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Efficient bioethanol production from sodium hydroxide pretreated corn stover and rice straw in the context of on-site cellulase production

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ABSTRACT

A novel and efficient bioprocess from sodium hydroxide pretreated corn stover (SHPCS) or sodium hydroxide pretreated rice straw (SHPRS) to ethanol was successfully established, where the on-site cellulase production by the mixed culture of *Trichoderma reesei* and *Aspergillus niger* was used, producing 3.63 ± 0.35 FPIU/mL cellulase from SHPCS and 2.56 ± 0.33 FPIU/mL cellulase from SHPRS, respectively. Then those cellulases were applied to the enzymatic saccharification of SHPCS and SHPRS respectively, leading to yields of 81.5 ± 1.2% and 70.5 ± 2.1% respectively. Subsequently, SHPCS and SHPRS enzymatic hydrolysates mainly containing glucose and xylose were fermented by *Saccharomyces cerevisiae*, producing 27.6 ± 1.5 and 21.7 ± 1.9 g/L ethanol respectively. Concurrent with the distillation of fermentation broths for ethanol separation, the residual xylose was concentrated about 3 times. Thereafter, the distillation residues from SHPCS and SHPRS containing xylose were fermented by the adapted *Pichia stipistis*, producing 16.6 ± 1.1 and 13.0 ± 0.9 g/L ethanol respectively. In sum, the bioprocess could produce 33.1 g ethanol from 113.20 g SHPCS and 26.0 g ethanol from 117.58 g SHPRS.

1. Introduction

Corn stover and rice straw are the representative agricultural residues in the North and South China, respectively, which are always treated improperly such as combusted directly, thereby not only wasting of energy but causing serious environmental problem. China is the largest producer of rice and the second largest producer of corn in the world [1]. Issues about how to utilize them in a sustainable and environment-friendly way, therefore, are becoming more and more important as the concerns on energy security and environment protection increase in China [2].

Bioethanol is considered as an important renewable fuel to partly replace fossil-derived fuels because it can be used as favorable and near carbon-neutral renewable fuel that can reduce CO_2 emissions and associated climate change, as octane enhancer in unleaded gasoline, and as oxygenated fuel-mix for a cleaner combustion of gasoline that can reduce tailpipe pollutant emissions and improve the ambient air quality [3]. Bioethanol production from agricultural residues, regarded as the 2nd generation bioethanol, has many advantages such as non-competitiveness with the food chain, but its process is more complicated compared with 1st generation ethanol production and involves many technical and economic challenges [4,5].

Reducing the production cost is the key to enabling the commercialization of 2nd generation bioethanol. Pretreatment, enzymatic hydrolysis as well as cellulase preparation are the greatest leverage for cost reduction of lignocellulosic ethanol [6–8]. Alkali pretreatments increase cellulose digestibility and they are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization than acid or hydrothermal processes [9,10]. It can be performed at lower temperature and pressure than acid hydrolysis [11], even at room temperature [9], hopeful for reducing the pretreatment cost. Sodium hydroxide is

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suitable pretreatment, which breaks the bonds between lignin and carbohydrate polymers, partly solubilizes lignin, causes swelling, increases the internal surface of cellulose, and decreases the degree of polymerization and crystallinity [9–11]. The concept of on-site enzyme production for cellulase autarky can save the costs of cellulase preparation because it uses inexpensive substrate and avoids enzyme separation and concentration, enzyme storage, and transportation [12–15]. More importantly, using lignocelluloses as substrate to induce cellulase production has an increased enzymatic hydrolysis specificity for the substrate itself than others [14,16], capable of increasing the yield of enzymatic hydrolysis and thus reducing the relevant cost.

In addition, efficient utilization of the hemicellulosic component of lignocellulose feedstocks, mainly composed of xylose, can reduce the cost of bioethanol production by 25% [17,18]. A variety of researches on xylose fermentation for bioethanol production were carried out, ranging from metabolic engineering of *Saccharomyces cerevisiae* for fermenting xylose [19–21] to process engineering using xylose-fermenting yeasts [22–26]. Further work has been done in order to improve the efficiency of xylose fermentation by recombinant *S. cerevisiae* [27–29]. Though *S. cerevisiae* is the safest and the most robust glucose-fermenting yeast and widely used for large-scale production of ethanol in industry [19,28–30], the recombinant *S. cerevisiae* with the heterologous xylose metabolism pathways cannot compete with those native xylose-assimilating yeasts such as *Pichia* and *Candida* species in industrial performance.

In this work, the lignocellulosic materials that are corn stover and rice straw were pretreated with sodium hydroxide. Then the sodium hydroxide pretreated corn stover (SHPCS) and rice straw (SHPRS) were used as the substrates. The on-site cellulase production by the mixed culture of *Trichoderma reesei* and *Aspergillus niger* was established to supply cellulase to the enzymatic hydrolysis of SHPCS and SHPRS for production of fermentable sugars. The enzymatic hydrolysates of SHPCS and SHPRS were fermented by different yeasts including *S. cerevisiae*, the recombinant *S. cerevisiae* with the ability of fermenting xylose, *Pichia stipistis*, and the adapted *Pichia stipistis* with the tolerance to inhibitors. Finally, the different processes from corn stover or rice straw to ethanol using different yeasts were compared to select the most efficient one.

2. Materials and methods

2.1. SHPCS and SHPRS

Corn stover was collected from Kaifeng, Henan Province. Rice straw was from Wuxi, Jiangsu Province. They were air-dried once collected and stored at room temperature. They were pretreated with sodium hydroxide as described by Chen et al. [10]. Then SHPCS and SHPRS were washed using distilled water with a ratio of 1:10 (g/mL). Subsequently, they were stored at 4 °C in fridge until use. The water contents of washed SHPCS and SHPRS were measured. The main composition of SHPCS (dry material) was as follows: 63.9 ± 1.1% glucan, 15.0 ± 0.5% xylan, 9.0 ± 0.4% lignin, and 12.1% others. The main composition of SHPRS (dry material) was as follows: 60.0 ± 0.9% glucan, 12.1 ± 1.0% xylan, 9.5 ± 0.8% lignin, and 18.4% others. The compositions were determined using the standard procedures by NREL (National Renewable Energy Laboratory, USA) [5].

2.2. Microorganisms and media

T. reesei, *A. niger*, *S. cerevisiae* and *P. stipistis* were obtained from the strain collection of National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University. The recombinant *S. cerevisiae*, capable of fermenting xylose, was engineered by introducing the heterologous genes, the xylose reductase gene *xyl1* and the xylitol dehydrogenase gene *xyl2* from *P. stipistis* as well as the gene *xks1* encoding xylulokinase from *S. cerevisiae* [17,31]. *P. stipistis* was adapted gradually from 10% to 80% the prehydrolysate resulted from pretreatment until it can tolerate inhibitors (cells could grow normally in the prehydrolysate, i.e. no decrease in the value of optical density at a wavelength of 600 or no decrease in cell counts).

The medium for pre-culturing *T. reesei* and *A. niger* was as follows (50 mL): 0.5 g glucose, 0.05 g/L peptone, 5 mL Mandels nutrients salts solution [32], 2.5 mL citrate buffer, 0.05 mL Mandels trace elements solution [32], 2 drops Tween 80. The fermentation medium for cellulase production was as follows (g/L): SECS (dry biomass) 30, glucose 1, $(NH_4)_2SO_4$ 6, KH_2PO_4 2, $CaCl_2$ 0.3, $MgSO_4$ 0.3, $FeSO_4$ 0.005, $MnSO_4$ 0.0016, $ZnSO_4$ 0.0014, and $CoCl_2$ 0.0037. The initial pH was 4.8. The media were autoclaved at 121 °C for 20 min (the pre-culture medium) or 30 min (the fermentation medium).

The seed medium for *S. cerevisiae* was as follows (g/L): glucose 20, peptone 5, yeast extract 3, pH natural. The seed medium for xylose-fermenting yeasts was composed of (g/L): xylose 30, peptone 5, yeast extract 3, pH natural. The medium used for adapting *P. stipistis* was as follows (g/L): xylose 30, peptone 5, yeast extract 3, pH natural, 10%, 20%, 30%, 40%, 50%, 60%, 70% or 80% prehydrolysate. The fermentation medium was consisted of (g/L): the enzymatic hydrolysates or the distillation residues, for *S. cerevisiae* and the recombinant *S. cerevisiae*, $MgSO_4$ 0.08, $ZnCl_2$ 0.08, $CaCl_2$ 0.20, urea 0.24 and initial pH 5.5; for *P. stipistis* and the adapted *P. stipistis*, peptone 0.5, yeast extract 0.3, $(NH_4)_2SO_4$ 2, KH_2PO_4 4, $MgSO_4 \cdot 7H_2O$ 1 and initial pH 6. The media were autoclaved at 121 °C for 20 min.

2.3. Mixed culture of T. reesei and A. niger

T. reesei was pre-cultured at 30 °C for 48 h and *A. niger* was pre-cultured at 30 °C for 36 h in the medium described above to prepare inocula. Different mixed culture forms of *T. reesei* and *A. niger* were compared, including 0 h/1:1 (*T. reesei* and *A. niger* were inoculated at the same time point and the inoculum ratio of *T. reesei* to *A. niger* was 1:1), 0 h/5:1 (*T. reesei* and *A. niger* were inoculated at the same time point and the inoculum ratio of *T. reesei* to *A. niger* was 5:1), 24 h/1:1 (*A. niger* was inoculated 24 h after *T. reesei* inoculation and the inoculum ratio of *T. reesei* to *A. niger* was 1:1), 24 h/5:1 (*A. niger* was inoculated 24 h after *T. reesei* inoculation and the inoculum ratio of *T. reesei* to *A. niger* was 5:1), 48 h/1:1 (*A. niger* was inoculated 48 h after *T. reesei* inoculation and the inoculum ratio of *T. reesei* to *A. niger* was 1:1) and 48 h/5:1 (*A. niger* was inoculated 48 h after *T. reesei* inoculation and the inoculum ratio of *T. reesei* to *A. niger* was 5:1). The mixed cultures were carried out in 50 mL Erlenmeyer flasks with a working volume of 50 mL and a shaking of 170 rpm at 30 °C on the first day and 28 °C from the second day. Enzymatic activities were measured to monitor the cellulase production process. At least triplicate samples ($n \geq 3$) were used in the analysis and data are shown in the form of means ± standard deviations.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out in 250 mL Erlenmeyer flasks with a working volume 50 mL consisted of 2.5 mL 1 M citrate buffer solution (pH 4.8), 100 g/L substrate SHPCS or SHPRS, 25 FPIU/g glucan cellulase and a supplementary amount of water to 50 mL. Then the flasks were incubated at 50 °C in an orbital shaker with a shaking of 140 rpm for 48 h. At least triplicate samples ($n \geq 3$) were used in the analysis and data are shown in the form of means ± standard deviations.