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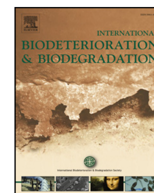

The improvement of sodium hydroxide pretreatment in bioethanol production from Japanese bamboo *Phyllostachys edulis* using the white rot fungus *Phlebia* sp. MG-60

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A B S T R A C T

The concentration range of sodium hydroxide solution (0.0–7.0% w/w) was used to determine the impact of chemical pretreatment in bioethanol production from Japanese bamboo by using the white rot fungus *Phlebia* sp. MG-60. A pretreatment of sodium hydroxide at 120 °C for 1 h demonstrated a significant effect on the removal of lignin and xylan components, leading to an increase in glucan composition for the pretreated bamboo. The saccharification rate was improved from 41.0% in the initial sample to 89.5% in the 7.0% NaOH pretreated sample. This first report on consolidated bioprocessing of Japanese bamboo points out that the highest ethanol yield was 12.8% in 7.0% NaOH pretreated samples, equivalent to 28.5% of polysaccharide volume converted to ethanol, while the conversion proportion in the initial bamboo sample was negligible. Bioethanol production by applying semi-simultaneous saccharification and fermentation showed the highest conversion rate: 58.9% in 7.0% NaOH pretreated samples. However, after considering the weight loss of bamboo samples during pretreatment, the 1.0% NaOH pretreated sample was indicated as the highest ethanol-producing efficiency with 38.1% conversion rate. These results show that sodium hydroxide is an effective pretreatment in combination with *Phlebia* sp. MG-60 in bioethanol production from Japanese bamboo, with or without commercial hydrolytic enzymes.

1. Introduction

Lignocellulosic biomass usually includes forest biomass and wastes, agricultural residues, and energy crops, which are mostly comprised of cellulose, hemicelluloses, and lignin components. These natural resources have been focused on as potential materials for the second generation of bioethanol production because of their beneficial characteristics, including high annual yield, abundance, and high concentrations of holocellulosic contents (Cai et al., 2017; Zabed et al., 2016). According to the previous researches, the second generation of bioethanol production is recognized as a key topic in energy science research because the results contribute not only to energy production, but also to the reduction of environmental pollution. The primary research goal of second-generation bioethanol production is to increase the polysaccharide conversion and fermentation yield (Zabed et al., 2017).

The first step in bioethanol production is pretreatment. Successful methods were identified as the technologies able to alter or remove compositional impediments and resistant structures of biomass in order to improve enzymatic saccharification by using mechanical, biological, or chemical methods. Among these approaches, alkaline pretreatment was documented as one of the most effective methods to remove lignin and hemicelluloses, as well as increase surface contact area in various materials (Kim et al., 2016; Mosier et al., 2005; Saha et al., 2016; Suhara et al., 2012; Suhardi et al., 2013; Tomak et al., 2013; Yang et al., 2015; Zhang et al., 2007, 2017). In bioethanol production, depending on pretreated sample features and experimental conditions, various strategies have been applied such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and consolidated bio-processing (CBP). In general, modified SSF and CBP were identified as preferred processes with high economic feasibility (Chen and Fu, 2016; Mitchell et al., 2002).

Phlebia sp. MG-60 is a hypersaline-tolerant basidiomycete and is reported as a powerful bioethanol producer because its enzyme system can participate in lignin degradation, hydrolysis of polysaccharides, and fermentation of reducing sugars. Several studies have emphasized that *Phlebia* sp. MG-60 can produce ethanol from various types of biomass, including sugarcane bagasse, wood, and residues of mushroom

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production (Kamei et al., 2012a, 2012b, 2014; Khuong et al., 2014) without addition of commercial hydrolytic enzymes.

Bamboo is the common name for more than 1200 species groups of large woody grasses which have been used for food, construction materials, and traditional arts for approximately 2.5 billion people around the world. With such wide distribution, huge annual biomass yield, and high holocellulose content, bamboo has been recently recognized as a high-potential material for bioenergy production (Darabant et al., 2014; Fei et al., 2016; He et al., 2014; Scurlock et al., 2000). To continue the focus on bioenergy performance from bamboo species, this study was conducted to 1) evaluate the effect of alkaline pretreatment on the chemical transformation of Japanese bamboo and 2) determine the efficiency of the integration of alkaline pretreatment and the white rot fungus *Phlebia* sp. MG-60 fermentation in bioethanol production from Japanese bamboo, *Phyllostachys edulis*, with additional commercial hydrolytic enzyme (sSSF) or without (CBP).

2. Materials and methods

2.1. Bamboo samples

The stems of Japanese bamboo, *Phyllostachys edulis*, were kindly donated by Sanuki Kasei Co., Ltd. Samples were minced into 40–100 mesh size particles; four hundred grams of which were extracted with solution of benzene and ethanol (ratio 2:1 v/v in total 2.1 L) for 16 h in a Soxhlet apparatus. Extracted bamboo – hereafter referred to as “initial” – was air-dried, then stored in plastic bags.

2.2. Preparation of fungal strain

White rot fungus *Phlebia* sp. MG-60 was used for this research. Phylogenetic analysis of internal transcribed spacers (ITSs) containing rRNA gene sequence (ITS-rDNA) clarified that this strain belongs to the genus *Phlebia* and is closely related to the fungus *Phlebia lindtneri*. This sequence was deposited to data base with the DDBJ accession number AB210077 (Kamei et al., 2005). Stock culture was maintained in Potato Dextrose Agar (PDA) slant at −80 °C. The mycelial disk was then inoculated on PDA plates to recover at 28 °C. The mycelia were cultured for 5 days in the PDA before being used in the fermentation steps.

2.3. Sodium hydroxide pretreatment

Three grams of initial bamboo were each added to 24 mL of sodium hydroxide (0.0, 0.5, 1.0, 3.0, 5.0 and 7.0%, w/v) aqueous solutions in individual 100 mL Erlenmeyer flasks. The mixtures were autoclaved at 120 °C for 1 h. After autoclaving, samples were filtered through glass filter type G3, and then washed with distilled water to achieve neutral pH. Pretreated samples were dried in the air for 72 h and then kept in plastic bags to prevent environmental effects. The pretreatment step was done by three time repeats.

2.4. Commercial enzymatic hydrolysis

Two hundred milligrams of initial and pretreated bamboo samples were placed in individual test tubes, each containing 20 mL sodium citrate buffer (50 mM, pH 4.8) and 200 mg of the commercial hydrolytic enzyme Meicelase (containing 0.437 g protein per 1 g of enzyme, Meicelase-P; Meiji Seika Co. Ltd, Tokyo, Japan). The test tubes were then incubated at 60 °C with 120 rpm for 72 h. One mL of hydrolysate was collected after 24, 48, and 72 h and used to determine concentration of glucose and xylose.

The saccharification rate of the sample was determined by the following equation:

$$SR\cdot(\%) = \frac{\text{Total weight of glucose and xylose in hydrolysate}}{\text{Theoretical weight of glucose and xylose in 200 mg of sample}} \times 100$$

2.5. Consolidated bioprocessing (CBP)

Erlenmeyer flasks (100 mL type), each containing 0.8 g of sample and 40 mL of a basal liquid medium, which is the fundamental nutrients to fungal growth (10 g L^{-1} yeast extract, 10 g L^{-1} KH_2PO_4, 2 g L^{-1} $(NH_4)_2SO_4$ and 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$) with pH scale was 6.0, were autoclaved at 120 °C for 15 min. To start fermentation, a 6 mm-diameter disc of *Phlebia* sp. MG-60 mycelium from PDA was transferred into each Erlenmeyer flask mentioned above. Flasks were sealed with silicone plugs and incubated at 28 °C in the dark room.

2.6. Semi-simultaneous saccharification and fermentation (sSSF)

SSSF was done through combination of pre-hydrolysis by commercial enzyme and fungal cultivation in the single bioreactor. In the pre-hydrolysis step, bamboo substrate (0.8 g of each individual sample) was added into 100 mL Erlenmeyer flasks containing 40 mL sodium citrate buffer (50 mM, pH 4.8) and 0.4 g Meicelase at 60°C with shaking (120 rpm) for 72 h. After 72 h, 1 mL of hydrolysate was taken to determine xylose and glucose contents. To start the fungal cultivation, a 6 mm-diameter disc of *Phlebia* sp. MG-60 mycelium (taken from PDA) was added into each flask, sealed with a silicone plug, and incubated at 28°C in the dark room.

2.7. Chemical composition analysis of bamboo

Determination of structural carbohydrate and lignin contents in initial and pretreated samples was done by three time repeats, following the Laboratory Analytical Procedure NREL/TP-510-42618 (Sluiter et al., 2008). The 300 mg of sample was placed in a 100 mL Erlenmeyer flask and hydrolyzed with 3 mL of 72% H_2SO_4 for 30 min at 60 °C. Eighty-four mL distillated water was then added to the flask to dilute the solution, resulting in a 4% H_2SO_4 concentration. All samples were hydrolyzed a second time by autoclaving at 120 °C for 1 h. After being separated by glass filter G3, solid residues were dried at 100 °C in an oven to determine lignin content. One mL of liquid was taken to determine glucose and xylose concentrations by high-performance liquid chromatography (ICSep ICE-coregel 87H3 column 7.8 × 300 mm; Refractive Index Detector RID-10A; Shimadzu, Kyoto, Japan) with a mobile phase consisting of 5.0 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}. The injection volume was 10.0 μL and oven temperature was maintained at 70 °C.

2.8. Ethanol production analysis

One mL of medium sample was taken from the incubated flask and then centrifuged at the 12,000 × *g* for 10 min at 4 °C to separate supernatant and mycelium. The resulting supernatant was used to measure concentration of produced ethanol by HPLC which is mentioned in section 2.7.

The ethanol yield and conversion rate were determined by the following equations:

$$Ethanol\cdot yield\,(\%) = \frac{\text{Weight of produced ethanol (g)}}{\text{Weight of oven-dried sample (g)}} \times 100$$

$$Conversion\cdot rate\,(\%) = \frac{\text{Concentration of produced ethanol (g/mL)}}{\text{Theoretical concentration of ethanol (g/mL)}} \times 100$$

Ethanol production from xylose is described as $3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2$. In this equation, 3 mol of xylose (molecular mass 150) are required to produce 5 mol of ethanol (molecular mass 46). So,

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