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Simultaneous saccharification and fermentation of hemicellulose to butanol by a non-sporulating *Clostridium* species

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HIGHLIGHTS

- Simultaneous saccharification and fermentation of xylan to butanol by new strain MF28.
- Simultaneous fermentation of glucose and xylose to produce 11.9 g/L butanol.
- High yields of butanol on xylan and xylooligosaccharide were obtained.
- No acetone/ethanol byproducts were formed in converting lignocellulose to butanol.
- Non-sporulating strain MF28 is capable of continuous industrial-scale fermentation.

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ABSTRACT

Production of lignocellulosic butanol has drawn increasing attention. However, currently few microorganisms can produce biofuels, particularly butanol, from lignocellulosic biomass via simultaneous saccharification and fermentation. Here we report discovery of a wild-type, mesophilic *Clostridium* sp. strain MF28 that ferments xylan to produce butanol (up to 3.2 g/L) without the addition of saccharolytic enzymes and without any chemical pretreatments. Application of selective pressure from 2-deoxy-D-glucose facilitated isolation of strain MF28, which exhibits inactivation of genes (*gid* and *ccp* genes) responsible for carbon catabolite repression, thus allowing strain MF28 to simultaneously ferment a combination of glucose (30 g/L), xylose (15 g/L), and arabinose (15 g/L) to produce 11.9 g/L of butanol. Strain MF28 possesses several unique features: (i) non-sporulating, (ii) no acetone/ethanol, (iii) complete hemicellulose-binding enzymatic domain, and (iv) absence of carbon catabolite repression. These unique characteristics demonstrate the industrial potential of strain MF28 for cost-effective biofuel generation from lignocellulosic biomass.

1. Introduction

Biobutanol produced from renewable biomass is a promising way to solve a growing demand for energy and the risks of climate change from increasing greenhouse gas emissions (Morone and Pandey, 2014). However, substrate cost remains a major factor influencing the economic viability of fermentative butanol production, accounting for up to 50% of the production cost (Green, 2011). Therefore, transition toward cheaper (non-edible) feedstocks offers the biggest opportunity for cost reduction and improved sustainability (Guan et al., 2016). Among the different types of biomass available, xylan, which is the second most abundant natural repository of xylose on Earth, is an attractive substrate for butanol production by microbial processes (Deutschmann and Dekker, 2012). Current industrial bioconversion of lignocellulosic biomass to biofuels, mainly ethanol, relies on separate hydrolysis process prior to fermentation, and requires extensive pretreatments, addition of costly enzymes, and detoxification to remove hydrolysate inhibitors generated from lignin and sugar degradation. Therefore, the development of consolidated bioprocessing (CBP) technology for converting cellulosic biomass into biofuels without adding enzymes is of high interest for economical biofuel production. However, the recalcitrance of hemicellulosic materials and the inability of microorganisms to efficiently ferment biomass hydrolysates still prevent commercial production of biofuels.

Development of engineered microbes or wild-type microbes for achieving saccharification and fermentation in a single bioreactor

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has a great potential for value-added products production from cellulose (Sizova et al., 2011; Yang et al., 2015), while most of the reported studies using hemicellulose compounds of lignocellulosic biomass as a fermentation substrate have been focused on ethanol or hydrogen production rather than on butanol (Tolonen et al., 2011). However, few wild-type strains are known to produce butanol from cellulose or xylan via simultaneous saccharification and fermentation, leaving a need for development of one-step strategies for biobutanol production from lignocellulosic materials. Furthermore, inefficient co-fermentation of the three major reducing sugars (i.e., glucose, xylose and arabinose) derived from lignocellulosic biomass typically leads to incomplete sugar consumption due to carbon catabolite repression (CCR) (Yu et al., 2015). Poor butanol production from lignocellulosic biomass is thereby a common phenomenon observed in solventogenic *Clostridium* species. The aim of this study is to discover novel species exhibiting high productivity and yield when converting hemicellulosic xylan into butanol, while also being resistant to the effects of carbon catabolite repression and easy to cultivate for industrial applications. Finding a bacterium possessing a broad range of hydrolytic and solventogenic enzymes is, therefore, a potential pathway to fulfill the needs of economic conversion of biomass to butanol.

This study describes the characterization of a novel, mesophilic, wild-type *Clostridium* sp. strain MF28 capable of simultaneous saccharification and fermentation of hemicellulose and raw plant biomass to butanol in a consolidated process. Strain MF28 can also ferment both glucose and xylose simultaneously without exhibiting carbon catabolite repression. An unusual repressor gene in strain MF28 disables the expression of a crucial sporulation initiation gene cluster in the cell, making continuous or semi-continuous (fed-batch) industrial-scale fermentation possible.

2. Materials and methods

2.1. Media and cultivation

Cultures were grown and maintained in anaerobic, MES-buffered (20 mM) mineral salts medium BCM1 (biobutanol clostridial medium 1) containing $MgCl_2{\cdot}6H_2O$ (0.3 g/L), KH_2PO_4 (0.75 g/L), K_2HPO_4 (0.75 g/L), NH_4Cl (0.3 g/L), KCl (0.3 g/L), $CaCl_2{\cdot}2H_2O$ (0.015 g/L), and NaCl (1 g/L), and reduced by addition of L-cysteine (0.2 mM), $Na_2S{\cdot}9H_2O$ (0.2 mM) and DL-dithiothreitol (0.5 mM). 1 ml trace element mixture, 1 ml selenite-tungstate solution, and 0.1 ml resazurin solution were added aseptically per liter of medium (Li et al., 2014). Bottles (160 ml) containing 3% (wt/vol) of xylan were filled with 50 ml medium and sealed with butyl stoppers, autoclaved for 20 min, cooled to room temperature, and supplemented aseptically with 1 ml yeast extract solution (150 g/L) at a final concentration of 3 g/L. The pH was adjusted to 6.5. Agar plates (2%) were prepared with the same medium but containing 1% (wt/vol) xylan as the sole carbon and energy source. Agar plates were stored in an anaerobic chamber (Coy, USA) with a gas mixture of 85% N_2, 10% H_2 and 5% CO_2. All chemicals were reagent grade and were obtained from Sigma (St. Louis, MO), unless indicated otherwise. Gases (air, nitrogen, helium, hydrogen, and nitrogen-hydrogen-CO_2 mixture) were supplied by National Oxygen Pte Ltd (Singapore). For carbon assimilation and bioconversion kinetics studies, all cultures (triplicate for each experiment) were incubated in moderate temperature (37 °C) on a rotary shaker at 150 rpm, and un-inoculated controls were included to monitor potential non-biological activity. For substrate assimilation tests, actively growing MF28 cells (OD of ~2) of 3 ml were inoculated into the above mentioned medium (with a final total volume of 50 ml) supplemented with 6% (wt/vol) of various carbon sources, as indicated, and maintained under identical conditions. Substrate assimilation tests were performed in biological triplicates and repeated at least twice to confirm results.

2.2. Enrichment of butanologenic microorganisms capable of assimilating xylan

The inocula for butanologenic microorganism screening were distinct aged spent mushroom substrate samples collected from a local farm. Enrichment was first carried out in 160 ml serum bottles filled with 50 ml anaerobic BCM1 medium containing xylan (3%, vol/wt) as the sole carbon source, and incubated for 5 days at 37 °C with a shaking speed of 100 rpm. After performing three subcultures of microorganisms tested, the resulting butanologenic culture (designated culture MF) was identified through detection of butanol in the medium.

2.3. Selection and isolation of xylan-assimilating butanologenic bacterium with resistance to carbon catabolite repression

Prior to isolation, enrichments in xylan-supplemented BCM1 medium were subjected to 2-deoxy-D-glucose (2-DG) (0.7 g/L) for 3 days in order to select a strain with resistance to 2-DG, thereby eliminating catabolite repression. Following three subcultures into fresh BCM1 medium, the culture demonstrating successive butanol production was spread directly on agar plates and incubated anaerobically at 37 °C for three days. Colonies arising on the plates were selected, re-streaked for further purification, and re-tested for butanol production and cell growth.

2.4. Conversions of xylan and plant biomass to butanol

Fermentation experiments with xylan as a substrate were carried out in a 3 L BIOSTAT® B plus bioreactor (Sartorius, Germany) (equipped with redox potential, temperature and pH probes) containing 1.5 L (working volume) of BCM1 medium at an agitation speed of 100 rpm and at 37 °C according to the cultivation method described previously (Li et al., 2014) with slight modifications. Briefly, 90 mL seed culture (OD = ~2) was inoculated into the bioreactor (6%, v/v). The initial pH of the medium was 6.2, and was allowed to drop to 5.3 as the culture progressed. Subsequently, the pH was automatically maintained at or above 5.3 by addition of 6 M NaOH or 3 M H_2SO_4. Similar experiments with autoclaved plant biomass (switchgrass, corncob and hardwood) as a substrate in BCM1 were conducted as described above. Plant biomass was washed and ground to 80–120 mesh size powder prior to autoclaving. All experiments were performed in biological triplicates without any other pretreatment of biomass.

2.5. Adherence assay

Insoluble xylan was prepared as described previously (Irwin et al., 1994). Briefly, beechwood xylan (5 g) was suspended in 100 ml of deionized water. The mixture was then adjusted to pH 10.0 using 6 M NaOH, and stirred at 100 rpm for 1 h at room temperature. The pellets were harvested by centrifugation at 3000×*g* for 10 min and resuspended in deionized water (adjusted to pH 7.0 using 1 M acetic acid) followed by washing twice with 10 volumes of deionized water. The resulting pellets were filtered with Whatman No. 1 paper and dried at 60 °C. An adhesion assay of cells grown on insoluble xylan was performed as described previously (Bayer et al., 1983) with slight modification. *In vitro* assays were performed inside an anaerobic chamber. Cells used for adherence tests were prepared by centrifugation at 8000 *g* for 5 min at the appropriate time of growth and washed 3 times with PBS (pH