pH range 2.6 - 3.0, hence concentrated HCl was added to the whole mixture to adjust the pH 3, the precipitate of a dark brown material was obtained, which is called the crude melanoprotein. These brown amorphous particles were dissolved in 10 M NaOH and then treated with 20% chloroform to deproteinise the pigment. The mixture was centrifuged at 10000 rpm for 15 min, and then supernatant was precipitated by adding concentrated HCl until the pH was adjusted to 3 and centrifuged. The base solubilization and acid precipitation was repeated 5 times until the supernatant of the acid wash was almost colourless. The crude melanin was washed with distilled water six times, once with 100% methanol, 70% ethanol and ether respectively then air-dried and lyophilized. This procedure results in a dark brown powder. (Liu et al., 2003; Wan et al., 2006; Zhang et al., 2007; Aghajanyan et al., 2007). This pigment was used further for chemical and spectroscopic analysis.

### 3.8.3 Chemical characterization of melanin

Chemical characterization of melanin was carried out by testing its solubility in water, 5 M HCl, ethanol, benzene, chloroform, and acetone, 1 M KOH and 1 M NaOH. The specific chemical tests for melanin were carried out by adding the purified powder in H₂O₂, FeCl₃, KMnO₄ and potassium ferricyanide (Aghajanyan et al., 2005; Zhang et al., 2007; Shrishailnath et al., 2010).

### 3.8.4 UV-Visible spectroscopy analysis

The absorption spectra of standard synthetic melanin (0.1 g l⁻¹) and extracted melanin (0.1 g l⁻¹) were obtained by wavelength scanning from 200 nm to 400 nm by using an UV-Visible spectrophotometer (Shimadzu, Japan).
3.8.5 FTIR analysis

The purified dark brown powder and standard synthetic melanin were analysed by Fourier transform infrared (FTIR) spectroscopy with potassium bromide (KBr) as blank using FTIR-8400S spectrophotometer (Shimadzu, Japan) (Meredith and Sarna, 2006; Wan et al., 2007).

3.8.6 EPR analysis

The Electron paramagnetic resonance (EPR) analysis was carried out at Department of Biophysics & Center for Imaging Research Medical College of Wisconsin, Milwaukee, WI, USA and National Physical Laboratory, New Delhi, India. EPR spectra of standard melanin and brown powder purified from bacteria were obtained by using previously described method (Enochs et al., 1993; Shrishailnath et al., 2010) with a Bruker ER 220D-SRC EPR spectrometer (IBM Instruments, Inc.; Danbeny, CT) operating at X-band frequency 9 GHz with 100 kHz modulation. Each spectrum was recorded at room temperature using a microwave power of 0.21 mW and a modulation amplitude of 4 Gauss, center field, 3350.0 G; sweep width, 400.0 G; and sweep time, 83.88 s.

3.9 L-DOPA characterization

3.9.1 L-DOPA and L-tyrosine assay

The L-DOPA produced in the reaction mixture was determined according to Arnow’s method (Arnow, 1937). The reaction mixture was centrifuged at 5000 rpm for 15 min., and 1 ml supernatant was added with 1 ml of 0.5 N HCl, 1 ml of nitrite molybdate reagent and 1 ml of 1 N NaOH, final volume adjusted to 5 ml by distilled water. The absorbance was measured at 530 nm using double beam UV-Visible spectrophotometer (Shimadzu, Japan) and concentration of L-DOPA was determined by Arnow’s standard curve of L-DOPA.

The L-tyrosine utilized was measured by estimating residual L-tyrosine in the reaction mixture by Arnow’s method, 1 ml of supernatant from the same reaction mixture was added with mercuric sulphate reagent and kept in boiling water bath for 10 min., cooled at room temperature, 1 ml of nitrite reagent was added and volume
was adjusted to 5 ml by distilled water and absorbance was measured at 530 nm. The L-tyrosine concentration was determined by Arnow’s standard curve of L-tyrosine.

3.9.2 High performance thin layer chromatography (HPTLC)

The HPTLC analysis was performed by using HPTLC system (CAMAG, Switzerland). The 2 µl of standard L-tyrosine (1 mg ml⁻¹), Standard L-DOPA(1 mg ml⁻¹), cell free broth and reaction mixture supernatant were loaded on pre-coated HPTLC plates (Silica gel 60 F 254, Merck, Germany), by using spray gas nitrogen and TLC sample loading instrument (CAMAG LINOMAT 5). The HPTLC plates were developed in solvent system n-butanol: acetic acid: water; 4:1:2 (Krishnaveni et al. 2009). After development the plate was observed in UV chamber and scanned at 280 nm with slit dimension 5×0.45 mm by using TLC scanner. The results were analyzed by using HPTLC software WinCATS 1.4.4.6337.

3.9.3 High performance liquid chromatography (HPLC)

The HPLC analysis was carried out (Waters model no. 2690) on C 8 column (symmetry, 4.6mm×250 mm) by using methanol as mobile phase with flow rate of 1 mg ml⁻¹ for 10 min. and UV detector at 280 nm. The standard L-tyrosine, standard L-DOPA, cell free broth and reaction supernatant were prepared in HPLC grade water and injected in HPLC column (Rani et al., 2007).

3.9.4 Gas chromatography mass spectroscopy (GC-MS)

The GC-MS analysis was carried out with a QP2010 gas chromatography system coupled with mass spectrometer (Shimadzu). The cell free broth and buffer were extracted twice with equal volume of chloroform in separating funnel. The chloroform fraction was recovered and evaporated. The residue was dissolved in methanol and used for GC-MS analysis. The analysis was performed in the temperature programming mode at an ionization voltage 70 eV. Temperature of the Restek column (0.25 mm, 60 m; XTI-5) was kept at 80°C for initial 2 min., and raised up to 280°C with rate of 10°C min⁻¹, and held for 7 min. The temperature of injection port and the GC/MS interface were maintained at 280 and 290°C respectively. The flow rate for helium as a carrier gas was 1.0 ml min⁻¹ NIST spectral library stored in
the computer software (version 1.10 beta, Shimadzu) of the GC-MS was used for
detection of mass peaks (Rani et al., 2007).

3.9.5 Kinetic studies

Kinetic parameters for the bioconversion of L-tyrosine to L-DOPA with initial
and optimized reaction conditions were studied as previously reported (Ali et al.
2007). The kinetic parameters were, product yield coefficient \( (Y_{p/s}) = \frac{\text{mg of L-DOPA}}{\text{mg of substrate consumed}} \),
volumetric rate for substrate utilization \( (Q_s) = \frac{\text{mg of substrate consumed}}{\text{ml} \cdot \text{h}} \)
and specific substrate consumption rate \( (q_s) = \frac{\text{mg of substrate consumed}}{\text{mg of cells} \cdot \text{h}} \). All the experiments were carried out in
triplicates and data were analyzed by One-way analysis of variance (ANOVA) with
Tukey–Kramer multiple comparisons test, by using GraphPad InStat Software, and
readings were significant when \( P < 0.05 \).

3.10 Purification of tyrosinase from Bacillus sp. JPJ

3.10.1 Preparation of crude enzyme

The one ml of 6 h grown culture of Bacillus sp. JPJ was inoculated in tyrosine
broth and incubated for 18 h at 30°C at 120 rpm. This broth was centrifuged at 10,000
rpm for 20 min. and cell free broth was used as crude enzyme source.

3.10.2 Tyrosinase assay

The tyrosinase activity was determined by the previously described method
(Kandaswami and Vaidyanathan, 1973). The final assay concentration in 3 ml
reaction mixture contained 50 mM potassium phosphate (pH 7.4), 0.17 mM catechol,
0.070 mM and L-ascorbic acid equilibrated to 25°C. The \( \Delta A_{265} \) nm was monitored
until constant, and then 0.1 ml of the supernatant from the cell free broth was added.
The decrease in the \( \Delta A_{265} \) was recorded for 1 min. The \( \Delta A_{265} \) nm was obtained using
the maximum linear rate for both the test and control. One unit of tyrosinase activity
was equal to a \( \Delta A_{265} \) nm of 0.001 per min. at pH 7.4 at 25°C in a 3.0 ml reaction
mixture containing L-catechol and L-ascorbic acid. Protein content in the reaction
mixture and growth medium was determined using Lowry’s method (Lowry et al.
1951). The protein concentration of fractions collected during column
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chromatography was monitored by absorbance at 280 nm. The enzyme activity was calculated by following formula,

\[
\text{U/mg of enzyme} = \frac{\Delta A_{265/\text{min Test}} - \Delta A_{265/\text{min control}}}{(0.001) \times (\text{mg of enzyme per reaction mixture})}
\]

3.10.3 Ammonium sulphate precipitation

The partial purification of tyrosinase was carried out by using ammonium sulphate precipitation method. The cell free broth was added with 85% of ammonium sulphate with slow stirring at 4°C and kept overnight at 4°C. The mixture was centrifuged and precipitated protein was dialyzed against 20 mM sodium phosphate buffer (pH 7.4).

3.10.4 Ion exchange chromatography

The crude enzyme obtained after centrifugation was loaded on to a DEAE-cellulose column (15 ×1.8 cm) equilibrated with sodium phosphate buffer 50 mM (pH 7.4). The column was washed with the same buffer and the enzyme was eluted with 0.05 to 0.25 M NaCl linear gradient. Fractions containing tyrosinase activity were pooled and dialyzed against 20 mM sodium phosphate buffer (pH 7.4).

3.10.5 Polyacrylamide gel electrophoresis (PAGE) analysis

Protein with bacterial tyrosinase activity was evaluated with non denaturing PAGE electrophoresis (12% separating and 4% stacking gel), run on a slab gel unit (Genetech Laboratories). Composition of 12% separating gel was 40 ml stock acrylamide solution (30% acrylamide, 0.8% bisacrylamide), 25 ml Tris-HCl (pH 8.8) and 33.20 ml distilled water. Composition of 4% stacking gel was 5.36 ml stock acrylamide solution, 10 ml Tris-HCl (pH 6.8) and 24 ml distilled water. After degassing 0.75 and 0.2 ml (10%) ammonium persulfate and 50 and 40 µl TEMED was added for separating gel and resolving gel respectively. Sample buffer composed of (ml⁻¹) distilled water, 0.5 ml Tris-HCl buffer (pH 6.8), 0.5 g sucrose, 0.1 ml bromophenol blue (0.5% w/v). Electrode buffer composed of (g l⁻¹): Tris 6, glycine
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14.4 (pH 8.3). The gels were stained with coomassive brilliant blue R-250 at 0.1% (w/v) in methanol: acetic acid: water (v/v) (4:1:5) for 3 h at room temperature followed by distaining using methanol: acetic acid: water (v/v) (4:1:5). In order to confirm tyrosinase, the activity staining of the gel was also carried by using L-DOPA.

3.10.6 Optimum pH and temperature

The optimum pH was studied by assaying the activity of purified tyrosinase where reaction mixtures have various pH ranges from 1 to 12. The various buffers were used like HCl–KCl buffers (20 mM) of the pH range 1 to 2, citrate-phosphate buffer (20 mM) of the pH range 3 to 6 and potassium phosphate buffer having pH range 7 to 8, glycine-NaOH buffer of pH range 9-12. The optimum temperature was studied by assaying the activity of purified bacterial tyrosinase after keeping enzyme for 30 min. at the respective temperatures of 10 to 60°C with optimum pH

3.10.7 Effect of metal ions

The effects of different metal ions MnCl₂, MgCl₂, CuCl₂, ZnCl₂, KCl, NaCl and HgCl₂ on tyrosinase activity were studied. The enzyme was incubated for 15 min. at optimum temperature in the presence of above mentioned metal ions (0.5 mM concentration) with proper control and the results were reported as relative activity.

3.10.8 Substrate specificity and enzyme kinetics

The substrates specificity of Bacillus sp. JPJ tyrosinase was determined spectrophotometrically at the specific wavelength of each substrate. The substrates used were catechol, L-DOPA and L-tyrosine. These substrates were used at a concentration of 1 mM. Michaelis constant (Km) was determined by using L-tyrosine with concentrations 0.2 mM to 1 mM at optimum pH and temperature.