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High biobutanol production integrated with *in situ* extraction in the presence of Tween 80 by *Clostridium acetobutylicum*

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ABSTRACT

This study investigates the improvement of butanol production in the presence – Tween 80 by *Clostridium acetobutylicum* NJ4. With supplementation of 15% Tween 80 (v/v), the final butanol titer could be significantly enhanced to 18.65 g/L from 70 g/L glucose, which is 38% higher than that in the control batch (13.47 g/L). With further *in situ* extraction using biodiesel, the butanol titer was finally improved to 33.90 g/L in the fed batch mode. The supplementation of Tween 80 could improve butanol tolerance of strain NJ4 up to 18 g/L compared to 10 g/L of control. Meanwhile, butanol dehydrogenase activity was improved to 3.89 U/mg, which is 80.93% higher than that of the control. These factors may attribute to the high butanol production. The finding of this study thus offers fundamental knowledge for the future development of alternative ABE production strategies.

1. Introduction

Butanol is not only an important chemical precursor for paints, polymers, and plastics, but also a promising next generation liquid fuel due to its superior characteristics over ethanol [1]. Biobutanol production through biological acetone-butanol-ethanol (ABE) fermentation process by solventogenic clostridia has gained increasing attention owning to concerns on the depletion of fossil fuels and environmental issues [2,3]. However, several obstacles impeded the scaling up application of fermentative butanol as an alternative fuel: (i) high substrate cost arising from the usage of edible biomass, (ii) low final butanol titer caused by the toxicity of butanol (< 15 g/L), and (iii) high cost for butanol recovery (conventional distillation is energy-intensive) et al. [4–7].

Improvement of the final butanol titer is one of the key issues in ABE fermentation process. According to the economic analysis, the separation cost will be reduced by half if the final butanol titer could reach above 19 g/L [8,9]. Although genetic modification of solventogenic *Clostridium* shows potential in improvement of butanol tolerance in molecular levels; however, there are few successful reports regarding improvement of butanol tolerance through genetic manipulation [2]. Alternatively, some non-ionic surfactants, such as Tween 80 have been shown to be effective stimulatory agents for the production of valuable metabolic products, including polysaccharides, enzymes and secondary products in some bacteria, fungi, and medicinal mushrooms [10–12]. It was reported that non-ionic surfactants can affect the microbial metabolism, which is closely associated with the cell structure integrity and transportation activity across the cell membrane. For instance, Tween 80 can upset the cytomembrane. It was reported that the fatty acid synthase subunit alpha protein could be upregulated in *Pleurotus tuberregium* by the supplementation of Tween 80 to promote the synthesis of long-chain fatty acids and their incorporation into the mycelial cell membranes, thus increasing the membrane permeability [12]. Tween 80 could also affect the fungal metabolism, which is associated with the cell structure stability and transportation activity across the mycelia membrane. The expression level of transmembrane transporter at 0.05 and 2% (v/v) Tween 80 increased significantly compared to the control [13]. Tween 80 could interact with the biosynthesis pathway and transmembrane transport at the gene transcription level, thereby facilitating the release of metabolites into the extracellular medium. However, the effect of Tween 80 on ABE production by solventogenic *Clostridium* sp. has not been comprehensively investigated.

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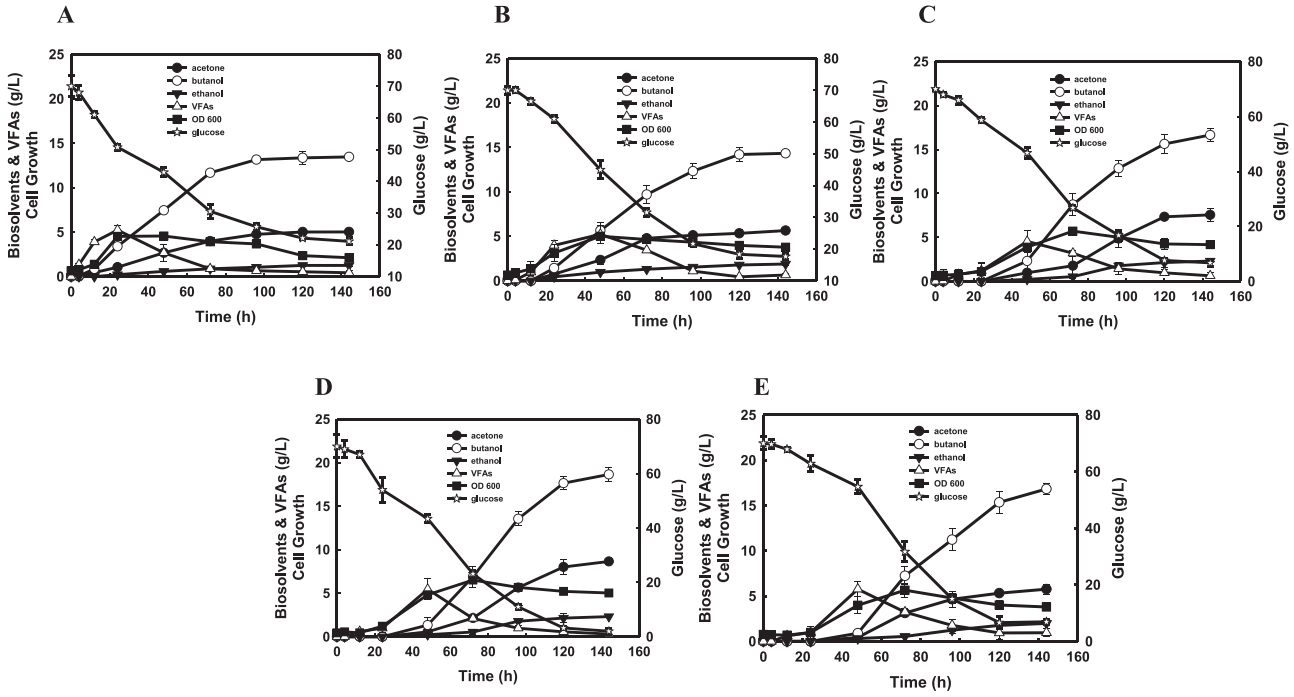

Fig. 1. Bacterial growth and fermentation profiles of *C. acetobutylicum* NJ4 in P2 medium amended with 70 g/L of glucose (A), 70 g/L of glucose and 5% (V/V) Tween 80 (B), 70 g/L of glucose and 10% (V/V) Tween 80 (C), 70 g/L of glucose and 15% (V/V) Tween 80 (D), and 70 g/L of glucose and 20% (V/V) Tween 80 (E). The pH values in the batch fermentation processes were controlled between 5.2–5.5.

Hence, the main aim in this study was to investigate the effect of the non-ionic surfactant – Tween 80 on butanol production by using *C. acetobutylicum*. Further improvement of the final butanol titer integrated with *in situ* extraction using biodiesel was also carried out.

2. Material and methods

2.1. Growth medium and culture conditions

All chemicals were purchased from Sigma-Aldrich with a purity of > 99%. Biodiesel was purchased from local company, Aoneng Co. Ltd., China. The main composition of biodiesel was fatty acid methyl ester. *C. acetobutylicum* NJ4 was isolated by our lab. Batch fermentation studies were conducted in 125 mL screw capped bottles containing 100 mL of P2 medium. Prior to autoclaving the medium, the pH was adjusted to 6.2 using 2 M NaOH. The medium containing carbon source and yeast extract (5 g/L; Sigma, USA) was sterilized at 121 °C for 15 min. On cooling to 35 °C under oxygen-free nitrogen atmosphere (in an anaerobic chamber), filter-sterilized P2 stock solutions [(buffer: KH_2PO_4, 50 g/L; K_2HPO_4, 50 g/L; ammonium acetate, 220 g/L), (vitamin: *para*-amino-benzoic acid, 0.1 g/L; thiamin, 0.1 g/L; biotin, 0.001 g/L), and (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)] were added (1 mL each). Batch fermentations were carried out in triplicates.

2.2. Fed batch fermentation with in situ extraction

Fed-batch fermentation was carried out in a 3.0-L bioreactor (BIOSTAT® B plus, Sartorius, Germany) at 35 °C with an agitation rate of 150 rpm 750 mL (O_2 free) biodiesel was added into 750 mL fermentation medium. When the glucose level dropped to approximately 10 g/L, fresh glucose stock solution was added to the fermentation broth to reach a final glucose concentration of 60 g/L.

2.3. Butanol tolerance experiments and enzymatic assays

When pre-culture OD_{600nm} of 1.0 ± 0.2 was achieved (approximately 12 h for the control and 24 h for the medium supplemented with 15% (v/v) Tween 80), each culture was added to different amount of butanol to achieve the concentration of 5, 10, 15, and 18 g/L butanol, respectively. The bacterial growth in the presence of different concentration of butanol was further monitored. The activities of butanol dehydrogenase (BDH) were measured by monitoring NADH consumption at 365 nm according to our previous method [14]. Samples were collected at the early exponential phase (48 h). Protein concentration in cell extract was determined by using the Bio-Rad protein assay kit with bovine serum albumin as the standard.

2.4. Analytic method

Bacterial growth was determined by measuring the optical density at 600 nm with appropriate dilution using a UV–visible spectrophotometer (Lambda-25, Perkin-Elmer, USA). 1 OD equals to 0.26 g/L of dry cell weight. Glucose was analyzed by a 1200 Series HPLC system (Agilent Technologies Inc.) equipped with an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) and a Refractive Index Detector (RID). The samples were run at 75 °C with 0.6 mL/min eluent of 5 mM sulfuric acid. Biosolvents (i.e., acetone, ethanol and butanol) and acids (i.e., acetic acid and butyric acid) were measured by a 7890A gas chromatography (Agilent Technologies, U. S. A.) on a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm; J&W, U.S.A.) equipped with a flame ionization detector (FID). The oven temperature was initially held at 60 °C for 2 min, increased to 230 °C at 15 °C/min, and held for 1.7 min. Helium was used as the carrier gas, with a flow rate of 1.5 mL/min.

3. Results and discussion

Non-iconic surfactants, such as Tween 80 have been widely used to display the maximum activities of various enzymes or even improve the