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Efficient acetone–butanol–ethanol (ABE) production by a butanol-tolerant mutant of *Clostridium beijerinckii* in a fermentation–pervaporation coupled process

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ABSTRACT

Butanol inhibition is one of the major obstacles limiting the economic viability of acetone–butanol–ethanol (ABE) fermentation. In this study, a butanol-tolerant mutant (*Clostridium beijerinckii* BT14) was generated by atmospheric and room temperature plasmas (ARTP). This mutant showed significant advantage over its parent strain in terms of butanol tolerance. Compared to its parent strain, batch fermentation by this mutant produced 25% higher butanol and 33% higher ABE solvents due to its efficient generation of intracellular NADH and high NADH-dependent butanol dehydrogenase activity. Furthermore, *C. beijerinckii* BT14 was applied to fed-batch fermentation with pervaporation (PV). As a results, *C. beijerinckii* BT14 grew to a high cell density and this process generated highly concentrated ABE solution with a high solvent productivity of 0.98 g/(L h) and glucose consumption rate of 2.64 g/(L h). Thus, this work provides an appropriate strategy to develop an efficient process for ABE production in the PV coupled fermentation.

1. Introduction

Butanol is an important industrial chemical with many applications in paints, polymers and plastics industries [8] and has recently received special attention as a renewable resource fuel alternative because of its attractive attributes, including high energy content, low vapor pressure, less corrosiveness, and ability to be mixed with gasoline and diesel oil in high proportions [2,14]. However, one of the major obstacles limiting the economic viability of ABE fermentation is the low butanol concentration caused by butanol inhibition. Since butanol producing strains cannot tolerate more than 2% butanol [24], simultaneous removal of solvents from fermentation broth has been employed to relieve product inhibition [5,7,28]. Among all the in situ removal techniques, pervaporation is a separation process where a binary or multi-component liquid mixture is separated by partial vaporization through a membrane and is regarded as a promising process for separating ABE solvents [15]. The butanol concentration in fermentation broth could be controlled at the level ranging from 4 to 9 g/L by pervaporation [31,33], but inhibition from butanol can be noticed at concentrations as low as 5 g/L in ABE fermentation [25]. Although pervaporation could relieve the inhibition from butanol, cells still suffer greatly, which could be seen from the low solvent productivity in PV coupled fermentations [4,10]. Thus, efforts have been taken to develop new pervaporation membrane with better performance. Silicalite-1 filled polydimethylsiloxane (PDMS)/polyacrylonitrile (PAN) composite membrane has been applied in ABE fermentation and the solvent productivity reached 0.51 g/(L h) in batch ABE fermentation [16]. Solvent productivity increased to 0.58 g/(L h) when using a poly ether-block-amide (PEBA)/carbon nanotubes (CNTs) membrane with high butanol flux [34]. However, the butanol removal efficiency of these membranes was still less than butanol production rate, which led to accumulation of butanol in fermentation broth and consequently cause inhibition. Therefore, the butanol tolerance of solventogenic *clostridia* should be improved.

Recently, traditional random mutagenesis and rational metabolic engineering have been applied to develop butanol-tolerant strains [11,23,30]. The atmospheric and room-temperature plasmas (ARTP) mutation system is becoming

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increasingly popular owing to the lower capital costs, low plasma temperature and flexible operations [22]. ARTP can generate diverse breakages of plasmid DNA and oligonucleotides with variations in plasma dosage [17], thus ARTP can be used as a rapid mutation tool for microbial breeding. The ARTP mutagenesis has been successfully applied in obtaining mutant of *Mortierella alpina* with high arachidonic acid production [20], *Escherichia coli* with increased substrate ultilization [13] and *Enterobacter aerogenes* with increased intracellular ATP level [9].

In the present work, a butanol-tolerant mutant, *Clostridium beijerinckii* BT14, was generated by ARTP mutagenesis. This mutant showed significant advantage over its parent strain in terms of solvent production and butanol tolerance. Cell density and ABE solvent production by *C. beijerinckii* BT14 during fed batch fermentation coupled with pervaporation were investigated. ABE solvents were highly concentrated and solvent productivity was significantly improved, suggesting that PV coupled fermentation with a butanol-tolerant strain could be helpful to develop an efficient process for ABE production.

2. Materials and methods

2.1. Strain and culture conditions

C. beijerinckii NCIMB 8052 was used in screening of butanol tolerant mutants. The cells of *C. beijerinckii* NCIMB 8052 were cultured in yeast extract/peptone/starch (YPS) medium composed of (per litre): 3.0 g yeast extract, 5.0 g peptone, 10.0 g soluble starch, 2.0 g CH_3COONH_4, 2.0 g NaCl, 3.0 g $MgSO_4{\cdot}7H_2O$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4 and 0.1 g $FeSO_4{\cdot}7H_2O$. The initial pH of culture medium was adjusted to 6.0 with 1 M HCl, then sterilized at 121 °C for 15 min and cooled to room temperature before inoculation. Solid medium for plates contained 1.5% agar. The inoculation level was controlled at 5% (v/v) in all the seed culture progress. After inoculation, the culture medium was purged with filtered nitrogen gas to remove dissolved oxygen. Cells were cultured at 35 °C for 12 h to the exponential phase and used as seed culture. The production medium used in ABE fermentation composed of (per litre): glucose 40.0 or 60.0 g, K_2HPO_4 0.50 g, KH_2PO_4 0.50 g, CH_3COONH_4 2.2 g, $MgSO_4{\cdot}7H_2O$ 0.20 g, $MnSO_4{\cdot}H_2O$ 0.01 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, NaCl 0.01 g, corn steep liquor 1 g. In all experiments, production medium was inoculated with a 10% (v/v) actively growing seed culture and was purged with filtered nitrogen gas to remove dissolved oxygen.

2.2. Screening of butanol tolerant mutants

The ARTP machine was from Tsinghua University [17]. 10 μl of the actively growing *C. beijerinckii* NCIMB 8052 (OD_{600} = 0.8–1.0) was dropped onto a sterilized stainless-steel plate (12 mm in diameter). The stainless-steel plate was placed into the vessel approximately 2 mm away the torch nozzle exit and the vessel. Helium gas flow rate of 10 standard liters per minute and radio-frequency power input of 100 W was used. After mutation, the stainless-steel plate was transferred to a test-tube containing 200 μl sterile saline and shaken for 30 s to resuspend cells. 50 μl of resuspend cells were spread over a selective agar plate containing 20 g/L butanol, which is the highest level of butanol produced by *C. beijerinckii* strains. Resazurin (a commonly used redox indicator) was added to the selective agar plate to screen mutants with strong deoxidizing activity. The mutagenic cells on sealed plates were cultured in an anaerobic workstation (Bug box; RUSKINN, UK, N_2:H_2:CO_2 = 8:1:1). Colonies that showed large haloes on the selective agar plate were selected for further study.

2.3. Batch fermentation

Batch fermentation was performed in a 5 L fermenter (Bioflo 110, USA) with a working volume of 2 L. After inoculation of 200 ml secondary seed culture, filtered nitrogen gas was bubbled through the production medium for 15 min to remove oxygen. The temperature of fermentation broth was maintained at 35 °C and the agitation speed was controlled at 150 rpm. Samples were taken regularly and centrifuged at 8000 rpm for 10 min. Supernatants were used for the analysis of ABE and glucose. All batch fermentations were triplicated, and averages of parameters were reported.

2.4. Fed batch fermentation with pervaporation

The PDMS/ceramic composite membrane used in this study was from the State Key Laboratory of Materials-Oriented Chemical Engineering, Nanjing Tech University. Pervaporation performance of the PDMS/ceramic composite membrane was tested in ABE mode solution and fermentation broth as described previously [18]. Fed batch fermentation with pervaporation was carried out in a glass fermenter with a working volume of 1 L. Concentrated production medium containing 300 g/L glucose was used for feed medium. The membrane was sterilized at 121 °C for 15 min before operation. The flow rate of fermentation broth was fixed at 15 L/h by a peristaltic pump (Longer BT300, China) during PV, and the permeation pressure was less than 400 Pa. Permeates were collected in a cold trap using liquid N_2 while the retentate was returned to fermenter. Samples were taken from the fermenter and permeate for the analysis of cell density, ABE solvents and glucose.

2.5. Analytical methods

The optical density at 600 nm (OD_{600}) was measured to monitor cell growth. Glucose was analyzed by an SBA-40C biosensor analyzer (Shandong Province, Academy of Sciences, China). Fermentation products (ABE) were quantified by gas chromatography (GC-2010, Shimadzu Scientific Instruments, Japan, equipped with a flame ionization detector and an InterCap WAX column (0.25 mm × 30 m, GL Sciences Inc., Japan). Isobutanol was used as an internal standard. If the permeate separated into two phases, the permeate sample was diluted with deionized water to one phase prior to injection. Total solvent was the sum of acetone, butanol and ethanol.

The intracellular concentrations of NADH and NAD^+ were determined using a cycling method [19]. The details about this method can be found in our recent work [12]. The NADH-dependent butanol dehydrogenase (NADH-BDH) was measured by procedures as described [27]. Cells were harvested and centrifuged at 12,000 rpm, 4 °C for 20 min. Cell pellets were resuspended in PBS buffer (50 mM, pH 7.2) containing 1 mM of reducing agent dithiothreitol (DTT). Cells were disrupted by ultrasonication on ice for 15 min with the following conditions: 5 s of sonication with a 10 s interval, set at 50% amplitude. After ultrasonication, the resulting lysate was centrifuged at 14,000 rpm at 4 °C for 20 min to remove cell debris. The supernatant was then used for enzyme analysis. The total protein concentration was measured using the Bradford method [3].

Enzyme substrates including butyraldehyde and butanol, cofactors were procured from Sigma–Aldrich. NADH-BDH activity was measured in the forward (butanol formation) direction at 25 °C. To measure the NADH-BDH activity, 2 mM of butyraldehyde and 0.5 mM of NADH in Tris–HCl buffer (50 mM, pH 6.0) were used. Final reaction volume was 3.0 ml and it contained 10% (by volume) of cell lysate except the blank control. The reaction was initiated by the addition of cell lysate, and the NADH-BDH activity was assayed by monitoring the oxidation of NADH at 340 nm using a UV–vis spec-

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