IMPROVING ENZYMATIC SACCHARIFICATION OF SUGARCANE BAGASSE BY BIOLOGICAL/PHYSICO-CHEMICAL PRETREATMENT USING *TRAMETES VERSICOLOR* AND *BACILLUS* SP.

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In this work, laccase biosynthesis of two microorganisms, *Trametes versicolor* TISTR 3224 and *Bacillus* sp. TISTR 908 isolated in Thailand, was investigated using sugarcane bagasse (SCB) as substrate. Two-stage biological/physico-chemical pretreatment of SCB on delignification and saccharification yield was studied. A two-level full factorial design was applied and 3 factors influencing delignification and saccharification processes of SCB were studied including C:N ratio (10:1 to 20:1), temperature (100 to 140°C), and alkali concentration (0 to 5% w/w NaOH). It was found that during biological pretreatment of SCB, a greater amount of laccase was produced from *T. versicolor* in the early stage of growth compared with *Bacillus* sp. Nitrogen supplement enhanced laccase biosynthesis of *T. versicolor*. By contrast, *Bacillus* sp. required a smaller amount of nitrogen source to produce laccase. Biological treated bagasse was subsequently subjected to a physico-chemical treatment. The results showed that the highest xylose and glucose yield of 51.97% w/w based on carbohydrate content was obtained from *T. versicolor* cultivation at a C:N ratio of 20:1, and consecutively treated in 5% w/w NaOH solution at 140°C for 1 h. Bacterial/alkali and alkali pretreatment yielded xylose and glucose in smaller degrees compared with fungal/alkali pretreatment. *T. versicolor* preferentially degraded lignin in sugarcane bagasse relative to cellulose and hemicelluloses constituents, while *Bacillus* sp. simultaneously attacked both lignin and carbohydrate moieties, as indicated by analysis of relative FT-IR intensities ratios of pretreated and untreated sugarcane bagasse.

Keywords: Trametes versicolor; Bacillus sp.; Sugarcane bagasse; Ligninolytic enzyme; Laccase; Xylose; Glucose; Experimental design; FT-IR spectroscopy

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INTRODUCTION

Sugarcane bagasse (SCB) is an agro-industrial residue produced in a great amount from the sugar industry in Thailand. SCB has a complex structure that is composed approximately of 25% lignin, 25% hemicellulose, and 40 to 50% cellulose. Carbohydrate constituents in SCB are made up of glucose, which is derived from cellulose depolymerization and other kinds of sugars *e.g*. xylose, mannose, arabinose, and galactose (Pandey *et al.* 2000; Krongtaew *et al.* 2010). Thus, SCB has been considered as a precursor for second-generation biofuels or other chemical building blocks. In order to

obtain sugars from SCB, suitable pretreatment and saccharification processes are required. The pretreatment step aims to remove lignin, reduce cellulose crystallinity, and increase the porosity of the material in order to make the hemicelluloses and cellulose more accessible to enzymes or acid in the saccharification step (Ohara 2003). Subsequently, saccharification by a mixture of cell wall degrading enzymes, including cellulases, β-glucosidase, and other related enzymes, is required to convert pretreated lignocellulosic material to sugars (Wyman 1999).

In the current work we were interested in biological pretreatment of SCB by microorganisms capable of producing lignin-degrading enzymes, as on-site SCB is partially sterilized from the processes of squeezing and washing with hot water to get rid of juice. Thus, the influence of the biological lignin removal step on enzymatic saccharification yield was investigated. Previous researchers have reported that several strains of white-rot fungi showed ability for either lignin degradation or lignin modification of lignocellulosic materials, *e.g*. *Ceriporiopsis subvermispora* (Sasaki *et al.* 2011), *Trametes versicolor, Echinodontium taxodii* (Zhang *et al.* 2007), *Pleurotus* spp. (Zadrazil and Puniya 1995), *Cerrena maxima, Coriolus hirsutus* (Koroleva *et al.* 2002), *Coriolus versicolor, Phanerochaete flavido-alba* (Lopez *et al.* 2002), *etc*. Some microorganisms preferentially degraded lignin; however some of them simultaneously deteriorated all components in lignocelluloses. Decolorization of lignocellulosic residues, pulp mill bleaching, or decolorization of effluent from pulping process by *Trametes versicolor* have been widely examined (Aloui *et al.* 2007; Modi *et al.* 1998; Archibald *et al.* 1997). It has been additionally reported that *T. versicolor* has substantial ability for degradation of lignin of wheat straw (Zafar *et al.* 1996) and bamboo culms (Zhang *et al.* 2007) by solid-state fermentation. For bacteria producing lignin-degrading enzymes, there have been reports on laccase production by *Bacillus subtilis, Bacillus licheniformis*, and *Streptomyces griseus* (Dwivedi *et al.* 2011). In the present study, laccase biosynthesis from two types of microorganisms, *T. versicolor* and *Bacillus* sp., grown on sugarcane bagasse, was investigated. These two microorganisms were considered to be strong decomposers predominantly present in the composting process (Kumar *et al.* 2011) and were isolated in Thailand. A two-level full factorial design was employed to screen significant factors in order to identify suitable conditions for biological pretreatment in a combination with physico-chemical pretreatment to improve the enzyme accessibility for the saccharification process of sugarcane bagasse.

EXPERIMENTAL

Microorganism

Trametes versicolor TISTR 3224 and *Bacillus* sp. TISTR 908 were obtained from Thailand Institute of Scientific and Technological Research, Thailand as a lyophilized form. *T. versicolor* inoculum was prepared by cultivation on potato dextrose agar (PDA) at 30°C for 10 days, and *Bacillus* sp. inoculum was grown on nutrient agar (NA) at 37°C for 3 days.

Experimental Design

Two-level full factorial design is a statistical tool that can be used to study the influence of factors in biological/physico-chemical pretreatment on delignification and

saccharification of sugarcane bagasse (SCB). Three factors were included in the design: C:N ratio, NaOH concentration based on dry SCB, and temperature of the alkali pretreatment, as shown in Table 1. Three center points were added for statistical computation. Thus, number of experiments was 2^3 plus 3 center points or 11 experiments in total. A quadratic regression model for xylose and glucose yield and analysis of variance (ANOVA) were calculated by Regression Toolbox (Microsoft Excel 2007).

Table 1. Coded and Actual Values of Factors from 2-level Full Factorial Design for SCB Pretreatment

Factors	Symbol	Coded and actual levels		
		-1	0	1
C:N ratio	X_1	10	15	20
Temperature (^{o}C)	X_2	100	120	140
NaOH (%w/w based on dry SCB)	X_3	0	2.5	5

Biological/Physico-Chemical Pretreatment of SCB

Sugarcane bagasse (SCB) was provided by Kornburi sugar factory, Thailand. Ten grams SCB (-20/+40 mesh) was added into a 500 mL-Erlenmeyer flask, and the moisture content was adjusted to 65% by adding a certain amount of deionized water calculated based on dry weight and mixed properly to obtain consistent moisture content of substrate. Urea at varying concentrations was added to obtain the C:N ratio according to the experimental design (Table 1). After sterilization of SCB at 121°C for 15 min in an autoclave, one plate of *T. versicolor* maintained on a potato dextrose agar was inoculated into sterilized SCB, mixed carefully with SCB, and incubated at 30^{o}C for 10 days. Fermented SCB was mixed prior to taking the sample during cultivation to determine laccase activities and weight loss. At the end of cultivation (10 days for *T. versicolor* and 3 days for *Bacillus* sp. similar to growth period in Petri dish), the rest of the fermented SCB was subjected to physico-chemical pretreatment, which was alkali pretreatment according to the experimental design at varying NaOH concentrations and temperatures (Table 1). The same procedure was applied for *Bacillus* sp., except for using nutrient agar (NA), 85% initial moisture content of SCB adjusted by deionized water based on dry material, and incubation condition at 37^{o}C for 3 days. The physico-chemical treatment of biological treated SCB was performed with a solid-to-liquid ratio of 1:20.

Laccase Activity

One g (wet basis) of fermented SCB sample was macerated in 2 mL sodium acetate buffer of pH 5.0 at 20^{o}C for 30 min. Extracellular laccase was able to be determined from this step. Afterward, the whole contents were ground and filtered to collect intracellular laccase extract. The procedure of grinding and filtering was repeated twice. Supernatants from every extraction step were mixed together and centrifuged at 4^{o}C with a rotational speed of 10,000 rpm for 10 min. Finally, the total volume was made up to 10 mL. Laccase activity was calculated from the ABTS oxidation at 420 nm (ε = 3.6×10^4 cm^{-1} M^{-1})(Levin *et al.* 2005). The reaction mixture contained 0.4 mL of 1 mM ABTS and 1.2 mL 0.1 M sodium acetate buffer of pH 5.0 and 0.8 mL aliquots of appropriately diluted enzyme extract described before. One laccase activity unit was defined as the amount of enzyme, which leads to the oxidation of 1 μM of ABTS per

minute. The activities were expressed in U per gram of extracted fermented substrate (U g^{-1}) (Mishra and Kumar 2007).

Enzymatic Saccharification and Sugar Analysis

Pretreated SCB (dry basis) was saccharified by adding 60 FPU/g dry substrate (National Renewable Energy Laboratory, NREL method no. NREL/TP-510-42629, USA) of Accellerase1500 (www.genencor.com) to 0.2 g of SCB in 50 mM Na-acetate buffer pH 4.8 with solid-to-liquid ratio of 1:100 and maintained at 50°C for 72 h in an arbitrary shaking incubator at 300 rpm. At the end of the enzymatic saccharification period, supernatant was taken to determine xylose and glucose content by high performance liquid chromatography (HPLC Younglin-YL9100, Korea) equipped with an Evaporative Light Scattering Detector (ELSD), (SofTA, USA). Deionized water was the mobile phase at a flow rate of 0.4 mL/min in isocratic mode using VertiSep SUGAR LMP column for HPLC 7.8×300 mm (Vertical Chromatography, Thailand).

Characterization of SCB

For extractive determination of untreated and pretreated SCB samples, 3.5 g milled sample (200 μm) was sequentially extracted using 1) cyclohexane-ethanol (2:1 v/v) for 6 h, 2) ethanol (95% v/v) for 1 h, and 3) water for 24 h according to TAPPI T 264 om-88. Acid-insoluble lignin contents (Klason lignin) of SCB samples was determined after acid hydrolysis (72% H_2SO_4, 20°C, 2 h and 3% H_2SO_4, 100°C, 4 h) according to modified TAPPI T222-om-06 (Schwanninger and Hinterstoisser 2002). Acid-soluble lignin was calculated from absorbance at 205 nm by UV-Vis spectrophotometer (TAPPI T222-om-02). Ash content was the residue after ignition of a known dry weight sample at 550±50°C for 2 h. Total carbohydrate content was calculated by subtraction of weight loss, total lignin, extractives, and ash contents from 100% untreated SCB.

The percentage of lignin removal was calculated from lignin reduction in pretreated SCB based on total lignin content in untreated SCB multiplied by 100. Saccharification yield was the amount of sugars (xylose + glucose) released from Accellerase 1500 saccharification of SCB divided by total carbohydrate content in untreated SCB multiplied by 100.

For Fourier-transform infrared (FT-IR) spectroscopy, 0.1 g dry SCB sample was milled to 100 μm, mixed with KBr (sample:KBr ratio of 1:99), and subsequently pressed by hydraulic press to form a transparent disc. Three FT-IR spectra of each sample were recorded between 4000 and 370 cm^{-1} in transmittance mode with 4 cm^{-1} resolution at 100 numbers of scans by FT-IR spectrometer (Perkin Elmer spectrum 2000, USA). All spectra were averaged and smoothed using 19 smoothing points for FT-IR spectral analysis (Savitzky-Golay Smoothing Algorithm).

RESULTS AND DISCUSSION

Laccase Production by *Trametes versicolor* and *Bacillus* sp.

The growth of *T. versicolor* was primarily monitored while it was being maintained on a PDA plate. At an incubation temperature of 30°C, *T. versicolor* required 10 days for full development of its hyphae on an 11-cm diameter Petri dish. On the other

hand, *Bacillus* sp. needed three days to form 350-370 CFU/plate on NA at 37°C. Their colonies were 0.2 to 0.4 mm in diameters.

The second inoculum seeds of both fungus and bacteria maintained on agar were transferred to sterile SCB with suitable moisture content for each microorganism, and laccase biosynthesis during microbial growth was investigated. There was no previous report of *T. versicolor* cultivation on SCB, only solid-state fermentation of *T. versicolor* on wheat straw (Zafar *et al.* 1996) and bamboo culms (Zhang *et al.* 2007) was investigated. Similarly, no information of lignin degrading enzymes excreted by *Bacillus* sp. was revealed, merely laccase production by *Bacillus subtilis* and *Bacillus licheniformis* has been reported (Dwivedi *et al.* 2011).

As illustrated in Fig. 1, the amount of inter- and extra-cellular laccase increased during the first three days of cultivation for *T. versicolor*; however, the laccase production decreased after three days of fermentation, as depicted in Fig. 1(a). This was mainly because the fungus needed carbon sources for their growth; thus the lignin-degrading enzyme was secreted in an early stage of growth to facilitate the removal of lignin seal from SCB, and successively hemicelluloses and cellulose were accessible for their hydrolytic enzymes. Therefore, laccase production by *T. versicolor* declined after three days of cultivation period when lignin was partially degraded and carbohydrate sources were susceptible. This is in agreement with Zafar and coworkers' work (1996) when 14 days of cultivation were necessary for *T. versicolor* growth on rice straw to achieve the highest ratio of lignin to cellulose degradation. Another possible reason is due to the production of secondary metabolite of *T. versicolor* when its growth reached the stationary phase, so laccase biosynthesis is inhibited, as reported earlier (Qiu and Chen 2008).

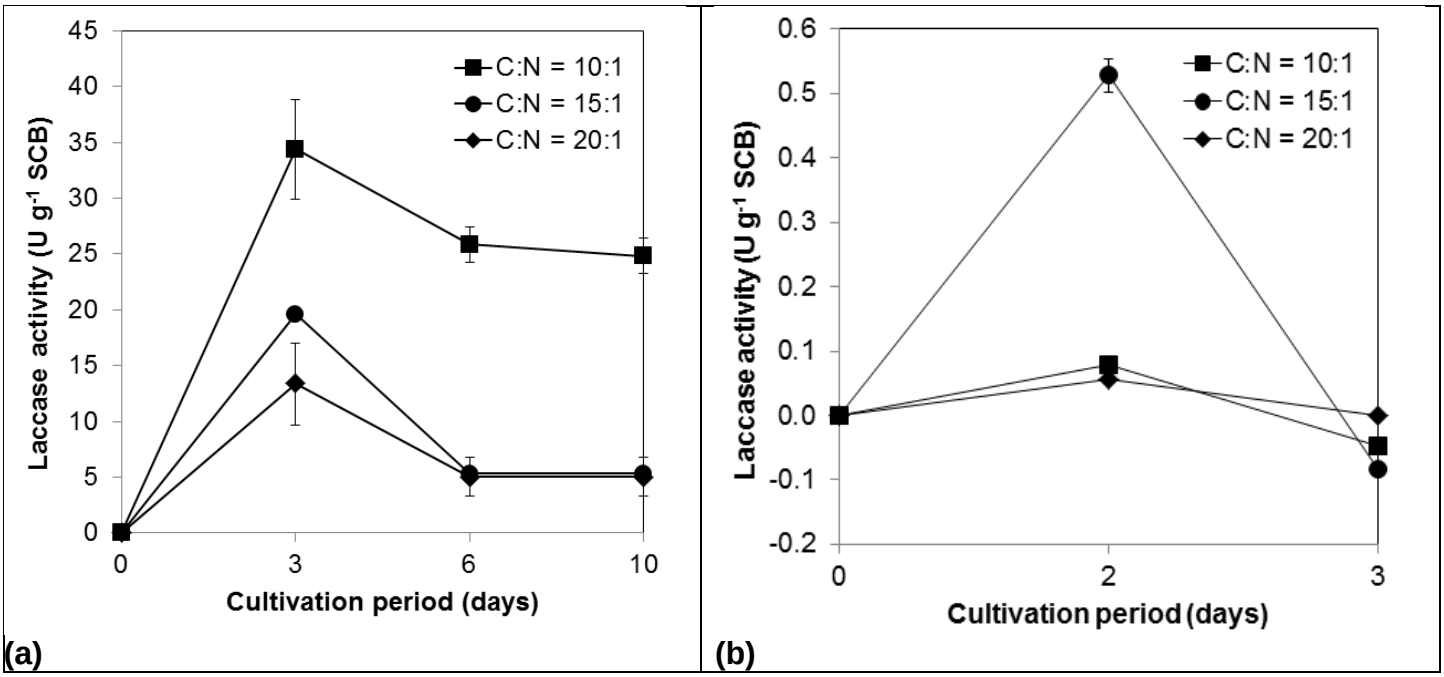

Fig. 1. Inter- and extra-cellular laccase enzyme from (a) *T. versicolor* and (b) *Bacillus* sp. during SCB fermentation at different C:N ratios of 10:1 (■), 15:1 (●) and 20:1 (◆)

The growth rate of *Bacillus sp*. was higher than *T. versicolor*, thus *Bacillus* sp. produced laccase in the early stage of its growth, and the amount of laccase decreased after two days (Fig. 1(b)). However, laccase produced by *Bacillus* sp. was mainly intracellular enzyme examined during laccase extraction procedure (data not shown), which is similar to a previous report (Martin *et al.* 2002).

When considering the effect of C:N ratios on laccase biosynthesis, the results showed that addition of a nitrogen source (C:N ratio of 10:1) significantly stimulated laccase production for *T. versicolor* on the third day of cultivation, whereas a little depletion of nitrogen supplement (C:N ratio of 15:1) led to enhanced laccase production for *Bacillus* sp. A similar circumstance was found when high C:N ratio or nitrogen starved condition fastened the ligninolytic process of *Coriolus versicolor*, another basidiomycete, when it was cultivated on wheat straw (Zafar *et al.* 1989). A previous study on fungal solid-state cultivation, *T. versicolor* and *Cerrena unicolor* on oat husk and waste from the paper industry, also reported an equivalent result of good manganese peroxidase activity level on the fifth days of *T. versicolor* cultivation. For laccase activity, the fungus needed 10 to 12 days to reach the maximum value. Medium composition played a vital role on lignin degrading enzyme production and the best medium for *T. versicolor* was only oat husk, whereas addition of fiber and de-inking sludge (FDS) to the medium decreased the activity of laccase (Winquist *et al.* 2008). Although manganese peroxidase was produced from fungal cultivation, its amount was only one third of the laccase enzyme. Consequently, in the present work we particularly considered laccase production from *T. versicolor* and *Bacillus* sp. from SCB.

Biological/Physico-Chemical Pretreatment of SCB

Untreated SCB consisted mainly of carbohydrates (cellulose and hemicelluloses), lignin, and extractives in respective degrees as shown in Table 2. Lignin removal of pretreated SCB was computed with respect to total lignin referred to summation of Klason lignin (acid-insoluble lignin) and acid soluble lignin of untreated SCB. Enzymatic saccharification yield of treated SCB was calculated based on and carbohydrate content of untreated SCB.

Table 2. Composition of Untreated SCB

Sample	Composition (%w/w)				
	Extractives	Klason lignin	Acid soluble lignin	Ash	Carbohydrates
Untreated SCB	12.28±0.49	28.02±1.04	3.79±0.02	7.84±0.34	48.07±1.89

Table 3 gives enzymatic saccharification results from alkali, fungal/alkali, and bacterial/alkali pretreatments of SCB expressed as glucose and xylose contents based on dry weight of SCB, as well as glucose and xylose yield based on carbohydrate content in SCB. From the experimental data of 2-level full factorial design, fungal/alkali and bacterial/alkali pretreated samples in most cases attained greater amounts of glucose and xylose contents compared with alkali pretreatment and untreated SCB. The highest glucose and xylose yield based on a carbohydrate content of 51.97% was achieved when SCB at C:N ratio of 20 was treated with *T. versicolor* for 10 days, followed by alkali treatment in 5% w/w NaOH solution at 140^{o}C (run#8) when untreated SCB reached only 5.6% glucose and xylose yield after enzymatic saccharification. Comparable results were

reported on sugarcane bagasse pretreated with *Ceriporiopsis subvermispora* and microwave hydrothermolysis. The maximum sugar recovery of 44.9% by weight based on dry material was achieved from microwave irradiation (180°C for 20 min) and fungal treatment, while the microwave treatment of raw sugarcane bagasse at the same condition gave only 28.6% by weight of sugar recovery (Sasaki *et al.* 2011). Zhang *et al.* (2007) investigated influence of white-rot fungal pretreatment for the enzymatic hydrolysis of bamboo culms, and the results showed that cultivation of *Echinodontium taxodii* 2538 for 120 days yielded the highest amount of fermentable sugar of 37% by weight based on dry material while *Trametes versicolor* G20 pretreatment for 120 days gave 22.5% by weight of sugar yield when using 20 FPU g^{-1} enzyme loading at hydrolysis time of 120 h. The fermentable sugar yields from biological pretreatment of these two fungal strains were substantially higher than that of untreated bamboo culms, which yielded only 2.8% by weight of fermentable sugar based on dry material at the same enzymatic hydrolysis condition. Thus, SCB is a recalcitrant lignocellulosic waste that requires delignifying pretreatment to enhance enzymatic accessibility of cellulose and hemicelluloses constituents. The more lignin removed, the better the yield of enzymatic hydrolysis of carbohydrates in SCB since lignin is considered an inhibitor having binding ability onto cellulase and hemicellulase enzymes, which then hinders the enzyme-substrate complex formation during the saccharification step (Alvira *et al.* 2010).

Table 3. Comparison of Amount of Sugars and Yield of Enzymatic Saccharification of Different Pretreated SCB

Run	Factors*			Glucose (mg/g SCB)			Xylose (mg/g SCB)			Yield of glucose and xylose based on carbohydrate (%)		
	x_1	x_2	x_3	Alkali	Fungal /alkali	Bacterial/ alkali	Alkali	Fungal/alkali	Bacterial/ alkali	Alkali	Fungal/ alkali	Bacterial/ alkali
1	1-	1-	1-	83.13	32.26	26.60	26.34	7.39	14.82	22.77	8.25	8.62
2	1-	1-	1	27.86	143.57	12.36	51.81	49.53	13.89	16.57	40.17	5.46
3	1-	1	1-	79.28	58.55	29.65	25.62	13.69	25.27	21.82	15.03	11.42
4	1-	1	1	56.90	225.31	77.23	65.53	18.34	55.44	25.47	50.69	27.60
5	1	1-	1-	55.59	46.85	12.43	17.85	0.16	13.84	15.28	9.78	5.46
6	1	1-	1	41.94	121.00	12.30	49.85	35.80	13.95	19.10	32.62	5.46
7	1	1	1-	10.49	70.67	44.43	20.00	9.83	14.03	6.34	16.74	12.16
8	1	1	1	64.49	184.69	73.40	66.74	65.15	53.39	27.30	51.97	26.38
9	0	0	0	89.42	61.48	24.03	9.93	13.98	18.87	20.67	15.70	8.92
10	0	0	0	11.64	137.44	14.73	26.15	28.21	33.08	7.86	34.46	9.95
11	0	0	0	67.92	75.27	12.40	26.37	14.89	13.92	19.61	18.76	5.47
Untreated SCB					12.78			14.14			5.6	

* x_1, x_2, and x_3 represent coded values of C:N ratios, temperatures, and NaOH concentrations, respectively.

Table 3 additionally shows that fungal/alkali pretreatment achieved significantly higher sugar yields compared with bacterial/alkali and alkali pretreatment, especially at run#8 (C:N ratio of 20:1, 140°C, and 5% NaOH), run#4 (C:N ratio of 10:1, 140°C, and 5% NaOH), run#2 (C:N ratio of 10:1, 100°C, and 5%NaOH), and run#10 (C:N ratio of 15:1, 120°C, and 2.5% NaOH), which achieved 51.97%, 50.69%, 40.17%, and 34.46% yield of xylose and glucose based on carbohydrate content in untreated SCB, respectively. Nonetheless, bacterial/alkali pretreatment gave considerably higher sugar yields than fungal/alkali pretreatment for run#1 (C:N ratio of 10:1, 100°C, and 0%

NaOH) and run#3 (C:N ratio of 10:1, 140°C, and 0% NaOH). This suggests that each microorganism requires an optimal amount of nitrogen supplement for growth and production of laccase to remove lignin from SCB, which in turn leads to enhanced enzymatic saccharification efficiency when subsequent hydrothermal treatment is applied without alkaline addition (0% w/w NaOH). The results were in agreement with a previous work reporting on enhancement of laccase production from *Trametes versicolor* and *Cerrena unicolor* when adding oat husk as nitrogen supplement in the medium (Winquist *et al.* 2008).

In accordance with experimental data, a quadratic model of glucose and xylose yield based on dry weight of SCB from fungal/alkali pretreatment was calculated as demonstrated in Eq. (1), where Z is glucose and xylose yield based on dry weight of SCB (%w/w). X_1, X_2, and X_3 are coded values of C:N ratio, temperature, and NaOH concentration, respectively.

$$Z = 11.05 - 0.17X_1 + 7.62X_3 + 2.69X_2 + 2.56X_3^2 + 1.04X_2X_3 \quad (1)$$

From ANOVA of the model, it was found that R^2 was 0.91 and the model was significant ($P<0.05$), indicating good agreement of the experimental data with the predicting model. In addition, Fig. 2 illustrates the response of the model depending on three factors from the experimental design. The surface plot showed the superior significant effect of NaOH concentration and temperature on sugar yield as depicted in Fig. 2(a). An increase of NaOH concentration from 0% to 5% w/w NaOH (-1 to +1 of coded values) illustrated considerable enhancement of glucose and xylose yield relative to an increase of temperature from 100 to 140°C. Moreover, temperature had greater influence on sugar yield than C:N ratio, as shown in Fig. 2(b). Though the C:N ratio gave the least significant effect on glucose and xylose yield of fungal/alkali pretreated SCB, the term X_1 attributed to C:N ratio was essentially included in the quadratic model (Eq (1)) to obtain the statistically significant model.

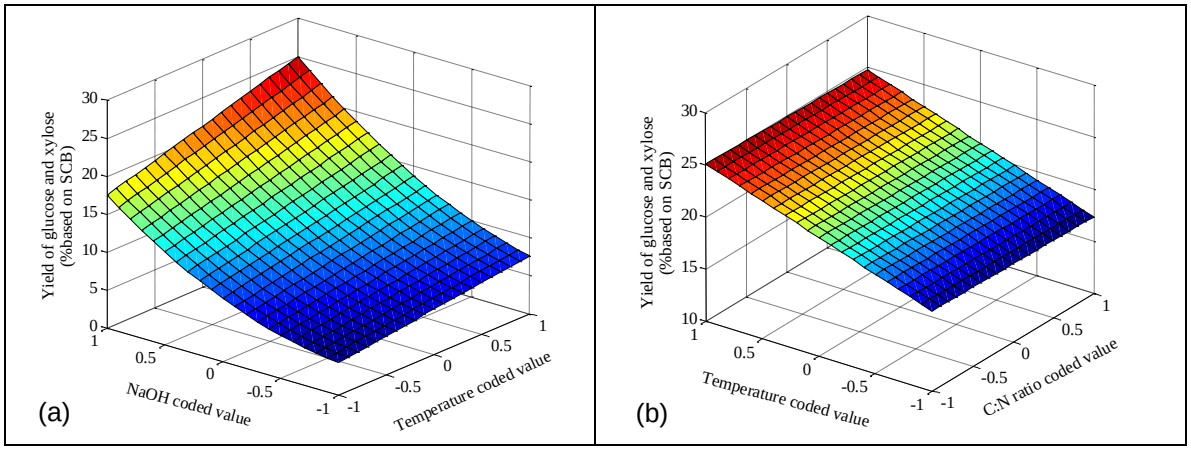

Fig. 2. Response surface plot of xylose and glucose yield from enzymatic saccharification of fungal/alkali pretreated SCB (a) effect of NaOH concentration and temperature and (b) effect of temperature and C:N ratio

Delignification of SCB and Characterization of Pretreated SCB by FT-IR Spectroscopy

Five pretreated SCB samples yielding low and high amounts of glucose and xylose from run#1, run#4, run#5, run#8, and run#9 demonstrated in Table 3 were analyzed for Klason lignin and acid-soluble lignin. Lignin removal based on lignin content in untreated SCB of each run was calculated as shown in Table 4. The results confirmed that delignifying efficiency influenced sugar yield from enzymatic saccharification, as an increase of lignin removal extent was related to an augmentation of glucose and xylose yield from enzymatic saccharification (Table 3). As demonstrated in Table 4, the maximum sugar yield for alkali, fungal/alkali, and bacterial/alkali pretreatments were reached from run#8 (C:N ratio of 20:1, 140°C, and 5% NaOH). The results of sugar yields from saccharification showed a good relationship with lignin removals from Table 4. From run#8, lignin was removed for 57.62%, 60.04%, and 48.18% for alkali, fungal/alkali, and bacterial/alkali pretreatment, respectively. These samples accomplished glucose and xylose yields based on carbohydrate content of 27.29%, 51.96%, and 26.37%, respectively.

Table 4. Delignification of SCB by Different Pretreatment Methods

Run	Condition	Lignin removal (%)		
		Alkali pretreatment	Fungal/alkali pretreatment	Bacterial/alkali pretreatment
1	C:N ratio of 10:1, 100°C and 0%NaOH	12.63	14.33	10.17
4	C:N ratio of 10:1, 140°C and 5%NaOH	58.62	58.14	50.73
5	C:N ratio of 20:1, 100°C and 0%NaOH	14.57	13.29	11.14
8	C:N ratio of 20:1, 140°C and 5%NaOH	57.62	60.04	48.18
9	C:N ratio of 15:1, 120°C and 2.5%NaOH	20.79	15.24	17.97

The lignin removal results were equivalent to FT-IR spectra that presented the changes of lignin moiety near 1510 cm^{-1}, which can be attributed to C=C aromatic structure (Zeng *et al.* 2012), as depicted in Fig. 3. The transmittance intensity of this peak in SCB sample substantially decreased when treated with fungal/alkali pretreatment. The finding suggests that the more lignin is removed, the higher will be the yield of sugars reached.

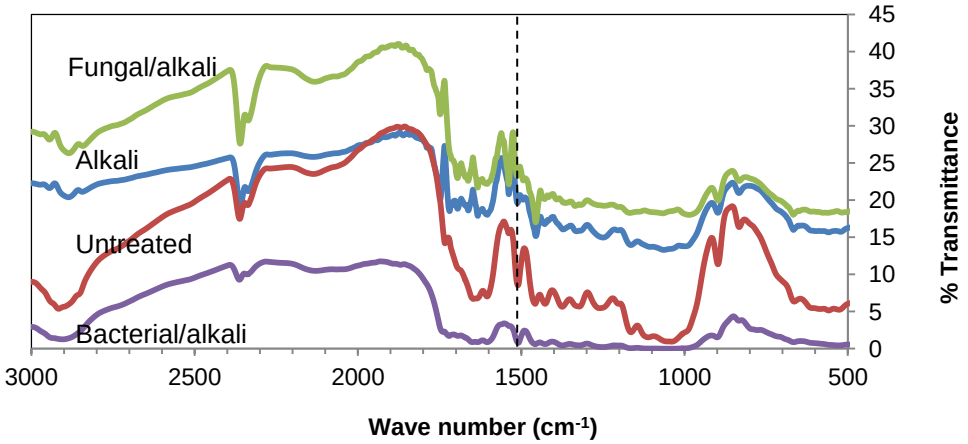

Fig. 3. FT-IR spectra of all pretreated sugarcane bagasse sample from run#8 (C:N ratio of 20:1, 5% w/w NaOH at 140°C) and untreated sugarcane bagasse

FT-IR analysis of lignocellulosic materials efficiently explained the small changes of lignin and carbohydrate quantities during decay. These data showed the selectivity of fungal and bacterial lignin degradation attributed to the aromatic skeleton vibration of lignin near 1510 cm^{-1} comparative to carbohydrate vibration peaks near 1734, 1373, 1161, and 898 cm^{-1}. Assignments of vibration peaks of carbohydrates were described previously: 1734 cm^{-1} for unconjugated C=O in hemicelluloses, 1373 cm^{-1} for C-H deformation in cellulose and hemicelluloses, 1161 cm^{-1} for C-O-C vibration in cellulose and hemicelluloses, and 898 cm^{-1} for C-H deformation in cellulose (Pandey and Pitman 2003; Zhang *et al.* 2007).

Table 5. Relative FT-IR Intensities of Lignin and Carbohydrate Moieties of Pretreated SCB from Pretreatment run#8 (C:N ratio of 20:1, 5% w/w NaOH at 140°C) and Untreated SCB Samples

Sample	Relative FT-IR intensities of lignin and carbohydrate moieties of sugarcane bagasse samples			
	$I_{1510/1734}$	$I_{1510/1373}$	$I_{1510/1161}$	$I_{1510/898}$
Fungal/Alkali treated sample	0.639	1.150	1.278	1.179
Bacterial/Alkali treated sample	0.025	0.100	5.000	0.033
Alkali treated sample	0.691	1.267	1.310	1.056
Untreated SCB	0.571	1.600	2.667	0.727

From Table 5, it has been postulated that an increase of relative FT-IR transmittance intensities ratio of lignin-to-carbohydrate vibration bands from pretreated SCB samples compared with that from untreated SCB represented either highly selective lignin degradation or lignin solubilization from SCB in relation to carbohydrate degradation. As a result, fungal/alkali pretreatment predominantly degraded lignin moieties in SCB associated with the increases of $I_{1510/1734}$ and $I_{1510/898}$ ratios. This indicated that unconjugated C=O of hemicelluloses and C-H deformation of cellulose were less damaged and remained in higher degree than lignin. With respect to untreated SCB, C-H deformation was attributed mainly to xylan, and the C-O-C vibration in cellulose and hemicelluloses was strongly attacked. The corresponding results were found from pretreated SCB by alkali pretreatment, which gave high values of $I_{1510/1734}$ and $I_{1510/898}$ ratios. Fungal/alkali pretreatment yielded superior results in terms of preserving cellulose, based on the fact that the peak located near 898 cm^{-1} was relatively untouched compared to alkali pretreatment.

In contrast, bacterial/alkali pretreatment preferentially degraded or removed cellulose and hemicelluloses relative to lignin, based on the FT-IR intensities ratios of $I_{1510/1734}$, $I_{1510/1373}$, and $I_{1510/898}$. However, it was interesting that C-O-C ether bonds in cellulose and hemicelluloses remained intact when using bacterial/alkali pretreatment, as indicated by the $I_{1510/1161}$ ratio. This peak intensity ratio was dramatically increased after bacterial cultivation on sugarcane bagasse, suggesting that 1) C-O-C ether linkages were non-degradable by laccase enzyme excreted by *Bacillus* sp., 2) inhibiting effect of ether bond degradation by intermediate substances produced by this kind of bacteria, and 3) etherification process during bacterial growth. The later was postulated to be regarded as ferulic acid ethers, which might form cross links between lignin and hemicelluloses by the simultaneous esterification of their carboxyl group to arabinose substituents of arabinoglucuronoxylan and etherification of their hydroxyl groups to phenyl hydroxyls of

lignin in the presence of peroxidase and water (Markwalder and Neukom 1976; Morison 1974). Consequently, bacterial etherification most likely the cause of a significant increase of the ratio $I_{1510/1161}$, as the subsequent alkali pretreatment step accounted solely for degradation of ester bonds but not ether linkages. Alkali acts on ester bond linkages between lignin and hemicelluloses, and a partial release of lignin substantially increases hydrophilicity of lignocellulosic material (Sjöström 1991; Krongtaew *et al.* 2010). Additionally, swelling effect of alkali pretreatment reduces crystallinity of cellulose and lead to higher sugar yields during enzymatic saccharification (Chandra *et al.* 2007; Krongtaew *et al.* 2010).

CONCLUSIONS

1. Laccase biosynthesis of *Trametes versicolor* and *Bacillus* sp. in the presence of sugarcane bagasse enhanced delignification and enzymatic saccharification of pretreated substrate.
2. Fungal lignin biodegradation showed superior delignification results than that of bacterial lignin biodegradation, which led to higher yield of sugars after enzymatic saccharification.
3. Biological pretreatment in addition to severe physico-chemical pretreatment of SCB worked together to enhance enzymatic saccharification efficiency.

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REFERENCES CITED

Aloui, F., Abid, N., Roussos, S., and Sayadi, S. (2007). "Decolorization of semisolid olive residues of "alperujo" during the solid state fermentation by *Phanerochaete chrysosporium, Trametes versicolor, Pycnoporus cinnabarinus, and Aspergillus niger*,"*Biochem. Eng. J.*35, 120-125.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., and Negro, M. J. (2010). "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review,"*Biores.Technol.*101, 4851-4861.

Archibald, F. S., Bourbonnais, R., Jurasek, L., Paice, M. G., and Reid, I. D. (1997). "Kraft pulp bleaching and delignification by *Trametes versicolor*,"*J. Biotechnol.* 53, 215-236.

Chandra, R. P., Bura, R., Mabee, W. E., Berlin, A., Pan, X., and Saddler, J. N. (2007). "Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics?"*Adv Biochem Eng Biotechnol.*108, 67-93.

Dwivedi, U. N., Singh, P., Pandey, V. P., and Kumar, A. (2011). "Structure-function relationship among bacterial, fungal, and plant laccases,"*J. Mol. Catal. B: Enzym.*68, 117-128.

Koroleva, O. V., Gavrilova, V. P., Stepanova, E. V., Lebedeva, V. I., Sverdlova, N. I., Landesman, E. O., Yavmetdinov, I. S., and Yaroplov, A. I. (2002). "Production of lignin modifying enzymes by co-cultivated White-Rot Fungi *Cerrena maxima* and *Coriolus hirsutus* and characterization of laccase from *Cerrena maxima*," Enzyme Microb. Technol. 30, 573-580.

Krongtaew, C., Messner, K., Ters, T., and Fackler, K. (2010). "Characterization of key parameters for biotechnological lignocellulose conversion assessed by FT-NIR spectroscopy. Part I: Qualitative analysis of pretreated straw,"*BioResources*5(4), 2063-2080.

Kumar, A. P., Kumar, C. D., Anil, P., and Johri, B. N. (2011). "Bacterial diversity in a bagasse-based compost prepared for the cultivation of edible mushrooms *Agaricus bisporus*,"*J. Agric. Technol.* 7(5), 1303-1311.

Levin, L., Forchiassin F., and Viale A. (2005). "Ligninolytic enzyme production and dye decolorization by *Trametes trogii*: Application of the Plackett-Burman experimental design to evaluate nutritional requirements," *Proc. Biochem.* 40, 1381-1387.

Lopez, M. J., Elorrieta, M. A., Vargas-Garcia, M. C., Suarez-Extrella, F., and Moreno, J. (2002). "The effect of aeration on the biotransformation of lignocellulosic wastes by white-rot fungi,"*Biores. Technol.* 81, 123-129.

Markwalder, H. U., and Neukom, H. (1976). "Diferulic acid as a possible crosslink in hemicelluloses from wheat germ,"*Phytochem.* 15, 836-837.

Martin, L. O., Soare, C. M., Pereira, M. M., Teixeira, M., Costa, T., Jones, G. H., and Henriques, A. O. (2002). "Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat,"*J. Biol. Chem.* 277, 18849-18859.

Mishra, A., and Kumar, S. (2007). "Cyanobacterial biomass as N-supplement to agro-waste for hyper-production of laccase from *Pleurotus ostreatus* in solid state fermentation," *Proc. Biochem.* 42,681-685.

Modi, D. R., Chandra, H., and Garg, S. K. (1998). "Decolorization of bagasse-based paper mill effluent by the white-rot fungus *Trametes versicolor*,"*Biores. Technol.* 66, 79-81.

Morison, I. M. (1974). "Structural investigation on the lignin-carbohydrate complexes of *Lolium perene*," *Biochem J.* 139, 197-204.

Ohara, H. (2003). "Biorefinery,"*Appl. Microbiol. Biotechnol.* 62, 474-477.

Pandey, A., Soccol, C., and Soccol, V. (2000). "Biotechnological potential of agro-industrial residues.I: Sugarcane bagasse,"*Biores. Technol.* 2, 69-74.

Pandey, K. K., and Pitman, A. J. (2003). "FT-IR studies of the changes in wood chemistry following decay be brown-rot and white-rot fungi,"*Int. Biodeter. Biodegr.* 52, 151-161.

Sasaki, C., Takada, R., Watanabe, T., Honda, Y., Karita, S., Nakamura, Y., and Watanabe, T. (2011). "Surface carbohydrate analysis and bioethanol production of sugarcane bagasse pretreated with the white rot fungus, *Ceriporiopsis subvermispora* and microwave hydrothermolysis,"*Biores. Technol.* 102, 9942-9946.

Schwanninger, M., and Hinterstoisser, B. (2002). "Klason lignin: Modifications to improve the precision of the standardized determination,"*Holzforschung* 56, 161-166.

Sjöström, E. (1991). "Carbohydrate degradation products from alkaline treatment of biomass,"*Biomass and Bioenergy* 1(1), 61-64.

Winquist, E., Moilanen, U., Mettälä, A., Leisola, M., and Hatakka, A. (2008). "Production of lignin modifying enzymes on industrial waste material by solid-state cultivation of fungi," *Biochem. Eng. J.* 42, 128-132.

Wyman, C. H. (1999). "Biomass ethanol: Technical progress, opportunities, and commercial challenges,"*Annu. Rev. Energy Environ.* 24, 189-226.

Zadrazil, F., and Puniya, A. K. (1995). "Studies on the effect of particle size on solid-state fermentation of sugarcane bagasse into animal feed using white-rot fungi,"*Biores. Technol.* 54, 85-87.

Zafar, S. I., Abdullah, N., Iqbal, M., andSheeraz, Q. (1996). "Influence of nutrient amendment on the biodegradation of wheat straw during solod state fermentation with *Trametes versicolor*,"*Int. Biodeter. Biodegrd.* 96, 83-87.

Zafar, S. I., Sheeraz, Q., and Abdullah, N. (1989). "Degradation of the lignocellulosic component on wheat straw-*Coriolus versicolor* solid state fermentation under nitrogen-starved conditions,"*Biol. Wastes.* 27, 67-70.

Zeng, Y., Yang, X., Yu, H., Zhang, X., and Ma, F. (2012). "The delignification effects of white-rot fungal pretreatment on thermal characteristics of moso bamboo,"*Bioresour. Technol.*, doi: 10.1016/j.biortech.2011.10.036.

Zhang, X., Yu, H., Huang, H., and Liu, Y. (2007). "Evaluation of biological pretreatment with white rot fungi for the enzymatic hydrolysis of bamboo culms,"*Int. Biodeter. Biodegr.*60, 159-164.

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