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Lalhlupuii
Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Banaras, Utter Pradesh, India

Dr. Amrita Poonia
Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Banaras, Uttar Pradesh, India

Correspondence
Dr. Amrita Poonia
Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Banaras, Utter Pradesh, India

Production of Laccase by *Ganoderma lucidium* NCIM 1091 from agro-industrial by-product (Banana Peel)

Lalhlupuii and Dr. Amrita Poonia

Abstract
The present study investigated the synergistic effect of nutritional supplements and inducer (gallic acid) and amino acids on laccase production by *Ganoderma lucidium* NCIM 1091 from utilizing agro-industrial by-product (banana peel) under submerged fermentation conditions. Further optimization of parameters i.e. amount of substrate, concentration of amino acids and inducers for maximum laccase production was studied by central composite rotatable design. From the present study, it can be concluded that *Ganoderma lucidium* NCIM 1091 can be used to produce laccase enzyme. Banana peel was proved to be a good substrate for laccase production. Alanine and gallic acid proved to increase laccase production significantly. The interactive effect of amount of banana peel and concentration of amino acid and gallic acid was also proved to be significant at some levels and maximum laccase production (4.32 U/ml) was obtained at 8.63 gm banana peel, 50.23 mg/ml concentration of alanine and 0.25 mM concentration of gallic acid.

Keywords: laccase, ganoderma lucidium, nutritional supplement, process optimization

Introduction
Laccases (E.C. 1.10.3.2, benzenediol: oxygen oxidoreductases,) are multi-copper enzymes belonging to the group of blue oxidases. They are among the important enzymes that have attracted tremendous attention in recent years due to their important applications in different industries. The ability of laccases to oxidize phenolic compounds as well as their ability to reduce molecular oxygen to water has led to intensive studies of these enzymes (Jolivalt *et al*., 1995) [1]. Fungal laccase is considered a key player in lignin degradation and/or the removal of potentially toxic phenols arising during morphogenesis, sporulation, or phytopathogenesis and fungal virulence (Gianfreda and Bollag, 1993) [2]. The role of laccase in lignin and phenolic compound degradations has been evaluated in a large number of biotechnological applications such as dye degradation (Wong, 1999; Mendoza *et al*., 2011) [3, 4] bioremediation of some toxic chemical wastes, wastewater and soil treatments and also biosensor developments (Nelson and Elisa, 2001; Nelson *et al*., 2002) [5, 6].
Production of laccases in cost-effective manner from microorganisms is a prerequisite for their use in industrial processes, which may be achieved by use of agro-industrial waste materials (Pandey *et al*., 2000) [7]. One such waste is banana peel which is available in bulk, and its disposal is a matter of concern for fruit-processing industries. Bananas belonging to the family *Musaceae* are one of the most important tropical fruits in the world market. Banana peels are rich in nutrients containing 59.51% carbohydrates, 7.87% protein, 11.6% fat 7.68% fiber (Essien *et al*.,2005) [8]. Because of the high nutrient content of banana peels it was selected as a possible alternative substrate for the production. Improper dumping of banana peels may result into ecological and environmental hazards. Global production of bananas is estimated to be around 48.9 MT. India is the largest producer of banana with a production figure of 54.5 thousand tonnes (Agricultural Statistics, 2012) [9]. The states of Tamil Nadu, Gujarat and Maharashtra in Western India, Karnataka in Southern India and Assam in the northeast are large banana growers. Banana is one of the highly consumed crops in the world and accounts 40% of the total world trade in fruits and fruit products.
In the present work, an attempt was made to enhance laccase production using *Ganoderma lucidium* NCIM 1091 under submerged fermentation process by optimizing the medium composition using an agro-industrial by-product (banana peel). Optimization of cultural was done by CCRD.

Materials and Methods

Bacterial strain and growth medium

Ganoderma lucidium NCIM 1091 used in the present study was procured from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL) Pune, India. The stock culture was maintained at 4 °C in 250 mL MRS broth as per specifications provided by NCIM on potato dextrose agar. It was allowed to grow in slants at 28 °C for 6 days. Growth culture was sub cultured monthly and slants prepared were kept at 4 °C.

Production medium and cultivation

Production medium was similar to growth medium and was prepared in 250 mL conical flasks. After autoclaving the broth, it was inoculated by transferring 2-3 loop full of fungal colonies slants to the broth. It was incubated at 28°C for 6 days and sub cultured after every 15 days and stored at 4 °C. PDA plates were spot-inoculated with fungal culture and incubated at 28°C for 7days. 2, 2-Azino-bis-3-ethyl-benzthiozoline-6-sulphonic (ABTS) solutions of 2 mM were freshly prepared and then agar plates were flooded with this solution. Colonies showing green color confirm the presence of laccase activity. Positive ligninolytic fungi turned ABTS from light green to dark green. For cultivation 10gm of fresh chopped banana peel was mixed with 0.3 g extract powder, 2.0 g glucose, 0.1 g ammonium chloride and 1ml salt solution. Salt contained (g/l distilled water): KH_2PO_4 2.0, $MgSO4.7H_2O$ 0.5, $CaCl_2.7H_2O$ 0.1, KCl 0.5. Solid-State Fermentation was carried out in 250 ml conical flask, containing production media. Flask was autoclaved (121^{o}C, 15psi) for 15 min in prior to inoculation with fungi. Inoculated flask was incubated at 28^{o}C for 14 days.

Enzyme Extraction

After seven days, the whole content of the flask were soaked in 100ml of acetate buffer (pH 4.5, 120mM) to flask and kept on a shaker for 2hrs at 28 ± 2^{o}C. Contents were transferred to muslin cloth and squeezed. Liquid extract obtained was centrifuged at 10,000 rpm at 4 ^{o}C for 10 min. and supernatant was analyzed for enzyme activity.

Enzyme Assay

Laccase activity measurement was determined by measuring the oxidation of (ABTS). Increase in absorbance for 3min was measured spectrophotometrically at 420nm (Niku-Paavola *et al.*, 1990) [10]. The reaction mixture contained 100µl of 50mM ABTS and 800µl of 20mM sodium acetate buffer (pH 4.5) and 100µl of appropriately diluted enzyme extract. Enzyme activity was expressed in units (U). One unit of laccase activity was defined as the amount of enzyme that oxidized 1µM of ABTS per min. The activity was expressed in U/ml.

$$\text{Enzyme activity} = \frac{A420nm \times Vt}{t \times € \times Vs}$$

Where,

Vt : final volume of reaction mixture (ml) : 1

Vs: sample volume (ml): 0.1

t :incubation time (min)

€ : extinction co-efficient of ABTS: 36000 cm-1 M-1 80

Colonies showing green color was observed which confirm the presence of laccase activity as laccase enzyme oxidized ABTS from light green to in tensed dark colour and was selected for further study.

Amino Acid Supplementation

Six different trials with different amino acids at three different combinations, viz., methionine (T1), alanine (T2), tryptophan (T3), tyrosine (T4), phenylalanine (T5), glycine (T6) and control (T7), without supplementation were used for the production of laccase at a concentration of (10mg/1ml salt solution) under solid state fermentation using banana peel as substrate and compared against the control. Effect of inducer gallic acid (mM/ml) salt solution was studied.

Effect of varying concentration of banana peel, amino acids and inducer on cell growth and laccase yield

Banana peel (fresh & chopped) concentration was varied between 95 5-25g/ml in order to check its effect on lacasse production. Amino acid concentration was varied in range of 0.5-3.5g/L. Inducer (gallic acid) varied in range of 0.5-3.0g/L.

Statistical Analysis

The data obtained from the various experiments were recorded and subjected to statistical by CCRD. The significance difference between the means was tested against the critical difference at 95% level of significance by using statistical tool Minitab 17 for data analysis. Three factors were chosen at five levels for optimization process, i.e., concentration of amino acids, inducer and banana peel concentration and one response, i.e., enzyme activity was observed. The data obtained from the design were analyzed by the response surface regression procedure using the following second- order quadratic equation:

$$Yi = \beta 0 + \Sigma \beta ixi + \Sigma \beta ii\ xi\ 2 + \Sigma \Sigma \beta ijxijj$$

Where Yi is the predicted response, xixj were independent variables, β0 is the offset term,

βi is the linear coefficient, βii is the quadratic coefficient, and βij the interaction coefficient.

Results and Discussion

Effect of nutritional supplements (banana peel, amino acids and inducer) on cell mass and laccase production

Initially, experiments were carried out to understand the effect of varying concentration of chosen variables, i.e., banana peel, amino acid and inducer on laccase production. It was observed that the fungus grew well attached to the banana peel. This may be due to the high hydrophobicity of banana peel, which eases the attachment of the fungus to the carrier (Kotrba *et al*., 2002) [11]. Highest activity was exhibited (2.5 U/ml) when 10.0g/ml of freshly chopped banana peel was used as substrate Figure 1a. High content of carbohydrate in banana peel makes it a suitable support substrate for laccase production and the maximum activity of laccase production from *Trametes pubescens* using banana peel was 1600 U/L at the end of the cultivation (Osma *et al*., 2007) [12]. *P. florida* produces two extracellular laccases and one of these regulates the vegetative growth as well as fruiting body production. As shown in Fig. 1.b. among all the six amino acids used, supplementation with alanine showed maximum enzyme activity (2.9 U/ml), followed by methionine and then tryptophan. Glycine, phenylalanine and tyrosine showed a negative effect on laccase production compared to the control. Dhawan and Kuhad, 2002 [13] reported that DL-methionine, DL-tryptophan, glycine and DL-valine stimulated laccase production, while alanine reduced laccase production and L-cysteine monohydrochloride completely inhibited the enzyme production but in the present study, glycine significantly reduced laccase production and alanine stimulated laccase

production. Inducers play a significant role in enhancing the production of laccase. Phenolic compounds that are related to the natural substrate lignin or lignin derivatives are good inducers. Aromatic inducers and phenolic compounds have been extensively used to extract improved laccase production by different organisms (D'Souza-Ticlo *et al*., 2009) [14] and the personality of the compound that induces laccase production differs significantly with the species. Supplementation with inducer showed enzyme activity (2.5 IU/ml) more than the control (2.3 IU/ml) and showed a positive effect on laccase production Figure.1c.

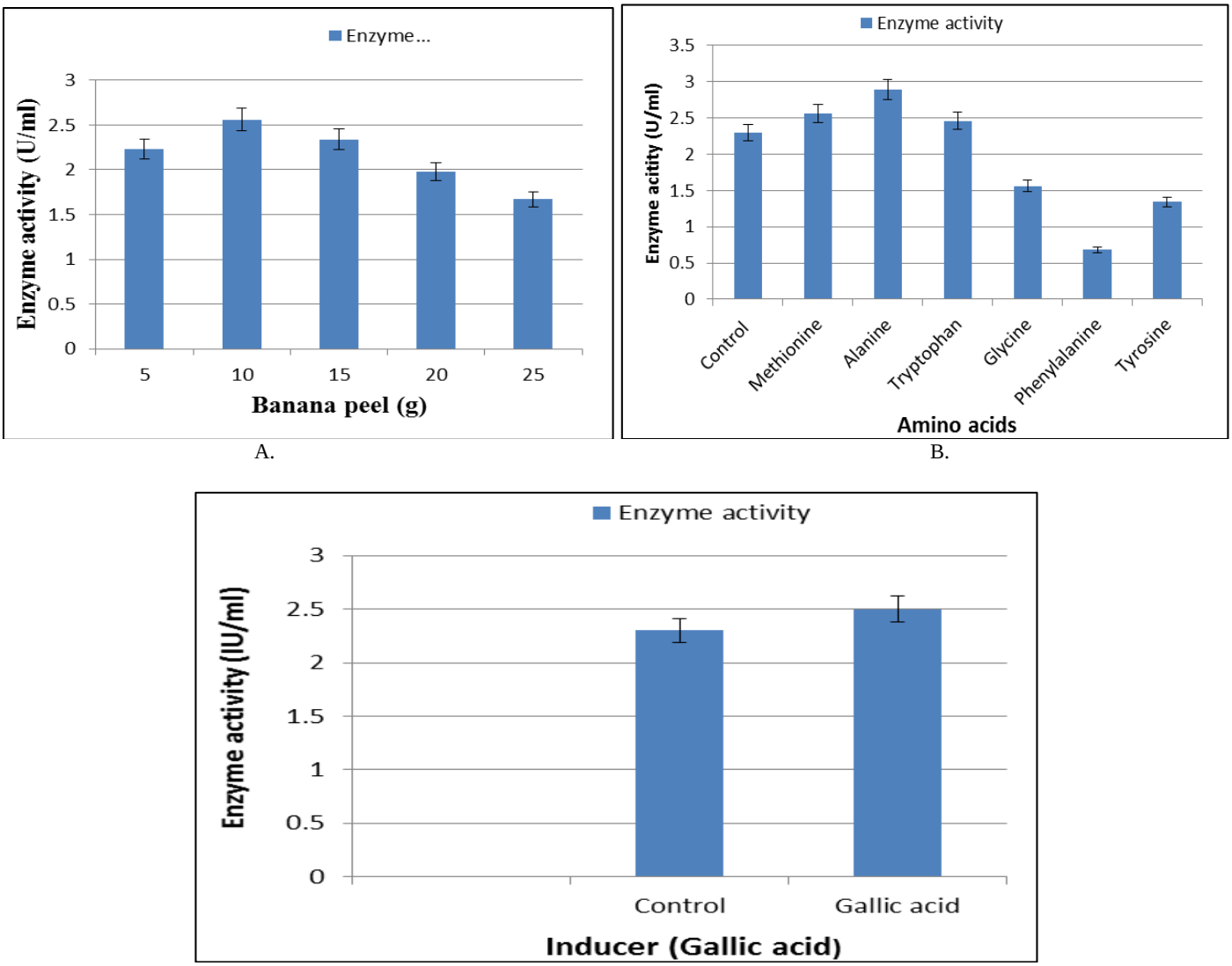


Fig 1: Graphs of laccase production by *Ganoderma lucidium* NCIM 1091showing effects of concentration of a level of substrate, b amino acids, c inducer (gallic acid)

Optimization of amount of banana peel and concentration of alanine (amino acid) and inducer concentration using RSM

The experiments with different combinations of amount of banana peel, amino acid and inducer concentration were run and assayed for laccase activity. Optimization was done using RSM for three parameters i.e. amount of banana peel, concentration of amino acid and inducer. A total of 20 experiments were run. The ranges and level of independent variables used in the design are given in (Table 1).

Table 1: Variables and their levels used for different parameters in RSM

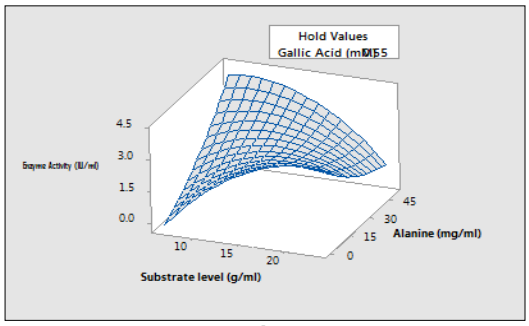
Variables	Low level	High level
A(x^1) Banana peel (g)	10	20
B(x^2) Amino acid (alanine; mg/ml)	10	40
C(x^3) inducer (gallic acid; mM)	0.1	1

Interactive effect of amount of banana peel and concentration of amino acid on enzyme activity and specific activity

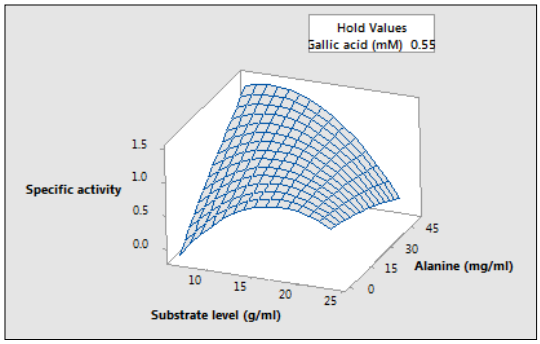
The enzyme activity obtained corresponding to each run are shown in (Table 2). It varies from 0.741 IU/ml to 3.704 IU/ml. According to the results obtained, the maximum enzyme activity was obtained in Run 14 containing 15g banana peel, 50.2mg/ml amino acid and 0.55mM gallic acid (Table 2). It was observed from Fig. 2a that with the increase in amount of banana peel, the enzyme activity increased exponentially and then beyond a certain level (20 g), the decreased with the further increase in amount of banana peel. Enzyme activity showed an increasing trend with increase in amino acid concentration till 45 mg/ml. It was also observed from Figure 2a that with the increased in amount of banana peel the specific activity first then decreased.

Table 2: CCRD design for optimization of three variables for production of lacccase by *Ganoderma lucidium* NCIM No. 1091

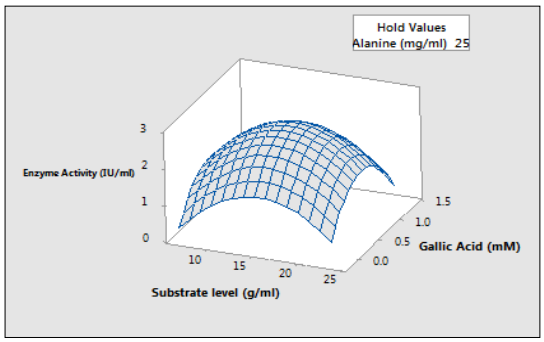
Run Order	Banana peel (g)	Alanine (mg/ml)	Gallic acid (mM)	Enzyme Activity(IU/ml)	Specific activity
1	20.0000	10.0000	1.00000	2.889	0.583
2	10.0000	40.0000	0.10000	2.870	1.137
3	6.5910	25.0000	0.55000	1.667	0.750
4	10.0000	40.0000	1.00000	2.426	0.989
5	15.0000	-0.2269	0.55000	2.796	0.820
6	15.0000	25.0000	0.55000	2.647	1.065
7	10.0000	10.0000	1.00000	1.296	0.452
8	15.0000	25.0000	-0.20681	1.759	0.648
9	20.0000	40.0000	0.10000	2.037	0.718
10	23.4090	25.0000	0.55000	1.759	0.445
11	10.0000	10.0000	0.10000	0.741	0.341
12	15.0000	25.0000	0.55000	2.669	1.070
13	15.0000	25.0000	0.55000	2.677	1.091
14	15.0000	50.2269	0.55000	3.704	1.277
15	20.0000	10.0000	0.10000	2.037	0.605
16	20.0000	40.0000	1.00000	1.315	0.338
17	15.0000	25.0000	1.30681	1.370	0.474
18	15.0000	25.0000	0.55000	2.667	1.069
19	15.0000	25.0000	0.55000	2.567	1.042
20	15.0000	25.0000	0.55000	2.667	1.078



A



B



C

Fig 2: Surface plots showing interactive effect of amount of banana peel (g) on laccase production by *Ganoderma lucidium* NCIM 1091 (enzyme activity) a concentration of alanine (mg/ml), b concentration of gallic acid (mM), c concentration of amino acid (mg/ml) & gallic acid (mM)

Interactive effect of amount of banana peel and concentration of inducer on enzyme activity and specific activity

Figure 2b and Fig 3b shows the surface plot showing interactive effect of amount of banana peel (g/ml) and concentration of gallic acid (mM) on laccase production (enzyme activity and specific activity, respectively) by *Ganoderma lucidium* NCIM 1091. It was observed from Fig. 2b that with the increase in amount of banana peel, the enzyme activity first increased exponentially and the beyond a certain level (15 g), the enzyme activity again decreased with the further increase in amount of banana peel. Similarly, enzyme activity showed an increasing trend with increase in gallic acid concentration till 0.5 mM and after that enzyme activity decreased. Gupta *et al*., 1990 [15] have reported the use of DL-2-amino-n-butyric acid, DL-alanine, l-lysine monohydrochloride and L-proline to stimulate xylanase production in *Staphylococcus* sp. Also, effect of gallic acidon laccase production studied showed positive effect. Results obtained was in agreement with the results of (Patel and Gupte, 2002) [16] who reported that laccase production by *Pleurotus ostreatus* increased approximately by 1.3 fold in presence of gallic acid at 1.0 mM concentration as compared to the control. According to the graph, due to the interactive effect, the maximum enzyme activity (2.5 IU) occurred at a level of 15 g banana peel and 0.5 mM of gallic acid, taking amino acid concentration constant at 25mg/10ml. Similarly, Figure 3b shows that with the increased in amount of banana peel and gallic acid concentration the specific activity first increased then decreased.

Interactive effect of concentration of amino acid and inducer on enzyme activity and specific activity

Figure 2c and Figure 3c shows the surface plot showing interactive effect of concentration of amino acid (mg/ml) and gallic acid (mM) on laccase production (enzyme activity and specific activity, respectively) by *Ganoderma lucidium* NCIM 1091. It was observed that with the increased in amino acid concentration (up to 45mg/ml), the enzyme activity increased exponentially whereas enzyme activity showed an increasing trend with increase in gallic acid concentration till 0.5 mM and after that enzyme activity decreased. Similarly, specific

activity 180 increased with increased in amino acid concentration whereas with the increased in gallic acid concentration specific activity first increased and then decreased.

Interactive effect of all the three parameters for enzyme activity

The enzyme activity obtained corresponding to each run are shown in (Table 2). It varies from 0.741 IU/ml to 3.704 IU/ml. According to the results obtained, the maximum enzyme activity was obtained in Run14 containing 15g banana peel, 50.2mg/ml amino acid 0.55mM gallic acid. The results (enzyme activity) obtained were analysed by ANOVA as appropriate to the experimental design used (Table 3) and the coded coefficient and VIF (very important factor) were determined (Table 4). The regression equation obtained after analysis gives the production of laccase. All the terms regardless of their significance were included in the second order polynomial equation:

Y = -5.117 + 0.6636 A + 0.1247 B + 3.415 C - 0.01489 A2 + 0.000761 B2 - 2.097 C2 -
0.008055 A + 0.0011 AC - 0.04765 BC

The determination coefficient (R2) of the model was calculated to be 0.9717 (a value of >0.75
indicates the fitness of the model). An R2 value can be between 0 to 1, and the closer value is to 1, the better the model fits the experimental data. Thus the study indicates that 97.17% of the variation in the response (enzyme activity) was attributed to the independent variable, whereas 2.83% of the total variance could not be explained by the model. An adjusted R2 was 0.9462, which accounted for the number of predictors in the model. Both of the obtained values suggested that the model fit the data well. From table 4.3, the square model was found to be significant (P<0.05). The values B, A2, B2, C2, AB and BC were found to be significant (P<0.05) (Table 4). For the first order effects, by evaluating the regression coefficients and t-value it could be concluded that the amino acid concentration (B) had the most significant effect on the laccase production followed by amount of banana peel (A). For the quadratic model, all the parameters found to have significant effect. For the 2-way interaction model, interaction of both amount of banana peel and concentration of amino acid (AB) and concentration of amino acid and concentration of gallic acid (BC) were found to be significant (P=0.000).

Table 3: Coefficient of quadratic model of coded factors for specific activity

Term	Effect	Coef	SE Coef	T-Value	P-Value
Constant		1.0714	0.0178	60.19	0.000*
A	-0.1740	-0.0870	0.0118	-7.36	0.000*
B	0.2884	0.1442	0.0118	12.21	0.000*
C	-0.1071	-0.0536	0.0118	-4.54	0.001*
A^2	-0.3631	-0.1815	0.0115	-15.79	0.000*
B^2	-0.0442	-0.0221	0.0115	-1.92	0.084
C^2	-0.3889	-0.1944	0.0115	-16.91	0.000*
AB	-0.3663	-0.1831	0.0154	-11.87	0.000*
AC	-0.0912	0.0456	0.0154	-2.96	0.014*
BC	-0.1542	-0.0771	0.0154	-5.00	0.001*

*Significant terms in the model; Coef: coefficient Model Summary: S 0.0.436472, R-sq: 98.88%, R-sq(adj): 97.88%, R-sq(pred): 91.93%

Table 4: Analysis of variance for RSM quadratic model for specific activity

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	9	1.68915	0.187684	98.52	0.000*
Linear:	3	0.42658	0.142193	74.64	0.000*
A	1	0.10333	0.103334	54.24	0.000*
B	1	0.28405	0.284051	149.10	0.000*
C	1	0.03920	0.039195	20.57	0.001*
Square:	3	0.93005	0.310018	162.73	0.000*
A^2	1	0.47497	0.474971	249.32	0.000*
B^2	1	0.00703	0.007033	3.69	0.084
C^2	1	0.54490	0.544895	286.02	0.000*
2-way Interaction	3	0.33252	0.110839	58.18	0.000*
AB	1	0.26828	0.268278	140.82	0.000*
AC	1	0.01665	0.016653	8.74	0.014*
BC	1	0.04759	0.047586	24.98	0.001*
Error:	10	0.01905	0.001905	-	-
Lack of fit	5	0.01774	0.003548	13.53	0.006*
Pure error	5	0.00131	0.000262	-	-
Total	19	1.70820	-	-	-

Interactive effect of all the three parameters for specific activity

The specific activity obtained corresponding to each run are shown in (Table 2). It varies from 1.277 to 0.065. According to the results obtained, the maximum specific activity was obtained in Run14 containing 15g banana peel, 50.2mg/ml amino acid and 0.55mM gallic acid which are same for enzyme activity. The results (specific activity) obtained were analysed by ANOVA as appropriate to the experimental design used and the coded coefficient and VIF (very important factor) were determined. The regression equation obtained after analysis gives the production of laccase. All the terms regardless of their significance were included in the second order polynomial equation:

Y = -2.068 + 0.2727 A + 0.05743 A + 1.527 B - 0.007262 A2 - 0.000098 B2 - 0.9602 C2 - 0.002442 AB - 0.02028 AC - 0.01143 BC

The determination coefficient (R2) of the model was calculated to be 0.9888 (a value of >0.75 indicates the fitness of the model). An R2 value can be between 0 to 1, and the closer value is to 1, the better the model fits the experimental data. Thus the study indicates that 98.88% of the variation in the response (specific activity) was attributed to the independent variable, whereas 1.12% of the total variance could not be explained by the model. An adjusted R2 was 0.9788, which accounted for the number of predictors in the model. Both of the obtained values suggested that the model fit the data well. From (Table 4) the square model was found

to be significant (P<0.05). The values A, B, C, A_2, B_2, C_2, AB and BC were found to be significant (P<0.05). For the first order effects, by evaluating the regression coefficients and t-value it could be concluded that the amino acid concentration (B) had the most significant effect on the laccase production followed by amount of banana peel (A). For the quadratic model, A2 and C2 were found to effect specific activity significantly (p< 0.05). For the 2-way interaction model, interaction of all, amount of banana peel and concentration of amino acid (AB), amount of banana peel and concentration of gallic acid (AC) and concentration of amino acid and concentration of gallic acid (BC) were found to be significant (P=0.000).

The optimization plot obtained after the analysis of results showed the optimized condition for maximum laccase production Figure 4. The amount of banana peel was varied from 6.59 g to 23.40 g; alanine was varied from -0.22 mg/ml to 50.22 mg/ml and gallic acid was varied from -0.21 mM to 1.31 mM in the experimental runs, but the maximum laccase production was obtained at banana peel amount of 8.62 g; amino acid concentration of 50.23 mg/10ml and gallic acid concentration of 0.25 mM. At this level, desirability obtained was one. According to this plot, it was clear that the enzyme activity decreased with increased in banana peel concentration whereas enzyme activity increased with increased in alanine concentration. In case of gallic acid, enzyme activity first increased with increased with gallic acid concentration and after attaining certain value the enzyme activity decreased with further increased in concentration. The change in enzyme activity with change in gallic acid concentration was less than that with peel and amino acid. It means that banana peel and amino acid had more pronounced effect on the enzyme production than gallic acid.

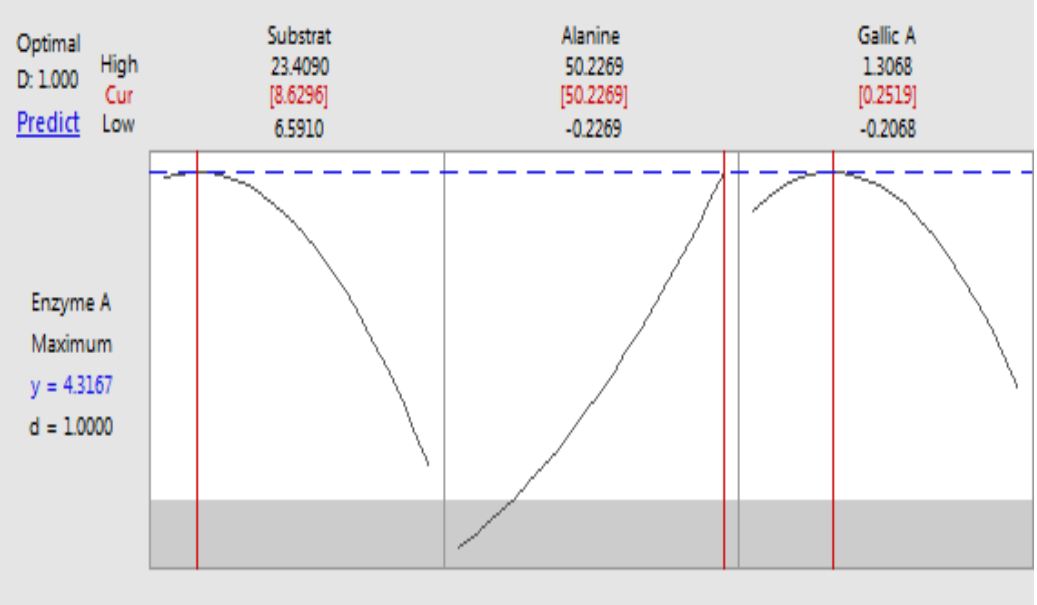


Fig 4: Optimization plot for production of laccase by *Ganoderma lucidium* NCIM 1091

Conclusions

From the present study done, it can be concluded that *Ganoderma lucidium* NCIM 1091 can be used to produce laccase. Also banana peel was proved to be a good substrate for laccase production. Alanine and gallic acid proved to increase laccase production significantly. The interactive effect of amount of banana peel and concentration of amino acid and gallic acid was also proved to be significant at some levels and maximum laccase production (4.32 U/ml) was obtained at 8.63 gm banana peel, 50.23 mg/ml concentration of alanine and 0.25 mM concentration of gallic acid.

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Conflict of Interest

The authors report no conflict of interest

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