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Impacts of the Combined Pretreatment Using NaOH and Ozone on Enzymatic Hydrolysis and Morphology of Rice Straw

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The combined pretreatment of rice straw using NaOH and ozone followed by enzymatic hydrolysis was investigated in comparison with NaOH pretreatment. In order to measure the composition variance, the surface morphology of rice straw was investigated by the scanning electron microscopy. The combined pretreatment of rice straw increased the specific surface area and total pore volume, which could degrade the lignin polymer, solubilize hemicellulose and neutral detergent solubles. During the combined pretreatment of rice straw using NaOH and ozone, no measurable quantity of furfural and hydroxymethyl furfural has been detected. These chemical and morphological changes of rice straw lead to the increased access of cellulase to rice straw surface and the enhanced susceptibility to enzymatic hydrolysis. The conversed percentage of cellulose and hemicelluloses presented in combined pretreated rice straw is 92.6 %, while those of NaOH pretreated and untreated rice straw are 73.8 % and 52.5 %, respectively.

Key Words: Rice straw, Ozone, Pretreatment, Enzymatic hydrolysis, Morphology.

INTRODUCTION

The ever growing interest in renewable energy sources is a result of many factors, including energy consumption increase, petroleum price instability and environmental concerns because of the increased greenhouse gas emission[1]. Replacing fossil fuels with other renewable and less polluting alternatives is the target set out in the European White Paper "European transport policy for 2010: time to decide", published in 2001 and subsequently endorsed by Directive 2003/30/CE that established a reference value of a 5.75 % market share for biofuels by 2010[2]. The United States government approved the energy independence and security act of 2007 (EISA) that mandated the production of 21 billion gallons of advanced biofuels by 2022, of which 16 billion gallons must be derived from lignocellulosic feed stocks[1]. Sustainable production of economically viable biofuels, such as bioethanol which can be produced from lignocellulosic materials, are considered to be one of the most suitable alternatives[3-5].

The production of bioethanol from lignocellulosic materials usually requires three steps. The first step is that an efficient depolymerization to convert cellulose and hemicellulose to soluble sugars by enzymatic hydrolysis is conducted. The second step is to carry out the fermentation of mixed-sugar-hydrolysates containing hexoses and pentoses, whereas the last step is the filtration and distillation, leading to the yield of 99 % ethanol. Prior to the enzymatic hydrolysis of lignocellulosic materials, such as rice and wheat straw, pretreatments of the materials are often necessary due to the stringent structure of β-(1,4)-linkages in cellulose and close association of lignin with cellulose and hemicellulose, leading to limited reactive sites available for enzyme attachment[6-9]. These pretreatments are to modify lignocellulosic structure, solubilize and/or degrade its different components and open up the crystalline structure in cellulose. Different approaches of lignocellulose pretreatment involve the use of physical, chemical, physico-chemical and/or biological methods, *e.g.* milling and grinding[10], steam explosion[3,11], hot water extraction[12-14], sulfuric acid[15-18], sodium hydroxide[19], hydrogenperoxide[20], organosolv[21], ozonolysis[2,22] and wet oxidation[6,9]. Pretreatments of lignocellulosic materials should not produce inhibitory compounds such as furfural and hydroxymethylfurfural from the carbohydrates, which would be a problem in the fermentation step[6,20].

Moreover, rice straw has attracted great interest in Asian countries as a potential source for bioethanol production[10,23,24]. Rice straw consists of heterogeneous complex of carbohydrate polymers. Cellulose and hemicellulose are densely packed by layers of lignin, which prevent them from enzymatic hydrolysis. Therefore, it is necessary to employ a pretreatment process to break down ligninseal and make cellulose and hemicellulose exposed for enzymatic reactions.

In this study, we propose ozonization as an effective chemical pretreatment approach and investigate its impacts and origins in details. Ozone is a powerful oxidant, which is soluble in water and can react with most organic compounds. In recent years, ozone has been used in the pretreatment process for enzymatic hydrolysis because ozone generation is cheaper and ozonolysis has shown to be efficient in degrading lignin polymers[2,21,22]. Lignin can be easily oxidized by ozone to form soluble compounds of less molecular weight, mainly organic aids such as formic acid and acetic acid[25]. The main advantage of ozonolysis pretreatment don't yield any degradated products[2]. Herein, the combined pretreatment of rice straw using NaOH and ozone was proposed and its impact on enzymatic hydrolysis and morphology of rice straw was investigated in details compared with NaOH pretreatment.

EXPERIMENTAL

Rice straw: Rice straw used in the experiments was harvested at maturity in October 2007 from Hunan Agricultural University experimental farm, P.R. China. It was first cut to 1-2 cm by hand and washed thoroughly with water until the filtration was clean and colourless and then dried at 60 ºC for 36 h. Then the dried straw was milled in a mini-type plant mill to pass through a 0.85 mm screen. The milled rice straw was stored in a sealed plastic bag at room temperature. The chemical compositions of rice straw are shown in Table-1.

TABLE-1
WEIGHT LOSS AND COMPOSITION OF RICE STRAW AFTER PRETREATMENT

Component	Dry solids (%, w/w)		
	Untreated rice straw	NaOH pretreated rice straw	Combined pre-treated rice straw
Cellulose	40.30	65.82	70.18
Hemicellulose	25.10	19.89	17.37
Lignin	4.87	2.62	2.56
Ash	3.41	0.66	0.68
NDS	26.32	11.01	9.21
Weight loss		41.70	45.60

NDS = Neutral detergent fibre

Pretreatments: NaOH pretreatment procedure was as follows: the milled rice straw was mixed with 2 % NaOH solution (8.3 %, w/v) and shaken by an incubator at 140 rpm at 30 ºC for 24 h. Then, the suspension was filtered and neutralized by washing with deionized water. Afterwards, the pretreated straw was dried at 60 ºC for 24 h and weighed. The combined pretreatment was shown as follows: after the NaOH treatment for 24 h, ozonolysis pretreatment was performed by continuously sparging ozone through the suspension for 4 h. Ozone was generated *in situ* by passing 1 L/min of oxygen through an ozonator. Finally, the suspension was filtered, washed and dried as described above. All three kinds of materials (including untreated rice straw) were used as substrates for cellulase.

Enzymatic hydrolysis: The cellulase enzyme used in this study was a commercial cellulase (E.C.3.2.1.4, from *trichodermaviride*) purchased from Sinopharm Chemical Reagent Co. Ltd., Prior to use, 1 g of cellulase preparation was dissolved in 200 mL of 20 mmol/L acetate buffer at pH 5.0 and 1:50 000 *N*-cetylpyridinum chloride was added to prevent microbial growth. Cellulase activity was 15 international units per mg of cellulase preparation. Enzymatic hydrolysis of rice straw was carried out in an incubator by shaking at 165 rpm at 45 ºC after adjusting the pH to 5.0 with HCl and adding cellulase prepared (using 6.2 mL cellulase preparation per gram of substrate). The hydrolysis lasted for 120 h. Reducing sugar was determined on samples drawn at different time intervals. The enzymatic hydrolysis experiments were performed in three replicates.

Scanning electron microscopy analysis: Scanning electron microscopy (SEM) analysis was conducted to analyze the micro structural changes on the rice straw samples pretreated by the integrated process of NaOH and ozone. The dried samples (including the untreated and pretreated rice straw) were coated with gold and then evaluated using SEM (JSM-6360LV, Jeol, Japan) at a voltage of 20 kV.

UV/VIS-spectra: The UV/VIS spectra and their first derivative were measured for pretreated solutions using an UV-2450 UV-VIS-spectrophotometer (SHIMADZU, Japan).

Analytical Methods: Hemicellulose, cellulose and lignin contents of milled rice straw and filter cakes were determined according to Van Soest's method by FIWE3 (made in Italy). The content of hemicellulose was estimated by the difference between neutral detergent fibre (NDF) and acid detergent fibre (ADF) and that of cellulose as the difference between acid detergent fibre and acid detergent lignin (ADL). The lignin content was estimated by the difference between acid detergent lignin and ash content. The ash content was determined in an oven at 550 ºC over 6 h. The total reducing sugar amount was ascertained by the 3,5-dinitrosalicylic acid reagent with glucose as the standard. Furfural and hydroxymethylfurfural were analyzed by high performance liquid chromatography (Agilent 1200 HPLC, VWD, Alltech Model 3300ELSD,XDB-C_{18}). Peaks were identified by comparison with the retention time of their standards.

RESULTS AND DISCUSSION

Impacts of pretreatment on composition and weight loss of rice straw: The results, expressed as weight loss, of the composition of cellulose, hemicellulose, lignin, ash, neutral detergent solubles in the NaOH pretreated rice straw and the combined pretreated rice straw are summarized in Table-1. The lost ratio of these components after pretreatment is presented in Fig. 1. It is found that pretreated results are both increase of cellulose and decrease of hemicellulose, lignin and neutral detergent solubles contents. The content of cellulose in the combined pretreated rice straw is higher than that in the NaOH pretreated rice straw, with the difference of about 4 % (70.18 % *vs*. 65.82 %). The content of hemicellulose and neutral detergent solubles in the combined pretreated rice straw is slightly lower than that in the NaOH pretreated rice straw. The loss ratio of cellulose is quite low, whereas loss ratios of lignin, hemicellulose and neutral detergent solubles are significantly higher. It is well known that many factors, like lignin content, crystallinity of cellulose and the specific surface area, markedly affect enzymatic hydrolysis of lignocellulosic biomass. We cannot simply distinguish which pretreatment of the two is better based on the content and loss ratio of lignin. Comparing the two pretreatments, we find that there is no much difference between the combined pretreatment and the NaOH pretreatment in the content and the loss ratio of lignin, but there are considerable differences between the two kinds of pretreatment on the weight loss of rice straw and loss ratios of hemicellulose and neutral detergent solubles. The weight loss of rice straw after the combined pretreatment is higher than that after the NaOH pretreatment and the loss ratios of hemicellulose and neutral detergent solubles have the same tendency, indicating that the combined pretreatment is more propitious to hemicellulose and other soluble substances in rice straw. Based on this, we can see that the combined pretreatment is better than NaOH pretreatment in decreasing polymerization of lignocellulosic materials, increasing the specific surface area for enzymatic reactions and thus enhancing the susceptibility to enzymatic hydrolysis. This point will be confirmed by the enzymatic saccharification and SEM results of pretreated rice straw below.

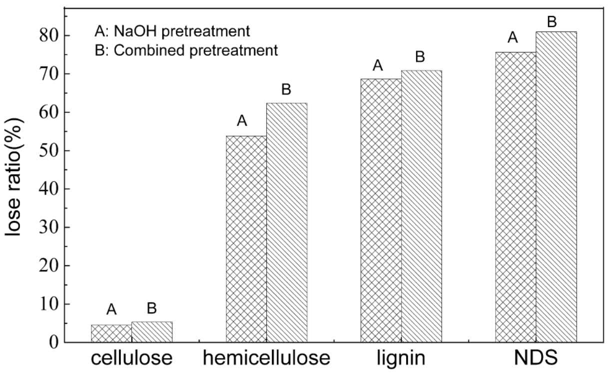

Fig. 1. Loss ratioof rice straw componentafter pretreatment

Effect of duration of enzymatic hydrolysis on saccharification of rice straw: Fig. 2 shows the time profile of enzymatic hydrolysis for the three categories of rice straw. The yield of the resultant sugar is dependent of the reaction time of enzymatic hydrolysis. The reducing sugar yield increases as the duration of enzymatic hydrolysis increases. There are some differences in the behaviour of the time profiles among the three kinds of rice straw. The reducing sugar amount from the pretreated rice straw increases rapidly within the first 36 h and then the pace slows down up to 120 h. The growth rate of the reducing sugar from the combined pretreated rice straw is higher than that of the NaOH pretreated rice straw. The reducing sugar from the untreated rice straw is found to increase rather slowly from beginning all the way to 120 h. The sugar yield from the combined pretreated rice straw and enzymatic saccharification (45 ºC, pH 5.0,120 h) using 6.2 mL enzyme preparation per gram of substrate is 902 mg/g of pretreated rice straw, suggesting that the conversion yield is 92.6 % of the total cellulose and hemicellulose presented in the pretreated rice straw. However, under the same enzymatic hydrolysis conditions, the reducing sugar yield from the NaOH pretreated rice straw is only 705 mg per gram of the pretreated rice straw (*i.e.*, 73.8 % of the conversion yield). The sugar yield from the untreated rice straw is only 382 mg/g of rice straw, corresponding the conversion rate of 52.5 %.

SEM of rice straw: Fig. 3a and 3b show the SEM images of the outer and internal surfaces of untreated rice straw, respectively. As observed, the outer surface of the untreated rice straw is coated tidily with silica cells, suberized cells and silica swelling. The internal surface of the untreated rice straw is covered with cutin and waxes. The untreated rice straw shows a flat, smooth and continuous surface with very little specific surface areas available for enzymatic hydrolysis. Fig. 4a and 4b display the SEM of the outer and internal surfaces of the NaOH pretreated rice straw, respectively. Fig. 5a and 5b display the SEM of the outer and internal surfaces of the combined pretreated rice straw, respectively. For comparison, it can be found that the original materials, such as silica cells and suberized cells, coated on the outer surface have been entirely removed after pretreated by the integrated process of NaOH and ozone and the mechanical tissue of rice straw was fully exposed (Fig. 5a). The inner substances of thin-walled cells were completely lost, causing cell walls to become loosened and the porosity increased (Fig. 5b). However, after pretreated by NaOH, parts of the coverings coated on the outer surface of rice straw were still remained (Fig. 4a). The inner substances of thin-walled cells are partially lost (Fig. 4b). As a result, the effective specific surface areas and total pore volumes of rice straw were substantially increased by the combined pretreatment, thus making the enzymatic saccharification significantly accelerated.

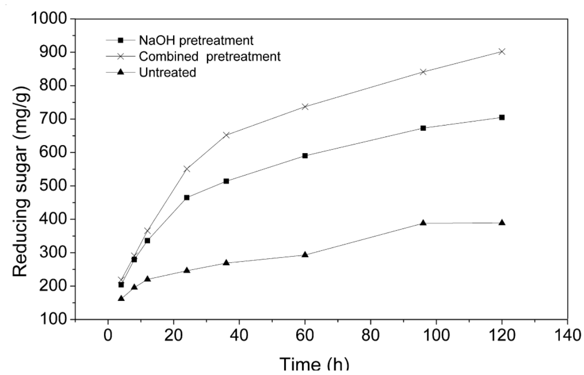

Fig. 2. Time course of enzymatic hydrolysis of three kinds of rice straw

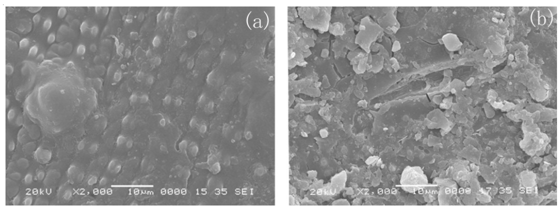

Fig. 3. SEM of the untreated rice straw. (a) The outer surface of the untreated rice straw. (b) The internal surface of the untreated rice straw

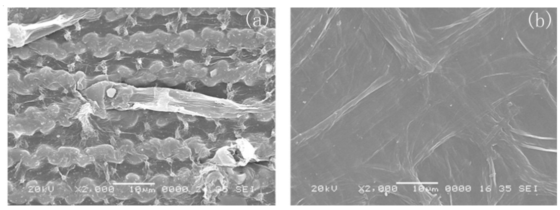

Fig. 4. SEM of the NaOH pretreated rice straw. (a) The outer surface of the NaOH pretreated rice straw. (b) The internal surface of the NaOH pretreated rice straw

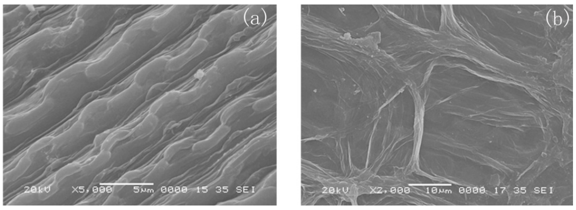

Fig. 5. SEM of the combined pretreated rice straw. (a)The outer surface of the combined pretreated rice straw. (b) The internal surface of the combined pretreated rice straw

Furfural and hydroxymethylfurfural analysis: A critical concern in the fermentation of the resulted sugar is the inability of the fermentative microorganism to withstand inhibitory compounds formed during pretreatment. A detoxification step is often required to improve ferment ability. In the UV/visible-spectra (Fig. 6a) of the combined pretreated rice straw solution, we only observed a maximum absorption peak at 199 nm. This peak is assigned to the absorbance of carboxyl group. The first derivative of UV/visible-spectra is more informative in assigning the chromophore composition. The first derivative of UV/visible-spectra (Fig. 6b) of the combined pretreated rice straw solution shows that no other chromophore exists in addition to carboxyl group in the solution, indicating that there is no furfural and hydroxymethylfurfural in the combined pretreated rice straw solution. This result was further confirmed by HPLC analysis. The detectable limit of both furfural and hydroxymethylfurfural was 1 µg/mL. These results suggest that there exists no inhibitor problem in the combined pretreatment of rice straw.

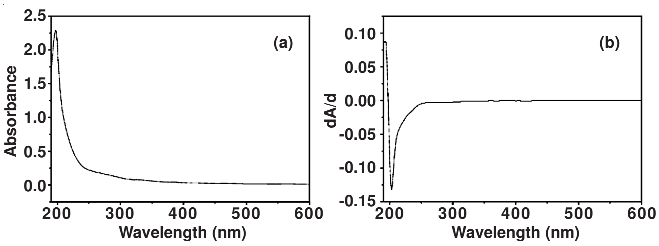

Fig. 6. (a) UV/VIS-spectra of the combined pretreated rice straw solution. (b) The first derivative spectra

Mechanism of treatment: O_3, as a kind of super oxidant, has strong oxidate, decomposite and osmotic abilities[20]. In the process of the pretreatment of lignocellulosic materials, O_3 can permeate into the plant cells and break down the structure of cell walls, which can cause the decomposition of the lignin, from C=C structure to C-C bond, detach the glucose from the side chain of hemicelluloses, so that it can reduce the content of lignin and hemicellulose in cell walls. Under the alkaline medium, the free-radical reaction is taking place: O_3, under the alkaline condition, releases free radicals, namely HO·, HO_2·, O_x^-·, which possess of strong oxidablity. Moreover, it is the free radicals that permeate into the plant cells, break down the structure of cell walls and finally decompose the lignin and hemicelluloses. The schematic of mechanism as showed in Fig. 7.

$$O_3 + OH^- \rightarrow HO_2\cdot + \cdot O_2;\ O_3 + OH^- \rightarrow HO_3\cdot + \cdot O_2^-$$
$$O_3 + \cdot O_2^- \rightarrow \cdot O_3^- + O_2;\ O_3 + HO_2\cdot \rightarrow HO\cdot + 2O_2$$
$$O_2^- + HO\cdot \rightarrow O_2 + OH^-$$

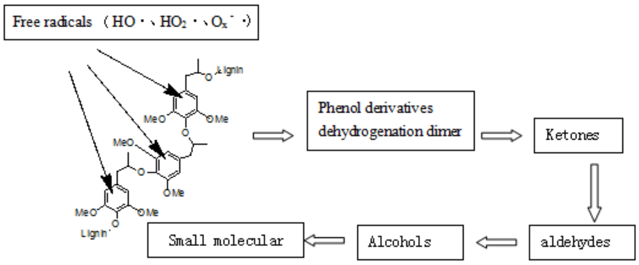

Fig. 7. Schematics of mechanism using O_3

Normally, the lignocelluloses in rice straw can hardly be take advantage as the raw material for fodder, fertilizer, or primary material of bio-ethanol (except for those was utilized by ruminant animals in low efficiency). However, most of the lignin and hemicelluloses in rice straw can be decomposed by O_3/NaOH pretreatment, which can hardly affect the cellulose. Hence, we present the O_3/NaOH pretreatment technology in this paper, hoping that it will break through the technical bottleneck of biological degradation of lignocellulose, solve the key problems in lignocelluloses degradation and accelerate the development of bioethanol industry.

Conclusion

To summarize, our present study demonstrated that the combined pretreatment using NaOH and ozone was an efficient approach for rice straw. The combined pretreatment induced morphological changes. In particular, we found that the untreated rice straw showed a flat, smooth and continuous surface, whereas the combined pretreated rice straw had a porous, rugged and unstructured surface. During the combined pretreatment, no furfural and hydroxymethylfurfural byproducts were produced. The removal of lignin, hemicellulose and neutral detergent solubles led to the increased surface areas and speed-up of the enzymatic hydrolysis. The conversion yield of cellulose and hemicellulose presented in the combined pretreated rice straw was 92.6 %, compared to 73.8 % and 52.5 % of the NaOH pretreated and untreated rice straw, respectively.

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