SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF ALKALI-PRETREATED COGONGRASS FOR BIOETHANOL PRODUCTION

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Simultaneous saccharification and fermentation (SSF) of alkaline pretreated cogongrass to ethanol was optimized using the commercial cellulase Accellerase 1500 and Ethanol Red dry yeast. Cogongrass was pretreated with 10% (wt) NaOH at room temperature for 24 hours, resulting in an increase in the cellulose percentage from 38.5% to 60.5%. Each SSF of alkali-pretreated cogongrass was carried out with 1 g/L of dry yeast loading at pH 5.0 under 150 rpm shaking. Response surface methodology (RSM) based on a three-level three-factor Box-Behnken design was employed to optimize the key variables within the following ranges: cellulase concentration per unit gram water-insoluble cellulose (WIS) (0.15-0.25 mL/g-WIS), substrate concentration (5-15 % WIS, w/w), and temperature (35-45°C) for the SSF process. The response surface model arrived at the optimum SSF conditions: cellulase concentration of 0.255 ml/g-WIS, temperature at 37.5°C, and substrate concentration of 7.28% WIS for obtaining 80.3 % ethanol yield in 72 h. The optimal conditions were verified experimentally with an average absolute relative deviation of 3.01 %. Also, the SSF was scaled up to a 5-L rotary drum reactor filled with 1 kg of substrate under the optimal conditions, and an ethanol yield of 76.2% was obtained.

Keywords: Bioethanol; Simultaneous saccharification and fermentation; SSF; Cogongrass; Alkaline pretreatment

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INTRODUCTION

Cogongrass [*Imperata cylindrical* (L.) Beauv. var. *major*. (Nees) C.E.Hubb] is an aggressive, perennial grass that is distributed worldwide in the tropical and subtropical regions. Although it has been used for forage and soil stabilization, cogongrass is considered to be one of the top 10 worst weeds in the world, reported by 73 countries as a pest in a total of 35 crops (Holm et al. 1977). Despite its being an invasive and exotic weed, it is regarded as a promising medicinal plant because secondary metabolites isolated from the rhizome of *I. cylindrical* can have medicinal uses (Matsunaga et al. 1994; Pinilla and Luu 1999; Yoon et al. 2006). Genomic and proteomic methods have been used to reveal the genetic and ecotypic variations among *Imperata cylindrica* ecotypes in Taiwan (Chou and Tsai 1999; Chang 2008). In Taiwan, cogongrass was used several decades ago as a material for making houses and raincoats for farmers. Because of the flat form and high density of its leaf, cogongrass has proved better and more

durable than *Miscanthus* as material for the roofs of huts. As cogongrass can be grown on land considered unsuitable for row crop production and it can grow all year long in tropical countries, this grass can be developed as a bioresource for renewable energy. Roots of cogongrass have potential for medicinal application, and its stems and leaves can serve as the feedstock for biofuels. The present work describes the use of cogongrass for the production of bioethanol by simultaneous saccharification and fermentation (SSF).

After pretreatment, lignocellulosic feedstock such as cogongrass undergoes enzymatic hydrolysis of cellulose and yeast fermentation of the hydrolyzates to yield ethanol. The SSF process combines the enzymatic hydrolysis and ethanol fermentation in a single stage. SSF is usually preferred over separate hydrolysis and fermentation because higher ethanol yield can be achieved by minimizing product inhibition. Also, SSF has an advantage of low potential costs because lower amounts of enzyme are employed, reducing the capital investment (McMillan et al. 1999; Karimi et al. 2006; Olofsson et al. 2008). SSF studies, for example, have been carried out to produce ethanol from lignocellulosic wastes (sugar cane leaves and *Antigonum leptopus* leaves) using *Trichoderma reesei* cellulase and yeast cells (Krishna et al. 2001). Other lignocellulosic residues such as bermudagrass, reed, and rapeseed have also been used as the raw materials for the production of bioethanol by SSF (Li et al. 2009). SSF process variables that can influence the ethanol production efficiency include enzyme concentration, substrate concentration, temperature, and reaction time. SSF of alkaline hydrogen peroxide pretreated *Antigonum leptopus* (Linn) leaves to ethanol was optimized by using response surface methodology (RSM) (Krishna and Chowdary 2000). Response surface methodology (RSM) is a powerful tool for the optimization of complex processes, because it can offer several advantages. These include (1) an understanding of how the process variables affect the selected process response, (2) determination of any possible interrelationship among the test variables, and (3) characterization of the combined effect that all process variables may have on the process response (Domingos et al. 2003). RSM was used here for optimizing the SSF of alkaline pretreated cogongrass for bioethanol production. The obtained optimal SSF condition was verified by experiments carried out in a flask as well as in a rotary drum reactor.

EXPERIMENTAL

Alkaline Pretreatment of Cogongrass

Cogongrass stems and leaves were collected from the campus of National Chung Cheng University. Air-dried cogongrass was pre-cut into sticks of ca. 2 cm long. For the alkaline pretreatment, cogongrass sticks were incubated with 10% (wt) NaOH at a solid-to-liquid ratio of 1:20 (w/v) at room temperature for one day and then washed with tap-water until the pH became neutral. The pretreated cogongrass was stored in sealed plastic bags at 4 °C. To calculate its dried weight, the pretreated cogongrass was dried in a forced-air oven at 65 °C for 24 h. The composition of the cogongrass before and after pretreatment was determined according to a previously reported method (Sluiter et al. 2008). The scanning electron microscope (SEM) micrographs of cross-section of the

cogongrass before and after pretreatment were taken with a Hitachi-S2400 SEM-EDX microscope (Japan). Samples were sputter-coated with gold prior to SEM observation.

Simultaneous Saccharification and Fermentation (SSF) of Pretreated Cogongrass

Pretreated cogongrass was simultaneously saccharified and fermented by using the commercial cellulase Accellerase 1500 (Genencor, USA) and Ethanol Red^{TM} dry *Saccharomyces cerevisiae* yeast (Fermentis, France), respectively. Cogongrass that was pretreated by alkali under optimal conditions was transferred to a 250-mL flask containing 0.05 M citrate buffer at pH 5.0, to produce a final water-insoluble-solids (WIS) concentration of 10% (w/w) and then autoclaved at 121 °C for 30 min (Hayward et al. 1995). In addition, cellulase and dry yeast (1 g/L) were loaded into each substrate mixture and then incubated in water bath at 150 rpm for three days. Preliminary study indicated that the ethanol concentration could approach to a steady value after 3-d SSF. Fifteen runs of SSF were carried out with different combinations of independent variable values as described in the sub-section of Design of Experiments. Samples (1 mL) of SSF were centrifuged at 8050 g for 10 min to remove denatured enzyme and insoluble residues. The ethanol yield was calculated based on the conversion of glucan to ethanol.

Analytical Methods

Concentrations of ethanol and sugars in the SSF mixture were analyzed by HPLC using a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm i.d.), operating at 65°C. The mobile phase was 5 mM H_2SO_4, and the flow-rate was 0.6 mL/min with an RI detector.

Design of Experiments

A three-level three-factor Box-Behnken design was adopted for the study. The important factors involved in ethanol production were substrate concentration, cellulase concentration, and temperature, which is in agreement with our previous study on the SSF of alkaline-pretreated rice straw, a similar raw material to cogongrass (Lin and Lee 2011). The amount of dry yeast loading was fixed at 1 g/L. The factors and their levels are given in Table 1, and the design of experiments employed is presented in Table 2. In Table 1 the level values of cellulase and substrate concentration were so chosen that at those conditions higher SSF efficiencies could be achieved. Temperature levels of 35, 40, and 45°C were chosen because that the yeast could ferment at higher temperature, and the elevated temperature favored SSF.

Table 1. Level of Variables Chosen for the Study

Symbol	Independent variables	Levels			Units
		-1	0	1	
X_1	Temperature	35	40	45	°C
X_2	Cellulase concentration	0.15	0.2	0.25	ml/g-WIS
X_3	Substrate concentration	5	10	15	(WIS %, w/w)

In Table 2, $Y_{experimental}$ is the experimental ethanol yield, while $Y_{predicted}$ is the calculated ethanol yield using the response surface model as described in the following section.

Table 2. Experimental Design Showing Coded Values of Variables, as well as the Experimental and Predicted Responses

Test number	X_1	X_2	X_3	$Y_{experimental}$	$Y_{predicted}$
1	-1	-1	0	35.15	41.41
2	1	-1	0	51.21	50.10
3	-1	1	0	71.67	72.79
4	1	1	0	60.67	54.41
5	-1	0	-1	73.57	69.66
6	1	0	-1	62.69	64.81
7	-1	0	1	26.42	22.96
8	1	0	1	12.86	18.11
9	0	-1	-1	72.05	70.37
10	0	1	-1	72.17	75.64
11	0	-1	1	14.55	11.09
12	0	1	1	39.84	41.52
13	0	0	0	67.28	68.41
14	0	0	0	67.90	68.41
15	0	0	0	70.06	68.41

Statistical Analysis

The experimental data (Table 2) were analyzed according to the response surface regression procedure to fit the following second-order polynomial equation, in which the level of significance (*P* value) of all coefficients was <0.05,

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1^2 + A_5X_2^2 + A_6X_3^2 + A_7X_1X_2 + A_8X_1X_3 + A_9X_2X_3 \quad (1)$$

where *Y* is the ethanol yield (%, w/w), A_0 is the intercept, A_1-A_3 are the linear coefficients, A_4-A_6 are the quadratic coefficients, A_7-A_9 are the cross-product coefficients, and X_i are the coded independent variables. The regression analyses, statistical significances, and response surfaces were carried out using STATISTICA software (version 8.0; StatSoft). Optimization of the reaction parameters for maximum ethanol yield was obtained through the software package.

SSF of Pretreated Cogongrass in a Rotary Drum Reactor

In a 5-L rotary drum reactor (the full volume was 7.7 liters), pretreated cogongrass was mixed with 0.05 M citrate buffer at pH 5.0, making up a total of 1 or 2 kg mixture with a WIS concentration of 10% (w/w). The pH value of the mixture was adjusted to 5 using 1N HCl. SSF was run with 0.258 mL/g-WIS of enzyme and 1 g/L dry

yeast. The reactor was operated at 37°C in a temperature controlled box and rotated at a speed of 5 rpm for 1 min at the beginning of SSF. Samples (1 mL) of SSF were taken every 24 h with large-mouth pipette tips and were centrifuged at 8050 g for 10 min. After each sampling point the reactor was also rotated for 1 min.

RESULTS AND DISCUSSION

Pretreatment with NaOH

After harvesting, a constant weight of cogongrass was reached in two days in an oven at 65 °C. A water content of 59.9% (wt) was determined in the fresh cogongrass. When the harvested cogongrass was air-dried to a constant weight, 1 kg raw material could yield 415 g of dried material. After NaOH pretreatment, 1 kg dry material was converted to 492 g WIS. Results as shown in Table 3 indicated that NaOH pretreatment at room temperature led to an increase in cellulose content (% glucan) by the removal of some hemicellulose and lignin. The fact that alkali pretreatment can decrease the proportion of hemicellulose and lignin has been reported for a similar raw material, rice straw (Zhang and Cai 2008). Figure 1 shows the morphology of cogongrass before and after pretreatment with 10%(wt) NaOH. Significant morphological changes of the cogongrass from sticks (Fig. 1a) to fibrous clusters (Fig. 1b) were observed. It was confirmed by SEM that changes in microstructure occurred, since a large fraction of lignin and some xylan were removed by the alkaline pretreatment. In contrast to the untreated sample that exhibited a cover of material on the microfibrils (Fig. 1c), the microfibrils of pretreated sample became exposed after removal of the cover material by the pretreatment (Fig. 1d). A similar behavior was observed in SEM of corn stover pretreated with aqueous ammonia (Kim and Lee, 2005).

The use of 10% NaOH caused solubilization of xylan from cogongrass at room temperature. In order to decrease the consumption of alkali and water, the alkaline solution was repeatedly used. No significant decrease in the pretreatment efficiency was observed on the use of alkali solution after five recycles of pretreatment (Table 3), suggesting that the consumption of NaOH and water could be saved by at least 75%. Pretreatment of cogongrass rendered it much more accessible to the enzymes for cellulose and hemicellulose degradation due to the removal of lignin and its structural changes.

Table 3. Composition of Cogongrass Before and After 10% NaOH Pretreatment

	% glucan	% xylan	% arabinan
Cogongrass	38.5	17.6	4.8
Pretreated-cogongrass	60.5	8.8	2.7
Five-times recycled pretreated-cogongrass	59.3	9.4	1.9

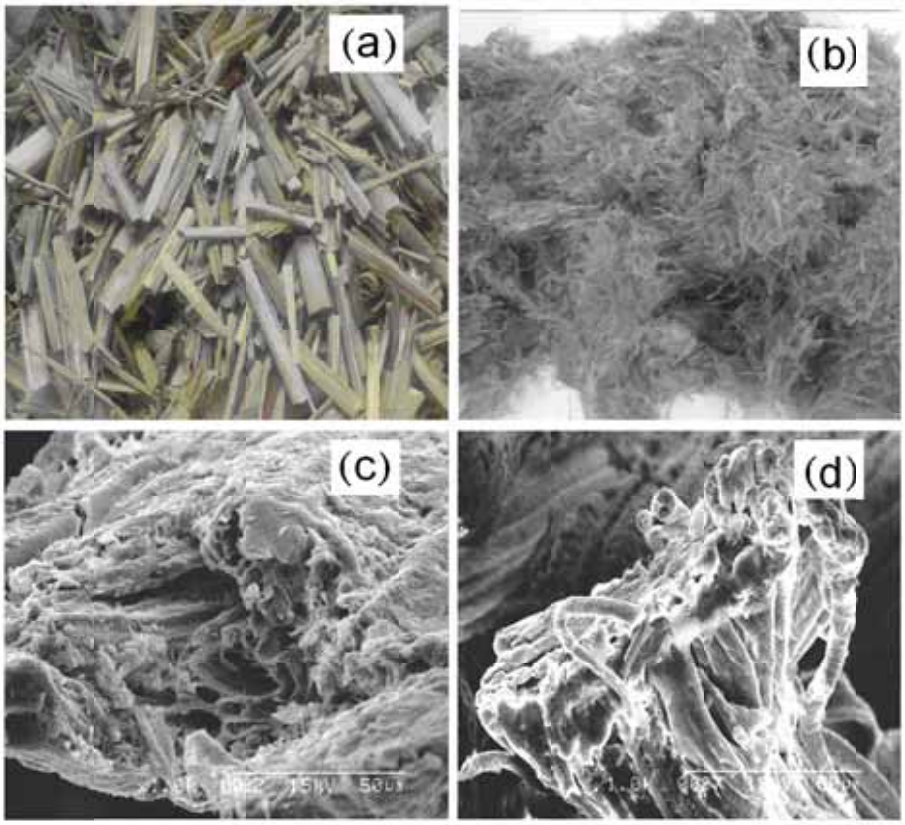

Fig. 1. Morphological (a, b) and microstructural (c, d) changes of cogongrass before (a, c) and after (b, d) pretreatment with 10% NaOH at room temperature

Cogongrass has a cellulose content that is similar to that of rice straw (37.2 %) (Vlasenko et al. 1997) but higher than coastal bermudagrass (25.6%) (Holtzapple et al. 1994) on a dry-weight basis. After alkaline pretreatment, cogongrass with high cellulose and hemicellulose content has a high potential for lignocellulosic ethanol production. The morphological changes in cogongrass suggest the exposure of microfibrils by the pretreatment. Results in the present paper indicate that NaOH-pretreated cogongrass can be used to efficiently produce ethanol via SSF. The advantage of this pretreatment method is that energy input is minimal, since the feedstock is kept at room temperature, and the alkali can be recovered for reuse. No significant loss of pretreatment efficiency was found after five-fold repeated use of the alkaline solution.

Optimization of SSF

The coefficient values and the analysis of variance (ANOVA) are presented in Table 4, indicating that the predictability of the model was at a 96.3% confidence level. The coefficient of determination (R^2) value was 0.9741, suggesting that the modeling equation is highly reliable. From the model, ethanol yield was predicted as a function of substrate concentration, cellulase concentration, and temperature. The final response function to predict the ethanol yield after eliminating the insignificant terms was obtained as the following equation.

$$Y = 68.4128 - 2.4229(X_1) + 8.9223(X_2) - 23.3514(X_3) +$$
$$9.7539(X_1^2) - 3.9838(X_2^2) - 14.7754(X_3^2) +$$
$$-6.7676(X_1 \cdot X_2) + 6.2921(X_2 \cdot X_3) \quad (2)$$

The fitted response surface for the production of ethanol by the above model was generated using STATISTICA and is given in Figs. 2 through 4. Figure 2 depicts the interaction of substrate concentration and temperature. Higher yield could be achieved at low substrate concentration (WIS was less than 10%) when the temperature was 40°C or lower. Figure 3 shows the effect of interaction of cellulase concentration and temperature.

As expected, the higher the dose of cellulose, the higher the ethanol yield and the highest ethanol yield could be achieved at about 38 °C. Figure 4 shows the interacting effects of substrate concentration and cellulase concentration. For a SSF with low substrate concentration, the ethanol yield increased slightly with the dose of cellulase. However, the ethanol yield dropped tremendously at higher substrate concentration, especially when the cellulase concentration was at a lower level.

Table 4. Values of Coefficients and ANOVA

R-sqr=0.9741; Adj:0.9275; MS Pure Error=2.1329							
Coefficients	Sum of squares	Degree of freedom	Mean sum of squares	Regression Values	Pure Err	t-Stat	p-value
A0				68.4128	0.843186	81.1362	0.000152
A1	46.965	1	46.965	-2.4229	0.516344	-4.6925	0.042538
A2	636.860	1	636.860	8.9223	0.516344	17.2798	0.003332
A3	4362.296	1	4362.296	-23.3514	0.516344	-45.2245	0.000489
A4	351.283	1	351.283	-9.7539	0.760037	-12.8335	0.006017
A5	58.598	1	58.598	-3.9838	0.760037	-5.2415	0.034525
A6	806.075	1	806.075	-14.7754	0.760037	-19.4403	0.002636
A7	183.203	1	183.203	-6.7676	0.730220	-9.2679	0.011443
A8	1.786	1	1.786	**-0.6682**	**0.730220**	**-0.9151**	**0.456738**
A9	158.361	1	158.361	6.2921	0.730220	8.6167	0.013202
Lack of Fit	169.954	4	42.488				0.036877
Pure Error	4.266	2	2.133				
Total SS	6662.391	14					

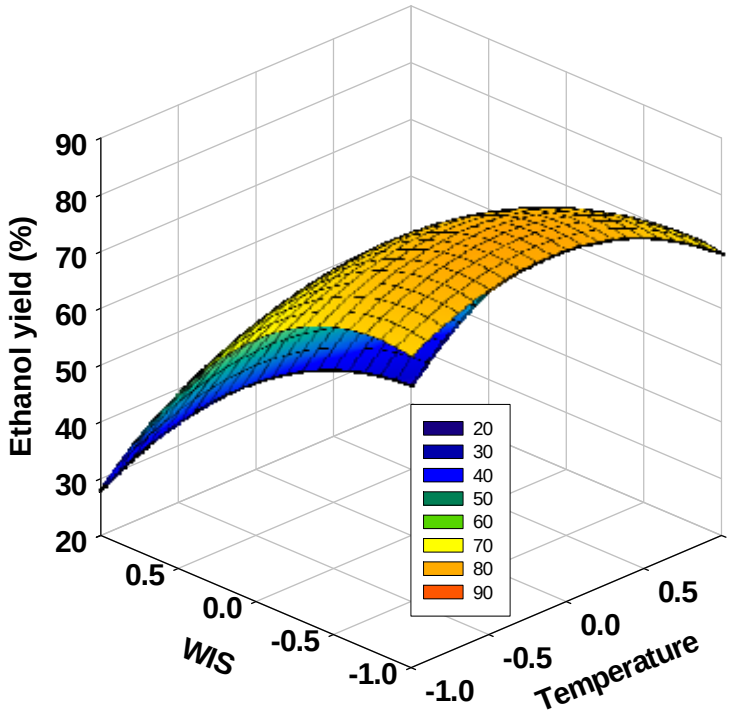

Fig. 2. Response surface of ethanol production by SSF 0.255 mL/g-WIS

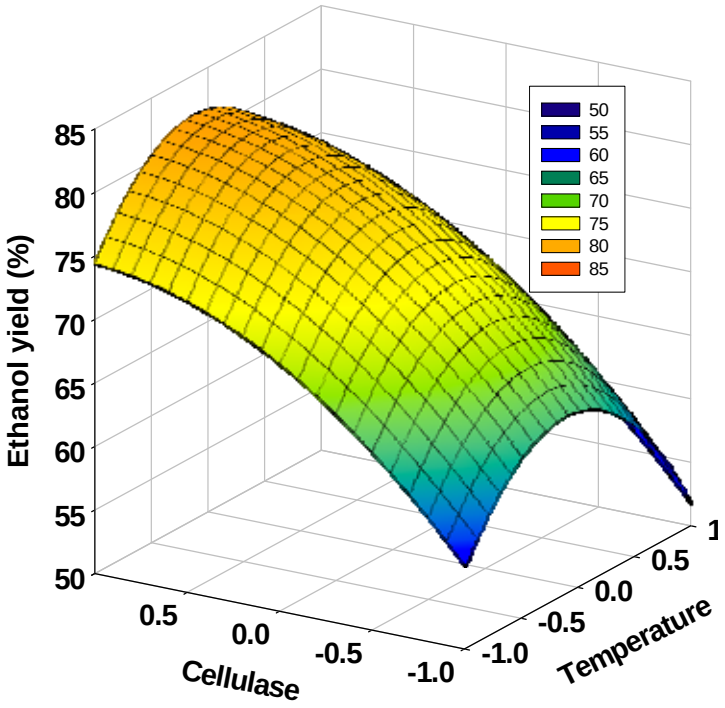

Fig. 3. Response surface of ethanol production as function of cellulase concentration and temperature at fixed substrate concentration of 7.28 wt%

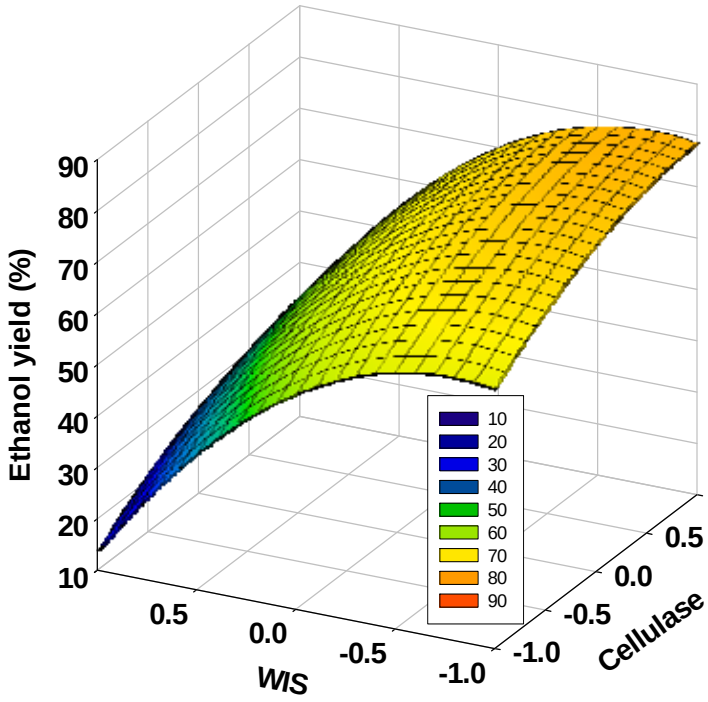

Fig. 4. Response surface of ethanol production as function of substrate concentration and cellulase concentration at fixed temperature of 37.5°C

The response surface model suggested a set of optimum SSF conditions (cellulase concentration of 0.255 mL/g-WIS, temperature at 37.5°C, and substrate concentration of 7.28% WIS) for obtaining 80.3% ethanol yield. The model was tested for validity and adequacy by carrying out additional experiments under the optimal conditions. Results (Table 5) show that the model predictions reasonably agreed with the flask experimental values, with an average absolute relative deviation (%AARD) of 3.01 %. The %AARD is defined by the following equation,

$$\%\text{DDRA} = \frac{100}{N}\sum_{i=1}^{N}\frac{\left|Y_{predicted,i} - Y_{experimental,i}\right|}{Y_{experimental,i}} \quad (3)$$

where N is the number of experiments.

Table 5. Model Validation Experiments

	Temperature (°C)	Cellulase (ml/g-WIS)	WIS (%, w/w)	Ethanol yield (%)
Predicted	37.5	0.255	7.28	80.3
Flask 1	37.0	0.258	7.30	74.0
Flask 2	37.0	0.258	7.30	77.0
1 kg rotary drum reactor	37.0	0.258	7.30	76.2
2 kg rotary drum reactor	37.0	0.258	7.30	75.0

The production of ethanol from pretreated cogongrass by SSF was influenced by the concentration of substrate, enzyme dose, and temperature. As the yeast concentration was kept at a sufficient level of 1 g/L, effects of these operational variables on the SSF efficiency were well predicted via the RSM analysis. The RSM results predicted not only the optimal SSF conditions but also the predicted effects of interacting factors. The last two terms in Equation 2 take into account effects of interacting factors. The coefficient for X_1X_2 is negative and the coefficient for X_2X_3 is positive, suggesting that a higher value of X_1 (temperature) is harmful for ethanol yield from SSF. SSF of alkali-pretreated cogongrass under optimal conditions predicted an ethanol yield of 80.2%, corresponding to 0.41 g/g-glucose. A 100% ethanol yield means one gram of glucose is converted into 0.51 g ethanol theoretically.

The RSM model was tested and validated with experiments in the flask and rotary drum reactor under predicted optimal conditions, which resulted in an average ethanol yield of approximately 76%, corresponding to 0.39 g/g-glucose. This level is comparable with those in the literature reported for the SSF of rice straw (Table 6). A 120-h SSF of ammonia-pretreated rice straw containing 3% (w/w) glucan with *Saccharomyces cerevisiae* D5A and hydrolytic enzymes (Celluclast 1.5L) at 15 FPU/g-glucan and β-glucosidase at a level of 30 CBU/g-glucan at 38 °C could bring out the yield and concentration of ethanol of 83.1% and 12.7 g/L, respectively (Ko et al. 2009). Another example is an SSF of dilute acid-pretreated rice straw using 50 g/L dry matter (ca. 3% w/w glucan), *Saccharomyces cerevisiae* and 25 FPU/g-cellulose of a commercial cellulase enzyme (BTXL). The yield and concentration of ethanol after a 72-h SSF at 38 °C and pH 5 were 60.8% and 10.2 g/L, respectively (Kargi and Curme 1985).

Table 6. Comparison of Results from SSF of Similar Raw Materials and Pretreatment Technology

Pretreatment method/raw material	Substrate conc.	Yeast	Cellulase/dose	Temp.	Time	Ethanol yield	Final ethanol concentration	Reference
ammonia pretreated rice straw	3% (w/w) glucan	*Saccharomyces cerevisiae* D5A	Celluclast 1.5L / 15 FPU/g-glucan	38 °C	120-h SSF	83.1%	12.7 g/L	Ko et al., 2009
dilute acid-pretreated rice straw	3% (w/w) glucan	*Saccharomyces cerevisiae*	BTXL / 25 FPU/g-cellulose	38 °C	72-h SSF	60.8%	10.2 g/L	Kargi and Curme, 1985
10% alkali-pretreated cogongrass	7.3% (w/w)WIS	*Saccharomyces cerevisiae* (Ethanol Red™)	Accellerase 1000/ 0.255 ml/g-WIS	37 °C	72-h SSF	76.2%	19.08g/L	This work

SSF at Optimal Conditions in a Rotary Drum Reactor

Figure 5 shows the time courses of SSF in a rotary drum reactor containing 1 kg and 2 kg pretreated cogongrass at the optimum SSF conditions predicted by the response surface model. When 1 kg substrate solution was used, a 72-h SSF from 7.3% WIS at 37°C with 0.258 mL/g-WIS and 1 g/L dry yeast resulted in a final ethanol concentration

of 19.1 g/L, corresponding to 76.2% of the theoretical ethanol yield from glucan. The SSF of 2 kg substrate solution in the rotary drum reactor led to almost the same results. These results from SSF in rotary drum reactor were close to those from SSF in flasks under the optimal conditions, suggesting that the scale-up of bioethanol production from cogongrass by SSF is achievable. Figure 5 also shows the time courses for the glucose and xylose concentrations. During the saccharification and fermentation process, glucose was produced and consumed. In the first few hours, a higher glucose concentration appeared in the reaction mixture because the rate of yeast fermentation could not keep up with that of enzyme hydrolysis. The glucose concentration reached the peak at about 6 h and decreased gradually because the fermentation rate approached the rate of enzyme hydrolysis. Xylose was also produced by the enzymatic hydrolysis at the early stage. Since xylose was not consumed by the yeast, its concentration remained flat during the later time period, as shown in Fig. 5.

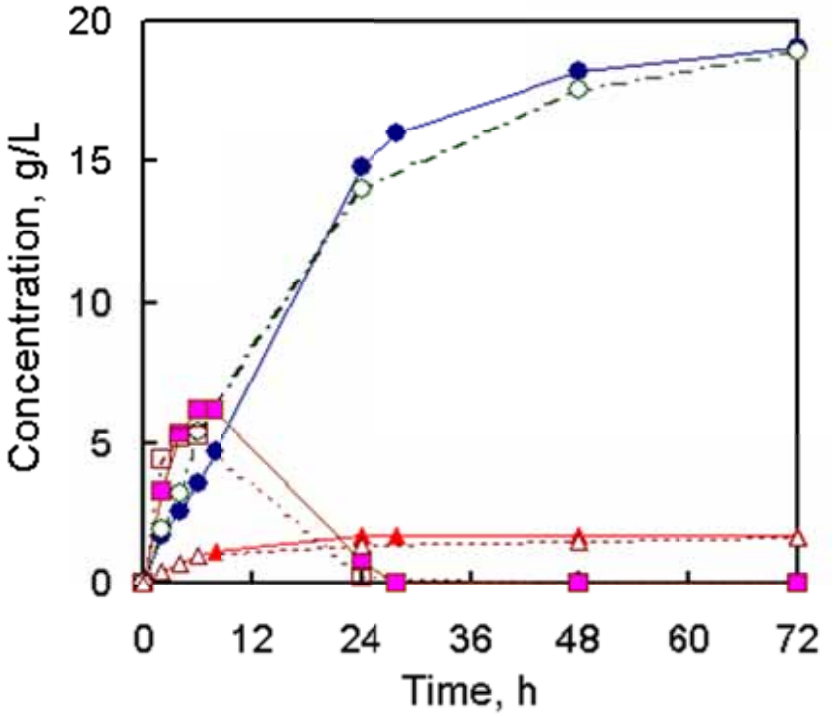

Fig. 5. Ethanol (circles), glucose (squares) and xylose (triangles) concentrations during SSF of alkaline-pretreated cogongrass in rotary drum reactor. SSF with a total volume of 1 kg (solid symbols) or 2 kg (open symbols) was carried out at 37°C with 7.3% WIS, 0.258 ml/g-WIS of enzyme and a yeast dose of 1 g/L.

SSF of alkaline-pretreated cogongrass was effectively carried out in a rotary drum reactor. For the SSF process, the reactor was slowly rotated at the beginning and subsequently rotated occasionally for mixing of enzyme and yeast with the substrate. According to a previously study (Kargi and Curme 1985) for the fermentation of chopped solid-sweet sorghum in a rotary drum fermentor, the rate of ethanol formation decreased with increasing rotational speed, and the maximum rate and extent of ethanol formation were achieved at 1 rpm rotational speed.

CONCLUSIONS

By the process of combining saccharification and fermentation together, alkaline pretreated cogongrass could be converted to ethanol with a predicted yield of 80.3 % in 72 h. The optimal conditions for the SSF of alkali-pretreated cogongrass were obtained based on the response surface methodology and verified experimentally. Ethanol yields of 75.0% and 76.2% were obtained from a scaled-up rotary drum reactor filled with 1 and 2 kg of substrate, respectively, under the optimal conditions. Since cogongrass can be planted and grown on lands that are unsuitable for row crop production, there is high potential to use this grass for ethanol production by SSF.

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