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ORIGINAL RESEARCH

Screening and optimization of parameters affecting fungal pretreatment of oil palm empty fruit bunch (EFB) by experimental design

Agarat Kamcharoen · Verawat Champreda · Lily Eurwilaichitr · Piyarat Boonsawang

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Abstract In the present study, various white-rot fungi were used for the pretreatment of oil palm empty fruit bunch (EFB) using solid-state cultivation. The results showed that *Trametes versicolor* TISTR 3224 gave the highest selectivity value (the ratio of lignin degradation to cellulose degradation) of 1.57. In comparison, *Trametes* sp. BCC 8729, *Phanerochaete chrysosporium* ATCC 24725, *Marasmius* sp. BCC 9542 and *Xylaria* sp. BCC 7749 gave selectivity of 0.60, 0.59, 0.30 and 0.06, respectively. Screening parameters for the fungal pretreatment of EFB using *T. versicolor* TISTR 3224 was studied by Plackett–Burman design (PBD). It indicated that the moisture content and co-substrate gave a positive effect on the lignin degradation, while EFB concentration had a negative effect on cellulose degradation. The optimum conditions for lignin degradation obtained from Box–Behnken statistical experimental design (BBD) were 80 % moisture content, 2.29 % wheat flour and 23.3 % EFB. Under this condition, 15.6 % of delignification was obtained. After an enzymatic hydrolysis, the digestibility of fungal treated EFB under the optimum condition achieved 1.34-fold compared with untreated EFB.

A. Kamcharoen · P. Boonsawang (✉)
Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
e-mail: piyarat.b@psu.ac.th

V. Champreda · L. Eurwilaichitr
Bioresources Technology Research Unit, Enzyme Technology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand

Keywords Box–Behnken design · Plackett–Burman design · Fungal pretreatment · Oil palm empty fruit bunch (EFB) · Deliginification

Introduction

Oil palm (*Elaeis guineensis*) is one of the most economical oil crops. The process of palm oil production has generated empty fruit bunches, fiber and palm shell as wastes [1]. With the increasing demand for energy, biofuel from renewable raw materials has been attractive because it is easily accessible, locally abundant and rich in lignocelluloses [2]. Recently, attempts have been made to apply EFB for bioethanol production [3–7]. Generally, bioethanol production from lignocellulosic materials employs three major steps: pretreatment for breakdown of lignin and opening up the crystalline structure of cellulosic materials; hydrolysis for fermentable sugar production; and bioconversion of fermentable sugar produced to bioethanol [8]. Although chemical and physicochemical pretreatments have been widely investigated, inhibitory compounds, e.g., furfural and hydroxymethyl furfural, are released and further affect the fermentation process [9, 10]. Therefore, biological pretreatment is interesting and has the additional advantages of simple technique and low pretreatment requirements resulting in low operating cost and environmentally friendly process [11]. White-rot fungi are important microorganisms involved in lignin degradation during pretreatment [12–14]. *Phanerochaete chrysosporium* [15–18] and *Trametes versicolor* [19–22] have been reported for the pretreatment of lignocellulosic materials. Moreover, Xylariaceous fungi and *Marasmius* sp. have been reported as lignin-degrading microorganisms [13, 23–25]. However, different white-rot fungi differed in their capabilities of cellulose and lignin

degradation from one biomass to another [26]. Some fungi not only degrade lignin effectively, but also consume cellulose simultaneously leading to low available cellulose for bioethanol production. A common measure of delignification efficiency is the selectivity value (SV) of a fungal pretreatment, defined as the ratio of lignin degradation (LD) to cellulose degradation (CD) [18, 27, 28]. A low SV means a relatively high cellulose loss during fungal pretreatment. Thus, the SV of a fungal pretreatment is used to screen white-rot fungi for biological pretreatment [28].

The limitation of biological pretreatment is a lower reaction rate and requires longer pretreatment time than chemical pretreatment [14]. Although the strategies of strain improvement may help resolve some of the drawbacks, the technical process is quite challenging. Another approach to improve the efficiency of biological pretreatment is through the optimization of nutrient and environmental cultivation to reach maximum lignin degradation and minimum cellulose degradation. Shi et al. [16] found that the moisture content and culture time affected the fungal pretreatment of cotton stalk using *P. chrysosporium*. Alam et al. [29] indicated that the moisture content, inoculum size and wheat flour as co-substrate affected ligninase production during the fungal pretreatment of oil palm biomass. Levin et al. [30] reported that the balance of cellulose and ligninolytic enzyme production during fungal pretreatment depended on pH, peptone and copper. Although, there have been many researches that studied the factors affecting fungal pretreatment, those involved a one-factor-at-a-time experiment or examination of only a few factors. Moreover, the screening of significant factors affecting the fungal pretreatment of EFB has not been studied. In this research, the systematic evaluation of the optimization for the fungal pretreatment was investigated. Statistically designed experiments are a powerful tool to get more information about the system being studied with a minimum number of experiments [31]. The Plackett–Burman design (PBD) has been frequently used for screening process variables that make the greatest impact on a process [32]. Response surface methodology (RSM) is a statistical and mathematical technique useful for developing, improving and optimizing the processes of an interest variable. RSM offers a large amount of information from a small number of experiments and reduces time [10, 33].

This research aims to identify the selective lignin-degrading white-rot fungus with high lignin degradation and low cellulose loss. Also, the optimum condition was studied to improve the efficiency of fungal pretreatment. PBD was used for screening the significant factors for fungal pretreatment during solid-state cultivation. Box–Behnken design was then applied to determine the optimum level of each of the significant factors for delignification with a high SV.

Methods

EFB preparation

EFBs were collected from Thai Tallow and Oil Co., Ltd., Thailand. The sample was dried and crushed into 5–10 mm fibrous length using a hammer mill, then ground to pass through a 1 mm screen (18 meshes) and kept for use in the whole experiment. The chemical composition of EFB, given on dry weight basis, was as follows: 37.6 % cellulose, 21.5 % hemicellulose and 19.0 % lignin [34].

Microorganisms

Phanerochaete chrysosporium ATCC 24725 was obtained from the Faculty of Agro-industry, Prince of Songkla University. *Xylaria* sp. BCC 7749, *Trametes* sp. BCC 8729 and *Marasmius* sp. BCC 9542 were received from the BIOTEC culture collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA). *Trametes versicolor* TISTR 3224 was purchased from Thailand Institute of Scientific and Technological Research (TISTR).

Inoculum preparation

Phanerochaete chrysosporium ATCC 24725 was maintained and grown on potato dextrose agar (PDA) at 37 °C for 7 days. *Xylaria* sp. BCC 7749, *Trametes* sp. BCC 8729, *Marasmius* sp. BCC 9542 and *Trametes versicolor* TISTR 3224 were maintained and grown on PDA at room temperature (28 °C) for 7 days [35].

Selection of fungi for the pretreatment of EFB

The fungal pretreatment was carried out in 250 ml Erlenmeyer flasks. The fermentation medium containing 4 g of dried EFB, 0.08 g of wheat straw and 15 ml of sodium acetate buffer (10 mM, pH 5.0) was sterilized at 121 °C for 15 min. Each flask was inoculated with four fungal pieces (1 cm^2) obtained from a PDA plate [35] and incubated for 14 days at the same temperature as mentioned for the inoculum preparation.

Screening parameters for the fungal pretreatment by PBD

PBD was employed to identify the significant factors for the fungal pretreatment by *T. versicolor* TISTR 3224. The experiment was conducted in 250 ml Erlenmeyer flasks. From PBD, 12 experimental runs with 7 variables including the initial moisture content (%), initial pH, inoculum

Table 1 Experimental definition for Plackett–Burman and Box–Behnken design for fungal pretreatment

Variables	Key	Plackett–Burman design		Box–Behnken design		
		Low level (−)	High level (+)	−1	0	+1
Moisture content (%)	M	60	80	60	70	80
Initial pH	P	4	6		5	
Inoculum size (%)	I	5	15		15	
Co-substrate (%)	C	0	3	1	2	3
EFB (%)	E	23	33	23	28	33
Incubation time (week)	T	1	3		3	
Mineral salt (%)	S	0	40		0	

size (%), wheat flour concentration (%), EFB concentration (%), incubation time (week) and mineral salt solution (%) were performed with low and high levels (Table 1). Each flask was inoculated with a final spore concentration of 1×10^5 spores/ml. The initial moisture content was adjusted with the addition of 10 mM sodium acetate buffer (pH 5). The pH and moisture contents were not adjusted during the fermentation process. All experiments were incubated at room temperature (28 °C) and repeated in triplicate.

PBD is based on the first-order model (Eq. 3) and the effect of each variable was determined using Eq. 3 [32].

$$Y = \beta_0 + \sum \beta_i x_i \tag{1}$$

$$E_{(x_i)} = \frac{2(\sum M_{i+} - \sum M_{i-})}{N} \tag{2}$$

where Y is the response, β_0 is the model intercept, β_i is the linear coefficient, x_i is the level of the independent variable, Ex_i is the standardized effect of the tested variable, M_{i+} and M_{i-} are the responses from trials where the variable (x_i) is presented at high and low levels, respectively, and N is the number of experimental runs. The analysis of linear regression was carried out using SPSS version 17.

Box–Behnken design (BBD) for the optimization of *T. versicolor* pretreatment

The significant variables from PBD analysis were subsequently optimized using the BBD with three levels (low, medium and high, coded as −1, 0, and +1). Table 1 shows the factor codes and values used in this experiment. All experiments were carried out in triplicate and the percentages of LD, CD and SV were taken as responses. The responses were fitted to the following second-order polynomial model (Eq. 3):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \tag{3}$$

where Y is the response, X_1 the concentration of co-substrate (%), X_2 the percentage of moisture content (%) and X_3 the concentration of EFB (%). β_0 is intercept β_1 β_2 β_3 β_{12} β_{13} β_{23} β_{11} β_{22} β_{33}.

Enzymatic hydrolysis condition

The enzymatic hydrolysis of pretreated EFB under the optimum condition obtained from BBD was carried out in triplicate to determine cellulose digestibility. The enzymatic hydrolysis was conducted in a 1.5 ml microtube with a final volume of 1 ml, containing 50 mg of pretreated EFB, 50 mM sodium citrate buffer of pH 4.8 and 50 µl of 5 % sodium azide (to inhibit microbial contamination). The samples were hydrolyzed by mixed enzymes: 10 FPU/g substrate of cellulase (Celluclast 1.5 L, Sigma) (with a cellulase activity of 61.7 FPU/ml and a xylanase activity of 1,307 U/ml), 267 U/g substrate of xylanases (Optimash BG, Japan) (with an enzyme activity of 1,374.6 U/ml) and 0.66 U/g substrate of β-glucosidase (Novozyme 188, Sigma) (with an enzyme activity of 13.2 U/ml). The reaction mixtures were incubated at 50 °C and taken after 72 h of saccharification. Then, the samples were centrifuged at 10,000 rpm for 10 min, separated and stored at − 20 °C for a reducing sugar determination.

Analytical methods

After the pretreatment, the residual biomass was filtered and dried at 105 °C to a constant weight. Weight losses in total solids were calculated from the initial and final dry weights. Then the samples were analyzed for acid detergent fiber, lignin and cellulose [34]. LD and CD were defined as the percentage of total lignin and total cellulose reduced during pretreatment [14, 18]. SV of degradation was calculated as the ratio of LD to CD [27].

Glucose concentration was analyzed using a high-performance liquid chromatography system (Ultimate-3000 RS) equipped with a refractive index detector (RI-Shodex) and an Aminex HPX-87H column (BioRad). The column temperature was set at 65 °C. Samples were eluted at a flow rate of 0.5 ml/min with 5 mM H_2SO_4. All analyses were performed in triplicate. Cellulose digestibility was calculated using the following equation [7]:

$$\text{Cellulose digestibility}(\%) = \frac{\text{amount of glucoseproduced} \times 0.9 \times 100}{\text{amount of cellulose in pretreated biomass}}$$

Table 2 Weight loss and degradation efficiency after 2 weeks of pretreatment of empty fruit bunch by different white-rot fungi

Microorganisms	Weight loss (%)	Lignin degradation (LD) (%)	Cellulose degradation (CD) (%)	Selectivity value (SV)
Marasmius sp. BCC 9542	$3.51^{a} \pm 0.08$	$0.95^{a} \pm 0.09$	$3.14^{a} \pm 0.09$	$0.30^{a} \pm 0.02$
Xylaria sp. BCC 7749	$13.03^{b} \pm 0.39$	$1.15^{a} \pm 0.53$	$19.67^{c} \pm 0.43$	$0.06^{b} \pm 0.03$
Trametes sp. BCC 8729	$12.65^{b} \pm 0.49$	$13.21^{c} \pm 0.58$	$20.85^{c} \pm 0.53$	$0.63^{c} \pm 0.01$
Trametes versicolor TISTR 3224	$4.97^{a} \pm 0.48$	$8.86^{b} \pm 0.70$	$5.68^{b} \pm 0.72$	$1.57^{d} \pm 0.08$
Phanerochaete chrysosporium ATCC 24725	$22.41^{c} \pm 0.76$	$18.79^{d} \pm 0.97$	$31.88^{d} \pm 0.81$	$0.59^{c} \pm 0.02$

Data are means of triplicates; letters on the right side of the data indicate significant levels, descending by alphabetical order

Statistical analysis

The software SPSS version 17 was used for statistical and linear regression analysis. All component degradation data were subjected to analysis of variance (ANOVA). Multiple comparison tests were performed with Turkey's test.

Results and discussion

Effect of fungal stains on the pretreatment of EFB

The weight and component losses of EFB pretreated with white-rot fungi are shown in Table 2. To select the selective lignin-degrading white-rot fungus, a high SV greater than 1.0 was considered [27]. Although *P. chrysosporium* ATCC 24725 had the highest cellulose and lignin degrading ability (31.9 and 18.8 %, respectively), it gave an SV of 0.59. It indicated that *P. chrysosporium* could simultaneously degraded both lignin and cellulose. It was presumed that cellulose might be hydrolyzed and consumed by *P. chrysosporium* during pretreatment of lignocellulosic biomass [14].

In this study, only *T. versicolor* TISTR 3224 was the selective lignin-degrading fungus with the highest SV of 1.57. A high SV means better prospects for preferential lignin degradation and a low SV means a relatively high cellulose degradation during biological pretreatment leading to low available cellulose [36]. However, a high amount of remaining cellulosic biomass after fungal pretreatment is desired for further hydrolysis and ethanol production. Consequently, *T. versicolor* TISTR 3224 was selected for further studying the optimization of environmental conditions for fungal pretreatment.

Screening parameters for fungal pretreatment by *T. versicolor* TISTR 3224 using PBD

The experimental matrix and the values of responses are presented in Table 3. The statistical analysis of the responses for the fungal pretreatment is shown in Table 4. All the examined factors did not significantly affect SV and had a low confidence value (<85 %). It indicated that the total variations were not satisfactorily explained by this model and these variables were considered insignificant. The factors that had a significant effect on the LD were moisture content and wheat flour as co-substrate, with a high confidence value (>95 %). In the case of CD, the co-substrate was the only significant factor with a high confidence value (>95 %). It was observed that the co-substrate affected both LD and CD with a standardized effect of 8.23 and 7.10, respectively. These positive effects mean that if the concentrations of wheat flour are increased, the cellulose loss can be increased as well. The efficiency of pretreatment is not considered but only lignin degradation; however, the availability of cellulose is also an important criterion for further hydrolysis and ethanol production. Consequently, the optimum value of wheat flour concentration was further studied.

Also, the moisture content was considered because it had a significantly positive effect on LD, with a standardized effect of 3.57. The moisture content was a key factor for fungal growth in solid-state cultivation. Too low moisture contents could limit fungal delignification without providing sufficient water to fungal growth [14]. Higher moisture contents cause clogging interparticle spaces, limited oxygen circulation, often inhibited aerobic solid-state cultivation and increased susceptibility to bacterial contamination [16, 37]. Another effect of high moisture content is reduced solid loading for the fungal pretreatment [14]. Therefore, the optimum amount of moisture content for the high LD was further investigated.

Although EFB concentration gave a low confidence value for both LD and CD (5.8 and 90 %, respectively), it gave the highest negative effect (Ex_i value of −3.24) for CD (Table 4). The negative effect means that if the EFB concentration was added on a decreasing trend, the CD could be improved. Accessibility of cellulose for enzymatic hydrolysis is one of the major factors influencing the hydrolysis process. The optimum EFB concentration was also examined.

According to statistical analysis, the initial pH, inoculum size, incubation time and mineral solution were the

Table 3 PBD variables in code levels with lignin degradation (LD), cellulose degradation (CD) and selectivity value (SV) as responses

Run	M	P	I	C	E	T	S	LD (%)	CD (%)	SV
1	+	−	+	−	−	−	+	10.45 ± 0.92	6.69 ± 1.86	1.64 ± 0.59
2	+	+	−	+	−	−	−	21.21 ± 2.63	13.82 ± 2.01	1.56 ± 0.42
3	−	+	+	−	+	−	−	10.43 ± 0.11	4.88 ± 0.12	2.14 ± 0.03
4	+	−	+	+	−	+	−	19.85 ± 1.56	16.45 ± 0.13	1.21 ± 0.10
5	+	+	−	+	+	−	+	16.52 ± 3.09	15.65 ± 1.43	1.07 ± 0.30
6	+	+	+	−	+	+	−	13.25 ± 2.44	2.35 ± 1.10	6.61 ± 4.12
7	−	+	+	+	−	+	+	17.20 ± 0.45	13.96 ± 1.43	1.24 ± 0.16
8	−	−	+	+	+	−	+	14.13 ± 2.36	11.76 ± 0.51	1.20 ± 0.15
9	−	−	−	+	+	+	−	16.04 ± 1.94	8.61 ± 0.92	1.89 ± 0.43
10	+	−	−	−	+	+	+	9.69 ± 2.17	5.97 ± 0.35	1.64 ± 0.46
11	−	+	−	−	−	+	+	6.65 ± 1.02	8.93 ± 1.56	0.75 ± 0.02
12	−	−	−	−	−	−	−	5.11 ± 1.56	8.81 ± 1.02	0.59 ± 0.25

Table 4 Level of the variables and statistical analysis of PBD on lignin degradation (LD), cellulose degradation (CD) and selectivity value (SV) as responses

Variables	Code	Effect (Ex_i)	Coefficient	*t*-value	*P* value	Confidence (%)
LD:						
Moisture content	M	3.57	1.784	4.065	0.015	98.5
pH	P	1.66	0.832	1.899	0.131	86.9
Inoculum size	I	1.68	0.841	1.916	0.128	87.2
Co-substrate	C	8.23	4.114	9.375	0.001	99.9
EFB concentration	E	−0.07	−0.034	−0.78	0.942	5.80
Incubation time	T	0.81	0.402	0.917	0.411	58.9
Mineral solution	S	−1.87	0.938	−2.136	0.100	90.0
CD:						
Moisture content	M	0.66	0.332	0.433	0.687	31.3
pH	P	0.22	0.108	0.141	0.894	10.6
Inoculum size	I	−0.95	−0.475	−0.620	0.569	43.1
Co-substrate	C	7.10	3.552	4.638	0.010	99.0
EFB concentration	E	−3.24	−1.62	−2.115	0.102	89.8
Incubation time	T	−0.89	−0.445	−0.581	0.592	40.8
Mineral solution	S	1.34	0.670	0.875	0.431	56.9
SV:						
Moisture content	M	0.99	0.493	1.389	0.237	76.3
pH	P	0.87	0.433	1.220	0.289	71.1
Inoculum size	I	1.09	0.545	1.534	0.200	80.0
Co-substrate	C	−0.87	−0.433	−1.22	0.289	71.1
EFB concentration	E	1.26	0.63	1.774	0.151	84.9
Incubation time	T	0.85	0.428	1.206	0.294	70.6
Mineral solution	S	−1.08	−0.538	−1.516	0.204	79.6

insignificant factors with low confidence levels (<90 %) for all responses, except mineral solution in the LD model (Table 4). However, this factor gave a negative effect on LD, which means that if a lower mineral solution was added LD could be improved. This might be due to the sufficient inorganic salts in natural lignocellulosic materials required for fungal growth [16]. For the inoculum size between 5 and 15 %, this study indicated that only a very small quantity of mycelium was enough to inoculate the substrate for fungal pretreatment. An increase of inoculum quantity could shorten the time required for substrate colonization and also aid the inoculated fungus to displace any other microbes that may be present [37]. However, a high inoculum size might lead to an exhaustion of nutrients in the fermentation medium [29].

Optimization of key parameters using Box and Behnken design

BBD was adopted to establish the optimum point of each variable affecting LD, CD and SV. The results of the observed and predicted responses are presented in Table 5. A second-order polynomial model was fitted to the experimental data and constructed using the Design Expert software. The models were developed in the following equations (Eqs. 3, 5, 3) with only significant coefficients ($P < 0.05$):

$$Y_1 = 12.21 + 3.61X_1 + 1.09X_2 + 0.11X_3 - 3.44X_1^2 - 0.08X_2^2 - 0.99X_3^2 - 0.62X_1X_2 + 1.08X_1X_3 - 2.29X_2X_3 \quad (4)$$

$$Y_2 = 13.29 + 3.98X_1 - 0.43X_2 - 0.74X_3 \quad (5)$$

$$Y_3 = 1.01 + 0.08X_1 + 0.12X_2 + 0.04X_3 - 0.27X_1^2 - 0.05X_2^2 - 0.19X_3^2 - 0.20X_1X_2 + 0.03X_1X_3 - 0.11X_2X_3 \quad (6)$$

where Y_1 = lignin degradation (%), Y_2 = cellulose degradation (%), Y_3 = selectivity value (%), X_1 = wheat flour concentration, X_2 = moisture content and X_3 = EFB concentration.

Table 6 shows the ANOVA for the response surface model. The significance of the models was also confirmed by high F values of the regression with low probability values (P value <0.05). However, a lack of fit F values for LD, CD and SV models was 0.43, 1.34 and 0.074, respectively, with high P value (>0.05). It implied that it was not significant relative to the pure error. There were 75.0, 44.7 and 97.0 % chances that a lack of fit F value could occur due to experimental errors from the LD, CD and SV models, respectively. Non-significant lack of fit would be appropriate for this experiment. Also, a low probability value (P value <0.05) obtained from the

Table 5 Box–Behnken design matrix along with the experimental (exp.) and predicted (pred.) values of lignin degradation (LD), cellulose degradation (CD) and selectivity value (SV) after the pretreatment of EFB by *T. versicolor* TISTR 3224

Trials	X_1	X_2	X_3	LD (%)		CD (%)		SV	
				Exp.	Pred.	Exp.	Pred.	Exp.	Pred.
1	1	60	28	3.16	3.37	10.41	9.75	0.30	0.29
2	3	60	28	11.55	11.82	14.39	17.70	0.80	0.85
3	1	80	28	7.06	6.79	7.15	8.88	0.99	0.94
4	3	80	28	12.97	12.77	19.16	16.84	0.68	0.69
5	1	70	23	4.20	5.13	9.41	10.06	0.45	0.46
6	3	70	23	9.34	10.20	15.47	18.01	0.60	0.55
7	1	70	33	4.07	3.21	9.34	8.57	0.44	0.49
8	3	70	33	13.50	12.57	19.10	16.53	0.71	0.70
9	2	60	23	8.78	7.65	17.87	14.47	0.49	0.50
10	2	80	23	15.07	14.41	16.39	13.60	0.92	0.96
11	2	60	33	11.79	12.45	14.14	12.98	0.83	0.80
12	2	80	33	8.93	10.06	10.64	12.12	0.84	0.83
13	2	70	28	11.37	12.21	14.89	13.29	0.76	1.01
14	2	70	28	15.01	12.21	13.38	13.29	1.12	1.01
15	2	70	28	12.91	12.21	10.00	13.29	1.29	1.01
16	2	70	28	9.55	12.21	10.93	13.29	0.87	1.01

X_1 wheat flour concentration (%), X_2 moisture content (%), X_3 EFB concentration (%)

Table 6 The ANOVA of Box–Behnken design for the lignin degradation (LD), cellulose degradation (CD) and selectivity value (SV) after the pretreatment of EFB by *T. versicolor* TISTR 3224

Source	Sum of squares	DF	Mean square	F value	P value	R^2	Adequate precision
LD:							
Model	192.26	9	21.36	5.58	0.0244	0.8932	7.243
Residual	22.98	6	3.83				
Lack of fit	6.86	3	2.29	0.43	0.7495		
Pure error	16.12	3	5.37				
Corrected total	215.24	15					
CD:							
Model	132.43	3	44.14	6.99	0.0057	0.6360	7.509
Residual	75.79	12	6.32				
Lack of fit	60.73	9	6.75	1.34	0.4474		
Pure error	15.05	3	5.02				
Corrected total	208.21	15					
SV:							
Model	0.85	9	0.094	3.09	0.0916	0.8225	5.230
Residual	0.18	6	0.031				
Lack of fit	0.013	3	0.004	0.074	0.9698		
Pure error	0.17	3	0.057				
Corrected total	1.03	15					

regression ANOVA demonstrated that the quadratic model of LD (P value = 0.0244) and linear model of CD (P value = 0.0057) were significant. In contrast, a probability value of the quadratic model of SV (P value = 0.0916) was not significant. For this reason, the SV model was not considered.

The goodness of fit of the model was checked using the determination coefficient (R^2). The models of LD and CD were given R^2 of 0.893 and 0.636, respectively. This explained the high level of correlation between the experimental and predicted values for the LD model. The adequate precision of 7.24 was high compared to the desirable value (greater than 4) [38, 39]. Therefore, this model can be used to navigate the design space. In contrast, the model of CD gave low R^2, though a high adequate precision (7.509) was obtained. Therefore, the CD model cannot be used for the prediction of fungal pretreatment.

Figure 1 shows the interactive effect of each variable on the percentage of LD in relation to the moisture content, wheat flour and EFB concentration. The highest LD of 14.7 % was predicted at the optimum condition of an initial moisture content of 80 %, wheat flour of 2.29 % and EFB concentration of 23.3 %. To verify the optimized results, a set of experiments was performed. LD of 15.6 % and CD of 12.5 % were obtained with SV of 1.25 and less than 5.0 % solid loss. The observed LD was slightly higher than the predicted LD with an error of 0.06 %. Physicochemical pretreatment of EFB such as ionic liquid treatment [7], NH_3 treatment [5], bisulfate treatment [40] and sequential acid/alkaline treatment [41] achieved an LD of 33.5–70.7 % at 60–121 °C. Although the delignification of EFB (15.6 %) obtained from this study was lower than from physicochemical pretreatments, this fungal pretreatment was conducted under low temperature (at 28 °C) leading to lower energy input and lower operating cost. Delignification of EFB using *T. versicolor* TISTR 3224 in this study seems rather average compared to other lignocellulosic biomasses, eucalyptus wood chip [21], oil palm trunk [22], wheat straw [35], rubber wood [36], corn straw [42], hornbeam wood chip [43] and bamboo [44], which achieved a delignification of 9.35–24.4 % in 10–32 days. Compared with other fungi for the pretreatment of EFB, the ability of *T. versicolor* TISTR 3224 for delignification was higher than that of *Pleurotus floridanus* (a lignin removal of 0.48 %) as reported by Isroi et al. [45] and *Phanerochaete chrysosporium* ATCC32629 (a lignin removal of 5.89 %) as reported by Hamisan et al. [46]. However, Harmini et al. [47] found that *Pleurotus floridanus* LIPIMC996 removed lignin from EFB in approximately 25–27 %. Different strains might have different abilities of delignification. Consequently, it indicated that *T. versicolor* TISTR 3224 has the potential to be used for biological pretreatment of EFB.

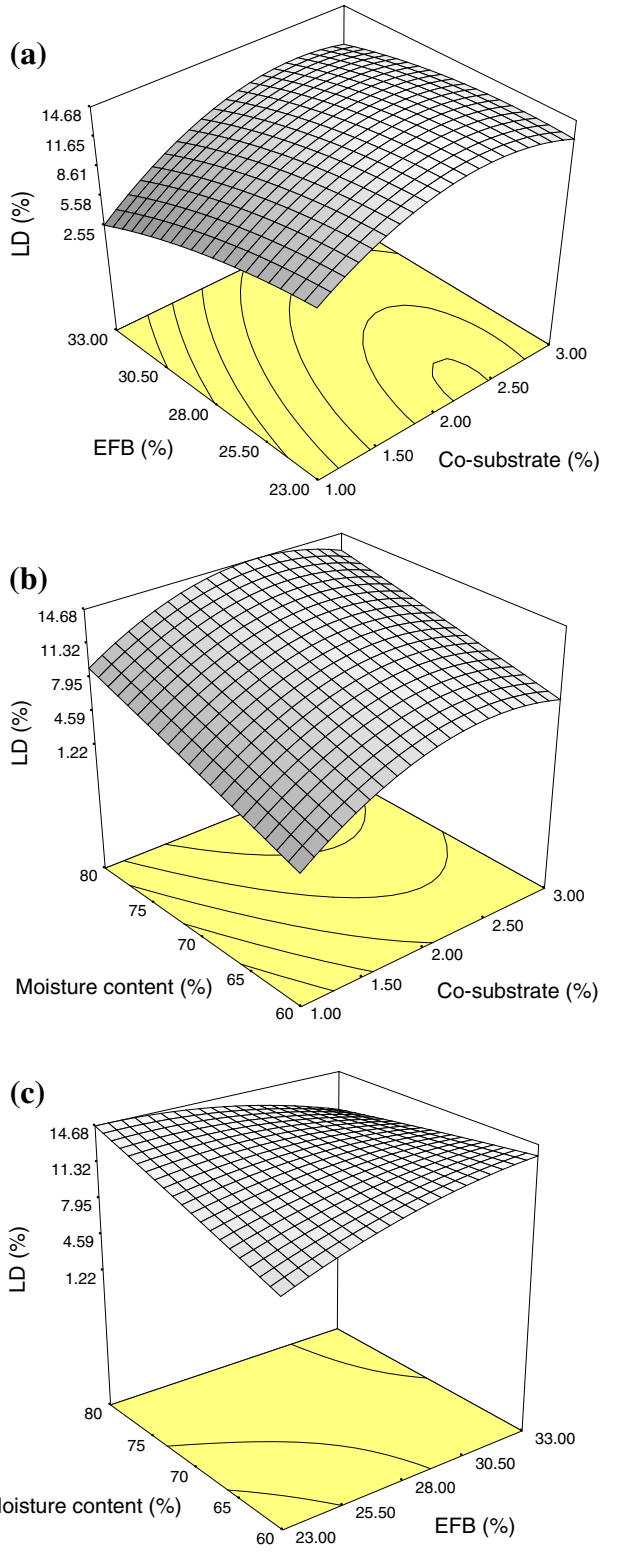

Fig. 1 Three-dimensional surface plot showing interactive effects between (**a**) EFB concentration and wheat flour at an initial moisture content of 80 %, (**b**) moisture content and wheat flour at EFB concentration of 23.3 % and (**c**) moisture content and EFB concentration at wheat flour 2.29 % on lignin degradation (LD)

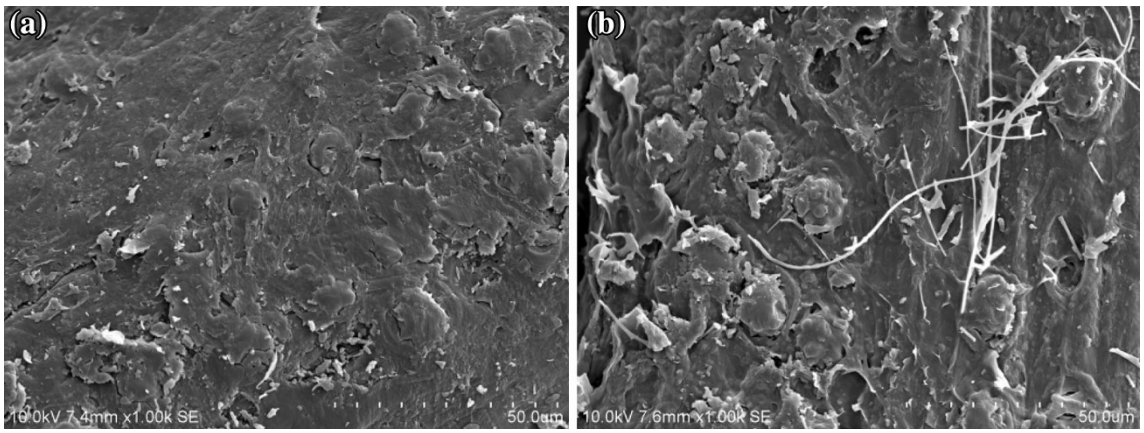

Fig. 2 Scanning electron micrographs (×1,000) of the untreated EFB (**a**) and EFB pretreated with *T. versicolor* TISTR 3224 (**b**)

In this study, SV cannot be predicted by the model and the optimized value cannot be statistically analyzed. However, the highest SV of 1.29 was observed with the 28 % EFB concentration and 2 % wheat flour concentration at 70 % moisture content (Trial 15) (Table 2). It indicated that there was no difference between the SV obtained under this condition and under optimum condition (23.3 % EFB concentration and 2.29 % wheat flour concentration at 80 % moisture content). For an economic approach, low wheat flour concentration and moisture content might be preferred.

Structure analysis and enzymatic digestibility of fungal-treated EFB (fEFB)

The surface morphology of the untreated EFB and fEFB was investigated by scanning electron microscopy (SEM). SEM images show the smooth and packed fibers of the untreated EFB (Fig. 2a). After fungal pretreatment, the mycelium covered and penetrated into the surface of EFB, which led to more porosity and roughness than the untreated EFB (Fig. 2b). After an enzymatic hydrolysis, the cellulose digestibility of the fungal pretreated EFB was higher than that of the untreated EFB (Fig. 3). After 72 h of saccharification, the untreated EFB and fEFB achieved 9.04 and 12.6 % of cellulose digestibility, respectively. It indicated that the fungal pretreatment increased cellulose digestibility by about 1.34-fold from the untreated EFB. According to Isroi et al. [45], fungal pretreatment of EFB affected the structural changes in the lignin and loss of aromatic units. Consequently, fungal pretreatment caused a further increase in the surface area and susceptibility of EFB to enzymatic saccharification.

However, a long fungal pretreatment (3 weeks) was required to achieve high lignin degradation. Physical or chemical pretreatments may take a shorter time than a biological method [14]. However, the solid loss from

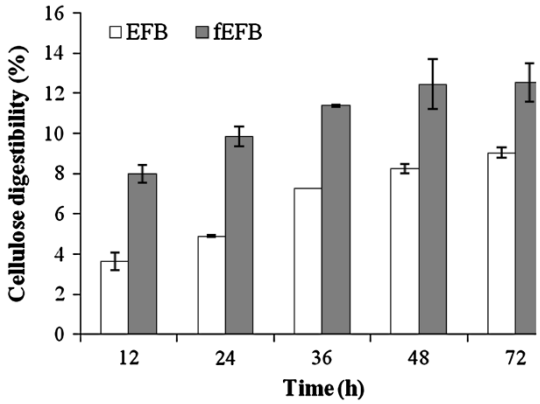

Fig. 3 Cellulose digestibilities of untreated EFB and fEFB after saccharification (50 °C, pH 4.8) of 10 FPU/g substrate with cellulase

those methods was high with generation of pollutants. Also, the high energy input or production cost must be taken into account [48]. The combination of fungal pretreatment with physical and/or chemical methods has been reported to synergistically improve enzymatic digestibility with an environmental friendly and energy-efficient process [27]. Therefore, a future study for the suitable combination of fungal pretreatment with other methods might be required.

Conclusions

Trametes versicolor TISTR3224 showed a great selective lignin-degrading ability on biological pretreatment of EFB under solid-state cultivation. With statistical analysis using PBD and BBD, maximum LD was achieved with 23 % EFB concentration and 2.29 % wheat flour concentration at 80 % moisture content. With the consideration of the high SV and an economic benefit, 28 % EFB concentration and 2 % wheat flour concentration at 70 % moisture content

might be preferred. However, a relatively low efficiency and long residence time were still the major disadvantages of e fungal pretreatment. New strategies should be used to overcome these weak aspects. A two-step pretreatment consisting of fungal pretreatment and other methods may be a good approach to enhance the efficiency of biological pretreatment and lower the requirements.

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Conflict of interest The authors declare that they have no competing interests.

Authors' contributions AK and PB co-conceived the idea and carried out the design of the study. AK carried out laboratory experiments, the experimental and statistical analysis and drafted the manuscript. VC, LE and PB supervised the work. PB corrected the manuscript. All authors read and eventually approved the final manuscript to be submitted.

References

1. Kun, Y.C., Abdullah, A.M.H.: Simulation of total dust emission from palm oil mills in Malaysia using biomass fuel composition and dust collector efficiency models. Int. J. Energy Environ. Eng. **4**, 19 (2013)
2. Alam, M.Z., Mamun, A.A., Qudsieh, I.Y., Muyibi, S.A., Salleh, H.M., Omar, N.M.: Solid state bioconversion of oil palm empty fruit bunches for cellulase enzyme production using a rotary drum bioreactor. Biochem. Eng. J. **46**, 61–64 (2009)
3. Lau, M.J., Lau, M.W., Gunawan, C., Dale, B.E.: Ammonia fiber expansion (AFEX) pretreatment, enzymatic hydrolysis, and fermentation on empty palm fruit bunch fiber (EPFEF) for cellulosic ethanol production. Appl. Biochem. Biotechnol. **162**, 1847–1857 (2010)
4. Han, M., Kim, Y., Kim, S.W., Choi, G.W.: High efficiency bioethanol production from OPEFB using pilot pretreatment reactor. J. Chem. Technol. Biotechnol. **86**, 1527–1534 (2011)
5. Jung, Y.H., Kim, I.J., Han, J.I., Choi, I.G., Kim, K.H.: Aqueous ammonia pretreatment of oil palm empty fruit bunches for ethanol production. Bioresour. Technol. **102**, 9806–9809 (2011)
6. Piarpuzán, D., Quintero, J.A., Cardona, C.A.: Empty fruit bunches for oil palm as a potential raw material for fuel ethanol production. Biomass Bioenergy. **35**, 1130–1137 (2011)
7. Katinonkul, W., Lee, J.S., Ha, S.H., Park, J.Y.: Enhancement of enzymatic digestibility of oil palm empty fruit bunch by ionic-liquid pretreatment. Energy **47**, 11–16 (2012)
8. Hamisan, A.F., Aziz, S.A., Kamaruddin, K., Shah, U.K.M., Shahab, N., Hassan, M.A.: Delignification of oil palm empty fruit bunch using chemical and microbial pretreatment methods. Int. J. Agric. Res. **4**, 250–256 (2009)
9. Goncalves, F.A., Sanjinez-Argandona, E.J., Fonseca, G.G.: Cellulosic ethanol and its co-products from different substrates, pretreatments, microorganisms and bioprocesses: a review. Nat. Sci. **5**, 624–630 (2013)
10. Marasabessy, A., Kootstra, A.M.J., Sanders, J.P.M., Weusthuis, R.A.: Dilute H_2SO_4-catalyzed hydrothermal pretreatment to enhance enzymatic digestibility of *Jatropha curcas* fruit hull for ethanol fermentation. Int. J. Energy Environ. Eng. **3**, 15 (2012)
11. Keller, F.A., Hamilton, J.E., Nguyen, Q.A.: Microbial pretreatment of biomass: potential for reducing severity of thermochemical biomass pretreatment. Appl. Biochem. Biotechnol. **105**, 27–41 (2003)
12. Howard, R.L., Abotsi, E., Jansen van Rensburg, E.L., Howard, S.: Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr. J. Biotechnol. **2**, 602–619 (2003)
13. Sánchez, C.: Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol. Adv. **27**, 185–194 (2009)
14. Wan, C., Li, Y.: Fungal pretreatment of lignocellulosic biomass. Biotechnol. Adv. **30**, 1447–1457 (2012)
15. Istek, A., Sivrikaya, H., Eroglu, H., Gulsoy, S.K.: Biodegradation of *Abies bornmulleriana* (Mattf.) and *Fagus orientalis* (L.) by the white rot fungus *Phanerochaete chrysosporium*. Int. Biodeterior. Biodegrad. **55**, 63–67 (2005)
16. Shi, J., Chinn, M.S., Sharma-Shivappa, R.R.: Microbial pretreatment of cotton stalks by solid state cultivation of *Phanerochaete chrysosporium*. Bioresour. Technol. **99**, 6556–6564 (2008)
17. Bak, J.S., Ko, J.K., Choi, I.G., Park, Y.C., Seo, J.H., Kim, K.H.: Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. Biotechnol. Bioeng. **104**, 471–482 (2009)
18. Shi, J., Sharma-Shivappa, R.R., Chinn, M., Howell, N.: Effect of microbial pretreatment on enzymatic hydrolysis and fermentation of cotton stalks for ethanol production. Biomass Bioenergy **33**, 88–96 (2009)
19. Hwang, S.S., Lee, S.J., Kim, H.K., Ka, J.O., Kim, K.J., Song, H.G.: Biodegradation and saccharification of wood chips of *Pinus strobus* and *Liriodendron tulipifera* by white rot fungi. J. Microbiol. Biotechnol. **18**, 1819–1825 (2008)
20. Dinis, M.J., Bezerra, R.M.F., Nunes, F., Dias, A.A., Guedes, C.V., Ferreira, L.M.M., Cone, J.W., Marques, G.S.M., Barros, A.R.N.: Modification of wheat straw lignin by solid state fermentation with white-rot fungi. Bioresour. Technol. **100**, 4829–4835 (2009)
21. Gupta, R., Saini, V.K., Bhatt, R.P., Thapliyal, B.P., Naithani, S.: Influence of mechanical operation on the biodelignification of *Eucalyptus tereticornis* by *Trametes versicolor*. Cellul. Chem. Technol. **47**, 759–764 (2013)
22. Singh, P., Sulaiman, O., Hashim, R., Peng, L.C., Singh, R.P.: Evaluating biopulping as an alternative application on oil palm trunk using the white-rot fungus *Trametes versicolor*. Int. Biodeterior. Biodegrad. **82**, 96–103 (2013)
23. Pointing, S.B., parungao, M.M., Hyde, K.D.: Production of wood-decay enzymes, mass loss and lignin solubilization in wood by tropical *Xylariaceae*. Mycol. Res. **107**, 231–235 (2003)
24. Liers, C., Ullrich, R., Pecyna, M., Schlosser, D., Hofrichter, M.: Production, purification and partial enzymatic and molecular characterization of a laccase from the wood-rotting ascomycete *Xylaria polymorpha*. Enzyme Microb. Technol. **41**, 785–793 (2007)
25. Ferreira Gregorio, A.P., Da Silva, I.R., Sedarati, M.R., Hedger, J.N.: Changes in production of lignin degrading enzymes during interactions between mycelia of the tropical decomposer basidiomycetes *Marasmiellus troyanus* and *Marasmius pallescens*. Mycol Res. **110**, 161–168 (2006)
26. Okano, K., Kitagawa, M., Sasaki, S., Watanabe, T.: Conversion of Japanese red cedar (*Cryptomeria japonica*) into a feed for ruminants by white-rot basidiomycetes. Anim Feed Sci Technol. **120**, 235–243 (2005)

27. Hakala, T.K., Maijala, P., Konn, J., Hatakka, A.: Evaluation of novel wood-rotting polypores and corticioid fungi for the decay and biopulping of Norway spruce (*Picea abies*) wood. Enzyme Microb. Technol. **34**, 255–263 (2004)
28. Zhang, X., Yu, H., Huang, H., Liu, Y.: Evaluation of biological pretreatment with white rot fungi for the enzymatic hydrolysis of bamboo culms. Int. Biodeterior. Biodegrad. **60**, 159–164 (2007)
29. Alam, M.Z., Mahmat, M.E., Muhammad, N.: Solid state bioconversion of oil palm biomass for ligninase enzyme production". Artif. Cell Blood Substit Biotechnol. **33**, 457–466 (2005)
30. Levin, L., Herrmann, C., Papinutti, V.L.: Optimization of lignocellulolytic enzyme production by the white rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. Biochem. Eng. J. **39**, 207–214 (2008)
31. Altekar, M., Homon, C.A., Kashem, M.A., Mason, S.W., Nelson, R.M., Patnaude, L.A., Yingling, J., Taylor, P.B.: Assay optimization: a statistical design of experiments approach. JALA **11**, 33–41 (2006)
32. Plackett, R.L., Burman, J.P.: The design of optimum multifactorial experiments. Biometrika. **33**, 305–325 (1946)
33. Baş, D., Boyaci, I.H.: Modeling and optimization I: usability of response surface methodology. J. Food Eng. **78**, 836–845 (2007)
34. Association of official analytical chemists: Official methods of analysis of the association of official analytical chemists. 15th edn, Washington D.C. (1990)
35. Arora, D.S., Chander, M., Gill, P.K.: Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. Int. Biodeterior. Biodegrad. **50**, 115–120 (2002)
36. Nazarpour, F., Abdullah, D.K., Abdullah, N., Zamiri, R.: Evaluation of biological pretreatment of rubberwood with white rot fungi for enzymatic hydrolysis. Materials **6**, 2059–2073 (2013)
37. Lee, J.: Biological conversion of lignocellulose biomass to ethanol. J. Biotechnol. **56**, 1–24 (1997)
38. Mannan, S., Fakhru'l-Razi, A., Alam, M.Z.: Optimization of process parameters for the bioconversion of activated sludge by *Penicillium corylophilum*, using response surface methodology. J. Environ. Sci. **19**, 23–28 (2007)
39. Singh, S.S., Dikshit, A.K.: Optimization of the parameters for decolourization by *Aspergillus niger* of anaerobically digested distillery spentwash pretreated with polyaluminium chloride. J. Hazard. Mater. **176**, 864–869 (2010)
40. Tan, L., Yu, Y., Li, X., Zhao, J., Qu, Y., Choo, Y.M., Loh, S.K.: Pretreatment of empty fruit bunch from oil palm for fuel ethanol production and proposed biorefinery process. Bioresour. Technol. **135**, 275–282 (2013)
41. Kim, S., Park, J.M., Seo, J.W., Kim, C.H.: Sequential acid-/alkali- pretreatment of empty fruit bunch fiber. Bioresour. Technol. **109**, 229–233 (2012)
42. Yu, H., Zhang, X., Song, L., Ke, J., Xu, C., Du, W., Zhang, Ji: Evaluation of white-rot fungi-assisted alkaline/oxidative pretreatment of corn straw undergoing enzymatic hydrolysis cellulase. J. Biosci. Bioeng. **110**, 660–664 (2010)
43. Solar, R., Kacik, F., Reinprecht, L., Laurova, M., Geffertova, J., Dibdiakova, J.: Effect of biotic and abiotic pretreatments of hornbeam wood on its properties interesting from the viewpoint of pulping in alkaline media. Part 2: chemical alterations. Bioresources **3**, 1291–1302 (2008)
44. Zhang, X., Xu, C., Wang, H.: Pretreatment of bamboo residues with *Coriolus versicolor* for enzymatic hydrolysis. J. Biosci. Bioeng. **140**, 149–151 (2007)
45. Ishola, M.M., Millati, R., Syamsiah, S., Cahyanto, M.N., Niklasson, C., Taherzadeh, M.J.: Structural changes of oil palm empty fruit bunch (OPEFB) after fungal and phosphoric acid pretreatment. Molecules **17**, 14995–15012 (2012). doi:10.3390/molecules171214995
46. Hamisan, A.F., Abd-Azziz, S., Kamaruddin, K., Md-Shah, U.K., Shahab, N., Hassan, M.A.: Delignification of oil palm empty fruit bunch using chemical and microbial pretreatment methods. Int. J. Agric. Res. **4**, 250–256 (2009)
47. Harmini, S., Hutomo, M.D., Bahruddin, L., Millati, R., Cahyanto, M.N., Niklason, C., Taherzadeh, M.J.: Fungal pretreatment of oil palm empty fruit bunch: effect of manganese and nitrogen. Cellul. Chem. Technol. **47**, 751–757 (2013)
48. Liu, J., Wang, M.L., Tonnis, B., Habteselassie, M., Liao, X., Huang, Q.: Fungal pretreatment of switchgrass for improved saccharification and simultaneous enzyme production. Bioresour. Technol. **135**, 39–45 (2013)