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Cell recycle batch fermentation of high-solid lignocellulose using a recombinant cellulase-displaying yeast strain for high yield ethanol production in consolidated bioprocessing

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HIGHLIGHTS

- Cell recycling significantly reduces costs associated with inoculum preparation.
- Two-phase separation enabled cell recycling fermentation of lignocellulose.
- Cell-surface engineering successfully improved ethanol yield.
- Ethanol yield through the repeated batch fermentation was 86.3% of theoretical.

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ABSTRACT

The aim of this study is to develop a scheme of cell recycle batch fermentation (CRBF) of high-solid lignocellulosic materials. Two-phase separation consisting of rough removal of lignocellulosic residues by low-speed centrifugation and solid–liquid separation enabled effective collection of *Saccharomyces cerevisiae* cells with decreased lignin and ash. Five consecutive batch fermentation of 200 g/L rice straw hydrothermally pretreated led to an average ethanol titer of 34.5 g/L. Moreover, the display of cellulases on the recombinant yeast cell surface increased ethanol titer to 42.2 g/L. After, five-cycle fermentation, only 3.3 g/L sugar was retained in the fermentation medium, because cellulase displayed on the cell surface hydrolyzed cellulose that was not hydrolyzed by commercial cellulases or free secreted cellulases. Fermentation ability of the recombinant strain was successfully kept during a five-cycle repeated batch fermentation with 86.3% of theoretical yield based on starting biomass.

1. Introduction

Numerous environmental and social benefits are anticipated from the replacement of petroleum-based transport fuels with bioethanol converted from lignocellulosic materials such as agricultural residues and industrial waste. However, establishing an economically feasible process for industrial cellulosic ethanol production requires markedly increased ethanol titers after fermentation due to the high energy demands of the subsequent ethanol distillation (Galbe and Zacchi, 2007). In addition, ethanol production from lignocellulosic materials is complicated because multi-step processes are required for ethanol production from lignocellulosic material (Taherzadeh and Karimi, 2007).

Consolidated bioprocessing (CBP), which combines enzyme production, saccharification and fermentation in a single step, has been expected to simplify the ethanol production process. Whereas natural microorganisms capable of both efficient saccharification and fermentation are not currently available, recent development of recombinant microorganisms represents significant progress toward CBP (Hasunuma and Kondo, 2011). The predominant way for engineering CBP microorganisms is to express genes encoding cellulolytic enzymes in the yeast *Saccharomyces cerevisiae*. Display of multiple cellulase components including endoglucanase, cellobiohydrolase and β-glucosidase on the yeast cell surface through genetic engineering has enabled direct conversion of cellulose to ethanol (Fujita et al., 2004; Yamada et al., 2011). Recently, high-yield ethanol production from high-solid lignocellulosic biomass was achieved by enhancement in the activity of cellulase displayed on the yeast cell surface in a drum-type rotary fermentation system (Matano et al., 2012).

Another strategy for effective ethanol production is cell recycle batch fermentation (CRBF), because cell recycling significantly reduces time and costs associated with inoculum preparation

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(Kavanagh and Whittaker, 1994; Shojaosadati et al., 1996). However, in the case of fermentation of lignocellulosic materials, it is impossible to collect only microbial cells by conventional cell collection procedures, because lignocellulosic residue, which is not utilized by microbes, is retained in the fermentation medium with yeast cells in the solid fraction after fermentation (Kiran et al., 2000; Ma et al., 2009; Suzzi et al., 1996). Lignin left as solid residue in the fermentation medium also inhibits cellulase activity. Therefore, the removal of lignin is required for the efficient saccharification of cellulose in the subsequent batch. Therefore, CRBF of lignocellulosic materials has not yet been realized in simultaneous saccharification and fermentation processes (Kiran et al., 2000; Ma et al., 2009; Suzzi et al., 1996).

In the present study, a two-phase separation consisting of rough removal of lignocellulosic residue and yeast cell collection has enabled cell recycling for the repeated batch fermentation of lignocellulosic materials. Moreover, cellulase display on the recombinant yeast cell surface improved ethanol yield from lignocellulose, which was successfully maintained during a five-cycle repeated batch fermentation.

2. Methods

2.1. Materials

A cellulosic material of hydrothermally pretreated rice straw was obtained as described previously (Matano et al., 2012). In order to determine its components, the cellulosic material was hydrolyzed by sulfuric acid (Sluiter et al., 2008). The composition of the cellulosic material was 43.0% (w/w) glucan, 2.0% (w/w) xylan, 42.3% (w/w) ash and lignin, and 12.7% (w/w) other materials.

2.2. Yeast transformation and enzyme activity assay

The plasmids used in yeast transformation are listed in Table 1. The plasmid for secretion of the *Aspergillus aculeatus* BGL gene (*BGL1*) was constructed as follows. A DNA fragment composed of the *TDH3* promoter, the secretion signal sequence of the *Rhizopus oryzae* glucoamylase gene, *A. aculeatus BGL1* and the *TDH3* terminator was amplified by PCR using pIBG13 (Katahira et al., 2006) as template with the primers 5′-aaggaaaaaagcggccgcaccagttctcacacggaacaccac-3′ and 5′-aaggaaaaaagcggccgctcaatcaatgaatcgaaaatgtcattaaaatag-3′. After digestion with *Not*I, the amplified fragment was ligated into pRS404 to yield pIWssBGL.

A DNA fragment that included the secretion signal sequence of *R. oryzae* glucoamylase and *Trichoderma reesei* endoglucanase (EG2) gene was amplified by PCR using plasmid pEG23u31H6 (Fujita et al., 2002) as template with the primers 5′-gctctagaatgcaactgttcaatttgccattgaaag-3′ and 5′-gctctagactactttcttgcgagacacgagctgac-3′. The amplified fragment was digested with *Xba*I and *Spe*I and cloned into pGK406 (Ishii et al., 2009) to yield pGK406-ssEG2. After digestion of pGK406-EG2 with *Apa*I and *Not*I, the resultant EG2 fragment was ligated into the pRS403 and pRS405 vectors (Yamada et al., 2010) to yield pGK403-ssEG2 and pGK405-ssEG2, respectively.

A DNA fragment composed of the *S. cerevisiae TDH3* promoter, the secretion signal sequence of the *R. oryzae* glucoamylase gene, the *T. reesei CBH2* gene, and the *TDH3* terminator was amplified by PCR using pFCBH2w3 (Fujita et al., 2004) as template DNA with the primers 5′-ccgctcgagaccagttctcacacggaacaccactaatgga-3′ and 5′-atagtttagcggccgctcaatcaatgaatcgaaaatgtcattaaa-3′. The amplified fragment was digested with *Not*I and then ligated into pGK403-ssEG2, pGK405-ssEG2, pGK406-ssEG2, pRS405, and pRS406 to yield pGK403-ssEG2-ssCBH2, pGK405-ssEG2-ssCBH2, pGK406-ssEG2-ssCBH2, pRS405-ssCBH2, and pRS406-ssCBH2, respectively.

The yeast transformants constructed in this study are summarized in Table 1. Transformation was performed as previously described (Matano et al., 2012). Cellulase activity was determined by hydrolysis of 5 g/L phosphoric acid-swollen cellulose (PASC) in 50 mM citrate buffer (pH 5.0) at 50 °C. PASC was prepared from Avicel PH-101 (Fluka Chemie GmbH, Buchs, Switzerland) as described previously (Fujita et al., 2004). After aerobic cultivation in YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/L dextrose) for 72 h at 30 °C, cells were pelleted by centrifugation at 6000*g* for 10 min and then washed once with distilled water. The yeast cells were resuspended in a reaction mixture with the optical density at 600 nm adjusted to 5.0. After the hydrolysis, the mixture was centrifuged at 20,000*g* for 5 min, and the amount of reducing sugar in the supernatant was measured using the Somogyi–Nelson method as described previously (Matano et al., 2012). For the determination of cellulase activity of culture supernatant, 4 ml of culture supernatant was mixed with 1 ml of 500 mM citrate buffer (pH 5.0) and 5 ml of 10 g/L PASC. The amount of reducing sugar in the mixture was measured as described above. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol reducing sugar per minute.

Table 1
Characteristics of the *S. cerevisiae* strains and plasmids used in yeast transformation.

Strain or plasmid	Description	Reference or source
S. cerevisiae strain		
NBRC1440	Wild type	NBRC[a]
NBRC1440 ΔHUWL	MAT α his3 leu2 trp1 ura3	Yamada et al., 2010
NBRC1440/B-EC3	NBRC1440 ΔHUWL (pIWBGL, pGK403EG2-CBH2, pGK405EG2-CBH2, pGK406EG2-CBH2)	Matano et al., 2012
NBRC1440/ssB-ssEC3	NBRC1440 ΔHUWL (pIWssBGL, pGK403-ssEG2-ssCBH2, pGK405-ssEG2-ssCBH2, pGK406-ssEG2-ssCBH2)	This study
Plasmid		
pIWssBGL	TRP1, secretion of BGL1	This study
pGK403-ssEG2-ssCBH2	HIS3, secretion of CBH2 and EG2	This study
pGK405-ssEG2-ssCBH2	LEU2, secretion of CBH2 and EG2	This study
pGK406-ssEG2-ssCBH2	URA3, secretion of CBH2 and EG2	This study

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2.3. Liquefaction and fermentation

Liquefaction of cellulosic material was performed in three 50 mL polypropylene tubes (Corning Inc., NY) for each experimental condition, which were set in a heat block (Thermo Block Rotator SN-06BN; Nissin, Tokyo, Japan). Cellulosic material with a dry weight (DW) of 2 g was mixed with 8 mL medium containing 10 g/L yeast extract, 20 g/L peptone, 50 mM citric acid buffer (pH 5.0), and cellulase (Novozymes Cellic CTec2; Novozymes Inc., Bagsvaerd, Denmark) at concentrations of 10 FPU/g-biomass by axially rotating the tube at 35 rpm under a controlled temperature of 50 °C. After 2 h liquefaction treatment, fermentation was initiated by the addition of yeast into the tube, which was closed with a silicon plug (AS ONE, Osaka, Japan) into which a hole had been bored using a disposable needle ($\varphi = 0.6$ mm) (Terumo Corp., Tokyo, Japan).

S. cerevisiae strains used for fermentation were propagated under aerobic conditions at 30 °C for 48 h in 500 ml of YPD medium. The yeast cells were collected by centrifugation at 4000*g* for 10 min at 4 °C, washed twice with distilled water, and then 2 ml