SIMULTANEOUS PRODUCTION OF CELLULASE AND REDUCING SUGAR FROM ALKALI-PRETREATED SUGARCANE BAGASSE VIA SOLID STATE FERMENTATION

Li Wan Yoon,[a] Gek Cheng Ngoh,[a,*] and Adeline Seak May Chua[a]

This study optimized alkali pretreatment of sugarcane bagasse (SCB) and investigated the potential of alkali-pretreated SCB in producing cellulase and reducing sugar by a white-rot fungus, *P. sanguineus*, via solid state fermentation (SSF). The fermentability of the reducing sugar produced during SSF was examined by co-culturing yeast, *Saccharomyces cerevisiae*, with *P. sanguineus*. Central composite design (CCD) was applied to optimize the pretreatment based on reducing sugar yield obtained from enzymatic hydrolysis of the pretreated SCB. The model developed from CCD fitted the data well, and the optimized conditions for alkali pretreatment were 128 °C, 0.62 M NaOH, and 30 min with a reducing sugar yield of 97.8%. The alkali-pretreated SCB after washing and drying was cultivated with *P. sanguineus* during SSF. It was found that cellulase and reducing sugar can be produced simultaneously from this SSF system. The maximum cellulase activities determined from filter paper assay (FPase), carboxylmethylcellulase (CMCase) assay and β-glucosidase assay were 0.02 IU/mL, 0.11 IU/mL, and 0.13 IU/mL on day 8, day 3, and day 6 of cultivation, respectively. The maximum reducing sugar concentration of 19.9 mg/g pretreated SCB was obtained on day 4 of SSF. The reducing sugar produced was converted into ethanol upon the addition of yeast into the SSF system. Evidently, the reducing sugar acquired can be further utilized to produce other valuable products in subsequent processes.

Keywords: Pretreatment; Sugarcane bagasse; Central composite design; Cellulase; Reducing sugar; Solid state fermentation

*Contact information: a: Department of Chemical Engineering, Faculty of Engineering, University Malaya, 50603, Kuala Lumpur, Malaysia;*Corresponding author: ngoh@um.edu.my*

INTRODUCTION

Bioproducts such as enzyme, fermentable sugar, organic acid, and biofuel have gained massive interest from various industries due to their vast and vital applications (Sun 2009; Yao *et al.* 2007; Bansal *et al.* 2011; Mirahmadi *et al.* 2010). These bioproducts are favourable products from agricultural residues due to the lignocellulosic nature of the residues (Sun 2009). Oil palm fronds, sugarcane bagasse, and rice husks are a few examples of low-cost agricultural residues that are rich in cellulose, hemicellulose, and lignin (Sousa *et al.* 2009). Nevertheless, most agricultural residues have complex structures that may negatively affect the bioconversion of these biomasses into value-added products. In order to facilitate bioconversion processes such as enzymatic hydrolysis and fermentation, pretreatment is often necessary to alter the structure of the biomass prior to the conversion process (Mirahmadi *et al.* 2010).

Pretreatment techniques are generally classified as physical, chemical, biological, or physicochemical (Galbe and Zacchi 2007; Yang and Wyman 2008; Karunanithy and Muthukumarappan 2011). Alkali pretreatment is one of the effective techniques applied to alter the structure of biomass. Operating conditions such as temperature, duration, and alkali concentration are among the most commonly investigated parameters in the application of alkali pretreatment on various biomasses (Nlewem and Thrash 2010; McIntosh and Vancov 2010; Xu *et al.* 2010; Zhang *et al.* 2010). The alkaline solution acts as a delignification agent, and it plays a role in disrupting the bond between lignin and carbohydrates, thereby removing the lignin from the biomass (Galbe and Zacchi 2007). Due to the removal of lignin, the accessible surface area of cellulose and hemicellulose for enzyme binding and microbial attack increases (Singh *et al.* 2011). Furthermore, most of the cellulose in biomass can be retained after alkali pretreatment (Singh *et al.* 2011; Mirahmadi *et al.* 2010). This further contributes to the significant improvement in reducing sugar (RS) yield after enzymatic hydrolysis of alkali-pretreated biomass (Sukumaran *et al.* 2009; Zhao *et al.* 2009). Alkali-pretreated biomass, which has a lower lignin content and higher percentage of cellulose, is able to facilitate the production of various enzymes, especially cellulase, through fermentation (Damisa *et al.* 2008; Singh *et al.* 2011; Soni *et al.* 2010).

Solid state fermentation (SSF), being one of the most extensively studied fermentation methods, is widely applied in the production of industrial enzymes such as cellulase and xylanase, bioethanol, and other secondary metabolites (Tengerdy and Szakacs 2003). SSF offers a number of advantages as compared to submerged fermentation (SmF). SSF is particularly suitable for filamentous fungi because SSF provides a condition that is closer to their natural habitat compared to SmF (Raghavarao *et al.* 2003). Furthermore, the cost of dewatering can be greatly reduced, since SSF is carried out in the absence of free flowing water (Pandey *et al.* 2000). Most importantly, SSF offers benefits in terms of having a higher concentration of end products, higher fermentation productivity, and lower demand on sterilization of the equipment (Hölker *et al.* 2004). All of these results make SSF a technique worthy of investigation for the bioconversion of agricultural residues into value-added products with the aid of filamentous fungi.

The objective of this work was to perform an optimization study on the alkali pretreatment of sugarcane bagasse (SCB) and to investigate the potential of a white-rot fungus, *Pycnoporus sanguineus*, in the production of cellulase and reducing sugar from alkali-pretreated SCB via SSF. Ethanol production was used to examine the fermentability of the reducing sugar produced by adding yeast to the SSF system. SCB was selected due to its relatively high cellulose content of approximately 50% (Pandey *et al.* 2000). The potential of *P. sanguineus* in producing cellulase and reducing sugar has also been confirmed (Quiroz-Castaneda *et al.* 2009; Teoh and Mashitah 2010). Alkali pretreatment is suitable for application to SCB since this pretreatment method is effective on agricultural residue, which contains a relatively lower amount of lignin compared to woody materials (Galbe and Zacchi 2007). In the optimization study, the effect of pretreatment temperature, duration, and sodium hydroxide (NaOH) concentration on RS obtained from enzymatic hydrolysis of pretreated SCB was examined using Central Composite Design (CCD). It was expected that a higher RS yield would be obtained with a more effective pretreatment condition. The ability of *P. sanguineus* to produce cellulase and reducing sugar simultaneously was further investigated by cultivating the fungus on

SCB pretreated under optimum conditions. Ethanol production as a result of co-culturing yeast and *P. sanguineus* was reported.

EXPERIMENTAL

Fungal Culture and Inoculum Preparation

White-rot fungus *Pycnoporus sanguineus* was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). It was maintained on malt extract peptone agar at 4 °C and was sub-cultured every month. The fungus inoculum was prepared by washing the surface of the fungus, which was grown on an agar plate, with 5 mL of sterilized water. The mycelia suspension obtained was then transferred to an Erlenmeyer flask that contained 50 mL of 2% malt extract culture medium. The mycelia suspension was incubated at room temperature at 100 rpm for 5 days. The fungal pellets obtained were washed with sterile water by centrifugation and homogenized before suspending the pellets into a sterile Mandel's medium prior to use in SSF. The Mandel's medium consisted of 2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L urea, 0.3 g/L $CaCl_2.2H_2O$, 0.3 g/L $MgSO_4.7H_2O$, 1 g/L peptone, 0.2% (v/v) Tween 80, 5 mg/L $FeSO_4.7H_2O$, 1.6 mg/L $MnSO_4.2H_2O$, 1.4 mg/L $ZnSO_4.7H_2O$, and 2 mg/L $CoCl_2.6H_2O$.

Alkali Pretreatment

SCB was collected from Purecane Manufacturing Sdn. Bhd., Malaysia. The SCB was thoroughly washed and sun dried. Alkali pretreatment of the SCB was carried out by adding NaOH of various concentrations to the test tube that contained the SCB. The mixture was then heated in an oil bath (Julabo, MC V.2, Germany) to the desired temperature. The operating conditions examined are shown in Table 2. After the reaction, the mixture was cooled and filtered. The pretreated solid was washed with deionized water and acetate buffer before drying at 60 °C prior to enzymatic saccharification.

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed by adding a cellulase solution with a loading of 30 FPU/g substrate to the pretreated SCB at a ratio of 2% (w/v) (Zhao *et al.* 2009). The mixture was buffered with a 50 mM acetate buffer solution with a pH of 4.8. The reaction was carried out at 50 °C for 48 hours and halted by heating the mixture in boiling water for five minutes. The mixture was then centrifuged at 6000 rpm for 20 min. The concentration of RS in the supernatant was determined using the DNS method (Miller 1959; Ghose 1987). Glucose solutions were used as standards and the absorbance reading was taken at 540 nm. The RS yield obtained from the enzymatic hydrolysis was determined using Equation (1) (Li *et al.* 2009).

$$\text{Yield, Y (\%)} = \frac{\text{Weight of Reducing Sugar (mg)}}{\text{Initial Weight of Substrate used in Enzymatic Hydrolysis (mg)}} \times 100\% \quad (1)$$

Optimization of Alkali Pretreatment

Design Expert 6.0.6 software (STAT-EASE Inc., Minneapolis, USA) was used to determine the optimum conditions for alkali pretreatment of SCB. Central Composite Design (CCD) of Response Surface Methodology (RSM) was employed to optimize the

pretreatment process. The effects of three independent variables; pretreatment temperature (°C), NaOH concentration (M), and duration (min) on alkali pretreatment were investigated (Table 1). The selection of the range of each independent variable was based on some preliminary studies. RS yield obtained after enzymatic hydrolysis was the response or the dependent variable. A total of 20 experimental runs were conducted to optimize the process. Analysis of variance (ANOVA) was used to evaluate the statistical significance of the model developed by the software.

Table 1. Levels of the Independent Variables in the Alkali Pretreatment

Variable	Coding	Units	Levels		
			-1	0	1
Temperature	A	°C	100	120	140
NaOH concentration	B	M	0.1	0.5	0.9
Duration	C	min	15	30	45

Solid State Fermentation (SSF)

SCB pretreated with alkali under the optimized operating conditions was subjected to SSF. The SSF was carried out in an Erlenmeyer flask with 2 g of pretreated-SCB inoculated with 1 mL of homogenized mycelia suspension. The moisture content of the SCB was adjusted to 75% with Mandel's medium, and SSF was conducted at room temperature. The content of the flask was extracted within 3 to 8 days of fermentation with 10 mL of a citrate buffer (50 mM, pH 4.8). The flask was then agitated at 150 rpm for 1 hour, and subsequently the content was centrifuged at 4 °C and 3500 rpm for 20 min. The supernatant obtained from centrifugation was filtered, and thereafter the cellulase activities, total soluble protein content, and reducing sugar concentration were determined from the filtrate.

Ethanol Production

Ethanol production was examined by co-culturing yeast, *Saccharomyces cerevisiae*, with *P. sanguineus*. *S. cerevisiae* used in this study was obtained from the Institute of Biological Science, Faculty of Science in University Malaya, Malaysia. After 4 days of SSF of *P. sanguineus* on the alkali-pretreated SCB, 19 mL of deionized water and 1 mL of the *S. cerevisiae* suspension with a cell concentration approximately 10^8 cells/mL were added to the fermented SCB. Ethanol concentration was analyzed after 2 days of fermentation under static conditions.

Analytical Methods

The reducing sugar concentration was determined using the DNS method (Miller 1959). The total soluble protein content was determined using a TP0300 total protein kit, which is based on micro Lowry Peterson's modification method (Sigma-Aldrich, USA).

The total cellulase (filter paper activity, FPase), carboxylmethyl cellulase (CMCase), and β-glucosidase activities were determined in accordance with the procedures reported (Ghose 1987). Whatman filter paper no.1, carboxylmethyl cellulose, and cellobiose were used as the substrates in the determination of FPase, CMCase, and β-glucosidase activities, respectively. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmolmL^{-1} min^{-1} of glucose (2 μmol mL^{-1} min^{-1} of glucose in the case of β-glucosidase) from the substrate under the assay conditions.

The ethanol concentration obtained from co-culturing *S. cerevisiae* and *P. sanguineus* was analyzed using high performance liquid chromatography (HPLC, Waters, USA) equipped with a Waters 410 refractive index detector. A Hi-Plex H column (7.7 mm x 300) (Agilent, USA) was adopted, and the analysis conditions were as follows: deionized water as the mobile phase at a flow rate of 0.6 mL min^{-1}, injection volume of 10 µL, and a column temperature of 65 °C.

RESULTS AND DISCUSSION

Optimization of Alkali Pretreatment

The alkali pretreatment of SCB was optimized before subjecting the substrate to the subsequent SSF process. Alkali pretreatment plays a major role in partial lignin and hemicellulose removal from the biomass (Brodeur *et al.* 2011; Galbe and Zacchi 2007).Other products such as phenolic acid, furfural, and aldehydes, which are known as fermentation inhibitors, might be produced during alkali pretreatment (Brodeur *et al.* 2011). Thus, it is necessary to perform a washing step prior to enzymatic hydrolysis or solid state fermentation to remove lignin, soluble hemicellulose, and other inhibitors that were deposited on the surface of alkali-pretreated biomass. While washing the alkali-pretreated biomass, a neutral pH is attained, and this creates a condition that is favorable for enzymes and microbial attack in the subsequent bioconversion processes. However, substantial amount of wastewater is inevitably produced from the washing step, and this incurs extra cost in the wastewater treatment, which is not beneficial in the economical point of view. To improve the cost effectiveness of this process, the alkaline solution could be pressed out from the biomass after pretreatment (Williams *et al.* 2013). Alternatively, the washed water can also be reused after the desired pH adjustment for alkaline pretreatment process.

The experimental design matrix and RS yields obtained from enzymatic hydrolysis of the alkali-pretreated SCB are presented in Table 2. RS yield as high as 97.1% was obtained from the alkali-pretreated SCB. The RS yield obtained under the operating conditions investigated was fitted to a second order polynomial equation. The final equation in terms of their coded factors after elimination of the insignificant model terms is shown as Equation (2).

$$Y = 94.77 + 1.85\ A + 22.75\ B + 1.54\ C - 2.17\ A^2 - 23.27\ B^2 - 2.12\ C^2 - 1.43\ AC + 2.52\ BC \quad (2)$$

The results of the analysis of variance (ANOVA) for the above reduced quadratic model are tabulated (Table 3). A, B, C, B^2, and BC are the significant model terms, since their probability > F values are less than 0.05 in 95% confidence interval. Among these terms, B and B^2 have the lowest probability > F values. This implies that changes in NaOH concentration have the greatest impact on the RS yield obtained from alkali-pretreated SCB. The model developed by the software was able to fit the experimental data well, as proven by a significant model term and an insignificant lack of fit term. This is further confirmed by the coefficient of determination, R-squared, value for the model (0.9952), which is close to 1.

Table 2. Experimental Design Matrix and RS Yield from Enzymatic Hydrolysis of Alkali-Pretreated SCB

Run	Independent Variables			RS Yield, Y (%)
	Temperature, A (°C)	NaOH concentration, B (M)	Duration, C (min)	
1	100	0.1	15	42.7
2	100	0.1	45	44.3
3	100	0.5	30	89.0
4	100	0.9	15	84.2
5	100	0.9	45	92.0
6	120	0.5	15	88.3
7	120	0.1	30	49.0
8	120	0.9	30	93.3
9	120	0.5	30	93.2
10	120	0.5	30	95.9
11	120	0.5	30	95.2
12	120	0.5	30	94.4
13	120	0.5	30	97.1
14	120	0.5	30	94.2
15	120	0.5	45	96.3
16	140	0.1	15	49.3
17	140	0.1	45	41.3
18	140	0.5	30	95.5
19	140	0.9	15	89.3
20	140	0.9	45	95.3

Table 3. ANOVA Results for the Reduced Quadratic Model of Alkali Pretreatment

Source	Sum of squares	DF*	Mean square	F-value	Probability>F
Model	8679.77	8	1084.97	283.95	< 0.0001
A	34.23	1	34.23	8.96	0.0122
B	5175.63	1	5175.63	1354.52	< 0.0001
C	23.72	1	23.72	6.21	0.0300
A^2	12.93	1	12.93	3.38	0.0930
B^2	1488.87	1	1488.87	389.65	< 0.0001
C^2	12.34	1	12.34	3.23	0.0998
AC	16.24	1	16.24	4.25	0.0637
BC	51.00	1	51.00	13.35	0.0038
Residual	42.03	11	3.82		
Lack of fit	32.53	6	5.42	2.85	0.1349
Pure error	9.50	5	1.90		
Cor total	8721.80	19			

* DF = degree of freedom

Figures 1 and 2 show the three-dimensional response surface plots and the interaction plots of the effect of the parameters investigated on RS yield. Both response surfaces can be best described as elliptical systems, as suggested by Wu and Hamada (2000). By comparing both figures, the interaction between duration and NaOH concentration was shown to have a larger effect on the RS yield. This can be

further proven by the lower F value of the AC term (F-value = 4.25) compared to the F-value of the BC term (13.35), as stated previously in Table 3.

At a lower temperature *i.e.* 100 °C, longer pretreatment duration was needed to improve the RS yield of the pretreated SCB [Fig. 1(b)]. This might be due to the fact that the amount of lignin removed during alkali pretreatment is proportional to the pretreatment duration. Higher lignin removal further facilitated the release of sugar from the pretreated SCB during enzymatic hydrolysis (McIntosh and Vancov 2010). On the other hand, pretreatment at 140 °C gave similar RS yields regardless of the duration employed. This might be the maximum amount of RS that can be derived from SCB, and hence prolonging pretreatment duration did not lead to any improvement in the RS yield. From Fig. 1(b), it seems that pretreatment temperature did not significantly influence the production of reducing sugar. Only little variation in the amount of RS was observed when the pretreatment temperature was increased from 100 °C to 140 °C.

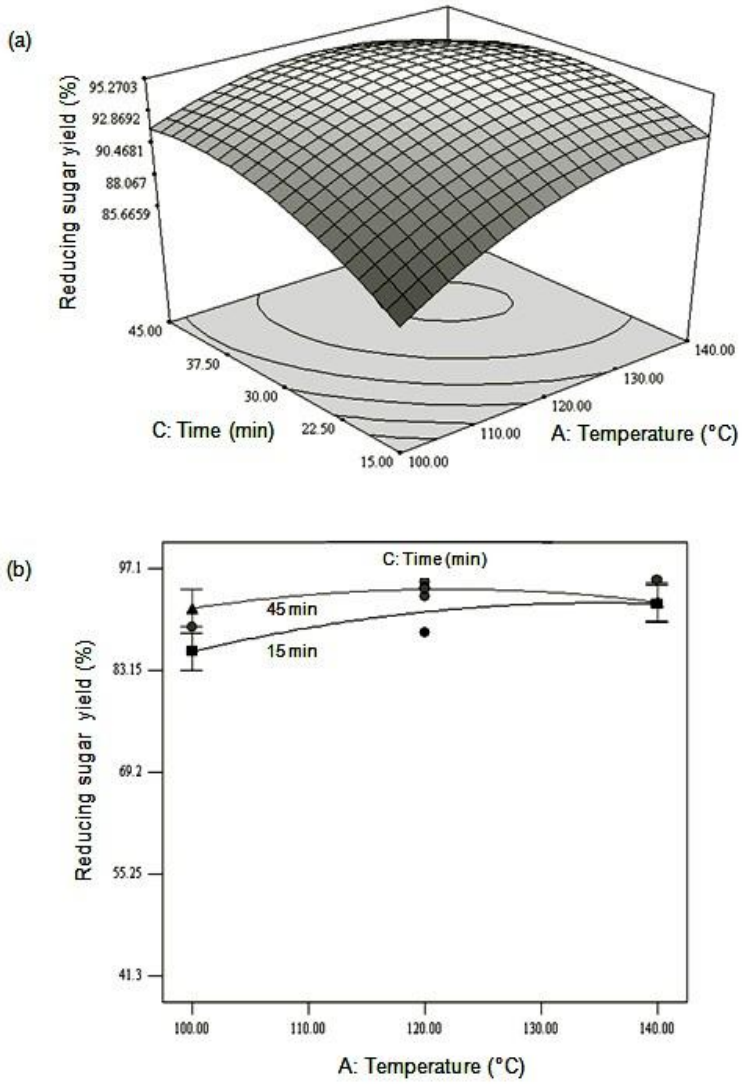

Fig. 1. (a) Three-dimensional response surface plot of the effect of pretreatment temperature and duration on the RS yield at 0.5 M NaOH; (b) Interaction plot between pretreatment temperature and duration on the RS yield at 0.5 M NaOH

As shown in Fig. 2(b), RS yield was significantly affected by the concentration of NaOH used in the pretreatment process. RS yield improved drastically when a NaOH concentration higher than 0.1 M was employed. There was a slight decrease in the RS yield when a NaOH concentration greater than approximately 0.65 M was applied in pretreatment. The decrease might be attributed to the loss of cellulose and hemicellulose during pretreatment by alkaline solution with a greater strength (Nlewem and Thrash 2010). Apart from that, longer pretreatment duration led to higher RS yield and this phenomenon was more apparent when an alkali solution with a higher concentration was used.

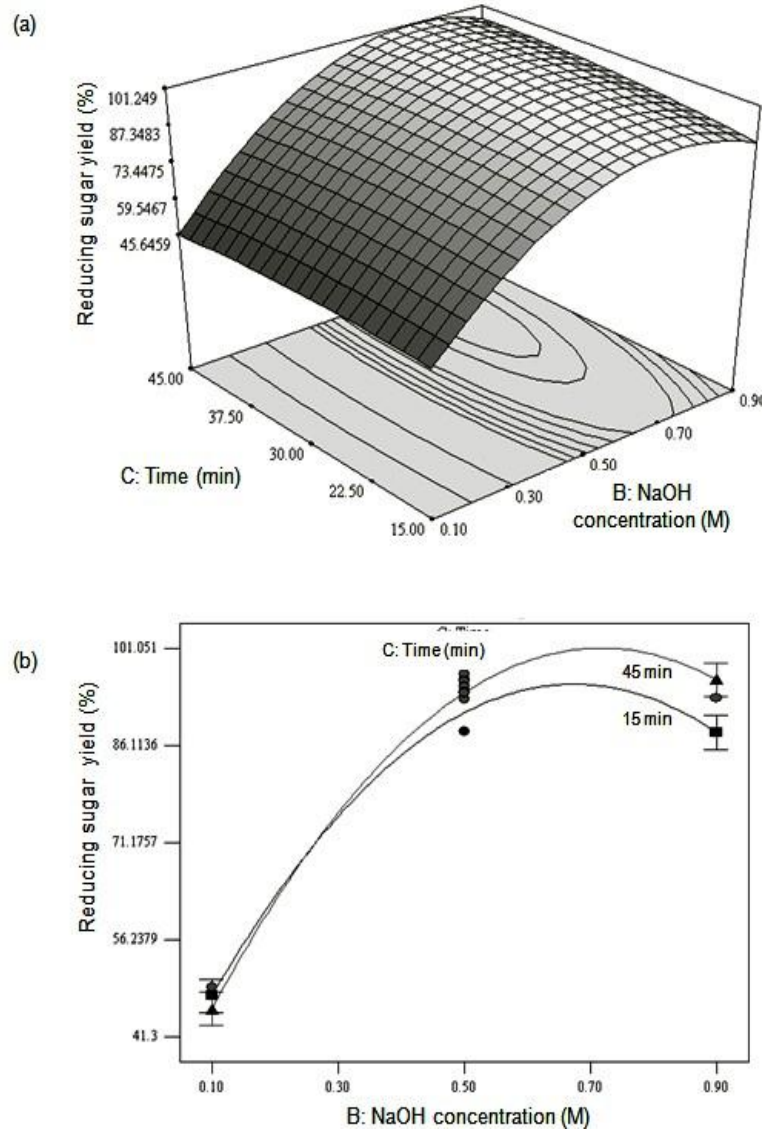

Fig. 2. (a) Three-dimensional response surface plot of the effect of pretreatment duration and NaOH concentration on the RS yield at 120°C (b) Interaction plot between pretreatment duration and NaOH concentration on the RS yield at 120°C

The optimum conditions for alkali pretreatment were obtained using numerical optimization features of Design Expert software. The optimized pretreatment conditions attained were 128°C, 30 min, and 0.62 M with a predicted RS yield of 99.8%. The

average experimental RS yield under these particular conditions was found to be 97.8%. The difference of 1.8% between the predicted and experimental values implies that the model is reliable in predicting the RS yield for the range of the examined pretreatment conditions.

Solid State Fermentation of Alkali-Pretreated SCB

Through SSF with the aid of a microorganism, lignocellulosic biomass can be further converted into other value-added products and consequently, the applicability of the biomass can be evaluated. SCB pretreated under optimized conditions was used as the substrate to cultivate *P. sanguineus* in SSF. The ability of *P. sanguineus* for simultaneous production of cellulase and reducing sugar from alkali-pretreated SCB was investigated. *P. sanguineus* was found to be one of the most suitable white-rot fungi to be cultivated on alkali-pretreated SCB in a prior screening test.

A complete cellulase system should consist of three cellulase components, namely endoglucanase, exoglucanase, and β-glucosidase (Cen and Xia 1999). Each component functions distinctively during the hydrolysis of lignocellulosic biomass. Endoglucanase hydrolyze cellulose chains to form new chain ends and these chain ends are further broken down into cellobiose and cello-oligosaccharides by exoglucanase (Dashtban *et al.* 2009; Kumar *et al.* 2008). Cellobiose is then further fractionated into glucose by the action of β-glucosidase. The cellulose content in the substrate can only be hydrolysed completely into its smallest basic unit, glucose, in the presence of all three cellulase components in significant amounts.

Figure 3 shows that all three cellulase components were detected in the cellulase system produced by *P. sanguineus*. Colonization of *P. sanguineus* on the pretreated SCB began after 2 days of SSF. Cellulase was detected on day 3 of fermentation, and these components of cellulase generally require different durations to reach their maximum activities. CMCase had the highest activity (0.11 IU/ mL) in the shortest fermentation duration (day 3) as compared to the two other cellulase components. On the other hand, FPase and β-glucosidase both had their highest activities recorded on day 8 and day 6 with titres of 0.02 IU/ mL and 0.13 IU/ mL, respectively.

From Fig. 3, it is obvious that β-glucosidase activity was the highest, followed by CMCase and FPase in the cellulase system produced by *P. sanguineus*. This finding is slightly different from the cellulase system of *P. sanguineus* as reported by Teoh and Mashitah (2010) and Quiroz-Castaneda *et al.* (2009). Teoh and Mashitah (2010) cultivated *P. sanguineus* on palm oil mill effluent and palm-pressed fiber under submerged fermentation and found that CMCase is higher than FPase and β-glucosidase during the course of fermentation. Quiroz-Castaneda *et al.* (2009) reported that the highest activity was recorded by CMCase, followed by β-glucosidase and FPase during SSF of *P. sanguineus* on wheat straw. This might indicate that substrates with different structural and chemical compositions are able to stimulate the production of different components in the cellulase system. On the other hand, the production of CMCase was always higher than FPase (Fig. 3), and this phenomenon is in accordance with other reported findings (Quiroz-Castaneda *et al.* 2009; Teoh and Mashitah 2010; Elisashvili *et al.* 2008; Kachlishvili *et al.* 2005; Khan *et al.* 2007).

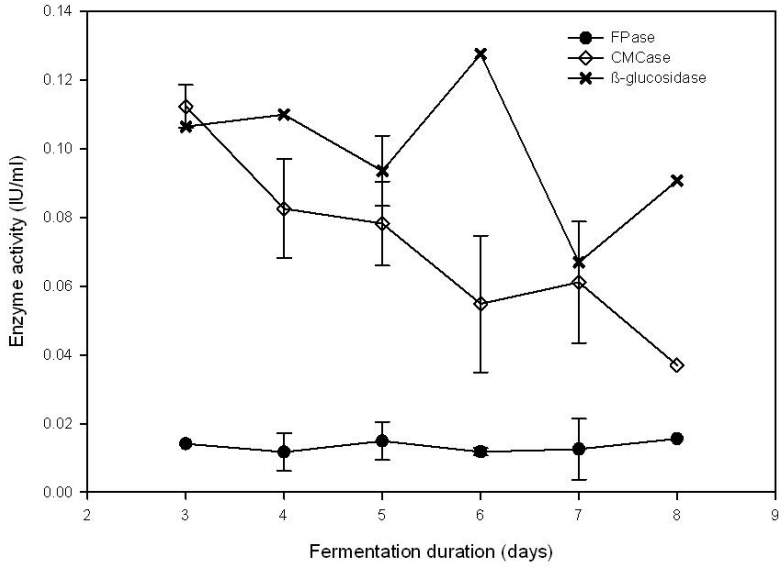

Fig. 3. Time profile of cellulase activities during SSF

The activity of cellulase obtained from SSF of *P. sanguineus* in this study was generally lower than other findings on cellulase production from white-rot fungi via SSF. Cellulase activities in the range of 0.9 to 3 IU/mL were obtained by Shi *et al.* (2008) and Khan *et al.* (2007) via SSF of *Phanerochaete chrysosporium* on cotton stalks and rice straw, respectively. The activity of CMCase is able to reach as high as 62 U/ mL when *Trametes versicolor* is cultivated on banana peel, as reported by Elisashvili *et al.* (2008). The results obtained from this study indicated that the combination of *P. sanguineus* and alkali-pretreated SCB might not be the best in terms of cellulase production. The substantial removal of lignin in SCB during alkali pretreatment could be one of the factors that affects the cellulase production from *P. sanguineus*, because the presence of lignin in biomass to some extent promotes the growth of fungi and aids the production of cellulase (Philippoussis *et al.* 2011).

Despite the low activities, the cellulase system of *P. sanguineus* was able to trigger hydrolysis of cellulose in pretreated SCB under the applied SSF conditions. In conjunction with this, cellulose in the SCB was broken down into its basic unit, reducing sugar. This is proven by the presence of reducing sugar in the fermentation broth, as demonstrated in Fig. 4.

The highest concentration of RS with a total of 19.9 mg/g substrate was obtained on day 4 of fermentation (Fig. 4). This amount of RS was found to correlate well with a report by Shrestha *et al.* (2008), which stated that RS in the concentration of 24 mg/g was obtained from SSF of *Phanerochaete chrysosporium* on corn fiber after 7 days of fermentation. It is worth noting that after 5 days of fermentation, the amount of RS decreased drastically until day 8 of fermentation, as shown in Fig. 4. The decrease might be due to the consumption of RS by *P. sanguineus* during fungus growth (Shrestha *et al.* 2010). This is evident from Fig. 4, in which the RS concentration was seen to be decreasing when fungus growth increased as indicated by the increasing total soluble protein content during the course of fermentation.

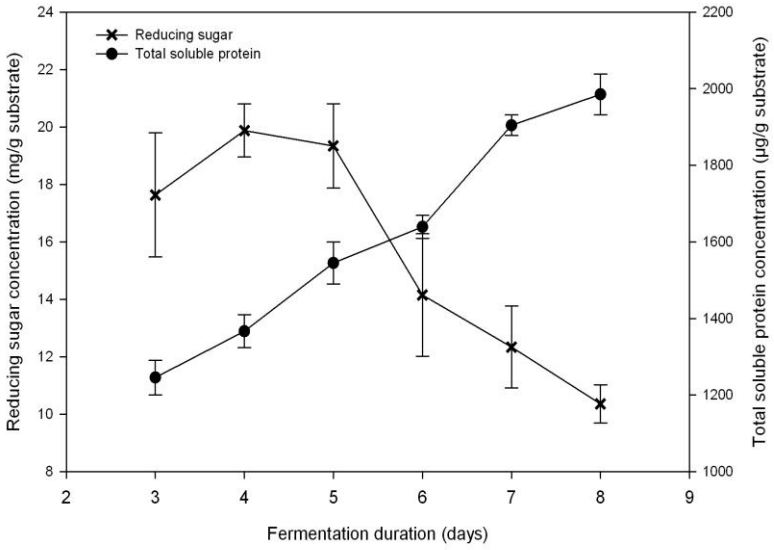

Fig. 4. Time profile of reducing sugar concentration and total soluble protein content during SSF

Ethanol Production

To examine the fermentability of RS produced from the cultivation of *P. sanguineus* via SSF, *S. cerevisiae* was added into the SSF system to convert the available RS into ethanol. No ethanol was detected when the alkali-pretreated SCB was cultivated with *P. sanguineus* alone. During the co-culture of *P. sanguineus* and *S. cerevisiae*, 14.8 mg ethanol/g SCB was obtained after 2 days of fermentation. This amount is equivalent to 1.48 g ethanol/100g SCB, and it is slightly lower compared to the reported values by Shrestha *et al.* (2008) and Rasmussen *et al.* (2010). Shrestha *et al.* (2008) obtained 3 g ethanol/100 g corn fiber after 2 days of fermentation when yeast was added to the co-culture with *Phanerochaete chrysosporium*. On the other hand, the co-culture of yeast and a brown-rot fungus, *Gloeophyllum trabeum*, has successfully produced a maximum of 4 g ethanol from 100 g corn fiber (Rasmussen *et al.* 2010). Ethanol production from the current SSF system could be further improved by optimizing the process.

In short, the low-cost lignocellulosic biomass can be effectively converted to more value-added products through SSF. In spite of the low cellulase activities obtained from SSF of alkali-pretreated SCB, the reducing sugar produced *in situ* could serve as a valuable intermediate for the production of other value-added products such as ethanol. Further study on the conversion of the reducing sugar produced *in situ* into ethanol could be conducted to improve the cost effectiveness of the SSF process.

CONCLUSIONS

1. An optimization study of the alkali pretreatment of sugarcane bagasse was conducted, and a model that gives an accurate prediction of the RS yield was attained. NaOH concentration was found to be the most significant factor that affects alkali pretreatment on sugarcane bagasse compared to the temperature and duration applied during pretreatment.

2. Cellulase and reducing sugar were produced simultaneously when *Pycnoporus sanguineus* was cultivated on alkali-pretreated SCB. Irrespective of the low cellulase activities, the activities are sufficient to hydrolyse the cellulose in pretreated-SCB into reducing sugar. This reducing sugar can be potentially converted to ethanol through the co-culture of yeast and *Pycnoporus sanguineus*. Thus, alkali-pretreated bagasse could serve as a potential feedstock for in-situ production of cellulase, reducing sugar, and ethanol via SSF.

ACKNOWLEDGMENTS

Part of the abstract was submitted to the 15th International Biotechnology Symposium and Exhibition 2012 (IBS), which will be held in EXCO, Daegu, South Korea from 16th to 21st September 2012. The authors thank the University of Malaya for supporting this research under the grants of UMRG RG006/09AET and PV076/2011B. The authors would also like to express their gratitude to Purecane Manufacturing Sdn. Bhd. for supplying the SCB.

REFERENCES CITED

Bansal, N., Tewari, R., Gupta, J. K., Soni, R., and Soni, S. K. (2011). "A novel strain of *Aspergillusniger* producing a cocktail of hydrolytic depolymerising enzymes for the production of second generation biofuels," *BioResources* 6(1), 552-569.

Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K. B., and Ramakrishnan, S. (2011)."Chemical and physicochemical pretreatment of lignocellulosic biomass: A review," *Enzyme Research*, 2011, 1-17.

Cen, P., and Xia, L. (1999). "Production of cellulase by solid-state fermentation," *Adv. Biochem. Engin. / Biotech.* 65, 69-92.

Damisa, D., Ameh, J. B., and Umoh, V. J. (2008). "Effect of chemical pretreatment of some lignocellulosic wastes on the recovery of cellulase from *Aspergillus niger AH3* mutant,"*Afr. J. Biotech*7(14), 2444-2450.

Dashtban, M., Schraft, H., and Qin, W. S. (2009). "Fungal bioconversion of lignocellulosic residues; Opportunities & perspectives," *Int. J. Biol. Sci.* 5(6), 578-595.

Elisashvili, V., Kachlishvili, E., and Penninckx, M. (2008). "Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes," *Ind. Microbiol. Biotechnol*. 35(11), 1531-1538.

Galbe, M., and Zacchi, G. (2007). "Pretreatment of lignocellulosic materials for efficient bioethanol production, *Biofuels*, Springer-Verlag Berlin, Berlin, 108, 41.

Ghose, T. K. (1987). "Measurement of cellulase activities," *Pure Appl. Chem*. 59(2), 257-268.

Hölker, U., Höfer, M., and Lenz, J. (2004). "Biotechnological advantages of laboratory-scale solid state fermentation with fungi,"*Appl. Microbiol. Biotech.* 64(2), 175-186.

Kachlishvili, E., Penninckx, M. J., Tsiklauri, N., and Elisashvili, V. (2005). "Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation," *World J. Microbiol. Biotech.* 22(4), 391-397.

Karunanithy, C., and Muthukumarappan, K. (2011). "Optimization of alkali, big bluestem particle size, and extruder parameters for maximum enzymatic sugar recovery using response surface methodology," *BioResources* 6(1), 762-790.

Khan, M. M. H., Ali, S., Fakhru'l-Razia, A., and Alam, M. Z. (2007). "Use of fungi for the bioconversion of rice straw into cellulase enzyme," *Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes* 42(4), 381-386.

Kumar, R., Singh, S., and Singh, O. V. (2008). "Bioconversion of lignocellulosicbiomass: Biochemical and molecular perspectives," *Journal of Industrial Microbiology & Biotechnology* 35(5), 377-391.

Li, Q., He, Y. C., Xian, M., Jun, G., Xu, X., Yang, J. M., and Li, L. Z. (2009). "Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment," *Bioresource Technol.* 100(14), 3570-3575.

McIntosh, S., and Vancov, T. (2010). "Enhanced enzyme saccharification of sorghum bicolor straw using dilute alkali pretreatment," *Bioresource Technol.* 101(17), 6718-6727.

Miller, G. L. (1959). "Use of dinitrosalicyclic acid reagent for determination of reducing sugar," *Analytical Chem.* 31(3), 426-428.

Mirahmadi, K., Kabir, M. M., Jeihanipour, A., Karimi, K., and Taherzadeh, M. J. (2010). "Alkaline pretreatment of spruce and birch to improve bioethanol and biogas production," *BioResources* 5(2), 928-938.

Nlewem, K. C., and Thrash, M. E. (2010). "Comparison of different pretreatment methods based on residual lignin effect on the enzymatic hydrolysis of switchgrass," *Bioresource Technol.*101(14), 5426-5430.

Pandey, A., Soccol, C. R., Nigam, P., and Soccol, V. T. (2000). "Biotechnological potential of agro-industrial residues. I: Sugarcane bagasse," *Bioresource Technol.* 74(1), 69-80.

Philippoussis, A., Diamantopoulou, P., Papadopoulou, K., Lakhtar, H., Roussos, S., Parissopoulos, G., and Papanikolaou, S. (2011). "Biomass, laccase and endoglucanase production by *Lentinula edodes* during solid state fermentation of reed grass, bean stalks and wheat straw residues," *World J. Microbiol. Biotechnol.* 27(2), 285-297.

Quiroz-Castaneda, R. E., Balcazar-Lopez, E., Dantan-Gonzalez, E., Martinez, A., Folch-Mallol, J., and Anaya, C. M. (2009). "Characterization of cellulolytic activities of *Bjerkandera adusta* and *Pycnoporus sanguineus* on solid wheat straw medium," *Electron. J. Biotechnol.* 12(4),1- 8.

Raghavarao, K., Ranganathan, T. V., and Karanth, N. G. (2003). "Some engineering aspects of solid-state fermentation," *Biochemical Engineering Journal* 13(2-3), 127-135.

Rasmussen, M. L., Shrestha, P., Khanal, S. K., Pometto III, A. L., and Leeuwen, J. (2010). "Sequential saccharification of corn fiber and ethanol production by the brown rot fungus *Gloeophyllum trabeum*," *Bioresource Technol.* 101(10), 3526-3533.

Shi, J., Chinn, M. S., and Sharma-Shivappa, R. R. (2008). "Microbial pretreatment of cotton stalks by solid state cultivation of *Phanerochaete chrysosporium*," *Bioresource Technol.* 99(14), 6556-6564.

Shrestha, P., Khanal, S. K., Pometto III, A. L., and Leeuwen, J. (2010). "Ethanol production via in situ fungal saccharification and fermentation of mild alkali and steam pretreated corn fiber," *Bioresource Technol.* 101(22), 8698-8705.

Shrestha, P., Rasmussen, M., Khanal, S. K., Pometto III, A. L., and Leeuwen, J. (2008). "Solid-substrate fermentation of corn fiber by *Phanerochaete chrysosporium* and subsequent fermentation of hydrolysate into ethanol," *J. of Agric. Food Chem.* 56(11), 3918-3924.

Singh, A., Tuteja, S., Singh, N., and Bishnoi, N. R. (2011). "Enhanced saccharification of rice straw and hull by microwave–alkali pretreatment and lignocellulolytic enzyme production," *Bioresource Technol.* 102(2), 1773-1782.

Soni, S. K., Batra, N., Bansal, N., and Soni, R. (2010). "Bioconversion of sugarcane bagasse into second generation bioethanol after enzymatic hydrolysis with in-house produced cellulases from*Aspergillus* sp. S4B2F," *BioResources* 5(2), 741-757.

Sousa, L. D., Chundawat, S. P. S., Balan, V., and Dale, B. E. (2009). "'Cradle-to-grave' assessment of existing lignocellulose pretreatment technologies," *Current Opinion in Biotechnology* 20(3), 339-347.

Sukumaran, R. K., Singhania, R. R., Mathew, G. M., and Pandey, A. (2009). "Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production," *Renew. Energy* 34(2), 421-424.

Sun, R. C. (2009). "Detoxification and separation of lignocellulosic biomass prior to fermentation for bioethanol production by removal of lignin and hemicelluloses," *BioResources* 4(2), 452-455.

Tengerdy, R. P., and Szakacs, G. (2003). "Bioconversion of lignocellulose in solid substrate fermentation," *Biochemical Engineering Journal* 13 (2-3), 169-179.

Teoh, Y. P., and Mashitah, M. D. (2010). "Cellulase production by *Pycnoporus sanguineus* on oil palm residues through pretreatment and optimization study," *Journal of Applied Sciences* 10(12), 1036-1043.

Williams, K., Zheng, Y., McGarvey, J., Fan, Z., and Zhang, R. (2013). "Ethanol and volatile fatty acid production from lignocellulose by *Clostridium cellulolyticum*," International Scholarly Research Network, 2013, 1-7.

Wu, J. C. F., and Hamada, M. (2000). *Experiments: Planning, Analysis and Parameter Design Optimization*, Wiley-Interscience, New York.

Xu, J. L., Cheng, J. J., Sharma-Shivappa, R. R., and Burns, J. C. (2010). "Sodium hydroxide pretreatment of switchgrass for ethanol production," *Energy Fuels* 24, 2113-2119.

Yang, B., and Wyman, C. E. (2008). "Pretreatment: The key to unlocking low-cost cellulosic ethanol," *Biofuels Bioprod. Biorefining* 2(1), 26-40.

Yao, R., Qi, B., Deng, S., Liu, N., Peng, S., and Cui, Q. (2007). "Use of surfactants in enzymatic hydrolysis of rice straw and lactic acid production from rice straw by simultaneous saccharification and fermentation," *BioResources* 2(3), 389-398.

Zhang, B., Shahbazi, A., Wang, L., Diallo, O., and Whitmore, A. (2010). "Alkali pretreatment and enzymatic hydrolysis of cattails from constructed wetlands," *American Journal of Engineering and Applied Science* 3(2), 328-332.

Zhao, X. B., Peng, F., Cheng, K., and Liu, D. H. (2009). "Enhancement of the enzymatic digestibility of sugarcane bagasse by alkali-peracetic acid pretreatment," *Enzyme and Microbial Technology* 44(1), 17-23.

Article submitted: August 2, 2012;Peer review completed: September 4, 2012; Revised version received and accepted: September 7, 2012; Published: September 13, 2012.