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Cascade Enzymatic Hydrolysis Coupling with Ultrafine Grinding Pretreatment for Sugarcane Bagasse Saccharification

Zheng-qiu Yuan[a,b], Jin-xing Long[a], Tie-jun Wang[a*], Yu-qin Li[c*], Qi Zhang[a], Long-long Ma[a]

a. Key Laboratory of Renewable Energy, Guangzhou Institute of Energy Conversion, Chinese Academy of Science, Guangzhou 510640, China
b. University of Chinese Academy of Science, Beijing 100049, China
c. School of Chemical Engineering, Xiangtan University, Xiangtan 411105, China

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The biorefinery process for sugarcane bagasse saccharification generally requires significant accessibility of cellulose. We reported a novel method of cascade cellulase enzymatic hydrolysis coupling with ultrafine grinding pretreatment for sugarcane bagasse saccharification. Three enzymatic hydrolysis modes including single cellulase enzymatic hydrolysis, mixed cellulase enzymatic hydrolysis, and cascade cellulase enzymatic hydrolysis were compared. The changes on the functional group and surface morphology of bagasse during cascade cellulase enzymatic hydrolysis were also examined by FT-IR and SEM respectively. The results showed that cascade enzymatic hydrolysis was the most efficient way to enhance the sugarcane bagasse saccharification. More than 65% of reducing sugar yield with 90.1% of glucose selectivity was achieved at 50 °C, pH=4.8 for 72 h (1200 r/min) with cellulase I of 7.5 FPU/g substrate and cellulase II of 5 FPU/g substrate.

Key words: Sugarcane bagasse, Ultrafine grinding pretreatment, Cascade enzymatic hydrolysis, Reducing sugars, Glucose

I. INTRODUCTION

Exploration of new sustainable and environment-friendly energy sources such as liquid biofuels (bioethanol and biodiesel) has been an increased interest in recent years due to current energy crisis and environmental issues. However, when producing ethanol from maize or sugarcane, the raw material constitutes about 40%−70% of the production cost [1] and glucose is estimated to account for around 80% of total medium costs for algal biodiesel [2, 3]. This is the major reason why biofuels from these feedstocks have not made its breakthrough yet. Therefore, seeking low-cost and effective feedstocks for liquid biofuels has received special attention.

Many lignocellulosic materials are considered as the feedstock for large-scale biological production in the future since they are abundant and cheap [4−7]. Many excellent reviews have been reported on the theme of biofuels production from lignocellulosic biomass [8−12]. One of the most promising lignocellulosic materials for conversion to biofuels in tropical countries is sugarcane bagasse (SCB) [13], which is primarily composed of approximately 50% cellulose and 25% each of hemicellulose and lignin [14]. Actually, around 50% of bagasse residue is burned to produce steam and electricity for distillery plants as a source of energy, or to be used as cattle feed during the harvest season, causing not only energy waste, but also environmental pollution. Therefore, in the approximately 80 sugarcane producing countries (such as Brazil, China, Macedo), there is a potential to develop biofuels from the important industrial waste-SCB to solve energy crisis and environmental issues in recent years [15].

However, this material is difficult to be used as biofuel feedstock because of its chemical structure and high pentose fraction, making it recalcitrant to saccharification (acid or hydrolysis) unless it is pretreated to be a more accessible form. Many pretreatment methods have been developed, including steam explosion [16] and liquid hot water pretreatment [17], pretreatments with alkali-peracetic acid [18], pretreatments with white rot fungi [19], along with a combination of these methods, such as wet oxidation [20−22]. Hereon, an effective and economical pretreatment should limit the formation of degradation products that can inhibit the growth of fermentative microorganism, minimize energy demands, and lower the cost of enzymes [23].

Ultrafine grinding pretreatment method is to make use of mince, burst, and mill physical force to achieve fine particles and reduce crystallinity of lignocellulosic materials to enhance the enzymatic digestibility. It is an attractive approach because it does not require the addition of chemicals such as sulfuric acid, lime and

*Authors to whom correspondence should be addressed. E-mail: wangtj@ms.giec.ac.cn, yuqinli2004@xtu.edu.cn

ammonia *etc.* And no hydrolysis or fermentation inhibitors, such as furfural or hydroxymethyl furfural are produced by this pretreatment method [24].

Hydrolysis is one of efficient and widely-used process for biofuel production from renewable biomass. For example, acid hydrolysis of biomass has been extensively studied [25, 26]. However, proton acid such as H_2SO_4 and HCl are generally required, resulting in labor-in tensive post separation and economatic environmental pollution. On the other hand, enzymatic hydrolysis is considered as another promising way for obtaining sugars from lignocellulosic materials [11, 20], for its high sugar yield and avoidance of the problems associated with the use of strong acid catalysts. Therefore, we investigate the SCB hydrolysis for the reducing sugar production using a cascade enzymatic hydrolysis mode coupling with ultrafine grinding pretreatment.

II. MATERIALS AND METHODS

A. Materials

Sugarcane bagasse was kindly supplied by Jiang men Sugar Industry Co. Ltd., Guangdong province. It contained about (41.6±0.04)% cellulose, (26.37±0.04)% hemicellulose, and (19.76±0.05)% lignin, in addition to (0.61±0.02)% water and (0.93±0.01)% ash. Commercial cellulase I (Denicell 101 L) was purchased from Advanced Enzyme Technologies Company, India. Its activity was measured as filter paper unit (FPU). According to the method presented by Ghose (1987) [27], the enzyme showed activity of $(1.72\pm0.01)\times10^4$ FPU/g. The cellulase II (Cellulase A "Amano" 3) showed activity of $(2.40\pm0.01)\times10^4$ FPU/g, supplied by Amano Enzyme Inc (Nagoya, Japan). Glucose, D-xylose, D-fructose, and cellobiose are from Sigma-Aldrich Co. St. Louis, MO, USA. Other chemicals were of analytical grade.

B. Methods

1. Ultrafine grinding pretreatment of sugarcane bagasse

The sugarcane bagasse was washed with distilled water, and then it was dried at 80 °C for 2 days. The dried sugarcane bagasse was grinded by ultra-mizer (BB 51, Retsch, Germany) at room temperature to achieve ultrafine powder that was used for enzymatic hydrolysis.

2. Enzymatic hydrolysis of ultrafine sugarcane bagasse

Single cellulase enzymatic hydrolysis: 500 mg ultrafine sugarcane bagasse was suspended in 10 mL of citric acid-sodium citrate buffer (pH=4.8, 50 mmol/L). Cellulase I of 7.5 FPU/g substrate or cellulose II of 5 FPU/g substrate was added respectively into citric acid-sodium citrate buffer and incubated in an air-bath shaker at 50 °C and 1200 r/min for 72 h to investigate the performance of single cellulose enzymatic hydrolysis.

Mixed cellulase enzymatic hydrolysis: 500 mg ultrafine sugarcane bagasse was suspended in 10 mL of citric acid-sodium citrate buffer (pH=4.8, 50 mmol/L). Both cellulase I of 7.5 FPU/g substrate and cellulase II of 5 FPU/g substrate was added together into citric acid-sodium citrate buffer and incubated in an air-bath shaker at 50 °C and 1200 r/min for 72 h to investigate the performance of mixed cellulase enzymatic hydrolysis.

Cascade cellulase enzymatic hydrolysis: 500 mg ultrafine sugarcane bagasse was suspended in 10 mL of citric acid-sodium citrate buffer (pH=4.8, 50 mmol/L). Firstly, cellulase I of 7.5 FPU/g substrate was added into citric acid-sodium citrate buffer and incubated in an air-bath shaker at 50 °C and 1200 r/min for 6 h. Then, cellulase II of 5 FPU/g substrate was added and further incubated at 50 °C and 1200 r/min for 72 h to investigate the performance of cascade cellulase enzymatic hydrolysis.

3. Sample and analysis

The aqueous samples of enzymatic hydrolysis were taken periodically. Firstly, the sample was centrifuged at 8000 r/min for 20 min. Then, 20 μL supernatant was subjected for HPLC analysis. The content of reducing sugars was analyzed by the 3,5-dinitrosalicylic acid method [28]. The concentration of glucose, xylose, fructose and cellobiose were determined by HPLC apparatus (Waters 600 Controller machine, 6.5 mm×300 mm sugar Pak column, Waters 2414 RID detector). The HPLC working temperature was 90 °C and the mobile phase flow rate was 0.5 mL/min. The mobile phase consisted of EDTA-Ca^{2+} (0.05 g/L) and 0.01 mol/L oligosaccharide standards, which was purchased from Sigma (St. Louis, MO).

The enzymatic digestibility was denoted as yield of reducing sugars ($Y_{\rm RS}$, %) and yield of monosaccharide ($Y_{\rm M}$, %) [18], which were respectively defined as:

$$Y_{\rm RS} = \frac{0.9w_{\rm RS}}{w_{\rm IS}} \times 100\% \quad (1)$$

$$Y_{\rm M} = \frac{0.9w_{\rm M}}{w_{\rm IS}} \times 100\% \quad (2)$$

where $w_{\rm RS}$ is the weight of reducing sugars produced by enzymatic hydrolysis. $w_{\rm IS}$ is the weight of initial solid. $w_{\rm M}$ is the weight of monosaccharide (glucose, xylose, or arabinose).

The hydrolysis residue was dried at 80 °C for 2 days and characterized. FT-IR spectrum was recorded on Perkin-Elmer Spectrum One B instrument using KBr pellet technique. Scanning electron micrograph (SEM) was obtained with a JEOL JSM-6700F instrument.

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TABLE I Y_{RS} and content of monosaccharides before and after enzymatic hydrolysis of SCB.

Mode	Y_{RS}/(mg/g)				
	Reducing sugar	Glucose	Xylose	Fructose	Cellobiose
Raw	97	41	32	5	4
Cellulase I	590	296	70	18	11
Cellulase II	571	311	32	21	
Mixed cellulase	656	426	110	34	2
Cascade cellulase	712	642	50	18	

III. RESULTS AND DISCUSSION

A. The effect of ultrafine grinding pretreatment on enzymatic hydrolysis

In order to investigate the effect of ultrafine grinding pretreatment on enzymatic hydrolysis, the single cellulase I enzymatic hydrolysis was adopted to produce reducing sugar. As shown in Fig.1, ultrafine grinding time had a significant influence on the yield of reducing sugar. When ultrafine grinding time was increased from 0 min to 40 min, the yield of reducing sugar was increased lineally from 8.7% to 53.1%. But further increasing ultrafine grinding time did not significantly improve reducing sugar yield. It indicated that ultrafine grinding of sugarcane bagasse for 40 min promoted significantly the single cellulase I enzymatic hydrolysis to produce reducing sugar. Under this condition, the yield of glucose, xylose, fructose, and cellobiose was obtained (Table I). The promotion role can be attributed to the fact that ultrafine grinding reduced the sugarcane bagasse particle size and destroyed the crystallinity, which enhanced the enzymatic digestibility.

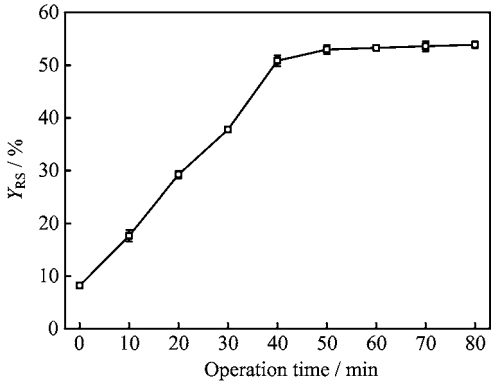

FIG. 1 Effect of ultrafine grinding pretreatment on the yield of reducing sugar.

B. The effect of different enzymatic hydrolysis process on the yield of reducing sugars

Three kind of hydrolysis processes (single cellulase enzymatic hydrolysis, mixed cellulase enzymatic hydrolysis and cascade cellulase enzymatic hydrolysis) were investigated to compare their performance of reducing sugar production.

For single cellulase enzymatic hydrolysis of ultrafine sugarcane bagasse, cellulase I of 7.5 FPU/g substrate and cellulase II of 5 FPU/g substrate were adopted respectively and their enzymatic hydrolysis performance was compared. As shown in Fig.2(a), both cellulase I and cellulase II exhibited sharp increase of reducing sugars yield during the first 6 h. Cellulase I (48.0% reducing sugar yield) was more active than cellulase II (41.1% reducing sugar yield). Further prolonging reaction time to 72 h, the yield of reducing sugar by cellulase I and cellulase II reached 53.1% and 51.4%, respectively.

For mixed cellulase enzymatic hydrolysis of ultrafine sugarcane bagasse, cellulase I and cellulase II were mixed and worked synergistically to hydrolyze ultrafine sugarcane bagasse. As shown in Fig.2(b), the yield of reducing sugar reached 59.0%, which was higher than that (53.1% for cellulase I, 51.4% for cellulase II) of single cellulase enzymatic hydrolysis. The yields of glucose, xylose, fructose, and cellobiose were obtained by this process (Table I). It implied that the selectivity of glucose in the total reducing sugar was 65%.

For cascade cellulase enzymatic hydrolysis of ultrafine sugarcane bagasse, cellulase I of 7.5 FPU/g substrate was added firstly into citric acid-sodium citrate buffer and incubated in an air-bath shaker at 50 °C and 1200 r/min for 6 h. Then, cellulase II of 5 FPU/g substrate was added and further incubated at 50 °C and 1200 r/min for 72 h. As shown in Fig.2(b), the yield of reducing sugar reached 65.0%, which was higher than that (59.0%) of mixed cellulase enzymatic hydrolysis. The yield of glucose, xylose, and fructose were obtained by this process (Table I). It implied that the selectivity of glucose reached 90.1%, which was higher than that (65%) of mixed cellulase enzymatic hydrolysis process.

C. The effect of ultrasonic treatment on cascade enzymatic hydrolysis

Ultrasonic treatment was used to promote the cascade cellulase enzymatic hydrolysis of ultrafine sugarcane bagasse. In order to investigate the effect of ultrasonic power on the yield of reducing sugar, the ultrasonic treatment time was kept 5 min. As shown

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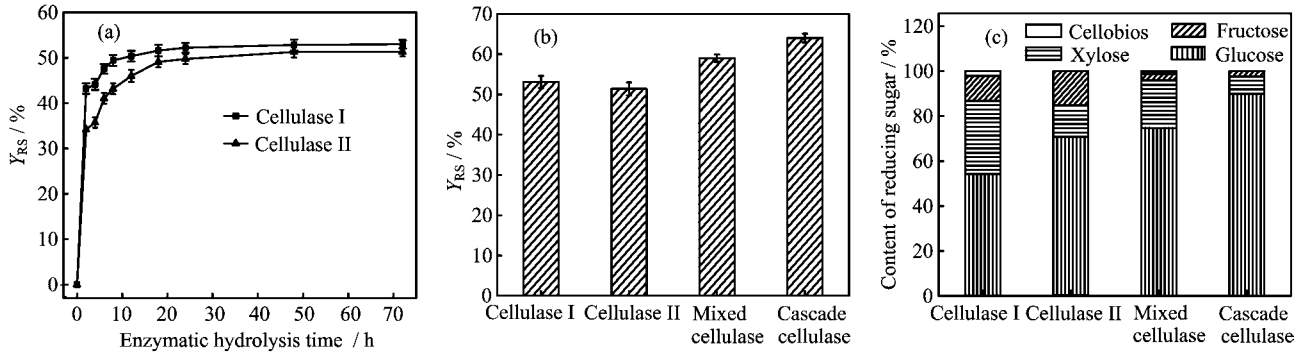

FIG. 2 (a) Effect of enzymatic hydrolysis time on the yield of reducing sugar. (b) Effect of enzymatic hydrolysis mode on the yield of reducing sugar. (c) Effect of enzymatic hydrolysis mode on the content of reducing sugar.

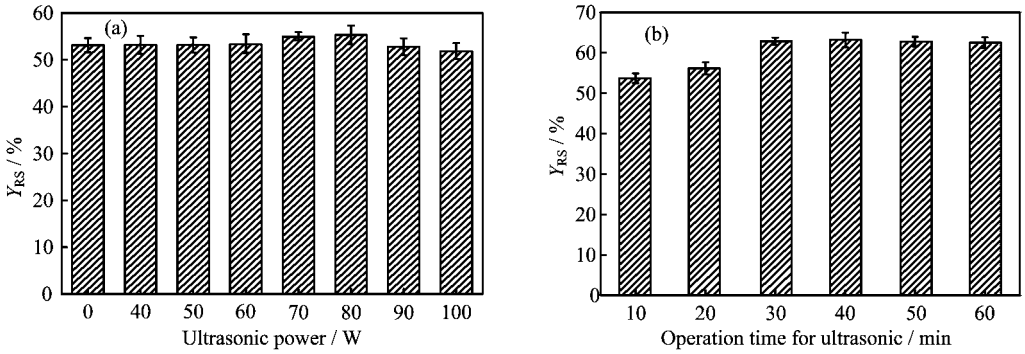

FIG. 3 Effect of ultrasonic treatment on the yield of reducing sugar. (a) Ultrasonic power. (b) Operation time of ultrasonic treatment.

in Fig.3(a), the yield of reducing sugar was almost constant (about 53.1%) when increasing the ultrasonic power. It indicated that ultrasonic power had little promotion on the cascade cellulase enzymatic hydrolysis of ultrafine sugarcane bagasse. When keeping the ultrasonic power of 50 W, increasing ultrasonic treatment time from 0 to 30 min resulted in the increase of reducing sugar yield from 53.1% to 63.8% (Fig.3(b)). Further increasing the ultrasonic treatment time did not increase the yield of reducing sugar. Under this condition, the yield of glucose, xylose, and fructose were 344, 90, and 22 mg/g bagasse, respectively. It implied that 30 min ultrasonic treatment promoted significantly the cascade cellulase enzymatic hydrolysis of ultrafine sugarcane bagasse to produce reducing sugar. The promotion role can be attributed to the fact that 30 min ultrasonic treatment promoted the contact between cellulase and ultrafine sugarcane bagasse particles, which improved the performance of enzymatic hydrolysis.

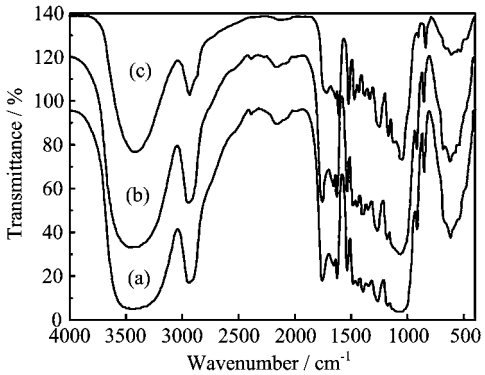

FIG. 4 FT-IR spectra of raw, pretreated and residual sugar bagasse. (a) Raw material of sugarcane bagasse, (b) the sugarcane bagasse by ultrafine grinding pretreatment for 50 min, and (c) the sugarcane bagasse residue by cascade enzymatic hydrolysis.

D. The structural analysis of cascade enzymatic hydrolysis sugarcane bagasse

Generally, the enzymatic hydrolyzed mixture was separated into filtrate (containing saccharides, Table II and Fig.2) and solid residue (38.64% of raw material) by filtration process. The structure of residue was analyzed as follows.

The FT-IR spectra of sugar bagasse with or without cascade enzymatic hydrolysis are shown in Fig.4, and the bands were assigned [29, 30]. The infrared band at about 1000−1200 cm^{-1} was ascribed to characteristic absorption band of the cellulose. The 1160

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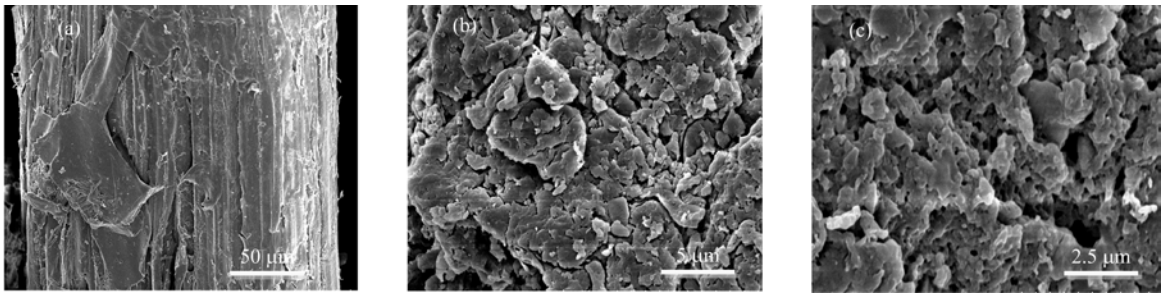

FIG. 5 SEM images of sugar bagasse with or without cascade enzymatic hydrolysis. (a) Raw material of sugarcane bagasse, (b) the sugarcane bagasse by ultrafine grinding pretreatment for 50 min, and (c) the sugarcane bagasse residue by cascade enzymatic hydrolysis.

and 1318 cm^{-1} peaks corresponded to C−O−C stretching vibration and -CH_2- flexural vibrations of the cellulose and hemicellulose. The peak appearing in the 1635−1640 cm^{-1} region corresponded to adsorption water flexural vibrations. It can be seen from Fig.4 that cellulose peak intensity was gradually weak after pretreatment and cascade enzymatic hydrolysis. The C=O stretching vibration of the cellulose and hemicellulose lead to obvious absorption peak at 1050 cm^{-1}, which indicated that ultrafine grinding operation caused cellulose to release from lignin and destroyed crystal structure of cellulose (Fig.4(b)). The absorption peak at about 1635 cm^{-1} was sharply weakened, indicating enzyme molecule penetrated cellulose molecule by cellulase treatment, leading to the content of absorbed water was decreased. Absorbed water was advantageous for cellulase operation (Fig.4(c)).

SEM image further showed that bagasse cellulose was breakdown after ultrafine grinding and the holes were formed on the solid surface of residues by cascade enzymatic hydrolysis (Fig.5 (b) and (c)). Compared with raw material (Fig.5(a)), the structure of the residues was loose, and no great changes in the basic framework were found. This phenomenon was presumed by present lignin ingredient (about 19.76%) in bagasse, nevertheless the cellulose and hemicellulose were completely digested, but lignin still existed.

IV. CONCLUSION

Compared with other enzymatic hydrolysis methods, cascade enzymatic hydrolysis coupling with ultrafine grinding pretreatment obtained high yield of sugars. In this process, cellulase I firstly degraded the cellulose by its ability of endo-acting and exo-acting, and following cellulase II abolished the inhibitory effect of cellobiose. And thus, the highest Y_{RS} of 65% with glucose selectivity of 90.1% was achieved under the optimized conditions.

V. ACKNOWLEDGMENTS

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