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Propionic acid production from glycerol by metabolically engineered *Propionibacterium acidipropionici*

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ABSTRACT

Large amounts of crude glycerol produced in the biodiesel industry can be used as a low-cost renewable feedstock to produce chemicals and fuels. Compared to sugars (sucrose, glucose, xylose, etc.), glycerol has a lower reducing level, which is of benefit to the production of reduced chemicals. In this work, glycerol as the sole carbon source in propionic acid fermentation by metabolically engineered *Propionibacterium acidipropionici* (ACK-Tet) was studied. It was found that the adapted ACK-Tet mutant could use glycerol for its growth and produced propionic acid at a high yield of 0.54–0.71 g/g, which was much higher than that from glucose (~0.35 g/g). In addition, the production of acetic acid in glycerol fermentation was much less than that from glucose. Thus, glycerol fermentation produced a high purity propionic acid with a high propionic acid to acetic acid ratio of 22.4 (vs. ~5 for glucose fermentation), facilitating the recovery and purification of propionic acid from the fermentation broth. The highest propionic acid concentration obtained from glycerol fermentation was ~106 g/L, which was 2.5 times of the highest concentration (~42 g/L) previously reported in the literature.

1. Introduction

Propionic acid is an important chemical intermediate in the synthesis of cellulose fibers, herbicides, perfumes, and pharmaceuticals. Propionic acid and its salts are also widely used as food and feed preservatives. As a weak organic acid, the non-dissociated propionic acid can pass through cell membrane into cytoplasm and release protons due to the intracellular alkaline environment. As a result, the pH gradient across the cell membrane is disturbed, which affects the nutrients transfer and inhibits cell growth [1]. Currently, propionic acid is almost exclusively produced via petrochemical processes. However, recent rising oil price and consumer's desire for biobased chemical products have generated large industrial interests in producing propionic acid and other chemicals from biorenewable feedstocks, including agricultural and industrial wastes [2]. Meanwhile, increased oil price has dramatically increased the market demand for biodiesel, which is mainly produced from vegetable oils and alcohols via transesterification with glycerol as the main byproduct [3–7]. The large amounts of crude glycerol produced in the biodiesel industry pose significant environmental concern if not properly treated. It is thus desirable to use waste glycerol as a renewable, low-cost feedstock to produce industrial chemicals and biofuels [8–10].

The goal of this work was to evaluate the feasibility of using glycerol as the sole carbon source in propionic acid fermentation by *Propionibacterium acidipropionici*, which has been extensively studied in the production of propionic acid from mainly carbohydrate-based feedstocks, including glucose and whey lactose [11–22]. However, relatively few studies have used glycerol as the carbon source in propionic acid fermentation [23,24]. Compared to glucose and other carbohydrates, glycerol has a much lower reducing state, which favors the production of more reduced metabolites [24] but could also cause redox imbalance in the metabolism and thus inhibit cell growth [25]. Furthermore, conventional propionic acid fermentation processes are limited by low productivity, yield, and final concentration due to strong end-product inhibition [13,14,26]. Consequently, current bioproduction of propionic acid cannot compete with commercially used petrochemical routes.

Therefore, increasing bacterial tolerance to propionic acid is critical to its economical production in the fermentation [27]. Advances in genetic engineering have provided new tools for creating mutants with improved fermentation capability [28]. Recently, Suwannakham et al. generated metabolically engineered mutants (ACK-Tet) that can tolerate and produce propionic acid at higher concentrations and yields [29]. Furthermore, by immobilizing and adapting cells in a fibrous-bed bioreactor (FBB), a high propionic acid concentration of ~72 g/L was produced from glucose by *P. acidipropionici* [30]. In this work, propionic acid production from glycerol by *P. acidipropionici* mutant ACK-Tet was

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investigated. The effects of cell immobilization and adaptation in the FBB on propionic acid fermentation were also studied and are reported in this article.

2. Materials and methods

2.1. Culture and media

A mutant strain of *P. acidipropionici* ATCC 4875 with *ack* gene (encoding acetate kinase) knock-out (ACK-Tet) was used in this study [29]. Unless otherwise noted, the bacterium was cultivated in a synthetic medium containing (in 1 L) 10 g yeast extract (Difco Laboratories, Detroit, MI), 5 g trypticase (BBL), 0.25 g K_2HPO_4, 0.05 g $MnSO_4$, and 20–40 g glycerol or glucose as the carbon source. The basal medium (without the carbon source) and the concentrated carbon source solution were sterilized separately at 121 °C and 15 psig for 30 min to avoid undesirable reactions. They were mixed aseptically before use in the fermentation study.

2.2. Fermentation kinetic studies

Both free-cell and immobilized-cell fermentations were carried out. Free-cell fermentations were conducted in a 5-L fermentor (Marubishi MD-300) containing 2 L of the medium at 32 °C and pH 7.0 (±0.02), which was controlled by automatically adding 6 N NaOH. Anaerobiosis was created by sparging N_2 through the medium for 30 min before inoculation and once for 10 min each day afterwards. The fermentor was inoculated with 100 mL of exponential phase cells ($OD_{600} \approx 2.0$) grown in a serum bottle at 32 °C. Liquid samples (3 mL each) were taken from the fermentor at proper time intervals throughout the fermentation.

Immobilized-cell fermentations were studied in a fibrous-bed bioreactor (FBB), which was connected to the 5-L fermentor for pH and temperature controls through a recirculation loop. The FBB was made of a glass column with a water jacket. A piece of cotton cloth was spirally wound with a stainless steel mesh and packed into the glass column. The FBB itself had a working volume of ~600 mL and the complete system contained ~2 L of the medium, unless otherwise noted. Detailed construction and operation of the FBB can be found elsewhere [30,31]. After seeding with the culture, the FBB was operated under the repeated batch mode for several batches with glucose as the substrate to increase the cell density in the reactor system. Then, fed-batch fermentation was carried out in the FBB system with glycerol as the substrate to study the fermentation kinetics and to gradually adapt the cells to tolerate and produce a high concentration of propionic acid from glycerol. At the end of the fed-batch fermentation, the adapted cells in the FBB were collected from the cotton cloth and subcultured in serum bottles for further analyses.

2.3. Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-mL cuvette using a spectrophotometer (Shimadzu, UV-16-1). One unit of OD was equivalent to ~0.87 g/L cell dry weight. The concentrations of carbon source (glycerol or glucose) and acid products (acetic, succinic, and propionic acids) in fermentation samples were analyzed by using a high-performance liquid chromatograph (HPLC) equipped with an organic acid column (Bio-Rad, HPX-87) operated at 45 °C with 0.01 N H_2SO_4 as the eluant at a flow rate of 0.6 mL/min.

3. Results and discussion

3.1. Fermentation kinetics with glycerol as the carbon source

Fig. 1 shows typical batch fermentation kinetics with glycerol as the sole carbon source. In general, glycerol can support cell growth and metabolism in propionic acid fermentation, with propionic acid as the main product and acetic and succinic acids as two byproducts, similar to the fermentation with glucose as the carbon source. However, cell growth on glycerol was very slow and it took 700 h to produce ~19 g/L propionic acid in the free-cell fermentation (Fig. 1A). The propionate yield from glycerol was 0.55 g/g (~69% of the theoretical yield), and there was almost no acetic acid production in the fermentation. This should not be a surprise since ACK-Tet (with *ack* knock-out) had an impaired acetic acid synthesis pathway [29]. The negligible acetic acid production from glycerol might also be attributed to the lower reducing state of glycerol, which favors the production of more reduced product (i.e., propionic acid) vs. more oxidized metabolite (i.e., acetic acid) in order to balance the intracellular redox potential [23]. It is noted that 24% of the initial glycerol remained unused after 700 h fermentation. Clearly, the glycerol fermentation had a low

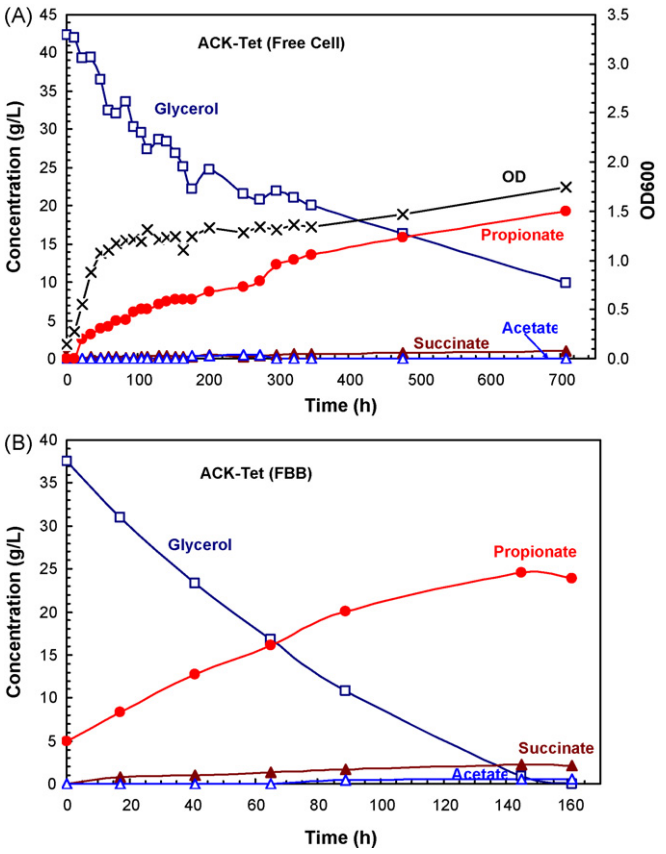

Fig. 1. Kinetics of batch fermentations of glycerol by *P. acidipropionici* ACK-Tet at pH 7.0, 32 °C. (A) Free-cell fermentation and (B) immobilized-cell fermentation in the fibrous-bed bioreactor.

productivity and final propionate concentration, which could be attributed to the low specific growth rate and cell density in the fermentation (see Table 1).

To increase the productivity, the fermentation was carried out with cells immobilized in a fibrous-bed bioreactor and the results are shown in Fig. 1B and Table 1. As expected, the FBB increased the fermentation rate significantly and all glycerol in the medium was consumed in ~160 h, with a propionate productivity ~6.5-fold of that in the free-cell fermentation. The improved fermentation rate was mainly attributed to the high cell density in the FBB, which was estimated at 30–60 g/L, depending on the feeding condition, at pseudo-steady state. Cell immobilization in the FBB did not seem to affect the propionate yield, but there was significantly more acetic acid production although the amount was still very low.

3.2. Fed-batch fermentation in fibrous-bed bioreactor

Fig. 2 shows the fed-batch fermentation kinetics with *P. acidipropionici* ACK-Tet immobilized in the FBB and glycerol as the carbon source. In this study, the FBB was initially operated under the repeated batch mode for several batches to increase the cell density in the reactor system. Then, the reactor was operated under a fed-batch mode to gradually increase the propionic acid concentration produced in the fermentation, which continued for 4 months until cells ceased to consume glycerol or produce propionic acid. At this point, the propionic acid concentration had reached 106 g/L, which was 2.5 times of the highest concentration (~42 g/L) from glycerol fermentation ever reported in the literature [24]. The high final concentration of propionic acid obtained in the fermentation indicated that the immobilized cells