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# A STATISTICAL APPROACH FOR ENHANCING LACCASE YIELDS FROM WHITE ROT FUNGI (WRF) USING RESPONSE SURFACE METHODOLOGY

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**Abstract-** A high demand for fungal laccases on account of innumerable biotechnological applications necessitates enhanced production. Three novel isolates of white rot fungi (WRF) designated NI-07, NI-09 and NI-04 were subjected to submerged cultivation in liquid basal medium for laccase production. Of a total of 23 factors evaluated, 22 factors were selected for GLM using Proc GLM procedure of SAS (Version 9.3). The first optimization step identified the significant factors for laccase production and were used to construct RSM using PROC RSREG of SAS along with Lack Fit to maximize laccase enzyme activity. The statistical software, Design-Expert (version 9.0.1.0) was used to analyze the data and generate Response Surface Graphs (3D) for statistical optimal condition given by SAS (RSM) to build an optimized response using Response Surface Methodology (RSM). The initial laccase activity of 737U/mL increased to 7833U/mL in isolate NI-07, from 700 U/mL to7480 U/mL in NI-09 and from 1132 U/mL to 11141 U/mL in NI-04. When compared to the conventional method, a 10 fold increase in laccase activity could be obtained in the three isolates of WRF (10.62 for NI-07, 10.68 for NI-09 and9.84 for NI-04) after statistical optimization employing RSM. Validation experiments proved that experimentally determined production values were in close agreement with statistically predicted ones, confirming the reliability of the model. The results of this study serve as reference for optimization of medium composition for enhancing laccase production in WRF in submerged fermentation. Through statistical optimization maximum yields of laccase could be achieved at a minimum production cost.

**Keywords-** Design-Expert, inducers, laccase, OFAT, pH, RSM, temperature

**Short title-** Enhancing laccase production in white rot fungi

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## Introduction

Laccases (benzenediol:oxygen oxidoreductases (EC1.10.3.2), catalyze the oxidation of both phenolic and non phenolic substrates with the concomitant reduction of molecular oxygen to water. As an oxidase, laccase is used in many agricultural, industrial, medicinal and biotechnological applications. Current investigations are focused on laccase-based biooxidation, biotransformation, biosensor, and enzymatic synthesis of organic compounds. Fungal laccases play a variety of roles, such as, morphogenesis, pathogenesis, and lignin degradation [1-3]. White rot fungi (WRF), especially the basidiomycetes, have been the focus of attention owing to the fact that they remain the most promising source of laccase production. As low levels of laccases are secreted in the native state by WRF it necessitates production of larger quantities of laccase to meet the growing demand on the one hand and cost effectiveness on the other.

The optimal design of the culture medium is a very important aspect as medium composition can significantly affect product yield and economics. Many efforts have already been directed towards enhancing laccase production by optimizing nutrient media composition in fungal fermentations [4,5]. Conventional optimization procedures in fungal fermentations usually employ OFAT (one factor at a time) method, altering one parameter at a time keeping all the other parameters constant, which is laborious and time consuming. This process also requires many experimental data sets and above all it cannot provide information about the mutual interactions of the different parameters employed [2].

Currently research focus is directed at optimization of culture media components using various statistical methods, based on data obtained through conventional optimization methods. For optimizing enzyme production using microorganisms a number of statistical experimental designs with Response Surface Methodology have been employed [6]. Response Surface method (RSM) is a statistical technique for designing experiments, building models, evaluating the effects of several factors, and searching optimum conditions for desirable responses [7]. The Response Surface Methodology (RSM) first described by Box & Wilson [8] is an effective strategy for seeking the optimum conditions for a multivariable system [9,10]. and the main advantage of this method is the number of experi-

ments trials to evaluate multiple parameters can be reduced. This is of great relevance to the industry, where experiments can be very expensive and time consuming. Results can continually be received from each run rather than having to wait until the entire experiment is completed [10]. RSM has successfully been demonstrated for its efficiency in evaluating and optimizing the interactions between various physicochemical parameters and process variables in fermentation [10-12]. The present study was directed at building an optimized response for different components viz. pH, temperature, fermentation duration, carbon sources, nitrogen sources and inducers used in the culture media to enhance production of laccase enzyme by three novel isolates of white rot fungi (WRF) designated NI-07, NI-09 and NI-04 using RSM. As far as our knowledge goes this is the first study wherein such a large number of parameters have been successfully utilized at one time for statistical optimization of enhanced laccase production employing RSM in three isolates of WRF.

## Material and Methods

### Fungal Isolates

Three isolates of WRF NI-07, NI-09 & NI-04 screened positive as potent laccase producers [13] were selected for monitoring the effect of growth (biomass) on laccase enzyme production. Cultures were grown in 100 mL Erlenmeyer flasks containing 20 mL of sterile liquid basal medium in five replicates. Inoculum disks were transferred aseptically into the flasks and fermentation was carried out 28ºC and 120 rpm for 7 days. Biomass and activity was monitored at regular intervals.

### Optimization Experiments Using One-Factor-at-a-Time (OFT) Method

Optimization experiments were carried out by altering the various culture conditions that affect growth of fungal biomass and laccase enzyme production so as to maximize production.

### Effect of Temperature on Laccase Activity

Laccase activity was monitored in all the three isolates of WRF NI-07, NI-09 & NI-04 after growing the cultures for seven days under continuous agitation (120 rpm) at four different temperatures viz. 25°C, 30°C, 35°C and 40°C in three replicates. The cultures were harvested after seven days and the mycelial free filtrate was used to determine laccase activity.

### Effect of pH on Laccase Activity

The three fungal isolates of WRF NI-07, NI-09 & NI-04 were grown in liquid basal media with pH ranging from 3.5 to 10 (gradation of 0.5) with three replicates for each pH. After eight days the mycelium free filtrate was used to determine the laccase activity.

### Effect of Carbon Sources on Laccase Activity

The three isolates of WRF NI-07, NI-09 & NI-04 were grown in a chemically defined liquid basal medium containing L-aspargine (0.1% w/v) as the nitrogen source and supplemented with different carbon sources at 1% w/v [14]. The carbon sources used for the study were glucose, maltose, fructose, lactose, cellobiose, sucrose and starch. Each of the carbon sources was replicated into three flasks to obtain the average of mycelium mass. After seven days of incubation at 28ºC under continuous agitation (120 rpm), the cultures were harvested and the mycelium free filtrate was used to determine laccase activity. The basal medium containing glucose (20 g·L) was used as the control. The dry weight of the mycelium was also recorded.

### Effect of Nitrogen Sources on Laccase Activity

The three WRF isolates NI-07, NI-09 & NI-04 were cultured in a chemically defined medium containing 1% glucose as carbon source and supplemented with different nitrogen sources at 1% (w/v). The nitrogen sources used included L-aspargine, yeast extract, ammonium sulphate, glycine and peptone. Each nitrogen source was replicated into three flasks to obtain the average of mycelium mass. All the flasks were incubated at 28ºC under continuous agitation (120 rpm) for a duration of 7 days. Dry weight of the mycelium was monitored along with laccase activity of the filtrate. The basal medium with L-aspargine (2.5 g ·L) was treated as control.

### Effect of Inducers on Laccase Production

To study the effect of inducers on laccase activity, different aromatic inducers such as p-anisidine (p-methoxy aniline), ferulic acid (4-hydroxy-3-methoxy benzoic acid), guaiacol, 1-hydroxybenzotriazole (HoBT), veratryl alcohol, Tween-80 were dissolved in 50% ethanol as stock solutions and sterilized by filtration. Catechol and vanillic acid were dissolved in sterile distilled water aseptically to give a concentration of 0.1 M. Three flasks were replicated for each inducer to obtain the average of mycelial mass. All the flasks were incubated at 28ºC under continuous agitation (120 rpm). On the fourth day of incubation 0.1 M inducer was added to the growing fungal cultures so that the final concentration in the flask was 1 mM. Dry weight of the mycelium was monitored along with laccase activity of the filtrate for a duration of 7 days.

### Statistical Methods

After the first step of media optimization employing the conventional one factor at a time method (OFT), 22 out of the 23 evaluated factors were selected for GLM using Proc GLM procedure of SAS (Version 9.3) [15].

GLM was considered to establish the Analysis of Variance (ANOVA) and least square means to check for the effect of different variables on mycelial growth (biomass) and laccase enzyme production.

### Optimization of Culture Media Components using RSM

Response Surface Methodology (RSM) was used to optimize the production of laccase enzyme from nine screened variables by GLM procedure. From these results a matrix design was constructed for RSM using PROC RSREG of SAS along with Lack Fit to maximize the activity levels of laccase. The statistical response of the combined effect of each individual factor as well as the individual effect on the quantity of laccase enzyme production was elucidated. To test the goodness of fit of the model, the regression equation and the determination coefficient $R^2$ were evaluated. The regression equation can be explained by the general equation [Eq-1] [16]:

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ii} X_i X_i \qquad (1)$$

Where Y represents the response variable, $\beta_0$ is the interception coefficient, $\beta_i$ the coefficient of the linear effect, $\beta_{ii}$ is the coefficient of quadratic effect and $\beta_{ij}$ is the coefficient of interaction effect.

### Statistical Software

Design-Expert (version 9.0.1.0) was the statistical software, em-

ployed to analyze the data and generate Response Surface Graphs (3D) for statistically optimized conditions given by SAS (RSM). Pareto charts were created using factorial design so as to illustrate the order of significance of variables for enhancing laccase production.

**Validation of the Model**

The optimized parameters obtained from RSM were validated experimentally in case of the three isolates of WRF, NI-07, NI-09 & NI-04.

**Results and Discussion**

Laccases are produced by wild-type filamentous fungi during secondary metabolism either by submerged and/or solid state fermentation [17]. Fungal laccases secreted by WRF, have been the focus of attention of many researchers owing to their high demand on account of their innumerable biotechnological applications [1]. As the levels of laccases secreted by most WRF in their native state are very low, it necessitates enhancing production of higher quantities of laccase through optimization of the media composition in order to meet the growing demand on the one hand and cost effectiveness on the other. Several factors which include: pH, temperature, carbon limitation, nitrogen source, and concentration of micro-elements influence laccase production [18]. Traditional optimization methods are comprised of changing one independent variable at a time and keeping all the other variables fixed at a designated level. In this study out of a total of 23 factors evaluated, 22 factors were selected for GLM performed to pick out significant factors for enhancing laccase production. As the fermentation duration is a very critical factor in laccase enzyme production this was directly considered for RSM studies without subjecting to initial screening from GLM. The 22 factors selected for GLM included temperature (25, 30, 35 and 40°C), pH (3.5 to 10 with a gradation of 0.5), 7 carbon sources (glucose, maltose, fructose, lactose, cellobiose, sucrose and starch), 5 nitrogen sources (L-asparagine, yeast extract, ammonium sulphate, glycine and peptone) and 8 inducers (p-anisidine (p-methoxy aniline), ferulic acid (4-hydroxy-3-methoxy benzoic acid), guaiacol, 1-hydroxybenzotriazole (HoBT), veratryl alcohol and Tween-80) were selected for optimization of enhanced laccase production by adoption of OFT. The least square means and the significant levels for each factor which contributed to laccase production were tabulated [Table-1]. [Table-1] summarizes the PROC-GLM report with p-values of different levels of temperature and pH, carbon sources, nitrogen sources and inducers used and their influence on the laccase activity (LS-means).

The first optimization step identified the significant factors for laccase production and were used to construct RSM using PROC RSREG of SAS along with Lack Fit to maximize laccase enzyme activity. The statistical software, Design-Expert (version 9.0.1.0) was then used to analyze the data and generate Response Surface Graphs (3D) for statistical optimal condition given by SAS (RSM) so as to build an optimized response using Response Surface Methodology (RSM).

**Effect of Temperature on Laccase Activity**

The optimum temperature reported for laccase production lies between 25°C and 30°C [19]. When cultivation of fungi was carried out at temperatures higher than 30°C the laccase enzyme activity was observed to get reduced [20]. The effect of different temperatures on production of laccase enzyme is given in [Fig-1a]. The distribution of laccase activity with respect to different temperature as seen from the Box plot shows that 30°C has a wide range of activity with a higher average which is in agreement with earlier reports. The effect of different temperatures on laccase productivity analysed using Proc GLM [Table-1] also showed that a temperature of 30°C was highly significant ($p$<0.001) at 99% CI with highest LS-means of 734. Increase in temperature beyond 30°C showed a gradual decrease in LS-means of laccase activity. This explains that 30°C is the optimum temperature for laccase production and higher temperatures have a negative effect on the laccase activity production. From [Table-1] it can also be seen that at temperatures beyond 55°C there is a depression in growth of the fungus.

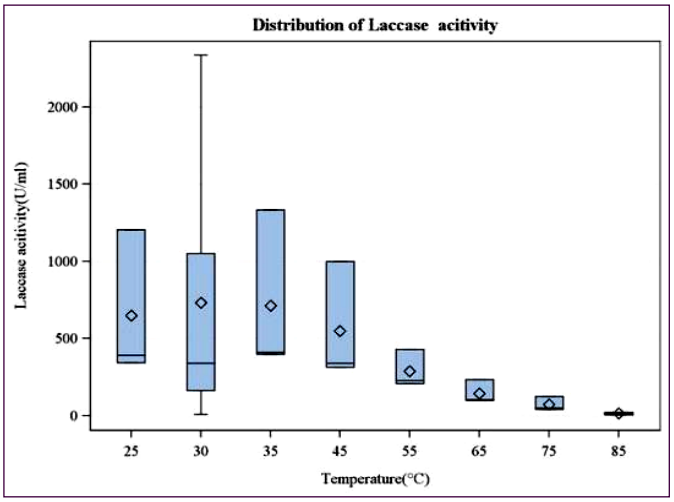


**Fig. 1a-** Box plot for distribution of laccase activity with respect to temperature

**Effect of pH on Laccase Activity**

Most of the available reports indicate that an initial pH between 4.5 and 6.0 is suitable for production of laccase enzyme [21]. The effect of different pH on production of laccase enzyme in our study is given in [Fig-1b].

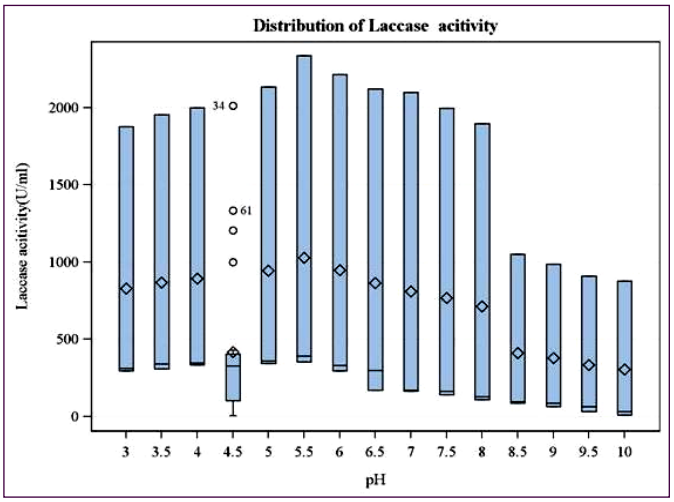


**Fig. 1b-** Box plot for distribution of laccase activity with respect to pH

The distribution of laccase activity with respect to different pH as seen from the Box plot shows that acidic pH of 5.5 had higher average and broad activity range when compared to others. The effect of a wide range of pH from 3 to 10 was tested to select the pH fa-

voring maximum laccase production. Enhanced laccase activity was obtained between pH 3 to 5.5 indicating that acidic pH was favourable for the production of laccase [Table-1]. A decrease in production was observed with increase in pH explaining the negative impact of alkaline pH. From the analysis it is clear that pH 4.5 was significant ($p$<0.001) with LS-means of 417 at CI of 99% and 5.5 had a higher LS-means of 542 with suggestive significance.

### Effect of Carbon Sources on Laccase Activity

High concentrations of glucose have been reported to inhibit laccase production in a number of fungal strains while excess sucrose also reduced laccase production by blocking its induction [22]. The effect of different carbon sources in our study, on production of laccase enzyme is given in [Fig-1c]. The distribution plot with respect to carbon source shows that fructose, glucose and sucrose as a carbon source had broader range of activity and higher averages compared to other carbon sources [Fig-1c]. From [Table-1], it can be seen that glucose and fructose showed a positive influence on the laccase activity with LS-means 723.20 and 888.53 showing significance ($p$<0.05) with 95% confidence Interval whereas sucrose gave suggestive significance at 90% confidence Interval (CI) with LS-means of 780.53.

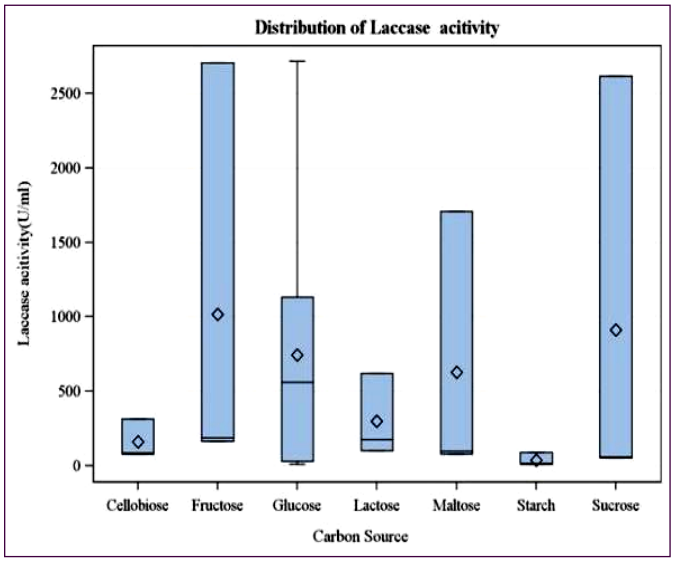


**Fig. 1c-** Box plot for distribution of laccase activity with respect to carbon sources

### Effect of Nitrogen Sources on Laccase Activity

Fungal laccases have been reported to be triggered by nitrogen depletion [23], though some studies reported nitrogen to have no effect on enzyme activity [24]. High laccase activity was reported by using low carbon to nitrogen ratio [25], while others reported higher laccase production by employing a high carbon to nitrogen ratio [26]. Some studies reported high laccase production upon cultivation of fungus in nitrogen rich rather than nitrogen-limited media [27]. The distribution of laccase activity obtained in our study with respect to different nitrogen sources is given [Fig-1d]. The distribution plot with respect to nitrogen source showed that L-aspargine, peptone and yeast extract as a nitrogen source had broader range of activity and higher averages compared to other nitrogen sources. Among the nitrogen sources shown in [Table-1], L-aspargine (LS-means of 558.24) was found to be highly significant ($p$<0.001) at 99% CI.

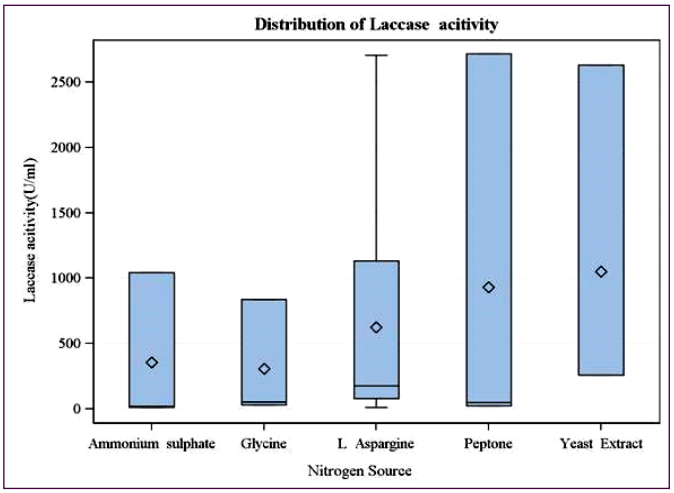


**Fig. 1d-** Box plot for distribution of laccase activity with respect to nitrogen sources

### Effect of Inducers on Laccase Production

Production of laccases can be enhanced to a great extent by the addition of various inducer compounds/ supplements to the culture media [28]. An increase and induction of laccase activity was reported upon addition of xenobiotic compounds like xylidine, lignin, and veratryl alcohol [29]. The addition of cellobiose was reported to induce appreciable laccase activity in some species of *Trametes* [30]. A low concentration of copper was also found to induce laccase production [31]. Addition of 2,5-xylidine to various basidiomycetes, ascomycetes, and deuteromycetes grown in sugar rich liquid medium were observed to induce laccase production. While in cultures of *Fomes annosus*, *Pholiota mutabilis*, *Pleurotus ostreatus*, and *Trametes versicolor* laccase production was stimulated by the addition of xylidine, in *Podospora anserina* an inhibition of laccase production was obtained [32]. The distribution of laccase activity in response to different inducers from our study is elaborated in [Fig-1e]. The distribution plot showed that catechol, veratryl alcohol and p-anisidine had higher averages compared to other inducers. All the tested inducers showed positive influence in enhancing the laccase activity levels [Table-1]. Among them catechol, veratryl alcohol and p-anisidine were highly significant compared to others with greater LS-means of 2329, 1887 and 3270 respectively at 99% CI.

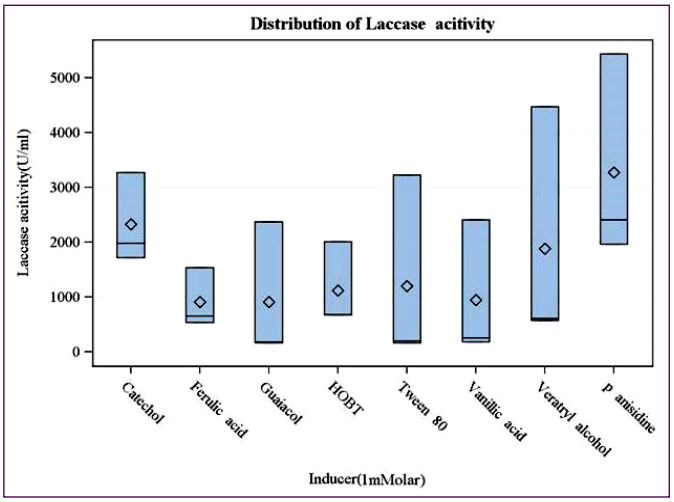


**Fig. 1e-** Box plot for distribution of laccase activity with respect to inducers

**Table 1-** Least Square mean values for laccase activity with regard to different temperatures, pH values, different carbon sources, nitrogen sources and inducers

| Factors | LS-Means ± SE | P value |
|---|---|---|
| Temperature | | |
| 25 | 478.91±312.79 | 0.1332 |
| 30 | 733.24±58.08 | <0.0001** |
| 35 | 545.91±312.79 | 0.0882+ |
| 45 | 382.24±312.79 | 0.2285 |
| 55 | 120.58±312.79 | 0.7018 |
| 65 | -23.09±312.79 | 0.9415 |
| 75 | -95.42±312.79 | 0.7618 |
| 85 | -154.09±312.79 | 0.6248 |
| pH | | |
| 3 | 342.96±308.04 | 0.2719 |
| 3.5 | 383.96±308.04 | 0.2195 |
| 4 | 408.96±308.04 | 0.1915 |
| 4.5 | 417.63±79.53 | <0.0001** |
| 5 | 460.96±308.04 | 0.142 |
| 5.5 | 542.63±308.04 | 0.0854+ |
| 6 | 462.29±308.04 | 0.1409 |
| 6.5 | 378.63±308.04 | 0.2259 |
| 7 | 326.29±308.04 | 0.2955 |
| 7.5 | 282.63±308.04 | 0.3641 |
| 8 | 227.29±308.04 | 0.4647 |
| 8.5 | -72.71±308.04 | 0.8146 |
| 9 | -105.38±308.04 | 0.734 |
| 9.5 | -149.71±308.04 | 0.6295 |
| 10 | -178.38±308.04 | 0.5656 |
| Carbon Source | | |
| Cellobiose | 30.86±427.49 | 0.943 |
| Fructose | 888.53±427.49 | 0.0481* |
| Glucose | 723.20±166.56 | <0.0001** |
| Lactose | 170.20±427.49 | 0.6939 |
| Maltose | 498.53±427.49 | 0.2545 |
| Starch | -90.46±427.49 | 0.8341 |
| Sucrose | 780.53±427.49 | 0.0798+ |
| Nitrogen Source | | |
| Ammonium sulphate | 62.57±416.43 | 0.8818 |
| Glycine | 11.57±416.43 | 0.9781 |
| L-Aspargine | 558.24±128.03 | <0.0001** |
| Peptone | 634.90±416.43 | 0.1399 |
| Yeast Extract | 958.39±492.04 | 0.0628+ |
| Inducers | | |
| Catechol | 2328.67±350.78 | <0.0001** |
| Ferulic_acid | 912.33±350.78 | 0.0209* |
| Guaiacol | 907.67±350.78 | 0.0215* |
| HOBT | 1121.67±350.78 | 0.0065** |
| Tween_80 | 1200.33±350.78 | 0.0041** |
| Vanillic_acid | 949.33±350.78 | 0.0170* |
| Veratryl_alcohol | 1887.00±350.78 | <.0001** |
| p_anisidine | 3270.67±350.78 | <.0001** |

+Suggestive significance ($0.05<p<0.10$); *Moderately significant ($0.01<p\leq0.05$); & **Strongly significant ($p\leq0.01$)

The Interaction plot for laccase activity [Fig-2] with respect to the effect of differnet inducers on the three WRF cultures exhibits that p -anisidine and catechol have a positive influence on culture NI-07(a) and NI-09 (b), whereas p-anisidine and veratryl alcohol were found to be effective inducers in case of NI-04 (c) for enhancing laccase production. It is clearly evident from the analysis that the factors glucose, fructose, L-aspargine, catechol, veratryl alcohol and p-anisidine are vital for enhancing laccase production as evident from the significance and LS-mean values.

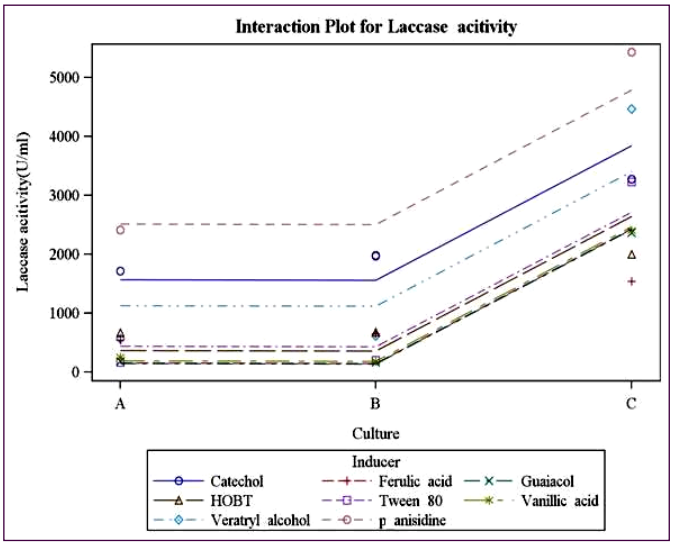


**Fig. 2-** The interaction plot for laccase activity with respect to different inducers in case of the three WRF cultures

### Effect of Biomass

Proc GLM for biomass LS means and significance with respect to different carbon sources and nitrogen sources has been summirized [Table-2] and the biomass distribution patterns for carbon and nitrogen sources have been shown in the [Fig-3](a&b) respectively. From the results it is clearly evident that only glucose and L-aspargine contributed positively towards build up of fungal biomass with LS means of 0.78±0.57 (glucose) and 0.65±0.56 (L-aspargine). All other carbon and nitrogen source evaluated in the optimization experiments failed to show a significant effect.

**Table 2-** Least Square mean values for biomass with regard to various carbon and nitrogen sources

| Factors | LS-Means ± SE | P value |
|---|---|---|
| Carbon Source | | |
| Cellobiose | -0.43±0.94 | 0.651 |
| Fructose | -0.27±0.94 | 0.773 |
| Glucose | 0.78±0.57 | 0.183 |
| Lactose | -0.13±0.94 | 0.893 |
| Maltose | -0.30±0.94 | 0.75 |
| Starch | -0.24±0.94 | 0.801 |
| Sucrose | 0.05±0.94 | 0.956 |
| Nitrogen Source | | |
| Ammonium sulphate | -0.66±0.94 | 0.489 |
| Glycine | 0.14±0.94 | 0.882 |
| L-Aspargine | 0.65±0.56 | 0.258 |
| Peptone | -0.32±0.94 | 0.735 |
| Yeast Extract | -0.23±0.94 | 0.805 |

### Statistical Optimization using RSM

Based on the results of our preliminary study on the production of laccase by the three WRF isolates NI-07, NI-09 & NI-04 [13] experiments were performed to identify the significant variables affecting enzyme production which were considered for RSM. A total of nine independent variables (8 from GLM+ incubation duration preselected based on its criticality) were considered for RSM, extrapolated from the GLM reports. The experimental range and levels of all the independent variables evaluated for optimization of enhanced laccase production are tabulated in [Table-3]. Experiments carried out by conventional method with the selected variables were consid-

ered for carrying out the RSM analysis to enhance laccase enzyme production in the three WRF cultures. The experimental design used for the analysis is elaborated in [Appendix-1]. matrix design was applied to the nine significant variables. For all three WRF isolates NI-07, NI-09 & NI-04 the carbon source1, nitrogen source1 and inducer1 were taken as glucose, L-aspargine and P- anisidine respectively, where as carbon source 2 for culture NI-07, NI-09 (a and b) was fructose and for culture NI-04 (c) it was sucrose. Nitrogen source 2 for all the cultures was yeast extract and Inducer 2 for culture a and b was catechol and in case of culture C it was veratryl alcohol. Selection for the RSM was based on the highest LS means for laccase activity and significance level with respect to different factors obtained from GLM procedure [Table-1].

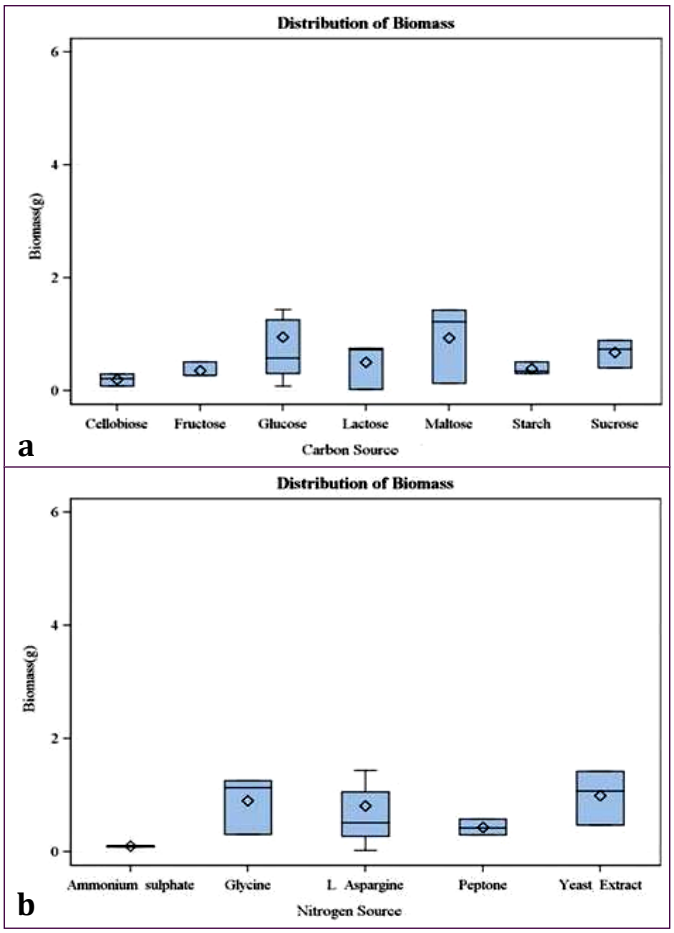


**Fig. 3-** Distribution of biomass with regard to different **(a)** carbon sources; and **(b)** nitrogen sources

**Table 3-** Experimental range and levels of all the independent variables evaluated for optimization of laccase production

| Factor | Name | Units | Minimum | Maximum |
|---|---|---|---|---|
| A | pH | | 3 | 10 |
| B | Incubation | Days | 1 | 9 |
| C | Temperature | °C | 25 | 85 |
| D | Glucose | % | 0 | 2 |
| E | Fructose/Sucrose | % | 0 | 2 |
| F | L-Aspargine | % | 0 | 0.25 |
| G | Yeast extract | % | 0 | 0.25 |
| H | P-anisidine | mM | 0 | 1 |
| I | Catechol/Veratryl alcohol | mM | 0 | 1 |

The fit of the model was checked by the determination coefficient ($R^2$) and coefficient of variation (CV). ANOVA of regression model demonstrated that the model adopted for optimization of lacccase production by the three WRF, NI-07 (a), NI-09 (b) and NI-04(c) was highly significant as was evident from the Fischer test with very low probability value [Table-4]. The model F values of 9.62, 14.28 and 31.10 for all the three WRF isolates NI-07, NI-09 & NI-04 indicated that the models were significant. Evaluated analysis of variance (ANOVA) from RSM for all the three WRF cultures NI-07, NI-09 and NI-04 is summarized in [Table-5]. Factors like pH and temperature were found to be significant contributors to enhancing laccase production. Glucose, L-aspargine and p-anisidine and catechol were observed to be significant only in case of the WRF cultures NI-07 and NI-09 while incubation period, sucrose, veratryl alcohol and p-anisidine were found to be significant in case of the WRF culture NI-04 [Table-5].

**Table 4-** Goodness of fit for RSM models adopted for optimization of lacccase production NI-07 (a), NI-09 (b) and NI-04(c

| Model | R-Square (%) | CV | Sum of squares | Mean square | F value | *p* value |
|---|---|---|---|---|---|---|
| A | 87.8 | 57.9 | 7467160 | 622263.3 | 9.62 | < 0.0001** |
| B | 87.3 | 58.1 | 6464363 | 538696.9 | 14.28 | <0.0001** |
| C | 78.3 | 39.1 | 48013981 | 4001165 | 31.1 | <0.0001** |

** Strongly significant ($p \leq 0.01$)

Significance of the factors were considered at 90% CI with $p<0.01$. Interaction of the factors and the squared factors seems to be insignificant and can be removed from the model without affecting the goodness of fit. The predicted and observed responses along with design matrix using Design-Expert (version 9.0.1.0) are given in [Appendix-2]. The comparison graphs for actual Vs predicted values for NI-07, NI-09 and NI-04 indicate a strong relationship between the experimental and the predicted responses [Fig-4a], [Fig-4b] & [Fig-4c].

$$Y = -534.11 + 19.03*A + 92.54*B - 8.36*C + 209.38*D + 88.0*E + 1675.04*F + 1000.0*G + 1984.24*H + 1294.24*I - 7.20*A^2 - 4.38*B^2 + 0.003*C^2 \quad (1a)$$

$$Y = -518.69 + 69.13*A - 21.14*B - 4.10*C + 189.76*D + 74.0*E + 1514.07*F + 956.0*G + 1566.48*H + 1590.48*I - 10.58*A^2 + 7.34*B^2 - 0.03*C^2 \quad (1b)$$

$$Y = -720.72 + 759.95*A + 266.12*B - 95.36*C + 132.47*D + 764.50*E + 89.87*F + 549.17*G + 25445*H + 35075*I - 67.59*A^2 - 56.18*B^2 + 0.52*C^2 \quad (1c)$$

Where 'Y' is the laccase activity (U/ml), A,B,C,D,E,F,G,H and I are pH, incubation temperature, glucose, fructose, L-aspargine, yeast extract, p-anisidine and catechol for the WRF culture NI-07 and NI-09, while pH, incubation temperature, glucose, sucrose, L-aspargine, peptone, p-anisidine and veratryl alcohol for culture NI-04.

3D response surface graphs were plotted based on the model equation for data obtained using SAS (Version 9.3) to investigate the interaction among the variables and determine the optimum concentration of each factor for maximum laccase production for the three WRF isolates NI-07, NI-09 and NI-04. The three dimensional response surface graphs generated using version 9.0.1.0 of Design-Expert based on the final model for studying the interaction among variables for maximizing laccase production, depicted that with increasing concentrations of glucose as carbon source and L-aspargine as nitrogen source there was a sequential increase in the activity of laccase in NI-07 [Fig-5a]. When both catechol and p-anisidine were present in the media, the presence of p-anisidine as inducer had a positive effect in increasing laccase production with an increase in the p-anisidine concentrations, whereas catechol

showed little or no effects on the activity levels with increasing concentrations. Decreasing trend in both pH and temperature showed an increase in the laccase activity levels indicating the very fact that lower pH activates the enzyme at around 30°C. Increasing the concentration of glucose beyond 2% would enhance laccase production, is a question which needs further validation [Fig-5b], but the effect of incubation period on laccase activity showed a steady increase up to 7 days and increase in enzyme production beyond this duration was negligible clearly demonstrating that 8 days incubation was ideal enough for enhancing laccase production in case of NI-09. Increase in the laccase production was monitored with decrease in the pH and increase in the glucose concentrations and a sharp decrease in activity above pH 7.0 was obtained. With addition of inducers into the culture medium, the incubation duration could be brought down drastically. After addition of p-anisidine into the medium a sharp increase was obtained in laccase production which increased by increasing the concentration of p-anisidine. The response surface graphs in [Fig-5c] demonstrate that maximum activity of laccase was recorded at a temperature of 30°C in culture NI-04. Increase in glucose concentration in the culture media had a positive effect with respect to increase in laccase production. However, increasing the yeast extract concentrations above 0.08 showed a depression in laccase production signifying that it was a less significant nitrogen source in presence of L-asparagine. Maximum laccase production was obtained within 1-5 days of fermentation. Higher values and lower values in all the graphs are explained with reference to already optimized activity depicted using RSM from SAS (Version 9.3).

**Table 5-** Evaluated Analysis of Variance (ANOVA) from RSM for WRF culture NI-07, WRF culture NI-09 and WRF culture NI-04

| Parameter | Degrees of freedom | Estimate | Sum of squares | Mean square | F value | P value |
|---|---|---|---|---|---|---|
| WRF culture NI-07 | | | | | | |
| Residual | 25 | | 1616829 | 64673.18 | | |
| Total | 38 | | 9083989 | | | |
| Intercept | 1 | -534.11 | | | -0.67 | 0.5107 |
| pH | 1 | 19.03 | 514542 | 257271 | 6.08 | 0.0073* |
| Incubation period (Days) | 1 | 92.54 | 199867 | 99933 | 2.36 | 0.1157 |
| Temperature (°C) | 1 | -8.36 | 364658 | 182329 | 4.31 | 0.0252* |
| Glucose (%) | 1 | 209.38 | 164322 | 164322 | 3.88 | 0.0604+ |
| Fructose (%) | 1 | 88 | 15488 | 15488 | 0.37 | 0.5508 |
| L-Aspargine (%) | 1 | 1675.04 | 164322 | 164322 | 3.88 | 0.0604+ |
| Yeast Extract (%) | 1 | 1000 | 31250 | 31250 | 0.74 | 0.3986 |
| p anisidine (mM) | 1 | 1984.24 | 3689364 | 3689364 | 87.21 | <0.0001** |
| Catechol (mM) | 1 | 1294.24 | 1569613 | 1569613 | 37.1 | <0.0001** |
| pH*pH | 1 | -7.2 | 17613.66 | 17613.66 | 0.272349 | 0.61 |
| Days* Days | 1 | -4.38 | 1853.344 | 1853.344 | 0.028657 | 0.87 |
| Temp*Temp | 1 | 0.003 | 1136.677 | 1136.677 | 0.017576 | 0.9 |
| WRF culture NI-07 | | | | | | |
| Residual | 25 | | 943352.3 | 37734.09 | | |
| Total | 38 | | 242016.3 | 11000.74 | | |
| Intercept | 1 | -518.69 | | | | |
| pH | 1 | 69.13 | 464898 | 232449 | 6.16 | 0.0067** |
| Incubation period (Days) | 1 | -21.14 | 163198 | 81599 | 2.16 | 0.136 |
| Temperature (°C) | 1 | -4.10 | 311619 | 155810 | 4.13 | 0.0282* |
| Glucose (%) | 1 | 189.76 | 135503 | 135503 | 3.59 | 0.0697+ |
| Fructose (%) | 1 | 74.00 | 10952 | 10952 | 0.29 | 0.595 |
| L-Aspargine (%) | 1 | 1514.07 | 134790 | 134790 | 3.57 | 0.0704+ |
| Yeast Extract (%) | 1 | 956.00 | 28560 | 28560 | 0.76 | 0.393 |
| p anisidine (mM) | 1 | 1566.48 | 2308552 | 2308552 | 61.18 | <0.0001** |
| Catechol (mM) | 1 | 1590.48 | 2379832 | 2379832 | 63.07 | <0.0001** |
| pH*pH | 1 | -10.58 | 29097.09 | 29097.09 | 0.77 | 0.388 |
| Days* Days | 1 | 7.34 | 9201.61 | 9201.61 | 0.24 | 0.626 |
| Temp*Temp | 1 | -0.03 | 929.20 | 929.20 | 0.025 | 0.876 |
| WRF culture NI-07 | | | | | | |
| Residual | 25 | | 3216383 | 128655.3 | | |
| Total | 38 | | 51230364 | | | |
| Intercept | 1 | 720.72 | | | -0.67 | 0.5107 |
| pH | 1 | 759.95 | 2689641 | 1344821 | 3.76 | 0.0327* |
| Incubation period (Days) | 1 | -266.12 | 12166017 | 6083008 | 17.00 | <0.0001** |
| Temperature (°C) | 1 | -95.37 | 10136141 | 5068070 | 14.16 | <0.0001** |
| Glucose (%) | 1 | 132.47 | 250869 | 250869 | 0.70 | 0.4078 |
| Sucrose (%) | 1 | 764.50 | 1948201 | 1948201 | 5.44 | 0.0252* |
| L-Aspargine (%) | 1 | -89.87 | 78190 | 78190 | 0.22 | 0.6429 |
| Yeast Extract (%) | 1 | 549.17 | 904752 | 904752 | 2.53 | 0.1203 |
| Veratryl alcohol (mM) | 1 | 25445 | 6162028 | 6162028 | 17.22 | 0.0002** |
| p anisidine (mM) | 1 | 35075 | 11708842 | 11708842 | 32.72 | <0.0001** |
| pH*pH | 1 | -67.59 | 1114602 | 1114602 | 8.663474 | 0.007 |
| Days* Days | 1 | 56.18 | 607916 | 607916 | 4.725154 | 0.039401 |
| Temp*Temp | 1 | 0.52 | 61731.96 | 61731.96 | 0.479824 | 0.494887 |

+Suggestive significance ($0.05<p<0.10$); *Moderately significant ($0.01<p\leq0.05$); & **Strongly significant ($p\leq0.01$)

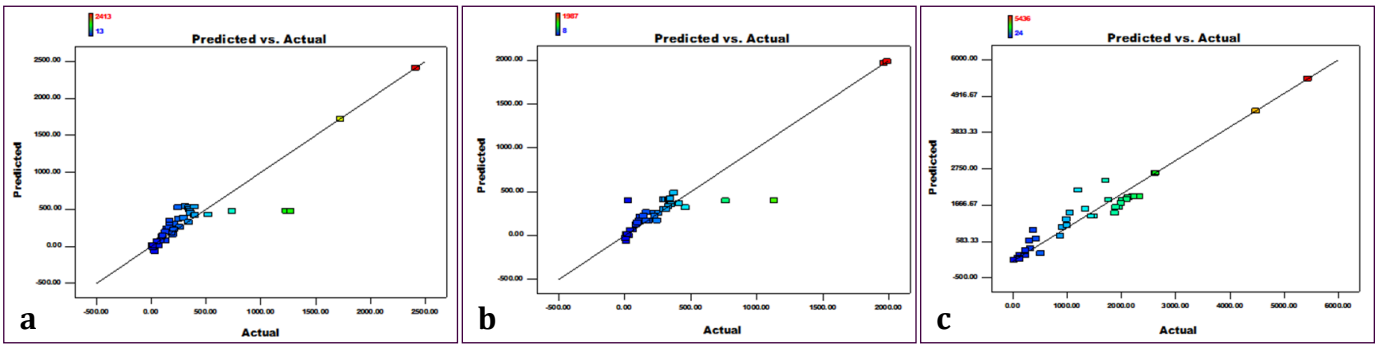


**Fig. 4-** Comparison graph for Actual Vs Predicted values for **(a)** NI-07; **(b)** NI-09; and **(c)** NI-04

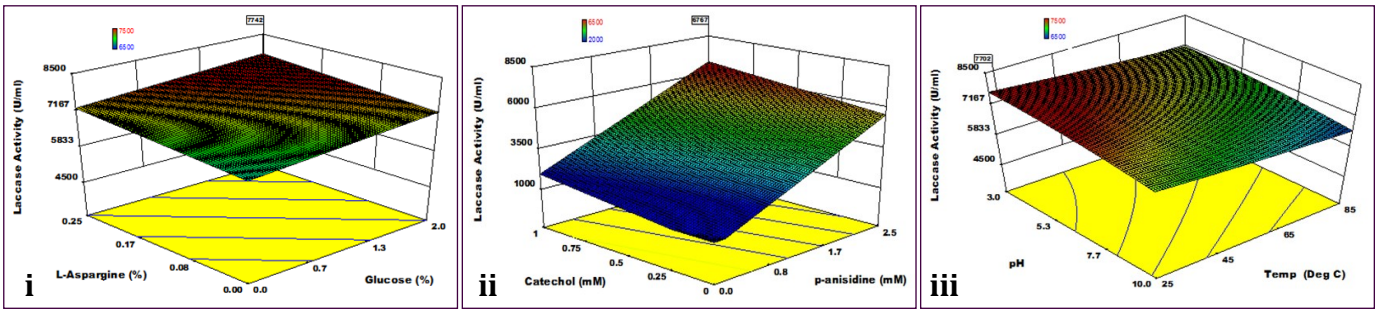


**Fig. 5a-** Three dimensional response surface plots for the effect of **(i)** L-aspargine and glucose, **(ii)** catechol and p-anisidine, **(iii)** pH and temp for WRF culture NI-07

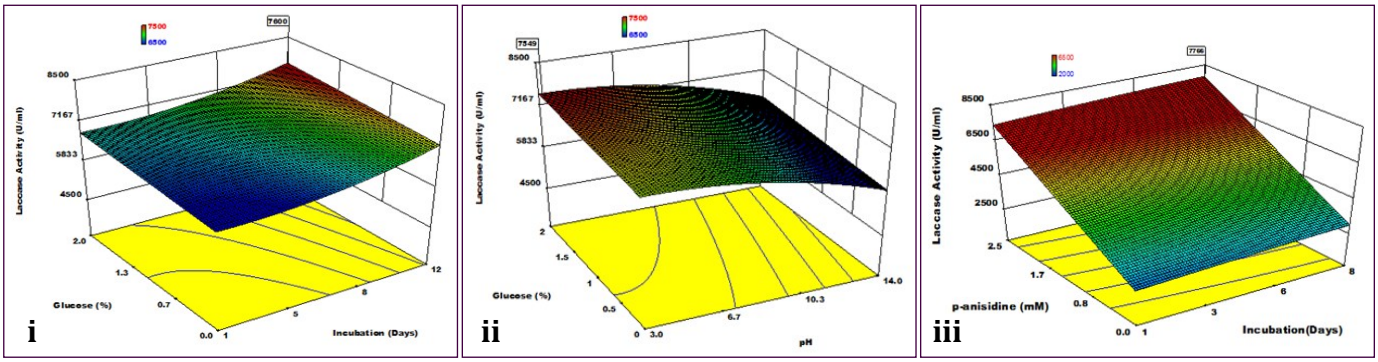


**Fig. 5b-** Three dimensional response surface plots for the effect of **(i)** glucose and incubation days; **(ii)** glucose and pH; **(iii)** p-anisidine and incubation days for WRF culture NI-09

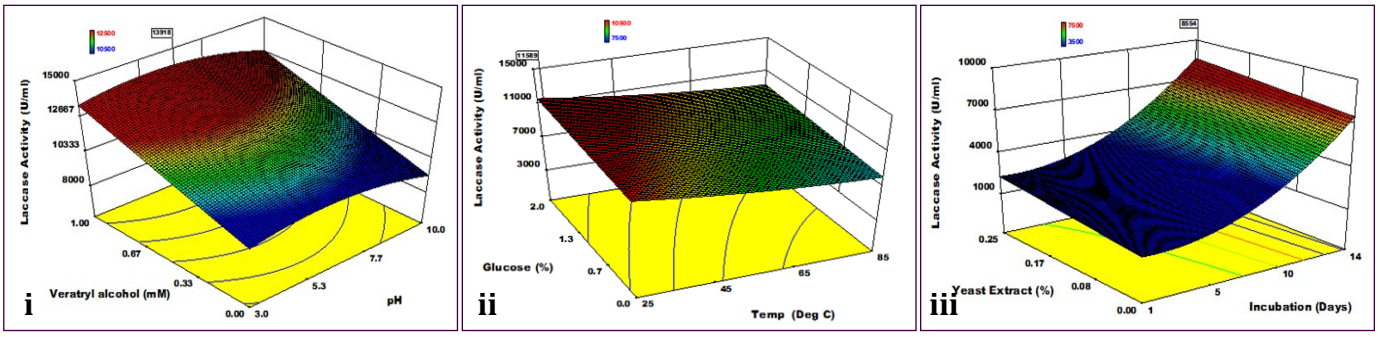


**Fig. 5c-** Three dimensional response surface plots for the effect **(i)** veratryl alcohol and pH; **(ii)** glucose and temperature; **(iii)** yeast extract and incubation days for culture WRF culture NI-04

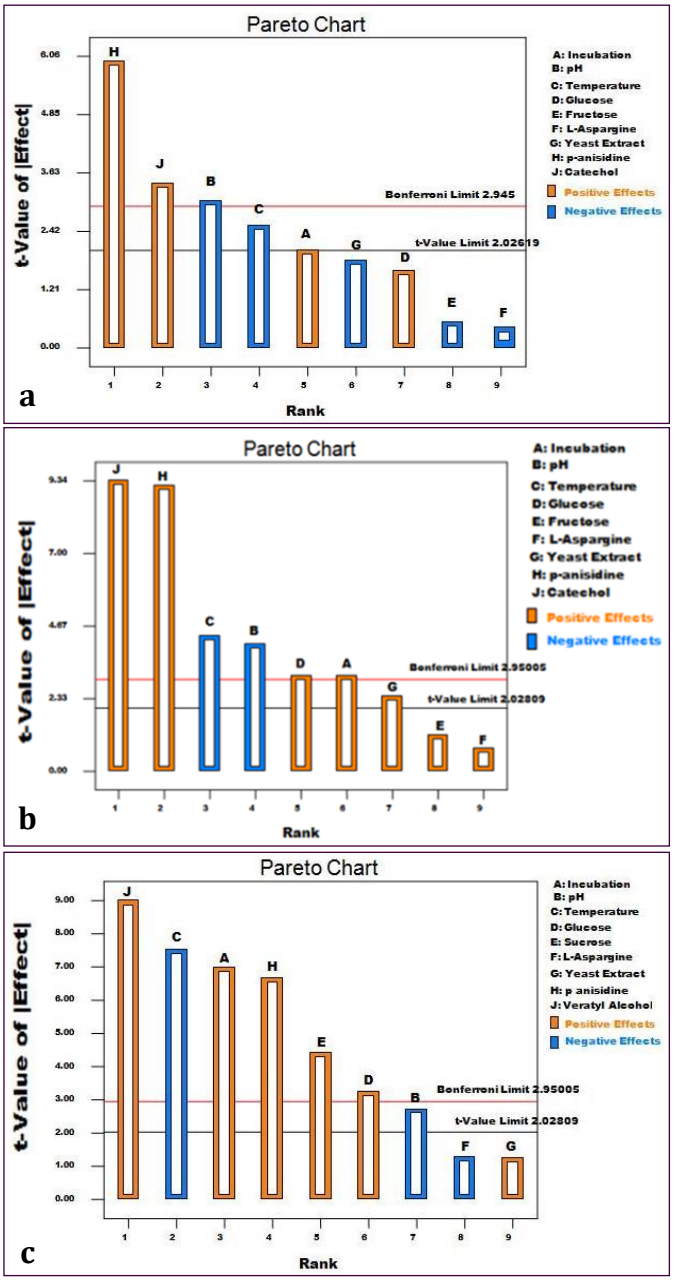


**Fig. 6-** Pareto Charts demonstrating the order of significance for different variables affecting laccase production for **(a)** NI-07; **(b)** NI-09; & **(c)** NI-04

The second order regression equation provided the laccase activity as a function of independent variables [Table-3] and can be presented in terms of mathematical expressions of coded factors as in the following equations for culture NI-07 [Eq-1a], NI-09 [Eq-1b] & NI -04 [Eq-1c]

The optimized parameters for enhancing laccase production obtained from RSM are given in [Table-6]. These parameters for enhancing laccase production in the WRF isolate NI-07using RSM gave a statistical optimal response of 4.3 for pH, 6.3 in case of days of incubation, an incubation temperature of 25.91°C, a glucose concentration of 1.8%, and a fructose concentration of 1.3% as the carbon sources, a L-aspargine concentration of 0.22% and a yeast extract concentration of 0.18% as nitrogen sources, a p-anisidine concentration of 2.45% and a catechol concentration of 1.77% as inducers. In the case of WRF isolate NI-09 the optimized parameters were obtained as pH of 4.8, 11.58 days of incubation, temperature of 33.37°C, a glucose concentration of1.7% and a fructose concentration off1.2%, and a L-asparagine concentration of 0.21%. For culture NI-04 the optimized factors for maximizing laccase production were obtained as a pH 6.1, 11.44 of incubation, temperature of 33.39°C, a glucose concentration of 1.05%, a sucrose concentration of 1.3% and a 0.96% concentration of L-asparagine.

RSM was employed [11] to find the best composition of factors such as inoculum (seed), carbon, nitrogen concentration to produce a maximum mycelium mass of *Schizophyllum commune* as a response. Of thirty set of different range medium compositions statistical analysis showed that optimum media containing 11.7% (v/v) of inoculum, 27g glucose and 1.2g yeast extract gave the maximum production of mycelium mass where the yields of mycelium mass increased from 6.148g/l (un optimized) to 15.68g/l in medium (optimized). RSM was applied [12] to determine laccase factor dependence in *Stenotrophomonas maltophilia* AAP56 using two substrates ABTS and DMP. The variables were dye (0 to 0.1mg $mL^{-1}$), Cu in Med(0 to 400µM), shaking(0 to 150rpm) and $CuSO_4$ in assay (0 to 0.2mM). A significant correlation was reported by them between the effects of these variables on ABTS and DMP oxidase activities. In the current study our RSM model depicted on an average a 10 fold enhanced yield in laccase production relative to that of the conventional method of media optimization, with enhanced activities of 7832 U/ml, 7479 U/ml and 11141 U/ml being recorded in case of the three WRF cultures NI-07, NI-09 and NI-04 respectively.

A maximum production of laccase during submerged fermentation was obtained at pH 8.0, 210 rpm and 100µM of $CuSO_4$ after 60 h of incubation employing RSM [10] for optimizing process conditions (pH, incubation time, agitation and $CuSO_4$ concentration). A 9.3 fold increase in laccase production was thus reported with experimental findings being in close agreement with the model predictions. In this study, as compared to the conventional method, a 10 fold increase in laccase activity was obtained in three isolates (10.62 for NI-07, 10.68 for NI-09 and9.84 for NI-04) of WRF after statistical optimization [Table-6].

**Table 6-** Statistically optimized parameters obtained from RSM for enhancing laccase production in the three cultures of WRF.

| Factors | Culture | | |
|---|---|---|---|
| | NI-07(A) | NI-09(B) | NI-04(C) |
| Incubation (days) | 6.3 | 11.58 | 11.44 |
| pH | 4.3 | 4.88 | 6.15 |
| Temperature (°C) | 25.9 | 33.38 | 33.39 |
| Glucose (%) | 1.82 | 1.74 | 1.05 |
| Fructose (%) | 1.35 | 1.28 | 1.30 (Sucrose) |
| L-Aspargine (%) | 0.23 | 0.22 | 0.96 |
| Yeast Extract (%) | 0.19 | 0.18 | 1.22 |
| p-anisidine (mM) | 2.45 | 2.02 | 0.08 |
| Catechol (mM) | 1.77 | 2.04 | 0.07 (veratryl alcohol) |
| Laccase activity (U/ml)* | 7833 | 7480 | 11141 |

*One unit of laccase activity was expressed as change in absorbance of 0.001/minute.

### Pareto Charts

The order of significance for different variables affecting laccase production was demonstrated using Pareto Chart for the three isolates of WRF NI-09, NI-07 & NI-04. Among the 9 variables tested for their effects, p-anisidine showed highest significance by showing

a high positive effect. pH showed a very high negative effect for NI-07, which beyond the optimal limit would prove inhibitory for laccase production [Fig-6a]. Catechol and p-anisidine showed significant positive effect and pH and temperature showed a negative effect for NI-09 [Fig-6b]. Veratryl alcohol and temperature showed a significant positive and negative effect simultaneously for WRF isolate NI-04 [Fig-6c].

### Validation of the Model

Employing RSM initial laccase activity of 737U/mL could be increased to 7833U/mL in WRF isolate NI-07, from 700U/mL to7480U/mL in NI-09 and from 1132U/mL to 11141U/mL in NI-04. These optimized predicted values obtained from RSM for enhancing laccase production were validated experimentally in case of the three isolates of WRF, NI-07, NI-09 & NI-04. The laccase activity obtained was 7606.36 U/mL in WRF isolate NI-07,7146.96 U/mL for NI-09 and 11026.9 U/mL in case of NI-04. The validation experiments proved that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the reliability of the model.

### Conclusion

It was possible to determine optimal media composition using one factor at a time method and RSM to maximize the production of laccase by the three WRF. Compared to the conventional method, a 10 fold increase in laccase activity was observed in all the three isolates NI-07, NI-09 and NI-04 after statistical optimization confirming that RSM could be effectively used to optimize the process parameters in complex processes using the statistical design of experiments. The results of this study provide useful information and reference for the optimization of medium composition for enhancing laccase production in other WRF in submerged fermentation processes. Through statistical optimization maximum yields of laccase could be achieved at a minimum production cost.

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**Appendix 1-** Matrix Design for RSM (Extrapolated from the conventional method)

| Run | Incubation (days) | pH | Temp (°C) | C* source 1 | C* source 2 | N** source 1 | N** source 2 | Inducer 1 | Inducer 2 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 2 | 2 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 3 | 3 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 4 | 4 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 5 | 5 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 6 | 6 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 7 | 7 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 8 | 8 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 9 | 7 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 10 | 7 | 4.5 | 30 | 0 | 2 | 0.25 | 0 | 0 | 0 |
| 11 | 7 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 12 | 7 | 4.5 | 30 | 2 | 0 | 0 | 0.25 | 0 | 0 |
| 13 | 7 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 1 | 0 |
| 14 | 7 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 1 |
| 15 | 7 | 3 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 16 | 7 | 3.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 17 | 7 | 4 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 18 | 7 | 4.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 19 | 7 | 5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 20 | 7 | 5.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 21 | 7 | 6 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 22 | 7 | 6.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 23 | 7 | 7 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 24 | 7 | 7.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 25 | 7 | 8 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 26 | 7 | 8.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 27 | 7 | 9 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 28 | 7 | 9.5 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 29 | 7 | 10 | 30 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 30 | 7 | 4.5 | 25 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 31 | 7 | 4.5 | 35 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 32 | 7 | 4.5 | 45 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 33 | 7 | 4.5 | 55 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 34 | 7 | 4.5 | 65 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 35 | 7 | 4.5 | 75 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 36 | 7 | 4.5 | 85 | 2 | 0 | 0.25 | 0 | 0 | 0 |
| 37 | 7 | 4.5 | 30 | 0 | 0 | 0.25 | 0 | 0 | 0 |
| 38 | 7 | 4.5 | 30 | 2 | 0 | 0 | 0 | 0 | 0 |

*C-carbon source, **N-nitrogen source

**Appendix 2-** Comparison table for actual Vs predicted Values

| Standard Order | Laccase activity (U/ml)* | | | | | |
|---|---|---|---|---|---|---|
| | A | | B | | C | |
| | Actual | Predicted | Actual | Predicted | Actual | Predicted |
| 1 | 134 | 76 | 181 | 171 | 136 | 186 |
| 2 | 197 | 159 | 245 | 172 | 221 | 166 |
| 3 | 142 | 237 | 118 | 187 | 511 | 232 |
| 4 | 212 | 307 | 116 | 218 | 320 | 382 |
| 5 | 250 | 371 | 217 | 263 | 301 | 617 |
| 6 | 517 | 428 | 459 | 322 | 379 | 936 |
| 7 | 737 | 479 | 764 | 397 | 1483 | 1340 |
| 8 | 245 | 523 | 373 | 485 | 1764 | 1829 |
| 9 | 1230 | 479 | 26 | 397 | 1712 | 2403 |
| 10 | 186 | 186 | 165 | 165 | 2616 | 2616 |
| 11 | 1273 | 479 | 1132 | 397 | 90 | 90 |
| 12 | 260 | 260 | 257 | 257 | 1440 | 1340 |
| 13 | 2413 | 2413 | 1963 | 1963 | 2630 | 2630 |
| 14 | 1723 | 1723 | 1987 | 1987 | 4473 | 4473 |
| 15 | 311 | 540 | 296 | 412 | 5436 | 5436 |
| 16 | 342 | 524 | 309 | 412 | 1876 | 1433 |
| 17 | 349 | 504 | 334 | 407 | 1955 | 1598 |
| 18 | 354 | 479 | 342 | 397 | 1998 | 1730 |
| 19 | 360 | 451 | 345 | 381 | 2011 | 1829 |
| 20 | 393 | 418 | 353 | 360 | 2132 | 1896 |
| 21 | 296 | 381 | 332 | 333 | 2336 | 1930 |
| 22 | 170 | 340 | 298 | 302 | 2213 | 1931 |
| 23 | 170 | 295 | 165 | 265 | 2122 | 1899 |
| 24 | 163 | 246 | 143 | 223 | 2098 | 1835 |
| 25 | 130 | 193 | 109 | 175 | 1996 | 1738 |
| 26 | 98 | 135 | 87 | 123 | 1897 | 1608 |
| 27 | 87 | 74 | 64 | 64 | 1051 | 1445 |
| 28 | 65 | 8 | 32 | 1 | 987 | 1249 |
| 29 | 32 | -62 | 11 | -68 | 908 | 1021 |
| 30 | 393 | 534 | 345 | 426 | 876 | 760 |
| 31 | 399 | 426 | 413 | 366 | 1206 | 2116 |
| 32 | 340 | 324 | 316 | 300 | 1333 | 1561 |
| 33 | 210 | 230 | 227 | 228 | 998 | 1076 |
| 34 | 105 | 142 | 99 | 149 | 432 | 664 |
| 35 | 53 | 62 | 42 | 64 | 234 | 322 |
| 36 | 13 | -12 | 8 | -27 | 126 | 52 |
| 37 | 13 | 13 | 17 | 17 | 24 | 24 |
| 38 | 15 | 15 | 18 | 18 | 126 | 52 |

*One unit of laccase activity was expressed as change in absorbance of 0.001/minute.

**Conflicts of Interest:** Authors report no conflict of interest.

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