Research Paper

Optimization of media components for laccase production by litter dwelling fungal isolate *Fusarium incarnatum* LD-3

Urvish Chhaya and Akshaya Gupte

Department of Microbiology, N.V. Patel college of Pure and Applied Sciences, Sardar Patel University, Vallabh-Vidyanagar, Gujarat, India

Laccase production by solid state fermentation (SSF) using an indigenously isolated litter dwelling fungus *Fusarium incarnatum* LD-3 was optimized. Fourteen medium components were screened by the initial screening method of Plackett-Burman. Each of the components was screened on the basis of 'p' (probability value) which was above 95% confidence level. Ortho-dianisidine, thiamine HCl and $CuSO_4 \cdot 5\ H_2O$ were identified as significant components for laccase production. The Central Composite Design response surface methodology was then applied to further optimize the laccase production. The optimal concentration of these three medium components for higher laccase production were (g/l): $CuSO_4 \cdot 5\ H_2O$, 0.01; thiamine HCl, 0.0136 and ortho-dianisidine, 0.388 mM served as an inducer. Wheat straw, 5.0 g was used as a solid substrate. Using this statistical optimization method the laccase production was found to increase from 40 U/g to 650 U/g of wheat straw, which was sixteen times higher than non optimized medium. This is the first report on statistical optimization of laccase production from *Fusarium incarnatum* LD-3.

Keywords: *Fusarium incarnatum* LD-3 / Laccase / Plackett-Burman / Central Composite Design / Solid State Fermentation

Received: June 24, 2009; accepted: October 07, 2009

DOI 10.1002/jobm.200900203

Introduction

Laccases (benzenediol : oxygen oxidoreductase, E.C.1.10.3.2) are multicopper containing enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates like diphenols, methoxy substituted monophenols, aromatic amines [1]. Laccases have received a lot of scientific attention over last couple of decade for their ability to oxidize phenolic and nonphenolic lignin related compounds as well as highly recalcitrant environmental pollutants, a fact which underscores its enormous practical implications for various possible biotechnological applications. Currently laccases are used in pulp delignification, textile dye bleaching, effluent detoxification, washing powder components, removal of phenolics from cork stoppers [2], transformation of antibiotics and steroids [3], as well as in nanobiotechnology for the development of biosensors to detect various phenolic compounds, oxygen or azides. [4]. Most of the studies conducted on laccasses so far have focused on the model white rot species *Trametes versicolor*, *Pleurotus ostreatus* and a few others. However, the ecological group of litter dwelling fungi (LDF), represented by species inhabiting the natural environment of soil and decaying litter, are very promising candidates for the production of ligninolytic activities. There are very few reports describing the presence of ligninolytic activities in these species [5, 6]. The application of laccase in biotechnological processes requires the production of high amount of enzyme at low cost and hence the current focus of laccase research is oriented towards the identification and optimization of such an efficient production system. Solid State Fermentation (SSF) is an important mode of fermentation where microorganisms grow on the substrates in the absence or near-absence of free water and

Correspondence: Dr. Akshaya Gupte, Department of Microbiology, N.V. Patel College of Pure and Applied Sciences, Vallabh-Vidyanagar-388120, Gujarat, India
E-mail: akshya_gupte@hotmail.com
Phone: +91-2692-235500
Fax: +91-2692-234111

excrete aimed product efficiently [7, 8]. Considering the lower energy requirements, simplicity of cultivation and media equipment, high product titres and lower waste water output, this traditional method ignites the interest of researchers again in the production of enzymes, fine chemicals, and antibiotics, [9–11] etc. *Fusarium incarnatum* LD-3 isolated from soil near decomposed litter (Panchmarhi forest, Madhya Pradesh, India) is found to produce laccase. There are large numbers of reports on the optimization of carbon and nitrogen source by the classical method of medium optimization that changes one independent variable, while fixing other variables at definite levels. Optimizing all the affecting parameters by statistical experimental design, Plackett-Burman and response surface methodology can eliminate the limitations of single-factor optimization process collectively [12–16].

The present work attempts to formulate a suitable production medium using statistical optimization that can increase the laccase production from *Fusarium incarnatum* LD-3 using Plackett-Burman and central composite design.

Materials and methods

Chemicals

2, 2-Azino-bis (3ethylbenzthiozoline-6-sulphonic acid) (ABTS) was purchased from Sigma (St. Louis M.O., U.S.A.). Yeast extract and peptone were procured from Hi-Media (Mumbai, India). Ortho-dianisidine was procured from CDH (Mumbai, India). Thiamine HCl, NH_4NO_3, $CuSO_4 \cdot 5\ H_2O$, $CoCl_2 \cdot 6\ H_2O$, $MnSO_4 \cdot H_2O$, ammonium ferric citrate, $MgSO_4 \cdot 7\ H_2O$, $CaCl_2 \cdot 2\ H_2O$, $ZnSO_4 \cdot 7\ H_2O$, KH_2PO_4, were purchased from SD Fine Chemicals, (Mumbai, India). Tween 80 was purchased from Merck, (Mumbai, India). All other chemicals were of analytical grade procured from Qualigens, (Mumbai, India). Wheat straw was collected locally and used as a lignocellulosic substrate.

Screening and isolation of fungal strain

The samples of soil with decomposed litter were collected from the region near the decomposed litter from the forest localities in Panchmarhi, Madhya Pradesh, India. Soil samples were suitably diluted and plated on 2% malt extract agar medium (MEA) supplemented with 50 μg/ml of streptomycin and incubated at 28 ± 2 °C. After 8 d of incubation fungal cultures were transferred on Sabouraud dextrose agar (SDA) incorporated with ortho-dianisidine (0.01% w/v). Fungal isolates showing positive Bavendamm's reaction were maintained on MEA at 28 ± 2 °C and stored at 4 °C. The cultures were transferred to fresh media once in a month.

Media preparation and inoculation

The basal medium described by Asther *et al.* [17] was modified and used to moisten the support material (5 g of wheat straw in 20 ml basal medium) in 250 ml Erlenmeyer flask. The requisite volume of media constituents were pipetted out from their stock solution of higher concentration and were mixed together before sterilization. Thiamine-HCl and ortho-dianisidine were both filter sterilized and added separately to the medium before inoculation. Wheat straw was separately sterilized at 15 psi for 30 min and mixed aseptically with the medium before inoculation. The pH of the medium was adjusted to 5.0 and sterilized by autoclaving at 15 psi for 15 min. Each flask was inoculated with four mycelial agar plugs of 8 mm in diameter, (cut from the edge of an actively growing colony on malt extract agar plates) and incubated under static condition at 28 ± 2 °C for 8 d. The media components for laccase production and their composition are given in Tables 1 and 2.

Enzyme assay

Laccase activity (E.C.1.10.3.2) was determined by measuring the oxidation of 2, 2-Azino-bis (3ethylbenzthiozoline-6-sulphonic acid) (ABTS). Increase in absorbance for 3 min was measured spectrophotometrically (Ellico BL-198, Hydrabad, India) at 420 nm (ε = 36 000 $cm^{-1}\ M^{-1}$) [18]. The reaction mixture contained 100 μl of 50 mM ABTS and 800 μl of 20 mM Na-Acetate butter (pH-5.0) and 100 μl of appropriately diluted enzyme extract. One unit of enzyme was defined as amount of enzyme that oxidized 1 μM of substrate per min.

Optimization procedure

(A) Identification of important nutrient components: Plackett-Burman design was followed to screen important medium components as shown in Table 2. Total fourteen components (variable k = 14) were selected for the study, with each variable being represented at two levels high (+) and low (–) and five dummy variables in 20 trials as shown in Tables 1 and 2. The numbers of positive and negative signs per trial are $(k + 1)/2$ and $(k - 1)/2$ respectively. Each row represents a trial and each column represents an independent (assigned) or dummy (unassigned) variable. The effect of each variable was determined by the following equation.

$$E(x_i) = 2(\sum M_i^+ - M_i^-)/N \quad (1)$$

where $E(x_i)$ is the concentration effect of the tested variable M_i^+ and M_i^- are the laccase production from the trial where the variable (x_i) measured was estimated by calculating the variance among the dummy variables as follows:

$$V_{\text{eff}} = \sum (E_d^2)/n \quad (2)$$

where V_{eff} is the variance of the concentration effect, E_d is the concentration effect for the dummy variables and n is the number of dummy variables. The standard error (S.E.) of the concentration effect was the square root of the variance of an effect and the significance level (p value) of each concentration effect was determined using student's t test.

$$t(x_i) = Ex_i/\text{S.E.} \quad (3)$$

where, Ex_i is the effect of variable x_i.

(B) Optimization of screened components: Response surface methodology was used to optimize the screened components for enhanced laccase production using central composite design. The behavior of the system was explained by the following quadratic equation.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (4)$$

where Y is predicted response, β_0 is offset term, β_i is linear offset, β_{ii} is squared offset and β_{ij} is interaction effect. x_i is dimensionless coded value of X_i. The above equation was solved by using the software Design-Expert (Version 7.0.2, Stat ease inc., USA). A 2^5 factorial design with five replicates at the center point with a total number of 20 trials were employed. The coded and uncoded values of the variables at various levels are given in Table 4.

Results

Screening and isolation of fungal cultures

Extracellular laccase activity was found in forty one isolates. The isolates showed a brown colored zone surrounding the growth on SDA plate containing ortho-dianisidine, which is a characteristic of phenol oxidase production on the solid medium. The culture designated as LD-3 showing 40 U/g of laccase was found to be the best amongst the forty one isolates tested. The identification of LD-3 was further corroborated by studies on its 18S rRNA, 5.8S rRNA and partial 28S rRNA gene sequencing carried out by Bangalore Genei, India. The isolate was identified as a *Fusarium incarnatum* LD-3 (Genbank Accession no EU426883). The boot strapped unrooted tree was structured by the neighbor-joining method from the distance data generated by multiple alignment of the nucleotide sequence.

Screening of important medium components for laccase production

Fusarium incarnatum LD-3 produces 40 U/g of laccase using medium according to Asther *et al.* at shake flask level under solid state fermentation. To enhance the production of laccase, statistical method of medium optimization was tried. Table 1 represents the independent variables and their respective high and low concentration used in the optimization study, while Table 2 shows the Plackett-Burman experimental design for 20 trials with two levels of concentrations for each variable and the corresponding laccase production in terms of units per gram of wheat straw. The variables X_1-X_{14} represent the medium constituents where as D_1-D_5 represent the dummy variables. Table 3 represents the effect, standard error, t (x_i), p value and confidence level for each component based on the units per gram of dry substrate of laccase in Table 2.

The components were screened at a confidence level of 95% on the basis of their effects. The confidence level for yeast extract, NH_4NO_3, peptone, $CoCl_2 \cdot 6\ H_2O$, $MnSO_4 \cdot H_2O$, ammonium ferric citrate, $MgSO_4 \cdot 7\ H_2O$, $CaCl_2 \cdot 2\ H_2O$, KH_2PO_4, $ZnSO_4 \cdot 7\ H_2O$, and Tween 80 were below 95% and hence were considered insignificant, while the remaining components ortho-dianisidine, thiamine HCl and $CuSO_4 \cdot 5\ H_2O$ showed confidence level at or above 95% and were considered to be significant. Here a positive effect meant an increase in the laccase production while negative effect means reduction in the laccase production. As a result the variables ortho-dianisidine, thiamine HCl and

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

Variables	Medium Components	+ Values (g/l)	– Values (g/l)
X_1	Ortho-dianisidine	0.5[a]	0.05[a]
X_2	Yeast extract	1.0	0.1
X_3	Thiamine HCl	12.5[b]	1.25[b]
X_4	NH_4NO_3	2.5	0.25
X_5	Peptone	5.0	0.5
X_6	$CuSO_4 \cdot 5\ H_2O$	0.07	0.007
X_7	$CoCl_2 \cdot 7\ H_2O$	0.035	0.0035
X_8	$MnSO_4 \cdot H_2O$	0.175	0.0175
X_9	$ZnSO_4 \cdot 7\ H_2O$	0.231	0.0231
X_{10}	Ammonium ferric citrate	0.425	0.0425
X_{11}	$MgSO_4 \cdot 7\ H_2O$	0.25	0.025
X_{12}	$CaCl_2 \cdot 2\ H_2O$	0.066	0.0066
X_{13}	KH_2PO_4	1.0	0.1
X_{14}	Tween 80	0.05[c]	0.005[c]

[a] mM, [b] mg/l, [c] ml/l

Table 2. Plackett-Burman design matrix of fourteen variables ($X_1 - X_{14}$) and five dummy variables (D1 – D5) along with observed response (laccase production).

Run No	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	D_1	D_2	D_3	D_4	D_5	Laccase (U/g)
1	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	220.92
2	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	231.17
3	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	497.7
4	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	271.65
5	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	−	146.16
6	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	−	286.00
7	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	−	96.00
8	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	+	71.70
9	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	−	296.55
10	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	+	289.76
11	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	−	682.78
12	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	+	195.00
13	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	+	549.08
14	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	+	258.30
15	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	+	277.29
16	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	−	184.86
17	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	−	225.29
18	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	+	108.96
19	+	−	−	+	+	+	+	−	+	−	+	−	−	−	−	+	+	−	+	150.78
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	62.64

Table 3. Statistical analysis of medium components in relation to laccase production as per Plackett-Burman design.

Factors	Medium Components	Effect	S.E.	t(xi)	P-value	Confidence level (%)
X_1	Ortho-dianisidine	167.70	45.20	3.70	0.01	99
X_2	Yeast extract	−35.22	45.20	−0.78	0.47	53
X_3	Thiamine HCl	127.60	45.20	2.82	0.03	97
X_4	NH_4NO_3	−55.38	45.20	−1.23	0.27	73
X_5	Peptone	−89.89	45.20	−1.99	0.103	89.7
X_6	$CuSO_4 \cdot 5\ H_2O$	−119.10	45.20	−2.63	0.04	96
X_7	$CoCl_2 \cdot 7\ H_2O$	4.035	45.20	0.089	0.9	10
X_8	$MnSO_4 \cdot H_2O$	20.8	45.20	0.46	0.66	34
X_9	$ZnSO_4 \cdot 7\ H_2O$	43.30	45.20	0.96	0.38	62
X_{10}	Ammonium ferric citrate	35.44	45.20	0.78	0.47	53
X_{11}	$MgSO_4 \cdot 7\ H_2O$	82.07	45.20	1.82	0.12	88
X_{12}	$CaCl_2 \cdot 2\ H_2O$	13.68	45.20	0.30	0.77	23
X_{13}	KH_2PO_4	−25.89	45.20	−0.57	0.59	41
X_{14}	Tween 80	−14.35	45.20	−0.32	0.76	24

$CuSO_4 \cdot 5\ H_2O$ showed confidence level of 99%, 97% and 96%, respectively, and were all considered significant.

These results indicate the effectiveness of the Plackett-Burman design in identifying the factors with a significant influence on the laccase production. There after the exact optimal values for the individual factors were determined using central composite design experiments.

Optimization of screened medium components for laccase production

The variables showing positive effect with a confidence level of 99% (ortho-dianisidine), 97% (thiamine HCl) and negative effect 96% ($CuSO_4 \cdot 5\ H_2O$) in the Plackett-Burman design were selected and further optimization was achieved using a central composite design, 3-D surface plots were obtained and analyzed based on feeding data on the laccase production in to the design expert software. The software allows the laccase production to be predicted within the studied range for the all three components of medium. Here each 3-D surface plot represents the effect of two medium components at their studied concentration range, when the other components have a fixed concentration. The values of the other components were then varied for that situation using the software to determine the optimum values. Based on the results of Plackett-Burman design the component with a significant confidence level (ortho-dianisidine, thiamine HCl and $CuSO_4 \cdot 5\ H_2O$) were set at their higher level, while the components with a

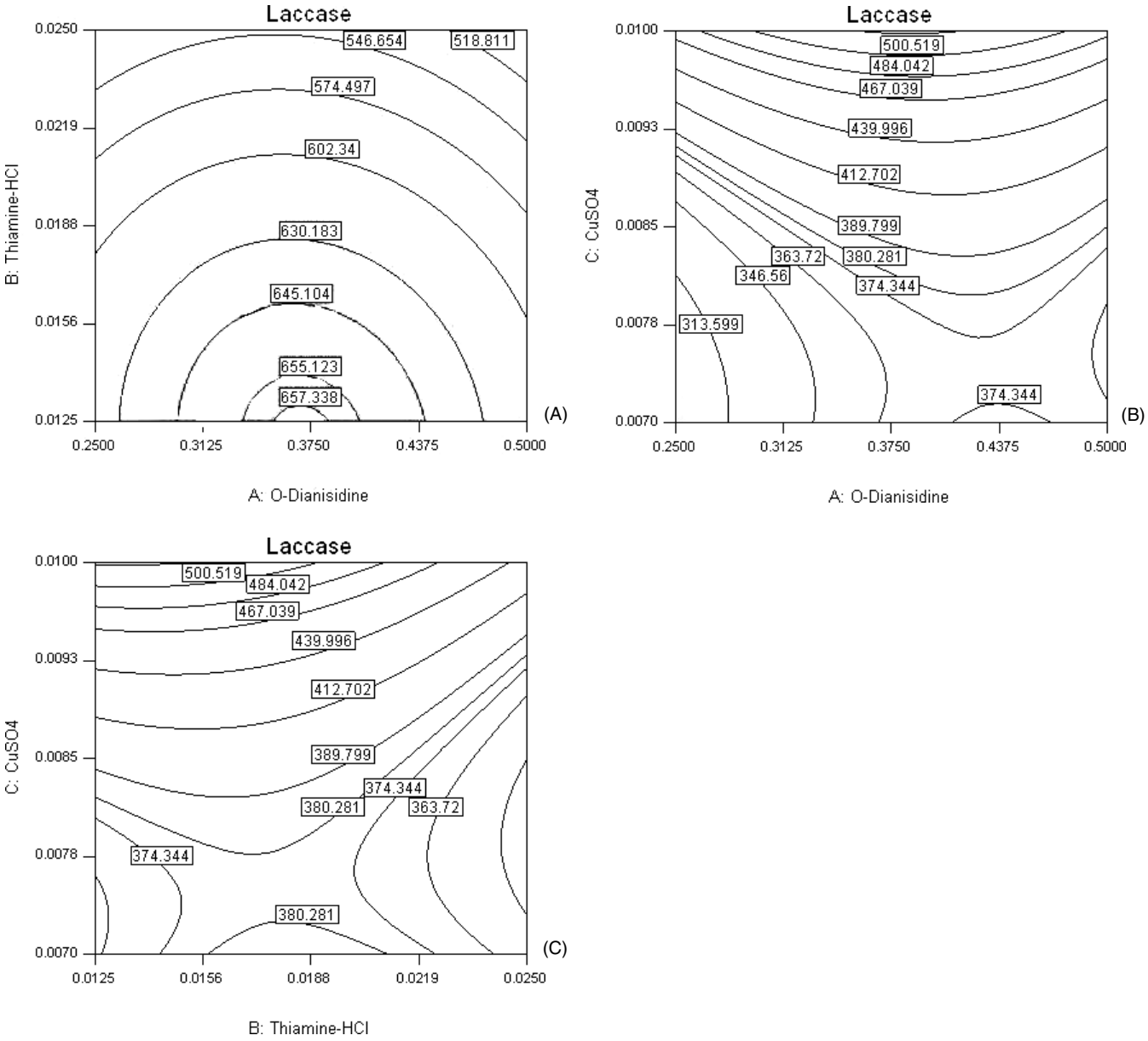

Figure 1. (A). Three dimensional plot showing the effect of Thiamine HCl and ortho-dianisidine at 0.01 g/l of $CuSO_4 \cdot 5\ H_2O$; (B) three dimensional plot showing effect of $CuSO_4 \cdot 5\ H_2O$ and ortho-dianisidine at 0.0136 (g/l) of thiamine HCl, and (C) three dimensional plot showing effect of $CuSO_4 \cdot 5\ H_2O$ and thiamine HCl at 0.388 mM of ortho-dianisidine.

confidence level below 95% were set at their middle level.

Table 4 represents the experimental design and the result obtained for laccase production. The variables used for factorial analysis were ortho-dianisidine, thiamine HCl and $CuSO_4 \cdot 5\ H_2O$ for laccase production. The actual and coded factor levels of laccase production are presented in Table 4. The data were analyzed by a quadratic multiple regression using a design expert (version 7.0.2, Stat ease Inc., USA) and the following equation was obtained.

$$Y = 394.14 + 19.32\ A - 23.14\ B + 60.23\ C - 4.87\ AB - 16.62\ AC - 20.49\ BC - 39.13\ A^2 - 25.42\ B^2 + 47.36\ C^2 \quad (5)$$

Here Y is the predicted response and A, B, C, are the coded variables for ortho-dianisidine, thiamine HCl and $CuSO_4 \cdot 5\ H_2O$, respectively.

To validate the regression coefficient, an analysis of variance (ANNOVA) of the laccase production was performed (Table 5). The values of the lack of fit, model F and model $P > F$ were found to be 0.5884, 8.99 and

Table 4. Central Composite Design matrix with coded values and actual values for laccase production.

Run No.	Ortho-dianisidine (mM)		Thiamine HCl (g/l)		$CuSO_4$, H_2O (g/l)		Laccase (U/g)	
	Coded Values	Actual Value	Coded Values	Actual Value	Coded Values	Actual Value	Actual Value	Predicted Value
1	−1	0.25	−1	0.0125	+1	0.01	465.08	473.24
2	0	0.375	0	0.0187	−2	0.006	441.66	426.80
3	0	0.375	0	0.0187	0	0.0085	386.64	394.14
4	0	0.375	0	0.0187	0	0.0085	378.00	394.14
5	−1	0.25	+1	0.025	−1	0.007	251.64	283.00
6	0	0.375	+2	0.029	0	0.0085	327.10	283.32
7	+1	0.5	+1	0.025	+1	0.01	381.96	391.37
8	0	0.375	0	0.0187	0	0.0085	406.09	394.14
9	−2	0.164	0	0.0187	0	0.0085	293.28	250.98
10	0	0.375	0	0.0187	0	0.0085	462.02	394.14
11	0	0.375	0	0.0187	0	0.0085	328.22	394.14
12	0	0.375	0	0.0187	+2	0.011	645.58	629.38
13	+1	0.5	+1	0.025	−1	0.007	331.35	345.15
14	−1	0.25	+1	0.025	+1	0.01	358.84	395.72
15	0	0.375	−2	0.0082	0	0.0085	348.43	361.16
16	+1	0.5	+1	0.025	+1	0.01	497.78	488.38
17	0	0.375	0	0.0187	0	0.0085	398.54	394.14
18	−1	0.25	−1	0.0125	−1	0.007	266.00	278.55
19	+2	0.585	0	0.0187	0	0.0085	304.72	315.97
20	+1	0.5	−1	0.0125	−1	0.007	375.10	360.19

Table 5. Analysis of variance (ANNOVA) for the quadratic model.

Source	Sum of Squares	Mean (df)	F Square	p-value	Prob > F
Model	13800	9	15337.88	8.99	0.0010
A-O-Dianisidine	5098.34	1	5098.34	2.99	0.1146
B-Thiamine-HCl	7313.76	1	7313.76	4.29	0.0653
C-$CuSO_4$	49538.58	1	49538.58	29.03	0.0003
AB	189.83	1	189.83	0.11	0.7456
AC	2210.79	1	2210.79	1.30	0.2816
BC	3359.95	1	3359.95	1.97	0.1909
A^2	22062.96	1	22062.96	12.93	0.0049
B^2	9313.59	1	9313.59	5.46	0.0416
C^2	32322.56	1	32322.56	18.94	0.0014
Residual	17067.37	10	1706.74		
Lack of Fit	7640.05	5	1528.01	0.81	0.5884
Pure Error	9427.32	5	1885.46		
Cor Total	15510	19			

R^2 = 0.8900, Adj R^2 = 0.7909, CV = 10.80%

<0.001, respectively, indicating that model was significant. The values of the adjusted determination coefficient (Adj R^2 = 0.7909) was also very high reconfirming the significance of the model. The lack of fit (0.5884) is found to be not significant. This indicates an excellent correlation between the experimental and predicted values of laccase production. At the same time relatively low coefficient variation (CV = 10.80%) confirms the precision and reliability of the experiment performed.

Fig. 2 represents the relationship between the actual laccase production and predicted values determined by the model equation (5) for *Fusarium incarnatum* LD-3.Clearly most of the points were near by the line adjustment which meant that the experimentally determined values were similar to those determined by the model. The model predicted that the maximum production of laccase using above optimum concentration of variables would be 657 U/g of wheat straw. To verify this, prediction experiments were carried out using optimized medium and the result showed a higher yield of laccase production i.e. 650 U/g which was 16 times higher than non optimized medium.

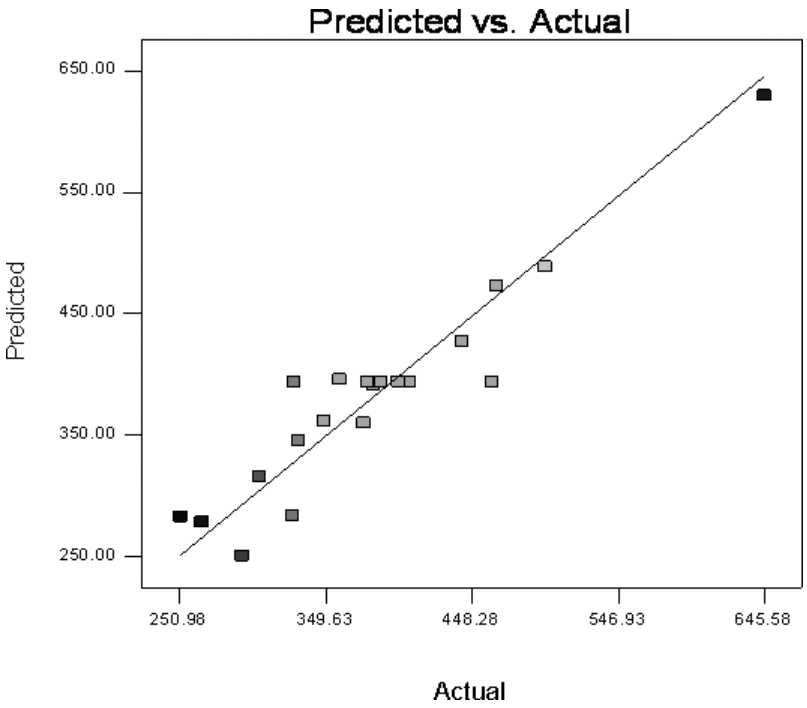

Figure 2. Predicted v/s actual laccase production from *Fusarium incarnatum* LD-3.

Discussion

There are few reports on the production of laccase from *Fusarium* sp. and litter dwelling fungi. A comparative study on the extra cellular lignolytic enzyme activity of five strains of *Fusarium solani* under carbon limited medium revealed a differential production of aryl alcohol oxidase and laccase i.e. 57 mU/ml and 8.6 mU/ml respectively [19]. Laccase, aryl oxidase and super oxide radicals were also detected in lignolytic cultures of *Fusarium proliferatum* during secondary metabolism [20]. These reports are in agreement with our findings that *Fusarium* sp. possesses lignolytic activities; however this is the first report of laccase production from *Fusarium incarnatum* LD-3.

In previous studies, different statistical methods for medium optimization has been employed to improve laccase production from white rot fungi. Improvement of laccase production from *Genoderma* sp. KU Alk 4 by medium engineering was done by Box-Behnken response surface methodology and 12 times higher laccase production was obtained compared to non optimized medium [21]. Central Composite Design using rice bran as a solid substrate was performed for the production of laccase from *Streptomyces psammoticus*, and three fold increase in the laccase activity as compared to unoptimized medium was observed [22]. The medium for laccase production was optimized by response surface methodology, and a four fold increase in the laccase activity (10,050 U/g) was achieved from indigenously isolated *Genoderma* sp. [23]. Report is also available for laccase production by white rot fungi *Trametes modesta*, where four fold increased production was observed. When final optimization was done using central composite design [24], laccase production has also been increased by 28.3% under submerged fermentation by Taguchi DOE methodology by *Pleurotus ostreatus* [25]. Optimization and modeling of laccase production by *Trametes versicolor* in a bioreactor using statistical experimental design resulted in 11,403 U/l laccase activity [26]. Response surface methodology was also applied for the decolorization of the azo dye reactive black 5 (RB-5) using purified laccase from *Pleurotus sajorcaju*. Box-Behnken design indicated that 0.5−2.5 U/ml of laccase was found to be optimum for the maximum (84.4%) decolorization [27].

Statistical methods for media optimization have been successfully utilized for production of other bioproducts as well. The statistical optimization method increased chitinase production by *Alcaligenes xylosoxydans* from 12 to 29 U/ml [28]. There was a 50.33% increase in lactic acid production by *Lactobacillus* sp. [29], and compactin production was increased from 250 μg/ml to 400 μg/ml in a *Penicillium* sp. [30]. Statistical optimiza-

tion of medium components for the production of biosurfactant by *Bacillus lichemiformis* K51 was carried out by Box-Behnken response surface methodology and yield of biosurfactant in terms of Critical Micelle Dilution (CMD) was increased ten times compared to non optimized medium [15]. Under solid state fermentation optimization of poly γ glutamate production was optimized and there was four times increase in the yield [31]. The optimization of phytase production was carried out by Box-Behnken design of experiments resulting in the maximum level of phytase production i.e. 9.18 U/g of substrate [32]. All these findings using statistical optimization are in close agreement with our results. However, there are no previous reports on the optimization of laccase production from *Fusarium incarnatum* LD-3, therefore this study attempts to formulate an optimized medium for laccase production from *Fusarium incarnatum* LD-3.

The methodology of Plackett-Burman was found to be very useful in determining the relevant variables for further optimization making it possible to consider large number of variables and avoid information loss, both of which are essential in the optimization process. As a result the important medium components with a significant effect on laccase production by *Fusarium incarnatum* LD-3 were identified. In conclusion the methodology of Plackett-Burman and Central Composite Design proved to be very effective in improving laccase production.

Acknowledgement

Authors are grateful to University Grants Commission (New Delhi) for providing financial support (Sanction No: F.47-052/6).

References

[1] Thurston, C.F., 1994. The structure and function of fungal laccases. Microbiology, **40**, 19–26.

[2] Brenna, O. and Bianchi, E., 1994. Immobilized laccase for phenolic removal in must and wine. Biotechnol. Lett., **24**, 35–40.

[3] Breen, A. and Singleton, F.L, 1999. Fungi in lignocellulose breakdown and biopulping. Curr. Opin. Biotechnol., **10**, 252–258.

[4] Roy, J.J., Abraham, T.E., Abhijit, K.S., Sujitkumar, P.V. and Thakur, M.S., 2005. Biosensor for the determination of phenol based cross linked enzyme crystals (CLEC) of laccase. Biosen. Bioelectron., **21**, 206–211.

[5] Steffen, K.T., Hofrichter, M. and Hatakka, A., 2000. Mineralization of C^{14} labelled synthetic lignin and ligninolytic enzyme activities of litter decomposing basidiomycetous fungi. Appl Microbiol. Bioeng., **54**, 736–744.

[6] Steffen, K.T., Hofrichter, M. and Hatakka A., 2003. Degradation of benzo{a}pyrene by the litter decomposing basidiomycete *Stropharia corrolina*: role of manganese peroxidase. Appl. Environ. Microbiol., **69**, 3957–3964.

[7] Pandey, A., Carlos, R.S. and David, M., 2000. New developments in solid state fermentation: I. Bioprocesses and products. Process Biochem., **35**, 1153–1169.

[8] Veronique, B.M., Olivier, O. and Pierre, C., 2003. Sensors and measures in solid state fermentation: a review. Process Biochem., **38**, 881–896.

[9] Zubeyde, B., Fikret, U. and Cetin, A., 2003. Solid state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot spring water. Process Biochem., **38**, 1665–1668.

[10] Kumar, D., Jain, V.K., Shankar, G. and Srivastava, A., 2003. Citric acid production by solid state fermentation using sugarcane baggasse. Process Biochem., **38**, 1731–1738.

[11] Adinarayana, K., Prabhakar, T., Srinivasulu, V., Anitha Rao, M., Jhansi Laxmi, P. and Ellaiah, P., 2003. Optimization of process parameters for cephalosporin C production under solid state fermentation from *Acremonium chrysigenum*. Process Biochem., **39**, 171–177.

[12] Gohel, V., Chaudhary, T., Vyas, P. and Chhatpar, H.S., 2006. Statistical screening of medium components for the production of chitinase by the marine isolate *Pantoea dispersa*. Biochem. Eng, J., **8**, 50–56.

[13] Montogomery, D.C., 2000. Design and Analysis of Experiments (5th Ed.). John Wiley & Sons, Singapore.

[14] Felse, P.A. and Panda, T., 1999. Self-directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. Process. Biochem., **34**, 563–566.

[15] Joshi, S., Yadav, S., Nerurkar, A. and Desai, A.J., 2007. Statistical optimization of medium components for the production of biosurfactant by *Bacillus lichemiformis* K51. J. Microbiol. Biotechnol., **17**, 313–319.

[16] Plackett, R.L. and Burman, J.P., 1944. The design of optimum multifactorial experiments. Biometrika, **33**, 305–325.

[17] Asther, M., Lesage, L, Drapron, R., Corrieu, G. and Odier, E., 1988. Phospholipid and fatty acid enhancement of *Phanerochaete chrysosporium* INA-12 in relation to ligninase production. Appl. Microbiol. Biotechnol., **27**, 393–398.

[18] Niku Paavola, M.L., Karhuneum, E., Kentelinen, A., Viikari, L., Lundell, T. and Hattaka, A., 1990. Detection of white rot fungi by a nontoxic stain. Mycology Res., **94**, 27–31.

[19] Saparrat, M.C.N., Martinez, M.J., Tournier, H.A., Cabello, M.N. and Arambarri, A.M., 2000. Production of lignolytic enzymes by *Fusarium solani* strains isolated from different substrata. World J. Microbiol. Biotechnol., **16**, 799–803.

[20] Rogalado, V., Parestelo, F., Rodriguez, A., Carnicero, A., Sosa, F.J., De la Fuente, G., *et al*., 1999. Activated oxygen species and two extracellular enzymes: laccase and aryl alcohol oxidase, novel for the lignin degrading fungus *Fu-*

sarium proliferatum. Appl. Microbiol. Biotechnol., **51**, 388–390.

[21] Churapa, T., Roberto, P., Christopher, B. and Lerluck, C., 2007. Improvement of laccase production from *Genoderma* sp. KU-Alk4 by medium engineering. World J Microbiol. Biotechnol., **23**, 1519–1527.

[22] Niladevi Narayanan, K., Sukumaran, R. and Prema, P., 2007. Utilization of rice straw for laccse production by *Streptomyces psammoticus* in solid state fermentation. J. Ind. Microbiol. Biotechnol., **34**, 665–674.

[23] Revankar, M., Desai, K. and Lele, S., 2007. Solid state fermentation for enhanced production of laccase using indigenously isolated *Genoderma* sp. Appl. Biochem. Biotechnol., **143**, 16–26.

[24] Nyanhongo, G.S., Gomes, J., Gubitz, G., Zvauya, R., Read, J.S. and Steiner, W., 2002. Production of laccase by a newly isolated strain of *Tremetes modesta*. Bioresource Tech., **84**, 259–263.

[25] Krishna, K., Prasad, S., Venkata Mohan, R., Sreenivas, Rao, Bikas Ranjan, Pati and Sharma, P.N., 2005. Laccase production by *Pleurotus ostreatus* 1804: optimization of submerged culture condition by Taguchi DOE methodology. Biochem. Eng. J., **241**, 7–26.

[26] Tavares, A.P.M., Coelho, M.A.Z., Agapito, M.S.M., Coutinho, J.A.P. and Xavier, A.M.B.R., 2006. Optimization and modeling of laccase production by *Tremetes versicolor* in a bioreactor using statistical experimental design. Appl. Biochem. Biotechnol., **134**, 233–248.

[27] Kumarasamy, M., Ankur, D., In-hyun, N., Youngo-Mo, Kim and Yoon-Seok, Chang, 2006. Decolourization of reactive black 5 by laccase: Optimization by response surface methodology. Dyes and Pigments, **20**, 1–9.

[28] Vaidya, R., Vyas, P. and Chhatpar, H.S., 2003. Statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans*. Enzyme Microb. Technol., **33**, 92–96.

[29] Chauhan, K., Trivedi, U. and Patel, K.C., 2006. Application of response surface methodology for optimization of lactic acid production using date juice. J. Microbiol. Biotechnol., **16**, 1410–1415.

[30] Konya, A., Jekkel, A., Suto, J. and Salat, J., 1998. Optimization of compactin fermentation. J. Ind. Microbiol. Biotechnol., **20**, 150–152.

[31] Xu, Jian, Chen, Shouwen and Yu, Ziniu, 2005. Optimization of process parameters for poly Y-glutamate production under solid state fermentation from *Bacillus subtilis* CCTCC202048. Process Biochem., **7557**, 1–7.

[32] Chanda, B.S., Harmeet, G., Mandeep, M., Saini, H.S. and Singh, N., 2004. Phytase production by the thermophilic fungus *Rhizomucor pusillus*. World J. Microbiol. Biotechnol., **20**, 105–109.

