Optimization of Ligninolytic Enzymes Production through Response Surface Methodology

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There is an increasing demand for green chemistry technologies that can cope with environmental waste management challenges. Agro-industrial residues are primarily composed of complex polysaccharides that support microbial growth for the production of industrially important enzymes such as ligninolytic enzymes. *Schyzophyllum commune* and *Ganoderma lucidum* were used alone, as well as mixed/co-culture, to produce crude ligninolytic enzymes extracts using corn stover and banana stalk as a substrate during solid state fermentation (SSF). In the initial screening, the extracted ligninolytic enzymes from *S. commune* produced using corn stover as the substrate showed higher activities of lignin peroxidase (1007.39 U/mL), manganese peroxidase (614.23 U/mL), and laccase (97.47 U/mL) as compared to *G. lucidum* and the mixed culture. To improve the production of ligninolytic enzymes by *S. commune* with solid state fermentation (SSF), physical factors such as pH, temperature, moisture, inoculum size, and incubation time were optimized by varying them simultaneously using response surface methodology (RSM) with a central composite design (CCD). The optimum SSF conditions were (for a 5 g corn stover substrate size): pH = 4.5; temperature = 35°C; inoculum size = 4 mL; and moisture content = 60%. Under optimum conditions, the activities of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase were 1270.40, 715.08, and 130.80 IU/mL, respectively.

Keywords: Schyzophyllum commune; Ganoderma lucidum; Corn stover; Ligninolytic enzymes; Optimization; Response Surface Methodology (RSM)

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INTRODUCTION

Industry and biotechnology demands for ligninolytic enzymes complexes from white-rot fungi (WRF) are increasing due to their versatile catalytic effects on various industrial processes. Ligninolytic enzymes have potential applications in a large number of fields, including bioremediation, biofuels, food, agriculture, paper & pulp, textile finishing, denim stone washing, cosmetics, biosensors, and many others (Wesenberg *et al.* 2003; Pazarlioglu *et al.* 2005; Asgher *et al.* 2008; Levin *et al.* 2008; Sadhasivam *et al.* 2008; Stoilova *et al.* 2010; Asgher *et al.* 2011, 2012 a, b, c). The robust non-specific and extracellular lignin-degrading enzymes, such as lignin peroxidase (LiP), laccase (Lac), and manganese peroxidase (MnP), are responsible for lignin degradation and bioremediation capabilities of WRF (Yang *et al.* 2011; Martorell *et al.* 2012). However, problems may arise associated with the direct application of the fungi, such as difficulties in satisfying the growth requirements on a large scale, long incubation times, and

adsorption of the pollutants on fungal mycelia (Casas *et al.* 2009; Kamei *et al.* 2009). In contrast, *in vitro* treatment with ligninolytic enzymes produced through WRF fermentation can minimize these problems (Wang *et al.* 2008; Torres-Duarte *et al.* 2009). However, the use of these purified and/or immobilized enzymes increases the cost of industrial processes.

In the recent past, some efforts have been made to investigate the potential of cell-free culture extracts of WRF for *in vitro* large-scale industrial and bioremediation treatment of recalcitrant and xenobiotic compounds (Rubilar *et al.* 2008). The process control of these enzymatic applications is simplified by the absence of the living bacterium, and has advantages, such as the ability to operate over a wider range of pollutant concentrations, pH, and temperatures. In addition, the biodegradative capacity of the enzymes may be unaffected by toxic compounds that could otherwise inhibit fungal cultures (Tsutsumi *et al.* 2001). Moreover, using an unpurified crude enzyme extract may also provide the enzyme with substances that mediate the catalytic cycle of WRF peroxidases and laccases, which are eliminated during enzyme isolation and purification.

However, the fungus must secrete high activities of ligninolytic and associated enzymes in the enzyme extract. To achieve this, enzyme production must be optimized (Champagne and Ramsay 2005). The ligninolytic machinery in most basidiomycetes is highly regulated by nutrients, such as nitrogen, copper, and manganese. Their production is also affected by fermentation factors, such as medium composition, nature of carbon source, concentration of carbon source, pH of fermentation broth, fermentation temperature, amount and nature of nitrogen source, as well as the presence of inducers, mediators and organic acids, such as citric, oxalic, and tartaric acids (Ryan *et al.* 2007; Wen *et al.* 2009; Iqbal *et al.* 2011).

Reducing the costs of enzyme production by using cheaper raw materials and optimizing the fermentation process for industrial applications is the ultimate target of basic research (Lee *et al.* 2011; Soni *et al.* 2012). The conventional classical optimization strategy (COS) of varying one-factor-at-a-time is time consuming and cannot guarantee the optimum physical and nutritional conditions, since the strategy does not consider the interactions among different variables/parameters (Lotfy *et al.* 2007; Hye *et al.* 2008; Kammoun *et al.* 2008; Gadhe *et al.* 2011; Tijani *et al.* 2011). In contrast, response surface methodology (RSM) can examine varying more than one-factor-at-a-time, at several different levels, to determine the interactions between two or more factors. This testing methodology can provide reliable optimization results (Benzina *et al.* 2012).

Among the various processes used for enzyme production, solid-state fermentation (SSF),which uses lignocellulosic biomass, appears promising because it has many advantages for fungal cultivations (Pointing 2001). SSF is an attractive option for bioconversion of lignocellulosic biomass and production of lignocellulolytic enzymes. The enzymes can be isolated and purified to different extents for diverse industrial applications, and the residual biomass can be utilized as animal feed (Elisashvili *et al.* 2009; Sharma and Arora 2010). Since SSF operates under low moisture conditions, bacterial contamination chances are also minimized (Basu *et al.* 2002; Li *et al.* 2006). SSF reproduces the natural conditions for the growth of WRF and has been shown to be more suitable for the production of industrial enzymes that do not need purification.

The present study was aimed at producing enzyme extracts by growing single and co-cultures of *G. lucidum* and *S. commune* utilizing SSF of lignocellulosic substrates. A

further goal was to optimize the production of ligninolytic enzyme extracts by selected fungal culture using the selected lignocellulosic substrate in RSM.

EXPERIMENTAL

All experimental and analytical work was carried out at the Industrial Biotechnology Laboratory (IBL) of the Department of Chemistry and Biochemistry at the University of Agriculture, Faisalabad (UAF) in Pakistan. Two indigenous white rot fungi (WRF), *G. lucidum* and *S. commune*, were isolated and used as individual cultures, as well as in co-cultures, to produce extracts of ligninolytic enzymes. The fungal strains/cultures were screened on different substrates in a SSF to select the hyper-producing fungal culture on the basis of higher ligninolytic enzymes activities in the crude enzyme extract. The physical and nutritional parameters were studied with response surface methodology (RSM) to optimize the production of ligninolytic enzymes by the selected fungal culture in a SSF on selected substrates.

Production of Crude Ligninolytic Enzymes Extracts

Lignocellulosic substrates

Corn stover and banana stalk were collected from local vegetable and fruit markets of Faisalabad, Pakistan. Substrates were sliced into pieces, oven dried at 50ºC, ground to 40 mm mesh particle size, and stored in airtight plastic jars to avoid moisture re-adsorption.

Ligninolytic enzymes producing white rot fungi

Pure cultures of indigenous strains of *G. lucidum* and *S. commune* were obtained from the Industrial Biotechnology Laboratory of the Department of Chemistry & Biochemistry at the University of Agriculture in Faisalabad, Pakistan. The fungi were raised on potato dextrose agar (PDA) slants at pH 4.5. The inoculated slants were incubated for five days at 30ºC for spore multiplication and stored in a refrigerator at 4ºC for subsequent use in production of crude enzyme extracts.

Inoculum preparation

Kirk's basal nutrient media (100 mL) was the inoculum medium used (Tien and Kirk 1988). It was prepared in three separate labeled Erlenmeyer flasks (500 mL) and adjusted at pH 4.5 with 1 M NaOH or 1 M HCl. After sterilization for 15 min. at 121ºC, the medium was supplemented with Millipore filtered (0.3 µm) 1% glucose. Spores of *G. lucidum* and *S. commune* were added to the respective sterilized inoculum media from slant cultures, and the flasks were incubated in a temperature-controlled still culture incubator (EYLA SLI-600ND, Japan) at 30ºC for 5 to 7 days to obtain a homogenous spore suspensions of fungi (1×10^6-10^8 spores/mL) to use as inoculum. Fresh inoculum was prepared for each experiment (Asgher *et al.* 2006).

Screening of fungi for production of enzyme extracts on different substrates in SSF

Two separate sets of flasks for respective lignocellulosic substrates were prepared in triplicate using 5 g of the respective substrate moistened with Kirk's basal medium (Tien and Kirk 1988) at pH 4.5 and 60% (w/w) moisture. All the reactor flasks were autoclaved in a laboratory scale autoclave (Sanyo, Japan) and inoculated with 5 mL

homogenous suspension of each strain. The co-culture flasks received 2.5 mL inoculum each of the two fungi. The inoculated flasks were incubated at 30ºC for 10 days. The culture flasks were harvested at different time intervals (48, 96, 144, 192, 240 h). To the fermented SSF culture flasks, 100 mL of 100 mM sodium succinate buffer (pH 4.5) was added; afterwards, the flasks were shaken (120 rpm) in an orbital shaker (Sanyo-Gallemkemp, UK) for half an hour. The biomass was filtered through a Whatman No.1 filter paper, and the resulting filtrates were centrifuged at 3000 g for 10 min. at 4ºC to remove fungal mycelia and cell debris. The clear supernatants were used to determine the activities of LiP, MnP, and laccase.

Optimization of Physical Factors though Response Surface Methodology

The physical parameters including inoculum size, temperature, pH, moisture content, and incubation time were optimized through response surface methodology (RSM), using a five factor-five level central composite design (CCD) with six center points (α =0.5) and six replicates for each center point. A total of 32 runs were employed (Table 1). The center point replicates were chosen to verify any change in the estimation procedure, as a measure of precision property.

Once the experiments were performed, a second order polynomial equation (1) shown below was used to describe the effect of variables in terms of linear, quadratic, and cross product terms

$$Y = b_0 + \sum_{i=1}^{k} b_i Xi + \sum_{i=1}^{k} b_{ij} X_i^2 + \sum_{i_i<j}^{k} \sum_{j}^{k} b_{ij} X_i X_j + e \quad (1)$$

where i and j are linear and quadratic coefficients, respectively, while 'b' symbols represent regression coefficients, Y is the ligninase yield, k the number of factors studied and optimized in the experiment, and 'e' is random error. When developing the regression equation, the test factors were coded according to the following equation (2),

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \qquad i= 1,2,3,\ldots..,k, \quad (2)$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_o is the real value of the independent variable at the center point, and ΔX_i is the step change value.

Table 1. Five Factor-Five Levels Central Composite Design (CCD) Using RSM for the Optimization of Crude Ligninolytic Enzymes by *S. commune* in a SSF from Corn Stover

Independent Variables	Symbols	Levels	
		Low	High
pH	A	4	6
Temprature (°C)	B	25	45
Inoculum size (mL)	C	2	6
Moisture (% w/w)	D	40	80
Incubation time (h)	E	48	240

Ligninolytic Enzyme Assays

Lignin peroxidase activity

LiP was assayed by the method of Tien and Kirk (1983). The LiP assay was performed by using 2.6 mL of reaction mixture containing 1 mL buffer of pH 3, 1 mL of 4 mM veratryl alcohol (3,4-dimethoxybenzylalcohol), 500 μL of 1 mM H_2O_2, and 100 μL of the enzyme aliquot. A blank contained 100 μL of distilled water instead of enzyme aliquot. The UV/Vis absorbance was read after a 10-minute reaction interval at 310 nm (ε_{310} =9300 $M^{-1}cm^{-1}$). Enzyme activity was defined as μM of veratraldehyde formed per min.

Manganese peroxidase Assay

The activity of MnP was measured by the method of Wariishi *et al.* (1992). The assay mixture (2.6 mL) contained 1 mL of 1 mM $MnSO_4$, 1 mL of 50 mM sodium malonate buffer of pH 4.5, and 100 μL of the culture supernatant. Five hundred microliters of 0.1 mM H_2O_2 was added as an oxidizing agent. Manganic ions Mn^{+3} form a complex with malonate that absorbs at 270 nm (ε_{270} = 11,590 $M^{-1}cm^{-1}$). MnP activity was defined as μM of $MnSO_4$ oxidized per min.

Laccase assay

Laccase activity in the crude enzyme extracts was measured by the method of Shin and Lee (2000) by monitoring the oxidation 2,2-azinobis(3-ethylbenzthiazoline-6 sulphonic acid) (ABTS) in a reaction mixture containing 1 mL of 1 mM ABTS in 1 mL of 50 Mm malonate buffer (pH 4.5) and 100 μL of the culture supernatant. The reaction mixture was incubated at 25ºC and absorbance was taken at 436 nm after a10 min interval (ε_{436} = 36,000 $M^{-1}cm^{-1}$).

A blank contained 100 μL of distilled water instead of enzyme solution or culture supernatant. Laccase activity was calculated as change in absorbance of the assay mixture after a ten-minute reaction interval.

RESULTS

Screening of WRF Cultures for Production of Ligninolytic Enzymes Extracts

Two white rot fungi, *S. commune* and *G. lucidum*, and their co-culture were used to produce ligninolytic enzymes extracts in the SSF of corn stover or banana stalks over a 10-day fermentation period. In case of *S. commune*, the maximum activities of LiP (1007.39 U/mL), MnP (614.23 U/mL), and laccase (97.47 U/mL) were noted on the ninth day of incubation in corn stover medium. *G. lucidum* also showed optimum activities of ligninolytic enzyme after nine days of SSF with banana stalk (Table 2).

However, the fungal co-culture of both strains showed lower activities of ligninolytic enzymes as compared to the individual cultures. The lower ligninolytic activities produced by the co-culture were unusual because both fungi have almost similar growth conditions (Irshad and Asgher 2011). The individual fungi may have secreted some metabolites that are antagonistic to each other leading to their growth inhibition.

Table 2. Production of Ligninolytic Enzymes by Single and Co-culture of *S. commune* and *G. lucidum* on Different Lignocellulosic Substrates in a SSF *

Time period (days)	Enzyme activity (IU/mL)	CORN STOVER			BANANA STALK		
		S. commune	*G. lucidum*	Co-culture	*S. commune*	*G. lucidum*	Co-culture
1	Laccase	44.69±7.31	14.86± 6.57	18.18±10.9	15.20±5.47	5.29±4.47	5.31±1.98
	Mnp	256.4±14.7	113.5±17.1	44.07±10.9	65.80±7.40	52.77±11.5	163.4±23.7
	LiP	261.0±13.1	31.53±12.3	76.33±25.1	46.03±6.47	64.55±25.8	119.0±13.4
2	Laccase	51.62±09.7	34.53±15.3	35.05±9.09	24.16±12.0	7.30±3.33	21.01±5.12
	Mnp	273.4±12.6	155.8±10.2	76.91±23.6	100.6±13.4	101.9±13.2	220.4±20.2
	LiP	272.1±10.5	276.7±11.7	80.99±16.2	83.42±11.0	105.2±24.6	146.9±10.5
3	Laccase	51.7±05.43	53.30±11.1	21.75±6.72	29.91±9.14	16.10±3.51	26.47±2.83
	Mnp	330.1±13.2	283.2±23.4	101.4±14.2	261.4±18.2	162.7±18.6	272.5±25.7
	LiP	370.5±14.6	356.2±17.4	102.9±14.8	135.5±20.3	211.7±24.9	196.41±5.2
4	Laccase	61.06±10.3	55.44±10.1	21.85±6.17	37.12±9.38	19,54±5.00	35.92±3.20
	Mnp	360.1±19.0	303.6±20.5	1380±25.7	418.9±20.7	231.5±27.7	306.2±24.4
	LiP	379.4±14.4	434.3±23.1	115.1±12.9	186.4±8.18	333.7±21.7	249.0±30.2
5	Laccase	64.62±10.0	55.82±11.4	36.47±2.47	47.42±16.8	32.49±6.11	36.20±4.01
	Mnp	420.7±12.4	312.4±28.5	135.1±16.5	563.4±12.4	277.5±20.8	346.0±19.2
	LiP	460.3±19.1	525.3±26.1	126.5±13.1	211.9±20.8	416.7±18.5	289.4±16.2
6	Laccase	88.29±12.9	57.94±10.4	39.43±3.70	58.01±7.54	57.68±8.58	46.01±4.08
	Mnp	471.6±17.5	438.1±23.0	138.2±15.5	660.6±22.9	314.0±13.9	455.7±22.0
	LiP	524.3±23.3	523.6±24.1	154.4±17.4	430.7±21.2	621.1±20.7	346.9±18.1
7	Laccase	75.55±12.5	64.10±9.58	41.74±3.21	90.46±14.3	67.58±11.2	48.51±5.24
	Mnp	528.2±26.4	291.5±26.9	190.7±27.8	729.3±7.13	390.1±19.9	404.7±16.3
	LiP	681.2±19.4	522.9±21.6	177.7±11.7	597.2±15.1	750.8±26.9	305.7±20.3
8	Laccase	80.22±7.29	79.64±14.6	41.44±3.62	95.36±16.4	84.64±13.0	46.84±5.98
	Mnp	563.8±22.6	450.5±27.4	193.6±29.3	853.8±10.0	424.3±17.7	331.0±19.0
	LiP	831.7±20.4	516.0±22.6	144.4±14.1	632.0±17.2	827.3±21.7	258.4±21.7
9	Laccase	97.47±11.1	72.85±8.48	34.88±7.92	91.31±15.3	91.47±15.8	33.09±7.16
	Mnp	614.2±20.2	556.7±15.5	139.7±10.9	952.6±16.2	552.1±25.4	286.3±13.6
	LiP	1007±21.0	448.1±8.06	123.6±7.91	661.6±12.2	942.3±22.4	182.5±26.1
10	Laccase	85.92±10.3	76.77±7.02	36.47±2.90	87.44±6.85	86.18±9.24	31.94±2.59
	Mnp	590.5±8.10	458.6±20.2	118.5±17.6	902.0±16.7	495.4±16.8	241.9±20.1
	LiP	915.3±25.7	418.5±23.2	102.62±6.8	655.4±10.4	902.7±10.3	164.10±16.93

*pH 4.5; Temperature, 30°C

The genetic variation among the fungi, and the nature and composition of the substrates used may be responsible for the better growth of fungi on different substrates (Giardina *et al.* 2000; Patel *et al.* 2009). Overall, the crude enzyme extract produced by *S. commune* in the SSF of corn stover had maximum activities of ligninolytic enzymes. On the basis of these results, *S. commune* was selected for the optimization of ligninolytic enzymes produced by the SSF of banana stalks and corn stover.

Optimization of Parameters for Enhanced Production of Ligninolytic Enzymes by *S. commume*

Optmization of physical factors through response surface methodology (RSM)

RSM was employed with a central composite design (CCD). The independent variables (*i.e.* the main effects) were: inoculum size (A), temperature (B), pH (C), moisture (D), and incubation time (E). The optimum ligninase activities were obtained with: inoculum size, 4 mL; temperature, 35°C; pH 4.5; moisture, 60%; and incubation time, 144 h. The activities of lignin peroxidase (1270.40 U/mL), mangenese peroxidase (715U/mL), and laccase (130.8 U/mL) were substantially enhanced by optimizing the process conditions (Table 3). LiP, MnP, and laccase are secreted by different fungi in different activity profiles even under the same growth conditions. Some fungi secrete more LiP and MnP, and low laccase activity, and some even lack laccase altogether. Others are better laccase producers with low or even negligible LiP or MnP activities. The production of all three enzymes by a particular white rot fungus is optimum under similar physical growth conditions such as pH, moisture, and temperature, but their activities are different. However, the optimum conditions for synthesis of individual enzymes might be variable under different nutritional conditions.

The effects of interactions among the variables on ligninases activities (dependant variables) were also studied. The model's large F-values of 633.56, 178.83, and 11.74 for LiP, MnP, and laccase, respectively, indicated the model's significance. There was statistically 0.01% chance that this large F-value of model could occur due to noise (Table 4 A, B, & C). Linear (A, B, C, D, E), interaction (AB, AC, AD, BC, BD, BE, CD, CE, DE), and quadratic (A^2,C^2,D^2) terms were statistically significant with *p*-values< 0.0001. Non-significant F-values for lack-of-fit for LiP (1.50), MnP (0.46), and laccase (0.0090), relative to the pure error, confirmed the model's predictability. A positive value of the *t*-statistic in the case of inoculum size, temperature, moisture, and incubation time indicated a positive linear effect, whereas it was found to be negative for pH. The coefficient for the interaction between temperature and incubation time (BE) was significant with *p*-value of 0.003, while all other interactions were non-significant with *p*-values higher than 0.05. The negative coefficient value for pH in the linear term showed that the production of laccase decreased with an increase in pH. The positive quadratic term indicated the existence of a minimum for these activities.

Justification of the variability in observed response values by the experimental factors (variables) and their interactions was measured by R^2 (coefficient of determination). The predicted R^2 values of 0.8158, 0.8062, and 0.4921 by the model for LiP, MnP, and laccase were in close agreement with the actual R^2 values of 0.9976, 0.9909, and 0.4921, respectively. The adjusted R^2 was very close to the actual R^2 value. LiP, MnP, and laccase R^2 values of 0.9991, 0.9968, and 0.9950, respectively, implied that the fitted linear, interaction, and quadratic terms could elucidate 99, 99.68, and 99.50% of variation, showing satisfactory representation of the process by the model.

The preciseness and reliability of conducted experiments were confirmed by lower values of coefficient of variation (CV) of 0.54, 0.96, and 6.86% for LiP, MnP, and laccase, respectively. The measured signal-to-noise ratio indicated adequate precision. A desirable ratio should be larger than 4. All the model ratios (106.81, 58.626, and 15.124 for LiP, MnP, and laccase, respectively) were greater than 4, indicating adequate signals that can be used for design space navigation. The standard deviation values of 5.36, 4.08, and 5.32 for LiP, MnP, and laccase indicated that the model showed strong compliance with predicted response.

Table 3. Optimization of Fermentation Parameters using the RSM with a Central Composite Design (CCD)

Runs	Inoculum size (mL)	Temperature (◦C)	pH	Moisture (%)	Incubation Time (h)	LiP	MnP	Lac
						(U/mL)		
1	6.00	25.00	4.00	40.00	48.00	910.25	381.69	54.49
2	2.00	45.00	4.00	40.00	48.00	998.09	371.68	48.91
3	2.00	25.00	6.00	40.00	48.00	897.25	403.49	71.67
4	6.00	45.00	6.00	40.00	48.00	822.82	350.59	37.92
5	2.00	25.00	4.00	80.00	48.00	850.13	432.45	75.49
6	6.00	45.00	4.00	80.00	48.00	744.50	425.08	58.12
7	6.00	25.00	6.00	80.00	48.00	848.48	383.65	80.04
8	2.00	45.00	6.00	80.00	48.00	700.06	381.23	53.36
9	4.00	35.00	5.00	60.00	96.00	1002.78	473.55	69.38
10	3.00	35.00	5.00	60.00	144.00	932.11	429.09	74.71
11	5.00	35.00	5.00	60.00	144.00	932.04	418.57	79.83
12	4.00	30.00	5.00	60.00	144.00	1061.29	398.25	85.44
13	4.00	40.00	5.00	60.00	144.00	1041.43	390.18	81.66
14	**4.00**	**35.00**	**4.50**	**60.00**	**144.00**	**1270.40**	**715.08**	**130.8**
15	4.00	35.00	5.50	60.00	144.00	1141.28	514.89	102.5
16	4.00	35.00	5.00	50.00	144.00	1048.25	420.42	72.08
17	4.00	35.00	5.00	70.00	144.00	1020.82	434.88	76.48
18	4.00	35.00	5.00	60.00	144.00	1056.33	462.84	77.91
19	4.00	35.00	5.00	60.00	144.00	1042.48	452.56	92.63
20	4.00	35.00	5.00	60.00	144.00	1048.51	449.41	86.42
21	4.00	35.00	5.00	60.00	144.00	1050.04	450.32	89.02
22	4.00	35.00	5.00	60.00	144.00	1046.29	451.97	72.86
23	4.00	35.00	5.00	60.00	144.00	1051.93	452.66	79.61
24	4.00	35.00	5.00	60.00	192.00	1102.87	480.64	83.80
25	2.00	25.00	4.00	40.00	240.00	1079.93	391.83	71.72
26	6.00	45.00	4.00	40.00	240.00	1013.87	325.55	70.80
27	6.00	25.00	6.00	40.00	240.00	1055.61	426.74	83.14
28	2.00	45.00	6.00	40.00	240.00	1058.10	432.68	79.53
29	6.00	25.00	4.00	80.00	240.00	1027.51	380.89	94.54
30	2.00	45.00	4.00	80.00	240.00	1078.76	411.51	96.47
31	2.00	25.00	6.00	80.00	240.00	1019.65	445.99	88.60
32	6.00	45.00	6.00	80.00	240.00	1049.22	411.37	87.16

Interaction among variables

Comparative effects of any two variables were explained by contour plots while holding the other factors fixed at their central point values. From response surface (3D) and contour plots (2D), the interactive effects of experimental factors on ligninolytic enzymes synthesis were determined.

Inoculum size vs.temperature

The response surface curves (Fig. 1) showed how LiP, MnP, and laccase production was a function of inoculum size and temperature by keeping the levels of pH, moisture, and incubation time at 4.50, 60%, and 144 h, respectively.

Table 4A. Analysis of Variance (ANOVA) for the Quadratic Polynomial Model for Lignin Peroxidase Production in SSF

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	3.646E+005	20	18230.15	633.56	<0.000significant
A-A	2666.24	1	2666.24	92.66	< 0.0001
B-B	3299.29	1	3299.29	114.66	< 0.0001
C-C	4301.47	1	4301.47	149.49	< 0.0001
D-D	17109.47	1	17109.47	594.62	< 0.0001
E-E	1.672E+005	1	1.672E+005	5811.86	< 0.0001
AB	2487.27	1	2487.27	86.44	< 0.0001
AC	10601.28	1	10601.28	368.43	< 0.0001
AD	3966.80	1	3966.80	137.86	< 0.0001
AE	53.47	1	53.47	1.86	0.2001
BC	1564.00	1	1564.00	54.35	< 0.0001
BD	946.64	1	946.64	32.90	0.0001
BE	4156.70	1	4156.70	144.46	< 0.0001
CD	450.18	1	450.18	15.65	0.0023
CE	2939.54	1	2939.54	102.16	< 0.0001
DE	12818.20	1	12818.20	445.48	< 0.0001
A^2	35013.99	1	35013.99	1216.86	< 0.0001
B^2	1.98	1	1.98	0.069	0.7979
C^2	27734.33	1	27734.33	963.87	< 0.0001
D^2	634.24	1	634.24	22.04	0.0007
E^2	13.86	1	13.86	0.48	0.5021
Residual	316.51	11	28.77		
Lack of Fit	203.44	6	33.91	1.50	0.3367 not
Pure Error	113.07	5	22.61		significant
Cor Total	3.649E+005	31			

Table 4B. ANOVA of CCD for the Quadratic Polynomial Model for Manganese Peroxidase Production in SSF

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	56622.87	20	2831.14	169.83	<0.0001significant
A-A	2200.79	1	2200.79	132.02	< 0.0001
B-B	1206.19	1	1206.19	72.36	< 0.0001
C-C	801.03	1	801.03	48.05	< 0.0001
D-D	2308.09	1	2308.09	138.46	< 0.0001
E-E	609.03	1	609.03	36.53	< 0.0001
AB	16.56	1	16.56	0.99	0.3403
AC	0.65	1	0.65	0.039	0.8473
AD	126.11	1	126.11	7.57	0.0189
AE	501.98	1	501.98	30.11	0.0002
BC	59.91	1	59.91	3.59	0.0846
BD	748.84	1	748.84	44.92	< 0.0001
BE	4.37	1	4.37	0.26	0.6188
CD	1815.61	1	1815.61	108.91	< 0.0001
CE	5585.32	1	5585.32	335.05	< 0.0001
DE	110.25	1	110.25	6.61	0.0260
A^2	1745.56	1	1745.56	104.71	< 0.0001
B^2	7847.45	1	7847.45	470.75	< 0.0001
C^2	10463.04	1	10463.04	627.65	< 0.0001
D^2	1277.52	1	1277.52	76.64	< 0.0001
E^2	1798.14	1	1798.14	107.87	< 0.0001
Residual	183.37	11	16.67		
Lack of Fit	65.62	6	10.94	0.46	0.8112 not significant
Pure Error	117.75	5	23.55		
Cor Total	56806.24	31			

Table 4C. ANOVA of CCD for the Quadratic Polynomial Model for Laccase Production in SSF

Source	Sum of Squares	df	Mean Squares	F Value	p-value Prob> F
Model	6599.70	20	329.98	11.67	<0.0001significant
A-A	17.47	1	17.47	0.62	0.4485
B-B	483.41	1	483.41	17.09	0.0017
C-C	6.34	1	6.34	0.22	0.6451
D-D	841.02	1	841.02	29.73	0.0002
E-E	2404.16	1	2404.16	85.00	< 0.0001
AB	52.56	1	52.56	1.86	0.2001
AC	5.93	1	5.93	0.21	0.6560
AD	61.70	1	61.70	2.18	0.1677
AE	20.66	1	20.66	0.73	0.4110
BC	118.48	1	118.48	4.19	0.0654
BD	5.625E-003	1	5.625E-003	1.989E-004	0.9890
BE	393.43	1	393.43	13.91	0.0033
CD	109.20	1	109.20	3.86	0.0752
CE	0.073	1	0.073	2.577E-003	0.9604
DE	3.57	1	3.57	0.13	0.7290
A^2	98.07	1	98.07	3.47	0.0895
B^2	5.097E-004	1	5.097E-004	1.802E-005	0.9967
C^2	961.00	1	961.00	33.97	0.0001
D^2	213.99	1	213.99	7.57	0.0189
E^2	120.51	1	120.51	4.26	0.0634
Residual	311.14	11	28.29		
Lack of Fit	30.28	6	5.05	0.090	0.9946not significant
Pure Error	280.86	5	56.17		
Cor Total	6910.84	31			

The production of lignin-modifying enzymes was affected by both factors and was maximized by the combination of the level of inoculum size and temperature. It was observed that inoculum size and temperature had strong interactive effect on production of lignin-modifying enzymes (LMEs) by *S. commune.* LiP, MnP, and laccase showed analogous results with maximum activities with 4 mL of inoculum at 35°C. The interaction of temperature and inoculum size showed that there is an increase in MnP and laccase production with an initial increase in temperature and inoculum size; however, high levels of these factors had inhibitory effects.

Inoculum size vs. pH

Countour and response surface plots (Fig. 2) showed that there was high production of LiP, MnP, and laccase at 4 mL of inoculum size and pH 4.5, but low yields at higher levels of these parameters. The contour plot for all lignin-modifying enzymes predicted that a pH range of 4 to 4.5 and inculum level of 2 to 4 mL was optimum. Response surface plot predicted that a pH range of 5 to 5.5 decreased the synthesis of the enzymes.

Inoculum size vs.moisture

At a moderate level of moisture (60%) and inoculum size (4 mL), *S. commune* showed optimum production of LMEs (Fig. 3). A further increase in both variables decreased the growth of fungus, and hence, the production of enzymes in the SSF. At

their initial levels, both inoculum size (2 to 4 mL) and moisture (56 to 64%) showed a profound effect on crude enzymes production. Conversely, at higher levels of these independent variables, the net LMEs yield decreased. Interaction of inoculum size and moisture had a significant effect on LiP, MnP, and laccase production in SSF by *S. commune* using corn stover.

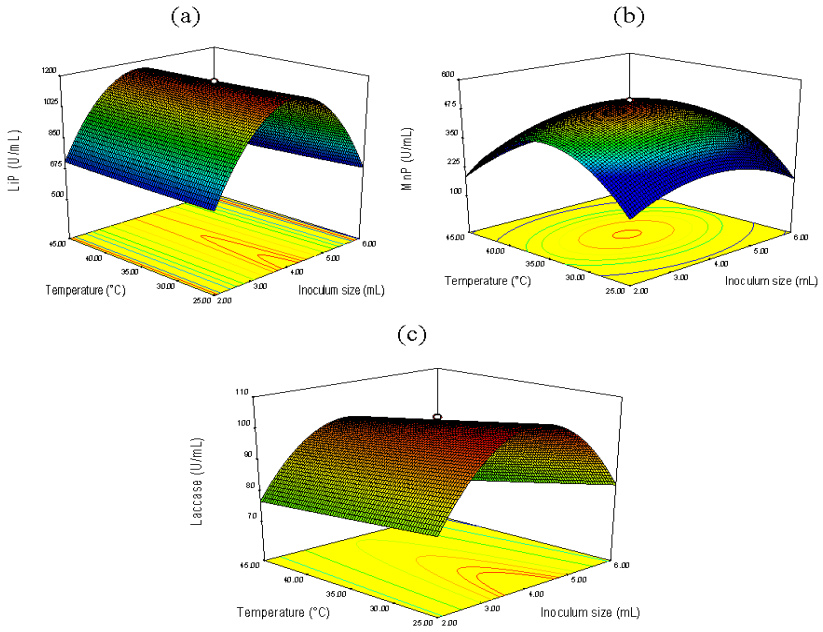

Fig. 1. Response surface plots showing the interactive effect of inoculum size and temperature on (a) LiP, (b) MnP, and (c) laccase production (hold value: pH, 4.5; moisture, 60%; and incubation time, 144 h)

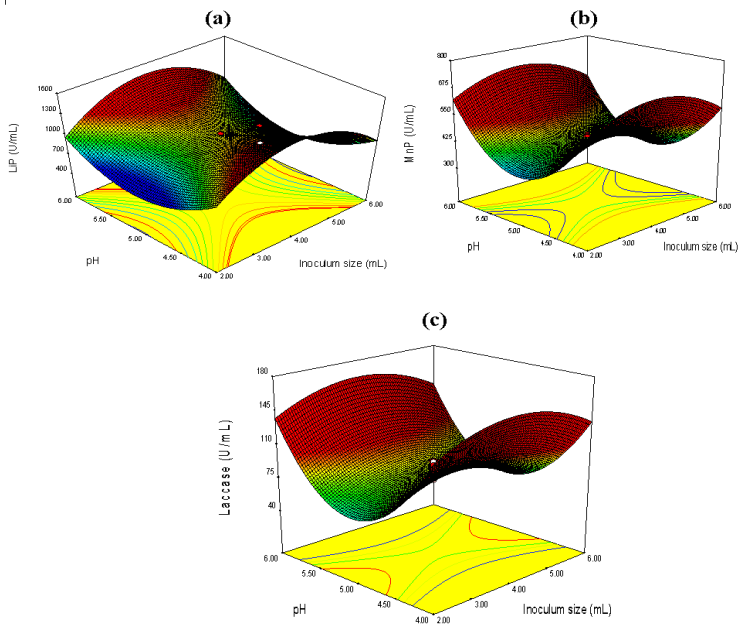

Fig. 2. Response surface plots showing the interactive effect of inoculum size and pH on (a) LiP, (b) MnP, and (c) laccase production (hold values: temperature, 35°C; moisture, 60%; incubation time, 144 h)

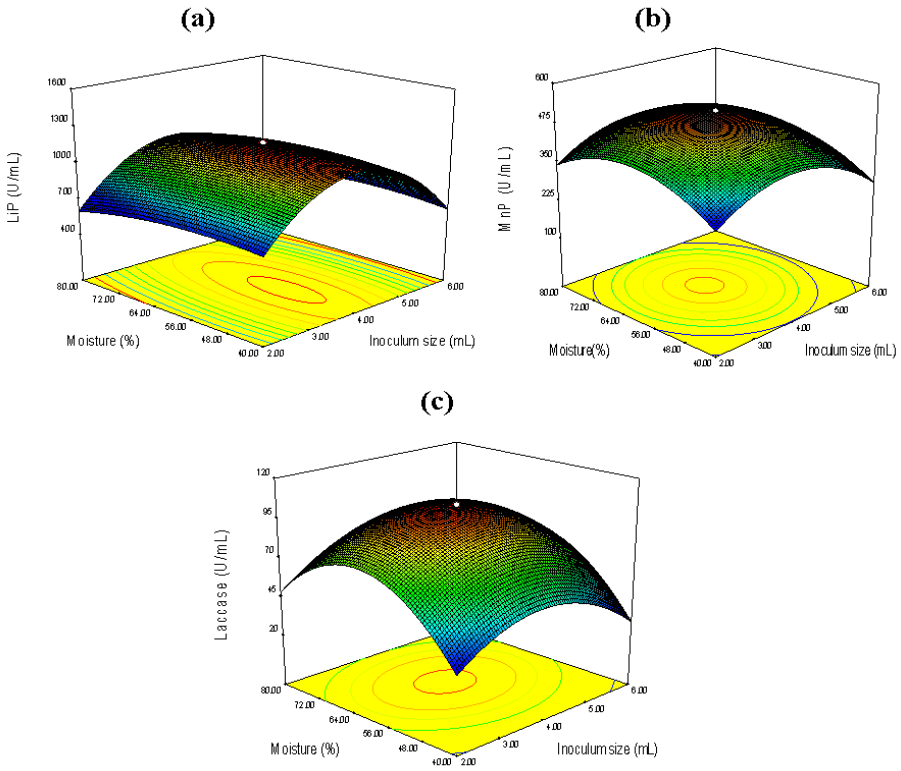

Fig. 3. Response surface plots showing the interactive effect of inoculum size and moisture on (a) LiP, (b) MnP, and (c) laccase production (hold value: temperature, 35°C; pH, 4.5; incubation time, 144 h)

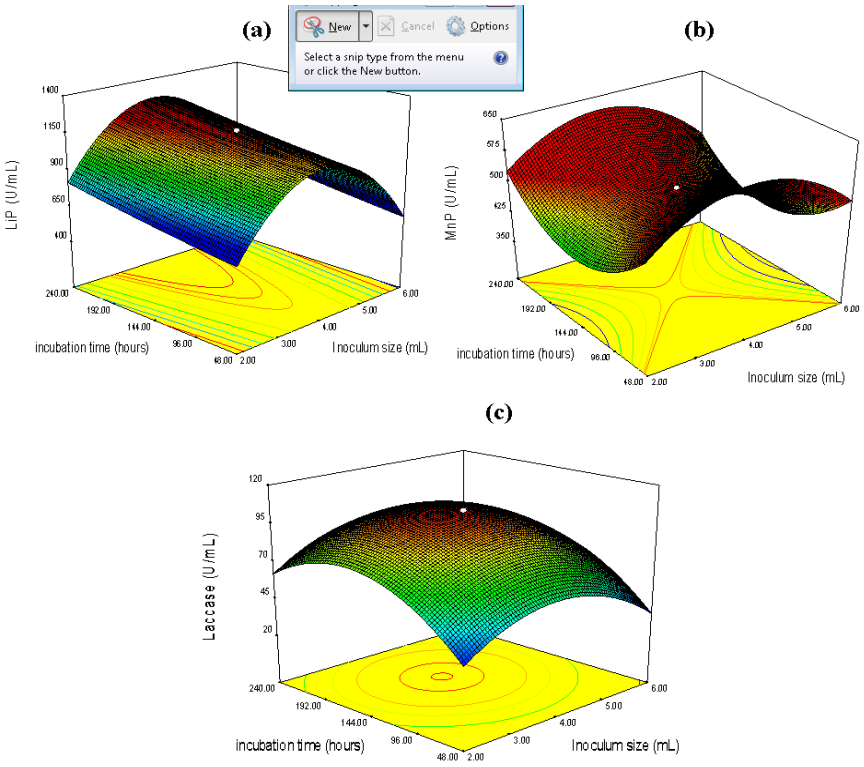

Fig. 4. Response surface plots showing interactive effects of inoculum size and incubation time on (a) LiP, (b) MnP, and (c) laccase production (at temperature, 35°C; pH, 4.5; moisture 60%)

Inoculum size vs. incubation time

As the incubation time and inoculum size were increased, the lignin-modifying enzymes (LiP, MnP, and laccase) production also increased to a certain extent; however, a further rise in both factors caused the inhibtion of enzymes synthesis by *S. commune* (Fig. 4). Inoculum size and incubation time interaction effect was accountable for this action. The response surface plot showed that incubation time ranging from 92 to 192 h was most effective in increasing the yield of LMEs. The contour plot predicted a strong interaction among the two variables for production of enzymes by *S. commune* in the SSF of corn stover.

Temperature vs. pH

While considering the pH and temperature, the saddle shape plots for LME production (Fig. 5) suggested the differential production of LiP, MnP, and laccase at varied pH and temperatures. On maximizing enzyme production, pH and temperature had antagonistic, as well as synergistic effects. The optimum temperature and pH for LMEs production were found to be 35°C and pH 4.

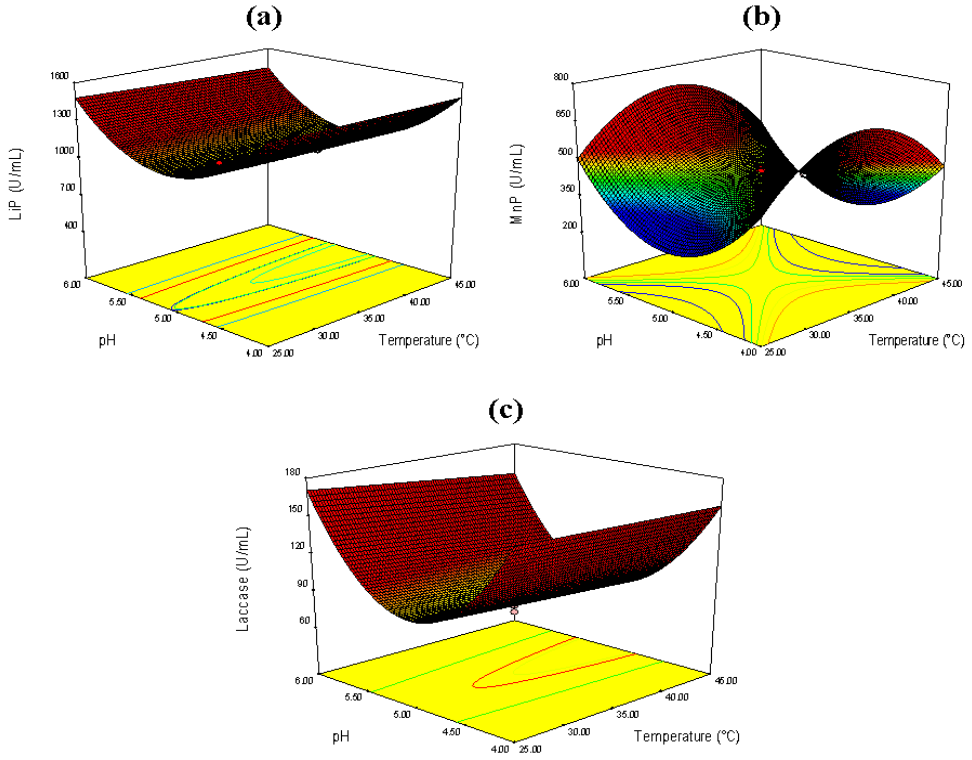

Fig. 5. Response surface plots showing the interactive effect of temperature and pH on (a) LiP, (b) MnP, and (c) laccase production (hold value: moisture, 60%; inoculum size, 4 mL and incubation time 144 h)

Temperature vs. moisture

Response surface plots (Fig. 6) showed that low temperature and high moisture enhanced LMEs yield; however, at higher moisture levels (above 64%), enzyme production decreased. Maximum activities of LiP, MnP, and laccase were obtained when the level of moisture and temperature was 60% and 35°C, respectively, while the hold values for inoculum size, pH, and incubation time were 4 mL, 4.5, and 144 h, respectively.

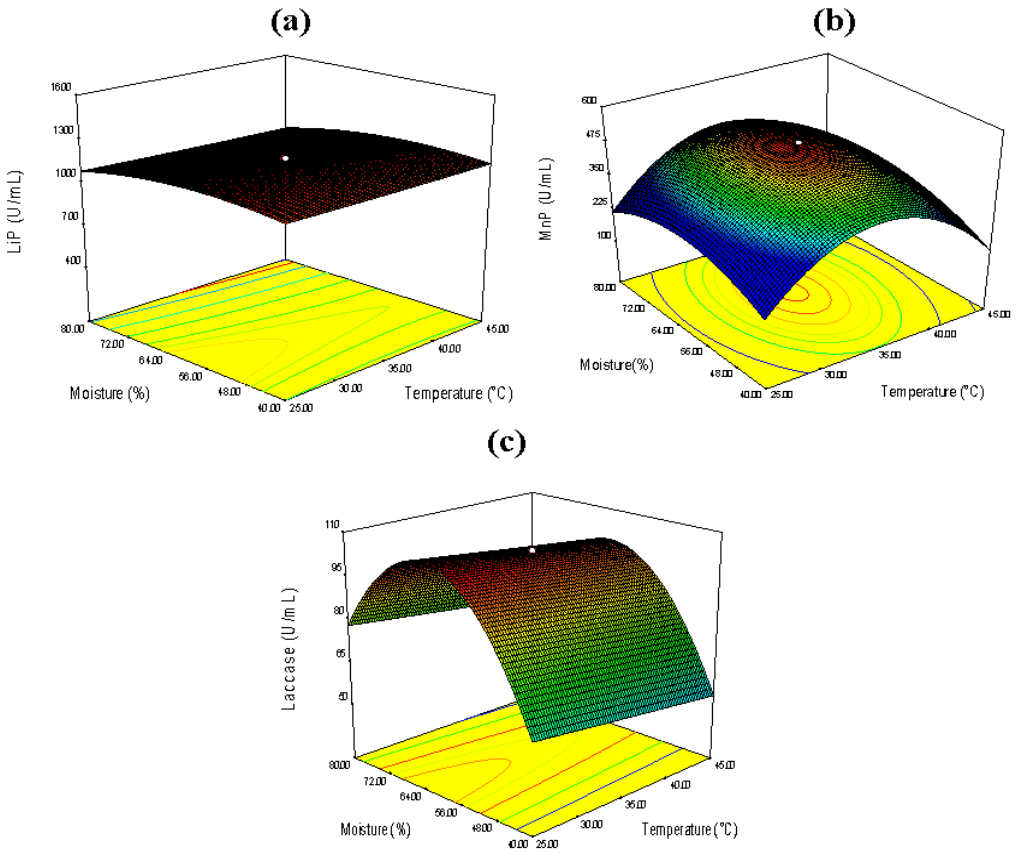

Fig. 6. Response surface plots showing the interactive effect of temparature and moisture on (a) LiP, (b) MnP, and (c) laccase production (hold value: inoculum size, 4mL; pH, 4.5; incubation time, 144 h)

Temperature vs.incubation time

The interaction between incubation time and temperature for the production of LiP, MnP, and laccase showed that an increase in temperature and incubation time enhanced enzyme yield; however, at higher temperatures (above 35°C), enzyme activity was low (Fig. 7). Optimum LiP, MnP, and laccase predicted yield was 1170.4, 515, and 103.8U/mL, respectively. The response surface graph predicted that at initial incubation time (48 to 96 h) and temperature (25 to 30°C), as well as high incubation time (192 to 240h) and temperature (40 to 45°C), the LMEs activity was low.

pH vs. moisture

Figure 8 shows the effect of pH and moisture on LME production. At the mid-range values for moisture content (56 to 64%) and pH (4 to 4.5), the optimum LiP, MnP, and laccase yield could be attained. From the contour plot, it could be noted that the optimum yield of LiP (1270.40 U/mL), MnP (715.08 U/mL), and laccase (130.80 U/mL) was obtained at pH 4.5 and 60% moisture content; the interaction among these two factors was significant.

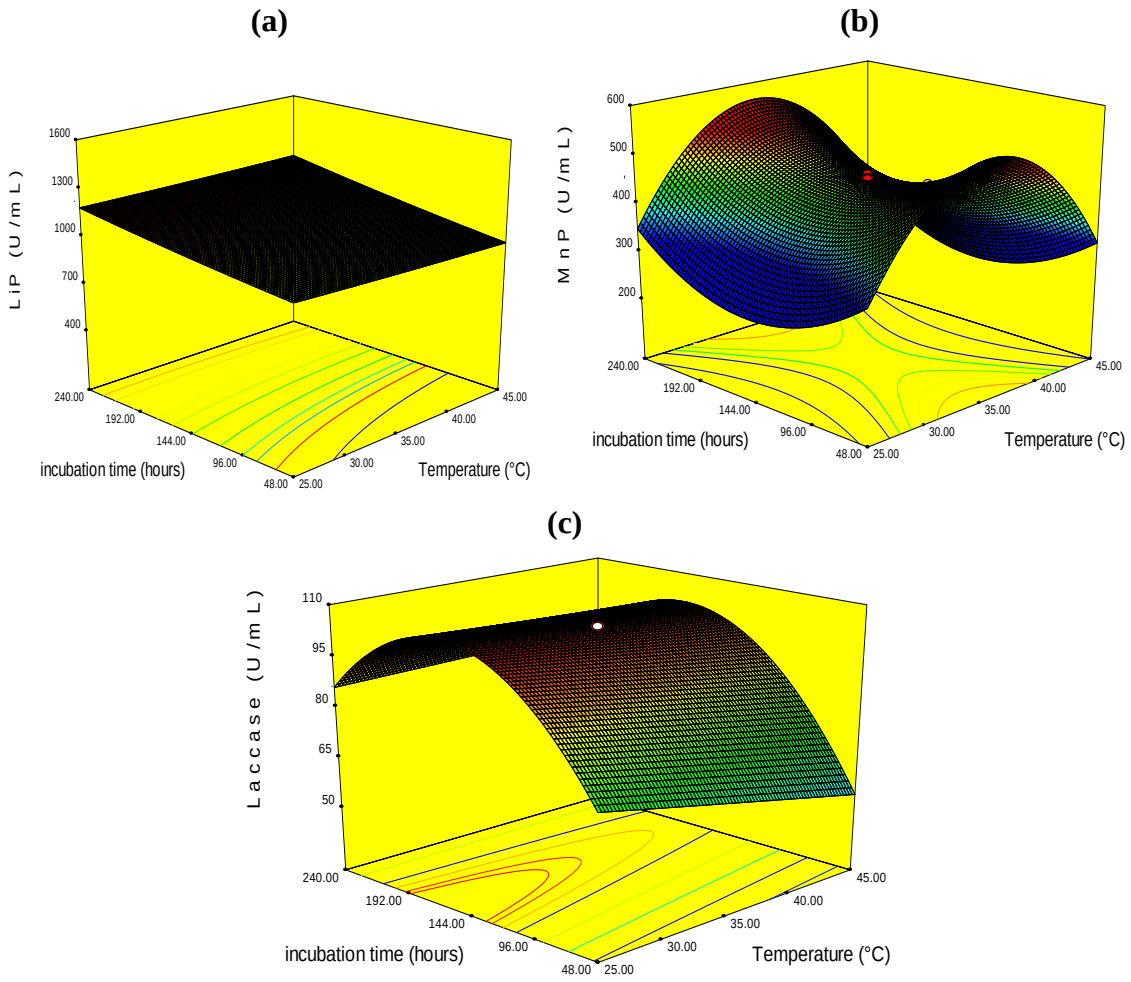

Fig. 7. Response surface plots showing the interactive effect of temperature and incubation time on (a) LiP, (b) MnP, and (c) laccase production (hold value: inoculum size, 4 mL; pH, 4.5, and moisture, 60%)

pH vs.incubation time

Figure 9 shows a response surface plot of pH against incubation time at fixed levels of the other three factors. The graph predicted that both factors had strong interactive influence on the growth of *S. commune* in the SSF medium and subsequent production of LiP, MnP, and laccase at their highest levels. Response surface plot showed that LME activity was high at incubation period of 96 to 144 h and pH 4 to 4.5. The optimum pH and incubation time for the maximum production of LMEs were 4.5 and 144 h, respectively, as shown in both plots.

Moisture vs.incubation time

Figure 10 shows the interaction between moisture and incubation time for the production of LiP, MnP, and laccase. The response surface graph showed that incubation time of 144 to 192 h was efficient for fungus cultivation and optimum yield of crude LMEs in the SSF with corn stover. The graphs showed that both factors played a significant role in the secretion of LMEs by *S. commune*. Optimum LME production was at 60% moisture after 6 days of incubation time; and at initial (40 to 48%) and higher (72

to 80%) level of moisture, the growth of *S. commune* was low and subsequent low production of crude LMEs was observed.

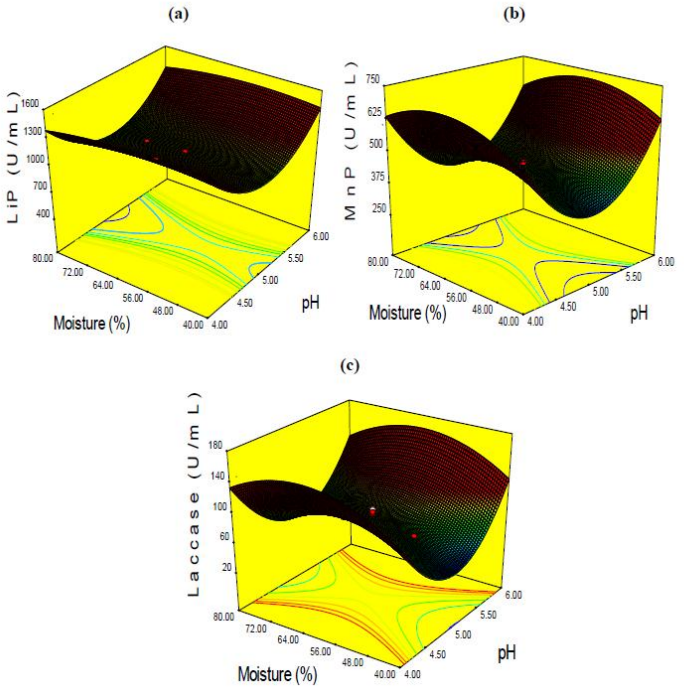

Fig. 8. Response surface plots showing the interactive effect of pH and moisture on (a) LiP, (b) MnP, and (c) laccase production (hold value: inoculum size, 4 mL; temperature, 35°C and incubation time, 144 h)

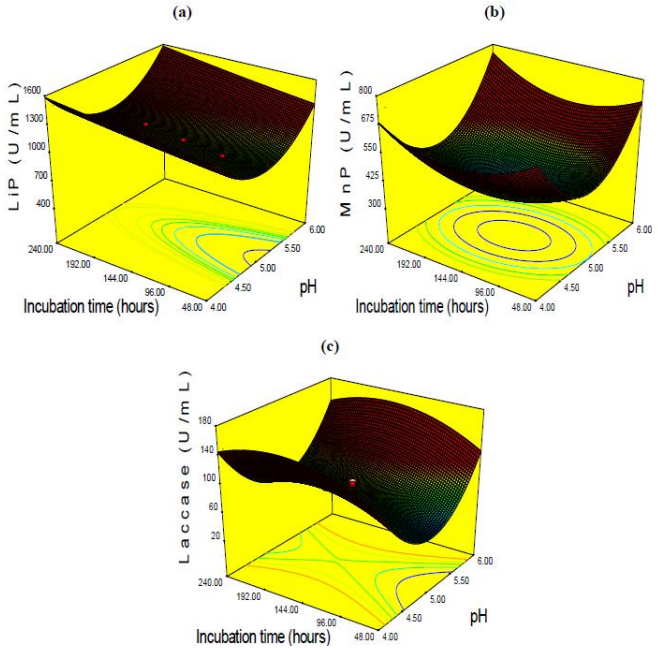

Fig. 9. Response surface plots showing interactive effect of pH and incubation time on (a) LiP, (b) MnP, and (c) laccase production (at: inoculum size, 4 mL; temperature, 35°C; moisture, 60%)

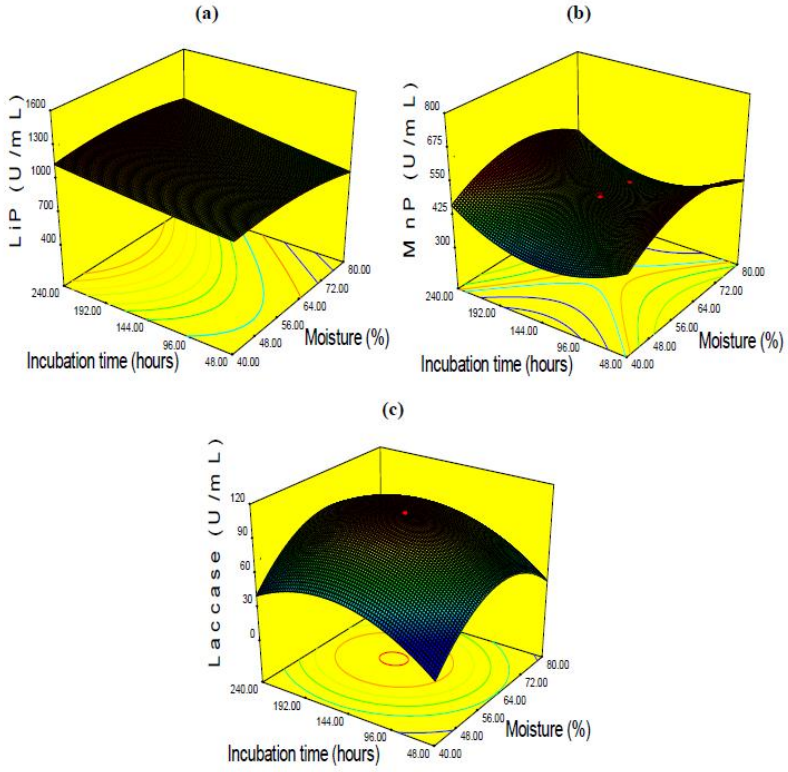

Fig. 10. Response surface plots showing the interactive effect of moisture and incubation time on (a) LiP, (b) MnP, and (c) laccase production (hold value: inoculum size, 4 mL; temperature, 35°C; pH, 4.5)

DISCUSSION

The culture conditions had significant influence on LMEs production. Maximum LME yield was obtained under the optimal conditions of inoculum size (4 mL), temperature (35°C), pH (4.5), moisture (60%), and incubation time (144 h (*i.e.* 6 days)). The highest LME activities of LiP, MnP, and laccase achieved were 1270.40, 715.08, and 130.80 U/mL, respectively, under these optimum conditions by *S. commune* in SSF of corn stover agro-substrate. The most significant factors for white rot reproduction growth and LME production in the SSF are oxygen supply, inoculum size, pH, incubation time, moisture, and temperature (Barlev and Kirk 1981). Among the factors that strongly influence metabolic activity and WRF strains growth were medium composition, moisture level, solid substrate (inert carrier), initial pH and temperature levels, and inoculum size (Couto *et al.* 2006). In order to initiate the growth of microorganism a lower level of inoculum may not be adequate, whereas competitive inhibition could be caused by higher level of inoculum (Sabu*et al.* 2005). *Phellinus robustus* (Songulashvili *et al.* 2006) and *Schizophyllum commune* (Bhatti*et al.* 2008; Li and Jia 2008) have been reported as good producers of LMEs. The synthesis of LMEs depends on growth conditions, nutritional factors, surfactants, and inducer/mediators (Praveen *et al.* 2011).

Enzyme production was decreased with a further increase in inoculum due to the depletion of nutrients, and consequentially, the decrease in metabolic activities of the

fungus (Patel *et al.* 2009). Moisture content is a crucial factor in any SSF process because this variable influences growth and biosynthesis of the microbe, as well as the secretion of different metabolites such as enzymes. At low or high water content beyond its optimal requirement, the production of LMEs is considerably affected. Optimization of moisture content could be employed for the management and the modification of microorganism metabolic processes (Pandey *et al.* 1994). Inoculum size and moisture, apart from their influence on the production of enzymes, also play a vital part in determining the ultimate morphological composition of fungal culture medium (Olsvik *et al.* 1993). Hence, for economical enzyme production, it is of immense importance that conditions should be optimized (Ustok *et al.* 2007). Galhaup and Hatrich (2001) and Asgher *et al.* (2006) reported that 2 and 5 mL of conidial suspension showed maximum LiP production with the SSF of lignoocellulosic substrates. Sung-Kwon *et al.* (2008) also reported that crude enzyme production decreased in the case of *P. chrysosporium* immobilized cultures with an increase in the inoculum level. Solid substrate porosity decreased at an elevated level of moisture, which eventually inhibits oxygen transport and leads to reduced yields of LMEs (Lonsane *et al.* 1985; Bhatti and Nawaz 2009). On the other hand, microbial growth is inhibited at very low moisture content, which leads to poor accessibility of WRF to nutrients present in the growth medium (Pandey 1992; Shaheen *et al.* 2008). LiP, laccase, and MnP production is favored at low moisture levels by degrading lignocellulosic agricultural waste substrates (Levin *et al.* 2008). In earlier studies (Iqbal *et al.* 2011), the maximum LME production was attained from rice straw when 5 mL of freshly prepared fungal spore suspension was added into the fermentation medium.

In any SSF method, a vital factor is the incubation time because it has strong effect on growth and biosynthesis of the WRF, as well as the production of various primary and secondary metabolites, such as enzymes (Vaithanomsat *et al.* 2010; Lee *et al.* 2011). Shinichi *et al.* (1996) reported that an incubation period of six days was optimum for LiP, MnP production, and a further increase of incubation time showed decreased enzyme activity. During the SSF of a lignocellulosic substrate, *Achraszapota*, by *Phanerochaete chrysosporium*, the LiP activity (2100 U/L) peaked on the seventh day of cultivation (Zahamatkesh *et al.* 2010). Our work is in accordance with Heinzkill *et al.* (1998) who observed that maximum MnP activity was obtained at pH 4.5, temperature 37°C, and incubation time of six days. Laccase and MnP high activities in the SSF medium were obtained after four and eight days, respectively, by cultivating *Datronia* sp. (Vaithanomsat *et al.* 2010). The time taken by WRF strains for the production of LMEs depends on the length of the lag phase and the primary metabolism (Zadrazil and punia 1995; Asgher *et al.* 2012). *Termitomyces clypeatus* was found to produce maximum LMEs by utilizing lignocellulosic substrates after an incubation period of six days (Bose *et al.* 2007). In line with our findings, high LME yields were obtained after an incubation period of five days by the cultivation of *T. versicolor* in a SSF medium utilizing sugarcane bagasse (Pal *et al.* 1995; Winquist *et al.* 2008). After an incubation period of six days, the maximum activities of LiP were obtained from *P. chrysosporium* and *S. ostrea*, followed by a declining trend (Baldrian and Snajdr 2006; Songulashvili *et al.* 2006; Praveen *et al.* 2011).

WRF showed enhanced growth and secretion of LMEs at medium temperature conditions (Toh *et al.* 2003). For optimum production of LME by various WRF, the temperatures optima of 25 to 37°C range have been reported (Zadrazil *et al.* 1999; Arora and Gill 2001; Tekere *et al.* 2001; Tripathi *et al.* 2008). Optimal pH and temperature are

important for development of microorganism and its metabolic action (Fatima *et al.* 2010). In SSF a crucial role is played by the initial pH and incubation temperature since it is not feasible to monitor and control medium pH due to the lack of free water and to control temperature due to heat and mass transfer issues. Consequently, in SSF, the successive regulation of pH and temperature is not possible. Asgher *et al.* (2006) studied optimum crude ligninolytic enzymes production in a SSF medium containing corncobs by white rot fungus *P. chrysosporium* after five days at pH 4 and 40°C. Low activity of LMEs at high pH and temperature is attributed to the fact that both factors may alter the three-dimensional structure of the enzymes (Kiran *et al.* 2012). Cheng *et al.* (2007) reported that *Schyzophyllum* sp. F17 grown on a SSF medium of pinewood produced optimum crude LMEs at a temperature of 35°C. Mishra *et al.* (2011) reported that a pH in the range of 5.0 to 7.0 and temperature in the range of 30°C to 35°C had a significant effect on laccase production. The yield of laccase was significantly affected by pH and temperature when the moisture content was 70%. Optimum yield of crude LMEs was obtained by *Phanerochaete sordida* in a SSF medium containing a lignin-extracted milled wood and a pH of 4.5 to 5.0 (Ruttimann *et al.*1993). The most appropriate conditions for the production of LiP and MnP are 32°C and ~4.5 pH, and optimum activities of both enzymes are produced under these conditions (Couto *et al.* 2006). Wen *et al.* (2010) reported the optimum pH of 4.8 for manganese peroxidase production from *P. chrysosporium*. Similar to our findings, the optimum pH of 4 and 5 has been reported for the maximum crude LME yield by *Coriolus hirsutus* and *Trametes villosa*, respectively (Yamanaka *et al.* 2008).

CONCLUSIONS

Schyzophyllum commune IBL-05 produced substantial activities of lignolytic enzymes in its enzyme extract when grown on corn stover in solid state fermentation. The production process could be further improved by optimizing different factors through RSM using CCD. The high activities of enzymes produced by *Schyzophyllum commune* IBL-05 under optimum conditions, suggests the possibility of commercialization of the ligninolytic enzymes production process through SSF.

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