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Full Length Research Paper

Lignocellulose degradation, enzyme production and protein enrichment by *Trametes versicolor* during solid-state fermentation of corn stover

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Microbial conversion of corn stover by white rot fungi has the potential to increase its ligninolysis and nutritional value, thereby transforming it into protein-enriched animal feed. Response surface methodology was applied to optimize conditions for the production of lignocellulolytic enzymes by *Trametes versicolor* during solid-state fermentation of corn stover, as well as enhance ligninolysis and increase the crude protein content. The effects of an additional carbon source (glucose), copper sulfate ($CuSO_4$) and initial moisture content on lignocellulolytic enzymes, changes in chemical constituents and the crude protein content of corn stover were investigated. *T. versicolor* produced high laccase, moderate xylanase, and low CMCase activity, whereas neither LiP nor MnP activity was detected. An overall 20-fold increase in laccase activity (45.1 U/g corn stover) was achieved under the optimized conditions. The maximum degradation of lignin and hemicellulose was up to 34.8 and 21.9%, respectively. However, the maximum cellulose loss was less than 10.5%. The crude protein content of the fermented corn stover was doubled under the optimized conditions. Therefore, *T. versicolor* is a potential organism for laccase production using solid-state fermentation, as well as the simultaneous enhancement of delignification and improvement of the crude protein content in corn stover.

Key words: Corn stover, central composite design, laccase, ligninolysis, *Trametes versicolor*, crude protein.

INTRODUCTION

Agricultural straw, which mainly consists of cellulose, hemicellulose and lignin, is the most abundant renewable lignocellulosic biomass. Most of this material is disposed by burning, which results in environmental pollution and only a small portion is used as feed for ruminants and feedstock for paper pulp or biofuels (Sanchez, 2009; Yang et al., 2001). Agricultural straw is rich in energy, low in crude protein (CP), digestible and poorly palatable, which prevents its use in feedlots. Ruminal microbial utilization of energy-rich cell walls in agricultural straw is hindered by the presence of lignin (Bisaria et al., 1997). Lignin is quite resistant to microbial degradation under natural conditions, thus, it plays a key role in limiting the quality of lignocellulosic biomass as animal feed. Faced with these problems, a potential solution is available, that is, the utilization of microorganisms, mainly fungi, to convert agroindustrial residues to obtain products with higher nutritive value, especially in terms of CP and enhanced ligninolysis (Villas-Bôas et al., 2002; Mukherjee and Nandi, 2004).

Solid-state fermentation (SSF) is a means for the microbial conversion of lignocellulosic biomass. SSF is defined as the growth of microorganisms in the absence or near absence of free water with inert or natural substrates as solid support (Pandey et al., 1999). SSF is an advantageous method for degrading lignin and

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Abbreviation: SSF, Solid-state fermentation.

improving the digestibility of agricultural straw. Compared with submerged fermentation, SSF possesses many advantages such as low effluent generation, requirement for simpler fermentation equipment and the direct applicability of the fermented product for feeding (Yang et al., 2001). During SSF, microorganisms produce lignocellulolytic enzymes, degrade components of the cell wall and synthesize microbial proteins. A number of microorganisms, mainly white rot fungi, have been used for producing microbial proteins and lignocellulolytic enzymes by SSF from different agricultural residues (Bisaria et al., 1997; Niladevi et al., 2007; Arora and Sharma, 2009; Zeng et al., 2011). White rot fungi are well known producers of lignocellulolytic enzymes, including lignin-modifying enzymes, hemicellulases, and cellulases. They reportedly degrade lignin more efficiently than any other group of microorganisms (Kuhad et al., 1997; Kuhar et al., 2008; Shabtay et al., 2009). However, most white rot fungi not only degrade lignin, but also cellulose, leading to significant cellulosic losses, which can be minimized with selective ligninolytic fungi (Sharma and Arora, 2010). Thus, choosing a microorganism that selectively degrades lignin over cellulose in the substrate and capable of synthesizing proteins with high nutritional value is necessary.

In this study, *T. versicolor* sdu-4, a selective ligninolytic fungus, was used to enhance the production of laccase, ligninolysis and the CP content of corn straw (CS). Central composite design was applied to optimize additional glucose, $CuSO_4$ and the initial moisture content during SSF.

MATERIALS AND METHODS

Substrate and organism

Locally collected CS was cut into uniform pieces (1.5 to 2.0 cm) and dried at 80°C. The chopped straws were ground by milling in a laboratory mill and sieved (40 mesh) for SSF. The fungus used in this study was stored on wheat bran extract medium with 15 g agar per liter at 4°C. One-week -old fully grown slants were used for inoculum preparation. The fungus denominated sdu-4 was identified as *T. versicolor* by the analysis of its internal transcribed spacer nucleotide sequence (Dhouib et al., 2005). The ITS1-5.8S rRNA-ITS2 gene sequence of the fungal strain was obtained using a phenol-chloroform extraction method (Sambrook and Russell, 2001). The 5.8S rRNA gene, in association with the two flanking internal transcribed spacers (ITS1 and ITS2), was amplified using universal primers ITS1 and ITS4 (Gardes and Bruns, 1993). The 646 bp (ITS1-5.8S rRNA- ITS2) gene sequences were submitted to GenBank under accession number of JF437649. The ITS1-5.8S rRNA- ITS2 gene sequence was compared and aligned with sequences deposited in the GenBank database.

Solid-state fermentation

About 4 g of dried CS was placed inside a 250 ml Erlenmeyer flask. A saline solution with 0.75% NH_4Cl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.004% $CaCl_2$, 0.0001% $MnSO_4$, 0.0001% $ZnSO_4$ and 0.0001% $FeSO_4$ was used as the moistening medium. Then, 4 ml of moistening medium and an appropriate amount of distilled water were added into the flask to reach the predetermined initial moisture content. The contents of the flask were mixed thoroughly and autoclaved at 121°C for 30 min. Then, 5 agar plugs (7 to 8 mm in diameter) cut from actively growing fungal mycelia were used as inocula and incubated in an atmosphere with controlled humidity at 30°C for 21 days.

Experimental design and data analysis

Three independent variables, an additional carbon source (glucose), copper sulfate ($CuSO_4$) and initial moisture content, were chosen based on the results obtained from earlier preliminary experiments. Response surface methodology using a central composite design was applied to optimize the selected variables. Each variable was studied at five different levels (-α, -1, 0, +1 and +α) and the variable ranges [(-)1–(+)1] were: glucose (8 to 10 mg/g CS), $CuSO_4$ (4 to 5 µM/g CS) and initial moisture content (70 to 90%). The experimental design included 20 flasks with six central points (Table 1). All experiments were performed in triplicate, which was necessary to estimate the experimental variability of measurements. The quadratic polynomial equation (Box and Behnken, 1960) which includes all interaction terms was used to evaluate the mathematical relationship of response Y (for each parameter) and independent variable X (X_1, glucose; X_2, $CuSO_4$; X_3, initial moisture content). The responses were: laccase, xylanase and carboxymethyl cellulase (CMCase) activities, as well as the changes in the lignin, hemicellulose, cellulose, crude protein content in the substrate.

$$Y = k_0 + k_1X_1 + k_2X_2 + k_3X_3 + k_{12}X_1X_2 + k_{13}X_1X_3 + k_{23}X_2X_3 + k_{11}X_1^2 + k_{22}X_2^2 + k_{33}X_3^2 \quad (1)$$

Where, Y is the predicted response; k_0 is the intercept term; k_1, k_2, k_3 are the linear coefficients; k_{12}, k_{13}, k_{23} are the interaction coefficients; k_{11}, k_{22}, k_{33} are the squared coefficients and X_1, X_2, X_3 are the coded levels of the independent factors glucose concentration, CuSO4 concentration and initial moisture content. This design was used to evaluate the main effects, interaction effects and quadratic effects and to optimize the levels of parameters for different responses. The statistical software package design expert version 7.0 (State-Ease, Inc, Minneapolis, USA) was performed for regression and graphical analysis of data obtained. The optimum levels of glucose concentration, CuSO4 concentration and initial moisture content for each response were obtained by solving the regression equation and also analysis the response surface model graphs. The whole experiment was repeated three times.

Enzyme extraction

At the end of the incubation period, the fermented material was extracted with sodium acetate buffer (20 mM, pH 4.8) to a final extraction volume of 100 ml. The contents were mixed thoroughly by keeping the flasks on a rotary shaker at 120 rpm for 2 h at 30°C. The mixture was centrifuged at 13,000 rpm for 20 min at 4°C. The supernate was collected and used for different enzyme assays, such as laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), xylanase and carboxymethyl cellulase (CMCase). The precipitate was dried at 80°C until a constant weight was obtained and the dried biomass was used to analyze the changes in lignin, hemicellulose, cellulose and CP content.

Table 1. Central composite design for variables and measured response.

Run	Variable			Enzyme activity (U/g CS)			Change in straw constituent (%)			
	Glucose (mg/g)	$CuSO_4$ (μM/g)	Moisture (%)	Laccase	Xylanase	CMCase	Lignin loss	HMCL loss	CL loss	CP
1	8.00	4.00	70.00	7.62	1.94	0.56	10.23	3.39	2.58	5.24
2	10.00	4.00	70.00	11.29	3.87	1.29	15.86	6.34	5.11	5.95
3	8.00	5.00	70.00	6.66	1.79	0.649	9.67	4.27	2.48	5.19
4	10.00	5.00	70.00	12.18	3.67	1.21	14.58	6.23	5.03	5.85
5	8.00	4.00	90.00	18.48	4.7	2.82	20.13	13.57	6.41	6.42
6	10.00	4.00	90.00	17.21	6.69	4.08	23.08	21.36	8.92	7.86
7	8.00	5.00	90.00	13.66	4.34	2.79	19.69	13.46	5.72	6.40
8	10.00	5.00	90.00	14.49	6.56	3.96	21.85	21.18	8.81	7.73
9	7.32	4.50	80.00	11.11	3.78	1.68	19.94	8.24	5.05	6.15
10	10.68	4.50	80.00	14.76	8.25	3.29	28.05	14.7	11.07	7.87
11	9.00	3.66	80.00	26.17	6.94	2.82	35.97	12.37	9.05	6.69
12	9.00	5.34	80.00	23.17	6.68	2.59	23.24	15.26	8.36	6.50
13	9.00	4.50	63.18	1.17	0.66	0.12	4.5	2.14	0.28	4.15
14	9.00	4.50	96.82	12.19	7.86	4.68	15.65	20.06	8.65	6.53
15	9.00	4.50	80.00	45.14	7.04	2.75	33.59	16.46	8.58	6.58
16	9.00	4.50	80.00	45.25	7.21	2.68	33.71	16.39	8.64	6.53
17	9.00	4.50	80.00	45.05	7.16	2.77	33.61	16.4	8.62	6.61
18	9.00	4.50	80.00	44.89	7.18	2.75	33.64	16.42	8.64	6.61
19	9.00	4.50	80.00	45.26	7.08	2.73	33.72	16.37	8.68	6.49
20	9.00	4.50	80.00	44.97	7.13	2.69	33.68	16.35	8.71	6.58

HMCL, Hemicellulose; CL, cellulose; CP, crude protein; CS, corn stover.

Enzyme assays

Laccase (E.C. 1.10.3.2) activity was determined by monitoring the A_{420} change related to the rate of oxidation of 1 mM 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, $\varepsilon = 36{,}000\ cm^{-1}\ M^{-1}$) (Bourbonnais and Paice, 1990). The assay mixture contained 1 mM ABTS, 20 mM sodium acetate buffer (pH 4.8) and enzyme to a total volume of 2.0 ml. LiP (1.11.1.14) and MnP (1.11.1.13) activities were determined using the methods described by Ryu et al. (2000). Lignocellulolytic enzyme activity (U) was defined as the amount of enzyme needed to oxidize 1 μM of substrate per minute. CMCase (3.2.1.4) and xylanase (3.2.1.8) activities were measured according to the methods by Xin and Geng (2010). One unit of activity was defined as 1 μM of glucose (CMCase) or xylose (xylanase) equivalent released per min per ml in the extracts recovered from the solid-state cultures using a xylose or glucose standard enzymatic activity curve and is reported in U/g CS.

Analytical methods

The fermented corn stover was analyzed in triplicate for CP (AOAC, 1995), hemicellulose, cellulose and lignin. The content of hemicellulose, cellulose and lignin in the corn stover were determined according to the procedures of Arora and Sharma (2009). Lignin, cellulose and hemicellulose loss are defined as the percentage of total lignin, cellulose and hemicellulose reduced during SSF, respectively.

RESULTS AND DISCUSSION

Strain identification

The GenBank accession number of the ITS1-5.8S rRNA-ITS2 gene sequence of the fungal strain used in this study was JF437649. As a result, the isolated strain was identified as *T. versicolor* and named as *T. versicolor* sdu-4.

ANOVA for response surface quadratic model

Regression analysis of the experimental data was performed for testing the adequacy of the proposed quadratic models based on Equation 1. The results of analysis of variance (ANOVA) are summarized in Tables 2 and 3, respectively. The P-values of the models were very low ($P < 0.001$) (Table 2), showing the high significance of the models. The F-values for all responses ranged from 11.16 to 17918.80 (Table 2), also indicating the response models are significant. There is only a

Table 2. Analysis of variance (ANOVA) for different response surface quadratic models.

Source	Laccase	Xylanase	CMCase	Lignin loss	HMCL loss	CL loss	CP
F-value	17918.80	11.16	66.73	22.76	101.15	19.30	546.76
P-value	<0.0001	0.0004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
R^2	0.9999	0.9094	0.9836	0.9535	0.9891	0.9456	0.9980
Adj R^2	0.9999	0.8280	0.9689	0.9116	0.9794	0.8966	0.9961
Adeq precision	364.355	10.794	29.052	15.931	33.453	15.592	97.865
CV	0.74%	16.87%	8.63%	12.34%	6.51%	12.83%	0.86%

HMCL, Hemicellulose; CL, cellulose; CP, crude protein; R^2, determination coefficient; Adj R^2, adjusted determination coefficient; adeq precision, adequate precision; CV, coefficient of variation. P-value less than 0.0500 indicate the model is significant.

Table 3. P-value of model coefficients for different responses.

Coefficient	P-value						
	Laccase	Xylanase	CMCase	Lignin loss	HMCL loss	CL loss	CP
X_1	<0.0001	0.0011	<0.0001	0.0199	<0.0001	0.0001	<0.0001
X_2	<0.0001	0.7185	0.5141	0.0404	0.1198	0.5523	0.0120
X_3	<0.0001	<0.0001	<0.0001	0.0005	<0.0001	<0.0001	<0.0001
X_1X_2	<0.0001	0.9469	0.6737	0.8559	0.6685	0.8263	0.3260
X_1 X_3	<0.0001	0.8824	0.0856	0.5179	0.0013	0.8492	<0.0001
X_2X_3	<0.0001	0.9587	0.7954	0.9837	0.6685	0.8207	1.0000
X_1^2	<0.0001	0.0180	0.0167	0.0003	<0.0001	0.0516	<0.0001
X_2^2	<0.0001	0.1240	0.1725	0.0158	0.0009	0.2258	0.2820
X_3^2	<0.0001	0.0003	0.0067	<0.0001	<0.0001	<0.0001	<0.0001

X_1, Glucose; X_2, $CuSO_4$; X_3, moisture; HMCL, hemicellulose; CL, cellulose; CP, crude protein. Value of P less than 0.0500 indicate model coefficient is significant. Value greater than 0.1000 indicate that the model coefficient is not significant.

0.01% chance that a "Model F-Value" this large could occur due to noise, for laccase and CMCase activities, lignin, hemicellulose and cellulose loss, crude protein content, respectively. For xylanase there is only a 0.04% chance that a "Model F-Value" this large could occur due to noise. The determination coefficient R^2 for all responses ranged from 0.9094 to 0.9999 (Table 2), indicating a high correlation between the experimentally observed and predicted values. This means that the quadratic models provided an excellent explanation of the relationship between the independent variables and the response (Pujari and Chandra, 2000; Sharma et al., 2009). The adjusted determination coefficient (Adj R^2, 0.8280 to 0.9999) also confirmed the significance of the models (Table 2). Lower values of coefficient of variation (CV, 0.74 to 16.87%) showed the experiments conducted were precise and reliable (Box et al., 1978). Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. All the models' ratios (10.794 to 364.355) were greater than 4 indicating adequate signals and they can be used to navigate the design space (Table 2).

The significance of each coefficient was determined by P-value which is listed in Table 3. The smaller the P-value, the bigger the significance of the corresponding coefficient (Lee and Wang, 1997). A P-value less than 0.05 indicate the coefficient is significant. A P-value greater than 0.1 indicates the coefficient is not significant.

Response for enzyme production

During the SSF of CS by *T. versicolor* sdu-4, LiP and MnP were not detected. The predicted responses for laccase (Y_1), xylanase (Y_2) and CMCase (Y_3) are listed as follows:

$$Y_1 = 45.10 + 1.09X_1 - 0.93X_2 + 3.27X_3 + 0.49X_1X_2 - 1.20X_1X_3 - 0.93X_2X_3 - 11.42X_1^2 - 7.27X_2^2 - 13.63X_3^2 \quad (2)$$

$$Y_2 = 7.18 + 1.14X_1 - 0.094X_2 + 1.69X_3 + 0.022X_1X_2 + 0.05X_1X_3 - 0.018X_2X_3 - 0.69X_1^2 - 0.41X_2^2 - 1.31X_3^2 \quad (3)$$

$$Y_3 = 2.74 + 0.47X_1 - 0.039X_2 + 1.29X_3 - 0.032X_1X_2 + 0.14X_1X_3 - 0.02X_2X_3 - 0.16X_1^2 - 0.082X_2^2 - 0.19X_3^2 \quad (4)$$

The effects of the linear and the squared coefficients (X_1, X_3, X_1^2, and X_3^2) were significant for all enzymes (Table 3). The linear effects of glucose and the initial moisture content were identified as the most significant factors for

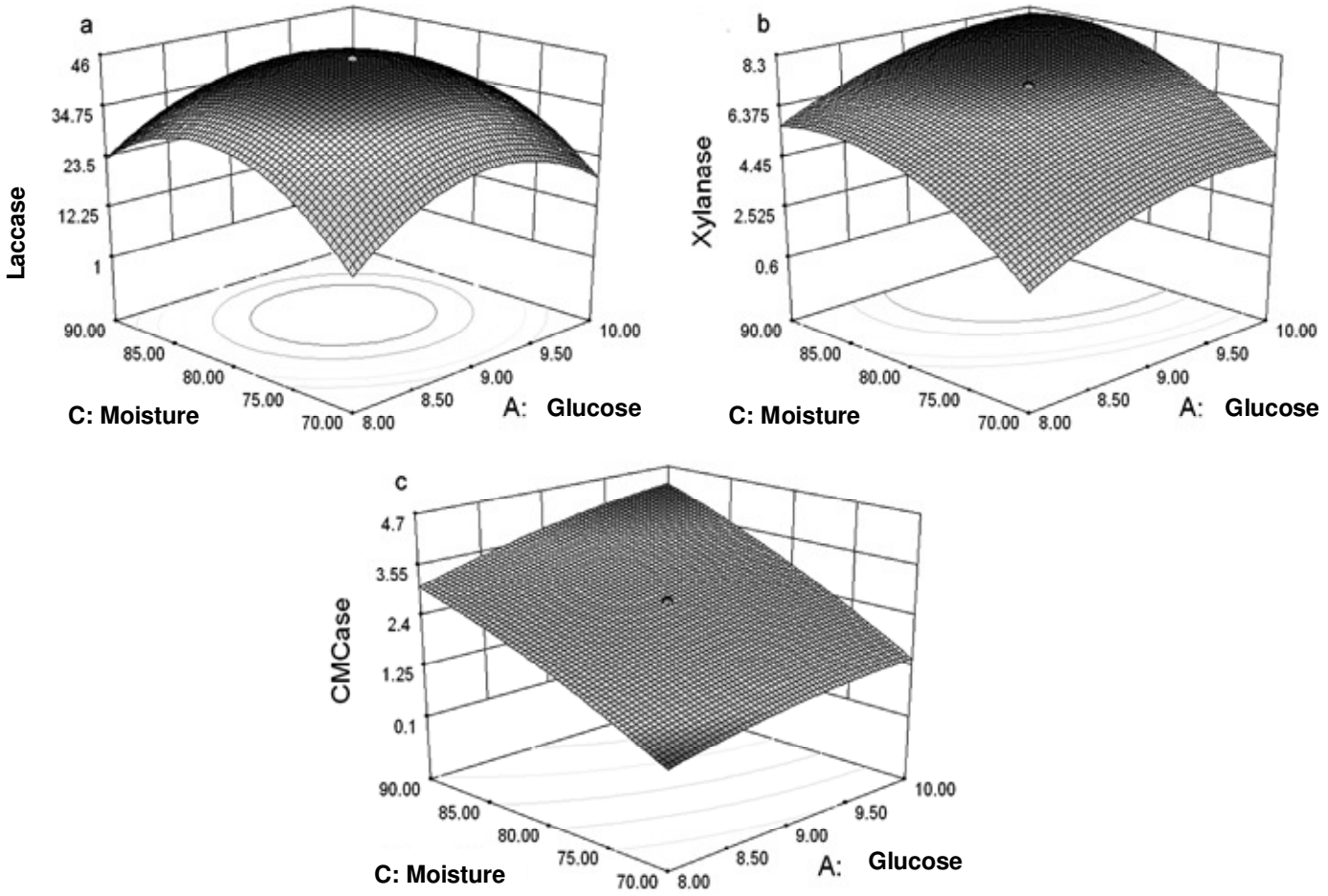

Figure 1. Response surface model graph for enzyme production. a. Graph for maximum laccase activity (A: glucose = 9 mg/g CS and C: moisture = 80%; hold value B: $CuSO_4$ = 4.5 µM/g CS). b. Graph for optimum xylanase activity (A: glucose = 10 mg/g CS and C: moisture = 87%; hold value B: $CuSO_4$ = 4.5 µM/g CS). c. Graph for optimum CMCase activity (A: glucose = 10 mg/g CS and C: moisture = 90%; hold value B: $CuSO_4$ = 4.5 µM/g CS).

all enzymes, as indicated by the *P* values less than 0.01 (Table 3). Glucose supplementation of the CS was performed for two reasons. First, it promotes the growth and rapid establishment of the fungus within the CS. Second, the white rot fungi needed an additional, easily metabolizable carbon source to degrade lignin from the lignocellulosic substrates (Kaal et al., 1995). Moisture is also a critical factor in SSF, which is required for new cell synthesis, growth and enzyme production (Niladevi et al., 2007; Pandey, 1992). The addition of glucose and increasing the initial moisture content enhanced enzyme production (Figure 1), whereas laccase production was reduced when the glucose concentration and moisture content were higher than 9 mg/g CS and 80%, respectively (Figure 1a). Maximum laccase production (45.1 U/g CS) was observed at a moderate glucose concentration (9 mg/g CS) and moisture level (80%), whereas the optimal glucose concentration and moisture level for xylanase and CMCase production were 10 mg/g CS, 87% and 10 mg/g CS, 90%, respectively (Figure 1). These observations are not consistent with previous findings (Sharma and Arora, 2010). In previous studies, maximum laccase activity was observed at higher moisture levels, whereas maximal xylanase (8.2 U/g CS) and CMCase (4.3 U/g CS) production were attained at moderate moisture levels. The difference may have been caused by the use of different substrates and fungi during SSF. The nature of the substrate and the organism under study play important roles in regulating the production of ligninolytic enzymes.

In order to further test the models, the actual values obtained experimentally were compared with the RSM models predicted values. The plot of predicted versus actual values for enzyme activity (Figure 2) showed that laccase was the most successfully optimized among the enzymes studied. The actual and predicted values of laccase activity were highly similar. This reflects the response surface model is reliable.

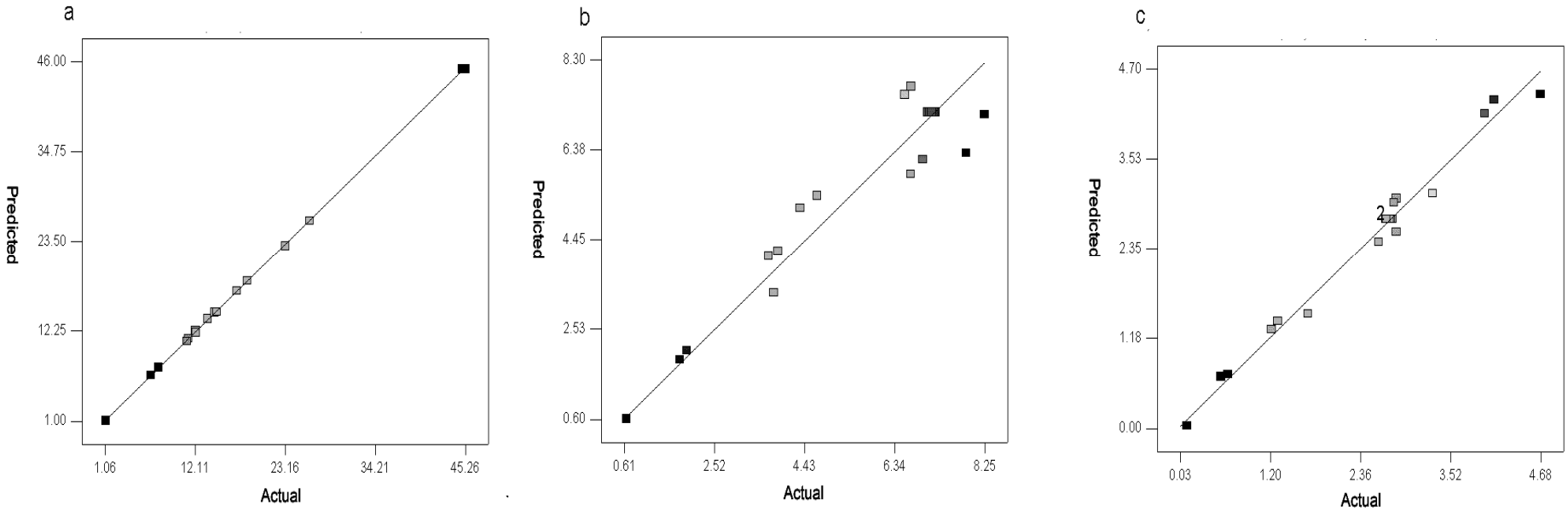

Figure 2. Plot of predicted versus actual values for enzyme activity. A. Plot of predicted versus actual laccase activity; B. plot of predicted versus actual xylanase activity; C. plot of predicted versus actual CMCase activity.

For laccase production, linear (X_2), squared (X_2^2) and interactive (X_1X_2, X_1X_3, and X_2X_3) effects were also significant, but they were not as important for xylanase and CMCase production. $CuSO_4$ is one of the widely reported inducers of laccases in many fungi (Niladevi et al., 2007; Palmieri et al., 2000; Palvannan and Sathishkumar 2010; Periasamy and Palvannan 2010). Cu^{2+}, as a micronutrient, plays a key role as a metal activator; it induces both laccase transcription and plays an important role in laccase production (Palmieri et al., 2000). In this study, laccase production was boosted by the addition of $CuSO_4$ to the medium. The optimal $CuSO_4$ concentration for laccase production was 4.5 µM/g CS. However, very high Cu^{2+} concentrations suppressed the growth of the strain; when the $CuSO_4$ concentration exceeded 4.5 µM/g CS, the laccase production began to decrease. This may be due to the interaction of metals with the white rot fungi; the involved defense mechanisms have been reviewed by Baldrian (2003). Optimal xylanase production was also achieved at 4.5 µM of $CuSO_4$/g CS, whereas the maximum CMCase activity was achieved at 4.0 µM of $CuSO_4$/g CS. Xylanase hydrolyzes the hemicellulose of lignocellulosic biomass and liberates simpler molecules and makes them freely available to the animals, thereby improving the nutritive quality of animal feed (Gilbert and Hazlewood, 1993; Beauchemin et al., 2003). It also plays an important role in the selective removal of lignin (Viikari et al., 1994). In recent years, xylanases have been applied in many industries, including animal feed, baking, pulp and paper, waste treatment and brewing (Qiu et al., 2010). Cellulase is another important animal feed additive that can significantly improve feed utilization and animal performance (Sharma and Arora, 2010; Beauchemin et al., 2003). Cellulase is also used for producing renewable biofuels, such as ethanol, butanol and hydrocarbons, from biomass sugars (Wilson, 2009).

Neither LiP nor MnP activity was detected during the SSF when $CuSO_4$ was added to the CS. High Cu^{2+} concentration reportedly inhibits LiP production (Levin et al., 2008). However, in this study, attempts to detect LiP and MnP during SSF without the addition of $CuSO_4$ were also unsuc

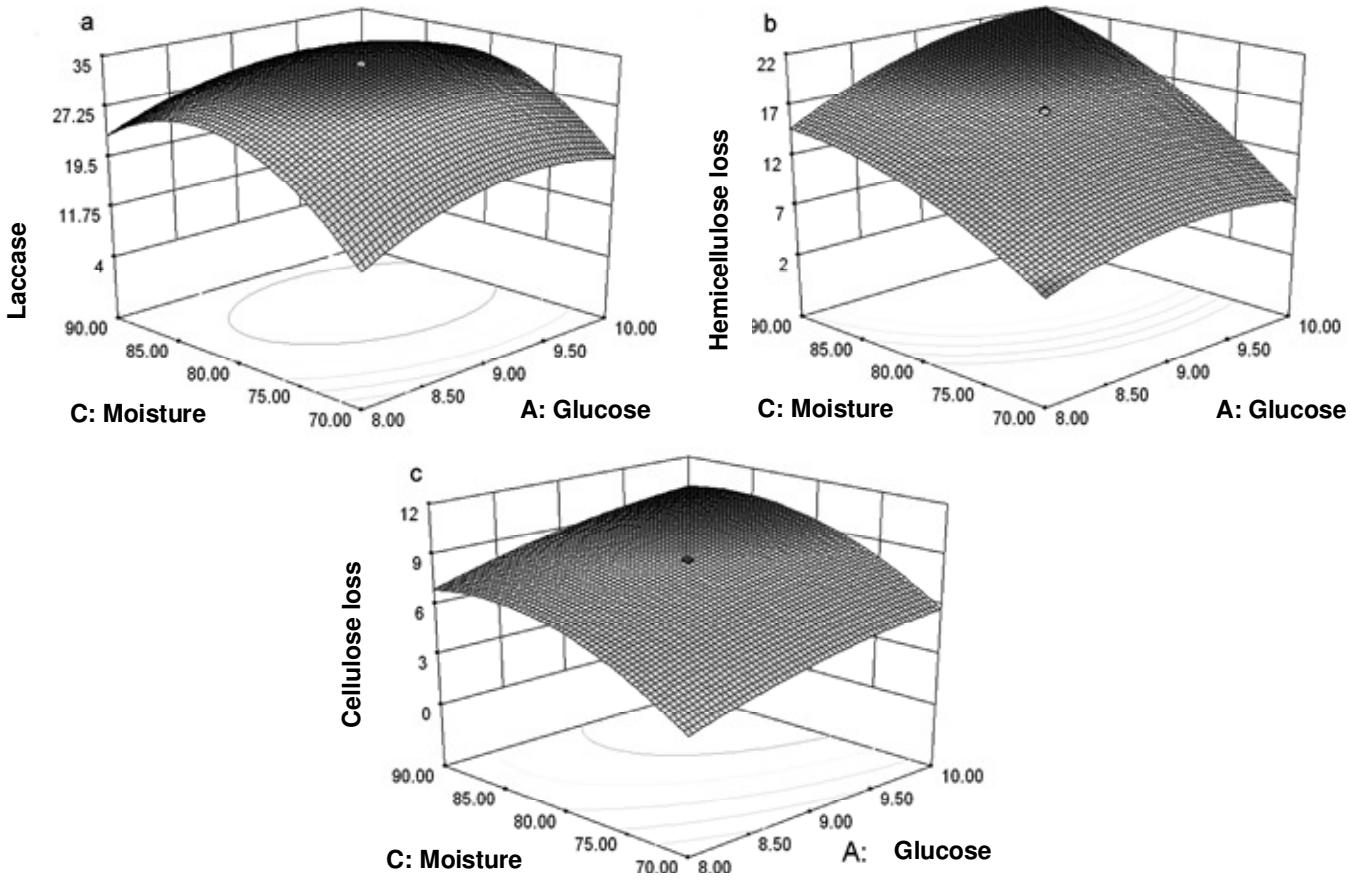

Figure 3. Response surface model graph for lignocellulolysis. A, Graph for maximum lignin loss (A: glucose = 9.2 mg/g CS and C: moisture = 82%; hold value B: $CuSO_4$ = 4.5 µM/g CS); b, Graph for maximum hemicellulose loss (A: glucose = 10 mg/g CS and C: moisture = 90%; hold value B: $CuSO_4$ = 4.5 µM/g CS); c, graph for maximum cellulose loss (A: glucose = 10 mg/g CS and C: moisture = 86%; hold value B: $CuSO_4$ = 4.5 µM/g CS).

cessful (data not shown). Thus, *T. versicolor* sdu-4 might be unable to secrete LiP and MnP or the levels of LiP and MnP it produced were too low to be detected. Laccase was the main ligninolytic enzyme. A 20-fold increase in laccase activity (45.1 U/g CS) was achieved under the optimized conditions, which indicates that the SSF of CS using *T. versicolor* sdu-4 may be an effective, economical and convenient way to produce laccase. Commercially, laccases have been used in the pulp and paper industry, the food industry, dye or stain bleaching, bioremediation, plant fiber modification, ethanol production, biosensors, biofuel cells, organic synthesis and drug synthesis (Mayer and Staples, 2002; Hadzhiyska et al., 2006; Tong et al., 2007).

Response for lignocellulolysis

The predicted responses for lignin loss (Y_4), hemicellulose loss (Y_5) and cellulose loss (Y_6) based on Equation (1) were as follows:

$$Y_4 = 33.77 + 2.14X_1 - 1.82X_2 + 3.89X_3 - 0.19X_1X_2 - 0.68X_1X_3 + 0.021X_2X_3 - 4.17X_1^2 - 2.19X_2^2 - 9.09X_3^2 \quad (5)$$

$$Y_5 = 16.42 + 2.29X_1 + 0.39X_2 + 5.82X_3 - 0.13X_1X_2 + 1.32X_1X_3 - 0.13X_2X_3 - 1.88X_1^2 - 1.05X_2^2 - 2.01X_3^2 \quad (6)$$

$$Y_6 = 8.69 + 1.52X_1 - 0.16X_2 + 2.10X_3 + 0.075X_1X_2 + 0.065X_1X_3 - 0.077X_2X_3 - 0.49X_1^2 - 0.26X_2^2 - 1.76X_3^2 \quad (7)$$

Ligninolysis was well optimized under the experimental conditions. All the linear (X_1, X_2 and X_3) and squared (X_1^2, X_2^2 and X_3^2) effects were significant for the lignin loss (Table 3). Maximal lignin (34.8%) was degraded with 9.2 mg of glucose and 4.3 µM of $CuSO_4$/g CS at 82% moisture content (Figure 3a). Hemicellulose (21.9%) was best degraded with 10 mg of glucose and 4.5 µM of $CuSO_4$/g CS at 90% moisture content (Figure 3b). All the three squared (X_1^2, X_2^2 and X_3^2), two linear (X_1 and X_3) and one interactive (X_1X_2) effects were important for hemicellulose loss. For cellulose degradation, linear (X_1 and X_3) and squared (X_3^2) effects were significant. The best conditions for cellulose loss (10.4%) were 10 mg of

glucose and 4.6 µM of $CuSO_4$/g CS at 86% moisture content (Figure 3c). Linear (X_1 and X_3) and squared (X_3^2) effects were important for lignocellulosic degradation, whereas none of the interactive effects were significant for lignin and hemicellulose degradation.

The result shows that maximal lignin loss occurred with moderate moisture content and glucose concentration, whereas maximal cellulose loss occurred with high moisture content and glucose concentration. Several reasons may account for this effect; among them is the oxygen concentration in the solid substrate. Lignin degradation is strictly an oxidative process, hence, it needs the presence of oxygen; in contrast, cellulose degradation is a hydrolytic process and it does not require oxygen. Barlev and Kirk (1981) reported that lignin degradation is affected by oxygen concentration at two stages: first, during stimulation of the transcription of the ligninolytic enzyme system; and second, during the oxidation of lignin. Moisture content has an important effect on the oxygen concentration in the substrate. Excessive water hinders the exchange of gases and creates anaerobic conditions inside the substrate, whereas insufficient water does not allow optimal fungal activity. Thus, more selective CS delignification by *T. versicolor* sdu-4 could be achieved by controlling the moisture content and additional glucose concentration during SSF. A maximum of 34.8% of the lignin was degraded after 21 days of fermentation, whereas under the same SSF conditions, cellulose and hemicellulose degradation was only 9.3 and 17.6%, respectively. A 1.4-fold increase in the cellulose/lignin ratio of the fermented CS (3.9) was achieved under the optimal ligninolysis conditions compared with that of raw CS (2.8). Previous authors concluded that the presence of highly lignified tissues form a physical barrier that limits the accessibility of the otherwise highly digestible tissues, that is, cellulose, through the action of hydrolytic enzymes (Ohgren et al., 2007). They have also shown that increased lignocellulosic biomass digestibility is associated with the degradation of lignin (Mukherjee and Nandi, 2004). The results of this study indicate that *T. versicolor* sdu-4 can selectively degrade lignin over cellulose in CS. The maximum hemicellulose loss was up to 22%. Hemicellulose degradation by white rot fungi also removes the physical protective coat of cellulose, which consequently improves cellulose digestibility (Ohgren et al., 2007). There was no direct correlation between the titers of enzymatic activities and the component weight loss of the substrate. These observations are in agreement with previous findings (Levin et al., 2008; Xu et al., 2009).

Plot of predicted versus actual values for lignocellulolysis (Figure 4) showed a similarity between the predicted and the actual results. The plots of the predicted and actual values were all approximately linear. This demonstrates that the response surface methodology has good predictability and accuracy in optimizing the multi-factor bioprocess.

The white rot fungus *T. versicolor* sdu-4 produces laccase as its main ligninolytic enzyme and neither LiP nor MnP was detected, even though it could effectively degrade lignin. This clearly indicates that laccase is essential for lignin degradation by *T. versicolor* sdu-4. This observation also confirms previous findings (Eggert et al., 1997). Thus, *T. versicolor* sdu-4 is an excellent model organism for elucidating the role of laccase in lignin degradation. Further research is needed to determine the ligninolytic mechanism of *T. versicolor* sdu-4.

Response for crude protein content

The experimental data was analyzed through multiple regression analysis, as derived from Equation 1. The predicted response for CP (Y_7) is listed further:

$$Y_7 = 6.57 + 0.51X_1 - 0.045X_2 + 0.75X_3 - 0.02X_1X_2 + 0.18X_1X_3 + 0.00X_2X_3 + 0.16X_1^2 + 0.016X_2^2 - 0.43X_3^2 \quad (8)$$

The CP content of CS was well optimized. The fermented straw contained the highest CP content (7.8%) with 10 mg of glucose and 4 µM of $CuSO_4$/g CS at 90% moisture content (Figure 5). All the three linear (X_1, X_2 and X_3), one interactive (X_1X_3) and two squared (X_1^2 and X_3^2) effects significantly affected the CP content of CS, whereas interactive (X_1X_2 and X_2X_3) and squared (X_2^2) effects were not significant (Table 3). The curve of predicted versus actual CP content (Figure 6) showed a high concordance between the predicted and the actual results, which confirms the accuracy and validates the response surface quadratic model in the optimization processes.

Fungi can be cultivated on agricultural straw; they grow fast and produce large amounts of cells rich in proteins that commonly contain all the essential amino acids, in addition to favorably high vitamin and mineral levels (Villas-Bôas et al., 2002). Thus, the microbial conversion of agricultural straw is a practical and promising alternative for increasing their nutritional value, transforming them into animal feed and ultimately producing a value-added product. In this study, *T. versicolor* sdu-4 grew fast, covering the entire surface of the CS within 5 to 6 days. Under optimal conditions, an overall 2-fold increase in CP content was achieved in the CS after 21 days of incubation compared with that in the raw CS (3.9%). Based on the results obtained, we can conclude that *T. versicolor* sdu-4 is an appropriate strain for use as inoculum in CS to increase its nutritional value, especially in terms of CP.

T. versicolor sdu-4 is an efficient organism for laccase production. LiP and MnP were not detected in solid culture of *T. versicolor* sdu-4 on CS. Moderate xylanase

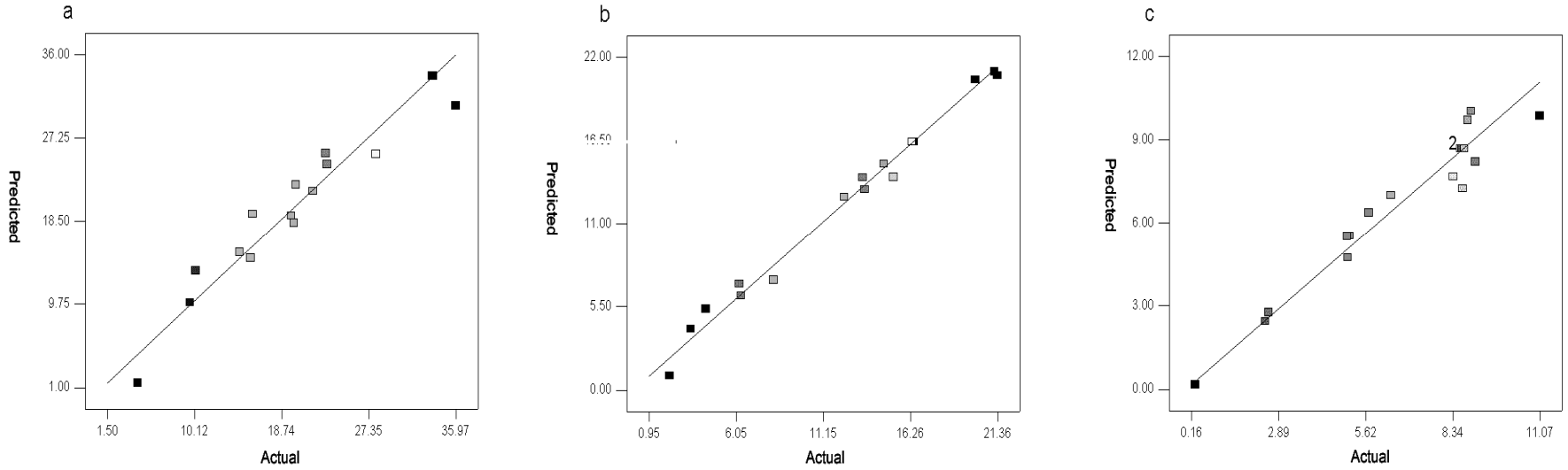

Figure 4. Plot of predicted versus actual values for lignocellulolysis. A, Plot of predicted versus actual lignin loss; B, plot of predicted versus actual hemicellulose loss; C, plot of predicted versus actual cellulose loss.

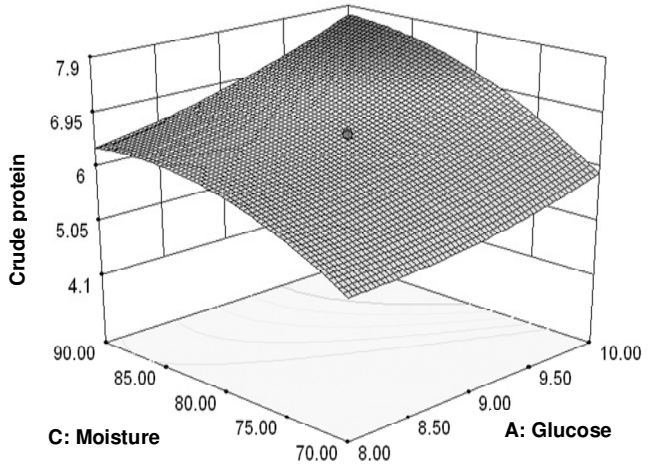

Figure 5. Response surface model graph for maximum crude protein content (A: glucose = 10 mg/g CS and C: moisture = 90%; hold value B: $CuSO_4$ = 4.5 µM/g CS).

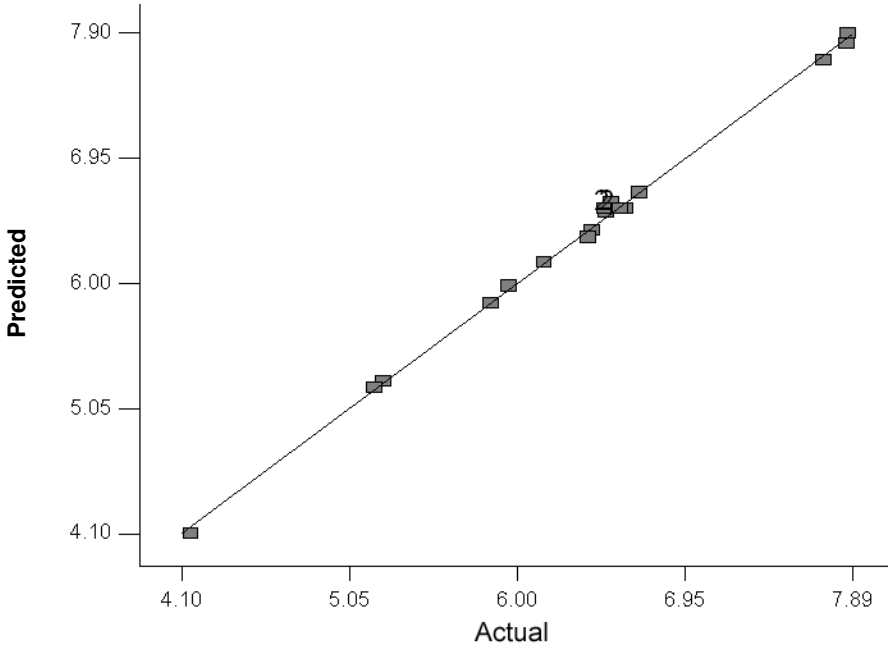

Figure 6. Plot of predicted versus actual crude protein content.

was produced during CS degradation but CMCase activity was very low. *T. versicolor* sdu-4 selectively degrades lignin in CS with minimal cellulose loss. A further increase in CP content was produced in the CS after SSF using *T. versicolor* sdu-4. This study may prove useful in designing an experimental setup for selectively degrading lignin and improving the nutritional value of agricultural wastes.

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