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Two-stage pretreatment with alkaline sulphonation and steam treatment of *Eucalyptus* woody biomass to enhance its enzymatic digestibility for bioethanol production

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ABSTRACT

This work proposed a two-stage pretreatment with alkaline sulphonation and steam treatment, and investigated its efficiency for converting *Eucalyptus* woody biomass to fermentable sugars and bioethanol. Comparing with steam pretreatment and subsequent sulphonation, this alkaline sulphonation-steam pretreatment improved carbohydrate recovery by maintaining a more neutral pH throughout the pretreatment process, while promoting the enzymatic digestibility of biomass through lignin removal and modification. Results showed that the alkaline sulphonation-steam pretreatment caused lignin removal of 69.37% and 120.28 mmol/kg acid groups incorporation into substrate, both of which could lead to significantly improved cellulose accessibility. About 80% of the sugars present in the original carbohydrate (cellulose and hemicellulose) were released, which could be recovered after pretreatment and enzymatic hydrolysis. The sugars released from enzymatic hydrolysis of substrate pretreated by alkaline sulphonation-steam pretreatment could be efficiently converted to ethanol, indicating that alkaline sulphonation-steam two-stage pretreatment is a promising pretreatment approach of lignocellulosic biomass for the production of biofuels.

1. Introduction

Biorefinery means the process to utilize lignocellulosic biomass for production of a wide range of fuels, chemicals and materials [1]. Among a large variety of lignocellulosic biomass, *Eucalyptus* is proposed to be a promising resource for biorefinery due to its fast growth, wide distribution and low cost [2]. However, the complex structure of cellulose-lignin-hemicellulose in raw biomass makes it recalcitrant towards enzymatic degradation to obtain fermentable sugars for downstream bioconverion. Therefore, proper pretreatment methods are needed to mitigate the complexity of lignocellulosic structure, and to maximize sugar production in subsequent enzymatic hydrolysis [3,4].

In the past two decades, various pretreatment methods have been proposed and investigated. Among them, steam pretreatment has been widely studied, due to a number of merits such as environmental friendliness, low energy requirement and high effectiveness for a large variety of lignocellulosic biomass [5]. In a typical steam pretreatment, the high temperature steam causes the release of acetyl groups to form acetic acid, mainly catalyzing hemicellulose solubilization. As a result, the enzymatic digestibility of biomass is increased. However, after steam pretreatment, the lignin content in water-insoluble fraction is higher than that in untreated biomass due to hemicellulose solubilization, which may adversely affect subsequent enzymatic hydrolysis [6,7]. Moreover, recent studies have shown that the lignin repolymerization during steam pretreatment aggravates inhibiting effects of lignin on enzymatic hydrolysis, primarily through intensive non-productive adsorption of cellulase enzymes [4,8].

To mitigate the limitation imposed by lignin, various second-stage pretreatments are applied on steam-treated pulps prior to enzymatic hydrolysis, including the use of alkali, hydrogen peroxide, oxygen delignification and sulphonation, etc., in order to remove or modify lignin [9–12]. Alkali second-stage pretreatments are commonly used, as they can remove lignin and increase biomass accessibility [11,13]. Besides, lignin modification, in which the chemical structure of lignin is

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modified, has also been proved as effective as lignin removal in enhancing the hydrolysis yield of steam pulps [4,14]. For instance, the acid groups incorporation by sulphonation has the potential to decreased non-productive adsorption of enzymes to residual lignin, resulting in improved ease of enzymatic hydrolysis [14].

Various two-stage pretreatments with different configurations have been assessed, aiming to maximize biomass saccharification [15–20]. It has been suggested that the sequence of two-stage pretreatment may also play a significant role on the enzymatic digestibility of biomass. For instance, the alkali-acid two-stage pretreatments have been reported to be much more effective than the acid-alkali two-stage pretreatments in terms of enhancing biomass saccharification and improving sugars recovery [18,21]. It is proposed that the alkaline first-stage pretreatment mostly leads to lignin removal, resulting in enhanced substrate porosity [13,19]. The enhanced porosity has a potential to raise the effectiveness of acidic or acid second-stage pretreatment that primarily causes hemicellulose solubilization [19,20].

In this study, two-stage pretreatment based on steam-sulphonation or sulphonation-steam was performed on *Eucalyptus* woody biomass. Initially, we investigated the influence of each strategy on enzymatic hydrolysis and total sugar yields. For each scenario, the impact of steam pretreatment severity on total sugar yield was also taken into consideration. Then, a comprehensive comparison between these two strategies was carried out. Through the comparison, we tried to figure out the role of lignin repolymerization during steam pretreatment in substrates' properties and total sugar yield. Finally, sodium bicarbonate was supplemented in the first-stage sulphonation pretreatment, in order to further enhance delignification selectivity and sugar yield from biomass. The fermentability of the resulting enzymatic hydrolysate was also evaluated.

2. Material and methods

2.1. Wood sample collection and chemicals used

Eucalyptus (wood processing residues) was kindly provided by Guangdong Academy of Forestry, China. Air-dried biomass was stored in sealed plastic bags at room temperature. The moisture content of the air-dried biomass was 8.73%. Sulphur dioxide (SO_2), sodium bicarbonate ($NaHCO_3$) and sodium sulfite (Na_2SO_3) were purchased from Sinopharm Chemical Reagent Co., Ltd.

2.2. Steam pretreatment followed by sulphonation

Biomass (20–100 mesh) was wrapped by 120-mesh screen and put into a steam gun (Jiangsu Kangwei Biological Co., Ltd., Jiangsu, China). Steam was then injected into the reactor and maintained at the temperature of either 200 °C or 210 °C for 5 min (Steam200 and Steam210). Pretreatment liquor and solid residues were collected after steam pretreatment. Subsequently, the solid residues were subjected to a sulphonation treatment (Steam200-Sulphonation and Steam210-Sulphonation) in a stainless steel batch reactor (NS-250I-C276, Anhui Kemi Machinery Technology Co., LTD, Anhui, China). Briefly, the steam treated substrate was resuspended in an aqueous solution containing sodium sulfite. The ratio of pretreatment liquor to dry weight of substrate was 10 (v/w). The sodium sulfite charge on dry weight of substrate was 12% (w/w). The mixture was heated to 130 °C and incubated for 1 h. At the end of sulphonation, the reactor was cooled down to room temperature by tap water. The slurry was separated into water-soluble fraction (WSF) and water-insoluble fraction (WIF). The water-insoluble fraction was washed with tap water and kept at 4 °C for further use. The water-soluble fraction and washing water were collected for determination of sugars concentration.

2.3. Sulphonation followed by steam treatment

Biomass was mixed with an aqueous solution containing sodium sulfite. The ratio of pretreatment liquor to dry weight of substrate was 5 (v/w). The sodium sulfite charge on dry weight of substrate was 12% (w/w). The mixture was cooked at 130 °C for 30 min. After the first-stage sulphonation, solid residues were collected and subjected to a second-stage steam treatment at either 200 °C (Sulphonation-Steam200) or 210 °C (Sulphonation-Steam210) for 5 min.

2.4. Alkaline sulphonation followed by steam treatment

Biomass were mixed with either water (Autohydrolysis) or an aqueous solution containing sodium sulfite and sodium bicarbonate (Na_2SO_3 Sulphonation-Steam). The ratio of pretreatment liquor to dry weight of substrate was 1 (v/w). The sodium sulfite and sodium bicarbonate charge on dry weight of substrate was 12% and 4% (w/w), respectively. Another set of two-stage pretreatment with alkaline sulphonation using SO_2 and sodium bicarbonate (SO_2 Sulphonation-Steam) was also carried out for comparison. Biomass was mixed with sodium bicarbonate solution in a plastic zipper bag. The ratio of pretreatment liquor to dry weight of substrate was 1 (v/w). The sodium bicarbonate charge on dry weight of substrate was 8% (w/w). Then, SO_2 was introduced into the plastic zipper bags with an amount of 6% (w/w). All samples were soaked in plastic zipper bags overnight, then transferred into the batch reactor. Water was added into the reactor to a final liquor-to-dry mass ratio of 5 (v/w). The mixture was subjected to a mild cooking at 130 °C for 30 min. After sulphonation, solid residues were collected and subjected to a second-stage steam treatment at 210 °C for 5 min.

2.5. Enzymatic hydrolysis

The enzymes used for the enzymatic hydrolysis were cellulase cocktail CTec 2 (filter paper activity of 191.25 FPU/g) and a β-glucosidase preparation NZ188 (β-glucosidase activity of 349.46 U/g), which were obtained from Sigma-Aldrich China Inc. Enzymatic hydrolysis of the pretreated substrates was performed in acetate buffer (50 mM, pH 4.8) at 50 °C with shaking at 180 rpm. Enzyme dosage was 20 FPU of cellulase and 10 U of β-glucosidase per gram cellulose. Enzymatic hydrolysis was conducted at substrate loading of 10% (w/v) in a 100 mL Erlenmeyer flask. Samples were taken after 48 h enzymatic hydrolysis. All experiments were run in duplicate.

2.6. Fermentation of the enzymatic hydrolysate

Following alkaline sulphonation-steam pretreatment using sodium sulfite and sodium bicarbonate and enzymatic hydrolysis, the enzymatic hydrolysate (liquid fraction of enzymatic hydrolysis) was separated from residual solids by filtration. A sequential fermentation using fermentable sugars in the enzymatic hydrolysate was applied as follows: after concentration of the enzymatic hydrolysate, *Saccharomyces cerevisiae* was added to the concentrated hydrolysate with an initial optical density (OD_{600}) of 10. Glucose fermentation was then performed at pH 5.5, 30 °C and 100 rpm, for 24 h. After glucose fermentation, *S. cerevisiae* was removed by centrifugation. The generated ethanol was distilled, using a rotary evaporator (BüCHI R-200, Swiss) at 70 °C, 160 mbar. Subsequently, xylose fermentation was carried out at pH 6.0, 30 °C and 150 rpm for 36 h, with initial dosage of *Pichia stipites* at OD_{600} 13. These yeast strains for glucose and xylose fermentation were obtained from Co-Innovation Center for Efficient Processing and Utilization of Forest Products, Nanjing Forestry University. After fermentation, the concentration of residual sugar and generated ethanol was determined by high performance liquid chromatography (HPLC). And the sugar-ethanol conversion rate (g/g) was calculated as gram ethanol produced per gram sugar consumed. All experiments were run