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EXPLORATION OF OPTIMUM OPERATING CONDITIONS FOR ENHANCED LACCASE ENZYME PRODUCTION BY *PLEUROTUS NEBRODENSIS* WC 850 THROUGH RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Laccase is a blue copper containing enzyme which is capable of catalyzing the oxidation of phenolic compounds to phenoxyl radicals. The purpose of recent study was to find optimum conditions for maximum laccase enzyme production by *Pleurotus nebrodensis* WC 850 by applying an important statistical technique response surface methodology (RSM) through solid state fermentation of lignocellulosic biomass. Firstly, screening experiments were conducted in order to select the best substrate for maximum laccase enzyme production among six different lignocellulosic substrates. Wheat straw was selected as best substrate with enzyme activity 145.10 U/mL after 6th day of fermentation. Different physical parameters including pH, temperature, inoculum size, incubation time and moisture were then optimized by response surface methodology under central composite design (CCD). RSM is an effective strategy which explores the optimum operating conditions for multivariable system as well as it also examines the simultaneous, systematic and effective variation of crucial components and determines the possible interaction among higher order effects which results in enhanced enzyme yield. The increase in laccase enzyme activity (171.63 U/mL) was observed at pH 5, temperature 30°C, inoculum size 4mL, incubation time 144mL and moisture 50% after optimization of different factors at different levels. This study reveal that optimization of different factors have significant effect on laccase enzyme production. Moreover, optimization of different parameters through RSM is time saving and good technique in biotechnology.

Key words: Laccase, *Pleurotus nebrodensis*, Response Surface Methodology

INTRODUCTION

Enzymes are multitalented catalysts that have ability to catalyze reactions in short time including those reactions which are difficult to perform using chemical methods. Enzymes have been preferred because of their unique catalytic nature and their specialty to work under complex and moderate conditions (Sarrouh *et al.,* 2012). Enzymes can be produced through fermentation biotechnology by utilization of many agro- industrial waste materials which are mostly supplied by paper-pulp industries and agro industries including forestry. These lignocellulosic waste materials cause environmental pollution when left untreated despite of the fact that they can be transformed into useful products like different organic chemicals, enzymes and biofuels. So it is essential to explore new industrial science which can efficiently utilize this waste material and convert it into beneficial products (Isroi *et al.,* 2011).

Fermentation biotechnology includes a series of steps which leads to conversion of complex substrates into simpler compounds by application of different microorganisms like fungi and bacteria (Robinson *et al.,* 2001). Fermentation technology is vast immensely used industrial science that deals with the formulation of variety of substances like antibiotics, single cell protein and enzymes. Fermentation technology has gained importance due to its economic and environmental benefits (Howard *et al.,* 2003). There are many types of fermentation but solid state fermentation (SSF) is considered reliable and useful than submerged fermentation (SmF) because of its greater product yielding ability, shortened energy prerequisites and easily performable downstream processing (Gupte *et al.,* 2007; Bhatnagar *et al.,* 2008).

Most of the white rot fungi produce laccase (Wong, 2009). Among different white rot fungi *Pleurotus* species have been described as best laccase enzyme producers because of its capability to secrete oxidative and hydrolytic enzymes which are responsible for lignocellulose degradation (Penninckx *et al.,* 2006; Kachlishvili *et al.,* 2008). There are many applications of *Pleurotus* species in environmental and biotechnology fields because of their ability to utilize lignocellulosic substrates via secreting hydrolytic and oxidative enzymes (Cohen, 2002; Mikiashvili *et al.,* 2004).

Laccases are multicopper (MCO) phenol oxidases responsible for oxidation of phenolic compounds to phenoxyl radicals. Laccase was first explored in *Rhus Vernicifera* and also found in insects,

bacteria and plants (Dittmer *et al.,* 2004; Giardina *et al.,* 2010; Maciel *et al.,* 2010). Fungal laccases are different as compared to bacterial and plant laccases due to its high redox potential (Brijwani *et al.,* 2010). Laccase are heme containing glycoproteins having molecular weight 60- 80 KDa (Hatakka, 2001).

There are many applications of laccase enzyme in paper and pulp industry, food industry and textile industry (Maciel *et al.,* 2010). High redox potential of fungal laccases makes them applicable in biotechnology as well as food industry (Mohammadian *et al.,* 2010). Laccases are being used for food processing, the stabilization of fruit juices and bioremediation of waste water (Morozova *et al.,* 2007; Ribeiro *et al.,* 2010; Ramachandran *et al.,* 2013) including production of hormone derivatives and delignification of wood pulps (Widsten and Kandelbauer, 2008; Koschorreck *et al.,* 2009; Brijwani *et al.,* 2010, Asad *et al.,* 2012).

Due to many applications of laccase enzyme in industry it is important to search new methods for laccase enzyme production by fermentation biotechnology. Extensive knowledge and understanding regarding recent technologies and scientific matters is essential in order to establish an industry including identification of exotic biological sources and their genetic manipulation through mutation for over-production, partial purification and stabilization for production of versatile enzymes (Azevedo *et al.,* 2012).

Response surface methodology (RSM) is a set of statistical and mathematical techniques which is mostly used to develop a model and analysis of problems in which a response of interest is affected by many variables and the main objective is to optimize this response (Montgomery, 2005). RSM has been applied for optimization purpose as a powerful statistical technique in biotechnology (Palvannan and Satishkumar 2010; Diwaniyan *et al.,* 2012). RSM is also used to study the interactions among different factors at changing levels (Vivekanadan *et al.,* 2014).

The objective of this study was to check the potential of new indigenous strain of *Pleurotus nebrodensis* WC 850 for laccase enzyme production through solid state fermentation of lignocellulosic materials. Moreover, this study involves the optimization of different physical parameters like pH, temperature, inoculum size, incubation time and moisture level of substrate for enhanced laccase enzyme production by a unique statistical technique response surface methodology under central composite design.

MATERIALS AND METHODS

Fungal Strain: Pure culture of *Pleurotus nebrodensis* WC 850 was obtained from Mushroom Laboratory, Department of Plant Pathology, University of Agriculture Faisalabad, Pakistan. Pure culture was maintained on Potato dextrose agar (PDA) slants at pH 4.4 and 28°C temperature. It was multiplied periodically for further use and preserved at 4°C.

Substrate Collection and Preparation: Six different substrates were used for laccase enzyme production. These include sugarcane baggase, wheat straw, rice straw, banana stalk, corn stover and corn cobs. These substrates were obtained from Industrial Biotechnology Laboratory and CPC Rafhan Mill Faisalabad. All these substrates were dried in oven at 80°C and then ground in an electric grinder to powder form of 40mm mesh size. These were stored in air tight plastic jars to keep them moisture free.

Inoculum Development: Homogeneous spore suspension of *P. nebrodensis* WC 850 was prepared by growing the fungus in inoculum medium for 5-8 days. The inoculum medium was of Kirk (Tien and Kirk, 1988) supplemented with 1% (w/v) glucose. The medium pH was adjusted to 4.5 and it was sterilized at 121°C temperature in laboratory scale autoclave (Sanyo, Japan) for 15 minutes. A loopful of *P. nebrodensis* WC 850 spores was then transferred to moderate cool sterilized inoculum medium under sterile conditions in laminar hood (Dalton, Japan). Inoculated flask was incubated at 31°C for 9 days in shaker (Sanyo- Gallemkemp, UK) with continuous shaking (120 rpm) to obtain the spore number of 1×10^6 to 1×10^8 spores/mL (Kay-Shoemake and Watwood, 1996).

Screening experiments for best substrate selection: *P. nebrodensis* was grown on all six substrates. Experiments were conducted in triplicate using 250 mL Erlenmeyer flasks in a temperature controlled incubator (EYLA SLI-600ND, Japan). The flasks contained 5g of respective substrates were moistened with 10mL of the basal medium without glucose. Each flask was sterilized at 121°C in an autoclave for 15 minutes and inoculated with 5mL of freshly prepared inoculum medium. The inoculated flasks were kept at 30°C for 1-10 days in temperature controlled incubator to determine the suitable substrate with high enzymatic activity. After the end of stipulated fermentation time period, the experimental and control flasks were harvested by adding 100 mL of distilled water and kept in shaker for 30 minutes. Filtrates were then centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatants were collected carefully and stored in sterilized glass bottles for enzyme activity determinations.

Laccase enzyme assay: Enzyme activity of supernatant collected at the end of screening experiments and optimization steps was determined using a spectrophotometer (T 60, PG, Instruments, UK). Laccase enzyme was assayed by monitoring 2, 2 azinobis 3-ethyl-benzthiazoline 6 sulphonate (ABTS) oxidation in 50mM sodium malonate buffer at 420 nm (Wolfendon

and Wilson, 1982). The enzyme activity was expressed as U/mL.

Optimization of culture conditions for enhanced laccase enzyme production by RSM under central composite Design: Five different parameters including pH, temperature, inoculum size, incubation time and moisture level were optimized by advance optimization method, response surface methodology RSM by using statistical software DOE, 9.0 (Design of Experts, 9.0). Central composite design was used for the optimization and improvement of laccase enzyme production by *P. nebrodensis* with six center points = 0.5 and six replicates for each center point. The full experimental plan with regard to their values in actual form is provided in (Table 1). The data obtained from RSM on laccase production were subjected to analysis of variance. A second degree of quadratic polynomial equation was selected to estimate the response of dependent variables and then it was fitted to data by multiple linear regression

$$Y = {}_{0} + {}_{1}X_1 + {}_{2}X_2 + {}_{3}X_3 + {}_{4}X_4 + {}_{5}X_5 + {}_{11}X_{12} + {}_{22}X_2^2 + {}_{33}X_3^2 + {}_{44}X_4^2 + {}_{55}X_5^2 + {}_{12}X_1X_2 + {}_{13}X_1X_3 + {}_{14}X_1X_4 + {}_{15}X_1X_5 + {}_{23}X_2X_3 + {}_{24}X_2X_4 + {}_{25}X_2X_5 + {}_{34}X_3X_4 + {}_{35}X_3X_5 + {}_{45}X_4X_5$$

Where Y is predicted response, X_1,X_2, X_3, X_4, X_5 are independent variables ${}_{0}$ is intercept, ${}_{1}$, ${}_{2}$, ${}_{3}$, ${}_{4}$, ${}_{5}$ are linear coefficients ${}_{11}$, ${}_{22}$, ${}_{33}$, ${}_{44}$, ${}_{55}$ squared coefficients and ${}_{12}$, ${}_{13}$, ${}_{14}$, ${}_{15}$, ${}_{23}$, ${}_{24}$, ${}_{25}$, ${}_{34}$, ${}_{35}$, ${}_{45}$ are interaction coefficients

Table 1. Experimental Units of independent variables in actual and coded form.

Independent Variables	Coded Levels	
	(-1)	(+1)
pH	2	6
Temperature (°C)	20	40
Inoculum Size (mL)	2	6
Incubation Time (h)	48	240
Moisture	40	60

RESULTS

Selection of best substrate for Laccase enzyme production: *P. nebrodensis* WC 850 was grown on six different substrates including sugarcane baggase, corn stover, banana stalk, wheat straw, rice straw, wheat straw and corn cobs for ten days for production of laccase enzyme. After every 48 hours, triplicate flasks were harvested and examined for laccase enzyme activity. Increased laccase enzyme activity of 145.10 U/ mL noted on wheat straw after six days of incubation. Second best substrate for laccase enzyme production was banana stalk with enzyme activity 136.16 U/mL after eight days of fermentation (Table 2). Laccase is produced during secondary metabolism by white rot fungi. Wheat straw was taken as most excellent substrate for laccase enzyme production by *P. nebrodensis* WC 850 after screening experiments.

Table 2. Screening experiment for best substrate selection for laccase enzyme production by *Pleurotus nebrodensis*

Substrates	Enzyme activities U/mL Incubation Days				
	2	4	6	8	10
Corn Cobs	113.34±1.12	117.76±2.81	120.56±3.03	116.22±1.63	117.10±4.01
Corn Stover	112.68±2.63	113.88±3.01	116.24±3.21	113.32±2.13	114.44±3.34
Sugarcane Baggase	118.32±4.80	118.90±1.19	127.78±4.70	116.10±6.51	120.56±5.32
Banana Stalk	91.40±1.16	104.06±1.10	136.16±5.11	128.16±2.33	123.21±1.11
Wheat Straw	105.01±2.18	125.10±2.30	145.10±3.20	122.12±2.10	130.68±1.02
Rice Straw	105.48±2.03	100.34±4.80	101.04±2.63	104.02±1.02	108.40±6.10

Process optimization for maximum production of Laccase enzyme by Response surface methodology under central composite design: Wheat straw was used further for the optimization of solid state fermentation production process. Physical parameters including pH, temperature, inoculum size, incubation time and moisture were optimized through response surface methodology (RSM) under central composite design (CCD). The optimum laccase enzyme production was obtained on pH 5, temperature 30°C, inoculum size 5 mL (1×10^6 to 1×10^8) spores/mL, incubation time (144 h) and (50%) moisture level (Table 3). The enhanced laccase enzyme activity was 171 U/ mL after optimization.

ANOVA to check adequacy of the Model: Data obtained from response surface methodology was subjected to analysis of variance. Analysis of variance (ANOVA) results of the quadratic models are presented in Table 4. The results of RSM were then used to fit second order polynomial equation i.e.

$$Y= (150.28) + (2.40\ A) + (-2.85B) + (-3.85\ C) + (2.03\ D) + (3.94\ E) + (-0.64\ AB) + (0.068\ AB) + (6.23\ AD) + (-3.55\ AE) + (0.34\ BC) + (0.17\ BD) + (2.28\ BE) + (-1.87\ CD) + (0.94\ CE) + (-0.87\ DE) + (82.16\ A^2) + (-73.66B^2) + (-34.18\ C^2) + (36.88\ D^2) + (-29.08\ E^2)$$

The effect of interactions among different factors on laccase enzyme production was also studied by

using design of experts 9.0 (DOE 9.0). The large F- value of model 171.62 indicates that the model is significant (Table 4). The t and P values are mostly used to evaluate the significance of each coefficient. Greater t-value and lowered p-value indicate that the corresponding coefficients are highly significant. Linear terms including (A, B, C, D, E) interaction terms (AD, AE, BE, CD, CE, DE) and quadratic terms A^2, B^2, C^2, D^2, E^2 were significant with p-values<0.0001. Non significant value of lack of fit 0.8842 for laccase proved the model's uniformity. Coefficient of variation R^2 is used to determine the variability in examined response by tested factors and their mutual interaction. The concluded R^2 value of 0.9597 is in correspondence with actual R^2 value of 0.9968. The adjusted R^2 value is close to actual R^2 value. Moreover, the low value of coefficient of variation is 0.95% confirms the preciseness and variability of experiments. The regular signal to noise ratio marked the adequate precision. Standard deviation value is used to indicate the model compatibility with predicted response.

Table 3. Process optimization for enhanced Laccase enzyme production by *Pleurotus nebrodensis* WC 850 through response surface methodology under central composite design.

Runs	pH	Temperature (°C)	Inoculum size (mL)	Incubation Time (h)	Moisture (%)	Laccase activity (U/mL)
1	4	35	4	144	50	130.06
2	5	30	4	144	50	171.63
3	6	20	6	48	60	127.88
4	6	40	6	240	60	137.12
5	2	40	6	48	60	141.69
6	4	30	4	144	50	154.28
7	6	20	6	240	40	142.24
8	2	40	6	240	40	108.51
9	2	20	6	48	40	127.23
10	6	20	2	240	60	148.66
11	2	40	2	48	40	123.89
12	6	20	2	48	40	134.49
13	4	30	4	144	50	150.85
14	4	30	4	144	45	140.14
15	4	30	4	96	50	157.85
16	4	30	4	144	55	144.96
17	2	20	2	240	40	130.61
18	2	20	6	240	60	126.96
19	6	40	2	240	40	144.22
20	4	30	4	192	50	160.23
21	4	30	4	144	50	149.8
22	4	30	4	144	50	150.1
23	3	30	4	144	50	169.09
24	4	30	3	144	50	143.03
25	6	40	2	48	60	127.07
26	4	30	5	144	50	139.52
27	6	40	6	48	40	116.86
28	4	25	4	144	50	132.75
29	4	30	4	144	50	150.1
30	4	30	4	144	50	150.88
31	2	40	2	240	60	137.17
32	2	20	2	48	60	144.15

Table 4. Analysis of variance table for quadratic polynomial model for laccase enzyme production by *Pleurotus nebrodensis.*

Source	Sum of Squares	df	Mean Square	F- value	P Value Prob> F	
Model	6130.76	20	306.54	171.62	<0.0001	Significant
pH	95.04	1	95.04	53.21	<0.0001	
Temperature	134.08	1	134.08	75.07	<0.0001	
Inoculum size	244.57	1	244.57	136.93	<0.0001	
Incubation Time	67.69	1	67.69	37.90	<0.0001	
Moisture	256.53	1	256.53	143.63	<0.0001	
AB	6.64	1	6.64	3.72	0.0800	
AC	0.074	1	0.074	0.042	0.8422	
AD	620.63	1	620.63	347.48	<0.0001	
AE	201.71	1	201.71	112.93	<0.0001	
BC	1.84	1	1.84	1.03	0.3316	
BD	0.49	1	0.49	0.27	0.6121	
BE	83.22	1	83.22	46.59	<0.0001	
CD	55.84	1	55.84	31.26	0.0002	
CE	14.01	1	14.01	7.84	0.0173	
DE	12.23	1	12.23	6.85	0.0240	
A2	1053.91	1	1053.91	590.06	<0.0001	
B2	847.03	1	847.03	474.23	<0.0001	
C2	182.37	1	182.37	102.10	<0.0001	
D2	212.37	1	212.37	118.90	<0.0001	
E2	132.00	1	132.00	73.91	<0.0001	
Residual	19.65	11	1.79			
Lack of Fit	5.79	6	0.97	0.35	0.8842	not significant
Pure Error	13.86	5	2.77			
Cor Total	6150.41	31				

Regression Coefficient for laccase production by *Pleurotus nebrodensis* WC 850: A coefficient is measure of any specified process. The regression coefficient table indicated that the positive t value in case of pH, incubation time and moisture has a significant effect on enzyme production while temperature and inoculum size has not significant influence on enzyme production with negative t value. The regression coefficient for interaction among pH and incubation time, temperature and moisture and inoculum size and moisture were significant having p-value 0.000 while other interaction terms were not significant having p-value greater than 0.05 (Table 5). While the positive value of regression coefficient for quadratic terms like pH*pH and Incubation time*Incubation time indicates the existence of a minimum for these activities. The negative value of regression coefficients for quadratic terms like temperature*Temperaure, Moisture*Moisture and Inoculum size*Inoculum size indicated the existence of maximum of laccase enzyme activity as a function of a certain levels of these factors. Beyond this point, these factors have inhibitory influences.

Table 5. Estimated regression coefficients for laccase enzyme production by *Pleurotus nebrodensis* WC 850 in SSF of Wheat straw.

Term	Coefficient	SE Coefficient	T	P
Constant	150.28	0.34	442	0.000
pH	2.40	0.33	7.27	0.000
Temperature	-2.85	0.33	-8.64	0.001
Inoculum Size	-3.85	0.33	-11.66	0.080
Incubation Time	2.03	0.33	6.15	0.000
Moisture	3.94	0.33	11.94	0.000
pH*Temperature	-0.64	0.33	-1.94	0.080
pH*Inoculum size	0.068	0.33	0.21	0.841
pH*Incubation Time	6.23	0.33	18.87	0.000

pH*Moisture	-3.55	0.33	-10.76	0.060
Temperature*Inoculum Size	0.34	0.33	1.03	0.333
Temperature*Incubation Time	0.17	0.33	0.52	0.611
Temperature*Moisture	2.28	0.33	6.91	0.000
Inoculum size*Incubation Time	-1.87	0.33	-5.66	0.050
Inoculum size*Moisture	0.94	0.33	2.85	0.000
Incubation Time* Moisture	-0.87	0.33	-2.64	0.010
pH* pH	82.16	3.38	24.31	0.021
Temperature*Temperature	-73.66	3.38	-21.79	0.081
Inoculum size*Inoculum size	-34.18	3.38	-10.12	0.023
Incubation Time*Incubation Time	36.88	3.38	10.91	0.000
Moisture* Moisture	-29.08	3.38	-8.60	0.009

Interaction among variables: Effect of varying physical parameters and their interaction was investigated on laccase enzyme production through RSM and results have been described under the following subheadings. Comparable effects regarding two variables were elucidated by response surface plots. Three dimensional response surface plots were used to determine the interactive effect of experimental parameters on laccase enzyme secretion.

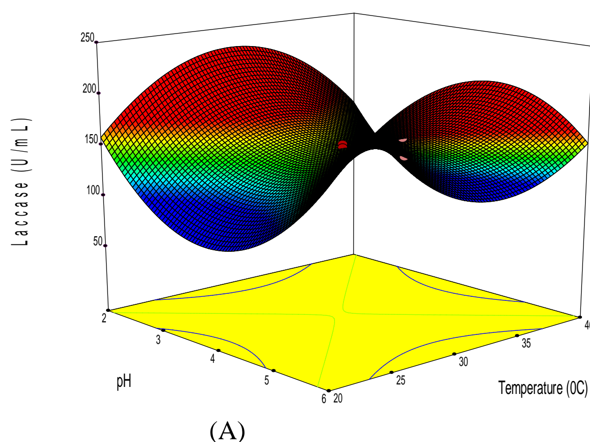
pH v/s Temperature: The 3D response surface curve shows how laccase enzyme production is influenced by pH and temperature (Fig 1 A). The optimum pH for laccase enzyme is 5 and optimum temperature for laccase enzyme production is 30°C. pH and temperature play an important role in enzyme production. Both these factors have antagonistic effect which means that with the increase in pH there is an increase in enzyme yield but the increase in temperature had an inhibitory effect on laccase enzyme production.

pH v/s Inoculum Size: The response surface curve showed maximum laccase activity at medium level of both factors (Fig 1 B). Response surface plot revealed that the pH range 4- 5 increased laccase enzyme activity but further increase in inoculum size caused an inhibitory effect. The optimum inoculum size was 4mL for laccase enzyme production.

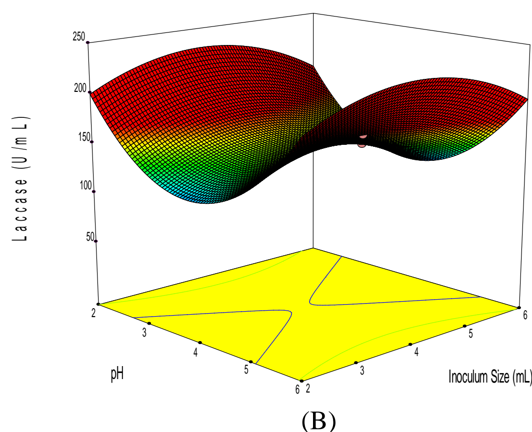
pH v/s Moisture: The 3 D surface graphs shows that the optimum yield of laccase obtained at pH 5 and 50 % moisture content (Fig 1 C). Both these factors have significant effect on laccase enzyme production which means that with the initial increase in pH and moisture level laccase enzyme yield increased.

pH v/s Incubation Time: The interactive effect of pH and incubation time (IT) indicated increase in enzyme production with the increase in pH and Incubation time (IT). The 3D response surface plot showed that at beginning level of pH (2-4) and high incubation time (192-240 h) the laccase activity was low. (Fig 1 D).

Temperature v/s inoculum size: Response surface plot revealed high yield of laccase at 30°C temperature and 4ml inoculum size but lower yield was observed at increased levels of these factors. Response surface graph showed that the temperature range (35-40°C) decreased synthesis of laccase enzyme (Fig 1 E).



(A)



(B)

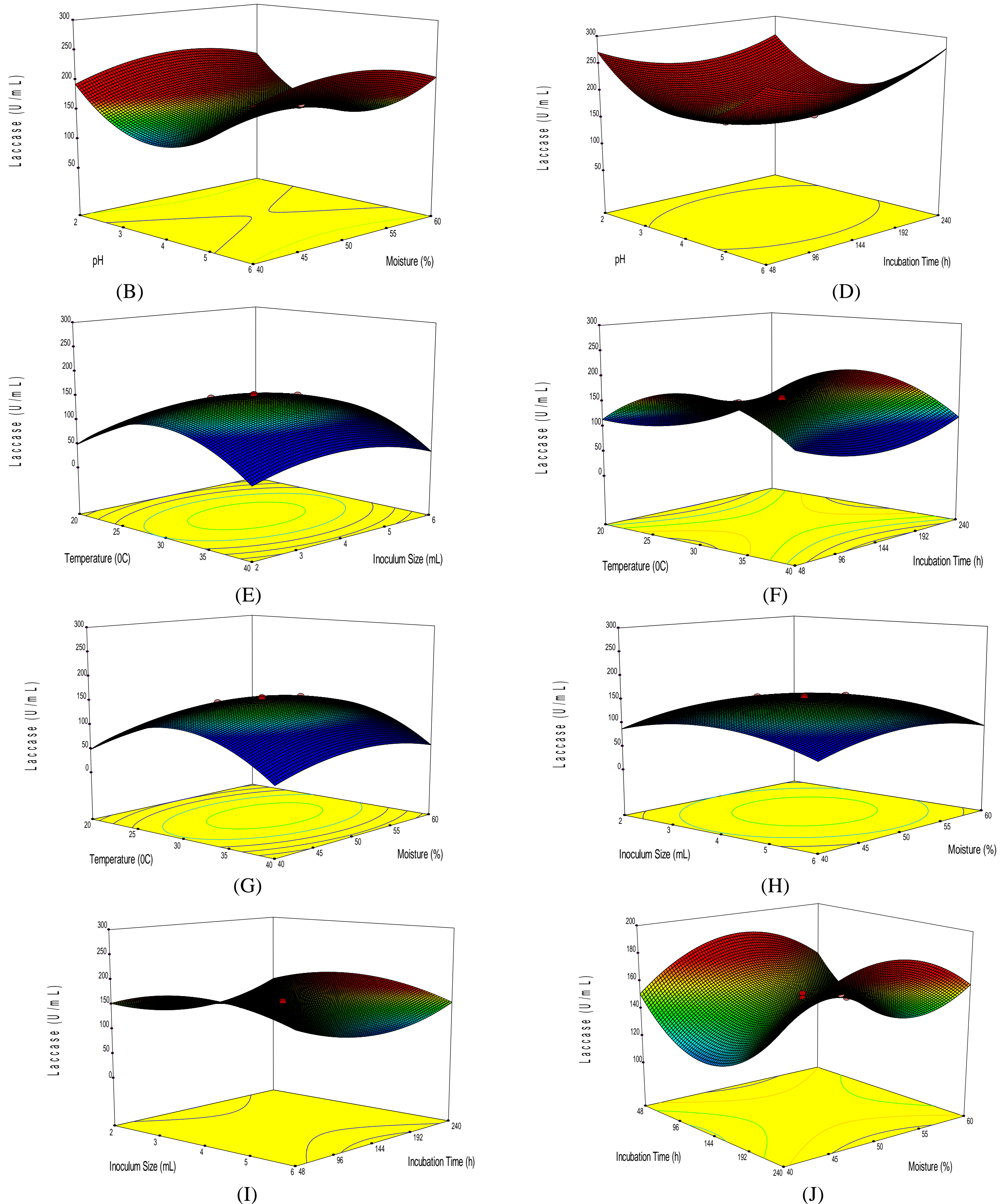


Fig 1. Three dimensional response surface graphs (Created by using DOE 9.0) for laccase enzyme production by *Pleurotus nebrodensis* showing the interactive effects (A) pH v/s Temperature (B) pH v/s Inoculum size (C) pH v/s moisture (D) pH v/s incubation time (E) Temperature v/s Inoculum size (F) Temperature v/s Incubation time (G) Temperature v/s Moisture (H) Inoculum size v/s Moisture (I) Inoculum size v/s Incubation Time (J) Incubation Time v/s Moisture

Temperature v/s incubation time: The saddle shape plot for laccase enzyme production showed the differential production of laccase at varied temperature and incubation time. Temperature and incubation time had antagonistic as well as synergistic effect on maximum enzyme production. The optimized temperature and incubation time (IT) for laccase secretion were 30°C and 144 h (Fig 1 F).

Temperature v/s Moisture: The production of laccase enzyme was affected by both factors. Combined effect of inoculum size and temperature play an important role in enhanced production of laccase enzyme. Temperature and moisture have strong interactive effect on laccase enzyme production. While higher levels of these factors have inhibitory effects on laccase enzyme production (Fig 1 G).

Inoculum size v/s Moisture: *P. nebrodensis* WC 850 revealed optimized laccase enzyme yield at balanced level of moisture i.e 50% and inoculum size 4mL. Additional rise in both levels results in lowered fungal growth and enzyme yield. The 3D surface plot showed that interaction among inoculum size and moisture had significant influence on laccase enzyme yield (Fig 1 H).

Inoculum size v/s Incubation time: 3 D surface plot showed that with the increase in inoculum size and incubation time laccase enzyme production increased but further increase in both factors had inhibitory effect (Fig 1 I). The interaction effect of inoculum size (IS) and incubation time (IT) was positive for laccase enzyme production. The three dimensional response surface plot is showing that the incubation time (IT) of 144 h is most effective in enhancing the yield of laccase enzyme.

Incubation Time v/s Moisture: The optimum yield of laccase can be obtained at incubation time 144 h and moisture level 50 %. (Fig 1 J). The graph shows that both factors play an important role on the secretion of laccase enzyme by *P. nebrodensis* WC 850. The graph also showed that at initial moisture level 45-55% growth of *P. nebrodensis* was high resulting in higher production of laccase enzyme in the solid state fermentation medium containing wheat straw.

DISCUSSION

In the present study the potential of an indigenous strain of *P. nebrodensis* for laccase enzyme production through solid state fermentation of lignocellulosic material was investigated. For this purpose, screening experiments were performed in order to select the best substrate suitable for laccase enzyme production. The maximum activity (145.10 U/mL) of laccase enzyme was observed when grown on wheat straw after six days of fermentation. Other findings of our research also revealed that varying culture processes play an important role in increased enzyme yield. The increase in enzyme activity (171.63 U/mL) was observed after optimization of different factors through RSM response surface methodology.

Varying levels of pH, temperature, inoculum size, incubation time and moisture were optimized by response surface methodology under central composite design to check their effect on laccase enzyme production. It was predicted by our results that the effect of pH is obvious and the amount of laccase increased with the initial increase in pH upto 5 but further increase in pH lowered the enzyme activity. High pH may affect the activity of enzyme due to conformational changes in enzyme structure (Tavares *et al.,* 2006; Kiran *et al.,* 2012; Vivekanadan *et al.,* 2014)). Response surface analysis showed that pH in range of 3.0- 5.0 has significant effect on increased laccase enzyme production by *Trametes versicolor* (Singh *et al.,* 2008). The optimized pH for laccase enzyme falls in the range 4.5-6.0 (Sivakumar *et al.,* 2010). The growth pattern of white rot fungus is tolerant to wide range of pH indicated that white rot fungus has flexibility to adapt according to different ecological conditions (Pandey *et al.,* 2001; Rinu *et al.,* 2012).

Temperature is also an important factor which play vital role in enhancement of enzyme activity. White rot fungi is capable of secreting the laccase enzyme under optimum conditions (Yasmeen *et al.,* 2013). Dhakar and Pandey, 2013 investigated that suboptimal temperatures 15- 35°C are suitable for enhanced laccase enzyme production. Our results are in close agreement with findings of Snajdar and Baldrain 2007 who reported maximum laccase enzyme activity by *Trametes versicolor* between 25 and 30°C. Yasmeen *et al.* (2013) reported that optimum temperature range of 25°C to37°C is best optimum range for the production of laccase enzyme. Optimum pH and temperature are crucial factors for the appropriate growth and metabolic action of microorganism (Couto *et al.,* 2006; Fatma *et al.,* 2010; Yasmeen *et al.,* 2013).

The growth of microorganism in solid substrate fermentation medium is also influenced by inoculum size. Low inoculum size is not suitable for growth of microorganism while large quantity of microorganism cause competitive inhibition (Sabu *et al.,* 2005). Large quantity of inoculum results in lowered metabolic activities of microorganism due to lack of nutrients (Patel *et al.,* 2009). Muhammad *et al.* (2012) found the lowered enzyme yield at higher and lower inoculum levels. In our case medium level of inoculum 4mL showed best laccase enzyme production by *P. nebrodensis* WC 850 when grown on wheat straw.

Moisture content is also an important factor to be considered for the growth of microorganism. According to our results moderate moisture level 50%

proved best for increased laccase enzyme production. High level of moisture content affects the substrate porosity and results in ceased oxygen transport leading to low enzyme yield (Chhaya and Modi, 2013). While low moisture content caused inhibition of microbial growth by limiting the access of white rot fungi to essential nutrients (Shaheen *et al.,* 2008; Chhaya and Modi, 2013).

Incubation time is also an important factor which affects the biosynthesis of fungus. Our results are in line with Nadeem *et al.* (2014) reported that maximum laccase activity in *Pleurotus ostreatus* after 6th days of incubation. Asgher *et al.* (2012) reported that the time taken by microorganism in order to produce laccase enzyme is due to long lag phase and primary metabolism. Wang *et al.* (2011) also reported the maximum activity of laccase enzyme after six days of incubation. Our current results suggest that response surface methodology is a unique and advance technique that not only considers one or more factors at one time but it also determines the interaction among different factors and their significant effect on increased laccase enzyme production in less time.

Conclusion: The production of laccase enzyme can be improved by optimization of different factors by response surface methodology using central composite design. All five parameters including pH, temperature, inoculum size, incubation time and moisture had significant effect on laccase enzyme production. It was observed that statistical optimization approach had helped to find out the most significant operating factors and optimum levels for laccase enzyme production by *P. nebrodensis*.

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