Optimization of laccase production from WRF-1 on groundnut shell and cyanobacterial biomass: By application of Box-Behnken experimental design

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ABSTRACT

Response surface methodology was employed for the optimization of different nutritional and physical parameters for laccase production by new isolates of white rot fungus (WRF-1) in the solid state fermentation. Initial screening of production parameters was performed using Plackett–Burman design and the variables with statistically significant effects on laccase production were identified. Cyanobacterial biomass, groundnut shell (GNS), % moisture content, pH and temperature were found to influence laccase production significantly. These variables were selected for further optimization studies using Box-Behnken design. The value of “R” (correlation coefficient) for the production of laccase was 0.9730 which indicates a good agreement between experimental and predicted values. On experimental validation of numerical and graphical optimization program within tested range the optimal combination of the physico-chemical factors for laccase production were obtained as cyanobacterial biomass, 2.03g; groundnut shell, 8.26g; pH, 5.42; temperature, 30.45°C and moisture content, 70.30%. This methodology facilitated analysis of the experimental data to establish the optimum conditions for the process and understand the contribution of individual factors to evaluate the response under optimal conditions. Thus application of Box-Behnken approach appears to have potential usage in process application.

Keywords: Laccase, WRF-1, Solid state fermentation, Cyanobacterial biomass, Response surface methodology.
INTRODUCTION

Extracellular laccases are copper containing oxidases that catalyze the oxidation of phenolic compounds by molecular oxygen [1]. They are capable to delignify wood pulp, decolorize and detoxify effluents generated by the pulp and paper industry and degrade toxic environmental pollutants and synthetic dyes, which are carcinogenic and hazardous to environment [2].

The white rot fungi are most efficient microorganisms capable of extensive aerobic lignin degradation and mineralization. Solid state fermentation (SSF) is considered as the most appropriate method for filamentous fungi cultivation and lignocellulolyltic enzyme production because they grow under condition close to their natural habitats due to which they may be more capable of producing certain enzymes with high productivity as compared to submerged fermentation [3]. The main limitation for the extensive industrial application of microbial enzymes is their higher cost, since the nutritive substances employed in the culture medium results in increase of total production costs, the reduction in the substrate expenses would thus increase the productivity of the processes [4].

During recent years, immense efforts are made to develop strategies to maintain the process under optimum condition, which can significantly increase the enzyme production. In SSF, since the fungi grow on heterogeneous substrate appropriation are essential in order to design and optimize SSF processes for hyper production of fungal metabolites of industrial significance [4]. For effective laccase expression, it is highly essential to optimize all the culture conditions and composition for production media, which further facilitates economic design of the full-scale fermentation operation system.

The one–at–time strategy of improving fermentation conditions is the most frequently used operation in biotechnology to obtain maximum cell density, high yields of the desired metabolic product or enzyme levels in the microbial system. This approach is not only time consuming, but also ignores the combined interactions between physico-chemical parameters.

Several statistical designs are currently available to predict the behavior of a reaction through response surface methodology (RSM). Such design of experiments explain the reaction completely and bringing out the finer details by carrying out just a few selected experiments. Basically this optimization process involves three major steps: performing statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model [5]. In the present study, effect of various factors on the production of laccase enzyme by new isolated WRF-1 on SSF is being studied by Box-Benhken experimental design.

The diazotrophic cyanobacteria are cosmopolitan microbes contributing significantly to nitrogen economy of the biosphere. The occurrence of water bloom leads to deterioration of water quality because some cyanobacteria release toxic compounds and dead cells cause an objectionable odour. However, besides rich source of nitrogen, cyanobacteria are rich source of sugars, lipids and proteins, needed for the production of useful compounds [6]. Therefore, an attempt was made to examine, the potential application of dry biomass of a diazotrophic cyanobacterium as a nitrogen supplement to raw carbon source (groundnut shell) for enhanced production of laccase.
by white rot fungus (WRF-1) in SSF. This makes the process economically attractive and eco-friendly [7].

**MATERIALS AND METHODS**

**Microorganism and culture conditions**

Fungus was isolated from the bark of tree of University campus, Varanasi, India in the month of July-August from the decaying wood. Samples were first put in a sterilized groundnut shell (GNS) plates after thorough washing, for an incubation period of one and half month. The plates were moistened with sterile distilled water at regular interval of 4-5 days. Distinct and predominant fungal colonies were isolated on plates, purified by subculturing. The fungus was grown on potato dextrose agar (PDA) plates at temperature 30°C and the stock culture were maintained at 4°C [8].

Groundnut shell was collected from local market. Water bloom was collected from river Ganges, Varanasi, India in summer season. Cyanobacterial water bloom sample showed typical morphological characteristics of *Microcystis* sp. under a microscope. Cyanobacterial biomass was harvested by centrifugation (13,000 x g, 10 min). Dry weight of cyanobacterial water bloom was determined by drying at 80°C until a constant weight was obtained [8, 9].

**Solid state fermentation**

The composition of SSF medium used for enzyme production was as follows: ground nut shell 8.0 g and 2.0 g dry cyanobacterial dry biomass. Medium was humidified with 10 ml of salt solution composed of (g/l) KH$_2$PO$_4$ 1.0; MgSO$_4$.7H$_2$O 0.5; CaCl$_2$.2H$_2$O 0.01; FeSO$_4$.7H$_2$O 0.01; MnSO$_4$.4H$_2$O 0.001; ZnSO$_4$.7H$_2$O 0.001 and CuSO$_4$.7H$_2$O 1mM on second day of fermentation. The pH of the medium was maintained at 5.0 with acetate buffer prior to sterilization. Roux bottle was autoclaved under 121°C and 15 psig pressure conditions for 20 min [9].

Four agar plugs cut from actively growing fungal colonies on potato dextrose plate were used as inoculum. The inoculated medium was incubated for 12 days at 30°C temperature with initial pH of 5.0 in solid state fermentation. Then 12 days old fermented medium was suspended in minimum amount of citrate buffer of pH 5.0 and was crushed by mortar and pestle. Then volume was made up to 50 ml with the help of buffer and fermented medium was centrifuged at 10,000×g for 10 min at 4°C. The supernatant was collected and used as laccase enzyme in the present study [7, 9].

**Analytical method**

Laccase activity was determined by measuring the oxidation of ABTS at 420 nm (ε = 3.6×104 cm$^{-1}$M$^{-1}$). The reaction mixture contained 1.0 ml ABTS (3 mM) and 0.8 ml sodium acetate buffer (0.1 M) of pH 5.0 and 0.2 ml aliquots of appropriately diluted enzyme extract at 60°C temperature for 10 min.

One laccase activity unit was defined as amount of enzyme, which leads to the oxidation of 1 mM of ABTS per minute. The activities were expressed in U per gram of extracted fermented substrate (U/gds). All experiments were performed in triplicate and results are mean of ±SD of triplicate experiments [10, 11].
Statistical analysis
In the present study the statistical software package Minitab 15 (trial version) was used for regression analysis of experimental data and to plot response surface. The randomized factorial design of the whole experiment consisted of eight factor variables (groundnut shell; dry powder of cyanobacteria; % moisture content; pH; temperature; CuSO$_4$; MgSO$_4$ and KH$_2$PO$_4$) [7, 12,13].

RESULTS AND DISCUSSION

Design of experiments
In the modeling problems involving multiple inputs, it becomes difficult to ascertain those inputs which are most influential in determining the model output. The Plackett-Burman (PB) design aims to select the most important variables in the system that influence overall enzyme productivity [14]. The PB methods allow evaluation of $N-1$ variables by $N$ number of experiments ($N$ must be a multiple of four). Each variable is variables for a desired response represented at two levels namely, “high and low”. This design assumes that there are no factor interactions between the different media constituents, $x_i$, in the range of variables under consideration. A linear approach is considered to be sufficient for screening.

$$Y = \beta_0 + \sum \beta_i x_i \text{ (i = 1, ...............k)}$$

where, $Y$ is the estimated target functions $\beta_0$ and $\beta_i$ are the regression coefficients of the model. The PB design is a factorial design and the main effect (the contrast coefficient) of such design may be calculated as the differences between the averages of the measurements made at high level (+1) and at low level (-1) as described in Table 1.

Table 1: Plackett-Burman design for medium optimization, positive (+1) and negative (-1) levels of independent variables used in trials

<table>
<thead>
<tr>
<th>Factor setting</th>
<th>Experimental Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content (%)</td>
<td>GNS (g/l)</td>
</tr>
<tr>
<td>High (+1)</td>
<td>80</td>
</tr>
<tr>
<td>Low (-1)</td>
<td>60</td>
</tr>
</tbody>
</table>

Contrast coefficients (CC) allow the determination of the effect of each constituent. A large contrast coefficient either positive or negative indicates that a factor has a large impact on titer, while a coefficient close to zero means that a factor has little or no effect. The P-value is the probability of magnitude of a contrast coefficient due to random process variability. The significance of each variable was determined by applying the student t-test [14, 15]. Eight variables [moisture content, (A); groundnut shell, (B); cyanobacterial biomass, (C); initial pH of the medium, (D); temperature, (E); CuSO$_4$, (F); MgSO$_4$, (G); KH$_2$PO$_4$, (H)] were used and for each variables a high (+1) and a low (-1) concentration were tested (Table 2). Their analysis showed that among the factor tested only dry cyanobacterial biomass, moisture content, groundnut shell, temperature and pH had a significant positive influence on laccase production as evaluated by their respective contrast coefficient value (b) (Table 2).
Table 2: Statistical calculation of P.B. experimental design

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Moisture content (%)</th>
<th>GNS (g/l)</th>
<th>CBM (g/l)</th>
<th>pH</th>
<th>Temp. (°C)</th>
<th>CuSO₄ (mM)</th>
<th>MgSO₄ (g/l)</th>
<th>KH₂PO₄ (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High level</td>
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<td>10.0</td>
<td>3</td>
<td>7.0</td>
<td>35</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Low level</td>
<td>60</td>
<td>6.0</td>
<td>1.0</td>
<td>3.0</td>
<td>25</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>Coefficient)</td>
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<td></td>
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<td></td>
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<td></td>
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<tr>
<td>P-score</td>
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<td>0.002</td>
<td>0.001</td>
<td>0.015</td>
<td>0.010</td>
<td>0.077</td>
<td>0.227</td>
<td>0.061</td>
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</table>

For medium optimization twelve different trials were made with process variables and laccase activity was measured after 12 days of fermentation at 30 °C temperature and pH 5.0 (Table 3). The row in the Table 3 represents the 12 different trials and each column represents a different variable.

Table 3: Plackett-Burman designs for medium optimization and measured response

<table>
<thead>
<tr>
<th>Run</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>Activity (U)</th>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<td>290.08</td>
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<td>-</td>
<td>+</td>
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<td>-</td>
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<td>-</td>
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<td>297.65</td>
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<tr>
<td>12</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>260.18</td>
</tr>
</tbody>
</table>

moisture content, (A); groundnut shell, (B); cyanobacterial biomass, (C); initial pH of the medium, (D); temperature, (E); CuSO₄, (F); MgSO₄, (G); KH₂PO₄, (H).

After determining the most significant factors for laccase enzyme production, their concentration optimizations were made by using Response surface methodology (RSM). It is a combination of experimental designs and statistical techniques for the empirical model building and optimization. By conducting experiments and applying regression analysis, a model of the response to some independent inputs variables can be obtained. Based on the model response, a near optimal point can then be deduced. Response surface methodology also quantifies the relationship between the controllable input parameters and the Response surface obtained [16].

If all variables are assumed to be measurable, the response surface can be expressed as follows:

\[ Y = f(x_1, x_2, x_3, \ldots , x_k) \]

where \( Y \) is the response and \( x_1 \) is the variables of action called factors. The goal is to optimize the response variable \( Y \). It is assumed that the independent variables are continuous and controllable by experiment with negligible errors. It is required to find a suitable
approximation for the true functional relationship between independent variables and the response surface. Usually a second order model is utilized in response surface methodology [16]

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + \varepsilon \]

where \( x_1, x_2, \ldots, x_k \) are the input factors which influence the response \( Y; \) \( \beta_0, \beta_{ii} (i=1,2,\ldots,k), \beta_{ij} (i=1,2,\ldots,k; j=1,2,\ldots,k) \) are unknown parameters and \( \varepsilon \) is the random error. The \( \beta \) coefficients, which should be determined in the second order model, are obtained by the least square method.

In the present study Box-Behnken experimental design was chosen for findings out the relationship between the response function (laccase activity) and the variables (moisture content, groundnut shell, cyanobacterial biomass, pH and temperature) designated as \( X_1, X_2, X_3, X_4 \) and \( X_5 \) (Table 4). There are several reports on use of Box–Behnken experimental design for production of metabolites in submerged fermentation but not much explored in case of solid state fermentation hence a systematic study was made on optimization of laccase production in SSF.

As central composite designs, Box–Behnken designs are response surface methods used to examine the relationship between one or more response variables and a set of quantitative experimental parameters [17]. Response surface methods are often used once preliminary screening has been carried out; using factorial designs such as Plackett-Burman (PB) to determine which factors significantly affect the response.

The significant variables were identified by the analysis of the Placket-Burman experiments and their levels were further optimized for enhanced laccase production by employing Box- Behnken design (Box and Behnken, 1960). Each selected variable was analyzed at three levels-low, medium and high coded as -1, 0 and +1 in a total of 46 runs (Table 5).

Box-Behnken design requires an experiment number according to \( N=k^2 + k + c_p \), where \( k \) is the factor number and \( c_p \) is the rotatable number of the central point [18]. Box-Behnken is a spherical, revolving design viewed as a cube, it consists of central point and the middle points of the edges. However, it can also be viewed as consisting of the three interlocking \( 2^3 \) factorial designs and a central point [18].

For the five level factorial Box-Behnken experimental designs, a total of 46 experimental runs, shown in Table 5, are needed.

The model is of the following form [18]:

\[
\begin{align*}
y &= \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \\
&+ \beta_{44} x_4^2 + \beta_{55} x_5^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{15} x_1 x_5 + \\
&+ \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{25} x_2 x_5 + \beta_{34} x_3 x_4 + \beta_{35} x_3 x_5 + \beta_{45} x_4 x_5.
\end{align*}
\]

where 'y 'predicted response; \( \beta_0 \) mode constants; \( X_1, X_2, X_3, X_4 \) and \( X_5 \) independent variables; \( \beta_1, \beta_2, \beta_3, \beta_4 \) and \( \beta_5 \) are linear coefficients; \( \beta_{12}, \beta_{13}, \beta_{14}, \beta_{15}, \beta_{23}, \beta_{24}, \beta_{25}, \beta_{34}, \beta_{35} \) and \( \beta_{45} \) are cross product coefficients, and \( \beta_{11}, \beta_{22}, \beta_{33}, \beta_{44} \) and \( \beta_{55} \) are the quadratic coefficients [18].
The simplest possible model (quadratic second order model) can be used to explain the mathematical relationship between the controllable variables and response. This design is preferred because relatively few experimental combinations of the variables are adequate to estimate potentially complex response functions. The regression equation obtained after analysis of variance gives the levels of laccase as a function of different concentration of moisture contents, groundnut shell, dry cyanobacterial biomass, pH and temperature. Table 4, represents the different level of variables selected for Box-Behnken design. Experiments were replicated three times. The statistical analysis was performed by the use of software’s Minitab 15.

Table 4: The level of variables chosen for Box-Benhken design

<table>
<thead>
<tr>
<th>Codedlevel</th>
<th>Experimental variables</th>
<th>Moisture content (%) (X₁)</th>
<th>GNS (g/l) (X₂)</th>
<th>CBM (g/l) (X₃)</th>
<th>pH (X₄)</th>
<th>Temp. (°C) (X₅)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td></td>
<td>60</td>
<td>6.0</td>
<td>1</td>
<td>3.0</td>
<td>25</td>
</tr>
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<td>0</td>
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<td>70</td>
<td>8.0</td>
<td>2</td>
<td>5.0</td>
<td>30</td>
</tr>
<tr>
<td>+1</td>
<td></td>
<td>80</td>
<td>10.0</td>
<td>3</td>
<td>7.0</td>
<td>35</td>
</tr>
</tbody>
</table>

Optimization of process parameters by Response surface methodology (RSM)  

Box-Behnken experimental design

Selection of physico-chemical parameters for maximum laccase productions, the one-at-a time strategy of improving fermentation media and physical conditions were successfully applied for the production of laccase enzyme. However, this one at time techniques of optimization have some major flaws. Due to these drawbacks the use of simultaneous optimization using experimental design has become more common and when using experimentation design, full factorial, partial factorial or central composite, where the techniques of choice is one of the most popular methods of optimizations of culture medium is response surface methodology [19]. Here the Box–Behnken experimental design was use for evaluating and optimizing the concentration of most significant factor for laccase production in SSF.

In order to search for efficient laccase production the optimum combinations of major components of the medium, for efficient laccase production, experiments were performed according to the Box-Behnken design plan (Table 4). Five nutrients had been identified as the most significant for promoting enzyme yields, which established that an optima could be found within the ranges of parameters studied the mathematical models, relating the production of laccase with the independent process variables (Table 5).
Table 5: Box-Behnken design matrix for optimization of 5 nutrients for laccase production by WRF-1 in solid state fermentation.

<table>
<thead>
<tr>
<th>Run</th>
<th>Moisture content</th>
<th>GNS</th>
<th>CBM</th>
<th>pH</th>
<th>Temp</th>
<th>Laccase activity (U/gds)</th>
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</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Observed</td>
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The coefficients of determination, $R^2$ was 0.9730, a value $> 0.75$ indicates the aptness of the model. For a good statistical model, the $R^2$ value should be close to one [20]. Results ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 97% of the variability in the dependent variables (laccase activity, U/gds) could be explained by this model and only 3.0% of the total variance could not explained by the model.
The Quadratic regression equation obtained for dependent variables. All the terms regardless to their significance are included in the following equation.

\[
\text{Laccase activity (U/gds)} = 159.97 + 21.91X_1 + 5.29X_2 + 5.12X_3 + 6.11X_4 + 6.66X_5 - 10.62X_1X_3 + 3.06X_1X_5 - 5.90X_2X_4 - 3.33X_2X_3 - 28.14X_1^2 - 55.90X_2^2 - 4.22X_3^2 - 28.14X_1^2 - 55.90X_2^2
\]

where \(X_1\), moisture content [60-80% with 70% as central value]; \(X_2\), groundnut shell [6-10 with 8 as central value]; \(X_3\), cyanobacterial biomass, [1-3 with 2 as central value]; \(X_4\), pH [3-7 with 5 as central value]; \(X_5\), temperature [25-35 °C with 30°C as central value].

The value of “R” (correlation coefficient) for the production of laccase is 0.9730 which indicates a good agreement between experimental and predicted values.

The corresponding analysis of variance (ANOVA) is presented in table (6). The \(F\)-value is a measure of variation of the data about the mean. Generally, the calculated \(F\) value should be several times greater than the tabulated value, if the model is a good prediction of their experimental results and the estimated factors effects are real [21]. Also the high \(F\)-value and a very low probability (\(P>F = 0.0001\)) indicates that the present model is in a good prediction of the experimental results [22]. The p-value serves as a tool for checking the significance of each of the coefficients. The pattern of interaction between the variables is indicated by these coefficients. The variables with low probability levels contribute to the model, whereas the others can be neglected and eliminated from the model. Values of prob >\(F\) less than 0.0500 indicates model terms are significant. In the present study terms are significant model terms [16]. High \(F\)-values and non significant lack of fit indicated that model was a good fit. The pred R-squared of 0.8951 is in a reasonable agreement with the adj R-squared value of 0.9514.

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R-Sq = 97.30%; R-Sq(pred) = 89.51%; R-Sq(adj) = 95.14%
Interaction among the nutrients
The 3D response surface and the 2D contour plot are the graphical representation of the regression equation. The main goal of response surface is to efficiently hunt for the optimum value of the variables such that the response is maximized. Response surface curve were made for variation in the yields of laccase production as a function of concentration of two nutrients and other nutrient being at their constant levels. From the response surface plot, it is very easy and convenient to understand the interactions among the nutrients and also to locate their optimum concentration.

Figure 1: Response surface plot (upper) and its contour plot (lower) of laccase production by white rot fungus (WRF-1) showing the interaction between cyanobacterial biomass (CBM) and groundnut shell (GNS) at constant levels of: moisture content 70 %, pH 5.0, temperature 30°C.
Figure 2: Response surface plot (upper) and its contour plot (lower) of laccase production by white rot fungus (WRF-1) showing the interaction between cyanobacterial biomass (CBM) and moisture content (%) at constant levels of: groundnut shell 8 g, pH 5.0, temperature 30°C.
Figure 3: Response surface plot (upper) and its contour plot (lower) of laccase production by white rot fungus (WRF-1) showing the interaction between groundnut shell (GNS) and moisture content (%) at constant levels of: cyanobacterial biomass 2 g, pH 5.0, temperature 30°C.
Figure 4: Response surface plot (upper) and its contour plot (lower) of laccase production by white rot fungus (WRF-1) showing the interaction between pH and cyanobacterial biomass (CBM) at constant levels of: moisture content 70 %, groundnut shell 8 g, temperature 30°C.
It can be observed that each of five variables used in present study has its individual effect on laccase activity by WRF-1 in solid state fermentation. Figure 1, showed the effect of cyanobacterial biomass (CBM) and groundnut shell (GNS) on laccase activity at temperature 30°C, pH 5.0 with 70% of moisture in the medium. It was observed that gradual increasing the concentration of CBM at low level 1.00 g (coded value -1) to its higher level 3.0 g (coded value1) and GNS at low level 6.00 g (coded value -1) to its higher level 10.0 g (coded value1) production of laccase enzyme increased. Similarly fig. 2 and fig 3. Shows that the production of laccase increased with increase of moisture content from 60 to 70 % thereafter further rise of leads to significant decrease of laccase activity from 385.27 U/gds to 350.21 U/gds.
Fig. 4 & 5, showed the cumulative effect of cyanobacterial biomass and pH; groundnut shell and pH on laccase activity. The maximum laccase activity of 385.27 U/gds was found to be in the pH range of 5.0 after increase and decrease of pH causes negative effect on laccase yields.

Fig. 6 & 7, represents the interactive effect of temperature with cyanobacterial biomass and groundnut shell on overall laccase yields. The temperature at its low level (25°C) with CBM (1.0 to 2.0 g) showed the activity as much as 371.10 U/gds as the temperature increased from 25°C to 30°C laccase production increased after that there was decline in laccase yields with increase in temperature. Similar trends was also observed from lower to higher level (6 to 10 g) of groundnut shell with temperature.

Figure 6: Response surface plot (upper) and its contour plot (lower) of laccase production by white rot fungus (WRF-1) showing the interaction between temperature and cyanobacterial biomass (CBM) at constant levels of: moisture content 70 %, groundnut shell 8 g, pH 5.0.
Figure 7: Response surface plot (upper) and its contour plot (lower) of laccase production by white rot fungus (WRF-1) showing the interaction between temperature and groundnut shell (GNS) at constant levels of: moisture content 70 %, cyanobacterial biomass 2 g, pH 5.0.
Figure 8: Response surface plot (upper) and its contour plot (lower) of laccase production by white rot fungus (WRF-1) showing the interaction between pH and moisture content (%) at constant levels of: groundnut shell 8 g, cyanobacterial biomass 2 g, temperature 30°C.
Figure 9: Response surface plot (upper) and its contour plot (lower) of laccase production by white rot fungus (WRF-1) showing the interaction between temperature and moisture content (%) at constant levels of: groundnut shell 8 g, cyanobacterial biomass 2 g, pH 5.0.
Fig. 8 and 9 shows the interaction of moisture content with pH and temperature, and it is evident that for higher laccase activity at temperature in the ranges of 30°C to 35°C and pH in the range of 5.0 to 7.0 have the greatest effect on laccase yields. The yield of laccase activity was
significantly affected by the pH and temperature when the concentration of CBM 2.0 g and moisture content 70%.

From the interaction of pH and temperature (Fig. 10) it shows that the maximum laccase yield can be obtained at pH 5.0 and temperature 30°C.

Therefore, the significant observation made from the interactions that influenced the laccase production was that as the CBM concentration increased from 1.00 to 2.00 g and moisture content from 60% to 70% resulted in the gradual increase in the laccase yields. Optimum pH and temperature for production of laccase by WRF-1 in SSF was 5.0 and 30°C respectively. When a numerical and graphical optimization program was run within the tested range, the optimum level of nutrients obtained were: CBM 2.03 g, pH 5.42, temperature 30.45°C and moisture content 70.30%. With these levels the model predicted maximum activity of 385.05 U/gds while on experimental verification the strain produced 384.15 U/gds.

CONCLUSION

Culture conditions and media composition optimization by a conventional one-at-the approach lead to a substantial increase in enzyme yield. However, this approach is not only cumbersome and time consuming but also has the limitation of ignoring the importance of interaction of various parameters. Box-Benhken experimental design for process optimization, involving a study of given system by set of independent variables (factors) over a specific region of interest (levels) by identifying the influence of individual factors. Result showed that the yield of ligninolytic enzymes largely varied in cultures of basidiomycetes when they were grown under different nutritional environment. In general, the yield of laccase was increased by supplementing the medium with dry cyanobacterial biomass. Observations suggest that dry biomass of diazotrophic cyanobacteria not only act as N-supplement to basic substrate but also help in maintaining optimal C:N ratio and porosity to sustain oxygen supply in SSF. In this study, Plackett-Burman (PB) and Box-Behnken matrix were used for optimization of culture media for laccase production by white rot fungus (WRF-1) in SSF. In addition, to establish optimal fermentation medium composition, the present methodology also helps to predict the yield if the composition of the medium components is altered in some way. The utilization of low cost raw material, for example, ground nut-shell and cyanobacterial biomass led to reduction in the culture medium cost for laccase production. Therefore, with the increase in yield and productivity and simultaneously cost reduction, the industrial laccase production by WRF-1 can be regarded as economically attractive process.

Acknowledgements
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REFERENCES