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**Regular Article**

# Laccase Production by *Phellinus noxius hp*F17: Optimization of Submerged Culture Conditions by Response Surface Methodology

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**In the present study, a laccase producing fungus, *Phellinus noxius* hpF17 was isolated and studied for its laccase production in submerged culture conditions. The fungus showed laccase activity of 545.50±6.7U/l in the defined liquid medium. Initial screening of production parameters was performed using Plackett–Burman design and the variables with statistically significant effects on laccase production were identified. Variables such as glucose, ammonium tartarate and tween 80 were found to influence the laccase production significantly. These variables were selected for further optimization using Response Surface Methodology. Optimum values of tested variables for maximum laccase production are glucose (20g/l), ammonium tartarate (2.25g/l) and tween 80 (2.08ml/l). By using this optimal fermentation medium, the laccase yield was increased up to 780U/l, an approximate 1.4 fold improvement as compared to the previous yield with un-optimized medium.**

**Key words:** *Phellinus noxius,* Response Surface Methodology, Plackett–Burman Design, ABTS, tween 80.

Laccase (EC 1.10.3.2) is a widespread copper containing enzyme able to oxidize various types of phenols and similar aromatic compounds through one-electron transfer mechanism. The enzyme has already found its way into the market as a biocatalyst. Because of its ability to be paired by electron mediators, the expectation for employing laccases in versatile processes is very high. Typically laccase mediated catalysis occurs with reduction of oxygen to water accompanied by oxidation of a substrate. Laccases can thus oxidize various polyphenols, methoxy-substituted phenols, aromatic diamines, and range of other compounds (Boubonnairth et al., 1990, Baldrian, 2006).

Laccases find wide commercial applications due their broad substrate specificity. Such applications include detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, polymer synthesis, bioremediation of contaminated soils, wine and beverage stabilization. Laccases are also used as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics. Recently, the utility of laccases has also been applied to nanobiotechnology (Gianfreda et al., 1999, Couto and Herrera,

2006, Mayer and Staples, 2002, Desai and Nityananda,2011).

Applications of laccase in biotechnological processes require its production in high amounts at low cost and hence current focus on laccase research is oriented towards the optimization of medium components by various statistical methods. Conventional optimization procedures involve altering one parameter at a time keeping all other parameters constant, which enables to assess the impact of those particular parameters on the process performance. These procedures are time consuming, cumbersome, require more experimental data sets and cannot provide information about the mutual interactions of the parameters (Desai and Nityananda,2011). To overcome these difficulties, statistical approaches such as response surface methodology can be used as alternative optimization tools.

Most laccases reported thus far are of fungal origin, especially from white rot fungi such as *Phlebia radiata*, *Pleurotus ostreatus* and *Tramates versicolor*. Many of the *Trichoderma* species extensively studied as sources of cellulases also been reported as sources of laccases. *T.atroviride*, *T. longibrachiatum*, *T. harzanium* are some of those *Trichoderma* sp. studied as laccase sources. Beside these, *Phanerochaete chrysosporium*, *Theliophora terrstrus*, *Stereum ostrea*, *Lenzitis betulina* and *Pycnoporus* species are some of the important basidiomycetes which have been reported as the sources of laccases (Desai and Nityananda,2011). However, there is no information available in the literature with regard to production of laccase in *Phellinus* species.

In the present study a basidiomycetous fungus, *Phellinus noxius* hpF17 was isolated and studied for its laccase production. The medium was optimized systematically for improving the production of laccase. Plackett-Burman design was adopted to determine the most important factors that affect enzyme production. Later Central Composite Design was used to optimize the levels of these controllable factors in order to formulate an optimal medium to increase the yield of laccase by *Phellinus noxius*-hpF17.

## MATERIALS AND METHODS

### Isolation, Screening and Production of laccase

The microorganism was isolated from the decaying wood source from a village forest of Karnataka (Near Mangalore). The isolate was then screened for its laccase production on solid media containing guaiacol as an indicator (Reddy et al., 2008). Laccase positive reaction was observed based on the visualization of brown zones in the plates due to the oxidative polymerization of guaiacol by the laccase. The isolate was maintained as a slant in 2% malt extract agar at $4^0$C and sub cultured every two weeks. The fungus was identified by morphological examination and partial sequencing of 18S rDNA and homology alignment as *Phellinus noxius* hpF17 (DDBJ Acc.No. AB639022). Mycelial agar plugs (4 plugs of 7mm diameter) from malt extract agar plates were used to inoculate 30ml of modified LMM medium (Dhouib et al., 2005) for the production of laccase in liquid culture. Composition of the medium was (g/l): glucose(10.0), ammonium tartarate(2.0),$KH_2PO_4$(1.0), $MgSO_4.7H_2O$(0.5), KCl(0.5), yeast extract(1.0), $CuSO_4.5H_2O$(150µM), and 10ml/l of trace elements containing per litre of distilled water: EDTA(0.5g), $FeSO_4$(0.2g), $ZnSO_4.7H_2O$(0.01g), $MnCl_2.4H_2O$(0.003g), $H_3BO_4$(0.03g), $CoCl_2.6H_2O$(0.02g), $CuCl_2.2H_2O$(0.001g), $Na_2MoO_4.2H_2O$(0.003g). The fungus was grown in 100ml Erlenmeyer flasks at $30^o$C on a rotary shaker (150 rpm).

The flasks were withdrawn in duplicates at an interval of every two days till fourteen days for the measurement of enzyme activities and biomass.

**Influence of Incubation temperature and medium pH**

Thirty milliliters of modified LMM medium was dispensed into 100ml Erlenmeyer flask, sterilized, and inoculated with four agar disc plugs (7mm) of the white-rot fungus and incubated at 23°C to 37°C for 6 days. To study the Influence of pH, twenty milliliters of the LMM medium was dispensed into 100ml Erlenmeyer flask and pH adjusted by using 0.1N HCl and 0.1N NaOH to 4.0, 5.0, 6.0, 7.0 and 8.0 and sterilized, then inoculated with three 7mm agar disc plug of the fungus and incubated at 30°C for 6 days (Adejoye and Fasid,2009). Thereafter the culture mycelium was harvested and the mycelium free filtrate was used to determine laccase activity.

**Statistical optimization of Laccase Production**

The optimization of medium components for laccase production was accomplished in two stages

**Plackett-Burman design (PBD)**

The Plackett-Burman design was used to find the nutrient components significantly influencing laccase production by *Phellinus noxius* hpF17. Total 11 components (variable k=11) were selected for the study with each variable being represented at two levels: -1 for low and +1 for high levels. The selected variables for the present study were carbon sources (glucose, sucrose, cellobiose); nitrogen sources (ammonium tartarate, potassium nitrate, ammonium nitrate, yeast extract and urea); and inducers (copper sulfate, 2,5-xylidine and tween 80) (table1). These eleven variables were selected based on the previous experiments and were evaluated in 12 experiments. However, process parameters such as medium pH and incubation temperature were optimized initially and hence were not included in the design. All experiments were carried out in duplicate and the average of laccase activity was taken as response. From the pareto chart, the variables showing highest positive effect on each category were considered to have greater impact on laccase production and hence selected for further optimization using central composite design of response surface methodology (Palvannan and Sathishkumar, 2010).

**Table.1. Variables showing medium components used in Plackett-Burman Design**

| Variables | Code | Low Level (-1) | High Levels (+1) | Effects |
|---|---|---|---|---|
| Glucose (g/l) | A | 5 | 10 | 818.4 |
| Sucrose (g/l) | B | 5 | 10 | -313.9 |
| Cellobiose (g/l) | C | 5 | 10 | 44.0 |
| Ammonium tartarate (g/l) | D | 0.2 | 2 | 1142.6 |
| Potassium Nitrate (g/l) | E | 0.2 | 2 | -537.9 |
| Ammonium Nitrate (g/l) | F | 0.2 | 2 | -21.9 |
| Yeast Extract (g/l) | G | 2.5 | 5 | -537.9 |
| Urea (g/l) | H | 2.5 | 5 | 527.8 |
| Copper sulphate (uM) | J | 300 | 600 | 63.4 |
| 2,5-Xylidine (mM) | K | 0.2 | 2 | 220.6 |
| Tween 80 (%) | L | 0.1 | 0.5 | 647.8 |

**Response Surface methodology (RSM)**

Response surface methodology was used to optimize the screened components for enhanced laccase production using Central Composite Design (CCD). The behavior of the system was explained by the following quadratic equation.

$$Y = \beta_0 + \Sigma \beta i\, Xi + \Sigma \beta ii\, Xi^2 + \Sigma \beta ij\, XiXj \quad (1)$$

Where Y represents response variable, βo is the interception coefficient, βi is coefficient of the linear effect, βii is the coefficient of quadratic effect and βij is the coefficient of interaction effect.

A $2^3$ factorial design augmented by 6 axial points (α = 1.682) was implemented in 17 experiments wherein the effect of each compound on laccase production was taken as a response. Design Expert Version 8.0 (Statease) was used for multiple regression analysis and to construct the plots of the obtained data. The coded and uncoded values of the variables at various levels are given in table 2. The coded variables were glucose (A), ammonium tartarate (B) and tween 80(C). These values were converted into their actual values to find out the optimum range of variables for the production of laccase as described by Palvannan and Sathishkumar, 2010.

**Table 2. Experimental range and levels of independent variables**

| Codes | Factors | Unit | Range and levels | | | | |
|---|---|---|---|---|---|---|---|
| | | | (-1.68) | (-1) | (0) | (+1) | (+1.68) |
| A | Glucose | (g/l) | 3.18 | 10 | 20 | 30 | 36.82 |
| B | Ammonium tartarate | (g/l) | 0.15 | 1 | 2.25 | 3.5 | 4.35 |
| C | Tween 80 | (ml/l) | 0.32 | 1 | 2 | 3 | 3.68 |

**Analytical methods**

The biomass of the cultures was determined by filtering the mycelia contents through oven dried, pre weighed Whatman No.1 filter paper. The dry weight of the fungus was determined by the difference in weight after drying the filter paper at 60°C until a constant weight.

**Laccase assay**

Laccase activities were measured spectrophotometrically (GBC) using 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (Sigma) as a substrate with an absorbance coefficient value of 36000/M/cm at 420nm. The reaction mixture consisted of 1.5 ml acetate buffer (1mM, pH 5.0), 1.5 ml ABTS (50 mM) and 1.5 ml culture filtrate. One unit (U) of laccase activity was defined as the amount of enzyme catalyzing the production of one micromole of colored product per min per ml (Tian et al., 2008).

**RESULTS AND DISCUSSION**

In solid media, the organism produced reddish brown zone which is due to the oxidative polymerization of guaiacol in the presence of extracellular fungal laccase (Fig.1). Reddish brown zone appeared on the second day of incubation period which showed the ability of the fungus to produce extracellular laccase.

In LMM medium, the fungus showed extracellular laccase activity of 545.50±6.7U/l on 6th day. At this point the mycelial mass was at its maximum level of 7.60±0.7g/l and reached its stationary phase afterwards. On day 8, similar laccase activity of 534.65±6.35U/l was obtained (fig.2). On

further incubation, the activity was reduced to 16.67±7.5U/l on 14th day.

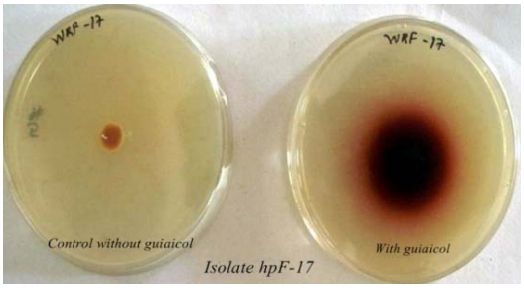



**Figure.1. Oxidative polymerization of guaiacol to form reddish brown zones in the medium by hpF-17**

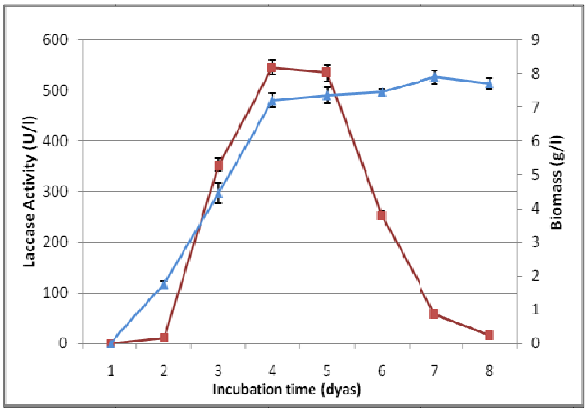



**Figure 2. Time course of biomass growth (—) and laccase activity (—) of *P.noxius* hpF17 in liquid culture. Error bar shows mean ± standard deviation.**

**Influence of Incubation temperature and medium pH**

Temperature showed significant effect on mycelial biomass yield and extracellular laccase activity of *P.noxius* hpF17. An increase in the temperature from 23°C to 28°C increased the laccase activity and biomass and further increment up to 37°C reduced both. Highest Laccase activity of 573.05±2.0U/l was observed at 28°C (Fig.3). A similar laccase activity of 558.9±1.2U/l was also observed at 30°C. At this temperature, a higher biomass yield (5.98±0.2g/l) was observed.

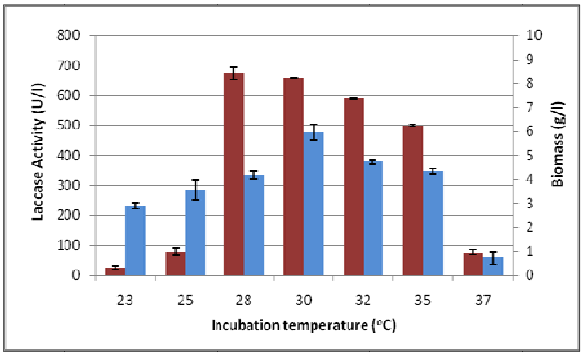



**Figure 3. Effect of incubation temperature on laccase (■) and biomass (■) production by *P. noxius* hpF17. Error bar shows mean ± standard deviation.**

Increase in laccase yield and biomass was observed form pH 4.0 to 6.0. Further increase in pH reduced the biomass as well as laccase activity (Fig.4). Highest laccase activity of 570.34±5.6U/l and mycelial biomass of 7.66±0.34g/l was observed at pH 6. In fact, biomass production and laccase activity of the fungus was not favored at low or high temperatures and pH. Similar observations were made by Gbolagade et al. 2006, Adejoy et al., 2009, Zadrazil et al., 1999 in different fungi.

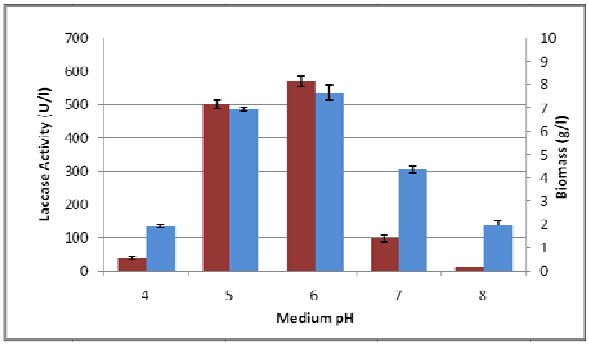



**Figure.4. Effect of medium pH on laccase (■) and biomass (■) production by *P. noxius* hpF17. Error bar shows mean ± standard deviation.**

**Statistical Optimization of Medium Components**

**Placket Burman Design (PBD)**

Variations ranging from 24.9 U/l to 2876.5 U/l in the production of laccase in 12 runs were observed in PB design experiments (Table 3). This variation reflected the importance of medium optimization to attain higher yields.

The pareto chart illustrates the order of significance for the variables affecting laccase production (Fig.5). Among the variables, ammonium tartarate showed highest significance by showing higher positive effect. However, for further optimization using CCD, the factors which showed highest positive influence in each of the category viz; carbon source, nitrogen source and inducers were selected. In the category of carbon sources, glucose showed higher significance as compared to sucrose and cellobiose and hence was included in CCD. Among Nitrogen sources, ammonium tartarate showed larger significance. In the category of inducers, tween 80 showed higher significance than copper sulfate and 2,5-xylidine, hence was selected as a variable for CCD.

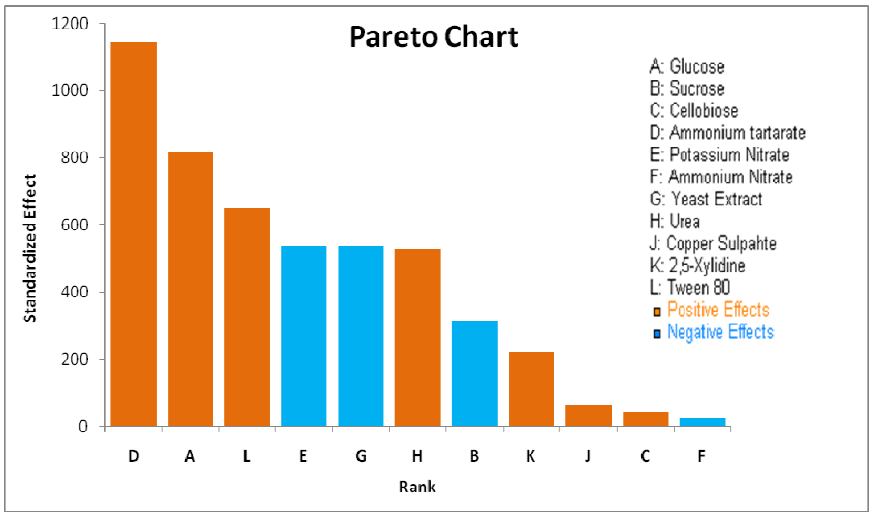



**Figure.5. Pareto chart of eleven-factor standard effects on laccase production.**

**Table.3. PBD variables (in coded) with laccase as response**

| Coded Variables | | | | | | | | | | | Laccase |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | B | C | D | E | F | G | H | J | K | L | Activity (U/l) |
| + | - | + | - | - | - | + | + | + | - | + | 1589 |
| + | + | - | + | + | - | + | - | - | - | + | 1239.2 |
| - | - | - | + | + | + | - | + | - | - | + | 1783.4 |
| - | - | + | + | + | - | + | + | - | + | - | 884.7 |
| - | + | + | + | - | + | + | - | + | - | - | 396.3 |
| + | + | + | - | + | + | - | + | - | - | - | 536.1 |
| + | - | + | + | - | + | - | - | - | + | + | 2876.5 |
| + | + | - | + | - | - | - | + | + | + | - | 2478.5 |
| - | + | + | - | + | - | - | - | + | + | + | 143.6 |
| - | + | - | - | - | + | + | + | - | + | + | 542.5 |
| + | - | - | - | + | + | + | - | + | + | - | 30.06 |
| - | - | - | - | - | - | - | - | - | - | - | 24.9 |

**Response Surface Methodology (RSM)**

Based on the PB design, glucose, ammonium tartarate and tween 80 were selected for further optimization using RSM-CCD. To examine the combined effect of these factors, a central composite design (CCD) was employed within a range of –α (-1.68) and +α (+1.68) in relation to the production of laccase. According to the design, 17 runs replicated three times at central points were performed and experimental and predicted responses were obtained (Table 5). Relationship between the variables was determined by fitting a second order polynomial equation to the data obtained from the 17 runs.

The predicted and observed responses along with design matrix are presented in Table 4 and the results were analyzed by ANOVA. The second-order regression equation provided the levels of laccase activity as a function of glucose, ammonium tartarate and tween 80 which can be presented in terms of coded factors as in the following equation:

$$Y\ (response) = +774.32 - 47.51A + 24.43B + 67.64C - 26.39AB - 89.12AC + 157.33BC - 124.64A^2 - 200.51B^2 - 231.86C^2$$

Where Y is the laccase activity (U/l), A, B, and C are glucose, ammonium tartarate and tween 80, respectively.

**Table 4. Full factorial central composite design matrix and their observed response**

| Run order | A (g/l) | B (g/l) | C (ml/l) | Laccase activity(U/l) Actual response | Predicted response | Residual Value |
|---|---|---|---|---|---|---|
| 1 | -1 | -1 | -1 | 213.33 | 214.295 | -0.965 |
| 2 | +1 | -1 | -1 | 269.12 | 350.231 | -81.111 |
| 3 | -1 | +1 | -1 | 1 | 0.855 | 0.145 |
| 4 | +1 | +1 | -1 | 10.96 | 31.251 | -20.291 |
| 5 | -1 | -1 | +1 | 242.5 | 212.9 | 29.642 |
| 6 | +1 | -1 | +1 | 1.5 | -7.705 | 9.205 |
| 7 | -1 | +1 | +1 | 720 | 629.538 | 90.462 |
| 8 | +1 | +1 | +1 | 313.75 | 303.434 | 10.316 |
| 9 | -1.68 | 0 | 0 | 435.83 | 502.326 | -66.496 |
| 10 | +1.68 | 0 | 0 | 395.83 | 342.586 | 53.244 |
| 11 | 0 | -1.68 | 0 | 197.08 | 166.842 | 30.238 |
| 12 | 0 | +1.68 | 0 | 205.42 | 248.910 | -43.490 |
| 13 | 0 | 0 | -1.68 | 70.83 | 5.478 | 65.352 |
| 14 | 0 | 0 | +1.68 | 154.3 | 232.904 | -78.604 |
| 15 | 0 | 0 | 0 | 787.5 | 774.215 | 13.285 |
| 16 | 0 | 0 | 0 | 795.83 | 774.215 | 21.615 |
| 17 | 0 | 0 | 0 | 741.67 | 774.215 | -32.545 |

**Table 5. Analysis of Variance (ANOVA) for response surface quadratic model for the production of laccase.**

| Source | Sum of Squares | Df | Mean Squares | F Value | p-value Prob>F | |
|---|---|---|---|---|---|---|
| Model | 1199000 | 9 | 133200 | 24.27 | 0.0002 | Significant |
| A-Glucose | 30820.04 | 1 | 30820.04 | 5.61 | 0.0497 | |
| B-Ammonium tartarate | 8133.62 | 1 | 8133.62 | 1.48 | 0.2630 | |
| C-Tween 80 | 62478.32 | 1 | 62478.32 | 11.38 | 0.0119 | |
| AB | 5569.35 | 1 | 5569.35 | 1.01 | 0.3474 | |
| AC | 63546.13 | 1 | 63546.13 | 11.57 | 0.0114 | |
| BC | 198500 | 1 | 198500 | 36.15 | 0.0005 | |
| A2 | 175100 | 1 | 175100 | 31.89 | 0.0008 | |
| B2 | 453200 | 1 | 453200 | 82.54 | < 0.0001 | |
| C2 | 6.06100 | 1 | 606100 | 110.37 | < 0.0001 | |
| Residual | 38437.10 | 7 | 5491.01 | 38437.10 | | |
| Lack of Fit | 36736.08 | 5 | 7347.22 | 8.64 | 0.1070 | Not significant |
| Pure Error | 1701.03 | 2 | 850.51 | | | |
| Cor Total | 1238000 | 16 | | | | |
| Model fitting | | | | | | |
| C.V= 22.67% | | R-Sq = 96.9% | | R-Sq(adj) = 92.9% | | |

The statistical significance of equation 1 was checked by F-test and the analysis of variance for response surface quadratic model is shown in table 5. ANNOVA of regression model demonstrates that the model is highly significant as it is evident from the Fischer test with very low probability value. The value of lack of fit, Model F and model P>F were found to be 8.64, 24.27 and <0.05 respectively, indicating that model was significant. Fisher F-test with a very low probability value (Pmodel >F = 0.0002) and also lack of fit was insignificant demonstrates a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient ($R^2$). The coefficient of regression ($R^2$) was calculated to be 0.969. The value of the adjusted regression coefficient (Adj $R^2$ = 0.929) was also high, which advocates for high significance of the model. At the same time relatively low coefficient variation (CV=22.67%) confirm the precision and reliability of the experiment performed. From the table 5, it can be seen that the factors with higher significance were A, C, AC, BC and squared terms of A2, B2 and C2. The interaction terms AB seems to be insignificant, which can be removed from the model without affecting the goodness of the model.

The relationship between the actual and predicted laccase activity (response) is shown in Figure 6. The cluster of

measurements near the diagonal line in the parity plot indicates a good fit of the model and demonstrates a satisfactory correlation between the actual and predicted values. The minimum response of 1U/l laccase activity was obtained with 10g/l of glucose 3.5g/l of ammonium tartarate and 1% tween 80. The maximum response of 795.83U/l laccase activity was obtained with 20 g/l glucose, 2.25g/l ammonium tartarate and 2% tween 80.

The three dimensional (3D) response surface graphs of laccase production based on the final model are depicted in figure 7 which were generated in pair-wise combination of the three factors while keeping the other one at its optimum level. The response at the central point corresponds to a maximum degree of achievable laccase activity for that factor. Almost all the interactions in the designed experiments produced a 'nearly spherical' variance function. This indicates that the variables have both individuals as well as interrelated/interaction effects, allowing for the prediction of optimum concentration levels for maximized laccase activity.

The predicted optimum levels of the tested variables, namely, glucose(A), ammonium tartarate(B) and tween 80(C) were obtained by applying regression analysis on Eq.(3). The optimal levels were as follows: A=0(20g/l), B=0(2.25g/l), C=0.08(2.08ml/l) with the corresponding, laccase Activity(Y) =785U/l. Verification of the predicted values was conducted by using optimal medium in inoculation experiments. The practical corresponding response was 780U/l. This result corroborated the validity and the effectiveness of this model.

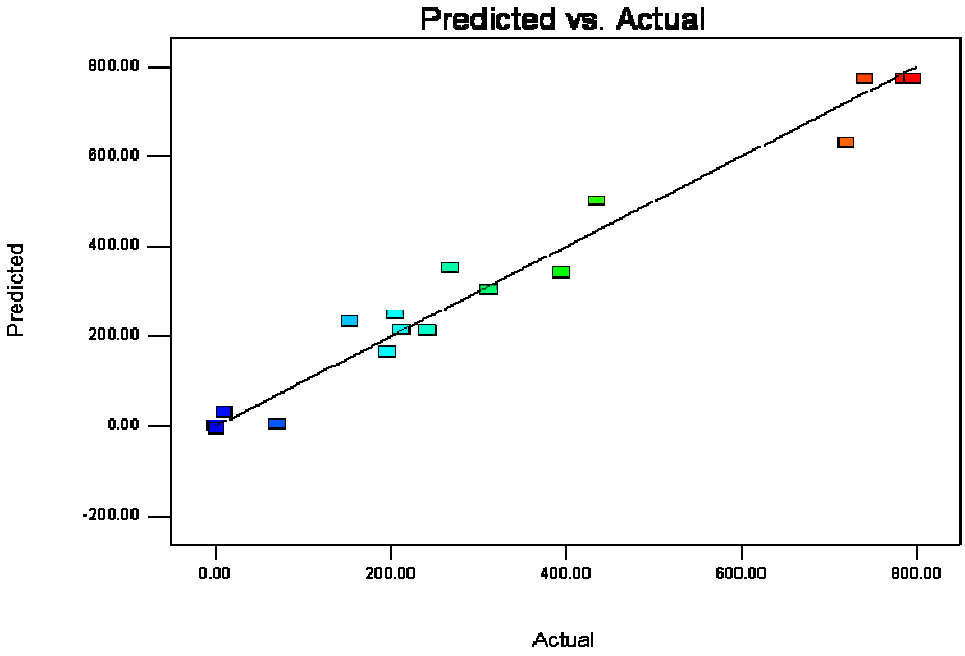
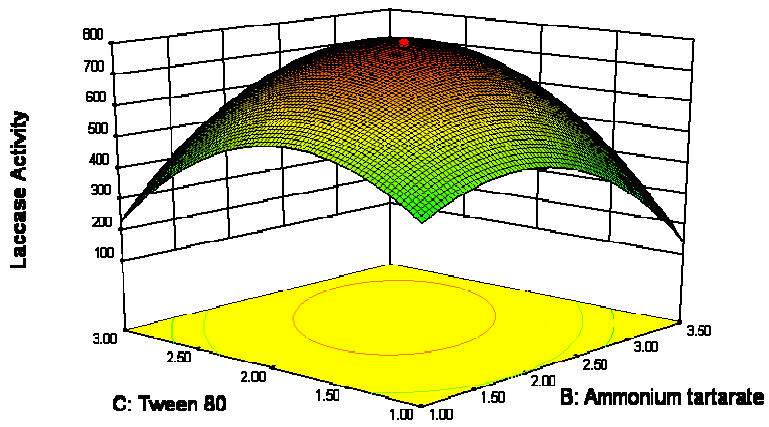
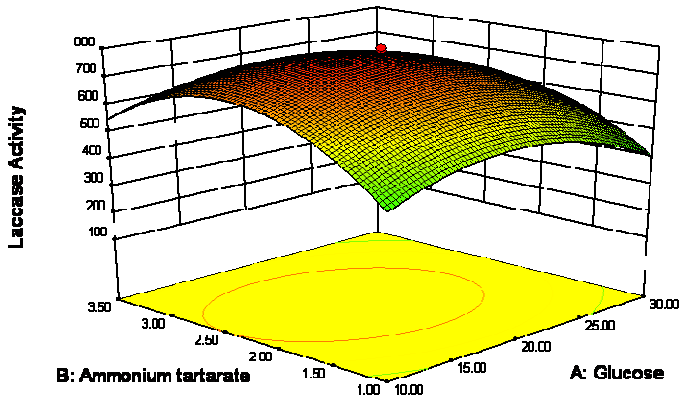



**Figure.6. Predicted v/s actual laccase production by *P.noxius* hpF17**




(a)

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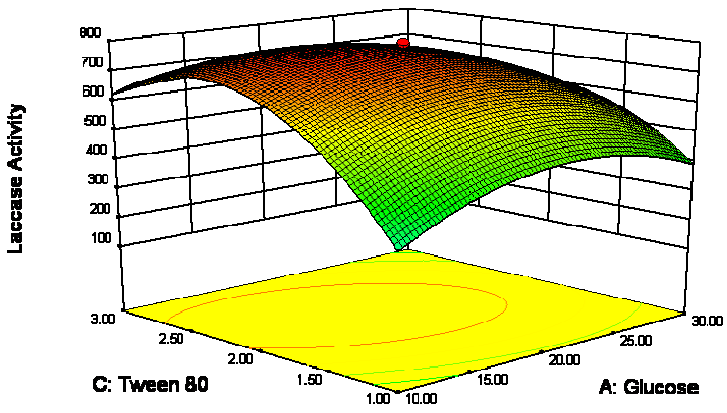


c)

**Figure 7. Three dimensional response surface plot for the effect of a) tween 80 and ammonium tartarate, b) ammonium tartarate and glucose and c) tween 80 and glucose for the production of laccase by *P. noxius hpF17***

## CONCLUSION

The effect of medium components on the production of laccase by *P. noxius* hpF17 was studied using Pb design. Medium components such as glucose, ammonium tartarate and tween 80 were found to influence the laccase production significantly. These variables were selected for further optimization studies using RSM. Optimum values of tested variables for maximum laccase production are glucose (20g/l), ammonium tartarate (2.25g/l) and tween 80 (2.08ml/l). By using this optimal fermentation medium, the laccase yield was increased up to 780U/l. Our result shows that we could achieve an approximate 1.4 fold improvement of laccase yield over the previous yield with un-optimized medium in *P. noxius* hpF17.

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