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Short Communication

Butanol production from alkali-pretreated rice straw by co-culture of *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum*

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HIGHLIGHTS

- Alkali pretreatment significantly reduced lignin content of rice straw up to 76%.
- Co-culture of cellulolytic and butanol producing *Clostridium* species was applied.
- The co-culture system produced 5.5 g/L of butanol from delignified rice straw.
- Addition of cellulase enhanced butanol titer (6.9 g/L) from delignified rice straw.
- Enhancement of exoglucanase is important for butanol production from rice straw.

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ABSTRACT

The co-culture of cellulolytic *Clostridium thermocellum* NBRC 103400 and butanol-producing *Clostridium saccharoperbutylacetonicum* strain N1-4 produced 5.5 g/L of butanol from 40 g/L of delignified rice straw pretreated with 1% (wt/vol) NaOH. The addition of cellulase (100 U/g biomass) in a co-culture system significantly increased butanol production to 6.9 g/L using 40 g/L of delignified rice straw. Compared to the control, this increase in butanol production was attributed to the enhancement of exoglucanase activity on lignocellulose degradation in experimental samples. The results showed that the co-culture system in conjunction with enhanced exoglucanase activity resulted in cost-effective butanol production from delignified rice straw.

1. Introduction

Biobutanol produced by *Clostridium* spp. can be used as biofuel and/or as chemical feedstock (Dürre, 2007). Lignocellulose, which is composed of cellulose, hemicellulose and lignin, is considered to be well suited for use as a cost-effective and sustainable substrate for the production of biobutanol, as it is both cheap and abundant around the world (Maki et al., 2009; Binod et al., 2010).

However, the utilization of lignocellulosic biomass for biobutanol production is limited by the inability of most butanol-producing clostridia to degrade cellulose, which is a major carbon component of lignocellulose. Thus, in order to achieve butanol production from lignocellulose, cellulose has to be hydrolyzed into di- or mono saccharides so that it can be utilized by the microorganisms. Although the cellulose can be hydrolyzed by cellulase, the hydrolysis of lignocellulose by the enzyme is limited by the presence of lignin, which physically inhibits access of cellulase to the cellulose fiber. To resolve this problem, delignification of the lignocellulose through acid, alkali, or thermochemical pretreatment, followed by hydrolyzation of cellulose, is required (Garcíaa et al., 2011; Baral et al., 2014). To date, butanol has successfully been produced from lignocellulosic materials after acid pretreatment and enzymatic hydrolysate (Qureshi et al., 2008; Amiri et al., 2014). However, delignification by acid pretreatment can produce inhibitors that affect the growth and butanol production capacity of butanol-producing clostridia (Pienkos and Zhang, 2009). In addition, in order to produce butanol cost-effectively, the hydrolysis step should be performed by cellulolytic microorganisms and not by commercial cellulase. This procedure is referred to as consolidated bioprocessing (CBP) and does not require the addition of cellulase enzyme (Lynd et al., 2005). This co-culture system has the potential

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for cost-effective consolidated bioprocessing because the cellulase secreted by *Clostridium thermocellum* hydrolyzes the cellulose in the rice straw and eliminates the need for a costly enzymatic hydrolysis step (Lynd et al., 2005).

We previously developed a co-culture system that used the cellulose-degrading *C. thermocellum* and butanol-producing *Clostridium saccharoperbutylacetonicum* that successfully produced butanol as a major fermentation product from crystalline cellulose (Nakayama et al., 2011, 2013). In those systems, the main component of cellulosic biomass, cellulose, was hydrolyzed by an endoglucanase, an exoglucanase, and a β-glucosidase, which originated from *C. thermocellum*, and the resulting hydrolysate was converted to butanol by *C. saccharoperbutylacetonicum*. These findings showed that co-culture of these strains can be used to produce butanol from lignocellulose cost-effectively as their system also eliminated the need for the addition of costly enzymes.

In this study, we aimed to use the co-culture system to produce butanol from rice straw, which is an abundant source of lignocellulose in East Asia (Rodríguez et al., 2008).

2. Materials

2.1. Bacterial strains and culture media

C. thermocellum NBRC103400 (ATCC 27405) was cultured anaerobically at 60 °C on National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC) medium 979 and *Clostridium saccharoperbutylacetonicum* strain N1-4 (ATCC 13564) was cultured anaerobically at 30 °C in TYA medium as previously described (Nakayama et al., 2011). The strains were cultured in test tubes sealed with a butyl rubber stoppers after substitution of the headspace with nitrogen gas.

2.2. Co-culture of C. thermocellum NBRC103400 and C. saccharoperbutylacetonicum strain N1-4

Co-culture experiments were performed in test tubes sealed with a rubber stopper as previously optimized method (Nakayama et al., 2011). The initial OD_{600nm} of *C. thermocellum* was adjusted to 1.0 and cultured at 60 °C for 24 h. After 24 h, the butanol-producing *C. saccharoperbutylacetonicum* strain N1-4 was collected by centrifugation, washed, suspended in TYA medium. After decreasing the incubation temperature to 30 °C, the cell suspension was added to the culture medium. The final OD_{600nm} of *C. saccharoperbutylacetonicum* strain N1-4 in the co-culture was adjusted to 1.0 and these co-cultures were incubated at 30 °C. Commercial cellulase derived from *Aspergillus niger* (Sigma–Aldrich Corp., St. Louis, MO) and commercial β-glucosidase (Sigma–Aldrich Corp.) derived from almond were added to the culture medium after *C. saccharoperbutylacetonicum* strain N1-4 was added to the *C. thermocellum* cultures.

2.3. Pretreatment of rice straw by sodium hydroxide

Rice straw was washed with water and cut into 2-cm lengths. Alkali-pretreated rice straw was produced by incubating rice straw in a seven-times volume of 1% (wt/vol) NaOH (14 g of rice straw per 1 L of 1% NaOH) at 100 °C for 1 h. The alkali-soaked rice straw was then washed in water until the pH was neutralized, followed by drying for a day at 60 °C. As a control, non-pretreated rice straw was soaked in 25-times volume of water and autoclaved for 30 min, and dried for a day at 60 °C. Dried alkali-pretreated and autoclaved rice straws were milled into a fine powder using a grinder (Labo Milser LM-PLUS, Osaka Chemical Co. Ltd., Japan) and sieved through a pass through 1.4 mm (0.055 in.) screen.

2.4. Analytical procedure

Fermentation products were quantified by HPLC (Aminex HPX-87H column, Bio-Rad Laboratories K.K., Japan) using a differential refractive index detector (Shimadzu Corporation, Japan) and an aqueous solution of 0.05 mM H_2SO_4 as the solvent at a flow rate of 0.7 ml/min. The lignin concentration was measured by the method of Bunzel et al. (2011).

2.5. Enzyme assays

The extracellular cellulase secreted by the *Clostridium* species was obtained as follows. *C. thermocellum* was cultured in NBRC979 medium containing cellobiose as a substrate at 60 °C, while *C. saccaroperbutylacetonicum* was cultivated in TYA medium at 30 °C. Both strains were cultivated for 24 h and centrifuged to remove cells. The crude extracellular enzyme was then collected by ultrafiltration of the culture medium supernatant (Amicon Ultra Ultracel-30 K; Millipore Ireland, Ltd., UK). The filtrate was then substituted with 50 mM Britton and Robinson's universal buffer (50 mM phosphoric acid, 50 mM boric acid, and 50 mM acetic acid: pH was adjusted to 5.0 with NaOH) (Kosugi et al., 2002). The protein concentration of each sample was measured by the Bradford method using Coomassie Protein Assay Reagent (Thermo Fisher Scientific, Inc., MA) and calculated using a standard curve plotted using bovine serum albumin.

Endoglucanase, exoglucanase, β-glucosidase, and xylanase were measured based on carboxymethyl cellulose (CMC) (WAKO pure chemical industries, Ltd., Japan), Avicel PH101 (Sigma–Aldrich Corp.), 4-nitrophenyl-β-D-glucopyranoside (Sigma–Aldrich Corp.), and beechwood xylan (Sigma–Aldrich Corp.) degradation activity at 30 °C. The enzymatic reaction was carried out using a modification of a method reported previously (Chundawat et al., 2011). One unit of specific activity of endoglucanase, exoglucanase, β-glucosidase, and xylanase activity was defined as one micromole of reducing sugars (as glucose equivalents) or 4-nitrophenol released per milligram protein per minute. A spectrophotometer (U-2001; Hitachi Ltd., Japan) was used for the enzyme assays.

3. Results and discussion

3.1. Improved butanol production using alkali-pretreated, delignified rice straw as a substrate

The co-culture of cellulolytic *C. thermocellum* and butanol-producing *C. saccharoperbutylacetonicum* was capable of producing 2.2 ± 0.2 g/L of butanol from non-pretreated rice straw for 14 days (Fig. 1A and B). However, the butanol titer was considerably lower than when Avicel cellulose was used as a reference substrate (7.9 g/L of butanol) (Nakayama et al., 2011), which does not contain lignin. The addition of 1000 U/g biomass of cellulase did not enhance the butanol titer (2.4 ± 0.1 g/L). One possible reason for the relatively low levels of butanol production typically observed in fermentation experiments is the presence of lignin, which may have limited the accessibility of the cellulase enzyme to the substrate and because lignin removal also promotes cellulase activity (Binod et al., 2010).

Alkali-pretreated rice straw was used as a substrate for confirming whether butanol production is improved by lignin removal because the lignin in plant biomass is known to be effectively removed by alkali-treatment, and that this delignification process improves saccharification of rice straw (Zhang and Cai, 2008; Rahnama et al., 2014). The lignin content of alkali-pretreated rice straw decreased from 23.3 ± 0.4% to 5.5 ± 0.1%, while that of non-pretreated rice straw remained at 24.1 ± 0.1%. As expected, the

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