CONVERSION OF LIGNOCELLULOSIC BIOMASS FROM GRASS TO BIOETHANOL USING MATERIALS PRETREATED WITH ALKALI AND THE WHITE ROT FUNGUS, *PHANEROCHAETE CHRYSOSPORIUM*

Yan Yee Liong,[a] Rasmina Halis,[b] Oi Ming Lai,[c] and Rozi Mohamed [a,*]

Grasses are abundant in many climatic regions of the world and have been regarded as weeds by many. This work investigated the use of *Pennisetum purpureum* (Napier grass) in the production of bioethanol. Two pretreated grasses were compared as the initial substance in the hydrolysis process followed by bacteria fermentation. For the purpose of breaking down lignin, alkali pretreatment, where grass was soaked in 7% NaOH, was used. For biological pretreatment, grass was incubated for 3 weeks with the white-rot fungus, *Phanerochaete chrysosporium*. Both types of pretreated materials were subjected to *Trichoderma reesei* ATCC 26921 enzyme hydrolysis. Glucose content from alkali-pretreated samples was 1.6-fold higher than fungus-pretreated samples. Hydrolysates from the pretreatments were fermented using the ethanol insensitive strain *Escherichia coli* K011. After 24 hours of fermentation, the ethanol yield from alkali-pretreated material was 1.5 times higher than the biological-pretreated material. It can be concluded that NaOH-pretreated enzyme hydrolysate had a better ethanol yield compared to biological-pretreated enzyme hydrolysate, but biological-pretreated enzyme hydrolysate had better ethanol conversion efficiency, which was 18.5 g/g. These results indicated that wild grass is capable of becoming an important biomass for small local bioethanol production.

Keywords: Basidiomycetes; Biological pretreatment; Cellulose; Escherichia coli; Fermentation

*Contact information: a: Department of Forest Management, Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia; b: Department of Forest Production, Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia; c: Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; *Corresponding author: rozimohd@putra.upm.edu.my*

INTRODUCTION

Grass is the world's cheapest lignocellulosic biomass, but people have not realized the importance of grasses and often treat grass species as weeds or feedstock for animals. However, grasses are capable of becoming a potential producer of lignocellulosic biomass. In general, grasses are available throughout every region of the world and throughout the seasons, even in winter. As a lignocellulosic perennial crop, grasses are a promising feedstock for producing bioethanol because of high yields, low costs, good suitability for low quality land, and low environmental impact. The lignin content for most materials is about 27%, but grasses contain distinctly lower levels in comparison to other lignocellulosic materials such as hardwood and softwood (~40%) (Hamelinck *et al.* 2005).

There has been some interest to convert perennial grasses such as switchgrass and ryegrass into bioethanol (Schmer *et al*. 2008; Martinez-Perez *et al.* 2007). Recent research showed that fructans in ryegrass can be converted to bioethanol through

microbial fermentation (Martel *et al.* 2010). Another study showed that saccharification of Kans grass using an enzyme mixture from *Trichoderma reesei* is capable of producing bioethanol (Kataria and Ghosh, 2011). Kans grass was first pretreated with acid, followed by enzymatic hydrolysis to produce fermentable sugars, which were then fermented to bioethanol using *Saccharomyces cerevisiae* (Kataria and Ghosh, 2011).

Pretreatment is always associated with bioethanol whenever ethanol yield is considered. It has always been a very crucial and important step in producing bioethanol because pretreatment can enhance the efficiency of sugar conversion during the hydrolysis process. The task of hydrolyzing lignocellulose to fermentable monosaccharides is still technically problematic because the digestibility of cellulose is hindered by many physico-chemical, structural, and compositional factors. Due to these structural characteristics, pretreatment is an essential step for obtaining potentially fermentable sugars during hydrolysis. The aim of pretreatment is to break down the lignin structure and disrupt the crystalline structure of cellulose for the purpose of enhancing enzymes' accessibility to the cellulose during hydrolysis (Mosier *et al.* 2005). Current pretreatment research is focused on identifying, evaluating, developing, and demonstrating promising approaches that primarily support the subsequent enzymatic hydrolysis of the treated biomass with lower enzyme dosages and shorter bioconversion times. A large number of pretreatment approaches have been investigated on a wide variety of feedstock types, and there are several recent review articles that provide a general overview of the field (Carvalheiro *et al.* 2008; Taherzadeh and Karimi 2008; Yang and Wyman 2008; Hendriks and Zeeman 2009). Some examples of pretreatment methods are steam explosion, alkaline pretreatment, and acid pretreatment. Alkali pretreatments increase cellulose digestibility, and they are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization than acid or hydrothermal processes. Besides that, alkali pretreatment also exhibits minor loss in cellulose amount and is more effective for hemicellulose solubilization than acid or hydrothermal processes (Carvalheiro *et al.* 2008). Alkali pretreatment can be performed at room temperature and for durations ranging from seconds to days. It has been known to cause less sugar degradation than acid pretreatments, and it was shown to be more effective on agricultural residues than on wood materials (Kumar *et al.* 2009). Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pretreatments. NaOH has been reported to increase hardwood digestibility from 14% to 55% by reducing lignin content from 24-55% to 20% (Kumar *et al.* 2009).

Although chemical pretreatment is the most commonly used method, it is very expensive and produces a lot of waste. Therefore biological pretreatment should be considered as an important alternative. Biological pretreatment, however, has not been very commonly used until recent years, because the fungi used are hard to monitor due to contamination problems and the long required period of time for such pretreatment. Biological pretreatment does not require a high amount of energy and can be carried out under normal conditions. Microorganisms such as brown- and white-rot fungi are used to degrade lignin and hemicellulose in waste materials such as agricultural waste due to their lignin-degrading abilities (Schurz 1978; Sun and Cheng 2002; Sánchez 2009; Halis *et al.* 2012). Brown-rot and white-rot fungi target different compounds (Fan *et al.* 1987); brown-rot mainly attacks cellulose, while white-rot fungus attacks both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for the biological pretreatment of lignocellulosic materials. Many white-rot fungi degrade lignin, and for this reason they have been utilized for ligninase production and lignocellulose degrada-

tion (Lee 1997). Lignin degradation by white-rot fungi occurs through the action of enzymes such as peroxidases and laccases (Kumar *et al.* 2009). Several white-rot fungi such as *P. chrysosporium*, *Ceriporia lacerata*, *Cyathus stercoreus*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnabarinus*, and *Pleurotus ostreatus* have been examined on different lignocellulosic biomasses showing high delignification efficiency (Shi *et al.* 2008; Kumar *et al.* 2009).

This work reports on the use of alkali- and fungus-pretreated grass for the process of converting lignocellulose into bioethanol. Fermentation efficiency and ethanol yield were compared between the two different pretreated samples.

EXPERIMENTAL

Plant Materials

Pennisetum purpureum Schumach. (Napier grass) was obtained from idle land near Bandar Baru Bangi, Selangor, Malaysia. Fresh green grass was collected during its flowering stage, at an average height of 1.5 m. The whole plant was cut 15 cm from the base of the stem. The sample was cut into smaller pieces (3 cm) and oven-dried at 70 ˚C for two days or until constant weight was achieved. The dried sample was milled using a Wiley's mill, and the grass powder was passed through a 1 mm sieve and stored for the alkaline pretreatment. For biological pretreatment, fresh grass was cut into 3 cm lengths and immediately cleaned with water. To surface-sterilize, the cut grass was quickly dipped in 100% alcohol, followed by washing in double distilled water before placing it into a sterilized aluminium tray.

Alkaline Pretreatment

Pretreatment was conducted by soaking the dried Napier grass in sodium hydroxide (NaOH) solutions at 7% for 4 h. The experiment was conducted with three replicates each time. The treated sample was then washed with water and later soaked in distilled water for 24 h, followed by washing. The treated material was then dried in the oven at 70 ˚C for two days or until constant weight was achieved.

Biological Pretreatment

A pure culture of *Phanerochaete chrysosporium* was obtained from the culture center of the Forest Research Institute of Malaysia. The culture was grown in Potato Dextrose Agar (PDA) slants, and spore suspension was prepared following Halis *et al.* (2012). A total of 20 mL of fungal inoculum at a concentration of 10^5 spores/mL was added into the grass material in the tray. The tray was then covered tightly with aluminium foil and kept at room condition for 3 or 4 weeks. At the end of the incubation period, samples were washed in hot water to remove the fungal layer and then dried in the oven before being ground and passed through sieve 1 mm. The experiment was repeated three times with each three replicates.

Scanning Electron Microscope (SEM)

SEM was carried out to examine the extent of fiber destruction by fungus. Freshly cut grass 3 cm in length was incubated with 4 mL of inoculum in a sterile petri dish for 3 or 4 weeks at room temperature, after which it was subjected to SEM analysis. For SEM, samples were first cut into 1 cm x 1 cm pieces and put into separate vials and

fixed in 4% glutaraldehyde for 12 to 24 h at 4 ˚C. They were then washed in 0.1 M sodium cacodylate buffer for 3 changes of 10 min each, after which they were post-fixed in 1% osmium textroxide for 2 h at 4 ˚C. The samples were washed again in 0.1 M sodium cacodylate buffer for 3 changes of 10 min each, after which the samples were dehydrated in a series of acetones at different concentrations. The first wash was 35% for 10 min, followed by 50%, 75%, and 95% for 10 min each. Finally, the samples were washed in 100% acetone with three changes of 15 min each, and dried in a critical dryer for 30 min. The dried sample was mounted onto the stub and coated in gold using the sputter coater and then viewed under the scanning electron microscope (Philips XL30 ESEM).

Response Surface Method (RSM)

The response surface method (Jeya *et al.* 2009) was used to design a series of experiments using temperature from 35 to 40 ^{o}C and agitation 150 to 200 rpm as the two parameters in enzymatic hydrolysis. Parameters that yielded the highest glucose concentration were chosen for the enzymatic hydrolysis step.

A factorial, central composite rotary design (CCRD) for two factors with replicates at the center point and star points were used in the investigation. The variables used were agitation and temperature. The actual levels of variables for CCRD experiments were selected based on the initial levels as the center points. A total of 13 experimental trials that included four trials for factorial design, four trials for axial points (two for each variable), and five trials for replication of the central points were performed. The response value, glucose yield (Y) is the average of triplicates.

Enzyme Hydrolysis

A commercial enzyme, cellulase, from *Trichoderma reesei* ATCC 26921 (700 units/g, Sigma, St. Louis, MO) was used. Hydrolysis was conducted in a 250 mL conical flask containing 1 g of either alkali-pretreated grass or biological-pretreated grass, 100 mL of distilled water, and 1 mL of cellulase. The reaction was conducted in an incubator shaker for 2 days at 38.5 ˚C and 175 rpm (Innova 40, New Brunswick, USA). After autoclaving the hydrolysate at 121˚C and 25 minutes, 1 mL of sample was collected in a 1.5 mL microcentrifuge tube and centrifuged at 10,000 g for 10 min and then filtered using a 0.45 µm nylon syringe filter (Sartorius). The supernatant was stored at -20 ˚C until sugar analysis.

Fermentation

Escherichia coli (*E. coli*) strain K011 was kindly provided by Professor Ingram, L.O. (University of Florida). The culture was grown in a modified Luria-Bertani (LB) broth containing the following per liter: 5 g NaCl, 5 g yeast extract, 10 g tryptone, and chloramphenicol (to a final concentration of 40 mg/L or 600 mg/L). For the 40 mg/L chloramphenicol plate, the antibiotic stock was made by adding 0.4 g chloramphenicol to 10 mL of 70% ethanol, then 1 mL/L of the stock solution was added into the sterilized agar medium. For the 600 mg/L chloramphenicol plate, 0.3 g of chloramphenicol was added directly to the agar mix and stirred well before plating. Culturing of the bacteria began with the 40 mg/L chloramphenicol plate and incubation at 30 ˚C for 12 h. Three large single colonies were picked and mixed well in 1 mL of LB broth. The bacteria were then spread on an LB plate containing 600 mg/L of chloramphenicol. Bacteria were

repetitively grown on the LB media by alternating them between the two chloramphenicol concentrations on a daily basis (Ingram, University of Florida).

Inoculum for the fermentation process was grown in 350 mL LB broth containing 5% glucose in a 1L conical flask and shaken in an incubator shaker at 35 ˚C and 120 rpm for 12 to 16 h. Batch fermentation contained the hydrolysate from the pretreatment material and bacteria, and was conducted in 500 mL conical flasks at 35 ˚C and 100 rpm for 48 h (Beall *et al.* 1991). Fermentation was started by adding the inoculum into the flask in drops until the fermentation broth reached an optical density (OD) of 0.3 at 550 nm.

Samples were collected from the flasks during the fermentation process at 0, 2, 4, 6, 8, 10, 12, 16, 24, and 48 h. For sampling, a total of 7 mL of the fermentation broth was collected from each flask at each time point and put into a 15 mL centrifuge tube. Sampling was conducted under the laminar flow to avoid contamination. The fermentation broth was tested immediately for pH and OD. Cell density was measured at 550 nm using an Implen Nanophotometer (Germany) and converted to dry cell weight based on a standard curve for *E. coli* K011. Samples for glucose and ethanol analyses were kept at -20 ˚C.

Glucose and Ethanol Analysis

Sugar composition was analyzed using a Shidmadzu Prominence HPLC System (Shimadzu Scientific Instruments Ins. Columbia, MD) with a refractive index detector. A Supelcosil LC-NH_2 (25 cm x 4.6 mm) with 5 μm internal diameter columns (Sigma Aldrich) was used with 10% (v/v) acetonitrile solution as the mobile phase.

The flow rate was controlled at 0.6 mL/min and the column temperature was 60 ˚C. Ethanol was measured using Gas Chromatography (GC) (Shimadzu GC-14B, Shimadzu Corporation, Japan), with nitrogen as the carrier gas and compressed air and hydrogen for combustion. GC was programmed with an initial temperature of 50 ˚C, ending with a final temperature of 150 ˚C with the temperature increasing at a rate of 5 ˚C/min.

The injector temperature was set at 150 ˚C and the detector temperature at 150 ˚C. The column used was Carbowax 20M, 30 m in length, 0.25 mm in internal diameter, and 0.25 μm film thickness.

Statistical Analysis

The statistical software package Design-Expert (Stat-Ease, Inc., Minneapolis, USA) was used for regression analysis of experimental data and to plot response surface. ANOVA was used to estimate the statistical parameters. RSM was used to test the results from the RSM experimental combination of enzyme hydrolysis.

Pretreated materials were subjected to chemical properties analysis, and glucose results were analyzed using SAS program version 9.1.3. (SAS Institute). Procedure Univariate was used in order to determine data normality by conducting Shapiro-Wilk W test and Kolmogorov-Smirnov (K-S) D test.

Data were transformed into square-root form prior to the normality test. Procedure General Linear Model (GLM) and Least Square Means (LSM) with probability difference were used to compare the significance level of chemical composition and sugar level.

RESULTS AND DISCUSSION

Alkali Pretreatment

The effectiveness of glucose conversion into bioethanol from grass materials was tested, using two types of pretreated grass. The pretreated grass had high levels of holocellulose (88.46%) and cellulose (82.21%) contents, with low lignin (9.77%) content, showing that it is an effective method for removing lignin. It has been shown that diluted alkali (7% NaOH) is a suitable pretreatment for grass (Liong *et al.* 2012). According to Taherzadeh and Karimi (2008) and Bjerre *et al.* (1996), diluted NaOH is effective for straws that have low lignin content. NaOH also causes swelling, increases the internal surface of cellulose, and decreases the degree of polymerization and crystallinity of cellulose. These provoke lignin structure disruption and made it easier for enzymes to access and disrupt the cellulose.

Biological Pretreatment

Biological pretreatment using a white rot fungus, *P. chrysosporium*, was tested on grass. Biological pretreatment is another method that uses lignin-degrading fungi, such as white-rot fungi and brown-rot fungi, to degrade the lignin and hence break down the recalcitrant linkage between lignin and hemicelluloses so that the cellulose is more exposed for hydrolysis. The white-rot fungus basidiomycete was used because it degrades lignin more rapidly and extensively than other microbial groups. In some studies, lignin is degraded at some distance from the hyphae and is removed progressively from the lumens towards the middle lamella (Blanchette 1991; Ruel and Barnoud 1985). Previous results (Table 1) have shown that the amount of lignin in the grass materials after biological pretreatment in the first to fourth week of incubation were in the range of 23% to 27% and cellulose in the range of 40% to 58% (Liong *et al.* 2012). Because cellulose content was the highest in the third (58%) and fourth week samples (56.6%), it led to an investigation on the action of *P. chrysosporium* on grass using SEM.

Table 1. Chemical Compositions of Napier Grass from Alkaline and Biological Pretreatment using TAPPI Standard Method (Liong *et al.* 2012)

Untreated Grass		NaOH Pretreatment NaOH Conc					Biological Pretreatment Treatment Period				
			1%	5%	7%	10%		1st week	2nd week	3rd week	4th week
Lignin	33.6	Lignin	14.8^{a}	11.3^{b}	9.8^{c}	9.7^{c}	Lignin	24.3^{ab}	23.1^{b}	25.0^{a}	25.9^{ab}
Holo	76.3	Holo	82.8^{c}	86.7^{a}	88.5^{d}	85.4^{b}	Holo	67.8^{d}	77.8^{b}	76.4^{a}	72.0^{c}
Cellu	38.2	Cellu	71.3^{d}	86.4^{b}	82.2^{c}	89.7^{a}	Cellu	52.9^{b}	40.8^{c}	58.3^{a}	37.5^{d}

*Holo = holocellulose * Cellu = cellulose
All of the chemical properties were calculated in percentages (%), and numbers with the same letter designation indicate that there was no significant difference between them.

Structural changes in the Napier grass pretreated with the fungus *P. chrysosporium* were imaged using SEM (Fig. 1). For the untreated grass, the texture was compact and covered in a thin wax layer commonly found in herbaceous biomass (Fig. 1a) (Yan *et al.* 2004). In a sample from the third week, the wax on the surface disappeared and the overall structure of this sample was disrupted (Fig. 1b). Most of the

leaf surface had been degraded, and hyphae grew out from the inner part of the cell wall. The fungus had formed conidia, and the microcell structure was starting to be revealed. Conidia spores were observed on the leaf surface, which indicates the partial breakdown of the lignin structure. By the fourth week (Fig. 1c), most of the lignin had been completely degraded and the materials were becoming thinner, indicating that lignin had perhaps been dissolved, exposing cellulose and hemicellulose from the inside. Hyphae could also be found inside the cell (Fig. 1c).

From these observations, it appeared that three weeks of incubation with *P. chrysosporium* is sufficient to break down the recalcitrant structure and release a larger amount of holocellulose and cellulose in the grass sample, while the four-week treatment revealed lower content of holocellulose and cellulose (Liong *et al*. 2012). The three-week treatment revealed the chance of having the hemicellulose to be freed from ferulic acid and the intramolecular lignin that bonds lignin and hemicellulose together (Sun and Cheng 2002). According to Hsu (1996), four weeks of treatment is not desirable because the fungus might over-degrade the lignin and consume cellulose, causing a loss of cellulose in the treated grass material. Therefore, the third week was judged to be the most suitable incubation period.

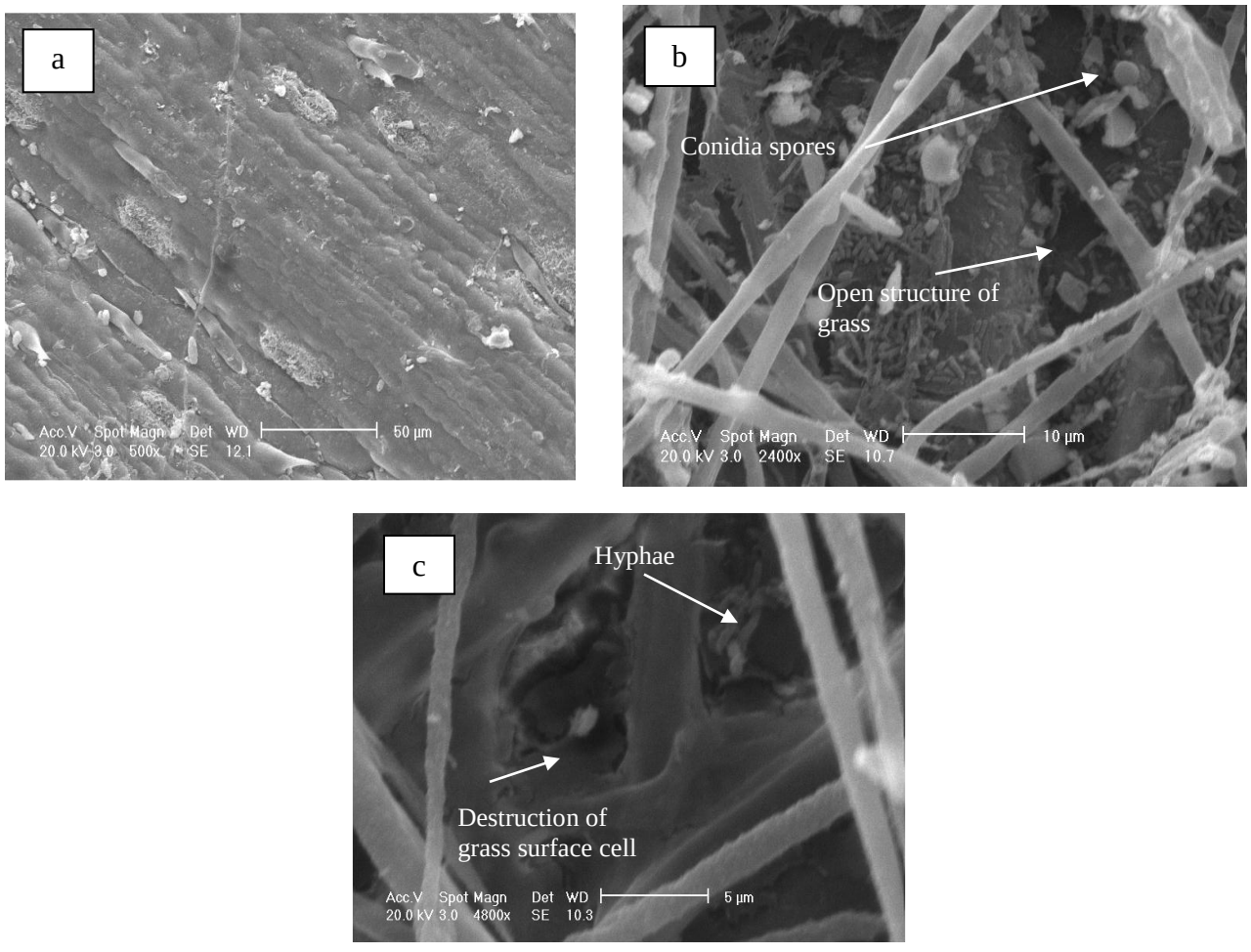

Fig. 1. SEM images of (a) raw and pretreated fiber of Napier grass after (b) 3 weeks and (c) 4 weeks of pretreatment with the white rot fungi, *Phanerochaete chrysosporium*.

Response Surface Method (RSM) Design

Grass materials that were pretreated with all four different concentrations of alkali were subjected to enzymatic hydrolysis. Response Surface Method (RSM) software was used in this study to create a series of experiments in combination of temperature and agitation. Through RSM design, several combinations of temperature and agitation were formed based on the range of temperature and agitation set.

Five best combinations that were chosen because of good glucose yield were 37 ˚C with 180 rpm, 38.5 ˚C with 170 rpm, 37 ˚C with 150 rpm, 37 ˚C with 200 rpm, and 32 ˚C with 165 rpm. ANOVA in RSM showed that in 1% NaOH, 5% NaOH, 7% NaOH, and 10% NaOH pretreated materials, temperature and the combination of temperature and agitation showed a significant effect on the glucose yield at $P<0.05$, respectively (Table 2). In this experimental design, 7% NaOH and 10% NaOH showed a better model and interaction with temperature and agitation, as both of the factors and the combination of two factors had significant effects on the glucose yield at $P<0.05$. Besides, both of the models had high coefficient of determination R^2 (96%). This indicated that the model could be used to explain the functional relationship temperature and agitation and glucose yield.

Table 2. Summarized ANOVA for Response Surface Quadratic Model for Alkali Pretreatment with Four Different Concentrations and their Interactions with Temperature and Agitation (Liong *et al.* 2012)

Alkali treatment	Sum of squares	Mean square	F-value	P-value Prob > F	R-Squared	Temp*RPM
1% NaOH	3.52	0.7	5.32	0.0246*	0.7915	0.0257*
5% NaOH	1.35	0.19	9.05	0.0138*	0.9268	0.5639
7% NaOH	1.04	0.15	22.32	0.0017*	0.969	0.0366*
10% NaOH	5.27	0.75	18.31	0.0028*	0.9625	0.0024*

* indicates significance

Figures 2a, 2b, 2c, and 2d show response surface plots of glucose yield against temperature and agitation. The graphs show a polynomial relationship. The observed variation in glucose yield when using these parameters may be due to the uneven treatment on the overall lignin-hemicellulose linkage by the alkaline solution and the possibility that some of the cellulose structures were not directly exposed to the hydrolysis process.

Figure 2 shows that 7% NaOH pretreated material yielded the highest glucose content (7.3 g/L). Looking at the overall trend of the glucose yield, 7% NaOH pretreated material was the best choice for enzyme hydrolysis because the glucose yield increased gradually from 1% to 7%. Based on the RSM experiment we concluded that 38.5˚C and 175 rpm was the best parameter combination for enzyme hydrolysis.

Enzymatic Hydrolysis on Pretreated Grass

Hydrolysis is an important step before fermentation as it has the role of yielding as much sugars as possible for the microbe to convert into ethanol during fermentation. However, enzymatic hydrolysis is often limited by compounds such as extractives, lignin, and other phenolic compounds. Through effective pretreatment, this limitation can be reduced greatly and hence enhance the hydrolysis process.

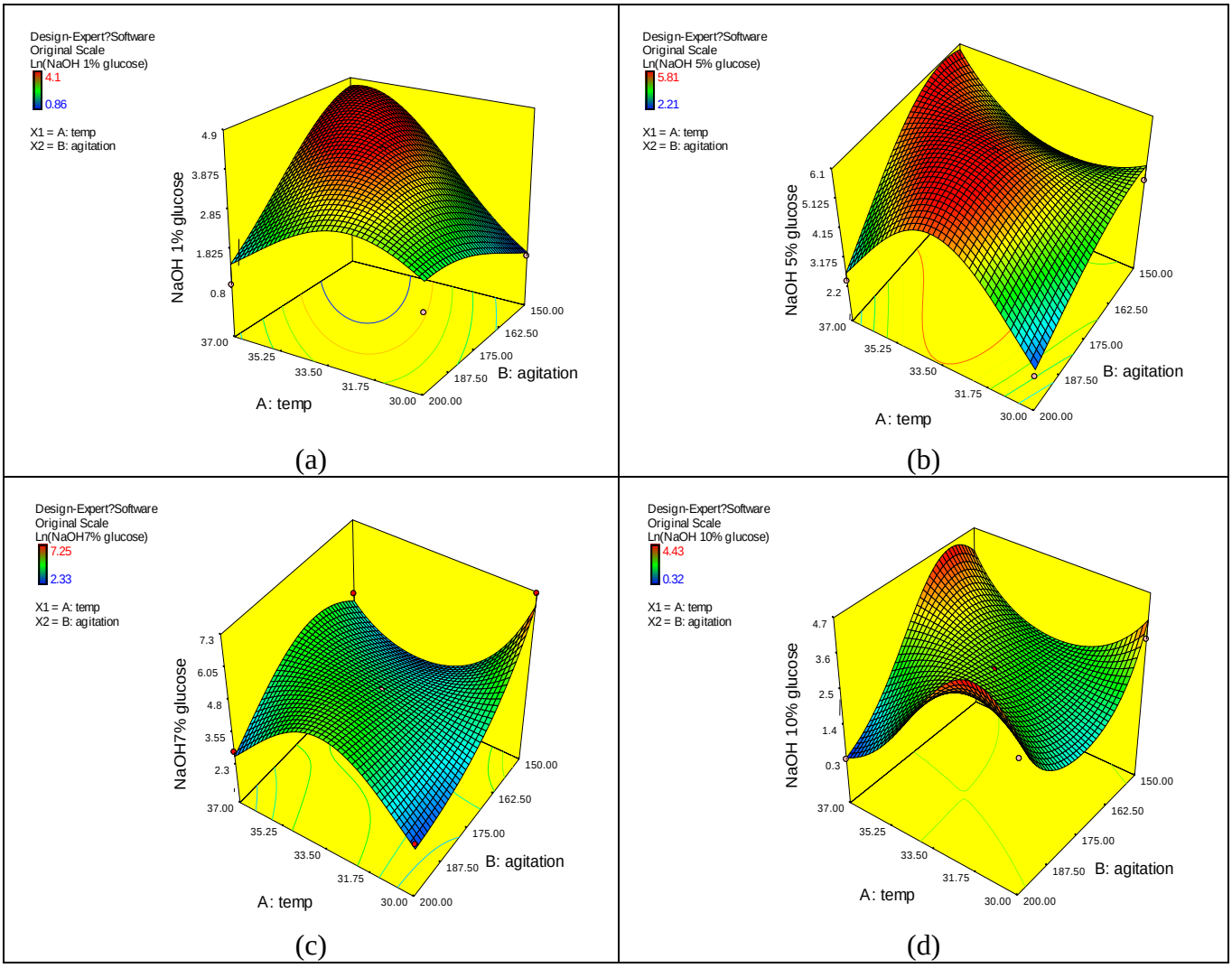

Fig. 2. Response surface plots showing the interaction between alkali pretreatment with agitation and temperature variables in the conversion of cellulose to glucose: (a) 1% NaOH; (b) 5% NaOH; (c) 7% NaOH; and (d) 10% NaOH.

In this study, cellulose from the grass material was converted into glucose using commercial cellulase enzyme, and the efficiency in glucose conversion between the two different pretreated materials was compared. On average, the three-week *P. chrysosporium*-treated grass yielded a lower amount of glucose (4.3 g/L) than that of the alkali-pretreated grass (7.4 g/L). The ratio of substance loading to enzyme loading applied was 1:1 in 100 mL of distilled water. Here, the sugar yield was expressed as grams of sugar released per 1 g (dry weight) of treated Napier grass. For 1 g of material from the 7% NaOH-pretreated grass, the glucose yield was 7.4 g/L or 0.74 g per 1 g of treated biomass. In this experiment, the percentage of potential yield (PPY) is used to define the percentage of potential amount of theoretical glucose to be yielded from the total biomass used. Here, the sugar yield was expressed as grams of sugar released per 1 g (dry weight) of treated Napier grass. For 1 g of material from the 7% NaOH-

pretreated grass, the glucose yield was 7.4 g/L or 0.74 g per 1 g of treated biomass. Therefore, in the alkali-pretreated material, the percentage of the maximum yields of glucose potentially released from Napier grass was 74%. On the other hand, biological-pretreated material yielded 0.43 g of glucose per 1 g of treated biomass, and therefore the maximum yield of glucose potentially released from 1g of this material was 43% (Hu and Wen 2008). In NaOH pretreatment, swollen fiber that breaks up the lignin-hemicellulose linkage in the amorphous crystalline of cellulose, effectively exposes the cellulose to cellulase digestion and conversion into glucose. In comparison, glucose yield of biologically pretreated material was obviously lower than chemical treatment as fungi take a long time to consume lignin. Upon the third week of incubation, there were still some crystalline linkage present in the grass structure and the consumption was not thorough in all materials (Fig. 1b). This perhaps contributes to the low glucose content from *P. chrysosporium*-treated grass.

Ethanol Conversion

Hydrolysates from enzymatic hydrolysis were subjected to fermentation to yield ethanol. The ethanol yield from glucose produced in hydrolysate originating from NaOH-pretreated material reached 38% at 24 h fermentation with *E. coli* strain K011, while the biological hydrolysate only reached 24% for the same period. From Fig. 3a, the ethanol curve showed a decrease at 12 h fermentation, after which ethanol yield increased significantly. The decrease can be due to the consumption of accumulated ethanol by the organism. It has been observed that when the ethanol accumulates in the medium, the microbial population adapts to simultaneously consume sugar and ethanol. However, after it has adapted to the ethanolic environment, the bacteria starts to grow again (Ramon-Portugal *et al.* 2004). Bacterial growth was slow, beginning from 4 to 5 h and almost reached a constant state after 5 h. On the contrary, ethanol content in the biological hydrolysate reached only 24% after 24 h of fermentation (Fig. 3b). Ethanol yield increased sharply during the first 6 h of fermentation and then it stayed constant at 21% until it reached 24% at the end of the fermentation. The pH in the medium dropped from 6.2 to 4.6. Although the *E. coli* strain was ethanol-tolerant, the acidic environment perhaps became a limiting factor for its growth and hence the conversion of glucose to ethanol.

Figure 3 shows that ethanol yield from the alkali-pretreated material was 1.5 times higher than the biological-pretreated material; however its ethanol conversion efficiency (15.2 g/g) was lower than biological-pretreated material (18.4 g/g). This could have possibly resulted from the initial pH of the biologically pretreated hydrolysate, which was at 6.2, a pH value that is favorable for bacterial growth. Most bacteria grow best in a narrow range of pH from 6.5 to 7.5 (Aminifarshidmehr 1996). Therefore, during fermentation using biologically pretreated hydrolysate, the bacteria was able to consume glucose and convert it into ethanol faster than when using alkali-pretreated hydrolysate, despite the fact that the ethanol obtained was not as high as expected. This again might be because the initial amount of substrate in the biologically pretreated hydrolysate was low. Meanwhile, the starting pH of the alkali-pretreated material was 5.3, which was already becoming acidic and could inhibit bacterial growth.

Other attempts to produce bioethanol from grass yielded mix results. The ethanol conversion of acid pretreated Kans grass upon fermentation of enzymatic hydrolysate was only 0.46 g/g (Kataria and Ghosh 2011). On the other hand, fermentation of Reed Canary grass with commercial *Trichoderma reesei* cellulase yielded high ethanol

concentration (20 g/L) and yield of 82% (Kallioinen *et al.* 2012). This showed that the performance of wild grass used in this study could be further improved experimentally to maximize yield and conversion rate.

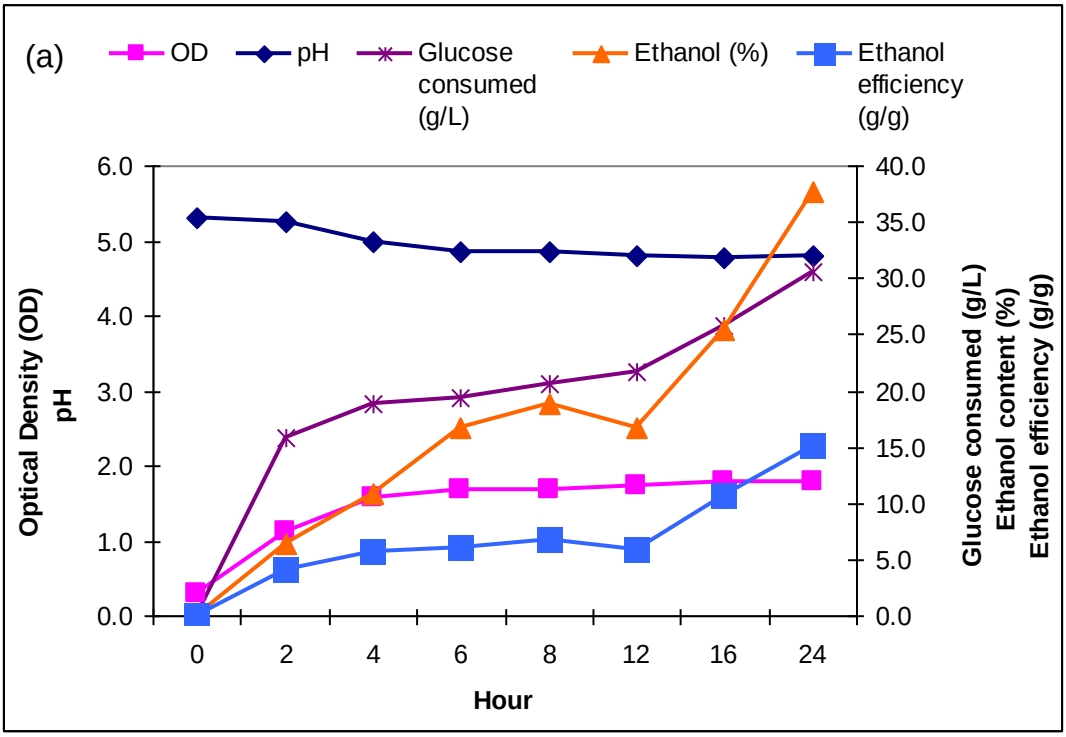

Fig. 3. Fermentation of cellulase-treated Napier grass supernatants after 24 h at 35 ˚C by *Escherichia coli* K011 using hydrolysate from (a) NaOH-pretreated material, and (b) biological-pretreated material

CONCLUSIONS

1. This work showed the potential for using Napier grass as a cheap and widespread source for lignocellulosic material in small local bioethanol production. Grasses as a source of biomass for small production can be cost effective relative to other materials such as oil palm empty fruit bunch because grasses are easier to process due to their soft fiber structure.
2. Diluted NaOH is a suitable pretreatment method to enhance glucose production during enzymatic hydrolysis. NaOH was sufficient to disrupt the recalcitrant structures of Napier grass. For 1 g of alkali-pretreated material, the maximum yield of glucose that was potentially released from Napier grass was 74% when using 7% NaOH pretreatment, compared to only 43% when using the biological pretreatment. However, in implementing a large-scale alkali pretreatment process, the optimal pretreatment conditions need to be balanced with cost saving.
3. Of the 74% glucose yield, only 15.2 g/g of the glucose was converted to ethanol when using NaOH as a pretreatment for grass biomass. Of the biological pretreated material of the 43% glucose yield, 24% ethanol was obtained and 18.4 g/g of the glucose was converted to ethanol. This showed that even though biological pretreatment yielded less glucose, it had better efficiency of ethanol conversion. This stage can be improved by conducting the experiment in a bioreactor. Bioreactors are better at controlling pH during the fermentation process, compared to shake flasks on a lab scale. pH is crucial in fermentation because it inhibits bacterial growth and thus is a limiting factor.

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