CHAPTER 3

EXPERIMENTAL METHODS AND ANALYSIS

3.1 GENERAL

3.1.1 Chemicals

All chemicals and reagents used in the Ph.D thesis experimental work were of the highest purity available and of the analytical grade. 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), syringaldazine, guaiacol, bovine serum albumin (BSA), dinitrosalicylic acid (DNS), glutaraldehyde, starch (from corn), triphenylmethane and reactive dyes were obtained from Sigma chemical Co., St Louis, USA. Glycerol, glucose, veratryl alcohol, yeast extract, tyrosine, sodium acetate, acetic acid, ethyl acetate, ammonium sulphate, tert-butanol, di sodium hydrogen phosphate, mono sodium hydrogen phosphate, methanol, ethanol, Tris HCl, EDTA, SDS, β-mercaptoethanol and all metal solutions were procured from SRL Chemicals India Private Limited, Chennai, India.

3.1.2 Instrumentation

All pH measurements were carried out with a microprocessor based pH meter model number HI 98107, Hanna Equipments Private Limited, Mumbai, India. The absorbance of the dye solution and enzyme activities were recorded on Shimadzu UV-1800 spectrophotometer model number (Tokyo, Japan). The shape and surface morphology of the immobilized enzymes were analyzed using a Leo Gemini 1530 SEM at an accelerating voltage of 10 kV. Purity of the enzyme was confirmed by RP-HPLC (Reverse Phase – High Performance Liquid Chromatography) analysis using Agilent
1100 HPLC system. The purified laccase was applied on to C-18 column (Zorbax C-18, 4.6 mm×250 mm i.d., 5 μm particle size, Agilent technologies).

3.1.3 Microorganisms

The various fungal species were obtained and maintained as pure cultures from the following culture collection centres. Aspergillus niger MTCC 1344, Aspergillus terrus MTCC 2580, Beauveria bassiana MTCC 6100, Beauveria feline MTCC 6294, Metarhizium anisopilae MTCC 892, Paecilomyces fumosoroseus MTCC 6292, Paceilomyces lilacinus MTCC 1422, Trichoderma harzianum MTCC 936, Trichoderma viridie MTCC 2535, Pleurotus ostreatus MTCC 1804 were obtained from Microbial Type Culture Collection Centre (MTCC), Punjab, India. Penicilium chrysogenum NCIM 709 was procured from National Collection of Industrial Micro organism (NCIM), Pune, India. Pleurotus platypus, Pleurotus florida, Pleurotus eous, Agaricus bisporus and Fusarium solani were obtained from Tamil Nadu Agriculture University (TNAU), Coimbatore, India. Aspergillus flavus and Penicillium decumbens were obtained from PSG Medical College (PSGMC), Coimbatore, India. Aspergillus sp and Penicillium sp were maintained on Czapeck dox agar slants and stored at 4°C in refrigerator. Beauveria sp, Metarhizium sp, Trichoderma sp, Fusarium sp were maintained on potato dextrose agar slants and stored at 4°C in refrigerator. Pleurotus sp and Agaricus sp were maintained on malt extract agar slants and stored at 4°C in refrigerator.
3.1.4 Dyes

Double distilled water was used for preparing solutions throughout the study. Stock dye solutions were stored in the dark at room temperature. Table 3.1 depicts the various types of dyes used in the present study and its maximum absorbance ($\lambda_{\text{max}}$ (nm)) values. Dyes were procured from the dyeing units of Kanchipuram, Tamilnadu, India. Textile mill effluent was obtained from the Tirupur, Tamilnadu, India.

Table 3.1 Type of dyes used in the present study

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Dyes</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Structure</th>
<th>CAS No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Azo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Reactive Black 18 (RB18)</td>
<td>435</td>
<td><img src="image" alt="Structure" /></td>
<td>12225-25-1</td>
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<tr>
<td>2</td>
<td>Reactive Blue 2 (RB2)</td>
<td>593.5</td>
<td><img src="image" alt="Structure" /></td>
<td>12236-82-7</td>
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<tr>
<td></td>
<td>Triphenylmethane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Malachite Green (MG)</td>
<td>618</td>
<td><img src="image" alt="Structure" /></td>
<td>569-64-2</td>
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<tr>
<td></td>
<td>Textile Mill Effluent (TME)</td>
<td></td>
<td></td>
<td></td>
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<td>4</td>
<td>TME</td>
<td>519.0</td>
<td><img src="image" alt="Structure" /></td>
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</tbody>
</table>
3.1.5 Solid Substrates

Sago hampas (SH) was used as a substrate for the maximum production of laccase. SH was chopped into small pieces and dried in oven at 60°C to 5% (w/w) moisture content. It was ground to powder form (40 mm particle size) and it was autoclaved at 121°C for 20 mins, prior to its use in SSF.

3.2 SCREENING OF LACCASE IN VARIOUS FUNGAL SPECIES

3.2.1 Plate Assay Method

Initially organisms were screened for laccase activity using guaiacol and syringaldazine as indicator compound. The PDA plates were supplemented with 0.1 % syringaldazine and 0.02 % guaiacol and on this the fungi was grown and evaluated for laccase production. The pale yellow color of syringaldazine is oxidized to a purple-colored compound in the presence of fungal colonies and was taken as the positive reaction for the presence of laccase enzyme activity as previously reported (Wilkołażka et al 2002, Ang et al 2010). In the presence of guaiacol, intense reddish brown color was produced in the medium around the fungal colonies and was taken as the positive reaction for the presence of laccase enzyme activity as previously reported (Coll et al 1993, Kiiskinen et al 2004, Vishwanath et al 2008, Ang et al 2010).

3.3 LACCASE PRODUCTION

3.3.1 Solid State Fermentation - Small scale

The cultures were carried out in Erlenmeyer flasks incubated in a tray chamber (50×50×70 cm) with temperature (27°C) and humidity automatically controlled. The 10 g SH was moistened with 20 mL of a MYG
medium containing in 1% malt extract; 0.4% yeast extract; 0.4% glucose and 100 mM acetate buffer (pH 6.0). Tyrosine, veratryl alcohol and copper sulphate (CuSO$_4$) acts as inducer. Ten grams of moistened SH was transferred to flasks (150 mL) and the flasks were closed with cotton plugs and autoclaved at 121°C and 15 psi for 15 min. During the cultivation, sterile, moist air was flowed over flasks. Inoculation was carried out directly in the Erlenmeyer flasks. Three agar plugs (diam., 3 mm), from an actively growing fungus on MEA, per Erlenmeyer were used as inoculum. The Erlenmeyer flasks were incubated statically in an incubator SCIGENICS, in complete darkness. This equipment allows maintaining an air atmosphere at 30 °C and 90% humidity, avoiding evaporation. Cultures were harvested at every two days interval for the quantification of extracellular enzymes.

### 3.3.2 Optimization of Fermentation Parameters

Several natural and synthetic substrates have been reported to enhance laccase activity in basidiomycetes. Based on the previous reports the following inducers were used in the present study: tyrosine (5 mM), veratryl alcohol (7 mM) and CuSO$_4$ (0.5 mM). For assessing the optimum inducer concentration, various concentration of copper sulphate (0.1 – 1 mM) was added to the MYG medium. The compounds tested as inducers were sterilized by filtration. The laccase-producing fungi were cultured for 15 days on previously mentioned media in the presence of inducers. For studying the effect of initial moisture level, the quantity of SH was kept constant and the moisture effect was studied by adding MYG solution in the range 5–50 mL per 10 g substrate and designated through 1:0.5 (10 gds:5 ml) to 1:5 (10 gds:50 mL). pH adjustment of solid medium was achieved by adjusting the pH of moisturizing medium before adding to the solid material. Temperature adjustment of the production was achieved by incubating the
Petri dishes containing SH at different temperature varying from 20 to 37°C. Results reported in this study are averages of triplicate findings.

3.3.3 Pilot Scale Solid State Fermentation

Pilot scale fermentation was carried out in enamel coated metallic tray (45 × 30 × 4 cm) bioreactor (Figure 3.1(a)). The culture medium composition was that determined as optimum in the experiments at flask scale. Inoculation was carried out directly in the bioreactor with 22 agar plugs (7mm diameter), from an actively growing fungus on MEA. The contents of the trays were mixed before and after inoculation (Figure 3.1 (b)). The bioreactor were covered with aluminium foil and incubated in a temperature and humidity control chamber kept at 30°C, with passive aeration and in complete darkness (Figure 3.1 (c)). At selected times duplicate trays were taken out of the chamber for the quantification of extracellular enzymes (Figure 3.1 (d)).

Figure 3.1 Pilot Scale Solid State fermentation in Tray Bioreactors
3.3.4  **Enzyme Extraction from Fermented Solids**

After fermentation the whole sample of each flask was extracted by the addition of 50 mL of 0.1 M sodium acetate buffer pH 4.6, following incubation at 30 °C and 150 rpm for 300 min. The cultures were then filtered through a nylon filter and centrifuged twice at 10,000×g to remove the fine particles. Enzyme activity by ABTS oxidation, protein concentration by Lowry’s method and total reducing sugar (TRS) by DNS method was assayed in the clear supernatant. All analyses were carried out in triplicates and standard deviation was lower than 5%. Different solvents were tested for extraction: tap water (pH 7.6), distilled water, sodium acetate buffer 0.1 M (pH 4.0, 4.6, 5.0) and sodium phosphate buffer (pH 6.0, 7.0).

3.4  **LACCASE PURIFICATION**

3.4.1  **Three Phase Partitioning**

TPP was performed in batch type (small scale – 20 mL in centrifuge tubes). The crude enzyme (clear supernatant after centrifugation) was saturated to 60 % (w/v) of ammonium sulfate and t-butanol was added in the ratio of 1.0:1.8 (v/v) and the tubes were kept at 42±3 °C for 1 h for complete phase formation (Kumar et al 2011). The tubes were centrifuged at 2,000 x g for 10 min and the three phases formed were collected separately. The enzyme is usually precipitated in the middle layer. Precipitate was then dialyzed for 24 h against 20mM sodium acetate buffer pH 5.0, then filtered through 0.22 μm microfilter membrane and used for the further applications.

3.4.2  **Optimization of Purification Parameters**

The purification conditions were optimized using one factor at a time (OFAT) method (Czitrom 1999; Friedman and Savage 1947) and response surface methodology (RSM) (Cochran and Cox 1992; Box et al
The experimental factors for the present study are ammonium sulphate saturation; \(20\text{-}80\% \text{ (w/v)}\), ratio of crude extract to t-butanol; \(1.0:1.0\) - \(1.0:3.0\) (v/v) and temperature; \(20\text{-}60\ \degree\text{C}\). All the experiments were done in triplicate and the yield and purity of laccase was taken as the dependent variable or response. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the fisher’s F-test (overall model significance), its associated probability \(P (F)\), determination coefficient \(R^2\) which measures the goodness of fit at regression models. For each variable, the quadratic model was represented as contour plates (3D) and response surface curves were generated using Design expert 8.0 trial version.

### 3.4.3 Pilot Scale TPP

Pilot scale (5 l separation flasks) purification of the laccase enzyme was performed by TPP in two-step purification based on a procedure reported by Kumar et al (2011). The crude enzyme solution was saturated with 30% w/v of ammonium sulfate and t-butanol was added in the ratio 1.0:1.8 v/v and the tubes were kept at \(42\pm3\ \degree\text{C}\) for 1 h for complete phase formation (First TPP). The tubes were centrifuged at 2,000 x g for 10 min and the three phases formed were collected separately. The enzyme usually gets precipitated in the middle layer. To enrich the enzyme concentration, middle layer obtained in the first stage was dissolved in acetate buffer and adjusted to pH 4.0 using 0.1 M HCl and again saturated with 30% salt (such that final concentration was 60% w/v) followed by the addition of an equal volume of t-butanol and the tubes were centrifuged and the interfacial precipitate was separated as described previously (Second TPP).
3.4.4 Laccase Purity Analysis

3.4.4.1 Reverse Phase – High Performance Liquid Chromatography

Purity of the enzyme was confirmed by RP-HPLC (Reverse Phase - High Performance Liquid Chromatography) analysis using Agilent 1100 HPLC system. The purified laccase was applied on to C-18 column (Zorbax C-18, 4.6 mm×250 mm i.d., 5 μm particle size, Agilent technologies). For elution, buffer A consisted of 0.1% (v/v) trifluoroacetic acid (TFA) in water and buffer B consisted of 0.1% (v/v) TFA in acetonitrile. After loading, the column was washed with 2% (v/v) buffer B for 2min, to elute any unbound protein. The bound proteins were eluted from column using a 40min linear gradient from 2 to 100% (v/v) buffer B at the constant flow rate of 1ml/min. Column temperature was maintained at 25 °C and column eluent was monitored at 280 nm.
3.4.4.2 SDS - PAGE

The purified laccase was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate the molecular weight of the protein. SDS-PAGE was carried out according to method of Laemmli (1971) with some modifications. A discontinuous gel containing 4 and 10% (w/v) of acrylamide in stacking (pH 6.8) and resolving gel (pH 8.8) were used respectively. The compositions of the systems are described below.

**Monomer solution (acrylamide-bisacrylamide solution, 30 %):**
29.2 g of acrylamide and 0.8 g of N’N’-bis-methylene-acrylamide was made to a final volume of 100 mL using DW. The stock solution was filter sterilized though 0.22 μm filter and stored at 4°C in dark.

**SDS-PAGE sample solubilizing buffer (6×):** contained 300 mM Tris-HCl (pH 6.8), 7.5 % β-mercaptoethanol, 10 % SDS, 60 % Glycerol, 0.6 % bromophenol blue prepared in autoclaved DW.

<table>
<thead>
<tr>
<th>Components</th>
<th>12 % Resolving gel (mL)</th>
<th>5 % Stacking gel (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW autoclaved</td>
<td>3.4</td>
<td>5.7</td>
</tr>
<tr>
<td>Monomer solution</td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td>Gel buffer</td>
<td>2.5†</td>
<td>2.5#</td>
</tr>
<tr>
<td>SDS (10 %)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>APS (10 %) freshly prepared</td>
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<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

† **Resolving buffer:** 1.5 M Tris-HCl, pH 8.8 adjusted with 6 N HCl.
# **Stacking buffer:** 0.5 M Tris-HCl, pH 6.8 adjusted with 6 N HCl.
**SDS-PAGE running buffer (pH 8.3, 10×):** 30.2 g Tris base, 144 g glycine and 100 mL of 10 % SDS, the final volume is made to 1 L using DW. After running the gel, the proteins were stained by Coomassie brilliant blue R-250.

**Fixative:** 250 mL methanol, 62.5 mL acetic acid & 500 μL formalin in 187 mL DW.

**Coomassie Blue Staining:** After electrophoresis was over, the gel was placed in Coomassie Blue stain for 2 h and then destained overnight using Coomassie Blue destain solution with several intermittent changes, until the background was cleared and only the protein bands stained blue.

**Coomassie Blue staining solution:** 40 % methanol, 10 % acetic acid, 0.25 % Coomassie brilliant blue R-250 prepared in milli Q water.

**Coomassie Blue destaining solution:** 40 % methanol, 10 % acetic acid, prepared in milli Q water.

### 3.4.4.3 Activity staining for laccase

After electrophoresis was over, the polyacrylamide gel was placed in a phosphate buffer (pH 6) solution containing 10 % guaiacol till staining occurs. Protein bands corresponding to laccase oxidize guaiacol to a deep brown colour and these bands retain the colour. The excess guaiacol was removed with repeated rinses in distilled water. The gel was placed in fixative. This removes the background and previously invisible laccase bands begin to appear. The gel was then placed in water for complete rehydration and the image was then documented using the gel documentation system.

**NOTE:** All incubation steps are carried out on a gel rocker.
3.5 LACCASE IMMOBILIZATION

3.5.1 Preparation of Conventional CLEAs Laccase

The CLEAs of laccase were prepared by the conventional method consisting of precipitation, cross-linking and washing according to Schoevaart et al. (2004). In a 50 mL centrifuge tube with a magnetic stirrer bar, 1 mL of free laccase in 0.1 M acetate buffer solution was added to 9 mL of t-butanol. After 15 min of stirring, the mixture was quenched with 9 mL of 0.1 M acetate buffer, samples were withdrawn from tubes before and after precipitation and assayed for laccase activity. The required (10 mM) volume of glutaraldehyde was added dropwise and allowed to react for 3 h under agitation and then 18 mL of 0.1 M acetate buffer pH 4.0 was added. The operations were performed in a water-ice bath, with the temperature in the reaction mixture never exceeding 4 °C. A sample was withdrawn from the resulting suspension which contained CLEAs as well as residual free enzyme and assayed for laccase activity. The CLEAs were resuspended in 0.1 M acetate buffer, pH 4.0, and centrifuged at 13000 g for 10 min; this operation was repeated four times and the biocatalyst was stored at 4 °C.

3.5.2 Preparation of p-CLEAs Laccase

Porous - CLEA preparation consisted of aggregation by TPP, cross-linking, enzymatic treatment of cross linked aggregates and washing. Ammonium sulphate was added to a final concentration of 60 % of saturation in a 10 mL of enzyme solution containing 2 mg/mL free laccase, 2 mg/mL BSA and 0.2 % gelatinized starch in 0.1 M acetate buffer, pH 4.0. Further addition of free laccase solution to t-butanol resulted in a ratio of, 1.0:1.8 (v/v) and the mixture was left standing for 1 h for complete phase formation (Kumar et al. 2011). Three phases were formed and the interfacial layer which contained the enzyme aggregates was separated.
Figure 3.3  Schematic Diagram of Conventional CLEAs and porous CLEAs
Glutaraldehyde was added slowly to a final concentration of 20 mM to the enzyme aggregates. At the end of the 3-h cross-linking reaction at room temperature, the suspension was centrifuged at 13,000 g for 10 min at 4 °C. On the next day, 1 mL of α-amylase was added and the mixture was incubated at room temperature. I$_2$–KI indicator was added to the p-CLEAs sample so as to detect whether starch was completely hydrolyzed (Wang et al 2010). The suspension containing the p-CLEAs was centrifuged at 4 °C for 10 min at 13,000 g and the supernatant discarded. The p-CLEAs were resuspended in 0.1 M acetate buffer, pH 4.0, and centrifuged at 13000 g for 10 min; this operation was repeated four times and the biocatalyst was stored at 4 °C.

3.5.3 SEM Analysis of CLEAs Laccase

The shape and surface morphology of the CLEAs were analyzed using a Leo Gemini 1530 SEM at an accelerating voltage of 10 kV. For the SEM studies, samples were mounted on metal stubs using double-sided adhesive and sputtered with gold. The micrographs were taken at various magnifications.

3.6 ANALYTICAL METHODS

3.6.1 Laccase assay

The activity measurements of laccase were estimated spectrophotometrically at 45 °C by monitoring the rate of product (dark green colour) formation due to the enzymatic oxidation of ABTS with a UV-vis spectrophotometer (Kumar et al 2011). The reaction was started by the addition of 45 mM substrate solution (for ABTS 0.1 M acetate buffer pH 4.6) to the enzyme solution. In a 5 mL of cuvette, the following components were added; 0.5 mL of 45 mM ABTS + 0.5 mL of enzyme was added, the same
buffer in which the ABTS was prepared in, was used to make up the total volume to 3 mL. The kinetic reaction was spectrophotometrically measured at 420 nm for 1 min at the desired temperature, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place. Activity assays were performed at least for two times.

\[
\text{Laccase activity (U/mL) = } \left( \frac{\Delta \text{OD}}{\Delta t \text{(sec)}} \right) \left( \frac{1}{\varepsilon} \right) \left( \frac{1000 \text{ mmol}}{\text{mmol}} \right) \left( \frac{3 \text{ mL reaction mixture}}{\text{mL enzyme solution}} \right) \left( \frac{60 \text{ sec}}{\text{min}} \right)
\]

where \( \Delta \text{OD} = \) change in the absorbance at 420 nm;

\( \Delta t = \) change in the time;

\( \varepsilon = \) molar extinction coefficient of the radical-cation ABTS, \( 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \).

One unit of the enzyme activity (U) was defined as the amount of the enzyme producing 1 \( \mu \text{mol} \) product per minute under the given reaction conditions.

The activity measurements of laccase were estimated spectrophotometrically at 30 °C by monitoring the rate of product (dark pink colour) formation due to the enzymatic oxidation of syringaldazine with a UV-vis spectrophotometer (Kumar et al 2011). In a 5 mL of cuvette, the following components were added; 0.5 mL of 10 mM syringaldazine prepared in ethanol + 2.0 mL of buffer (0.1 M phosphate buffer) + 0.25 mL of laccase was added, buffer, was used to make up the total volume to 3 mL. The kinetic reaction was spectrophotometrically measured at 525 nm for 1 min at 30°C, as an increase in absorbance.
Figure 3.4 Laccase assay using (a) ABTS and (b) Syringaldazine

The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

\[
\text{Laccase activity (U/mL)} = \left( \frac{\Delta \text{OD}}{\Delta t (\text{sec})} \right) \left( \frac{1}{\varepsilon} \right) \left( 1000 \frac{\mu \text{mol}}{\text{mmol}} \right) \left( 3 \frac{\text{mL reaction mixture}}{\text{mL enzyme solution}} \right) \left( 60 \frac{\text{sec}}{\text{min}} \right)
\]  

(3.2)

where \( \Delta \text{OD} \) = change in the absorbance at 525 nm;

\( \Delta t \) = change in the time;

\( \varepsilon \) = molar extinction coefficient of the radical-cation syringaldazine, \( 6.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \).

Laccase was expressed as enzyme units per liter i.e. \( \text{U mL}^{-1} \) or \( \mu \text{mol min}^{-1} \text{ mL}^{-1} \). One unit of the enzyme activity (U) was defined as the amount of the enzyme producing 1 \( \mu \text{mol} \) product per minute under the given reaction conditions.
In the case of CLEA, samples were taken from the reaction mixture and immediately filtered into a cuvette, whereas, the reaction of the free enzyme with substrate was conducted directly in the cuvette. The activity recovery in CLEAs was calculated as given in equation (3.3)

\[
\text{Activity recovery} (%) = \frac{\text{Total activity of CLEA (U)}}{\text{Total free enzyme activity used for CLEA production (U)}} \times 100
\]

(3.3)

The specific activity is the activity of laccase in Units per mg of total protein. The purification factor refers to the specific activity of redissolved aggregates obtained using a particular precipitant divided by the specific activity of the sample of free enzyme that they were derived from. It is a measure of how effective the precipitation was for a given enzyme sample protein using different precipitants.

3.6.2 Estimation of Biomass, Protein and Reducing Sugar Concentration

The fungal biomass was estimated as its dry weight. The culture broth was filtered though pre-weighed Whatman No.1 filter paper. The paper was dried at 60 °C, until a constant weight was achieved. This was then conditioned at room temperature and then reweighed. The difference in weight was considered as the dry weight and expressed as g L\(^{-1}\) of culture medium.

Protein concentration was determined by the Lowry’s method using bovine serum albumin as the standard (Lowry et al 1957). Bovine serum albumin (BSA); 1 g was dissolved in 1 mL distilled water and used as stock for the preparation of protein standards for protein estimation by Lowry’s
method. The various dilutions were prepared in distilled water. To 1000 μL of each protein dilution, 2100 μL of reagent C was added and incubated at RT for 10 minutes. Then add 0.2 mL of folin cioclateau regent, mix well and incubate at RT in the dark for 30 minutes. Blue color is developed, the absorbance was recorded spectrophotometrically at 660 nm and a standard graph for the same was plotted.

Calculations

\[
\text{Protein (mg mL}^{-1}\text{)} = \frac{A_{660}}{0.002}
\]

where \( A_{660} \) = Absorbance at 660 nm

Protein was expressed as mg mL\(^{-1}\) or g L\(^{-1}\)

Reducing sugar (glucose) was estimated by the Dinitrosalicylic acid (DNS) method using D-glucose as the sugar standard (Miller 1959). Samples and glucose at different concentration were dissolved in 0.1 M acetate buffer, pH 4.5 at 30°C for 1 h. One mL of this sample was mixed with one mL of DNSA reagent was incubated in a boiling water bath for 10 min. It was then
cooled to room temperature, the total volume was brought to 10 mL with distilled water and the absorbance was spectrophotometrically recorded at 540 nm. The blank was enzyme free.

Calculations

**Amount of reducing sugar**

The concentration of reducing sugar was determined as mg mL$^{-1}$, from the following equation obtained from the above Std. graph;

\[
y = 0.0011x - 0.0211
\]

\[
R^2 = 0.9967
\]

where \(X = 'A' \text{ mg reducing sugar mL}^{-1}\)

\(Y = \text{Absorbance of sample at 540 nm}\)

Total Reducing sugar: \(A \times \text{total vol of reaction mixture (mL)} = 'B' \text{ mg total reducing sugars}\)
3.7 BIOCHEMICAL CHARACTERIZATION OF FREE AND IMMOBILIZED LACCASE

3.7.1 Effect of pH on Activity and Stability of Laccase

The effect of pH on the activity of the free and immobilized laccase was determined by assaying the enzyme activity at different pH values ranging from 3.0 to 9.0 using 0.1 M of the following buffer systems: acetate (3.0, 4.0, 4.6 and 5.0), phosphate (pH 6.0 and 7.0) and Tris–HCl (pH 8.0 and 9.0) buffer systems. The pH stability of laccase was investigated in the pH range of 4.5–9.5. Therefore, 1 ml of the enzyme was mixed with 1ml of the buffer solutions mentioned above and incubated at 45 °C for 60, 120, 180, 240 and 300 min. Afterwards aliquots of the mixtures were taken to measure the laccase activity (%) with respect to control, under standard assay conditions.

3.7.2 Effect of Temperature on Activity and Stability of Laccase

The effect of temperature on the activity of the native and immobilized laccase was determined by performing the standard assay procedure at different temperatures ranging from 30 to 70 °C (30, 40, 50, 60 and 70 °C). Before the addition of enzymes, the substrate ABTS was preincubated at the respective temperature for 10 min. Thermal stability studies were conducted without any additives. The enzyme solution was incubated at various temperatures ranging from 25–75 °C (25, 37, 45, 55, 65 and 75 °C) in a temperature-controlled water bath for various times and the relative enzyme activity was measured at regular intervals of time. The relative activity was calculated as the percentage ratio of activity at a given temperature to the activity at optimum temperature.
3.7.3 Effect of Metal Ions and Various Compounds on Laccase Activity

Metal ions (K$^+$, Na$^+$, Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Hg$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Cu$^{2+}$ and Fe$^{2+}$), and various compounds (EDTA, glycerol, SDS and β-mercaptoethanol) were tested for their effect on free laccase activity in acetate buffer, pH 4.0. The enzyme was preincubated for 30 min at 40 °C with 1 mM and 10 mM of listed metal ions as a final concentration prior to the substrate addition.

3.7.4 Kinetics and Catalytic Activity of Free, CLEAs and p-CLEAs Laccase

The kinetic constants (K_m, V_max and V_max / K_m) were determined using a Lineweaver–Burk double reciprocal plot, where different concentrations of ABTS were used as substrate (0.1–2.5 mM) at respective optimum pH and temperature.

V_max (maximum velocity): A reciprocal plot of velocity of reaction (1/V) (y axis) and substrate concentration (1/S) (x-axis) [Line Weaver burke Plot] is plotted. Upon extrapolating the line, the reciprocal of the intercept on the positive Y-axis is the Vmax (µmol min-1).

K_m (Michaelis constant): From the above plot, the reciprocal of the intercept on the negative X-axis is the Km (µM) [the –ve sign of the value is ignored].

3.7.5 Storage Stability of Laccase

For testing storage stability, free enzyme, conventional CLEAs and p-CLEAs laccases in 0.1 M acetate buffer without substrate were stored in refrigerator at 4 °C for several months. Every week, CLEAs were separated
from the buffer and washed with distilled water. The remaining activities of
the immobilized enzymes were measured under standard conditions.

3.7.6 Operational Stability of Laccase

Operational stability was determined by carrying out a standard
CLEA activity test. After the reaction the conventional CLEAs and p-CLEAs
laccases were washed three times using 0.1 M acetate buffer. After washing
another activity assay was carried out and residual activity was determined.

3.7.7 Laccase Spectroscopic Studies

Purified enzyme was concentrated by lyophilization and dialyzed
against deionized water. The concentration of purified laccase preparation
was adjusted to 1mg protein per ml in 0.1 M acetate buffer (pH 4.0). The
absorption spectrum of purified laccase preparation was determined at room
temperature (28 °C) on UV spectrophotometer in the range of 300 -700 nm.

3.8 DYE DECOLORIZATION BY CLEAs LACCASE

Decolorization of dyes and effluents was examined using the
laccase. Unless otherwise indicated, all experiments were performed in 50 mL
centrifuge tubes in a 10 mL final reaction volume. The reaction mixture
contained 0.1 M acetate buffer pH 4.5, initial dye concentration (100 ppm for
reactive dyes; 1000 ppm for TPM dyes), and 10 U/ml CLEAs laccase in static
condition. The reaction was initiated by the addition of laccase and incubated
in the dark at 30 °C. Decolorization of dyes was followed by measuring the
\( \lambda_{\text{max}} \) using UV visible spectrophotometer at 10 min intervals. Controls used
heat killed enzyme solution whereas blanks contained all components of the
reaction mixture except the dyes. The percent decolorization was calculated
using the following relation;
3.8.1 Decolorization of Addition of Dye Aliquots

The effect of repeated MG addition on the decolorization performance of CLEA laccase was studied in batch mode. Dye (MG -1000 ppm and RB 18 – 100 ppm) was repeatedly added to a batch of CLEAs laccase (10 U/ml) after decolorization of the dye. The percentage decolorization and time were monitored after each cycle.

3.8.2 Decolorization and Degradation of Model Dyes and Effluent by Laccase

Various structurally different dyes like MG, RB2, RB18 and effluents were used in this study. The decolorization reaction was carried out at 37 °C for 6 h in 2 ml reactions mixture containing 1000 ppm dye prepared in 0.1 M acetate buffer (pH 4.5) and 10 U/mL laccase. Control containing heat-denatured enzyme was used to measure decolorization of dye at different time interval. The decolorization was monitored by scanning the UV–vis spectrum between 190–900 nm. The laccase decolorized dye solution was centrifuged at 1000×g for 1 min and the metabolites of dye were extracted by adding ethyl acetate. The extract was dried over anhydrous Na₂SO₄ and solvent was evaporated in rotary evaporator. The dried residues obtained were dissolved in small volume of HPLC grade methanol and the sample was used for further analysis. The biodegraded metabolites were characterized by HPLC (Agilent Technologies) and compared with control dye. HPLC analysis was performed in an isocratic Waters 2690 system equipped with dual absorbance detector, using C₁₈ column (4.6×250 mm) and HPLC grade methanol as a mobile phase.
3.9 TOXICITY STUDIES

3.9.1 Phytotoxicity Studies

Phytotoxicity tests were performed in order to assess the toxicity of the untreated and treated dye (by CLEAs laccase). The ethyl acetate extracted products of dye and effluent degradation were dried and dissolved in sterile distilled water to make a final concentration of 500 ppm for phytotoxicity studies. The phytotoxicity study was carried out (at room temperature) on Phaseolus mungo and Triticum aestivum important in the Indian agriculture (Parshetti et al 2006). Ten seeds of Phaseolus mungo and Triticum aestivum under study were regularly supplied with distilled water as a control. MG, RB2, RB18 (500 ppm) and effluents and their degradation products for 7 days. Volumes for supplement of water, dye and degraded dye metabolites were kept same, i.e. 10 ml per day for seeds taken in watch glasses. Toxicity effect was measured in terms of percent germination, lengths of shoot and root of the plant after 7 days. The germination percentage is an estimate of the viability of seeds (Parashetti et al 2006) and it is found by;

\[
\text{Seed Germination} \, (\%) = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100
\]

3.10 STATISTICAL ANALYSIS

All the experiments were carried out in triplicate and standard deviation for each experimental result was calculated using Excel spreadsheets (Microsoft office 2003 package). The error bars in the figures represent standard deviation (n = 3).