Factorial design for optimization of laccase production from *Pleurotus ostreatus* IMI 395545 and laccase mediated synthetic dye decolorization

P Pratheebaa, R Periasamy and T Palvannan*
Laboratory of Bioprocess and Engineering, Department of Biochemistry, Periyar University, Salem 636 011, India

Received 27 February 2012; revised 23 May 2012; accepted 10 August 2012

In the present study, Plackett–Burman design (PBD) followed by response surface methodology (RSM) was used to optimize the culture medium composition for laccase production from *Pleurotus ostreatus* IMI 395545. Influence of carbon, nitrogen (sources) and inducer were evaluated by Plackett–Burman design. The factors that induce positive effect on enzyme production were further optimized using central composite design of RSM. The multiple regression equation was used to optimize the values of carbon, nitrogen and inducer for maximum enzyme production. The optimized values were found to be 1.8 g L\(^{-1}\), 0.76 g L\(^{-1}\) and 164.95 µM for glucose, ammonium nitrate and copper sulphate, respectively. The maximum enzyme activity of 2.7 U mL\(^{-1}\) obtained experimentally was very close to the predicted response, 2.5 U mL\(^{-1}\). Furthermore, optimized laccase was used for decolorization of synthetic dyes, such as, reactive blue 172 and reactive red 22, which are routinely used in textile industries. Fourier transform infrared spectroscopy was performed to confirm the decolorization of the studied dyes by laccase. *Allium cepa* toxicity test was performed, which indicated that root growth inhibition was dependent upon the concentration of dye.

**Keywords:** Laccase, Plackett–Burman design, response surface methodology, dye decolorization, medium optimization.

**Introduction**

Laccases (*p*-diphenol:dioxygen oxidoreductase; EC 1.10.3.2) are part of a larger group of enzymes termed as multicopper enzymes. Laccase was first described by Yoshida\(^1\) and characterized as a metal containing oxidase by Bertrand\(^2\). This makes it one of the oldest enzymes ever described. These enzymes are able to bring about the mono-electronic oxidation of substituted phenols with simultaneous reduction of dioxygen to two water molecules\(^3\). The substrate range is fairly broad and includes polyphenols, methoxy-substituted monophenols, aromatic amines, polymethoxybenzenes, benzenethiols and easily oxidizable inorganic substrates\(^4,5\). In addition, laccases can oxidize non-phenolic compounds and degrade residual lignin in kraft pulps in the presence of appropriate substrates termed as mediators\(^4\).

Laccases are gaining increasing attention due to their possible use in food\(^6\) and textile\(^7\) industries, pulp and paper manufacturing\(^8\), wastewater treatment\(^8\), bioremediation\(^5\) and nano-biotechnology\(^9\). The use of laccase at a commercial scale is constrained by the ability to obtain the enzyme in high yields from microbial processes. For a broad application, the cost of enzymes is one of the main factors determining the economics of a process\(^10\). Reducing the costs of enzyme production by optimizing the fermentation medium is the basic research for industrial application. The use of different statistical designs for medium optimization has been recently employed for xylanase, amylase, lipase, glucansucrase, inulinase, nattokinase, and laccases\(^11-18\). One of the promising sources for production of laccase has been white-rot fungus, *Pleurotus ostreatus* and it has the ability to decolorize different textile dyes\(^19-21\). *P. ostreatus* is a commercially important edible mushroom, famous for its delicious taste and high quantities of proteins, carbohydrates, minerals (calcium, phosphorus, iron) and vitamins (thiamin, riboflavin and niacin) but low fat\(^22,23\). The medicinally beneficial activities of *P. ostreatus*, such as, anticancer activity, immunomodulatory effect, antiviral, antibiotic, anti-inflammatory and cholesterol lowering activity, has been known worldwide\(^24-26\).

Statistical experimental designs are useful tools for screening nutrients with significant impact on enzyme production as they can provide statistical models, which aid in understanding the interactions among the process parameters at varying levels. Furthermore, calculations of the optimal level of each parameter for

*Author for correspondence:
Tel: +91-427-2345766/2345520; Fax: +91-427-2345124
E-mail: pal2912@yahoo.com
a given target can be performed\textsuperscript{27}. Plackett-Burman design (PBD) as compared to the common ‘one-factor-at-a-time’ method has been proved to be powerful and useful tool\textsuperscript{28}. Response surface methodology (RSM) is widely used in order to improve product yield, reduce development time and overall process costs\textsuperscript{29,30}. In the present study, authors have applied PBD and RSM in order to optimize culture medium composition for obtaining maximum production of laccase. In addition, ability of optimized enzyme from RSM to decolorize the synthetic dyes, reactive blue 172 (RB 172) and reactive red 22 (RR 22), was reported.

\section*{Materials and Methods}

\subsection*{Strains and Chemicals}

\textit{P. ostreatus} IMI 395545 used in the present study was isolated in our laboratory. The strain was cultured on malt extract agar slants for 2 wk at 28\textdegree C. The stock culture was stored at 4\textdegree C and sub-cultured every month. The 2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was purchased from Sigma-Aldrich Chemical Corporation, St. Louis, MO, USA and all other chemicals were of analytical grade. RB 172 and RR 22 were procured from commercial textile dyeing industry at Erode, Tamil Nadu, India.

\subsection*{Inoculum}

The fungal inoculum was prepared by growing \textit{P. ostreatus} IMI 395545 on malt extract agar plates. Approx 5 pieces (2 mm \times 2 mm) of actively growing mycelium were transferred to 100 mL of potato dextrose broth and kept in static for 6 d at 28\textdegree C. Then, 2\% (v/v) of culture was used as inoculum for the medium optimization.

\section*{Laccase Assay}

Laccase activity was determined with ABTS as the substrate\textsuperscript{32}. The reaction mixture contained 0.5 mM ABTS, 20 mM sodium acetate buffer (pH 5.6) and the culture filtrate. Oxidation of ABTS was monitored by an absorbance increase at 420 nm ($\varepsilon_{420}=36,000$ M$^{-1}$ cm$^{-1}$) at 30\textdegree C. One unit of laccase activity was defined as the amount of enzyme that oxidized 1 $\mu$mol of ABTS per min. The activities are expressed in U mL$^{-1}$. Protein content of the samples was determined according to the method of Lowry \textit{et al}\textsuperscript{33}.

\subsection*{Plackett-Burman Design (PBD)}

PBD was employed for screening the most significant culture parameters affecting laccase production by \textit{P. ostreatus}. In the study, a 12-run PBD was applied to evaluate 11 variables. Each independent variable was tested at two levels, high and low, which were denoted by (+) and (-), respectively (Table 1)\textsuperscript{28}. Table 2 shows the details of the design. From the pareto chart analysis, the

<table>
<thead>
<tr>
<th>No.</th>
<th>Pattern</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>$X_4$</th>
<th>$X_5$</th>
<th>$X_6$</th>
<th>$X_7$</th>
<th>$X_8$</th>
<th>$X_9$</th>
<th>$X_{10}$</th>
<th>$X_{11}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>++++++++</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>2</td>
<td>+++++++-</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>++++++++</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>4</td>
<td>+++++++-</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>5</td>
<td>++++++++</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>6</td>
<td>+++++++-</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>7</td>
<td>++++++++</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>8</td>
<td>+++++++-</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
</tr>
<tr>
<td>9</td>
<td>++++++++</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
</tr>
<tr>
<td>10</td>
<td>+++++++-</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>11</td>
<td>++++++++</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>12</td>
<td>+++++++-</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables (factors)</th>
<th>Laccase activity (U mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_1$</td>
<td>0.60</td>
</tr>
<tr>
<td>$X_2$</td>
<td>0.03</td>
</tr>
<tr>
<td>$X_3$</td>
<td>0.74</td>
</tr>
<tr>
<td>$X_4$</td>
<td>0.27</td>
</tr>
<tr>
<td>$X_5$</td>
<td>0.84</td>
</tr>
<tr>
<td>$X_6$</td>
<td>0.74</td>
</tr>
<tr>
<td>$X_7$</td>
<td>0.27</td>
</tr>
<tr>
<td>$X_8$</td>
<td>0.84</td>
</tr>
<tr>
<td>$X_9$</td>
<td>0.74</td>
</tr>
<tr>
<td>$X_{10}$</td>
<td>0.27</td>
</tr>
<tr>
<td>$X_{11}$</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Table 2—Plackett-Burman design for medium optimization and measured response
variables which show highly positive effect on each group were considered to have greater impact on laccase production.

Response Surface Methodology (RSM)

To describe the nature of the response surface in the optimum region, a central composite design (CCD) of RSM was performed. The range of coded and uncoded variables was given in Table 3. A five level, three factor CCD requiring 20 experiments was employed in the study (Table 4). The CCD consists of a $2^k$ factorial runs with $2k$ axial runs and $x_0$ number of center points (six replicates). The number of experimental runs was calculated from the following equation:

$$N = 2^k + 2k + x_0$$

Where $N$ is the number of experimental run required, $k$ is the number of variables and $x_0$ is the number of central points. Thus for this design total number of experimental runs will be 20 ($k = 3$, $x_0 = 6$).

The relationships of variables were determined by fitting a second order polynomial equation to data obtained from the 20 runs.

The quadratic model was established as Equation (2) as follows:

$$\hat{Y} = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \sum_{j=1}^{i} \beta_{ij} X_i X_j$$

Where $\hat{Y}$ is the predicted response (laccase production), $\beta_0$ is a constant; $\beta_i$ linear terms coefficients; $\beta_{ii}$ quadratic terms coefficients and $\beta_{ij}$ interaction coefficients. The relation between the coded forms of the input variable and the actual values of chosen variables are described as follows:

$$X_i = \frac{Z_i - Z_i^*}{\Delta Z}$$

Where $X_i$ is the coded value of the variable, $Z_i$ is the actual value (uncoded) of the variable, $Z_i^*$ is the center point value and $\Delta Z$ is the step change between the levels.

Statistical Analysis

The data from the experiments performed were analyzed and interpreted using the Design Expert Software (Version 7.0) from Stat-Ease, Inc., Minneapolis, MN, USA.

Purification

The method for the laccase purification was adapted from a protocol described by Palvannan and Sathishkumar. Crude filtrate was collected at the maximum laccase activity, then filtered and the supernatant was clarified by centrifugation at 8,000× g for 15 min. The supernatant was then fractionated with ammonium sulphate at 40-80% saturation and centrifuged at 80,000× g for 30 min at 4°C. The supernatant was discarded, the pellet was resuspended with Buffer A [10 mM of sodium acetate buffer (pH 5.6)] and dialysed (membrane mol wt cut off 10,000 Da) at 4°C against the same buffer. The dialysate was loaded onto a pre-equilibrated DEAE-Sephacl anion exchange chromatography column, the column was washed with the Buffer A until the A$_{280}$ reading was less than 0.02. Bound protein was eluted with a linear gradient (0-1 M KCl) at a flow rate of 1 mL min$^{-1}$; the eluted fractions were assayed.
for laccase activity. Active fractions were dialysed against Buffer B (100 mM sodium acetate buffer, pH 5.6). After 12 h, dialysed fractions were subjected to Sephadex G 50 gel filtration. The fractions containing laccase isoenzyme was eluted at a flow rate of 4 mL h\(^{-1}\).

**Decolorization Experiment**

Purified laccase was used to perform the *in vitro* decolorizing experiments\(^{37}\). The reaction mixture consisted of 100 ppm dye (RB 172/RR 22), 2.5 U mL\(^{-1}\) of laccase enzyme and 100 mM of sodium acetate buffer (pH 5.6), in a final volume of 2 mL. All the reactions were incubated at room temperature, without shaking and in complete darkness. A control test containing the same amount of a heat-denatured laccase was performed in parallel. The residual dye concentration was measured spectrophotometrically and was associated with the decrease in the absorbance at the peak of maximum visible wavelength (590 nm for RB 172 and 540 nm for RR 22). The absorbance was calculated by measuring the area under the plot. A spectrophotometer was used in all the experiments. Dye decolorization was calculated by means of the formula:

\[
D = \left( \frac{A_{\text{ini}} - A_{\text{obs}}}{A_{\text{ini}}} \right) \times 100 \quad \ldots \ (4)
\]

Where \(D\) is the (percentage) decolorization of RB 172 and RR 22, \(A_{\text{ini}}\) is the area under the curve of the absorption spectrum at 590 and 540 nm at time zero and \(A_{\text{obs}}\) is the area under the curve of the absorption spectrum at 590 and 540 nm at a determined time.

**Fourier Transform Infrared (FTIR) Spectroscopy**

FTIR analysis was used to examine the surface functional groups that were involved in decolorization of the dye. FTIR analysis was carried out using Perkin Elmer Spectrophotometer and changes in percentage transmission at different wavelengths were observed. The spectra were collected within a scanning range of 4000-400 cm\(^{-1}\) with scan speed of 16. The samples were mixed spectroscopically with pure KBr in the ratio of 5:95, pellets were mixed in sample holder and the analysis was carried out.

**Allium cepa Assay**

The *A. cepa* test provides a rapid screening procedure for chemicals and environmental agents, which may represent environmental hazards. Root growth inhibition assay was performed as a 96 h semi-static exposure test\(^{38}\). Healthy and equal size of common onions were obtained from local vegetable market, Salem, Tamil Nadu, India. The dried outer scales were carefully removed leaving the ring of the root primodial intact. *A. cepa* was exposed for 96 h to different dilutions of the synthetic dye (RB 172 & RR 22) in presence of laccase as follows: RB 172 and RR 22 dye concentration was 25, 50, 75, 100, 125,150 and 200 ppm.

Each concentration was set-up in three replicates. The base of each of the onion bulbs was grown on each of the concentration of the environmental agents inside a 30 mL beaker and placed away from sunlight for 4 d. After which the root length was measured. Growth inhibition was estimated as EC\(_{50}\) (the effective concentration of a chemical producing 50% of the total effect).

**Results and Discussion**

**PBD**

Statistical designs for medium optimization have proved to be a potent and functional tool for biotechnology. Heinzkill and Schinner\(^{39}\) and Galhaup *et al.*\(^{40}\) reported that the culture conditions could affect the fungal physiology and expression of ligninolytic enzymes. Therefore, an attempt has been made to improve the composition of the medium by simultaneous comparison between two levels of several factors, applying the PBD. The factors tested include different concentration of the medium components, namely, sucrose, galactose, raffinose, starch, glucose, fructose, yeast extract, asparagine, ammonium nitrate, veratryl alcohol and copper sulphate for the laccase production. Table 2 shows the effects of these components on the laccase production. Pareto chart (Fig. 1) shows the most significant factors determining the laccase production.

![Fig. 1—Effect of eleven factors on laccase production as shown in Pareto chart.](image-url)
Of the carbon sources, glucose showed the maximum positive effect when compared to other sources. The supplementation of glucose has two reasons. First it promotes the growth and rapid establishment of the fungus. Second, in the presence of lignin, the white rot fungus utilizes carbon sources more easily.

The nature and concentration of nitrogen sources are the factors regulating ligninolytic enzyme production by wood-rotting basidiomycetes. Of the various nitrogen sources tested (Fig. 1), ammonium nitrate and yeast extract showed positive effect, whereas asparagine showed negative effect. The ligninolytic enzymes have been regulated by the usable concentration of nitrogen in the medium. The low nitrogen level can stimulate the ligninolytic enzyme production, whereas a high nitrogen level represses it.

The supplementation of the nutrient medium with an appropriate inducer maximized the yield of ligninolytic enzyme. The production of laccase by white rot fungi was induced by aromatic compounds, such as, veratryl alcohol, vanillic acid, 2,5 xylidine, ferulic acid and copper. In the present study, copper sulphate showed high positive effect (Fig. 1), while veratryl alcohol had negative effect. Addition of copper in the growth medium had a positive impact on laccase production. It is worth mentioning that laccase mRNA levels in several white-rot species were reported significantly increased in medium containing copper, while increasing concentration of copper suppressed the growth of the strain. This is possibly due to the interaction of metals with white-rot fungi and defense mechanism therein involved. The matter has been reviewed by Baldrian.

Based on the statistical analysis, the effects of glucose, ammonium nitrate and copper sulphate were found to have positive effect on laccase production. By the experimental design, a maximum productivity of about 0.86 U mL⁻¹ laccase was obtained.

### RSM

RSM was adopted to determine a suitable direction by increasing or decreasing the concentrations of variables according to the results of PBD. The enzyme production was found to be the maximum on 10⁸ d after inoculation. Table 4 shows full factorial central composite design matrix and their observed responses for laccase production.

A regression model having a $R^2$ value more than 0.9 was considered to be having a very high correlation. Therefore, the present $R^2$ value reflected a very good fit between the observed and predicted responses, and implied that the present model is reliable for laccase production. The $F$ test (8.79) also demonstrates a high significance for the regression model. Each of the observed values ($Y_j$) are compared with the predicted value ($\hat{Y}_j$) calculated from the model in Table 4. The comparison of the residuals ($Y_j - \hat{Y}_j$) with the residual variance (0.94) indicates that none of individual residual exceeds twice the square root of the residual variance. All of these considerations indicate a good adequacy of the regression model.

Table 5 shows the ANOVA of the regression model. The goodness of fit of the model was checked by several criteria. A $P$-value of less than 0.0001 indicates that the model terms are significant. In this case, the nitrogen source, ammonium nitrate had a significant effect on laccase yield ($P<0.0001$). Vikineswary et al. reported that nitrogen content had significant effect on fungal growth and enzyme production. These observations further confirm the results obtained in the present study. Kaal et al. reported that nitrogen sufficient media resulted in enhanced production of ligninolytic enzymes including laccase in many fungi. The fitness of the model was examined by the coefficient of determination $R^2$, which was found to be 0.89 indicating that the sample variation of 88% was attributed to the variables and only less than 12% of the total variance could not be explained by the model.

In Table 6, it can be seen that the variables with higher effect were squared terms of ammonium nitrate...
concentration ($X_2^2$), followed by linear terms ($X_2$). Higher significance of the squared terms ($X_2^2$) over corresponding linear terms ($X_1, X_2$ and $X_3$) shows that the optimum values for enzyme production lies within the experimental values chosen. The interaction terms $X_1^2X_3$ seems to be insignificant, which can be neglected from the model without affecting the goodness of the fit. Ammonium nitrate was significant, whereas glucose and copper sulphate interact insignificantly. The adjusted determination coefficient ($R^2=0.79$) was also satisfactory to confirm the significance of the model. Finally, the lower value of coefficient of variation ($C=6.34\%$) shows that the experiments were precise and reliable.

The model can be shown as follows:

$$
\hat{Y} = 2.5006930 - 0.095517X_1 + 0.230308X_2 - 0.046258X_3 + 0.092909X_1^2 - 0.181275X_2^2 - 0.092909X_3^2 - 0.05000X_1X_2 - 0.02500X_1X_3 - 0.1000X_2X_3 \ldots (5)
$$

The above equation can be converted in to the uncoded unit where,

$$
X_1 = \frac{Z_1 - 1.5}{0.5} \ldots (6)
$$

$$
X_2 = \frac{Z_2 - 0.6}{0.2} \ldots (7)
$$

$$
X_3 = \frac{Z_3 - 150}{50} \ldots (8)
$$

In order to gain a better understanding of the effects of the variables on the production of laccase, the predicted model was presented as response surface graphs. The interaction between variables can be seen in the contour plots (Figs 2a-c). The interaction between variables means that change in level of one variable affects the level of other variable for fixed level of enzyme production. Figs 2a-c show the optimum level of glucose, ammonium nitrate and copper sulphate to obtain maximum production of laccase enzyme activity. Figs 2a and b show that the optimum level of glucose was around 1.8 g L$^{-1}$ to obtain enzyme activity of 2.5 U mL$^{-1}$ with the ammonium nitrate and copper sulphate at the levels of 0.76 g L$^{-1}$ and 164.95 µM, respectively. Fig. 2c also shows that optimum level of ammonium nitrate and copper sulphate to be around 0.76 g L$^{-1}$ and 164.95 µM, respectively for the maximum enzyme activity.

Maximum and minimum principle of differential calculus was used to maximize the equation (5) with respect to individual tested variables. The partial differential equations obtained are:

$$
\frac{\partial \hat{Y}}{\partial X_1} = -0.095517 - 0.05X_2 - 0.025X_3 \ldots (9)
$$

$$
\frac{\partial \hat{Y}}{\partial X_2} = 0.232909 - 0.05X_1 - 0.36255X_3 + 0.1X_3 \ldots (10)
$$

$$
\frac{\partial \hat{Y}}{\partial X_3} = 0.046258 - 0.025X_1 - 0.1X_2 + 0.18518X_3 \ldots (11)
$$

The second order differential equations are:

$$
\frac{\partial^2 \hat{Y}}{\partial X_1^2} = -1.858 \ldots (12)
$$

Fig. 2—Response surface plot showing the effects of glucose and ammonium nitrate (a), glucose and copper sulphate (b), and copper sulphate and ammonium nitrate (c).
\[ \frac{\partial^2 Y}{\partial X_2} = -0.36255 \quad \ldots (13) \]
\[ \frac{\partial^2 Y}{\partial X_3} = -0.185818 \quad \ldots (14) \]

The negative values of second order partial differential equations (12-14) indicate the absence of local maximum and applicability of maximization. The equations 9-11 are equated to zero and solved for \( X_1, X_2 \) and \( X_3 \) which give the maximum value of \( Y \).

\[ -0.095517 - 0.185818X_1 - 0.05X_2 - 0.025X_3 = 0 \quad \ldots (15) \]
\[ 0.232909 - 0.05X_1 - 0.36255X_2 - 0.185818X_3 = 0 \quad \ldots (16) \]
\[ 0.046258 - 0.025X_1 - 0.1X_2 - 0.185818X_3 = 0 \quad \ldots (17) \]

The algebraic solution to the above equations (15-17) were \( X_1 = -0.77, X_2 = 0.82 \) and \( X_3 = 0.29 \). These values correspond to the uncoded values of \( Z_1 = 1.8 \, \text{g L}^{-1}, Z_2 = 0.76 \, \text{g L}^{-1} \) and \( Z_3 = 164.95 \, \mu M \). At these optimum values, laccase production was maximum. An enhancement of laccase from 0.86 U mL\(^{-1}\) (PBD) to 2.5 U mL\(^{-1}\) was gained after optimization using RSM. In a recent work, Palvannan and Sathish kumar\(^ {18} \) have also shown that the maximum production of laccase by \( P. \) flo\( \text{r} \)ida (4.8 U mL\(^{-1}\)) was obtained using RSM. Thus, it is evident from the present study that carbon, nitrogen and inducer when mixed in appropriate ratio can serve as a complete source of nutrient pool without any additional supplements. Optimum laccase production by \( P. \) cyanopus sanguineus was also reported by Pointing \textit{et al}\(^ {18} \) in culture condition with high carbon and low nitrogen medium.

**Purification and Dye Decolorization**

To study the enzyme activity, laccase was purified from \( P. \) ost\( \text{re} \)tus. The crude enzyme (100 mL) had a specific activity of 13.8 U mg\(^{-1}\) and yield of 100% with the purification of 1-fold. At the end of the purification process (10 mL), the specific activity was increased to 137.9 U mg\(^{-1}\) and the yield was 10% with purification factor of 16-fold (data not shown). Further in the study, the ability of optimized enzyme to decolorize synthetic dyes (RB 172 and RR 22) was assessed. The RB 172 dye was maximally decolorized (≈95%) at 2 h treatment, followed by RR 22 (≈64%). Increasing concentration of dye leads to decrease percentage of dye decolorization by laccase (Table 7). This may be interrelated to molar extinction coefficients and purity of each particular dye. Moreover, it is known that the nature and position of the dye substituent group strongly affect the decoloration extent\(^ {49,50} \).

**FTIR Characterisation**

The FTIR spectra of RB 172 dye and laccase-treated RB 172 dye are shown in Figs 3a and 3b. The untreated dye showed peak at 3910, 3376 and 3280 cm\(^{-1}\). This may be due to O-H stretch, representing the presence of hydroxyl bond. However, after the laccase treatment, the peak shifted to the higher wave number 3405 cm\(^{-1}\), suggesting that there is N-H stretching. This may be due to the formation of primary amines and secondary amines. Further, peaks in untreated dye were seen at 2082 and 138 cm\(^{-1}\), which may be due to C-O-C (isopropylether), C-O and phenol group. However, a significant change was observed in the corresponding peaks after laccase treatment. Hence, these corresponding groups might be involved in the decolorization process. In untreated dye (RB 172), other peaks were found at 1414, 1116 and 1018 cm\(^{-1}\), which may be due to C-O-C (isopropylether), C-O and phenol group. However, a significant change was observed after laccase treatment, suggesting that isopropylether, C-O and phenol groups are involved in decolorization process. In addition, peak at 657 cm\(^{-1}\) in RB 172 dye disappeared after laccase treatment, suggesting that corresponding peaks at 659 cm\(^{-1}\) might be involved in the decolorization process by laccase.

The FTIR spectra of RR 22 dye and laccase treated RR 22 dye are shown Figs 3c and d. The untreated dye showed peaks at 3667 cm\(^{-1}\), suggesting that there is O-H stretching frequency in RR 22. After laccase treatment, the peak had shifted to higher wave number 3378 cm\(^{-1}\), suggesting that this may due to O-H group representing the formation of hydroxyl bond. Further, peaks were found at 3165 cm\(^{-1}\), this may be due to

<table>
<thead>
<tr>
<th>Conc. of dye (ppm)</th>
<th>% decolorization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RB 172</td>
</tr>
<tr>
<td>25</td>
<td>100 ± 0.00</td>
</tr>
<tr>
<td>50</td>
<td>100 ± 0.00</td>
</tr>
<tr>
<td>75</td>
<td>98 ± 0.20</td>
</tr>
<tr>
<td>100</td>
<td>95 ± 0.34</td>
</tr>
<tr>
<td>125</td>
<td>87 ± 0.50</td>
</tr>
<tr>
<td>150</td>
<td>66 ± 0.72</td>
</tr>
<tr>
<td>200</td>
<td>32 ± 0.83</td>
</tr>
</tbody>
</table>

**Table 7—Decolorization of reactive dyes at various concentrations**
O-H stretch representing the carboxylic acid. However, a significant change was observed in the peaks after laccase treatment, suggesting involvement of O-H group in the decolorization process. Other peaks in untreated RR 22 were seen at 2090, 1639 and 1410 cm\(^{-1}\), this may be due to NH\(_3\), C=C and O-H stretching. Only slight change was observed in the corresponding peaks after laccase treatment. In addition, peaks at 1118 and 670 cm\(^{-1}\) disappeared after laccase treatment, hence corresponding peaks at 1120 and 669 cm\(^{-1}\) in RR 22 dye might be involved in the decolorization process by laccase.

**Toxicity Assessment Using Allium Test**

Figs 4a and b show the root growth inhibitory effect of A. cepa exposed to dyes. The estimated EC\(_{50}\) dose of dyes RB 172 and RR 22 against roots of exposed A. cepa with optimized laccase was found to be 200 and 123 ppm, respectively (Fig. 4a & b). Very little root growth was observed in A. cepa exposed to RB 172 and RR 22 concentration greater than 200 and 123 ppm, respectively with unoptimised laccase. The results obtained from optimized laccase decolorization revealed a dye concentration dependent decrease in root length. As the concentration increases from 25 to 200 ppm, the root length significantly decreased (p<0.05) as compared with the control. This result is similar to the other reports\(^{51,52}\).

Thus, present study proves that statistical experimental designs offer an efficient and feasible approach for laccase medium optimization. The results of laccase in decolorization process suggest its potential field application for the removal of dyes from industrial effluents.
Acknowledgement
The authors are grateful to the Department of Biotechnology, Ministry of Science & Technology, Government of India, New Delhi for financial support to the corresponding author (Project No.: BT/PR8973/GBD/27/57/2006 Dt 14/08/07).

References
1 Yoshida H, Chemistry of lacquer (urushi), J Chem Soc, 43 (1883) 472-486.
21 Palmieri G, Cennamo G & Sannia G, Remazol Brilliant R decolorization by the fungus Pleurotus ostreatus and its...


