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Amphipathic lignin derivatives to accelerate simultaneous saccharification and fermentation of unbleached softwood pulp for bioethanol production

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HIGHLIGHTS

- A new, fed-batch simultaneous saccharification and fermentation (SSF) was proposed.
- Effects of amphipathic lignin derivatives (A-LDs) on SSF were investigated.
- Addition of A-LDs did not inhibit but accelerate the yeast fermentation of glucose.
- Addition of A-LDs increased the yield of bioethanol from soda pulp by 30%.

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ABSTRACT

Amphipathic lignin derivatives (A-LDs) were already demonstrated to improve enzymatic saccharification of lignocellulose. Based on this knowledge, two kinds of A-LDs prepared from black liquor of soda pulping of Japanese cedar were applied to a fed-batch simultaneous saccharification and fermentation (SSF) process for unbleached soda pulp of Japanese cedar to produce bioethanol. Both lignin derivatives slightly accelerated yeast fermentation of glucose but not inhibited it. In addition, ethanol yields based on the theoretical maximum ethanol production in the fed-batch SSF process was increased from 49% without A-LDs to 64% in the presence of A-LDs.

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1. Introduction

Bioethanol as a renewable fuel and a platform compound for chemicals has drawn much public attention in this century. Lignocellulosic biomass is considered to be a promising candidate as a second generation biomass for bioethanol production (Olofsson et al., 2008), because bioethanol production from lignocelluloses does not compete with food production. Cedar wood (*Cryptomeria japonica*) is representative plantation softwood in Japan, which accounts for over 90% of plantation softwood, and piles of cedar wood is left unused in the forest after thinning-cut for timber production. Thus, it is an urgent issue to use such low-quality cedar (Baba et al., 2011). One of the possible ways to utilize such unused wood biomass is bioethanol production.

There are mainly two methods to produce bioethanol from lignocelluloses with cellulolytic enzymes, cellulase. One is separate hydrolysis and fermentation (SHF) to produce fermentable sugars with the enzymes sequentially followed by fermentation with yeast. The other is simultaneous saccharification and fermentation (SSF). In the former process, several additives to enhance enzymatic saccharification efficiency, such as surfactants, were developed since 1980's (Castanon and Wilke, 1981; Eriksson et al., 2002; Seo et al., 2011). We have also developed such additives from lignin in 2001 from the viewpoint of lignin utilization (Uraki et al., 2001). The background of our research work is summarized as follows. Lignin is an abundant, natural aromatic polymer, and it has a huge potential as a feedstock for polymeric materials (Goldstein, 1975; Jairo and Wolfgang, 2002). However, it is utilized merely as an energy resource in pulping industry. Therefore, development of value-added materials from lignin is

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an urgent subject in woody biomass refinery. In 2010's, isolated lignins, lignosulfonate (Wang et al., 2013; Zhou et al., 2013; Lou et al., 2014), organosolv lignin (Lai et al., 2014), and extractable lignin (Lai et al., 2014) were also demonstrated to improve enzymatic saccharification of lignocelluloses without further modification.

Our lignin-based additives were prepared by the coupling of several kinds of isolated lignins, such as kraft, soda and organosolv lignins, with epoxylated polyethylene glycol (PEG) (Aso et al., 2013). As a result, the reaction products were soluble in water and some organic solvents. Therefore, they were termed as amphipathic lignin derivatives (A-LDs). A-LDs significantly improved enzymatic saccharification of unbleached pulp from wood and empty fruit bunch of oil palm, but their effect on the bleached material, like filter paper, was not so drastic (Uraki et al., 2001; Bardant et al., 2010). In addition, A-LDs maintained cellulase activity for a long time even after a saccharification process (Winarni et al., 2013). This result would probably suggest that A-LDs enable repeated use of the enzymes and successive charge of the substrate to the saccharification media. Actually, it was found possible to use the enzyme repeatedly four to five times for the saccharification of unbleached pulp without further enzyme addition (Uraki et al., 2001; Winarni et al., 2014).

In 2007, it was reported that PEG with the molecular mass of more than 4000 Da had an ability to improve enzymatic saccharification (Börjesson et al., 2007). We investigated the interaction of CBH II, one of the major components of cellulase, with PEG 4000 and A-LDs, using Biacore, a biosensor utilizing surface plasmon resonance. Our results showed that A-LDs were directly associated with the enzyme, while PEG 4000 was not (Winarni et al., 2013). It was suggested that the improvement mechanism of A-LDs for the enzymatic saccharification was quite different from that of PEG 4000; PEG interacted with substrate to inhibit non-productive binding of cellulase to substrate (Börjesson et al., 2007), while A-LDs was associated with cellulase to assist the release of the enzyme from substrate after the hydrolysis, probably due to their significant surface activity. However, a detailed mechanism has not been clarified yet. Thereby, we are continuing investigation on this point together with the interaction of A-LDs with other cellulase components.

The aim of this study is to clarify the effect of A-LDs, prepared from black liquor of soda–anthraquinone pulping of cedar wood, on bioethanol production from the cedar wood by a fed-batch SSF process after the soda–anthraquinone pulping as a delignification process, where the fed-batch process means that a substrate is successively charged to the SSF media (Ballesteros et al., 2002; Rudolf et al., 2005; Hoyer et al., 2010; Kang et al., 2011; Lu et al., 2013; Lan et al., 2013). As expected from the aforementioned function of A-LDs for a single enzymatic saccharification process, A-LDs must also be useful for a fed-batch SSF process. However, non-ionic surfactant such as Triton X-100 reported previously to improve enzymatic saccharification (Eriksson et al., 2002) did not necessarily contribute to the fermentation (Lee et al., 1996). Accordingly, an influence of A-LDs on glucose fermentation of yeast was first investigated in this study.

2. Methods

2.1. Preparation of A-LDs

Soda lignin (SL) was obtained from the spent liquor of the soda pulping of cedar wood chips (200 g of cedar chips; 1 g of anthraquinone; 52 g of NaOH in 1 L of water; heating time: raised from room temperature to 170 °C for 90 min, and then maintained at the final temperature [170 °C] for 90 min) by precipitation with aqueous HCl solution to pH 2. Then, the crude lignin was washed several times with distilled water, and lyophilized to yield SL powder. SL was reacted with epoxylated PEGs, dodecyloxy poly (ethylene glycol) glycidyl ether (DOPEG) and ethoxy (2-hydroxy)propoxy poly (ethylene glycol) glycidyl ether (EPEG) to obtain A-LDs as follows. DOPEG, commercially available, was kindly supplied from Nagase Chemtex Corp. (Osaka, Japan), and EPEG was synthesized according to our previous report (Homma et al., 2008). Ten grams of SL was dissolved in 100 mL of 1 M aqueous NaOH solution, and then 45 g of DOPEG and 30 g of EPEG was separately added to the solution at 70 °C for 2 h with stirring. After the reaction, the reaction solution was acidified with glacial acetic acid to pH 4 to stop the reaction, and purified by ultrafiltration (cut-off molecular mass: 1000 Da, Advantec, Japan) (Homma et al., 2010). The ultrafiltration residue was lyophilized to yield A-LDs. PEG content in A-LDs was determined by modified Morgan method (Homma et al., 2008). Water surface tension in the presence of A-LDs was measured by Du Noüy ring method (Aso et al., 2013).

2.2. Enzymatic saccharification

EPEG-SL and DOPEG-SL (10% of substrate on dry weight basis) were separately dissolved in 100 mL of 50 mM citrate buffer (pH 4.8). A commercial cellulase, Genencor GC220 (liquid form; Genencor International Inc., USA; Lot # 4901121718) (10 filter paper unit (FPU)/g pulp), was added each to the solutions, and the mixture was stirred for 1 h at room temperature. Then, to the solutions was added 1 g of dry unbleached cedar pulp, which was prepared at a test plant of Forestry and Forest products Research Institute, Japan (Akita, Japan), under aforementioned pulping conditions. The suspension was shaken at 50 °C for 48 h. After saccharification, the suspension was filtered through a G4 glass filter. The precipitate was washed three times with the buffer solution, and weighed after completely dried at 105 °C. All the experiments were performed in duplicate. The sugar yield (%) was calculated according to the following equation:

$$\text{Sugar yield}\,(\%) = (\text{WS} - \text{WR})/\text{WS} \times 100$$

where WS (g) is the initial weight of substrate, and WR (g) is the weight of the filtered residue.

Finally, the enzyme solution was recovered from the filtrate by ultrafiltration (Vivaspin 20, with 10 kDa cut-off membrane: Satorius Co., Göttingen, Germany) in accordance with the previous report (Winarni et al., 2013). FPU of the recovered cellulase solution was measured according to the National Renewable Energy Laboratory (NREL) technical report, NREL/TP-510-42628 (Ghose, 1987; Adney and Baker, 2008), and a recovered activity of cellulase was calculated by the following equation.

$$\text{Recovered activity}\,(\%) = \text{FPU after saccharification} / \text{FPU of the initial cellulase} \times 100$$

2.3. Glucose fermentation by Japanese sake yeast

Japanese sake yeast, *Saccharomyces cerevisiae*, (2 g of dry powder, Mauri Yeast Australia Pty Ltd., Queensland, Australia) was dispersed in 100 mL of YP medium [0.05 M of citrate buffer (pH 4.8) including 10 g/L of yeast extract and 20 g/L of peptone] (Dowe and McMillan, 2001) together with 3 g of glucose, and incubated for 2 days at 38 °C with gentle shaking to give a pre-incubated yeast suspension.

Ten grams of glucose was dissolved in 80 mL of YP medium, and 20 mL of pre-incubated yeast suspension and 0.25 g of A-LDs were added to the glucose solution. The mixture was further incubated for 4 days at 38 °C with gentle shaking at 100 rpm. An aliquot was sampled out every day, and ethanol concentration in the

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