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Enhanced butanol production by coculture of *Clostridium beijerinckii* and *Clostridium tyrobutyricum*

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HIGHLIGHTS

- Free and immobilized-cell coculture greatly improved butanol productivity.
- The highest ABE productivity was obtained in continuous immobilized-cell coculture.
- This continuous coculture may be suitable for industrial ABE production.

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ABSTRACT

Cocultures of *Clostridium beijerinckii* and *Clostridium tyrobutyricum* in free-cell and immobilized-cell fermentation modes were investigated as a means of enhancing butanol production. The immobilized fermentation was performed in a fibrous-bed bioreactor (FBB). The results demonstrated that two-strain coculture significantly enhanced butanol production, yield and volumetric productivity compared with those in pure culture with or without butyric acid. Further, continuous immobilized-cell cocultures in two FBBs using glucose, cassava starch, or cane molasses were conducted at a dilution rate of 0.144 h^{-1}. The butanol production (6.66 g/L), yield (0.18 g/g), and productivity (0.96 g/L/h) were obtained with cassava starch as the substrate. Meanwhile, the acetone–butanol–ethanol (ABE) yield (0.36 g/g) was the highest among all processes investigated, suggesting that this continuous coculture mode may be suitable for industrial ABE production with no need for repeated sterilization and inoculation.

1. Introduction

Butanol is an important chemical of substantial industrial interest, as it can be used as a solvent in the production of hormones, drugs, antibiotics, cosmetics, hydraulic fluids, and vitamins (Lee et al., 2008). It is also a potential fuel or fuel additive and has advantages compared to ethanol: it has a higher energy content and lower volatility and is less hydroscopic, less corrosive, and less flammable (Qureshi and Ezeji, 2008). It was reported that an important worldwide market for butanol of 2.9 billion pounds had developed in the United States by 2007 (Ezeji et al., 2007). Historically, it has been produced mainly by the petrochemical industry; however, with increasing oil prices and the depletion of fossil fuels, bio-butanol has become an economic, attractive, and sustainable biofuel (Rude and Schirmer, 2009).

Despite the remarkable advantages of butanol as a fuel, currently there are some limitations to bio-butanol production, including low yield, low volumetric productivity, and low final product concentration, as well as degeneration of the butanol-producing strains (Dürre, 1998; García et al., 2011). Many strategies have been adopted to reduce the costs of acetone–butanol–ethanol (ABE) fermentation, including the use of inexpensive substrates, development of microbial hyperproducing strains, and optimization of fermentation conditions to improve the efficiency of converting substrate to ABE. Significant progress has been made towards genetically engineering clostridia to utilize a variety of substrates and to reduce the need for pretreatment processes (Dürre, 1998; Papoutsakis, 2008).

Butanol fermentation (so-called ABE fermentation) possesses two distinct characteristic phases, acidogenesis and solventogenesis (Al-Shorgani et al., 2012; Kumar and Gayen, 2011). Many factors are thought to be related to the transition between acidogenesis and solventogenesis, including extracellular or intracellular pH, acid content, nutrient limitation, temperature, available oxygen (Jones and Woods, 1986), and so on.

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The conventional feedstocks for ABE fermentation are corn, millet, wheat, and rye (Jones and Woods, 1986). As the cost of feedstock is a major factor determining the total economics of the ABE fermentation industry, research has focused on alternative inexpensive substrates, including corn stover, thin stillage, switchgrass, wood powder, cassava flour, and molasses (Ahn et al., 2011; Lee et al., 2009; Lépiz-Aguilar et al., 2011; Qureshi et al., 2010a,b; Wang and Chen, 2011). Among these, cassava starch and molasses are probably two of the most economically feasible choices due to low cost and availability.

Different processes have been adopted for ABE fermentation: batch fermentation, fed-batch fermentation, free-cell continuous fermentation, continuous fermentation with immobilized cells, and cell recycling and bleeding (Ezeji et al., 2007; Qureshi et al., 2008; Tashiro et al., 2004, 2005). Although most products in industrial biotechnology are produced by only one bacterium, coculture fermentation has also frequently been employed. Generally, coculture was defined as anaerobic or aerobic incubation of different specified microbial strains under aseptic conditions and it seems to be advantageous compared to pure culture fermentation due to the potential for synergistic utilization of the metabolic pathways of all involved microorganisms in a coculture system. Thus, it may result in increased yield, improved product qualities, and more efficient substrate utilization. It has been widely used in the production of bio-chemicals, biofuel, biopolymers, and enzymes (Bader et al., 2010).

Clostridium tyrobutyricum was used as butyric acid producer in several studies (Jiang et al., 2009; Zhu et al., 2002) due to its simple medium requirements for strain growth and relatively high product yield (Michel-Savin et al., 1990a,b). *C. beijerinckii* can use butyric acid as a substrate to produce butanol/ABE and, thus, it is usually employed in butanol/ABE production (Huang et al., 2004).

The aim of this work was to use *C. tyrobutyricum* ATCC 25755 in a coculture system to produce butyric acid, instead of adding butyric acid to the medium, so that the butyric acid generated can be utilized by *C. beijerinckii* ATTCC 55025 to produce butanol. The coculture system was studied in free-cell and immobilized-cell modes, and subsequently continuous immobilized-cell coculture was performed by using different inexpensive substrates. The results showed that this coculture system can significantly improve butanol yield and volumetric productivity and the continuous immobilized-cell coculture has the potential for industrial butanol production with no need for repeated sterilization and inoculation.

2. Methods

2.1. Strains and medium

C. beijerinckii ATCC 55025, an asporogenic strain, was purchased from American Type Culture Collection. The stock culture was maintained in the form of a cell suspension in 25% (v/v) sterile glycerol at −80 °C. The organism was grown in a Reinforced Clostridial Medium (Oxoid CM149) (3.0 g/L yeast extract, 10.0 g/L "Lab-Lemco" powder, 10.0 g/L peptone, 1.0 g/L soluble starch, 5.0 g/L glucose, 0.5 g/L cysteine hydrochloride, 5.0 g/L sodium chloride, 3.0 g/L sodium acetate, and 0.5 g/L agar) for 10–12 h at 37 °C before inoculation.

C. tyrobutyricum ATCC 25755 was supplied by Professor Shang-Tian Yang from Ohio State University and maintained in anaerobic serum bottles on *Clostridium* growth medium at 4 °C (Huang and Yang, 1998). The bacteria were then cultured in Reinforced Clostridial Medium (RCM) (Huang et al., 1998) for 12–24 h at 37 °C before inoculation.

P2 medium was used for the fermentation [glucose: 50 g/L; stock solution: (buffer: KH_2PO_4 50 g/L, K_2HPO_4 50 g/L, ammonium acetate 220 g/L; minerals: $MgSO_4{\cdot}7H_2O$ 20 g/L, $MnSO_4{\cdot}H_2O$ 1 g/L, $FeSO_4{\cdot}7H_2O$ 1 g/L, NaCl 1 g/L; vitamins: para-aminobenzoic acid 0.1 g/L, thiamin 0.1 g/L, biotin 0.001 g/L)]. The medium was sterilized at 121 °C for 20 min and the stock solution was sterilized by filtration. After cooling the medium to room temperature, 10 mL of each stock solution were added to 1000 mL of medium. After filtration, butyric acid was added to the sterilized P2 medium to obtain a butyric acid reinforced medium. When inexpensive biomass was used as the substrate, glucose was replaced by hydrolysate of cassava starch/cane molasses in P2 medium.

2.2. Cassava starch and cane molasses hydrolysis

Cassava starch powder was bought from a market (Guangzhou, China). The powder was hydrolyzed by 0.25 M HCl with a solid-to-liquid ratio of 1:10 (w/w) at 121 °C for 45 min. After centrifugation at 4500 rpm for 30 min, the pH value of the supernatant was adjusted to 6.5 with NaOH particles. The concentration of glucose in the cassava starch hydrolysate was 81 g/L at the end of the hydrolysis.

Cane molasses was obtained from Jiang-men sugar refinery (Guangdong, China), and its components have been described elsewhere (Jiang et al., 2009). The crude molasses was diluted with distilled water to a final sugar concentration of 60 g/L. For acid hydrolysis, the pH of the molasses solution was adjusted to 2 with concentrated HCl and incubated at 60 °C for 24 h. The pH value of the supernatant was adjusted to 6.5 with NaOH particles after centrifugation at 4500 rpm for 30 min. At the end of the hydrolysis, the concentrations of glucose and fructose in the solution were 29 and 43 g/L, respectively.

2.3. Free-cell and immobilized-cell fermentations

The free-cell fermentation was carried out in a 5-L bioreactor (BIOSTA A plus, Sartorius Stedim Biotech, Germany) with a working volume of 2 L. During the fermentation, the temperature was maintained at 37 °C, the pH was controlled at 5.0 by the addition of 6 M NaOH, and the mixture was agitated at 150 rpm. High purity nitrogen was introduced at the bottom of the bioreactor to maintain anaerobic conditions. The inoculum of each strain was 5% (V/V) in the pure culture and the coculture fermentation.

The immobilized-cell fermentation culture was carried out in a 5-L bioreactor (BIOSTA A plus, Sartorius Stedim Biotech, Germany), which was connected to a 500-mL fibrous-bed bioreactor (FBB) and recirculated via a loop. The FBB was made of a jacketed glass column packed with a spirally wound cotton towel (35 × 40 cm; ~5 mm thickness; with >95% porosity), and a detailed description of the bioreactor construction has been given elsewhere (Jiang et al., 2009). This fermentation was conducted under the same conditions as the free-cell culture. At the beginning of the fermentation, 100 mL of cell suspension in the exponential phase was inoculated into the fermentor containing 2 L P2 medium (at a ratio of 5%, V/V). When the cell density reached ~4 (OD_{600}), cell immobilization was initiated by turning on the peristaltic pump and lasted for 24–48 h. In the coculture fermentation, *C. tyrobutyricum* grew as free cells. When the concentration of butyric acid in the fermentation broth reached about 4 g/L, the FBB immobilized with *C. beijerinckii* was connected to the fermentor and began circulating with a pumping rate of 80 mL/min. Samples were taken at time intervals during the fermentation.

2.4. Continuous immobilized-cell fermentation

The continuous immobilized-cell fermentation system was composed of a medium tank, two FBBs (FBB-A and FBB-B), and a product tank. Fresh medium was stored in the medium tank and