Sequential Acid and Alkaline Pretreatment of Rice Straw for Bioethanol Fermentation

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Pretreatment is a prerequisite step for increasing the enzymatic digestibility of agricultural residues for conversion to fuels and chemicals in biorefineries. In this study, a sequential acid and alkaline process was developed for pretreatment of rice straw for ethanol fermentation. Effects of key parameters in acid pretreatment were studied using a full factorial design model, which showed the higher influence of time compared to acid concentration and temperature on reducing sugar yields. The combined sequential process involved an initial hemicellulose solubilization by dilute acid using 1% (w/v) H_2SO_4 at 125 °C for 10 min, followed by alkaline delignification using 1.25% NaOH at 90 °C for 10 min. Under these conditions, a glucose recovery yield of 70.9% from saccharification of the cellulose enriched fraction was obtained with 2- to 4-fold savings in chemical usage as compared with single-step processes. Scanning electron microscopy revealed modification of biomass micro-structure and increases in reactive surface area. Simultaneous saccharification and fermentation of the solid residues by *Saccharomyces cerevisiae*, using 25 FPU/g Accellerase® 1500, led to a final ethanol concentration of 21.0 g/L with the productivity of 0.27 g/L/h, equivalent to 84.6% theoretical yield. The results indicate the potential of the sequential process for increasing pretreatment efficiency and allowing stepwise separation of lignocellulose components for multi-product biorefineries.

Keywords: Alkaline pretreatment; Dilute acid pretreatment; Ethanol; Lignocellulose; Rice straw

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INTRODUCTION

During the past decade, the ever-increasing demand for fossil fuels has led to increased petroleum prices and environmental damage. In response to this problem, much attention has been focused on biorefinery as an alternative sustainable platform for conversion of renewable lignocellulosic biomass to fuels and other carbon compounds (FitzPatrick *et al.* 2010). Sugar platform biorefinery is mostly reliant on pretreatment of lignocellulosic materials and subsequent enzymatic hydrolysis of biomass to sugars, which are then further converted to valorized products by fermentation, catalytic, or biocatalytic processes (Ghatak 2011). Several biorefinery models have been proposed for co-production of bulk fuels from cellulose with high value chemicals and materials as co-products from hemicelluloses and lignin for establishing economically feasible integrated

processes (Browne 2011). Development of an efficient pretreatment technology that can effectively improve digestibility of the recalcitrant lignocellulose, while retaining the ability to separate its components for further processing, is thus a challenge for the biorefinery industry.

Lignocellulosic plant biomass is comprised of (i) cellulose, a linear glucose polymer linked by β-1,4-glycosidic linkages, which is organized into a highly crystalline structure; (ii) hemicellulose, a heterogeneous amorphous branched polysaccharide composed of pentose (*e.g.*, xylose and arabinose) and hexose (*e.g.*, mannose) sugars along with sugar acids and alcohols; and (iii) lignin, a recalcitrant polymer comprising phenylpropane units (*p*-coumaryl, coniferyl, and sinapyl alcohol), which shields the polysaccharides and provides rigidity to the plant cell wall (Fengel and Wegener 1984). Owing to their complex structures, a pretreatment step is needed for physical and chemical modification of the biomass prior to enzymatic hydrolysis for obtaining practically useful sugar yields.

The relevant outcomes of pretreatment include removal of lignin and hemicelluloses, reduction of cellulose crystallinity, and increased porosity of the materials. These alterations to the physical state of the biomass result in enhanced enzyme accessibility and efficiency on cellulose degradation (Hendricks and Zeeman 2009). Many physical, chemical, thermal, and biological methods have been used for pretreatment of various agricultural by-products; however, each method has limitations in technical and economic aspects (Mosier *et al.* 2005). Chemical pretreatment by acid, alkaline, and oxidative reagents are simple and efficient processes for increasing biomass digestibility.

Dilute acid pretreatment by mineral acids is effective for hydrolysis of hemicelluloses and has been widely used as a basic pretreatment method for various agricultural by-products (Saha *et al.* 2005; Hsu *et al.* 2010). In contrast, alkaline pretreatment by sodium hydroxide, ammonia, or lime results in delignification with minimal hydrolysis of the polysaccharide fractions, leading to increasing biomass digestibility (Kim *et al.* 2008; Zhu *et al.* 2010). Combined processes using sequential pretreatment steps have received increasing attention as a promising strategy to reduce the process severity factors, *i.e.*, chemical dosages and temperatures in each step. Combinations of pretreatments with different effects on biomass have been reported. For example, alkaline/peracetic acid (Zhao *et al.* 2011), formic acid/aqueous ammonia (Zhang *et al.* 2010), dilute acid/alkali (Zhang *et al.* 2010), liquid hot water/ammonia (Kim *et al.* 2006), and fungal delignification/phosphoric acid (Isroi *et al.* 2012). These combinations lead to overall increased pretreatment efficiency as well as chemical and energy savings, while retaining the ability to separate different lignocellulosic components in sequential steps.

In this study, a sequential acid/alkaline pretreatment process has been developed for pretreatment of rice straw. Highly digestible enriched cellulose was obtained by this process with saving in chemical usages and efficient separation of hemicelluloses and lignin in the sequential steps. The pretreated cellulose fraction was converted to ethanol by simultaneous saccharification and fermentation with high conversion efficiency. The work provides an alternative for pretreatment of biomass for an integrated multi-product biorefinery.

EXPERIMENTAL

Materials

Rice straw was collected from a local field in Pathum Thani province, Thailand. It was physically processed using a cutting mill (Retsch ZM2000; Haan, Germany) and sieved to retain particles 250 to 420 µm in diameter. The processed biomass was then used as a starting material for experimental studies. Chemical compositions (% lignin, cellulose, hemicelluloses, and ash) were analyzed using the standard NREL method (Sluiter *et al.* 2008). Commercial *Saccharomyces cerevisiae* (Thermosacc® Dry yeasts; Lallemand, Milwaukee, WI) was maintained on YPD agar containing 1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose, and 1.6% w/v agar.

Methods

1st step dilute acid pretreatment

A full factorial design (3x4x3) was employed for optimization of dilute acid pretreatment conditions. The design comprised 36 combinations with 3 independent variables. The independent variables considered in this study included (X_1) H_2SO_4 concentration (0.5, 1, and 2% w/v), (X_2) temperature (80, 95, 110, and 125 °C), and (X_3) operating time (10, 20, and 40 min). The reactions were performed in an autoclave (Tomy autoclave SS-325, Tomy Digital Biology, Tokyo, Japan). The pretreated rice straw was recovered by paper filtration (Whatman No.5) and washed with tap water until the pH was neutral. The pretreated substrate was dried at 70 °C and kept at room temperature for further experimental study. All the experiments were performed in triplicate and the average of reducing sugar yield from the enzymatic hydrolysis step was used as the response (Y). The experimental design and statistical analysis of the data were done by using SPSS for Windows version 11.5 (Softonic International S.A.; Barcelona, Spain). Regression analysis and analysis of variance (ANOVA) were used to evaluate the statistical significance of the model. The dataset was fitted to Eq. 1 as a second-order polynomial equation involving main effects and interaction effects for each variable. The fitting quality of the polynomial model equation was expressed by the coefficient of determination R^2,

$$Y = \beta_o + \sum_{i=1}^{3} \beta i x i + \sum_{i=1}^{3} \beta i i x i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta i j x i x j \quad (1)$$

where Y is the predicted response, o indicates the constant coefficient, i indicates the linear coefficient, ii indicates the quadratic coefficient, and ij indicates the interaction coefficient.

Sequential acid/ alkaline pretreatment

Three dilute acid pretreatment conditions representing conditions with varying severity levels from the first step were selected and combined with the second step alkaline pretreatment. In total, nine combinations of the sequential pretreatment process were studied in a complete matrix design. The selected conditions for the acid pretreatment step were: (1) 2% w/v H_2SO_4 at 125 °C for 10 min; (2) 1% w/v H_2SO_4 at 125 °C for 10 min; and (3) 0.5% w/v H_2SO_4 at 125 °C for 10 min. The conditions for the alkaline pretreatment step were: (1) 5% w/v NaOH at 90 °C for 20 min; (2) 2.5% w/v NaOH at 90 °C for 10 min; and 1.25% w/v NaOH at 90 °C for 10 min. The pretreated biomass from each step was then recovered by paper filtration (Whatman No.5) and washed with tap water before the subsequent pretreatment. The pretreated substrate was dried at 70 °C and kept at room

temperature. Chemical compositions (% lignin, cellulose, hemicelluloses, and ash) of the solid fractions were analyzed using the standard NREL method (Sluiter *et al*. 2008). Digestibility of the biomass was determined based on the reducing sugar yield obtained from enzymatic hydrolysis of the pretreated biomass. The sugar yield was reported based on the native biomass. The % sugar recovery were calculated as the percentage of the glucose and pentoses recovered based on the percent cellulose (×1.11) and hemicellulose (×1.13), respectively in the native rice straw on a dried weight basis.

Enzymatic Hydrolysis

The sugar yields from enzymatic hydrolysis of the pretreated substrates were evaluated. The hydrolysis reactions of 1 mL total volume contained 5% (w/v) pretreated substrate, with 25 FPU/g Accellerase®1500 (Danisco, Rochester, NY) in 50 mM sodium citrate buffer, pH 4.8, and 0.1% sodium azide. The reactions were incubated at 50 °C for 72 h with vertical rotation at 30 rpm. The experiments were done in triplicate. The released reducing sugar concentration was analyzed based on the amount of liberated reducing sugars using the 3,5-dinitrosalisylic acid (DNS) method (Miller 1959). The fermentable sugar profiles were analyzed on a high performance liquid chromatograph (SPD-M10A DAD, Shimadzu, Kyoto, Japan) equipped with a refractive index detector using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operating at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 mL/min.

Scanning electron microscope

Structures of the native and pretreated rice straw under various conditions were analyzed by scanning electron microscopy (SEM) using a VP-SEM S-3400N and EDX scanning electron microscope (Hitachi, Krefeld, Germany) operating at an accelerating voltage of 10 kV. The samples were dried and coated with gold for analysis.

Simultaneous saccharification and fermentation (SSF)

The SSF was performed in a 2.0-L reactor (Biostat®b, B. Braun (Thailand), Bangkok, Thailand) with a total operating volume of 1.2 L. The fermentation medium contained 5 g/L $(NH_4)_2SO_4$, 0.025 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L yeast extract, pH 4.8 with 6.25% (w/v) rice straw pretreated by the selected acid/alkaline pretreatment conditions. The medium was sterilized at 121 °C for 15 min. The pretreated substrate was pre-digested with 25 FPU/g Accellerase® 1500 at 50 °C with mixing at 500 rpm for 6 h. Thermosacc® yeast culture was grown at 30 °C for 24 h in YPD medium and then inoculated at 10% (v/v) into the Accellerase®-digested substrate, which had been allowed to cool to fermentation temperature. The fermentation mixture was incubated at 40 °C with pH controlled at 4.8 by H_3PO_4 and NH_4OH. Fermentation was operated for 72 h with continuous mixing at 300 rpm. Samples were collected periodically to analyze the content of ethanol, glucose, and xylose on a high performance liquid chromatograph equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) as described above.

RESULTS AND DISCUSSION

The native rice straw used in this study was found to contain 35.3% cellulose, 20.7% hemicelluloses, and 26.7% lignin. Compared with other widely studied biorefinery feedstocks, *i.e.*, sugarcane bagasse, corncob, corn stover, and palm wastes (Imman *et al.*

2013), rice straw contains a relatively lower content of cellulose with a relatively high ash content (14.2%), which is mainly composed of silica. The differences in physical structures and chemical compositions thus make rice straw a biorefinery feedstock likely to show unique characteristics in response to pretreatment and susceptibility to enzymatic hydrolysis. The native biomass showed low sugar yield from enzymatic hydrolysis, resulting in the reducing sugar yield of 76.0 mg/g of the substrate comprising glucose as the major sugar (55.6 mg/g) and pentoses as the minor product (26.0 mg/g).

Dilute Acid Pretreatment

The effect of dilute acid pretreatment on enzymatic digestibility of rice straw was systematically studied using an experimental design approach in the first step. Influences of acid concentration, temperature, and time on the reducing sugar yield after enzymatic hydrolysis of the solid fraction were studied (Fig. 1). Overall, pretreatment by dilute acid led to a 1.3- to 6.9-fold improvement in rice straw digestibility compared with the native biomass. The solid recovery yields were in the range of 44.0 to 85.7% from the initial biomass on a dried weight basis, which were varied depending on the severity of pretreatment conditions. This resulted in reducing sugar yields from subsequent enzymatic hydrolysis ranging from 86.0 to 288.0 mg/g_{native}. Increasing operating temperature and acid concentration led to a marked increase in reducing sugar yield, which also increased with longer time of pretreatment at low severity conditions. The effects were less observed under conditions with higher severity because of the increased decomposition of cellulose and hemicellulose which was also reflected in lower solid recovery and thus resulted in no marked improvement in sugar yield from the native biomass.

Statistical testing of the model was performed by the Fisher's statistical test for analysis of variance (ANOVA), and the results are shown in Table 1. In this case, the value of the determination coefficient (R^2) indicates that 89.00% of the variability in the response could be explained by the model. The regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation (Eq. 2). The response, reducing sugar yield (Y), can be expressed in terms of the following regression second order equation:

$$Y = -338.680+104.642X_1+5.188X_2+6.472X_3-8.335X_1^2-0.007X_2^2+0.0030.013X_3^2-0.413X_1X_2-0.592X_1X_3-0.056X_2X_3 \quad (2)$$

in which X_1 is H_2SO_4 concentration, X_2 is temperature, and X_3 is operating time.

According to the *P*-values, all three variables showed significant influence ($P < 0.05$) to the reducing sugar yield. Operating time (X_3) had a slightly stronger influence on the sugar yield obtained ($P = 0.003$) than the acid concentration (X_1) and temperature (X_2). The interaction of terms between temperature and time (X_2X_3) showed the most significant influence on the response as shown by the lowest *P*-value (< 0.001).

The maximum reducing sugar yield was obtained with 2% H_2SO_4 at 125 °C for 10 min, which led to the highest reducing sugar yield of 288.0 mg/g_{native}. Glucose was the major constituent (193.1 mg/g_{native}), while xylose and arabinose represented the major pentose sugars (16.3 mg/g_{native}). The results indicated that 49.7% of the glucose from the available cellulose in the native rice straw was recovered, while only 6.9% of pentoses were recovered owing to extensive hydrolysis of the xylan fraction under the selected dilute acid pretreatment conditions. This condition was chosen as the stringent condition for one-step acid pretreatment based on the maximal sugar yield from the pretreated biomass. Less

stringent conditions using 0.5% and 1% H_2SO_4 at the same pretreatment temperature and time that gave lower reducing sugar yields of 201.9 and 227.1 mg/g_{native}, respectively, were selected as the mild and intermediate pretreatment conditions, respectively, for further two-step acid/alkaline pretreatment study.

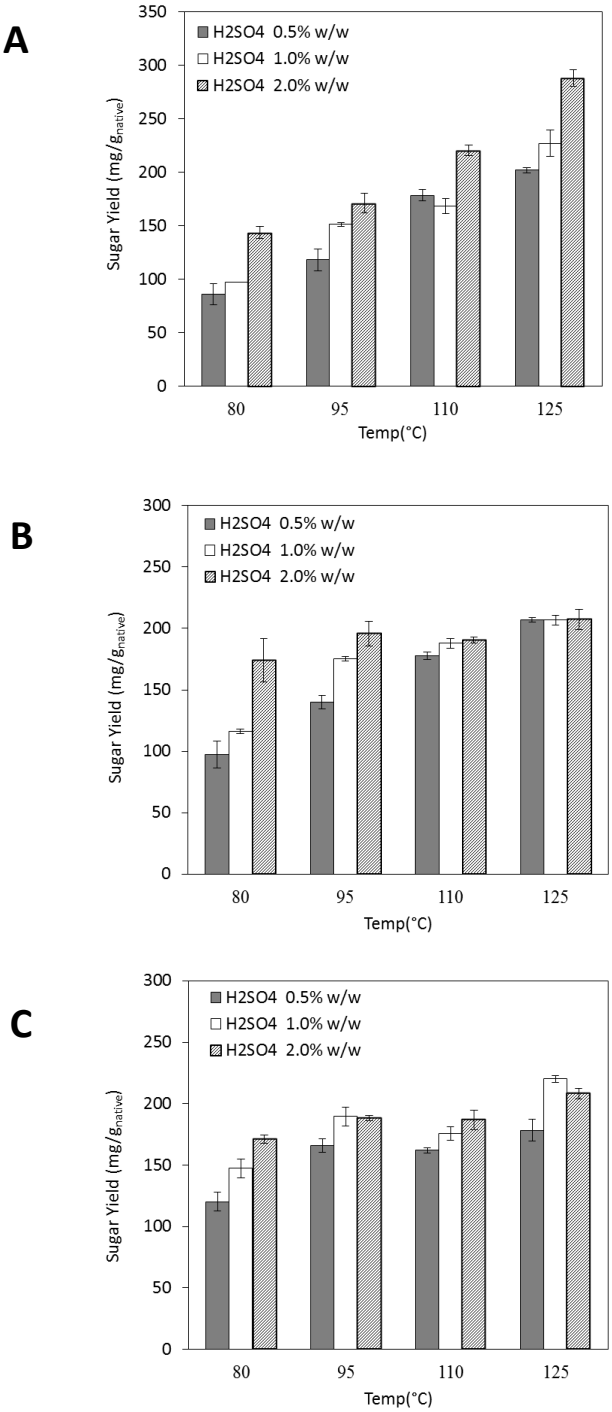

Fig. 1. Effects of dilute acid pretreatment on sugar yields from enzymatic hydrolysis of pretreated rice straw. The rice straw was pretreated at varying temperature (80 to 125 °C) and acid concentration (0.5 to 2% w/v) at a solid: liquid ratio of 1: 5 (w/v) for (A) 10 min, (B) 20 min, and (C) 40 min. The pretreated substrates were hydrolyzed with 25 FPU/g Accellerase® 1500 in 50 mM sodium citrate buffer, pH 4.8 and incubated at 50 °C for 72 h. Data shown are the mean from triplicate experiments and error bars represent the standard deviation (see Appendix)

Table 1. Regression Model Analysis of Reducing Sugar Yields from Rice Straw Pretreated using Dilute Acid Pretreatments under Different Conditions

Source	Coefficient	t-value	*P*-value
Constant	-338.680	-2.668	0.013
Acid conc.	104.642	2.675	0.013
Temperature	5.188	2.195	0.037
Time	6.472	3.309	0.003
Acid conc^2	-8.335	-.753	0.458
Temperature^2	-.007	-.646	0.524
Time^2	.003	.097	0.923
Acid * Temp	-.413	-1.673	0.106
Acid * Time	-.592	-1.807	0.082
Temp * Time	-.056	-4.574	<0.001

R^2 = 0.89 Adjusted R^2 = 0.84

Sequential Acid-Alkaline Pretreatment

The combined effects of sequential pretreatment were investigated in which an initial dilute acid step was followed by an alkaline step for improving rice straw digestibility. In total, nine conditions were evaluated from crossing each of the selected acid and alkaline conditions in a complete matrix. The reducing sugar yields and composite sugar profiles from the 2-step pretreatments are shown in Table 2. The maximal reducing sugar yield was achieved by combining the intermediate acid pretreatment condition (1% w/w H_2SO_4 at 125 °C for 10 min) and the mild alkaline pretreatment conditions (1.25% w/w NaOH, 10 min, 90 °C) (condition 12). This led to the highest yield of reducing sugars (353.1 mg/g$_{native}$), whereas only 227.1 mg/g$_{native}$ and 285.0 mg/g$_{native}$ reducing sugar yields were obtained from the enzymatic hydrolysis after the single step H_2SO_4 or NaOH pretreatments, respectively. The sequential acid-alkaline pretreatment thus led to greater glucose yields with less consumption of pretreatment agents, equivalent to 2-fold and 4-fold reduction in usages of H_2SO_4 and NaOH, respectively, as compared with the most effective conditions for the single step pretreatments.

The effects of varying the acid and alkaline pretreatment steps on rice straw composition were tested by chemical component analysis (Table 2). The main effect of acid pretreatment was a reduction in hemicellulose content, which is due to susceptibility of amorphous xylan in hemicellulose to acid hydrolysis (Brodeur *et al.* 2011). In addition, partial hydrolysis of the cellulose fraction and slight reduction in lignin content was observed after acid pretreatment. In contrast to acid, the major effect of alkaline pretreatment is on lignin removal and biomass swelling by solvation and saponification. Hemicellulose could be partially hydrolyzed by alkaline hydrolysis, while the attack on cellulose is more limited (Hendricks and Zeeman 2009; Kumar *et al.* 2013). The different effects of acid and alkaline pretreatments are thus complementary, and rice straw pretreated by the two-step process showed increased cellulose content with lower hemicellulose and lignin contents. The reduction of the latter could thus be attributed to the effects of acid and alkaline pretreatments, respectively. The sequential pretreatment under the most effective conditions (No. 12) led to the final enrichment of cellulose in the solid residue (72.0 wt% in the pretreated biomass) with small contents of hemicelluloses (6.4%) and lignin (14.5%). Upon enzymatic hydrolysis of the solid fraction, a maximum glucose yield of 275.4 mg/g$_{native}$ was obtained. This was equivalent to 70.9% recovery of glucose from the native biomass (Fig. 2). This indicated nearly 100% cellulose conversion to glucose by the 2-step process compared to 69.3 and 87.7% by the dilute acid (No. 3) and alkaline (No.

6) pretreatment, respectively, suggesting higher susceptibility of the biomass pretreated by the sequential process to enzymatic hydrolysis.

Table 2. Effects of Sequential Acid/ Alkaline Pretreatment on Composition of Rice Straw and Sugar Recovery

No.	Condition 2-step pretreatment						Residual composition [a]				Sugar yield		
	H_2SO_4			NaOH			C	H	L	A	Reducing sugar	Glc	Pen
	Conc	T	t	Conc	T	t							
	%w/v	°C	min	%w/v	°C	min	wt%	wt%	wt%	wt%	mg/g_{native}	mg/g_{native}	mg/g_{native}
Native	-	-	-	-	-	-	35.3	20.7	26.7	14.2	76.0	55.6	26.0
1	0.5	125	10	-	-	-	26.5	4.8	12.5	5.9	201.9	146.2	22.8
2	1	125	10	-	-	-	26.6	2.7	13.9	5.8	227.1	186.0	19.5
3	2	125	10	-	-	-	25.3	1.9	14.2	5.6	288.0	193.1	16.3
4	-	-	-	1.25	90	10	24.7	11.3	7.5	3.1	285.0	199.2	54.4
5	-	-	-	2.5	90	10	25.8	9.3	6.8	2.2	348.2	220.4	48.8
6	-	-	-	5	90	20	28.4	6.3	2.6	0.3	371.6	273.8	36.6
7	0.5	125	10	5	90	20	22.1	1.6	2.3	0.0	187.8	167.9	11.3
8	0.5	125	10	2.5	90	10	20.7	2.2	3.3	0.4	265.7	199.6	15.0
9	0.5	125	10	1.25	90	10	21.8	2.9	4.2	4.3	232.1	148.4	15.0
10	1	125	10	5	90	20	20.7	0.9	1.8	0.0	236.1	202.0	11.6
11	1	125	10	2.5	90	10	20.1	1.4	2.9	0.0	225.8	198.6	17.7
12	1	125	10	1.25	90	10	24.9	2.2	5.0	2.5	353.1	275.4	15.6
13	2	125	10	5	90	20	17.9	0.0	2.0	0.1	216.4	176.5	0.0
14	2	125	10	2.5	90	10	19.9	1.0	3.1	2.9	228.4	189.3	12.5
15	2	125	10	1.25	90	10	20.5	1.4	4.4	0.2	209.9	171.0	14.2

[a]The residual composition is reported as a residual %wt of the solid before and after pretreatments (on a dried weight basis). C, Cellulose; H, Hemicellulose; L, Lignin; A, Ash

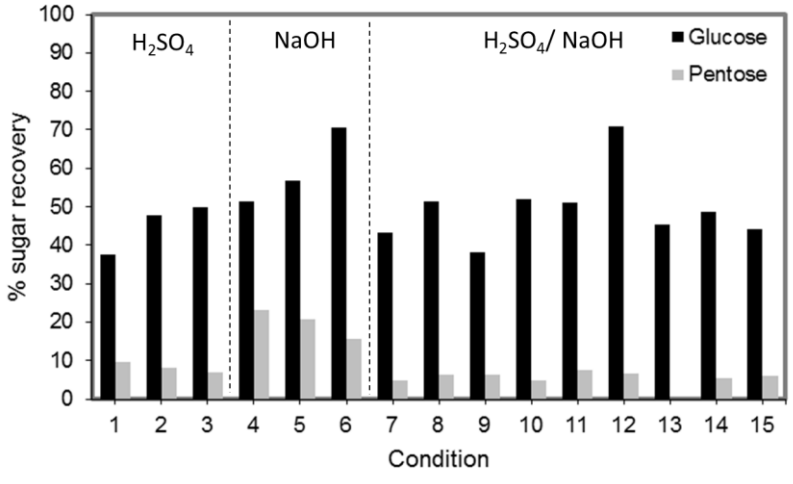

Fig. 2. Effects of sequential dilute acid/ alkaline pretreatment on sugar yield from enzymatic hydrolysis. The pretreated substrates were hydrolyzed with 25 FPU/g Accellerase®1500 in 50 mM sodium citrate buffer, pH 4.8, and incubated at 50 °C for 72 h. The data represented averages from triplicate experiments with SD ≤ ±5%. The conditions are according to Table 2

Several combinations of different pretreatment processes have been reported, *e.g.* alkaline/peracetic acid pretreatment (Zhao *et al.* 2009, 2011), formic acid/aqueous ammonia (Zhang *et al.* 2010), sodium hydroxide/lime (Xu and Cheng 2010), and dilute acid/steam-explosion (Chen *et al.* 2011), as means to increase pretreatment efficiency under mild conditions and generate a cellulose fraction with high homogeneity. Compared to these previous works, our work presented the combination of simple dilute acid and alkaline pretreatment methods into a sequential process with lower chemical requirements. Glucose recovery from the native biomass in this study was in the same range as reported in previous works using different combined two-step approaches, *e.g.*, ultrasonic or H_2O_2 pretreatment followed by fungal delignification (Yu *et al.* 2009), alkali/ peracetic acid (Zhao *et al.* 2009), and sodium hydroxide/ lime (Xu and Cheng 2010), from which variation in glucose and combined sugar yields were reported (50 to 70% of the theoretical recovery yield). The sequential acid/ alkaline pretreatment process in this study also led to enrichment of cellulose in the solid fraction containing 72.0% glucan with less cross-contamination of hemicelluloses and lignin compared with the respective single-step pretreatments employed in several previous reports, in which cellulose contents of 40 to 65% were obtained from various pretreated biomasses using dilute acid, alkaline, and liquid hot water pretreatment (Chen *et al.* 2009; Wan *et al.* 2011; Imman *et al.* 2013). Sequential acid/alkali pretreatment has also been reported for pretreatment of corncob in which pretreatment was performed using 2% H_2SO_4 at 121 °C for 45 min followed by 2% wt NaOH at 80 °C for 6 h (Zhang *et al.* 2010). Under these conditions, substrates were enriched with 91.1% cellulose; however, higher chemical dosages, temperature, and pretreatment time were used compared to those applied in our study. A single step catalytic aqueous-organosolv fractionation of eucalyptus pulp was also recently reported, which led to glucan content enrichment of the cellulose fraction to 68.5% (Klamrassamee *et al.* 2013). These works thus highlight the advantages of the two-step, or modified one-step, processes on increasing biomass digestibility under mild conditions, with added savings in chemical and energy consumption while also allowing separation of biomass components for valorization to chemical co-products or energy (Floebela *et al.* 2008; Pandey and Kim 2011).

Scanning Electron Microscopy Analysis

Scanning electron microscopy was used to determine the structural changes of rice straw resulting from the pretreatment process. The native rice straw showed intact surface and physical structure (Fig. 3a). Dilute acid pretreatment resulted in structural changes on biomass morphology with increasing papillae and wart-like structures owing to destruction of the cuticle wax and silica layers (Fig. 3b). The physical changes were coupled to extraction of the hemicellulose fraction in the substrate related to the increases in surface area. Alkaline pretreatment led to peeling of the surface lignin, allowing accessibility to the inner cellulose and hemicellulose components in the biomass (Fig. 3c). Less distinct papillae structures were found in the alkaline pretreated rice straw compared with those that underwent acid pretreatment. The combined two-step pretreatment led to extensive damage of the silicified waxy surface of the biomass and disruption of the cell wall structures (Fig. 3d). The majority of micro-fibrous cellulose structures were highly preserved while the lignin-hemicellulose complex was removed. Extensive surface peeling effects and the formation of papillae structures were found. These changes in physical structure are consistent with the combined pretreatment process conferring greater

susceptibility of the cellulose fibers to enzymatic action in the rice straw as compared with the native biomass and single-step (acid or alkaline) pretreatment.

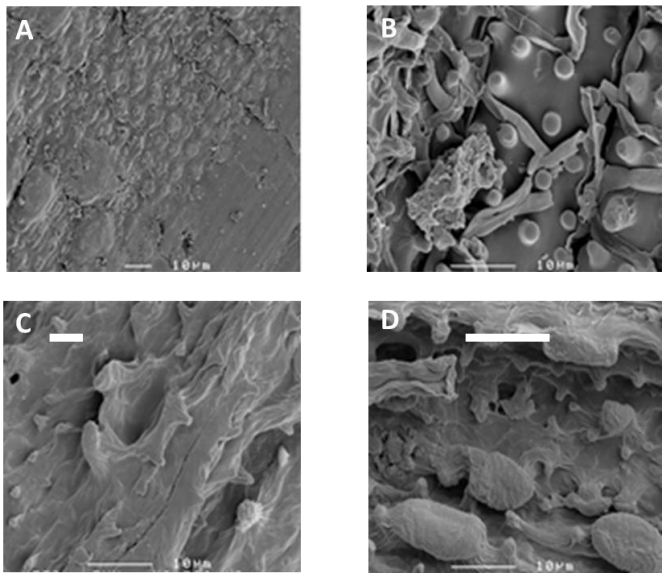

Fig. 3. Physical structures of native and pretreated rice straw as analyzed by SEM. (a) Native rice straw; (b) pretreated with 1.0% (w/w) H_2SO_4 at 125 °C for 10 min; (c) pretreated with 5.0% (w/w) NaOH at 90 °C for 20 min; and (d) 1.0% (w/w) H_2SO_4 at 125 °C for 10 min followed by 1.25% (w/w) NaOH at 90 °C for 10 min. Scale bars represent 10 μm

Ethanol Fermentation

The most effective sequential pretreatment condition obtained in this study (condition 12) was applied for preparation of the cellulose enriched substrate for ethanol production using a simultaneous saccharification and fermentation process in a laboratory-scale bioreactor. The initial content of pretreated rice straw was 62.5 g/L, equivalent to 45.1 g/L glucan. The substrate was pre-digested with Accellerase® 1500, and then inoculated with *S. cerevisiae* for fermentation.

In the pre-hydrolysis step, the pretreated rice straw was rapidly saccharified to glucose with the highest mean accumulated glucose concentration of 13.72 g/L at 6 h, while xylose was slowly released from the substrate. Glucose was continually converted in the fermentation step, while xylose was not assimilated by the yeast. The highest mean ethanol concentration of 21.0 g/L was achieved at 78 h, equivalent to 84.6% of the theoretical yield based on available glucan in the pretreated substrate with a production efficiency of 0.27 g/L/h (Fig. 4). The residual glucose at the end of fermentation was 0.49 mg/L, while xylose was not assimilated into the yeast cells, resulting in a residual concentration of 2.06 g/L.

Variation in ethanol concentration from fermentation of lignocellulosic biomass depends on various factors including the nature of substrates, pretreatment methods, ethanologenic microorganisms, and physical parameters of the fermentation processes. The final ethanol concentration obtained in this study was comparable to those previously reported on fermentation of rice straw pretreated with dilute acid (Karimi *et al.* 2006) and microwave/alkaline pretreatment (Zhu *et al.* 2005), which was in the range of 8.0 to 25.8 g/L of ethanol. Preparation of highly digestible cellulose-enriched substrate by the two-step pretreatment process in this study could provide an approach for increasing available carbon substrate in a fermenter, compared to pretreated biomass from a single step process

which contains lower fermentable content on a weight basis. This would be advantageous for increasing ethanol concentration in the fermentation mixture and hence lower cost in downstream processing. The fractionated hemicelluloses from the sequential pretreatment are also promising for conversion to value-added commodity chemicals by subsequent catalytic or biocatalytic reactions, *e.g.*, organic acids, furans, and bio-plastic monomers (Carvalheiro *et al.* 2008), while lignin could be used as fuel for on-site energy production or for conversion to aromatics (Yuan *et al.* 2013). This co-production of multi-products from biomass will lead to improved economic feasibility of the integrated biorefinery process.

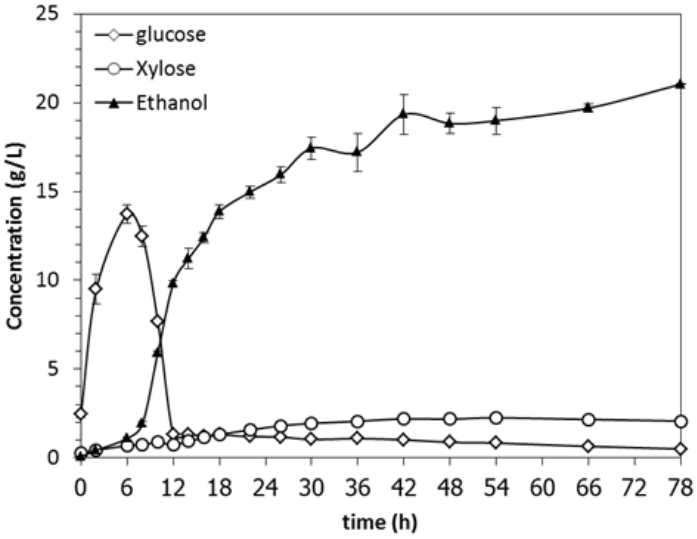

Fig. 4. Fermentation of cellulose-enriched substrate to ethanol. The fermentation mixture contained 6.25% w/v rice straw pretreated with the sequential acid/ alkaline process. The substrate was pre-digested with 25 FPU/g Accellerase® 1500 at 50 °C for 6 h with mixing at 500 rpm before inoculation of *S. cerevisiae* at 10% (v/v) and incubated at 40 °C for 72 h. Data shown are the average of triplicate experiments and error bars represent the standard deviation

CONCLUSIONS

1. A sequential dilute acid/ alkaline process was developed for efficient pretreatment of rice straw, resulting in high enzymatic digestibility and high sugar recovery from the solid residues.
2. The sequential pretreatment process led to improved pretreatment efficiency with less chemical consumption resulting in lower chemical cost and less chemical waste generation.
3. The developed process can allow step-wise separation of hemicellulose-derived sugars and lignin which can lead to further valorization to co-products in integrated biorefineries.

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APPENDIX

Table A1. Effects of dilute acid pretreatment on sugar yields from enzymatic hydrolysis of pretreated rice straw. The rice straw was pretreated at varying temperature (80 to 125 °C) and acid concentration (0.5 to 2% w/v) at a solid: liquid ratio of 1: 5 (w/v) for 10-40 min.

Reaction	Time (min)	Temperature (^oC)	H_2SO_4 conc. (%w/v)	Reducing sugar (mg/g_{native})	SD
1	10	80	0.5	86.0	9.7
2		95	0.5	118.1	10.2
3		110	0.5	178.6	5.3
4		125	0.5	201.9	2.3
5		80	1	97.2	0.0
6		95	1	151.2	1.8
7		110	1	168.2	7.0
8		125	1	227.1	12.4
9		80	2	143.7	5.6
10		95	2	171.3	8.9
11		110	2	220.5	4.9
12		125	2	288.0	7.8
13	20	80	0.5	97.4	4.8
14		95	0.5	139.8	11.1
15		110	0.5	177.8	5.4
16		125	0.5	206.7	3.1
17		80	1	116.1	1.9
18		95	1	175.3	1.8
19		110	1	188.0	4.0
20		125	1	206.7	3.7
21		80	2	174.0	17.6
22		95	2	195.6	9.9
23		110	2	190.3	2.5
24		125	2	207.3	8.2
25	40	80	0.5	120.1	7.7
26		95	0.5	165.8	5.5
27		110	0.5	161.9	1.9
28		125	0.5	178.4	9.1
29		80	1	147.3	7.7
30		95	1	189.5	7.4
31		110	1	175.8	5.6
32		125	1	220.2	2.8
33		80	2	170.9	3.4
34		95	2	188.0	2.2
35		110	2	186.7	7.8
36		125	2	208.1	4.5