RESEARCH ARTICLE

Biodegradation of agrowastes by lignocellulolytic activity of an oyster mushroom, *Pleurotus sapidus*

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Abstract: Lignocellulosic biomasses derived from dedicated crops and agro-industrial waste materials are promising renewable resources for the production of fuels and other value added bio-products. The production of ligninolytic and cellulolytic enzymes from different lignocellulosic agricultural wastes by *Pleurotus sapidus* WC 529 was investigated in the current study. The production pattern of the enzymes was examined during the growth of the organism for a period of 10 days, and the enzyme activities were expressed in units/mL. Cultivation in the solid-state culture of banana stalk (BS) gave rise to higher levels of laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) activities compared to the cellulolytic enzymes. Response surface methodology (RSM) was adopted to optimise the culturing conditions for maximum enzyme secretion. Optimal conditions yielding the highest enzyme activities were: initial pH, 4; temperature, 35 °C; moisture level, 60 %; inoculum size, 4 mL, and incubation time, 120 hours. The crude lignocellulolytic enzyme extract presented potential efficiency for the delignification of different lignocellulosic substrates within 48 hours. The results suggested the feasibility of lignocellulolytic enzyme production using cost effective agro-industrial residues that can be effectively used for lignin biodegradation.

Keywords: Lignin degradation, lignocellulolytic activity, optimisation, *Pleurotus sapidus*, response surface methodology, solid-state fermentation.

INTRODUCTION

Lignocellulose is a macromolecular complex comprising lignin, cellulose and hemicellulose. Lignin is composed of heteropolymers resulting from the oxidative coupling of three *p*-hydroxycinnamyl alcohol monolignols: *p*-coumaryl, coniferyl and sinapyl alcohols. The recalcitrance of lignin against biodegradation is due to its bulky and aromatic nature that limits the accessibility of microbial enzymes and decreases its biodegradability. Therefore, the degradation of lignin is the key step to lignocellulose transformation (Silva *et al*., 2014).

White-rot basidiomycetous fungi are the principal organisms that possess remarkable potential to decompose and degrade wood lignocellulose (including recalcitrant lignin) efficiently. The extracellular enzymes, lignin peroxidase (LiP EC 1.11.1.14), manganese peroxidase (MnP EC 1.11.1.13), versatile peroxidase (VP EC 1.11.1.16) and laccase (EC 1.10.3.2) secreted by white-rot fungi (WRF) are considered to be crucial for biodegradation of lignin and lignocelluloses (Munir *et al*., 2015). LiP is able to oxidise various aromatic compounds, while MnP oxidises Mn^{+2} almost exclusively to Mn^{+3}, which in turn degrades phenolic compounds. Laccases are blue copper-containing oxidases that reduce molecular oxygen to water and oxidise phenolic compounds. Similarly, the conversion of delignified cellulosic biomass to fermentable sugars requires the synergistic action of cellulolytic enzymes, including endo-1, 4- *β*-glucanase (EC 3.2.1.4; endo-cellulase), cellobiohydrolase (CBH, EC 3.2.1.91; exo-cellulase), and β-glucosidase (EC 3.2.1.21) (Ferreira *et al*., 2009; Goyal & Soni, 2011; Mtui, 2012).

Compared with direct application of WRF, enzymatic treatment appears potentially better due to shorter treatment time, absence of lag phase, reduced sludge volume and ease of controlling the process. Further, the use of purified and/or immobilised ligninolytic enzymes raises the cost of industrial processes (Bilal & Asgher, 2015). The crude fermented enzyme extract may also provide some accessory enzymes and mediators that

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mediate the catalytic cycle of WRF peroxidases and laccase that are otherwise removed during enzyme purification (Asgher *et al*., 2013a). There are several benefits of using WRF lignocellulolytic enzyme extracts for delignification of plant biomass:

i. The ligninolytic enzyme extracts from WRF also contain other accessory enzymes and mediators required for the action of these enzymes for complete degradation of lignin.

ii. The enzyme extracts may also contain cellulase enzymes that can simultaneously hydrolyse the exposed cellulose fibers of de-lignified biomass.

The present study focused on the elevated production of lignocellulolytic enzymes by *P. sapidus* utilising different agricultural waste substrates. The enzymes were subsequently employed for the biodegradation of lignin.

METHODOLOGY

Biomass and chemicals

Lignocellulosic biomass such as, wheat straw (WS) and rice straw (RS) were collected from Students Research Farms, University of Agriculture, Faisalabad, Pakistan; corn stover (CS) and corncobs (CC) were collected from Rafhan Maize Products Co. Ltd., Faisalabad, Pakistan; and sugarcane bagasse (SCB) and banana stalk (BS) were procured from the local fruit market and Crescent Sugar Mills, Faisalabad, Pakistan, respectively. The raw materials were milled (less than 5 mm mesh), washed, air dried, and stored in air tight containers at room temperature. All the chemicals and reagents were procured from Sigma-Aldrich (USA) and Merck (Germany) through local distributors and suppliers.

Organism and inoculum preparation

A pre-screened pure culture of *P. sapidus* WC 529 strain was obtained from Dr Nasir Ahmad Khan, Fungal and Mushroom Laboratory, Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. Fungal inoculum was prepared by growing the fungus using a rotary shaker (Sanyo Gallenkemp, UK) at 150 rpm and 30 °C, in a 250 mL flask filled with 100 mL of Kirk's basal medium containing: 10.0 g/L glucose; 0.2 g/L ammonium tartrate; 0.21 g/L KH_2PO_4; 0.05 g/L $MgSO_4.7H_2O$; 0.01 g/L $CaCl_2$; 0.001 g/L thiamine; 10 mL Tween 80 (10 %); 10 mL 100 mM veratryl alcohol; and 10 mL trace element solution; initial pH 4.5 (Yasmeen *et al*., 2013). After 5 days of cultivation, mycelia were homogenised using a Waring laboratory blender.

Solid-state fermentation for enzyme production

Solid-state fermentation (SSF) of designated biomasses was performed in triplicate fermentation flasks (250 mL) containing 5 g of lignocellulosic substrate, moistened with 10 mL of the standard basal medium. Prior to sterilisation in autoclave (Sanyo Labo Autoclave, Japan), pH of the medium (WTW pH-meter; InoLab pH 730) was maintained at 4.5. About 5 mL of *P. sapidus* spore suspension was used to inoculate the flasks containing lignocellulosic substrates (wheat straw, corncobs, corn stover, rice straw, banana stalk and sugarcane bagasse). The inoculated flasks were placed in still culture incubator at 30 °C for 10 days. After every alternate day, a sterile sample (1 mL) from each flask was taken and analysed for enzyme activities (Yasmeen *et al*., 2013).

Enzyme activity assays

Ligninolytic enzymes

LiP enzyme was assayed spectrophotometrically using the method reported by Tien and Kirk (1988) using veratryl alcohol as the substrate at 310 nm ($έ_{310}$ = 9300). The activity of MnP was monitored by following the methodology of Wariishi *et al*. (1992) by using H_2O_2 as the reaction initiator at 270 nm. Laccase activity was assessed by determining the oxidation of 2, 2 azinobis 3-ethylbenzthiazoline 6 sulphonate (ABTS) at 436 nm (Wolfenden & Willson, 1982).

Cellulolytic enzymes

Endo-1, 4-ß-glucanases was assayed using carboxymethyl cellulose (CMC) as the substrate and 3, 5-dinitrosalisylic acid (DNS) as the coupling reagent by the method of Gadgil *et al*. (1995). Exo-1,4-ß-glucanase activity was determined using 1.0 % salicin as the reaction substrate with DNS as the coupling reagent (Gielkens, 1999). ß-glucosidase was assayed according to Gadgil *et al*. (1995) using 1.0 % avicel as the assay substrate.

Optimisation of culture conditions

Response surface methodology (RSM) with central composite design (CCD) was employed to determine the effect of each parameter on enzyme activities. The independent parameters like pH, temperature, inoculum size, incubation time and moisture were used in the range of 2.0 − 6.0, 20 − 40 ºC, 1.0 − 5.0 mL, 24 − 120 hours and 40 − 60 %, respectively. A total number of 15 runs were carried out to estimate the coefficients for the production of lignocellulolytic enzymes. The data were subjected to analysis of variance (ANOVA) and 3D response

surface graphs were constructed using DOE 8.0.7.1 trial version software to study the responses and interactions between the variables. The quality of the fit of this model was expressed by the coefficient of determination (R^2) in the same programme. The quadratic model equation for predicting the response function, i.e. optimum ligninolytic and cellulolytic activities (U/mL) was expressed using second order polynomial according to equation 1 (Yasmeen *et al.*, 2013).

$$Y(x) = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{j=2}^{k} \sum_{i=1}^{j-1} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ij} x^2 + e \qquad ...(1)$$

where *i* and *j* are linear and quadratic coefficients, respectively, while *b* represents regression coefficient, Y is the ligninolytic or cellulolytic yield, *k* is the number of factors optimised in the experiment and *e* is the random error.

Delignification of substrates and lignin determination

Different volumes (5, 7.5, 10, 12.5, 15.5 mL) of the ligninolytic extract in combination with Na-malonate buffer (50 mM, pH 4.5) was applied at 35 °C for different time periods to degrade lignin. The percentage of lignin before and after treatment was calculated from each substrate according to Asgher *et al*. (2013a; b). The sample was dissolved in 10 mL (25 % w/v) of acetyl bromide in glacial acetic acid (GAA ≥ 99 %) by heating at 70 °C ± 2 °C for 30 min. After 30 min, the dissolved sample was transferred to a volumetric flask (200 mL) containing a mixture of acetic acid and caustic soda in 1:1 ratio. The sample was diluted to 15 mL with 99 % acetic acid, and the absorbance was read at 280 nm.

Statistical analysis

The experiments were performed in triplicate and the data obtained was subjected to ANOVA to study the interaction between variables and the responses (enzyme activities). Tukey's honestly significant difference test was used to compare the means when statistical differences were observed at p < 0.05. CoStat computer package (version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analyses.

RESULTS AND DISCUSSION

Different lignocellulosic substrates; wheat straw, corn cobs, corn stover, rice straw, banana stalk and sugarcane bagasse, were evaluated for ligninolytic and cellulolytic enzyme activities of the oyster mushroom *P. sapidus*. Among the substrates investigated the highest ligninolytic enzyme activities (LiP, MnP and laccase) were detected in banana stalk on the 4th day after inoculation through solid-state fermentation (SSF). The crude extract contained 228.2, 153.4 and 59.2 U/mL of laccase, MnP and LiP, respectively (Table 1). The detected enzyme activities were in agreement with earlier reports (Kalmis *et al.,* 2008; Singh *et al.,* 2012) highlighting that *P. ostreatus*, *P. eryngii* and *P. sajor caju* exhibit considerable laccase and MnP activities with only traces of LiP. The literature survey revealed that a large number of microorganisms, including bacteria, fungi and yeast produce lignin degrading enzymes (LDEs) but WRF, particularly *Pleurotus* species and *Phanerochaete chrysosporium* generally exhibit the most desirable quantities (Munir *et al.*, 2015). However, the selection of a particular strain remains a tedious task, especially when commercially competent enzyme yields are needed to be attained; and it depends upon a number of factors, in particular upon the composition and nature of the lignocellulosic substrate and environmental conditions. The selection of a suitable substrate for enzyme production in a SSF process depends upon the cost and availability of the substrate, and thus may involve screening of several agro-industrial residues (Yasmeen *et al.*, 2013).

In the case of cellulases, the maximum enzyme activities were recorded in fermented culture of banana stalk *i.e.,* endoglucanase; (0.158 U/mL), exoglucanase (0.098 U/mL) and ß-glucosidase (0.118 U/mL) (Table 2)*.* Tellez *et al.* (2008) and Singh *et al.* (2012) have encountered very low concentrations of cellulolytic enzymes from all strains of *Pleurotus*. Among those, *P. florida* has been reported as the best cellulolytic enzyme producer exhibiting 0.48, 0.102 and ß-glucosidase, respectively (Goyal & Soni, 2011). In a previous study, banana fruit stalks have been confirmed to be excellent solid substrates for the production of cellulolytic enzymes by *Neurospora sitophila* at optimised growth conditions of pH 5.0 and 35 °C (Asad *et al.*, 2006).

Optimisation of lignocellulolytic enzymes

Response surface methodology (RSM) is a powerful statistical approach, which is widely used to optimise different factors for enzyme production during fermentation processes (Kotzamanidis *et al.*, 2013). In comparison to the laborious one-factor optimisation at a time strategy, RSM is regarded as a more time saving and cost effective method. The application of this methodology is expected to provide the optimal values of

Table 1: Production of ligninolytic enzymes by *P. sapidus* cultivated on different lignocellulosic substrates in SSF[*]

Substrates	Enzymes	Enzyme activity after incubation (days) (U/mL)				
		2	4	6	8	10
WS	Lac	168 ± 2.72 [a]	193 ± 3.27 [b]	179.3 ± 2.49 [c]	199.1 ± 2.16 [d]	213.25 ± 3.13 [e]
	MnP	118 ± 1.46 [a]	116 ± 2.94 [a]	142.1 ± 3.64 [b]	136.7 ± 1.33 [bc]	125.2 ± 2.21 [c]
	LiP	44.4 ± 1.37 [a]	56.8 ± 1.71 [a]	58.11 ± 1.73 [b]	45.1 ± 0.27 [b]	39.15 ± 2.42 [c]
CC	Lac	186 ± 3.92 [a]	165 ± 2.22 [b]	160.9 ± 3.15 [b]	147.1 ± 2.51 [c]	132.1 ± 1.55 [d]
	MnP	105 ± 2.11 [a]	111 ± 2.12 [b]	125.2 ± 2.53 [c]	105.1 ± 1.41 [c]	95.2 ± 1.58 [d]
	LiP	50.1 ± 1.43 [a]	52.1 ± 0.41 [a]	41.12 ± 1.08 [b]	47.1 ± 1.32 [b]	52.25 ± 0.37 [c]
CS	Lac	100 ± 1.94 [a]	183 ± 3.42 [b]	173.1 ± 3.03 [b]	175.3 ± 1.76 [c]	138.26 ± 3.34 [d]
	MnP	131 ± 1.23 [a]	99.1 ± 1.92 [b]	118.4 ± 3.14 [c]	99.2 ± 1.12 [c]	72.1 ± 1.12 [d]
	LiP	45.1 ± 1.11 [a]	46.2 ± 1.37 [b]	48.1 ± 1.82 [b]	55.15 ± 0.6 [b]	46.12 ± 0.70 [b]
RS	Lac	174 ± 2.13 [a]	190 ± 3.13 [b]	186.4 ± 3.21 [c]	100.1 ± 1.12 [d]	165.11 ± 1.31 [e]
	MnP	121 ± 1.16 [a]	132 ± 2.16 [b]	130.2 ± 3.62 [c]	105.1 ± 1.16 [c]	121.1 ± 1.26 [d]
	LiP	30.1 ± 1.61 [a]	44.2 ± 0.64 [b]	34.25 ± 1.34 [c]	38.22 ± 0.47 [d]	45.2 ± 0.81 [e]
BS	Lac	167 ± 2.12 [a]	228.2 ± 2.21 [b]	200.2 ± 3.42 [c]	162.3 ± 1.92 [cd]	165.1 ± 2.13 [d]
	MnP	140 ± 1.91 [a]	153.4 ± 3.18 [b]	120.1 ± 3.30 [c]	118.2 ± 1.26 [c]	98.1 ± 2.42 [d]
	LiP	42.2 ± 1.18 [a]	59.2 ± 1.52 [b]	43.25 ± 1.19 [b]	41.22 ± 0.54 [c]	39.02 ± 0.17 [d]
SCB	Lac	110 ± 1.27 [a]	172 ± 2.12 [a]	172.4 ± 3.63 [b]	163.2 ± 2.54 [b]	155.05 ± 2.13 [c]
	MnP	126 ± 2.13 [a]	104 ± 2.13 [b]	105.2 ± 1.24 [b]	86.16 ± 1.03 [c]	65.2 ± 2.11 [d]
	LiP	38.2 ± 0.19 [a]	43.1 ± 1.12 [ab]	37.15 ± 0.32 [bc]	39.13 ± 0.38 [c]	41.12 ± 1.81 [c]

Data are means of three replicates. Means within rows having different superscripts are significantly different at p < 0.05
[*]pH - 4.5; Temperature - 30 °C
LiP - lignin peroxidase; MnP - manganese peroxidase; Lac - laccase; WS - wheat straw; CC - corncobs; CS - corn stover; RS - rice straw; BS - banana stalk; SCB - sugar cane bagasse

Table 2: Production of cellulolytic enzymes by *P. sapidus* cultivated on different lignocellulosic substrates

Substrates	Enzymes	Enzyme activity after incubation (days) (U/mL)				
		2	4	6	8	10
WS	EG	0.07 ± 0.01 [a]	0.094 ± 0.07 [b]	0.083 ± 0.05 [bc]	0.071 ± 0.06 [bc]	0.08 ± 0.01 [c]
	CBH	0.062 ± 0.02 [a]	0.053 ± 0.04 [a]	0.052 ± 0.02 [b]	0.048 ± 0.03 [bc]	0.043 ± 0.01 [c]
	BGL	0.05 ± 0.01 [a]	0.078 ± 0.03 [a]	0.055 ± 0.03 [b]	0.041 ± 0.02 [b]	0.033 ± 0.03 [c]
CC	EG	0.087 ± 0.03 [a]	0.099 ± 0.01 [ab]	0.084 ± 0.03 [ab]	0.068 ± 0.02 [ab]	0.074 ± 0.01 [b]
	CBH	0.067 ± 0.01	0.069 ± 0.04	0.058 ± 0.02	0.043 ± 0.02	0.045 ± 0.02
	BGL	0.053 ± 0.02	0.067 ± 0.03	0.046 ± 0.01	0.042 ± 0.04	0.056 ± 0.03
CS	EG	0.086 ± 0.02 [a]	0.118 ± 0.08 [ab]	0.082 ± 0.04 [ab]	0.069 ± 0.06 [ab]	0.082 ± 0.05 [b]
	CBH	0.058 ± 0.04	0.075 ± 0.02	0.057 ± 0.02	0.044 ± 0.05	0.039 ± 0.03
	BGL	0.054 ± 0.02 [a]	0.086 ± 0.04 [ab]	0.066 ± 0.01 [abc]	0.05 ± 0.01 [bc]	0.035 ± 0.05 [c]
RS	EG	0.073 ± 0.03 [a]	0.098 ± 0.03 [ab]	0.081 ± 0.03 [b]	0.073 ± 0.02 [b]	0.083 ± 0.02 [b]
	CBH	0.054 ± 0.02 [a]	0.066 ± 0.05 [b]	0.061 ± 0.02 [c]	0.057 ± 0.01 [c]	0.049 ± 0.02 [d]
	BGL	0.036 ± 0.03	0.044 ± 0.02	0.054 ± 0.01	0.047 ± 0.02	0.035 ± 0.03
BS	EG	0.122 ± 0.02 [a]	0.128 ± 0.02 [b]	0.158 ± 0.09 [bc]	0.137 ± 0.12 [bc]	0.139 ± 0.03 [c]
	CBH	0.063 ± 0.06 [a]	0.074 ± 0.09 [ab]	0.098 ± 0.01 [ab]	0.071 ± 0.06 [ab]	0.067 ± 0.04 [b]
	BGL	0.068 ± 0.05 [a]	0.086 ± 0.01 [ab]	0.118 ± 0.08 [ab]	0.101 ± 0.04 [b]	0.099 ± 0.01 [b]
SCB	EG	0.092 ± 0.07 [a]	0.075 ± 0.03 [b]	0.079 ± 0.02 [c]	0.07 ± 0.03 [d]	0.081 ± 0.03 [d]
	CBH	0.072 ± 0.03 [a]	0.059 ± 0.02 [ab]	0.068 ± 0.04 [c]	0.043 ± 0.02 [d]	0.038 ± 0.01 [e]
	BGL	0.049 ± 0.02	0.047 ± 0.02	0.045 ± 0.02	0.039 ± 0.02	0.036 ± 0.01

Data are means of three replicates. Means within rows having different superscripts are significantly different at p < 0.05
EG - endo 1, 4-*β* glucanase, CBH - cellobiohydrolase, BGL - *β* glucosidase
WS - wheat straw; CC - corncobs; CS - corn stover; RS - rice straw; BS - banana stalk; SCB - sugar cane bagasse

the key parameters affecting the production of enzymes in a straightforward approach. Therefore, during the last few years, many researchers have attempted this technique to enhance the enzyme production by different microbial strains (Yasmeen *et al.*, 2013). The ligninolytic enzyme activities have been widely investigated in *P. sapidus* than that of cellulolytic enzymes.

In the present study, optimum enzyme production was tested under the design of RSM with central composite design (CCD) using DOE 8.0.7.1 trial version software (Silva *et al.*, 2014). Banana stalk that resulted in the highest production of lignocellulolytic enzymes was solely optimised through RSM. An experimental design of 15 runs with 5 factors and 5 levels i.e. pH (2, 3, 4, 5 and 6), temperature (20, 25, 30, 35 and 40 °C), inoculum size (1, 2, 3, 4 and 5 mL), incubation time (24, 48, 72, 96 and 120 hours) and moisture (40, 45, 50, 55 and 60 %) was adopted. The results shown in Table 3 indicate that maximum lininase activities were obtained under optimum conditions of pH 4.0; temperature 30 °C; inoculum size 4 mL; moisture content 60 %, and incubation time 5 days. The activities of LiP (98.76 U/mL), MnP (219.60 U/mL) and laccase (313.55 U/mL) were substantially enhanced by optimising the culture conditions. Trial 7 presented greater crude cellulase activities, i.e., endoglucanase (0.212 U/mL), exoglucanase (0.187 U/mL) and

ß-glucosidase (0.217 U/mL), resulted by the medium pH 4, temperature 30 °C, inoculum size 4 mL and moisture content 60 %, after 5 days of incubation (Table 3).

ANOVA (Table 4) for the response surface model was carried out to evaluate the predictability of the polynomial model at 95 % confidence interval. The fit of developed models can be determined from the lack of fit values, which was significant and this condition indicated that the employed model fitted well to the lignocellulolytic enzyme production. In a previous study, 12 days incubation and 1.0 % carboxyl-methyl cellulose (CMC) as carbon source supported greater production of cellulases. A temperature range of 35 − 40 °C and pH 5.0 was optimum for exo and endoglucanase production while β-glucosidase production was optimum at 30 °C and pH 4.5 (Goyal & Soni, 2011).

Figure 1 shows that the predicted value of the response (Y) from the model is in agreement with actual values over the selected range of independent variables, and a straight line confirms the normality of the data. The high degree of agreement between predicted and actual values indicated that the CCD can be employed successfully as an effective and reliable tool for optimisation of conditions for maximum production of lignocellulolytic enzymes. Response surface plots for laccase activities

Table 3: Optimisation of the fermentation parameters using central composite design (CCD) under RSM

| Runs	pH	Temp. (°C)	I.S. (mL)	I.T. (hrs)	Moisture (%)	LiP	MnP	Lac	EG	CBH	BGL
1	4	40	2	72	40	81.04	162.22	243.05	0.088	0.097	0.187
2	3	30	5	48	50	62.5	106.3	163.61	0.103	0.069	0.062
3	2	20	5	120	60	77.24	139.24	233.01	0.094	0.056	0.187
4	2	40	3	24	50	78.42	192.48	156.66	0.109	0.165	0.075
5	6	40	1	24	60	75.87	161.36	262.77	0.092	0.067	0.187
6	6	20	1	72	50	79.56	124.65	162.38	0.093	0.066	0.087
7	4	30	4	120	60	98.76	219.6	313.55	0.212	0.187	0.217
8	4	20	3	48	55	85.87	101.31	151.66	0.104	0.168	0.109
9	6	30	3	96	45	86.29	71.73	251.86	0.092	0.068	0.079
10	2	35	1	24	40	67.24	95.93	181.38	0.088	0.064	0.085
11	4	40	2	72	40	77.54	178.13	154.45	0.093	0.167	0.185
12	2	40	4	96	55	55.24	106.56	240.56	0.092	0.068	0.086
13	2	20	1	24	60	84.97	180.43	113.61	0.092	0.168	0.095
14	6	40	5	120	60	59.65	146.76	237.56	0.173	0.175	0.111
15	3	30	5	48	50	79.35	165.32	207.37	0.109	0.169	0.083

An average absorbance of the three repetitions was used in the calculations. Temp - temperature; I.S. - inoculum size; I.T. - incubation time; LiP - lignin peroxidase; MnP - manganese peroxidase; Lac - laccase; EG - endo 1, 4-*β* glucanase, CBH - cellobiohydrolase, BGL - *β* glucosidase

Table 4: Analysis of variance (ANOVA) for laccase production in solid-state fermentation

Source	SS	Df	MS	F-value	p-value
Model	5.767	20	2.884	4.95	0.0064 significant
A	4.555	1	3.831	6.57	0.0282
B	2.97	1	4.555	0.78	0.3974
C	4.017	1	2.97	5.1	0.0476
D	7.236	1	4.017	6.89	0.0254
E	1.642	1	7.236	12.42	0.0055
AB	5.413	1	1.642	2.82	0.1242
AC	4.717	1	4.717	9.29	0.0123
AD	7.982	1	7.982	8.09	0.0174
AE	4.169	1	4.169	1.37	0.2691
BC	4.727	1	4.727	7.15	0.0233
BD	2.429	1	2.429	8.11	0.0173
BE	3.421	1	3.421	4.17	0.0685
CD	5.746	1	5.746	5.87	0.0359
CE	5.57	1	5.57	9.86	0.0105
DE	1.061	1	1.061	9.56	0.0114
A2	1.474	1	1.474	1.82	0.2071
B2	6.537	1	6.537	2.53	0.1429
C2	2.175	1	2.175	0.11	0.7446
D2	6.781	1	6.781	3.73	0.0822
E2	5.829	1	5.829	1.16	0.3061
Residual	5.429	1	1.086		
Lack of Fit	4	1	8	13.57	0.0062 significant
Pure Error	6.35	1			
Cor Total		1			
		1			
		5			
		30			

SS - sum of squares; Df - degree of freedom; MS - mean square

[Figure 2 (A-D)] indicated that low temperature within the range tested had a significant influence on the lignocellulolytic enzyme production. Maximum enzyme activities were recorded when the temperature tended to be 35 °C. Nonetheless, *P. sapidus* strain was not able to grow when incubated at temperatures below 20 °C. Fernandez-Fueyo *et al*. (2014) reported that extreme temperature (10 °C and 37 °C) ranges led to marked reduction in enzyme activities. Yasmeen *et al*. (2013) also described a maximum ligninolytic enzyme secretion by *Ganoderma luciduma* and *Schyzophilum commune* at 35 °C and 4.5 pH. When considering the pH, optimum activities of LDEs were achieved at pH 4.0 with a moderate temperature level (35 °C). Beyond this optimal pH, the activities of LDEs substantially declined. Fernandez-Fueyo *et al*. (2014) observed reduced *P. ostreatus* enzyme profiles at extreme pH values (3.0 and 8.0), whereas Wang *et al*. (2014) noted enhanced enzyme production by *T. versicolor* in an
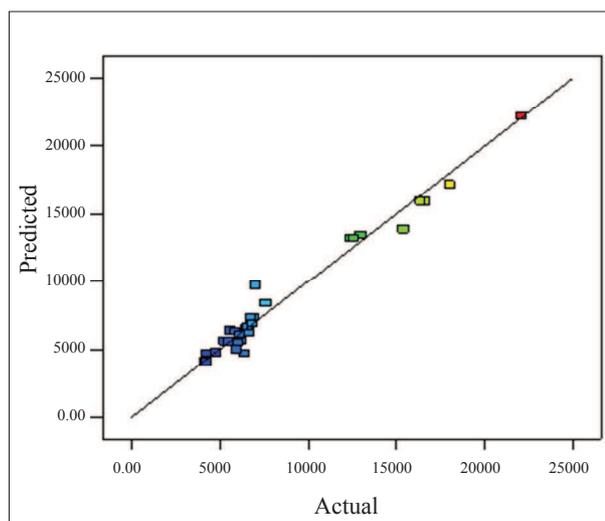
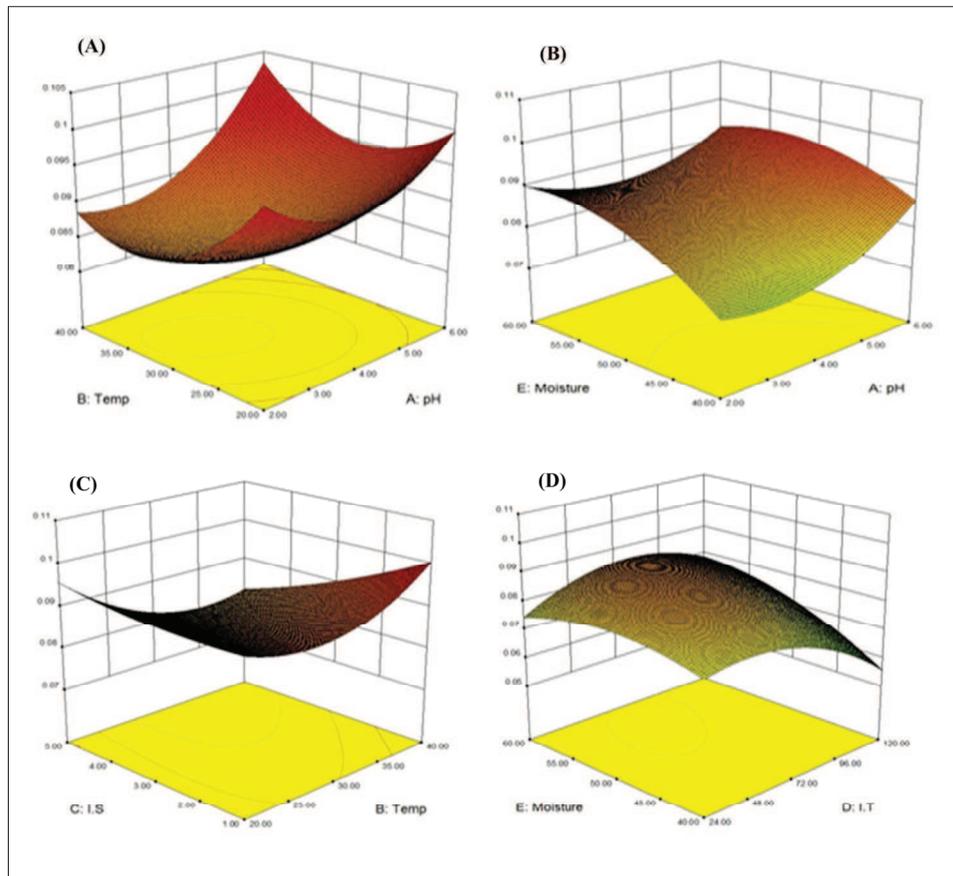**Figure 1:** Predicted and actual values for laccase enzyme production

Figure 2: Response surface plots showing the interactive effect of (A) pH *vs* temperature; (B) pH *vs* moisture; (C) temperature *vs* inoculum size, and (D) incubation time *vs* moisture for optimisation of laccase enzyme

acidic pH of 4. Solid-state fermentation (SSF) of plant biomasses promoted the production of ligninolytic enzymes than submerged fermentation (SmF) by several *Pleurotus* strains (Yasmeen *et al*., 2013). Vassilev *et al*. (2009) found the highest activities at 37 °C temperature, 60 % humidity and pH 5.0 after a 21 day process by *P. chrysosporium*. Using *Trametes villosa* with optimised cultivation conditions, Silva *et al*. (2014) determined a 15 days best time for the production of MnP, after which it declined until approaching zero on the 30th day.

Lignin degradation

The crude lignocellulolytic extract in varying dose levels (5, 7.5, 10, 12.5, 15.5 mL) was used for the delignification of selected agricultural wastes for different time periods, and the responses are illustrated in Figure 3. The lignin content in all the substrates was reduced after ligninolytic treatment and maximum delignification was recorded in rice straw, followed by sugarcane bagasse, corn cobs and wheat straw after 48 hours. Different percentages of

lignin removal in different substrates may be attributed to differences in cell wall assembly, the complicated structure of lignin and lignin-carbohydrate complex. Nevertheless, the lignin degradation trend achieved in the present study was in agreement with previous studies. Taniguchi *et al*. (2005) achieved 41 % klason lignin removal from pretreated rice straw by *P. ostreatus*. Asgher *et al*. (2013a) managed to achieve 33.5 % reduction in the lignin content of sugarcane bagasse and Wang *et al*. (2014) degraded lignin up to 34.7 % in rice straw. Silva *et al*. (2014) by applying crude enzyme extract from *Trametes villosa* (Sw.) Kreisel CCMB 651 caused 35.05 %, 63.11 % and 39.61 % reduction in lignin content for sugarcane bagasse, sisal fiber and coconut shell, respectively. Although all cited reports showed good results, none of the studies reached the degree of lignin removal achieved in the present ligninolytic based treatment that caused commendable delignification of all studied agricultural wastes under mild and eco-friendly processing conditions, which are safer and more stable than various alkali, acidic or other pre-treatment techniques.
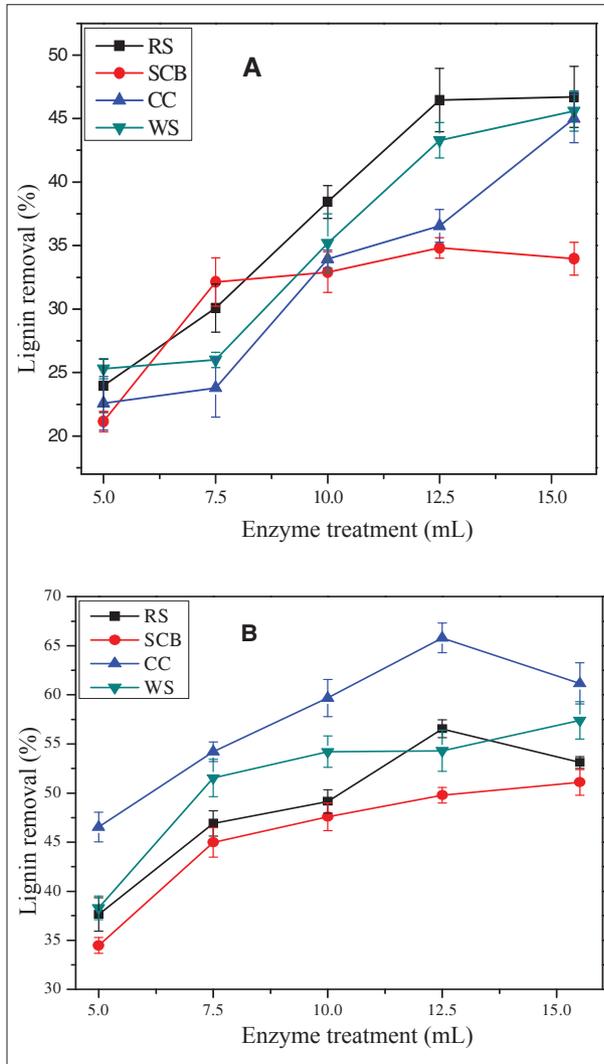
Figure 3: Delignification of different agricultural wastes by crude enzymatic extract from *P. sapidus* after (A) 24 hours and (B) 48 hours; RS - rice straw; SCB - sugarcane bagasse; CC - corncobs; WS - wheat straw

CONCLUSION

The present study has shown that the production of lignocellulolytic enzymes can easily be enhanced by optimisation of different process parameters through response surface methodology. The crude enzyme extract showed encouraging results for bio-delignification of various agricultural wastes.

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