3. MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Substrates

2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid] (ABTS), guaiacol, syringaldazine (SGZ), were purchased from Sigma-Aldrich (USA). Catechol, p-phenylenediamine (PPD), pyrogallol, tyrosine, ferulic acid, syringaldehyde, vanillin, vanillic acid were purchased from Hi-Media (India).

3.1.2 Reagents for polyacrylamide gel electrophoresis (PAGE)

Acrylamide, bis-acrylamide, ammonium persulphate (APS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich (USA). Coomassie brilliant blue R-250 was purchased from Hi-Media (India)

3.1.3 Chromatography gel Matrices

DEAE-Cellulose and sephadex G-100 was purchased from Sigma-Aldrich (USA) of molecular grade.

3.1.4 Protein marker

Prestained protein ladder (BLUeye) was purchased from Gene DireX, Inc

3.1.5 Grey hair

Grey hair of unknown person were procured from salon

3.1.6 Cell lines

Following cell lines were purchased from National Center for Cell Science (NCCS) Pune, India

- Raw 264.7 macrophage cell line
- SK-1 skin epithelial cell line

Other chemical such as ethylenediaminetetraacetic acid (EDTA), sodium azide, sodium citrate and media components were purchased from Hi-Media (India). Cysteine monohydrate, β-mercaptoethanol, inducer veratryl alcohol were purchased from Sigma-Aldrich (USA).

3.1.7 Instruments used

Major instruments used in this study were scanning electron microscope (SEM) [Model JSM6100 (Jeol) USA], FT-IR/FT-FIR [Perkin Elmer-spectrum 400], UV-3000 spectrophotometer [Lab India], polyacrylamide gel electrophoresis-mini PROTEAN tetra system [Bio-red] and small angle X-ray scattering- SAXSpace instrument [Anton Paar GmbH, Austria].

3.1.8 Media

3.1.8.1 M162 basal medium	**(L^{-1})**
*M162 medium (10X)	100ml
Yeast extract	2.0g
Tryptone	2.0g
$CuSO_4.5H_2O$ (from 100mM stock)	1.0ml
Phosphate buffer (pH 7.0; 1M)	100ml

Solid medium consisted of M162 basal medium + 2% agar

**** M162 medium (10X)***	**(L^{-1})**
#Micronutrient solution (10X)	1.0ml
Calcium sulphate dehydrate	0.4g
Nitriloacetic acid	1.0g
Magnesium chloride hexahydrate	2.0g
Ferric citrate (0.01M)	5.0ml

#Micronutrient solution (10X)	**(L^{-1})**
Boric acid	5.0g
Cobalt chloride hexahydrate	450mg
Copper sulphate pentahydrate	250mg

Manganese sulphate monohydrate	2.28g
Sodium molybdate dihydrate	250mg
Zinc sulphate heptahydrate	5.0g
Concentrated Sulphuric acid	5.0ml

3.1.8.2 Luria-Bertani (LB) medium (Sambrook and Russell, 2001)

Casein enzyme hydrolysate	10 ($g1^{-1}$)
Sodium chloride	5.0 ($g1^{-1}$)
Yeast extract	5.0 ($g1^{-1}$)

LB agar consisted of LB broth and 2.0% agar.

3.1.9 Solutions and Buffers

3.1.9.1 Stock solutions

- **HCl:** 1N solution was made by mixing 8.47ml HCl in distilled water and final volume was made to100ml.
- **KCl:** 1M solution was made by dissolving 7.46g in distilled water and the final volume was made to 100 ml.
- **Glycine:** 1M solution was made by dissolving 7.5g glycine in distilled water and final volume was made to100ml.
- **Sodium acetate:** 1M solution was made by dissolving 8.2g sodium acetate in distilled water and final volume was made to100ml.
- **Acetic acid:** 1M solution of acetic acid was prepared by adding 5.775ml acetic acid in 94.225ml distilled water.
- **Disodium hydrogen phosphate (Na_2HPO_4):** 1M solution was made by dissolving 14.1g in distilled water and final volume was made to100ml
- **Sodium bicarbonate (NaH_2CO_3):** 1M solution was made by dissolving 8.4g NaH_2CO_3 in distilled water and final volume was made to100ml
- **Sodium dihydrogen phosphate (NaH_2PO_4):** 1M solution was made by dissolving 11.9g in distilled water and final volume was made to100ml.

- **Sodium carbonate (Na_2CO_3):** 1M solution was made by dissolving 10.5g Na_2CO_3 in distilled water and final volume was made to100ml.
- **Tris-HCl:** 1M solution was made by dissolving 15.7g Tris-HCl in distilled water and the final volume was made to 100ml.
- **Tris base:** 1M solution was made by dissolving 12.1g tris base in distilled water and final volume was made to100 ml.
- **NaCl:** 1M solution was made by dissolving 5.8g NaCl in distilled water and the final volume was made to 100ml.
- **NaOH:** 1M solution was made by dissolving 4.0g NaOH in distilled water and final volume was made to100ml.
- **$CuSO_4.5H_2O$:** 100mM solution of copper sulphate was prepared by adding 1.2484g in distilled water and final volume was made to 100ml.
- **Ethylenediamdietertraacetic acid (EDTA):** 0.5M solution was made by dissolving 14.6g EDTA in distilled water and final volume was made to100 ml.
- **SDS:** 20% SDS solution was made by dissolving 20.0g SDS in distilled water and final volume was made to100ml.

3.1.9.2 Buffers

In this study different buffers were prepared according to methods given by Gomori, (1955) in Methods of Enzymology. Stock solutions of acidic and basic components were mixed to get the desired pH.

HCl-KCl buffer:

(A) 1N stock solution of HCl

(B) 1M stock solution of KCl.

Appropriate volume of A and B were mixed to obtain final pH range of 1.0 - 2.0.

Glycine-HCl buffer:

(A) 1M stock solution of glycine

(B) 1N stock solution of HCl

Appropriate volume of A and B were mixed to obtain final pH range of 2.5- 3.5.

Acetate buffer:

(A) 1M stock solution of sodium acetate

(B) 1M stock solution of acetic acid

Appropriate volume of A and B were mixed to obtain final pH range of 4.0-5.5.

Phosphate buffer:

(A) 1M stock solution of sodium dihydrogen phosphate

(B) 1M stock solution of disodium hydrogen phosphate

Appropriate volume of A and B were mixed to obtain final pH range of 6.0- 7.5.

Tris-HCl Buffer:

(A) 1M stock solution of tris base

(B) 1N stock solution of HCl

Appropriate volume of A and B were mixed to obtain final require pH.

Carbonate-bicarbonate buffer:

(A) 1M stock solution of sodium carbonate

(B) 1M stock solution of sodium bicarbonate

Appropriate volume of A and B were mixed to obtain final pH of 9.5 and 10.0.

3.1.9.3 Solutions for DNA analysis (Sambrook and Russell, 2001)

DNA gel loading dye

• Bromophenol blue	2.5 mg
• Glycerol	6.0 ml
• EDTA	50 mM (from stock solution)

Final volume was made to 10 ml with distilled water.

Ethidium bromide stock solution

• Ethidium bromide	10.0 mg
• Distilled water	1.0 ml

Solution was stored in dark bottle in refrigerator.

Tris-EDTA buffer (100 ml)

Ingredients	Final required concentration	Amount of stock solution
• Tris-Cl (pH 8.0)	10 mM	1.0 ml (from 1M stock)
• EDTA (pH 8.0)	1.0 mM	200 µl (from 0.5M stock)
• Distilled water	--	98.8 ml

Sodium Chloride-Tris EDTA buffer (100 ml)

Ingredients	Final required concentration	Amount of stock solution
• Tris-Cl (pH 8.0)	10 mM	1.0 ml (from 1M stock)
• EDTA (pH 8.0)	1.0 mM	200 µl (from 0.5M stock)
• NaCl	100 mM	10 ml
• Distilled water	--	88.8 ml

Tris-Acetate-EDTA buffer (50X)

• Tris base	242 g
• Glacial acetic acid	57.1 ml
• EDTA (0.5 M, pH 8.0)	100 ml

Final volume was made to 1000 ml with distilled water.

Saturated phenol solution (Sambrook and Russell, 2001)

8-hydroxyquinoline was added to distilled phenol at a final concentration of 0.1% (w/v) and mixed till dissolved, equal volume of 0.5 M Tris-HCl (pH 8.0) was added, mixed for 15 min, the phases were allowed to separate, the top layer siphoned and discarded. This step was repeated twice. Equal volume of 0.1M Tris-HCl (pH 8.0) was added to the phenol and the above procedure was repeated till the pH of the aqueous phase reached 8.0. 1 cm layer of 0.1M Tris-HCl (pH 8.0) was left over the phenol and stored in dark brown bottle at 4°C.

Phenol: Chloroform: Isoamylalcohol solution

Saturated phenol was mixed with chloroform and isoamyl alcohol in the ratio of 25:24:1.

3.1.9.4 Solutions for protein estimation (Lowry et al., 1951)

Reagent A

Sodium potassium tartrate	1.0g
Copper sulphate	0.5g
Distilled water	100ml

Reagent B

Sodium carbonate	2.0g
Sodium hydroxide	0.4g
Distilled water	100ml

Reagent C (working reagent)

Reagent A	1ml
Reagent B	49ml

Freshly prepared at the time of use

Reagent D

Folin ciocalteau's reagent	10.0ml
Distilled water	10.0ml

Freshly prepared at the time of use; kept in dark

3.1.9.5 Solutions for poly acrylamide gel electrophoresis (PAGE)

Polyacrylamide mix

Acrylamide	30g
Bis-acrylamide	0.8g

Final volume was made to 100ml with distilled water and filtered through whatman filter 1.

Ammonium per sulphate

Ammonium persulphate	100 mg
Distilled water	1.0 ml

TEMED

N,N,N',N'- tetramethylethylenediamine was added as per the protocol for PAGE as explained in section 3.3.5.1.

Lower Tris buffer (1.5 M Tris-Cl, pH 8.8) (4X) for PAGE

Tris base	18.17 g
HCl	1N (from stock solution)

Tris-base was dissolved in 70 ml distilled water. 1N HCl was used to adjust the pH to 8.8. The final volume was made to 100 ml with distilled water.

Upper Tris buffer (0.5 M Tris-Cl, pH 6.8) (4X) for PAGE

Tris base	6.05 g
HCl	1N(from stock solution)

Tris-base was dissolved in 70 ml distilled water. 1N HCl was used to adjust the pH to 6.8. The final volume was made to 100 ml with distilled water.

Tank buffer (0.1M Tris –glycine Buffer)

Tris	0.3g
Glycine	1.44g
SDS (10%)	1.0ml

Final volume was made to 100ml with distilled water

Sample buffer (3X) for SDS -PAGE

Tris-HCl (0.5M, pH 6.8)	2.4ml
Bromophenol blue	6.0mg
SDS (20%)	3.0ml
Glycerol	3.0ml

Final volume was made to 10ml

Staining solution

Coomassie Brilliant Blue	200mg
Methanol	40ml
Acetic acid	10ml
Distilled water	50ml

Destaining solution

Methanol	40ml
Acetic acid	10ml
Distilled water	50ml

All the reagents except the buffers were kept in brown bottles at 4°C and brought to room temperature 1 hour prior to use.

3.2 METHODS

3.2.1 Collection of soil samples

Samples were collected from different environmental sites such as effluent from dyeing industry of Ludhiana Punjab. Soil from landfill areas of Chandigarh and wood decaying sites of the forest area near Sukhna Lake, Chandigarh and Himachal Pradesh.

3.2.2 Isolation of laccase producing bacteria

Samples were enriched in M162 medium (prepared as discussed in section 3.1.8.1) containing *****2mM guaicol. Appropriate dilution of the enriched samples was plated on M162 agar plates containing 2mM guaiacol. The plates were incubated at 37°C for 48-72h. Isolates showing colonies of reddish brown color were selected.

*****20µl of guiacol from sigma (8M) in $100ml^{-1}$ medium

3.2.3 Screening of isolates

Extracellular laccase producing bacteria

Selected bacterial isolates were screened for the production of extracellular laccase. Selected isolates were grown in M162 medium and 0.1% of 24h old bacterial culture was inoculated in M162 basal medium. After 48 h, the culture supernatant was obtained by centrifugation at 7286g for 15min and was used as crude extracellular enzyme. Presence of laccase activity in crude enzyme was evaluated by doing enzyme assay with syringaldazine (SGZ) as explain in section 3.2.7. The strains exhibiting extracellular laccase activities were selected.

Evaluation of true laccase activity

Presence of true laccase activity in the crude enzymes was confirmed by doing**:**

a) Oxidation of ABTS and SGZ (as explain in section 3.2.7)

b) Evaluation of effect of laccase specific such as β-merceptoethanol, sodium azide and cysteine (as explain in section 3.3.11)

c) Evaluation of tyrosinase activity (as explain in section 3.2.7)

3.2.4. Selection of the isolate

Oxidation of p-phenylenediamine (PPD)

Enzymes from strains showing extracellular enzyme activity were tested for the oxidation of dye PPD (p-phenylenediamine) in alkaline range by doing the assay as explain in section 3.2.7

Temperature profile

Activity of crude extracellular laccase from the selected bacterial strains was analyzed in the temperature range of 35-70°C under standard assay conditions using SGZ and PPD as explained in section 3.2.7. The enzyme from the isolate showing activity in wide temperature range was selected.

pH profile

pH profile of laccase from selected bacterial strains was analyzed by doing enzyme assay as explained in section 3.2.7 with substrates of SGZ and PPD in the pH range of 6.0-9.5 by using buffers 0.1 M phosphate buffer (pH 6.0-7.5), 0.1M Tris-HCl buffer (pH 8.0-9.0) and 0.1M carbonate-bicarbonate buffer (pH 9.5)

The strain producing more enzyme yield and enzyme giving effective oxidation of PPD at alkaline pH and having temperature optima in wide range was selected for further work.

3.2.5 Evaluation of hair dyeing with PPD oxidized by enzyme from the selected strain

Dyeing of hair was done by mixing PPD and crude enzyme from the selected strain as explained in section 3.5. Hair coloring performance was evaluated by comparing the dyed hair with that of grey undyed hair.

3.2.6. Identification of selected strain

3.2.6.1 Morphological, physiological and biochemical characterization

The morphological, biochemical and physiological characterization of the selected strain was carried out in nutrient agar medium as per Bergey's Manual of Determinative Bacteriology (Kampfer and Glaeser, 2012). Gram reaction was determined by Gram staining. Cell morphology and arrangement were examined by light microscopy. All the biochemical tests were performed as per standard methods (Clarke and Cowan, 1952). Growth of the organism was checked at different pH (6.0- 10.0) and temperature (20-45°C).

Screening for industrially important enzymes

Xylanase: Selected Isolate was grown on nutrient agar containing 0.5% xylan to check the presence of xylanase. The presence of the enzymes was evaluated by zone of clearance around the colonies on staining with 0.2% congo red and then destaining by 1N NaCl.

Protease: Selected Isolate was grown on nutrient agar containing 0.5% casein to check the presence of protease. The presence of the enzymes was evaluated by zone of clearance around the colonies on staining with with 1N HCl.

Mannanase: Selected Isolate producing laccase was grown on nutrient agar containing 0.5% LBG to check the presence of mannanase. The presence of the enzymes was evaluated by zone of clearance around the colonies on staining with 0.2% congo red and then destaining by 1N NaCl.

Lipase: Selected Isolate was grown on nutrient agar containing 0.5% trybutyrin to check the presence of lipase. The presence of the enzymes was evaluated by zone of clearance around the colonies.

3.2.6.2 Phylogenetic analysis

Genomic DNA extraction (Ausubel et al., 1992)

25 ml of LB medium was inoculated with 1% of overnight grown cells. The flask was incubated at 37°C for 4 h at 150 rpm. The cells were centrifuged at 7286g at 4°C for 10 min and resuspended in 2.5ml of ice cold STE buffer. 300µl of lysozyme (5mgml^{-1} in TE buffer) was added and incubated at 37°C for 15 min. 250µl of SDS was added and incubated at 37°C with intermittent shaking. To this, 20µl of RNAase (10mgml^{-1}) was added and incubated at 37°C for 1h. The sample was mixed with equal volumes of saturated solution of phenol:choloroform:isoamyl alcohol (25:24:1) and the aqueous phase was extracted following centrifugation at 7286g for 15 min at 4°C. The extracted aqueous phase was mixed with 2 volume ethanol and 0.1 volume of 3M sodium acetate and kept in freezer overnight. Precipitated DNA was pelleted by centrifugation at 7286g for 20min. The pellet was then rinsed with 70% ethanol, thoroughly dried in an incubator at 37°C and resuspended in 200µl TE buffer.

DNA concentration

Concentration of genomic DNA was estimated by agarose gel electrophoresis and by comparing it with standard concentrations of DNA and also by measuring absorbance at 260 nm. Its purity was checked by measuring ratio of absorbance at 260 nm and 280 nm.

16S rRNA sequencing (Mullis and Faluna, 1987)

16S rRNA sequencing was carried out by using method (Mitra and Roy, 2010). A 1386 base pair long rDNA fragment of the genomic DNA was amplified using universal primer.

EzTaxon analysis (Chun et al., 2007)

The sequence obtained was analyzed by EzTaxon search to identify sequences from the database that resembled query sequence.

Construction of phylogenetic tree

Sequences with high query coverage were selected for phylogenetic analysis. Multiple sequence alignment was done using multiple sequence alignment (MultAlin) software version 5 with hierarchical clustering. Molecular Evolutionary Genetics Analysis (MEGA) software version 7 was used for the construction of phylogenetic tree. The evolutionary distance was calculated according to Kimura two parameter model (Kimura, 1980) and maximum composite likely hood method (Tamura et al., 2007). Clustering was done with neighbour joining (Saitou and Nei, 1987) and maximum parsimony (Eck and Dayhoff, 1966) methods. Bootsrap values based on 1000 replications were determined (Felsenstein, 1985).

The selected Isolate was identified as strain of *Bacillus subtils* and was designated as *Bacillus subtils* DS.

Sequence submission

The 16S rRNA sequence of the *Bacillus subtilis* DS was submitted to Genebank database with an accession number MF359736.

Maintenance of strain

Bacillus subtilis DS was deposited in Microbial Type Culture Collection (MTCC) CSIR-IMTECH, Chandigarh with MTCC Number 12618.

In addition, strain was maintained in 25% glycerol at -70°C and with routine culturing on M162 basal medium plates.

3.2.7. Laccase estimation

Assay procedure

Laccase activity was determined by doing enzyme assay in reaction volume of 1ml as explained in Fig. 3.1. Optimum reaction pH, reaction time and optical density of measurement was varied from substrate to substrate as explain in Table 3.1.Substrates were used at a final concentration of 2mM in the reaction mixture (using 100µl of substrate from a stock of 20mM)

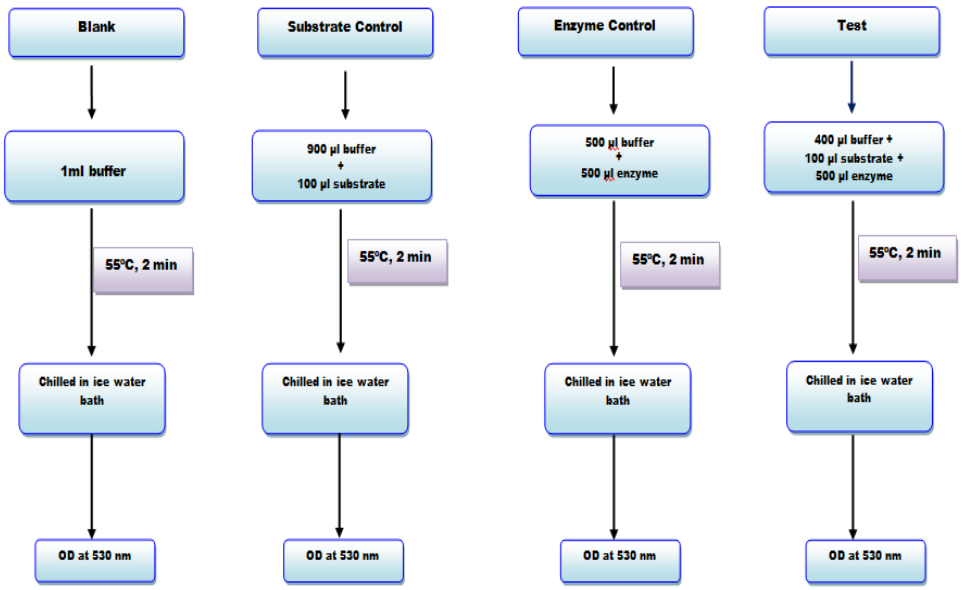

Fig. 3.1 Assay procedure for determination of laccase activity*#

*Enzyme reaction for PPD was done by following the same procedure a in Fig. 3.1. However assay was carried out in reaction volume of 4 ml using 1ml of substrate+ 1ml of enzyme +2ml of buffer. Substrates were used at a final concentration of 2mM in the reaction mixture (using 1ml of substrate from a stock of 8mM)

Most of the optimization and enzyme characterization was done using SGZ as substrate. Assay for other substrates was done where ever it was required

Table 3.1: Substrates and their assay parameter

Substrate	ε (M^{-1} cm^{-1})	λ_{max} (nm)	Assay pH	Reaction time (min)
ABTS	36000	420	3.5	2
SGZ	64000	525	6.5	2
Tyrosine	12000	278	-	5
PPD	14685	450	8.5	10

Enzyme units

Laccase activity was expressed in international units (IU). One unit of enzyme activity was defined as micromoles of product formed from substrate per minute by one ml of the enzyme under the assay conditions. Determination of laccase activity

$$Laccase\ activity\ (IUml^{-1}) = \frac{OD_{net} * 10^6 * \text{D.F}}{\varepsilon * \text{V} * \text{T}}$$

……Eq. (3.1)

Where,

ε = Extinction coefficient of the substrate (64000 $M^{-1}cm^{-1}$)

OD_{net} = Net optical density (OD_{net} = Test- (EC+SC)

T= Incubation time of reaction in min

D.F. = Dilution factor of the enzyme

(Initial dilution of enzyme+ total reaction volume/volume of enzyme added)

V = Volume of enzyme used

3.2.8 Hyper production of laccase from *Bacillus subtilis* DS

Laccase production from *Bacillus subtilis* DS was optimized by one variable at a time method (OVAT) as well as by statistical methods using Plackett-Burman (PB) and Response Surface Methodology (RSM).

3.2.8.1 Optimization of laccase production by one variable at a time method (OVAT)

Influence of different physiochemical factors on enzyme production was carried out by varying one factor at a time keeping the other constant. The optimized condition in each step was kept constant in subsequent steps.

Time profile of growth and enzyme production

Growth profile of *Bacillus subtilis* DS was studied by inoculating 1% of 24h old seed culture in M162 basal medium and incubating it at 30°C, 150rpm. Samples were withdrawn at regular time intervals and growth were measured in term of; colony forming unit (cfu) by plating appropriate dilution of the culture on M162 basal agar plate and optical density at 600nm. Extracellular activity was assayed using cell free supernatant according to the standard assay procedure (given in 3.2.7).

Production pH

The optimum pH for laccase production was determined by making M162 basal medium in the buffers of different pH (6.0-9.0). Medium was inoculated with 1% of 24h old seed culture and incubated at 30°C, 150rpm for 96h. After incubation, the culture was centrifuged at 7286g for 10min and cell free supernatant obtained was assayed for laccase activity.

Incubation temperature

Temperature optimum for enzyme production was determined by inoculating the M162 basal medium with 1% of 24h old seed culture and incubated in the temperature range of 20-42°C for 96h at 150rpm. After incubation, the culture was centrifuged at 7286g for 10min and cell free supernatant obtained was assayed for laccase activity.

Effect of inoculum density

Optimum inoculum concentration for enzyme production was determined by inoculating M162 basal medium with different concentration (0.1-5.0%) of 24h old seed culture and incubating at 37°C, 150rpm for 96h. After incubation, the culture was centrifuged at 7286g for 10min and cell free supernatant obtained was assayed for laccase activity.

Effect of different carbon sources

Different carbon sources *viz.* maltose, starch, lactose, fructose, mannitol, glucose, cellulose, xylose, galactose, raffinose, and arabinose were added in M162 basal medium at a concentration of 0.2%. The medium was inoculated with 1% of 24h old seed culture and kept at 37°C, 150 rpm for 96h. After incubation, the culture was centrifuged at 7286g for 10min and cell free supernatant obtained was assayed for laccase activity.

Effect of different concentration of copper sulphate

$CuSO_4.5H_2O$ was added in M162 basal medium at a concentration of 0-500µM*. The medium was inoculated with 1% of 24h old seed culture and kept at 37°C, 150rpm for 96h. After incubation, the culture was centrifuged at 7286g for 10min and cell free supernatant obtained was assayed for laccase activity.

* This $CuSO_4.5H_2O$ was over and above than what was present in M162 basal medium as a component of M162 micronutrient solutions

Effect of different nitrogen sources

M162 basal medium containing 0.2% yeast extract+0.2% tryptone as nitrogen source was taken as a control. In test flasks nitrogen source of M162 basal medium (0.2% yeast extract+0.2% tryptone) was replaced with different nitrogen sources *viz* yeast extract, tryptone, beef extract, gelatin, glycine, urea and peptone at a concentration of 0.4%. The medium was inoculated with 1% of 24h old seed culture and kept at 37°C, 150rpm for 96h. After incubation, the culture was centrifuged at 7286g for 10min and cell free supernatant obtained was assayed for laccase activity

Effect of different metal ions

To find out the effect of metal ions. Stocks solution of salt of different metal viz $Al_2SO_4.16H_2O$, $CaSO_4.2H_2O$, $CoSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $NiSO_4.5H_2O$, and $ZnSO_4.7H_2O$ were prepared and added in M162 basal medium at a concentration of 100µM. The medium was inoculated with 1% of 24h old seed culture and kept at 37°C, 150rpm for 96h. After incubation, the culture was centrifuged at 7286g for 10min and cell free supernatant obtained was assayed for laccase activity.

Effect of different concentration of xylose

Effect of xylose concentration on enzyme production was determined by varying xylose concentration from 0-1.6% in the M162 basal medium. The medium was inoculated with 1% of 24h old culture and incubated at 37°C, 150rpm for 96h. After incubation, the culture was centrifuged at 7286g for 10min and cell free supernatant obtained was assayed for laccase activity.

Effect of different $MnSO_4$ concentration

To find out optimum $MnSO_4$ concentration for enzyme production, varying concentration of $MnSO_4$ (50-300 µM) was added to the M162 basal medium. The medium was inoculated with 1% of 24h old culture and kept at 37°C, 150 rpm for 96h. After incubation, the culture was centrifuged at 10,000 rpm for 10 min and cell free supernatant obtained was assayed for laccase activity.

3.2.8.2. Statistical optimization

Selection of significant parameters by Plackett-Burman

The variables which highly influenced the laccase yield were evaluated by Plackett-Burman (PB) design of Design expert 8.0.7.1(Stat-Ease, Inc., Minneapolis, USA).Based on literature search and OVAT results, 19 different parameters: A: $FeSO_4.7H_2O$: B: $CuSO_4.5H_2O$: C: $MnSO_4.H_2O$: D: $MgSO_4.7H_2O$: E: $CoSO_4.7H_2O$: F: $Al_2SO_4.16H_2O$: G: $NiSO_4,H_2O$: H: yeast extract: J: xylose: K: 2,6,xylidine: L: catechol: M: ferulic acid: N: tryptone: O: vanillic acid: P: vanillin: Q: methanol: R: $CaSO_4.7H_2O$: S: $(NH_4)SO_4$: T: ethanol were evaluated by PB design. Concentration levels were decided on the basis of literature reports and OVAT results on laccase production (Table 3.2). Each factor was investigated at two levels, high (+) and low (-).

Table 3.2: Different level of variables for screening by Plackett-Burman design

Factor	Name	Unit	Low Level	High Level
A	$FeSO_4.7H_20$	µM	0	100
B	$CuSO_4.5H_2O$	µM	50	200
C	$MnSO_4.H_2O$	µM	50	300
D	$MgSO_4.7H_2O$	µM	0	100
E	$CoSO_4.7H_2O$	µM	0	100
F	$Al_2SO_4.16H_2O$	µM	0	100
G	$NiSO_4,H_2O$	µM	0	100
H	Yeast Extract	%	0.2	1
J	Xylose	%	0	0.4
K	2,6,xylidine	µM	0	100
L	Catechol	µM	0	100
M	Ferulic acid	µM	0	100
N	Tryptone	%	0	0.2
O	Vanillic acid	µM	0	100
P	Vanillin	µM	0	100
Q	Methanol	%	0	2
R	CaSO4.7H20	µM	0	100
S	(NH4)SO4	%	0	0.2
T	Ethanol	%	0	2

A design of total 20 experiments was generated (Table 4.2.2). Experiments were carried out in triplicates. The average of enzyme activity obtained was taken as response. The effect of individual factors on enzyme activity was calculated according to the following equation:

$$E_i = \frac{\sum P_{i+} - \sum P_{i-}}{N}$$

……Eq. (3.2)

where Ei is the effect of parameter i under study, Pi+ and Pi- are responses (enzyme activity) of trials at which the parameter was at its high and low level respectively, and N is the total number of trials.

Factors were analyzed by using half-normal probability plot or pareto chart of model. In case of half normal probability chart, factor were opened from biggest to smaller effect i.e. from right to left until the line runs through points nearest zero. Alternatively, on the pareto chart factor were opened from biggest to smaller effect i.e. from left to right until all other effects fall below the bonferroni and/or t-value limit.

Factors showing highest positive effects were selected for optimization using central composite design of response surface methodology.

Response Surface Methodology (RSM)

Variables which showed high positive effect on the production of laccase were optimized using central composite design (CCD) of RSM. Four factors yeast extract, tryptone, vanillic acid, and $MgSO_4$. $7H_2O$ which were highly influential variables were selected for CCD.

CCD was employed using Design Expert software, variables were studied at five different levels, low (-2, -1), medium (0) and high (+1, +2) (Table 3.3). The experimental plan consisted of 30 trials (Table 4.2.4).

Table 3.3 Experimental range and levels of independent factors for RSM design

Codes	Factors	Units	Levels				
			-2	**-1**	**0**	**+1**	**+2**
A	Yeast extract	%	0.4	0.8	1.2	1.6	2.0
B	Vanillic acid	µM	50	100	150	200	250
C	Tryptone	%	0.5	0.2	0.35	0.50	0.65
D	$MgSO_4.7H_2O$	µM	50	100	150	200	250

Regression analysis was performed on the data obtained. A second-order polynomial equation was used to fit the data by multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

$$Y = \beta 0 + \Sigma \beta i\ Xi + \Sigma \beta ii\ Xi2 + \Sigma \beta ij\ Xi\ Xj$$

……. Eq. (3.3)

where Y represents response variable (laccase activity), β0 is the interception coefficient, βi, coefficient of the linear effect, βii, the coefficient of quadratic effect and βij, the coefficient of interaction effect.

Validation experiments

To validate the statistical model, experiment was carried out using the optimal conditions predicted by the model and response (laccase yield) was measured as described earlier and compared to the predicted values. Each experiment was done in triplicates and data presented as mean ± SD.

3.2.9 Cost evaluation of laccase production from *Bacillus subtilis* DS

Tentative cost of the laccase production was evaluated using method given by Osma et al., (2011).

The cost involved is divided in three parts:

(i) *Cost of chemicals* = C_{CM},

(ii) *Cost of equipments* = C_{Eq}

(iii) *Cost of operating* = C_{op}.

For estimate the cost of chemicals (C_{CM}), most of the market prices for reagents or chemicals were obtained from Sigma-Aldrich (USA) and Hi-media (India).

The costs of equipment and operation (C_{Eq} and C_{Op}) were estimated by taking a standard autoclave, centrifuge and lab incubator as model equipments. Their lifetime (LT) was determined equal to their warranty time i.e two years for autoclave and one year each for the incubator and centrifuge. Capacity and power consumption of these equipments was

calculated as per manufacturer detail. The equipment cost was calculated as per equation 3.4 as follows:

$$C_{Eq} = C_I + C_A + C_C = \frac{D_{max}/365}{Cap_I}\left(\frac{P_I}{LT_I} + \frac{P_A}{LT_A} + \frac{P_C.Cap_C}{LT_C}\right)$$

….Eq. (3.4)

Where,

(C_A), (C_C) and (C_I) = autoclave, centrifuge and lab incubator respectively

D_{max} (days) = incubation period for obtaining maximum laccase activity;

Cap_I = capacity of the incubator;

P_I, P_A and P_C = prices of incubator, autoclave and centrifuge respectively;

LT_I, LT_A and LT_C = life times of incubator, autoclave and centrifuge respectively.

The incubator taken in case of submerged fermentation was the orbital temperature controlled shaking incubator. Operating cost was estimated as follows:

$$C_I = \frac{E_I.(24.D_{max})}{Cap_I}$$

…. Eq. (3.5)

$$C_A = \frac{E_A}{Cap_A}$$

…. Eq. (3.6)

$$C_C = \frac{E_C}{Cap_C}$$

…. Eq. (3.7)

The cost of laccase production ($Cost_{lac}$) was calculating as the ratio between the sum of the culture medium, equipment and operating costs and the produced enzyme activity (equation 3.8). The latter was calculated as the maximum laccase activity (Lac_{max}) multiplied by the volume of the extracted crude enzyme (Lac_V) that represents the maximum laccase unit.

$$Cost_{lac} = \frac{C_{CM} + C_{Eq} + C_{Op}}{Lac_{max}.Lac_V}$$

…. Eq. (3.8)

The cost of manpower was not considered in the analysis, since it will depend on the automation and monitoring of the process, the experience of the worker and the volume of laccase to be produced.

3.3 Purification

The laccase from *Bacillus subtilis* DS was purified as per standard protein purification procedures including various steps like ammonium sulphate precipitation, ion filtration chromatography and gel exchange chromatography. After each step of purification, enzyme activity, protein content and volume of enzyme was estimated.

3.3.1 Ammonium sulphate precipitation

Enzyme production was done under standardized conditions (section 4.2.1.3). Precipitation of protein was done by first excluding the proteins with 40% ammonium sulfate concentration and then salting out the proteins containing desired enzyme (laccase) [form the supernatant of 40% ammonium sulphate precipitated enzyme] with 80% ammonium sulfate. The precipitated proteins were harvested by centrifugation (7286g) for 15min at 4°C, and collected pallet was suspended in 3 ml phosphate buffer (100mM, pH 7.0) and dialyzed against the same buffer.

3.3.2 Ion exchange chromatography

DEAE-cellulose matrix was activated as per manufacturer instructions. The enzyme from ammonium sulphate precipitation (40-80%) was applied to a DEAE-cellulose column (12 x 1.2 cm^2). The bound laccase was eluted with 0–1.0M NaCl gradient at a flow rate of 0.8ml min^{-1} in fractions of 3ml each. The active fractions were pooled and concentrated by polyethylene glycol (PEG) (Degerli and Akpinar, 2001).

3.3.3 Gel filtration chromatography

Further purification of the laccase was done by gel filtration chromatography. The 3ml of sample was loaded on a sephadex G-100 column (30 x 1.5 cm^2) equilibrated with phosphate buffer (100mM, pH7.0) and the protein (laccase) was eluted at a flow rate of 1.0ml min^{-1}. 30 fractions of 3ml each were collected. The active fractions were pooled and concentrated by PEG.

3.3.4 Determination of laccase activity

Laccase activity after each step was estimated according to the method given in section 3.2.7.

3.3.4.1 Protein estimation

Quantity of protein present in the collected fractions during chromatography was estimated by measuring the absorbance at 280 nm.

Total protein in pooled fractions was estimated by Lowry method (Lowry et al., 1951) as explained in Fig. 3.2.

1ml sample of appropriate dilution was taken. To this, 5ml of freshly prepared alkaline copper sulphate reagent (reagent C; prepared as given in section 3.1.4.4) was added. The solution was mixed properly with vortex and kept for 10 min incubation at room temperature. Now, added 0.5ml of reagent D (folin ciocalteau's reagent as given in section 3.1.4.4).The mixture was incubated at room temperature under dark condition for 30min. Blank was taken by adding distilled water instead of sample. Absorbance was taken at 600nm. Bovine serum albumin was used as standard in the range of 100-1000µgml^{-1} for estimation of protein content (Appendix 1).

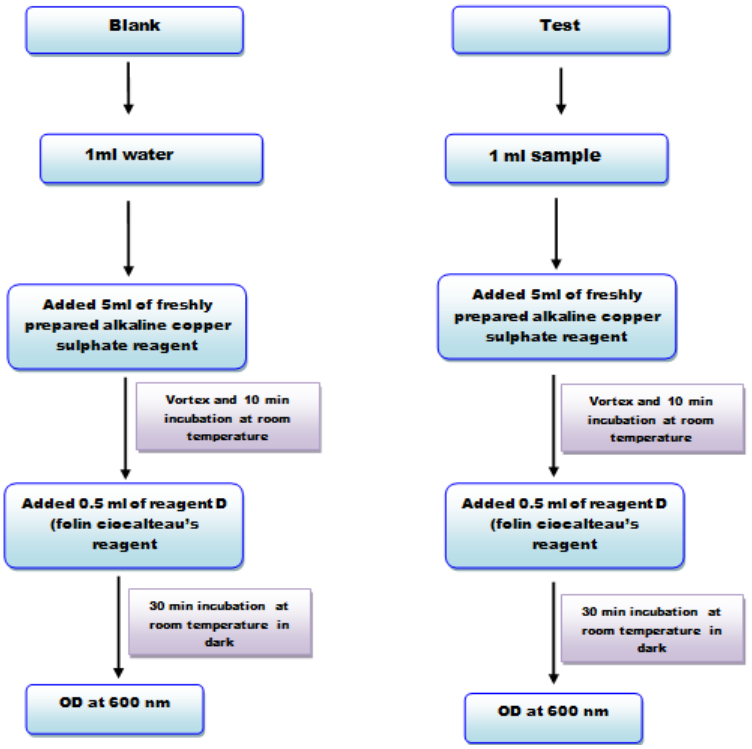

Fig. 3.2 Assay procedure of protein estimation by Lowry method

3.3.4.2 Specific activity

Specific activity of the protein was estimated by following formula:

$$Specific\ activity\ (U\ mg^{-1}) = \frac{Total\ enzyme\ units}{Total\ protein}$$

…Eq. (3.9)

3.3.4.3 Fold purification

At each step fold purification was calculated by following formula:

$$Fold\ Purification = \frac{Specific\ activity\ of\ current\ step}{Specific\ activity\ in\ step\ 1}$$

…Eq. (3.10)

3.3.4.4 Protein yield

At each step protein yield was calculated by following formula:

$$Yield = \frac{Enzyme\ activity\ of\ sample\ in\ current\ step}{Enzyme\ activity\ at\ step\ 1} * 100$$

… Eq. (3.11)

3.3.5 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed using 12% (w/v) gel, according to the method given by Laemmli (Laemmli, 1970). The electophoretic plates were washed, cleaned and dried before use. The plates were held in the gel casting stand with spacers (0.5 mm) sandwiched on both the sides and comb at the top end. The separating gel solution was poured carefully upto 2.0cm below the comb and overlaid with water to prevent diffusion of air into the gel. After polymerization of separating gel; water was removed and stacking gel solution was poured upto the brim of the plates. After the polymerization of stacking gel, combs were taken out and wells were washed with water to remove any unpolymerized residues.

3.3.5.1 Composition of gel

Component	Stacking gel (5%)	Separating Gel (10%)
(30%) Acrylamide Mix	650 µl	1.7 ml
Lower Tris buffer (pH 8.8)	-	1.3 ml
Upper Tris buffer (pH 6.8)	1.25ml	-
Ammonium per sulphate	50 µl	50 µl
TEMED	5 µl	2 µl
SDS	20 µl	50 µl
Distilled water	3.05 ml	1.95 ml

3.3.5.2 Loading

The prepared plates were kept in the electrode assembly provided with the apparatus. The upper and lower tanks were filled with tank buffer. Sample was mixed with 3X gel loading dye (3:1), kept in water bath (100°C) for 3-5 min, centrifuged and loaded in the wells.

3.3.5.3 Electrophoresis

After loading the samples a constant current of 20mA was applied from the power supply, till the dye front reached the bottom of the gel. The plates were then, carefully taken from the apparatus, and the gel was removed from the plates, washed with distilled water and transferred into staining solution.

3.3.5.4 Staining and destaining of gel

The gel was immersed in the coomassie staining solution (prepared as given in section 3.1.4.5) for overnight at room temperature on a gel rocker. Staining solution was removed and gel was immersed in destaining solution (prepared as given in section 3.1.4.5) till the protein bands were visible and background became clear.

3.3.5.5 Zymogram

Zymogram was done according to the protocol of Sondhi et al., 2014. Samples of purified enzyme were prepared without β-mercaptoethanol with heating at 70°C for 5min. Samples were resolved on SDS –PAGE after electrophoresis, the gel was dipped in phosphate buffer (100mM; pH 7.0) containing 2mM guaiacol for 30min at 37°C and looked for the development of reddish brown colored band.

3.3.5.6 Determination of molecular weight

Molecular weight of the purified laccase was determined by ploting a standard curve of R_f vs. Log MW of protein marker [R_f value for the standards and purified laccase was determined by following formula:

$$R_f = \frac{Distance\ migrated}{Gel\ length} \quad \text{…Eq. (3.12)}$$

Log MW was plotted against R_f for standards and extrapolated to determine the log molecular weight of the purified laccase.

3.3.6 Absorbance spectrum analysis of LAC-DS

Purified LAC-DS in 0.1M phosphate buffer was scanned at wavelength from 200-800nm. 0.1M phosphate buffer without enzyme was taken as blank. The plot was analyzed for characteristics peaks of various copper-centers**.**

3.3.7. Effect of temperature on activity and stability of LAC-DS

The optimal temperature of laccase for SGZ and PPD activity was determined by performing the enzyme assay (as per section 3.2.7) at temperature ranging from 35-70°C at an interval of 5°C at pH 7.0. The maximum enzyme activity was taken as 100% and relative activities were calculated as per equation 3.13.

Thermostability of the enzyme was measured by incubating the enzyme at different temperatures (45-70°C) at pH 7.0 for a time period of 24h. Samples were withdrawn sequentially at different time intervals and assay for laccase activity was performed using SGZ as substrate. Residual activity was calculated as per equation 3.14.

$$Relative\ Activity = \frac{Enzyme\ activity\ of\ sample}{Maximum\ enzyme\ activity} * 100$$

……Eq. (3.13)

$$Residual\ Activity = \left(100 - \frac{Enzyme\ activity\ of\ sample}{Enzyme\ activity\ of\ control}\right) * 100$$

……Eq. (3.14)

3.3.8 Effect of pH on activity and stability of LAC-DS

Stock solutions of substrates were prepared (PPD in water;ABTS and SGZ in DMSO) and diluted in buffer of appropriate pH. The optimal pH for laccase activity was determined by performing the enzyme assay in the pH range of 6.0-10. Enzyme was also diluted in the buffer of appropriate pH. The maximum enzyme activity was taken as 100% and relative activities were calculated as per equation 3.13.

Stability of the enzyme was measured by diluting the enzyme in the pH range of 6.0-9.0 and incubating it for 24h at room temperature Samples were withdrawn sequentially at different time intervals and assay for laccase activity was performed using SGZ as substrate. Residual activity was calculated as per equation 3.14.

Buffers used were 0.1M phosphate buffer (pH 6.0-7.5) and 0.1M Tris-HCl buffer (pH 8.0-9.0).

3.3.9 Effect of halides on LAC-DS stability

Effect of different halides i.e. sodium fluoride (NaF), sodium chloride (NaCl), sodium bromide (NaBr) and sodium iodide (NaI) on purified LAC-DS activity was studied by incubating the enzyme for 30 min at 37°C, with 1mM, 5mM, 10mM, 100mM and 500mM concentration of various halides. For this, enzyme was suitably diluted in the appropriate final concentration of various halides using stock solutions (1M) in buffer (0.1M phosphate, pH 7.0) and preincubated at room temperature for 30min. For control equal amount of enzyme was diluted in buffer and incubated under same conditions. Assay for laccase activity was performed as described in section 3.2.7. Residual activity was calculated as per equation 3.14.

3.3.10. Effect of metal ions on LAC-DS

Effect of metal ions on the activity of laccase was studied. 1M stock solutions of salts of various metal ions viz. $CuSO_4$, $NiSO_4$, $CoSO_4$, $MnSO_4$, $MgSO_4$ and $Al_2(SO_4)_3$were prepared in 0.1M phosphate buffer (pH 7.0). Metal ions were used at a final concentration of 0.1mM, and 0.5mM. For this, enzyme was suitably diluted using stock solutions of salts and buffer; preincubated at room temperature for 30min and assay for laccase activity was performed as described in section 3.2.7. Residual activity was calculated as per equation 3.14. For control equal amount of enzyme was diluted in buffer and incubated under same conditions.

3.3.11. Effect of inhibitors on LAC-DS

Effect of various inhibitors on the activity of laccase was studied. 0.1M stock solutions of sodium azide (NaN_3), sodium citrate ethylenediaminetetraacetic acid (EDTA), cysteine monohydrate, and β-mercaptoethanol were prepared in 0.1M phosphate buffer (pH 7.0). Inhibitors were used at a final concentration of 1mM, 5mM and 10mM. For this, enzyme was suitably diluted using stock solutions of inhibitors and buffer; preincubated at room temperature for 30 min and assay for laccase activity was performed as described in section 3.2.7. Residual activity was calculated as per equation 3.14. For control equal amount of enzyme was diluted in buffer and incubated under same conditions.

3.3.12. Effect of surfactants on LAC-DS

Effect of various surfactants on the activity of laccase was studied. 1M stock solutions of ionic (cationic CTAB and anionic SDS) and non ionic (tween-20, tween-80, triton X-100) were prepared in 0.1M phosphate buffer (pH 7.0). Inhibitors were used at a final concentration of 0.1mM, and 0.5mM. For this, enzyme was suitably diluted using stock solutions of surfactants and buffer; preincubated at room temperature for 30 min and assay for laccase activity was performed as described in section 3.2.7. Residual activity was calculated as per equation 3.14. For control equal amount of enzyme was diluted in buffer and incubated under same conditions.

3.3.13. Kinetic characterization of LAC-DS

Kinetic characteristics of LAC-DS were studied for various substrates viz. ABTS, SGZ, and PPD. Enzyme assay was done at different substrate concentrations ABTS (100-5000µM), SGZ (10-200µM) and PPD (100-5000µM). Michaelis-Menton coefficient (K_m) was determined by plotting Line-Weaver Burk plot for each substrate. K_{cat} and k_{cat}/K_m were also calculated for each substrate. The data was analyzed using the non linear regression program of Graph pad prism software and MS excel 2007.

3.4 Application of laccase (LAC-DS) from *Bacillus subtilis* DS for hair dyeing

3.4.1 Standardizing for the optimal oxidation of PPD with LAC-DS by one variable at a time (OVAT) method

Oxidation of p-phenylenediamine (PPD) was carried out with LAC-DS as explained in section 3.2.7. Optical density at 450nm was taken as a measure of amount of PPD oxidized. Optimal oxidation of PPD was standardized by one variable at a time method (OVAT) as well as by statistical method such as Response Surface Methodology (RSM).

Optimization of conditions for optimal PPD oxiation by one variable at a time (OVAT) method

In order to find the optimum conditions for oxidation of PPD by LAC-DS, various parameters such as enzyme dose, pH, reaction time and substrate concentration were optimized by varying one variable at a time method. In each experiment, Conditions optimized in previous step were kept constant in subsequent steps.

Effect of enzyme dose

To determine the effect of enzyme dose, oxidation reaction of PPD was carried out by varying concentration of LAC-DS in the range of 2-14 $IUml^{-1}$ of reaction mixture (8 IU-56 IU in 4ml reaction mixture) and substrate concentration of 2mM, pH-8.0, reaction time 10min at room temperature. Oxidation of PPD was determined by taking absorbance at 450nm.

Effect of pH

To determine the effect of pH, oxidation reaction of PPD was carried out in the pH range of 6.5-9.0 and enzyme dose of 10 $IUml^{-1}$(40 IU in 4ml reaction mixture), substrate concentration 2mM and reaction time 10 min at room temperature. Oxidation of PPD was determined by taking absorbance at 450nm.

Effect of reaction time

To determine the effect of reaction time, oxidation reaction of PPD was carried out for various time intervals (2-60min) and enzyme dose of 10 $IUml^{-1}$(40 IU in 4ml reaction mixture) of reaction mixture, substrate concentration 2mM, and pH 8.0 at room temperature. Oxidation of PPD was determined by taking absorbance at 450nm.

Effect of substrate concentration

To determine the effect of substrate concentration, oxidation reaction of PPD was carried out by varying substrate concentration in the range of 1mM to 12 mM (4mM- 56mM in 4ml reaction mixture) and enzyme dose of 10 $IUml^{-1}$ (40 IU in 4ml reaction mixture) of reaction mixture, reaction time 20 min, and pH 8.0 at room temperature. Oxidation of PPD was determined by taking absorbance at 450nm.

3.4.2 Optimization of conditions for optimal PPD oxidation by response surface methodology (RSM)

The optimum conditions for PPD oxidation was further standardized using central composite design (CCD) of RSM. Four factors *viz.* enzyme dose, substrate concentration, incubation time, and pH were optimized through central composite design (CCD). CCD was employed using Design Expert software, variables were studied at five different levels, low (-2, -1), medium (0) and high (+1, +2) (Table 3.4). The experimental plan consisted of 30 trials (Table 4.4.1).

Table 3.4 Experimental range and levels of independent factors for RSM design

Codes	Factors	Units	-2	-1	0	+1	+2
A	Enzyme dose	$IUml^{-1}$	8	10	12	14	17
B	pH		6	7	8	9	10
C	Reaction time	min	17	20	25	30	35
D	Substrate concentration	mM	7.5	8.0	8.5	9.0	9.5

Response data was analyzed by the software as discussed in section 3.3.2.

Validation experiments

To validate the statistical model, experiment was carried out using the optimal conditions predicted by the model and response (PPD oxidation/Absorbance at 450 nm) was measured as described earlier and compared to the predicted values. Each experiment was done in triplicates and data presented as mean ± SD.

3.5 Dyeing of hair with enzymatically oxidized dye (PPD)

3.5.1 Hair coloring performance of dye oxidized with LAC-DS in comparison to H_2O_2

A bunch of grey hair was dyed with PPD and LAC-DS under the conditions optimized in section 4.4.2. A coloring mixture was prepared containing PPD (8.5 mM) and LAC-DS (12 IU ml^{-1}) in phosphate buffer of pH 8.0 and was applied on bunch of grey hair for 25min. After coloring hair were washed with de-ionized water until the water became clear.

Another bunch of hair was dyed with PPD oxidized by H_2O_2. A coloring mixture was containing PPD (8.5mM) and H_2O_2 (0.2%; as recommended by Takada et al., 2003) in phosphate buffer of pH 8.0 and was applied on bunch of grey hair for 25 min. After coloring hair were washed with de-ionized water until the water became clear.

Coloring performance was evaluated by visual comparison of hair dyed with enzymatically oxidized dye with that of undyed grey hair and hair dyed with H_2O_2 oxidized dye.

3.5.2 Structural analysis of the hair dyed with enzymatically and H_2O_2 oxidized dye by Fourier Transform infrared (FTIR) spectrum

For FTIR analysis, hair was embedded in KBr disk during preparation of samples atmosphere exposure was avoided to prevent water absorbing. Samples were analyzed using Perkin

Elmer-spectrum 400 FT-IR/FT-FIR spectrometer in the wavelength range of 600 to 4000 cm^{-1}. Analysis of samples was done with the help of Essential-FTIR software.

3.5.3 Structural analysis of the hair dyed with enzymatically and H_2O_2 oxidized dye by scanning electronic microcopy

For scanning electron microscopy analysis. A small piece of hair was air dried and placed on the stubs, mounted with silver tape and sputter coated with gold using fine coat (S-3400N from Hitachi) and examined under scanning electron microscope.

3.5.4 Retaining ability of the LAC-DS oxidized dye on hair with respect to repeated washing

Retaining ability of color on hair dyed with enzymatically oxidized dye was evaluated with respect to repeated washing of the hair with shampoo. Dyed hair were washed with shampoo for 10 min and then thoroughly washed with tap water till all the detergent was removed. The whole process was repeated for six cycles. Retaining ability of color was evaluated by comparing washed hair with unwashed dyed hair and grey undyed hair.

3.6 Application of LAC-DS using commercial dye preparations

3.6.1 Hair coloring performance of commercial dyes oxidized with LAC-DS

Three bunchs of grey hair were taken and dyed with three different commercial dyes oxidized with LAC-DS a coloring mixture was containing 1 ml of commercial dye and LAC-DS (12 IU ml^{-1}) in phosphate buffer of pH 8.0 and was applied on bunch of grey hair for 25 min. After coloring hair were washed with de-ionized water until the water became clear.

Coloring performance was evaluated by visual comparison of hair dyed with enzymatically oxidized commercial dye/s with that of undyed grey hair.

3.6.2 Retaining ability of the commercial dyes oxidized with LAC-DS on hair with respect to repeated washing

Retaining ability of color on hair dyed with enzymatically oxidized commercial dye/s was evaluated with respect to repeated washing of the hair with shampoo. Dyed hair were washed with shampoo for 10 min and then thoroughly washed with tap water till all the detergent was removed. The whole process was repeated for ten cycles. Retaining ability of color was evaluated by comparing washed hair with unwashed dyed hair and grey undyed hair.

3.6.3 FTIR analysis of the hair, dyed with commercial dyes preparations oxidized with LAC-DS

FTIR analysis hair dyed with various commercial dye/s was done as explained in section 3.5.2 and was compared with undyed hair.

3.7 Development of enzyme based process for hair dyeing

3.7.1 Formulation of dye to be used in laccase based process

Stability/activity of LAC-DS in the presence of components of dye formulation

Effect of various ingredients on stability/activity of laccase was studied. Enzyme was suitably diluted with phosphate buffer pH -7.0 and appropriate ingredients so that its final concentration came to 0.01% and 0.1% (Wherever required stock solutions were prepared in appropriate solvents) (Table 3.5). Diluted enzyme was preincubated at room temperature; samples were withdrawn after 2h, 4h, and 6h and assay for laccase activity was performed as described in section 3.2.7.

Table3.5 Different components of dye formulation and their solvent

S. No.	COMPONENTS	Solvents
1	Ethanol	-
2	Glycerol	-
3	Cetryl Alcohol	Ethanol
4	Sodium Benzoate	*Buffer
5	SDS	Buffer
6	Olive oil	-
7	Ammonium Hydroxide	-
8	Coconut oil	-
9	Polyquaternium-6	Buffer
10	Proline	Buffer
11	Threonine	Buffer
12	Silicon Dioxide	Buffer
13	Titanium Dioxide	Buffer
14	Carbopol-940	Buffer
15	Lauric Acid	Buffer
16	Ethanolamine	Buffer
17	Rose water extract	-
18	Ascorbic acid	Buffer

Phosphate Buffer 0.1M pH-8.0

Activity of the enzyme immediately after adding the ingredient was taken as control and relative activities after different incubation periods were calculated taking the control activity as 100%.

Composition of dye formulation

Dye formulation for enzyme based hair dyeing process was prepared in phosphate buffer (pH -8.0; 0.1M). PPD was added at a concentration of 1.6%* and all other components are added at a concentration of 0.1%*. Final pH of dye preparation was found to be 8.0-8.5.

*[As before use dye preparation was mixed with equal proportion with developer (Section 4.5.3). final concentration of PPD was 0.8% and all other components were 0.05%]

3.7.2 Formulation of laccase based developer for hair dyeing

Stability/activity of LAC-DS in the presence of ingredients of enzyme based developer

Effect of ethanol, glycerol and cetryl alcohal on stability/activity of laccase was studied. Enzyme was suitably diluted with phosphate buffer pH -7.0 and ethanol/glycerol/cetryl alcohol so that the final concentration of these agents came to 0.01% and 0.1%. Diluted enzyme was preincubated at room temperature; samples were withdrawn after 1day, 4day, 8day, 15day and 30 day and assay for laccase activity was performed as described in section 3.2.7.

Activity of the enzyme immediately after adding the ingredient was taken as control and relative activities after different incubation periods were calculated taking the control activity as 100%.

Composition of laccase based developer formulation

Enzyme based developer was prepared in phosphate buffer (0.1M; pH -8.0), LAC-DS was added at a concentration of *24 $IUml^{-1}$ and all other components are added at a concentration of *0.1%. Final pH of developer was found to be 8.0-8.5.

*[As before use, developer was mixed with equal proportion with dye preparation (section 4.5.3). final concentration of enzyme was 12 $IUml^{-1}$ and all other components were 0.05%]

3.7.3 COLORZYME

The complete enzyme based hair dyeing kit including dye and developer formulation was designated as ‘COLORZYME’

3.7.4 Dyeing of hair with enzyme based process and its comparison with commercial H_2O_2 based process

3.7.4.1 Hair coloring performance

Dye and developer formulation of 'COLORZYME' were mixed in equal proportions and was applied on bunch of grey hair for 15 min. After coloring hair were washed with de-ionized water until the water became clear.

Another bunch of hair was dyed with commercial dye preparations and commercial H_2O_2 based developer (commercial dye preparation-1 used in section 3.6.1). Dye and developer formulation were mixed and applied on bunch of grey hair as per user instructions. After coloring hair were washed with de-ionized water until the water became clear.

Coloring performance was evaluated by visual comparison of hair dyed with 'COLORZYME' with that of undyed hair and commercial preparation.

3.7.4.2 Retaining ability of hair color

Retaining ability of color on hair dyed with 'COLORZYME' and commercial preparation was evaluated with respect to repeated washing of the hair with shampoo. Dyed hair were washed with shampoo for 10 min and then thoroughly washed with tap water till all the detergent was removed. The whole process was repeated for ten cycles. Retaining ability of color was evaluated by comparing washed hair with unwashed hair and grey undyed hair.

3.7.4.3 Structural analysis of hair dyed with enzyme based process by

Structure of hair dyed with 'COLORZYME' and commercial H_2O_2 based process was analyzed and compared by using various techniques such as fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and small angle X-ray scattering (SAXS).

To see the effect of long term usage; structural analysis was done with hair which were dyed for 4 dyeing cycles (dyeing of hair was done after that hair were washed for 20 times and whole cycle of dyeing and washing was repeated four times).

Structure analysis by FTIR

FTIR analysis hair was done as explained in section 3.5.2

Structural analysis by SEM

SEM analysis was done as explained in section 3.5.3.

Structural analysis of hair dyed with enzyme based process by Small angle X-ray scattering (SAXS)

SAXS analysis was done using SAXS Space instrument (Anton Paar GmbH, Austria). The instrument had a sealed tube X-ray source, a line collimated X-ray beam and a 1D CMOS Mythen detector (Dectris, Switzerland). The wavelength of X-rays was 0.154 nm and the sample to detector distance was 317.6 mm. Each hair sample was exposed for 10 minutes (1 frame each) to the x-ray beam. The data was calibrated for the beam position using SAXS treat software. The SAXSquant software was then used to set the usable q-range, and desmear the data using the beam profile. The data was analyzed in terms of scattering and intensity profile.

3.7.5 Toxicity evaluation and commercial assessment of 'COLORZYME'

'COLORZYME' was evaluated for cytotoxicity and commercial viability with respect to shelf life and cost estimation

3.7.5.1 Cytotoxicity evaluation of enzyme based process in comparison of H_2O_2 based commercial process

Safety evaluation of enzyme based hair dyeing process was done using SK-Mel-1 skin epithelial cell line and Raw 264.7 macrophage cell line and it was compared with that of commercial H_2O_2 based process.

Cell line maintenance

SK-Mel-1 skin epithelial cell line and Raw 264.7 macrophage cell line were procured from NCCS, Pune and maintained in serum supplemented DMEM media at 37°C in a 5% CO_2 humidified incubator. Cell lines were sub cultured and checked for their sterility periodically.

MTT Cell cytotoxicity Assay

The cell cytotoxicity test was done as per the guidelines of GB/T 16886.5-2003 (ISO 10993-5: 1999).

- SK-Mel-1 and RAW 264.7 cell line were cultured at 37°C with 5% CO_2 in 25 mm culture flasks containing DMEM culture medium with fetal calf serum to obtain a confluent monolayer of cells prior to use.
- Confluent layer of cells was trypsinized by using 1 ml of 0.25% of trypsin after decanting off the media and washed with PBS (pH 7.4)
- 1ml of media was added to the flask to neutralize the effect of trypsin and the mixture was centrifuged at 2,500 rpm for 5 minutes. Supernatant was discarded and fresh media was added. Cell count was performed with hemocytometer and cell number was adjusted to 50000 cells/ml
- 100µl of the cell suspension containing cell approximately 50,000 cell/well, were seeded in flat-bottomed 96-well micro-culture plates (Greiner Cellstar, Merck, USA) and incubated with appropriately diluted (Table 3.6)100µl of various component/s of hair dye in control well 100µl of PBS for 24 hrs at 37°C in a 5% CO_2 humidified incubator.
- For evaluation of viability of cultured cells, MTT assay was performed using the method of Mosmann, 1983; modified by Tada et al., 1986. Briefly, at the end of culturing time, 10 µl of MTT solution (5 mg/ml) was added to each well. After 2 hrs of incubation at 37°C in a 5% CO_2 humidified incubator, MTT solvent (4 mM HCl, 0.1% Nondet P-40 (NP40) in isopropanol) was added to dissolve formazan crystals, the optical density of each well was determined using a microplate reader (Bio-Rad, Model 680) at 570 nm. The percentage cell viability of samples compared to the control was calculated.

Table 3.6: Dilution of various component/s of hair dye for MTT assay

S. No	Hair dye component/s	Dilution used
1	Enzyme LAC-DS	$20IUml^{-1}$
2	Enzyme based developer	1:100 dilution
3	Dye formulation (from enzyme based process)	1:100 dilution
4	Enzyme based developer+ Dye for enzyme based process	1:100 dilution
5	Commercial H_2O_2 based developer	1:100 dilution
6	Commercial dye	1:100 dilution
7	Commercial H_2O_2 based developer + commercial dye	1:100 dilution

3.7.5.2 Shelf life of enzyme based developer

Shelf life of enzyme based developer was evaluated by incubating the developer at room temperature and 4°C for one month and laccase activity was determined periodically.

Activity of the enzyme immediately after mixing the various components of developer was taken as control and relative activities after different incubation periods were calculated taking the control activity as 100%.

3.7.3.6 Cost estimation of 'COLORZYME'

To check the commercial viability, approximate cost for one unit (for 50ml of developer + 50ml of dye of various components of enzyme based process was estimated under laboratory conditions.

Cost of enzyme was evaluated as given in section 3.2.9.To estimate the cost of PPD and other chemicals most of the market prices were obtained from Sigma-Aldrich (USA), Hi-media (India) and SRL (India).

The cost of manpower was not considered in the analysis, since it will depend on the automation and monitoring of the process, the experience of the worker and the volume of product to be produced. Moreover costs of packaging, marketing etc .was also not consider.

3. 8 Use of LAC-DS for synthesis of dye/s from natural precursors

Natural precursors such as catechol, gallic acid, syringaldehyde, syringic acid, ferulic acid and pyrogallol were used for the color development (Jeon et al., 2010). Oxidation reactions was carried for 30 min under same conditions as explained in section 3.2.7 for PPD. Appropriate substrate control (Substrate + Denatured Enzyme + Buffer), and enzyme control (Enzyme + Buffer) were used. After the completion reaction an aliquot of reaction mixture was taken in microtiter plate for comparing the color of different reactions.