Appl Biochem Biotechnol (2008) 151:43–50
DOI 10.1007/s12010-008-8177-4

Sugarcane Bagasse Mild Alkaline/Oxidative Pretreatment for Ethanol Production by Alkaline Recycle Process

Ke-Ke Cheng · Jian-An Zhang · Wen-Xiang Ping · Jing-Ping Ge · Yu-Jie Zhou · Hong-Zhi Ling · Jing-Ming Xu

Received: 8 November 2007 / Accepted: 11 February 2008 /
Published online: 8 March 2008

Abstract In order to decrease the alkali and water consumptions in the sugarcane bagasse alkaline/oxidative pretreatment for ethanol production, an alkaline recycle process was carried out. Two recycles of $NaOH/H_2O_2$ pretreatment did not decrease the pretreatment and enzymatic hydrolysis efficiencies and the consumptions of NaOH and water would be saved by 26% and 40%, respectively. A simultaneous saccharification and fermentation (SSF) culture with pretreated bagasse as substrate was developed giving 25 g ethanol l^{-1} with a yield of 0.2 g g^{-1} bagasse and productivity of 0.52 g l^{-1} h^{-1}.

Keywords Bagasse · Cellulase · Enzymatic hydrolysis · Ethanol · Fermentation · Pretreatment

Introduction

Lignocellulosic biomass conversion to valuable products such as glucose and other simple fermentable sugars has been considered to be an attractive route for ethanol production. However, hydrolysis of cellulose to glucose is difficult, and some forms of pretreatment are necessary [1, 2]. The objective of pretreatment of biomass is to alter the structure of the lignocellulosic matrix to increase cellulose digestibility using cellulase, which can be done by removing lignin, hemicelluloses, or their combinations. To address this problem, several types of pretreatment of various lignocellulosic materials have been proposed, including steam explosion, acid hydrolysis, dilute alkali pretreatment, and wet oxidation. All of these processes enhanced the enzymatic digestibility of biomass to a certain extent [3–7]. Most of the processes reported, however, have several drawbacks. In many cases, considerable

K.-K. Cheng · J.-A. Zhang (✉) · Y.-J. Zhou · J.-M. Xu
Division of Green Chemistry and Technology, Institute of Nuclear and New Energy Technology, Tsinghua University, Beijing 100084, People's Republic of China
e-mail: zhangja@tsinghua.edu.cn

W.-X. Ping · J.-P. Ge · H.-Z. Ling
Key Laboratory of Microbiology, College of Life Sciences, Heilongjiang University, Harbin 150080, People's Republic of China

byproducts occur, or the delignification of the starting material is not satisfactory. Specialized equipment capable of withstanding high temperatures and pressures have also been required, which increase the energy consumption and costs of equipment. Furthermore, these processes still leave most of the lignin in the material and limit the complete bioconversion of cellulose to sugar.

Alkaline/oxidative has been recognized as a powerful oxidizing agent and is quite selective toward the lignin structure [8, 9]. The enzymatic digestibility of alkaline/oxidative pretreated biomass was effectively enhanced. Curreli found that alkaline/oxidative treatment could obviously decrease the crystallinity and lignin content of milled wheat straw and enhance its hydrolysis [10]. Krishma studied alkaline/oxidative pretreatment of sugarcane leaves and its simultaneous saccharification and fermentation (SSF) to produce ethanol. The pretreated samples had a greatly enhanced enzymatic digestibility. A 92% conversion of 2.5% substrate to sugar was achieved [11]. However, these processes needed too much alkali and water due to high liquid/solid ratio, leading to the increase of pretreatment cost. An increased concentration of dry matter would perhaps solve this problem, but a mixing problem was encountered, which adversely decreased the pretreatment and enzymatic hydrolysis efficiencies [12].

Among the various agricultural crop residues, sugarcane bagasse is the most abundant agricultural material in Southern China. In this study, sugarcane bagasse was chosen as the raw lignocellulosic material. An alkaline recycle process was carried out to decrease the alkali and water consumptions in the sugarcane bagasse alkaline/oxidative pretreatment for ethanol production. Their performances of pretreatment were evaluated by ethanol fermentation using pretreated bagasse by *Kluyveromyces marxianus* DW08.

Materials and Methods

Alkaline/Oxidative Pretreatment

Sugarcane bagasse from Guangxi province in Southern China was used as raw material. Particles in the size ranged from 0.45 to 0.9 mm (20–40 mesh) were used in the experiments. The compositions of sugarcane bagasse were determined according to corresponding Chinese National standards. The data were shown in Table 1 [13].

The pretreatment was carried out in 1,000-ml glass flasks shaken in a shaker at 160 rpm and 30 °C for 20 h. The biomass at a solid loading of 8% (*w*/*w*) was packed into the flasks, and certain volume of prepared $NaOH/H_2O_2$ solution was added. After pretreatment, the

Table 1 Compositions of sugarcane bagasse.

Component	Values (%)	Methods
Benzene-ethanol extractives (%, *w*/*w*)	3.2±0.08	GB/T 2677.6-1994
Cellulose (%, *w*/*w*)	42.7±0.37	Nitric acid-ethanol method
Holocellulose (%, *w*/*w*)	77.1±0.55	GB/T 2677.10-1995
Hemicellulose	34.3±0.46	Calculated value
Klason lignin (%, *w*/*w*)	17.5±0.41	GB/T 2677.8-1994
Acid-soluble lignin (%, *w*/*w*)	0.8±0.04	GB/T 747-2003
Total lignin(%, *w*/*w*)	18.2±0.33	GB/T 2677.8-1994, GB/T 10337-1989

The values were the mean of four independent samples. Cellulose content was determined by the nitric acid–ethanol method [13]. Hemicellulose is calculated by difference between hollocellulose and cellulose.

bagasse was washed with water until neutrality and dried at 105 °C for 4 h. The oven-dried samples were stored for further analysis and enzymatic hydrolysis.

Alkaline Recycle Process

The biomass at a solid loading of 8% (*w*/*w*) was suspended in a solution containing 1% NaOH and 0.6% H_2O_2 and pretreated in a shaker at 30 °C and 160 rpm for 20 h. The liquid fraction was separated by filtration and the unhydrolyzed solid residue was washed with 20 ml warm water (45 °C). The residues were collected for further analysis and enzymatic hydrolysis. The filtrate and wash liquid were pooled together and then used in the next stage alkaline/oxidative pretreatment. Before every stage subsequent to the previous one, the concentration of NaOH was determined by sulfuric acid titration with methyl orange as an indicator. The concentration of H_2O_2 was determined by sodium thiosulfate titration with KI and starch as indicators. The NaOH and H_2O_2 are reintegrated to keep the same concentration and liquid/solid ratio as those in the first stage.

Enzymatic Hydrolysis

The cellulase used in the experiment was Cellulase ZC-1700, which was produced by CTA-TEX Chemical Co. LTD in China. The cellulase activity was determined by the method recommended by Ghose [14], and expressed in filter paper units (FPU). One FPU was defined as the amount of enzyme capable of producing 1μmol of reducing sugars in 1 min.

Before the enzymatic hydrolysis, the cellulose content in the treated samples was determined. Then, the samples were digested by cellulase loading of 20 FPU g^{-1} cellulose. The enzymatic digestibility tests were conducted as follows: 50 °C, pH of 4.5 (0.1 M citrate buffer), 140 rpm in an air-bath shaker. The digestibility, denoted as conversion ratio of cellulose (CRC), was defined as the percentage of cellulose converted to glucose after 72 h of incubation with cellulase. The sugar yield was calculated assuming that 1 g of cellulose gives 1.11 g of glucose and 1 g of hemicellulose gives 1.13 g of pentose [12].

Microorganism and Fermentation Experiments

Kluyveromyces marxianus DW08 was grown on the preculture medium containing 5 g KH_2PO_4 l^{-1}, 2 g $(NH_4)_2SO_4$ l^{-1}, 1 g peptone l^{-1}, 3 g yeast extract l^{-1}, 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 50 g glucose l^{-1}. The pretreated bagasse, supplemented with 5 g KH_2PO_4 l^{-1}, 2 g $(NH_4)_2SO_4$ l^{-1}, 0.2 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 1 g peptone l^{-1}, 5 g yeast extract l^{-1}, 10 ml 0.1 M citrate buffer (pH 4.5), was used as the fermentation medium. The seed cells were prepared in 500 ml flasks containing 100ml preculture medium. The flasks were incubated at 35 °C for 14 h and subsequently inoculated into the fermentation flasks at 5% (*v*/*v*). The cellulase solutions were not sterilized and added with the inoculum. The SSF were conducted in 500 ml flasks with 200 ml working volume. All SSFs experiments were carried out at 40 °C and 140 rpm.

Analytical methods

The liquid samples were analyzed by HPLC, equipped with RI detectors. Glucose, xylose, arabinose, cellobiose, and glycerol and ethanol were analyzed using refractive index detector and Aminex HPX-87 H column at 65 °C with 5 mM H_2SO_4 as mobile phase at 0.8 ml min^{-1}.

Results

Evaluation of Pretreatment

Lignocellulosic biomass requires pretreatment, mainly because the lignin in the plant cell walls forms a barrier against enzyme attack. An ideal pretreatment reduces the lignin content and crystallinity of the cellulose and increases the surface area. In this study, different pretreatment methods were compared (Table 2). It can be seen that 0.6% H_2O_2 pretreatment only dissolved 11.9% of the raw materials and 12.9% of the original lignin. NaOH pretreatment dissolved 19.9% of the raw materials with a great removal of hemicelluloses; however, the residue still had a relatively high lignin content. Alkaline/oxidative pretreatment gave the highest degree of delignification with relatively low degradation of hemicelluloses. When the treated bagasse was digested by cellulase, the alkaline/oxidative treated sample gave the highest saccharification rate and CRC. The H_2O_2-treated sample only gave a CRC less than 20%. NaOH pretreatment could remove the most hemicelluloses, but the CRC was lower than that of alkaline/oxidative pretreatment. The enzymatic hydrolysis of bagasse treated with 1% NaOH and 0.6% H_2O_2 was found to be optimal and finally selected for the further study.

Sugarcane Bagasse Alkaline/Oxidative Pretreatment

Five stages of alkaline/oxidative recycle treatments were carried out. The compositions of the pretreated bagasse and the consumptions of NaOH and H_2O_2 in each stage were showed in Table 3. It must be emphasized that this pretreatment only altered cellulose lightly, allowing its high recovery (about 95%). An interesting phenomenon was observed that in each stage the consumption of NaOH was almost the same (about 60%), and the consumption of H_2O_2 was almost entire except in the first stage. The highest hemicellulose and lignin removals were seen in the second stage and then with the increase of recycle stage, the contents of hemicellulose and lignin in solid residue increased, which indicating the removals of hemicellulose and lignin decreased gradually (Table 3). An obvious decrease in pretreatment efficiency was observed at the third recycle treatment (the fourth stage) and only 61% of lignin was removed in the solid residue. The results of enzymatic

Table 2 Compositions of sugarcane bagasse pretreated by different methods and their enzymatic hydrolysis.

	Untreated	Pretreatment methods			
		0.6% H_2O_2	1% NaOH	1% NaOH+0.3% H_2O_2	1% NaOH+0.6% H_2O_2
Dissolved (%)	0	11.9±0.4	19.9±0.5	22.4±0.3	26.6±0.3
Cellulose content (%)	42.7±0.4	48.3±0.3	52.7±0.8	53.3±0.4	55.8±0.6
Cellulose removed (%)	0	0.3±0.01	2.4±0.03	3.5±0.06	4.2±0.2
Hemicellulose (%)	34.3±0.5	38.3±0.3	31.0±0.5	33.0±0.9	36.5±0.3
Hemicellulose removed (%)	0	1.3±0.02	27.7±1.8	25.4±1.4	21.5±0.9
Total lignin (%)	18.2±0.3	17.9±0.4	10.0±0.7	8.2±0.3	7.2±0.3
Lignin removed (%)	0	12.9±0.6	55.6±0.3	65.5±1.7	70.1±1.6
CRC (%)	6.3±0.3	18.6±1.1	55.2±0.7	70.2±1.0	78.1±1.2

The enzymatic digestibility tests were conducted as follows: pretreated bagasse loading of 4%, 50 °C, pH of 4.5, cellulase loading of 20 FPU g^{-1} cellulose, 140 rpm. The values were the means of three independent samples.

Table 3 Compositions of pretreated bagasse, $NaOH/H_2O_2$ consumption in alkaline recycle process and enzymatic hydrolysis.

Stage	Composition (%)			Consumption (%)		CRC (%)
	Cellulose	Hemicellulose	Lignin	NaOH	H_2O_2	
2	55.9±0.9	35.3±0.2	7.1±0.3	58.5±0.1	92.2±0.1	79.1±1.1
3	55.1±0.4	35.8±0.9	7.2±0.2	61.1±0.3	98.5±0.1	78.0±1.2
4	54.7±0.7	36.4±0.3	8.0±0.6	58.8±0.2	98.6±0.2	70.4±0.2
5	53.9±1.5	36.8±0.5	9.2±0.4	59.3±0.3	99.1±0.3	58.5±0.9

The enzymatic digestibility tests were conducted as follows: pretreated bagasse loading of 4%, 50 °C, pH of 4.5, cellulase loading of 20 FPU g^{-1} cellulose, 140 rpm. The values were the means of three independent samples.

hydrolysis of pretreated bagasse in different stages were shown in Table 3 and Fig. 1. It was clear that the untreated bagasse was hard to be digested. Nearly no more sugar was formed after 12 h and the CRC was only 6.3% (Table 2). However, the enzymatic digestibility of pretreated bagasse was greatly enhanced and a relatively high saccharification rate was maintained in the first 36 h, and no obvious variance was found among the first, second, and third stage pretreated bagasse. So two recycles of alkaline/oxidative treatments could be reasonable. Compared with NaOH consumption of 0.125 g g^{-1} bagasse in the single stage alkaline/oxidative pretreatment, the consumption of NaOH in two recycles of alkaline/oxidative treatments decreased to 0.092 g g^{-1} bagasse, saved by 26%. At the same time, the consumption of water decreased to 7.5 g g^{-1} bagasse, saved by 40%.

Enzymatic Hydrolysis

Single-factor experiments were performed to investigate the effects of enzyme concentration, substrate concentration, and temperature on the sugar yield in enzymatic hydrolysis. In these experiments, pretreated bagasse in the third stage were used. It can be concluded from Fig. 2a that the sugar yield increased with increasing enzyme concentration. However, with the enzyme loading increasing from 20 to 30 FPU g^{-1} cellulose, the sugar yield only increased by 3%. Considering the enzyme cost, we chose the enzyme loading of 20 FPU g^{-1} cellulose in others enzymatic hydrolysis experiments. Low substrate loading also increased the sugar

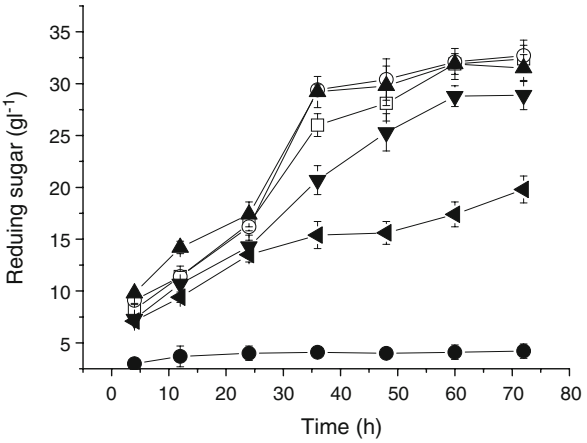

Fig. 1 Time course of enzymatic hydrolysis with different stage substrates in alkaline recycle process: bagasse in the first stage (*open square*), bagasse in the second stage (*open circle*), bagasse in the third stage (*filled triangle*), bagasse in the fourth stage (*inverted filled triangle*), bagasse in the fifth stage (*arrowhead*), bagasse untreated (*filled circle*). The values were the means of three independent samples. The reducing sugar is the sum of glucose, xylose, and arabinose

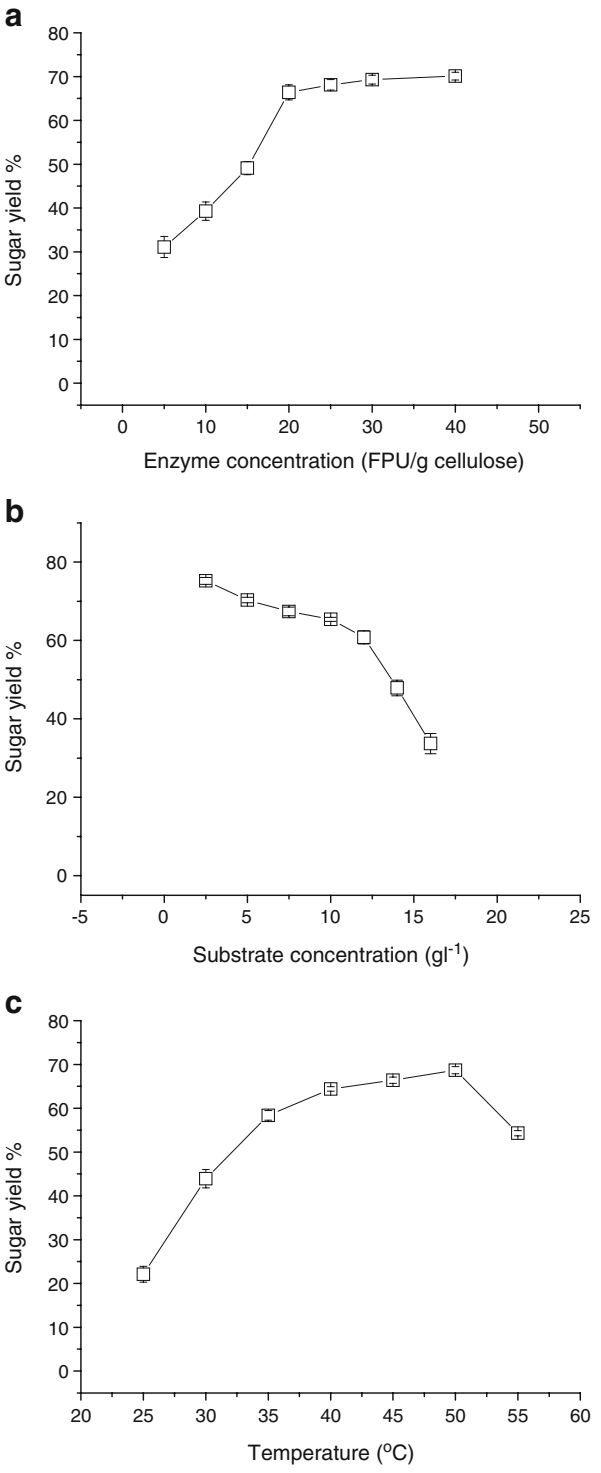

Fig. 2 The effects of cellulase loading, substrate concentration, temperature on sugar yield in enzymatic hydrolysis. The enzymatic hydrolysis conditions were: **a** pretreated bagasse loading of 4%, 50 °C, pH of 4.5, 140 rpm; **b** cellulase loading of 20 FPU g^{-1} cellulose, 50 °C, pH of 4.5, 140 rpm; **c** pretreated bagasse loading of 4%, cellulase loading of 20 FPU g^{-1} cellulose, pH of 4.5, 140 rpm. The values were the means of three independent samples. The reducing sugar is the sum of glucose, xylose, and arabinose

yield (Fig. 2b), but its drawbacks were low concentrations of sugars for fermentation. To maximize the sugar concentration, the substrate concentration was increased to 140 g l^{-1}, but then, a sharp decrease of sugar yield was observed. The sugar yield increased with an increase in temperature from 35 to 50 °C. A lower sugar yield could be obtained when a higher temperature was used. Normally, the optimal culture temperature for yeast was 30–40 °C. So in the further SSF studies, the fermentation was conducted at 40 °C.

SSF

The fermentations were performed under nonaseptic conditions with uncontrolled pH starting at 4.5 at the beginning of the fermentation and dropping steadily to 4 at the end. The loading of cellulase was 20 FPU g^{-1} cellulose. In this study, *K. marxianus*, a thermotolerant yeast, was used. It was capable of growth and producing ethanol with a high yield at 40 °C. However, a significant amount of pentoses remained unmetabolized. Figure 3 presents ethanol production after 48 h of SSFs with pretreated bagasse at a solid loading of 10 % (*w*/*w*). The ethanol concentration in the broth reached its highest value of 25 g l^{-1}. No obvious variance in ethanol production was found among the first, second, and third stage pretreated bagasse.

Discussion

In order to study the effectiveness of alkaline/oxidative pretreatment, we compared it with H_2O_2 or NaOH pretreatment under the same conditions. The experimental results indicated that in alkaline/oxidative pretreatment, the enzymatic digestibility was increased due to removal of hemicelluloses and lignin, and removal of lignin was more helpful for enzymatic hydrolysis than removal of hemicelluloses. It was found that reasonable alkaline recycle process to have no or negative effect on the enzymatic hydrolysis efficiencies and overall ethanol yield, and the consumption of alkali would be saved by 26%. Furthermore, the alkaline recycle process was helpful to decrease the water consumption at the same time.

The highest 25 g ethanol l^{-1} was reached in the SSF with 10% pretreated substances. This concentration corresponded to 79.4% ethanol yield based on the glucose content in the raw material. However, if the xylose and arabinoise presented in the broth at the end of the

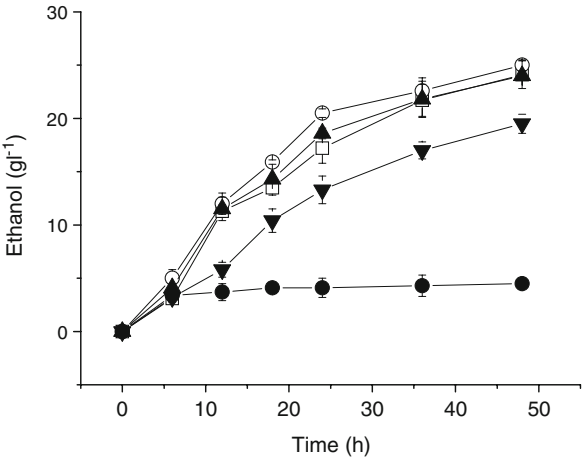

Fig. 3 Time course of SSF with different stage substrates in alkaline recycle process: bagasse in the first stage (*open square*), bagasse in the second stage (*open circle*), bagasse in the third stage (*filled triangle*), bagasse in the fourth stage (*filled inverted triangle*), bagasse untreated (*filled circle*). The values were the means of three independent samples

SSF could be fermented to ethanol, another 8.6 g ethanol l^{-1} could theoretically be produced (0.51 g ethanol/g pentose). When the substrate concentration is higher than 10%, a substrate saturation limit is reached which leads to a mixing problem and further limits the activity of the enzymes. Similar trends were observed by Varga [12]. In view of the high cost of ethanol recovery from broth, an economical production of ethanol from bagasse requires the improvement of both product concentration and productivity, so a fed-batch SSF procedure was in progress to solve this problem.

Acknowledgement This study was supported by the National Basic Research of China (2004CB719703), State 863 Program (2007AA100702) and Open Fund of Key Laboratory of Microbiology, College of Life Sciences, Heilongjiang University.

References

1. Duff, S. J. B., & Murray, W. D. (1996). Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. *Bioresource Technology*, *55*, 1–33.
2. Wyman, C. E. (1999). Biomass ethanol: technical progress, opportunities, and commercial challenges. *Annual Review of Energy and the Environment*, *24*, 189–226.
3. Grethlein, H. E. (1991). Common aspects of acid prehydrolysis and steam explosion for pretreating wood. *Bioresource Technology*, *36*, 77–82.
4. Kaar, W. E., Gutierrez, C. V., & Kinoshita, C. M. (1998). Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol. *Biomass & Bioenergy*, *14*, 277–287.
5. Saha, B. C., Iten, L. B., Cotta, M. A., & Wu, Y. V. (2005). Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. *Biotechnology Progress*, *21*, 816–822.
6. Martín, C., Klinke, H. B., & Thomsen, A. B. (2007). Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. *Enzyme and Microbial Technology*, *40*, 426–432.
7. Playne, M. J. (1984). Increased digestibility of bagasse by pretreatment with alkalis and steam explosion. *Biotechnology and Bioengineering*, *26*, 426–433.
8. Gould, J. M. (1984). Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. *Biotechnology and Bioengineering*, *24*, 46–52.
9. Mishima, D., Tateda, M., Ike, M., & Fujita, M. (2006). Comparative study on chemical pretreatments to accelerate enzymatic hydrolysis of aquatic macrophyte biomass used in water purification processes. *Bioresource Technology*, *97*, 2166–2172.
10. Curreli, N., Fadda, M. B., Rescigno, A., Rinaldi, A. C., Soddu, G., Sollai, F., Vaccargiu, S., Sanjust, E., & Rinaldi, A. (1997). Mild alkaline/oxidative pretreatment of wheat straw. *Process Biochemistry*, *32*, 665–670.
11. Krishna, S. H., Prasanthi, K., Chowdary, G. V., & Ayyanna, C. (1988). Simultaneous saccharification and fermentation of pretreated sugar cane leaves to ethanol. *Process Biochemistry*, *33*, 825–830.
12. Varga, E., Klinke, H. B., Reczey, K., & Thomsen, A. B. (2004). High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. *Biotechnology and Bioengineering*, *88*(5), 567–574.
13. Shi, S. L. (2003). *Analysis and detection of pulping and papermaking* (1st ed.). Beijing, China: Chinese Light Industry Press.
14. Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and Applied Chemistry*, *59*, 257–268.