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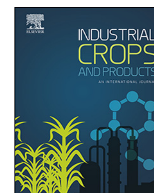

Microbial lipid production from enzymatic hydrolysate of corn stover pretreated by combining with biological pretreatment and alkalic salt soaking

Xiaojun Huang[a], Yun Ding[a], Xiaolong Liao[b], Bo Peng[b], Yucai He[a,b,*], Cuiluan Ma[a,b,*]

[a] Advanced Catalysis and Green Manufacturing Collaborative Innovation Center, Jiangsu Key Laboratory of Advanced Catalytic Materials and Technology, Changzhou University, Changzhou, PR China

[b] Hubei Collaborative Innovation Center for Green Transformation of Bio-resources, Hubei Key Laboratory of Industrial Biotechnology, College of Life Sciences, Hubei University, Wuhan, PR China

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ABSTRACT

The conversion of lignocellulose for biofuel production requires an effective pretreatment to disrupt the recalcitrant plant cell walls and to make the cellulose accessible to cellulases. In this study, sequential biological pretreatment (BP) with *Galactomyces* sp. CCZU11-1 under the mild condition and alkalic salt pretreatment (AP) with Na_2CO_3 by autoclaving was used to pretreat untreated corn stover (CS) for enhancing its enzymatic saccharification. After the optimization, the optimum pretreatment conditions were obtained. The crystallinity, porosity, and morphology of the pretreated solid residue of CS were correlated with the enhancement of enzymatic saccharification. After 72 h, the yields of glucose from the hydrolysis of 50 g/L BP-AP-CS with a cocktail of *Galactomyces* sp. CCZU11-1 cellulases in the absence and presence of Na_2CO_3 (0.82 wt%) could be obtained at 47.2% and 46.1% respectively. Using BP-AP-CS-hydrolysates containing 25.6 g/L glucose as carbon source, microbial lipids effectively produced by *Rhodococcus pyridinivorans* CCZU-B16 in the presence of Na_2CO_3 (0.82 wt%). Four fatty acids including palmitic acid (C16:0; 23.5%), palmitoleic acid (C16:1; 19.0%), stearic acid (C18:0; 16.2%), and oleic acid (C18:1; 24.8%) were distributed in total fatty acids. In conclusion, this combination pretreatment strategy has potential application for the production of microbial lipids from biomass in future.

1. Introduction

Lignocellulosic biomass is regarded as one of the most promising feedstocks, which has gained great interest for the clean production of chemicals and biofuels (Ding et al., 2016; Donohoe et al., 2008; Falls et al., 2011; He et al., 2014, 2016b; Hu et al., 2016; Li et al., 2012; Maurelli et al., 2013; Xu et al., 2015; Wang et al., 2016). Corn stover (CS), one kind of lignocellulosic byproduct left after harvest of corn, has been used for chemicals and biofuels production owing to its abundance as well as low cost (Cayetano and Kim, 2017; Gao et al., 2012; He et al., 2015; Liu et al., 2015; Kuhn et al., 2016; Xu et al., 2016a). To produce ethanol effectively, CS should be enzymatically hydrolyzed first to soluble sugars by cellulases (Katahira et al., 2014). Forthis purpose, various physical, chemical, physical-chemical and biological strategies including ammonia fiber expansion, ammonia recycle percolation, concentrated acid, dilute acid, concentrated alkali, dilute alkali, hot water, ionic liquid, lime, *N*-methyl-morpholine-*N*-oxide, and steam explosion have been employed to improve the accessibility of cellulases to the sugar matrix (Alvira et al., 2010; Cianchetta et al., 2014; Díaz et al., 2010; Dien et al., 2015; Haykir and Bakir, 2013; He et al., 2013; Jiang et al., 2015; Jin et al., 2013; Khaleghian et al., 2015; Mosier et al., 2005; Shuai et al., 2010; Strassberger et al., 2014; Wang et al., 2009; Xu et al., 2016b; Zhu et al., 2009).

Recently, alkalic salts as weak bases including Na_2CO_3, Na_2S, NaClO, CH_3COONa and Na_3PO_4 have been used to pretreat lignocellulosic materials (Kim et al., 2014; Nakashima et al., 2016). These salt types result in the dissolution and disruption of hemicellulose and lignin structures, restructuring and conversion of lignin, deesterification of intermolecular ester bonds, and altering the crystallinity of cellulose. Glucose yields obtained by the enzymatic saccharification of straw pretreated with 1 M Na_2CO_3 at 100 °C for 7 h was 92.5% (Khaleghian et al., 2015). Sulfite (SO_3^{2-}) pretreatment could convert

* Corresponding authors at: Advanced Catalysis and Green Manufacturing Collaborative Innovation Center, Jiangsu Key Laboratory of Advanced Catalytic Materials and Technology, Changzhou University, Changzhou, PR China.

E-mail addresses: heyucai2001@163.com (Y. He), macuiluan@163.com (C. Ma).

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water-insoluble lignin to water-soluble lignosulfonate (Jiang et al., 2015; Zhu et al., 2009). This pretreatment could decreased the lignin inhibitory effect on cellulases, and effective saccharification was conducted with low loading of cellulases (Shuai et al., 2010). 89% of xylan removal in pulp was achieved with 12% sulfite at 170 °C for 2 h, and the saccharification of pretreated pulp was 74% (Jin et al., 2013).

It is known that biological pretreatment (BP) is mostly associated with the action of microbes (e.g., bacteria and fungi) that are capable of producing enzymes to degrade hemicelluloses, lignin, and polyphenols in biomass (Cianchetta et al., 2014; Dai et al., 2015; Sindu et al., 2016). BP has gained a considerable interest due to its high substrate specificity, low energy consumption, and no formation of toxic compounds (Larran et al., 2015; Sindu et al., 2016). However, BP is a very slow pretreatment process that requires careful control of growth and pretreatment conditions. It was found that the combination pretreatment showed better than one step pretreatment (Dai et al., 2015; Ma et al., 2010). Sequential pretreatment with E. taxodii and 0.25 wt% H_2SO_4 was more effective than single pretreatment (Ma et al., 2010). The combination pretreatment of rice hulls with 2 wt% H_2O_2 for 2 d and *P. ostreatus* for 18 d was more effective than single pretreatment with *P. ostreatus* for 60 d. Combination of *Sphingobacterium* sp. LD-1 pretreatment with NaOH/Urea pretreatment was conducted to enhance enzymatic hydrolysis of rice straw (Dai et al., 2015). These studies indicated that combination of biological and other pretreatment is a promising strategy for improvement of enzymatic hydrolysis.

Recently, there is an increasing interest to employ lignocellulosic materials for producing microbial lipids with oleaginous microorganisms as a non-food oil feedstock for biodiesel production (Qin et al., 2017a; Xie et al., 2015). To effectively utilize CS, an efficient pretreatment of CS was developed and systematically investigated using combination pretreatment by sequential biological pretreatment (BP) with *Galactomyces* sp. CCZU11-1 and alkalic pretreatment (AP) with dilute alkalic salt Na_2CO_3, resulting in a noticeable enhancement of enzymatic saccharification with compexed cellulases of *Galactomyces* sp. CCZU11-1. Moreover, the cellulose structural changes of pretreated CSs were characterized with Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), and scanning electron microscopy (SEM). Finally, the fermentability of the recovered hydrolysates containing glucose from the enzymatic hydrolysis of BP-AP-CS was tested using microbial lipids producing strain *Rhodococcus pyridinivorans* CCZU-B16.

2. Materials and methods

2.1. Materials

Corn stover (CS) milled to a size < 3 mm was obtained from the suburb in Liaoning province (P.R. China). The cellulose, hemicelluloses, lignin, other components of raw CS were 36.0%, 24.2%, 14.4%, and 25.4%, respectively. Na_2CO_3 and other chemicals were purchased from Sinopharm Group Chemical Reagent Co. Ltd. (Shanghai, China). Novozyme 188 (665 CBU/mL) were purchased from Sigma (St. Louis, MO, USA). Xylanase (280 U/mg) was Genencor (Shanghai, China). The microbial lipids producer, *Rhodococcus pyridinivorans* CCZU-B16, was isolated from soil (Chong et al., 2018). The cellulase-producing strain, *Galactomyces* sp. CCZU11-1 (an accession number: CGMCC No. 5561) (He et al., 2013) has been deposited at China General Microbiological Culture Collection Center.

2.2. Biological pretreatment of CS with Galactomyces sp. CCZU11-1

A pre-seed culture was prepared by inoculating a single colony of *Galactomyces* sp. CCZU11-1 (CGMCC No. 5561) into 250 mL Erlenmeyer flask containing 100 mL growth medium (CS 0.5 g, $(NH_4)_2SO_4$ 0.050 g, K_2HPO_4 0.10 g, $MgSO_4{\cdot}7H_2O$ 0.050 g, $CaCl_2$ 0.050 g, pH 5.4) and incubated in flask shaker (180 rpm) at 30 °C for 72 h.

After 2 g dry CS was mixed with 4 mL medium ($(NH_4)_2SO_4$ 0.50 g/L, K_2HPO_4 1.0 g/L, $MgSO_4{\cdot}7H_2O$ 0.50 g/L, $CaCl_2$ 0.50 g/L, pH 5.4) for 1 min, the mixture was autoclaved at 121.5 °C for 20 min. After being cooled at room temperature, the seed culture of *Galactomyces* sp. CCZU11-1 was inoculated into the mixture at 0.1 mL seed/g dry CS. After the fermentation was conducted for 1–6 d at 30 °C in a shaker (80 rpm), 20 mL sodium acetate buffer (50 mM, pH 4.8) was added into the system, and then the mixture was further shaken for 1 h at 30 °C and 180 rpm. Cellulase solution was obtained by centrifuge and stored for the further use at 4 °C. The solid as the biological pretreatment of CS (BP-CS) was collected and washed with deionized water thoroughly, and then dried at 65 °C for the following experiments.

2.3. Pretreatment of CS with alkalic salt Na_2CO_3 by autoclaving

2.3.1. Optimization of dilute Na_2CO_3 pretreatment

At solid/liquid ratio of 1/10 (wt/wt), 2.0 g CS was mixed with 20.0 g dilute alkalic salts (0.5%–1.5% Na_2CO_3) and distilled water in a 100 mL conical flask sealed by a lid. After the pretreatment in an autoclave (LMQ.C-80E, Jinan Zhankang Medical Devices Co., Ltd) at 100–120 °C for 10–60 min, 4.48 g acetic acid-sodium acetate (HAc-NaAc) buffer (0.038 g sodium acetate, 0.029 g acetic acid, 4.41 g distilled water, pH 4.8) as anti-solvent was added to the pretreatment media for precipitating CS, and then the precipitate was filtered and washed two times with total amount of 73.44 g distilled water for removing Na_2CO_3. In an air drier, the obtained CS residual was dried at 90 °C for the enzymatic hydrolysis and other analyses. All experiments were performed in triplicate. Error bars represent standard deviation from the mean. In this study, the pretreatment severity (Log*Ro*) factor was applied to evaluate pretreatment conditions.

$$\text{Log}Ro = \text{Log}t \times \exp\left(\frac{T_H - T_R}{14.75}\right) \quad (1)$$

where t is the pretreatment time in minutes, T_H is the pretreatment temperature in °C, and T_R is the reference temperature in °C (100 °C).

2.3.2. Enzymatic in situ saccharification in the presence of Na_2CO_3

At solid/liquid ratio of 1/10 (wt/wt), 2.0 g BP-CS was mixed with 20.0 g dilute alkalic salts Na_2CO_3 (1 wt%) in a 100-mL conical flask sealed by a lid in an autoclave (LMQ.C-80E, Jinan Zhankang Medical Devices Co., Ltd) at 120 °C for 30 min, the pretreatment media was cooled at 90 °C, and then HAc-NaAc buffer (0.038 g sodium acetate, 0.029 g acetic acid, 4.41 g distilled water, pH 4.8) as anti-solvent was added to the pretreatment media to form the mixture containing 28.4 g/L cellulose, 15.1 g/L hemicellulose, and 3.9 g/L lignin in the presence of Na_2CO_3 (0.82 wt%). After the regeneration, HAc-NaAc buffer (50 mM, pH 4.8) and enzymes were added to this mixture to form the enzymatic in situ saccharification media containing alkalic salts Na_2CO_3 (0.82 wt%). All experiments were performed in triplicate. Error bars represent standard deviation from the mean.

2.4. Enzymatic hydrolysis of CS in the absence and presence of Na_2CO_3

The enzymatic hydrolysis media contained 50 g/L of CS sample, 100 mL of HAc-NaAc buffer (50 mM, pH 4.8), 40 μL of tetracycline hydrochloride (10.0 g/L) and a cocktail of cellulase (15 mg/g CS) + β-glucosidase (3.5 mg/g CS) + xylanase (1.5 mg/g CS). Enzymatic hydrolysis of CS was conducted in a shaker (180 rpm) at 50 °C in the absence and presence of Na_2CO_3. During the biotransformation, aliquot of 800 μL supernatant was withdrawn periodically and immediately kept in boiling water for 60 s to inactivate cellulases. All experiments were performed in triplicate. Error bars represent standard deviation from the mean.

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