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Efficient ethanol production from corncob residues by repeated fermentation of an adapted yeast

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HIGHLIGHTS

- A robust yeast strain was obtained by adaptation to lignocellulosic hydrolysate.
- High ethanol titer and productivity were reached by repeated fed-batch fermentation.
- Ethanol was produced efficiently without external nutrient addition or detoxification.

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ABSTRACT

For economically feasible lignocellulosic ethanol production, it is crucial to obtain a robust strain and develop an efficient fermentation process. An earlier-screened yeast strain *Pichia guilliermondii* was adapted to corncob residues (CCR) hydrolysate and used for high titer ethanol production without any detoxification or external nutrient supplementation. With an optimized fed-batch strategy, the maximum ethanol titer and productivity reached 56.3 g/l and 0.47 g l^{-1} h^{-1}, respectively. To further increase the ethanol productivity, the fed-batch process was repeated three times with cell reuse, and the maximum ethanol titer and productivity reached 51.2 g/l and 1.11 g l^{-1} h^{-1}, respectively. The results demonstrated that the combination of fed-batch with repeated fermentation was effective in improving the fermentation efficiency and achieving high ethanol productivity from CCR. The reported system is considered promising for commercial production of bioethanol from biomass hydrolysate in the future.

1. Introduction

Due to the limitation of fossil fuel reserves and increasing concerns about carbon dioxide emission, bioethanol has become an alternative liquid fuel to gasoline (Geddes et al., 2011). For industrial production of bioethanol, lignocellulosic materials are the most abundant renewable feedstock (Almarsdottir et al., 2012; Bajwa et al., 2009). Corncobs are an abundant agricultural residue in China (Qu et al., 2006; Fang et al., 2010), and the hemicellulose fraction is extracted by dilute-acid pretreatment for xylose and xylitol production, but its remaining residues are usually burned as boiler fuels (Cheng et al., 2011). The latter cause environmental contamination and a net loss of energy (Cheng et al., 2010). In fact, corncob residues (CCR) are a potential feedstock for cellulosic ethanol production because of their high cellulose content (Qu et al., 2006). However, a bioethanol process with lower cost and higher productivity is urgently required for its industrial production from CCR hydrolysate (Liu et al., 2012).

During ethanol production using CCR as feedstock, sulfite or alkali pretreatment and washing procedures were used to remove the lignin fraction (Zhang et al., 2010; Liu et al., 2010; Cheng et al., 2011), which increased the cost and reduced the efficiency of the entire process. Also, unlike other biomass feedstocks, most lignocellulosic substrates are nutrient-deficient (Kadam and Newman, 1997). Therefore, external nutrient supplementation is usually unavoidable during hydrolysate fermentation (Lau et al., 2008). In laboratory studies, rich media containing up to 6 g/l yeast extract and 10 g/l peptone were used to supplement CCR hydrolysates (Cheng et al., 2011; Liu et al., 2012). Meanwhile, some compounds such as furfural, 5-hydroxymethyl furfural (5-HMF) and acetic acid are formed during pretreatment, and they may have inhibitory effects on the fermentation by various microorganisms (Bellido et al., 2011; Hawkins and Doran-Peterson, 2011). Detoxification is usually necessary for optimal fermentation of lignocellulosic hydrolysates (e.g., Zhang et al., 2013). Therefore, techniques

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for efficient ethanol production from non-detoxified CCR hydrolysates without external nutrient addition would have practical significance in reducing the cost and improving the economic feasibility of the bioprocess.

For lignocellulose-based ethanol production to be economically viable on an industrial scale, high ethanol titer (above 40–50 g/l) and high ethanol productivity (over 1 g l^{-1} h^{-1}) are required (Galbe et al., 2007). Fed-batch fermentations were proven effective to achieve an ethanol titer higher than 50 g/l in CCR fermentation (Zhang et al., 2010; Liu et al., 2010). However, the fermentation processes were of relatively long duration and the ethanol productivity was not high enough. To increase the fermentation productivity, repeated fermentation with cell recycle may be used to reduce time and costs required for inoculum preparation (Matano et al., 2013; Watanabe et al., 2012; Jin et al., 2012). However, there have been no reports on repeated fermentation with cell-recycling process for ethanol production from corncobs.

In this work, a previously self-screened yeast strain of *Pichia guilliermondii* was adapted to CCR hydrolysates for better performance in lignocellulosic ethanol production. Inoculum size was then optimized and a fed-batch strategy was applied to the hydrolysate fermentation without any detoxification or external nutrient supplementation. Finally, cell recycle combined with fed-batch fermentation was established to achieve highly efficient ethanol production. The obtained strain and established strategies in this study might be useful for commercial bioethanol production in the future.

2. Methods

2.1. Materials

CCR hydolysates were provided by Futaste Co., Ltd. (Yucheng, Shandong, China) and stored at room temperature. All chemicals used in the experiments were of analytical grade. A commercial enzyme mixture Novozyme NS 22074, provided by Novozyme Co., Ltd. (Beijing, China), was used for enzymatic hydrolysis.

2.2. Medium and cultivation

Yeast strains were maintained on agar plates which contained 10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, and 20 g/l agar. Flask culture was carried out in sterilized YPG medium which contained 3.0 g/l yeast extract, 5.0 g/l peptone, and 50 g/l glucose at 30 °C and 200 rpm.

Minimum mineral (MM) medium was used for seed culture in hydrolysate fermentation experiments, which contains (per liter): 5.0 g $(NH_4)SO_4$, 3.5 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 15.0 mg EDTA, 4.5 mg $CaCl_2{\cdot}2H_2O$, 4.5 mg $ZnSO_4{\cdot}7H_2O$, 3.0 mg $FeSO_4{\cdot}7H_2O$, 1.0 mg H_3BO_3, 0.4 mg $Na_2MoO_4{\cdot}2H_2O$, 0.3 mg $CoCl_2{\cdot}2H_2O$, 0.3 mg $CuSO_4{\cdot}5H_2O$, 0.1 mg KI, 50 μg D-biotin, 0.2 mg *p*-aminobenzoic acid, 1.0 mg nicotinic acid, 1.0 mg calcium pantothenate, 1.0 mg pyridoxine HCl, 1.0 mg thiamine HCl, and 25.0 mg myoinositol. Twenty gram per liter glucose was supplemented as the carbon source. All media were adjusted to pH 5.0 before inoculation.

2.3. Strain adaptation

An inhibitor tolerant strain of *P. guilliermondii*, which was isolated in our laboratory (Zou et al., 2010), was used in the adaptation experiments. Evolutionary adaptation was conducted in CCR hydrolysates without external nutrient addition. Strains were transferred in increasing proportion (25–100%, in increments of 25%) of CCR hydrolysates as the procedure described previously with *Pichia stipitis* (Stoutenburg et al., 2011). The inoculum size of each transfer to the next hydrolysate medium was 2% (v/v). Colonies that grew faster on plates (25% hydrolysate (v/v) with 20 g/l agar) were selected and transferred to a higher concentration hydrolysate. Finally, the adapted strain was cultured in 100% CCR hydrolysate and stored in 20% glycerol at −80 °C.

2.4. Enzymatic hydrolysis and CCR hydrolysate concentration

Before enzymatic hydrolysis, the CCR hydrolysates were milled by grinder to a particle size range of 0.45–0.9 mm (20–40 meshes) and sterilized at 115 °C for 30 min. Water content of CCR was determined by weighing the CCR before and after drying at 65 °C for sufficient time. Enzymatic hydrolysis was performed in 250 ml baffle flasks with 60 ml sterile citrate buffer (50 mM, pH 5.0). The initial solid loading was set at 18% (18 g dry CCR/100 ml) with cellulase loading of 15 FPU/g. Flasks were incubated on a rotary shaker (200 rpm) at 50 °C for 72 h. After enzymatic hydrolysis, the mixtures were filtered and the liquid fraction was collected. Hydrolysates were sterilized at 115 °C for 30 min and then used for ethanol fermentation.

Concentrated CCR hydrolysates were prepared by vacuum evaporation. The evaporation was carried out under vacuum at 0.5 bar and 65 °C for about 20 min. After evaporation, the concentrated hydrolysates were sterilized at 115 °C for 20 min and then used for fed-batch fermentation. Before concentration, there were 74.9 g/l glucose, 8.82 g/l xylose and 1.53 g/l acetic acid in CCR hydrolysates. After evaporation, the concentrations of glucose, xylose and acetic acid were increased to 255.2, 28.2 and 4.42 g/l, respectively.

2.5. Batch and repeated-batch fermentation

Seeds were cultured in MM medium for 24 h. Cells harvested by centrifugation (8000×*g*, 10 min) were washed twice with sterile deionized water and then transferred to 20 ml CCR hydrolysates in 100 ml self-designed fermentation flasks (Zou et al., 2010). Fermentation was conducted anaerobically at 30 °C with an inoculum size of 4 g DCW (dry cell weight)/l. No external nutrients were added into the fermentation medium for all experiments. Samples for metabolite analysis were taken every 12 h. After centrifugation at 10,000×*g* and 4 °C for 5 min, the resulting supernatant was filtered through 0.22 μm filters (Rephile Bioscience and Technology, Shanghai, China) and stored at −20 °C for the determination of glucose, xylose, ethanol and acetic acid concentrations.

Repeated-batch hydrolysate fermentation was also performed anaerobically at 30 °C with non-enriched CCR hydrolysates. The inoculum size of the first cycle was set as 30 g/l. After 16–18 h of batch fermentation described above, cells were harvested by centrifugation at 8000×*g* for 10 min. The collected cells were then transferred into fresh hydrolysate to start a new cycle of fermentation. The process was sequentially repeated five times. Samples were taken at regular intervals.

Triplicate experiments were carried out for all experiments and error bars represent the standard deviation from the mean. Statistical analysis was carried out by students *t*-test with $p < 0.05$ considered as statistically significant.

2.6. Fed-batch and repeated fed-batch fermentation

For fed-batch hydrolysate fermentation, the inoculum size was set as 30 g/l. Initially, 16 ml CCR hydrolysates were added into shake flasks. After 14 h of fermentation, different feeding strategies with concentrated CCR hydrolysates were tested. Samples for metabolite analysis were taken at regular intervals.

Repeated hydrolysate fermentation with fed-batch strategy was also performed in shake flasks containing 16 ml hydrolysate with