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Microbial removal of carboxylic acids from 1,3-propanediol in glycerol anaerobic digestion effluent by PHAs-producing consortium

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ABSTRACT

Anaerobic fermentation of glycerol to 1,3-propanediol (1,3-PDO) is conceived as an economic feasible pathway to handle with increasing crude glycerol from biodiesel industry. However, glycerol anaerobic digestion effluent (ADE) consists of carboxylic acids and 1,3-PDO, imposing difficulties for separation. The objective of this study was, therefore, to investigate microbial removal of carboxylic acids from 1,3-PDO in glycerol ADE by polyhydroxyalkanoates (PHAs) producing consortium. Growth tests on carbon sources showed *Corynebacterium hydrocarbooxydans* had preference for butyrate while *Bacillus megaterium* for acetate and glycerol. Consequently, their consortium had a higher cell density and a faster substrate utilization rate than single strain grown in glycerol ADE. Acidic pH at 6.0 and 5.2 strongly inhibited cell growth and activity, while C:N ratio (w/w) at 8:1 could balance nitrogen demand for cell growth and PHA synthesis. Kinetic study further revealed over 80% of fed 1,3-PDO was preserved after depletion of carboxylic acids. Correspondingly, total organic carbon (TOC) contribution from 1,3-PDO rose from initial 55.8% to 84%. Produced PHAs comprised 3-hydroxybutyrate (3-HB) units. The results showed this study as the first attempt to provide a win-win solution to remove carboxylic acids from 1,3-PDO in glycerol ADE and converted them into PHAs as a secondary value-added product.

1. Introduction

Driven by increasing energy demand and awareness of sustainability, biodiesel produced via transesterification developed fast and expanded rapidly in the past decade [1]. Besides the target product, i.e. fatty acid methyl esters (FAMEs), glycerol is produced as a by-product at a glycerol-to-biodiesel ratio of 1:10 (w/w). Anaerobic fermentation of glycerol to 1,3-propanediol (1,3-PDO) is considered as a promising pathway to handle with increasing crude glycerol, because of high 1,3-PDO yield and wide application of 1,3-PDO in polymer industry [2–4]. Theoretically, the maximum yield of 1,3-PDO from glycerol was predicted to be 0.72 mol/mol [5]. 1,3-PDO is a monomer for commercial polyesters such as DuPont's Sorona®, CDP Natureworks® or Shell Chemical's Corterra™, and annual consumption of 1,3-PDO is more than 100 million pounds [6]. Although the microbial conversion of glycerol to 1,3-PDO was technically feasible, however, carboxylic acids such as acetic and butyric acids were inherently accompanied with 1,3-PDO production due to redox balance [7]. The separation of 1,3-PDO from carboxylic acids is necessary for downstream applications. Some chemical processes based on ion exchange had been patented [8], but its application was not reported yet. Inspired by the chemical separation based on distinct properties between alcohols and carboxylic acids, the microbial separation based on substrate preference could also be possible. It is well known that the microbes have preferences for certain substrates and these discriminations lead to sequential utilization of different categories of substrates. For glycerol ADE, it is a mixture comprising alcohols and carboxylic acids. If there were some bacteria with higher preferences for carboxylic acids than alcohols, it would be possible for them to consume carboxylic acids first but retain 1,3-PDO.

Polyhydroxyalkanoates (PHAs) producing bacteria could be good candidates for microbial removal of carboxylic acids from 1,3-PDO in glycerol ADE because some strains had been reported to take carboxylic acids as substrates [9,10]. Furthermore, aerobic PHAs-producing bacteria differ from other aerobes by the capability to

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synthesize and accumulate intracellular PHAs as stored carbon and energy material [11], which makes further cell separation from liquid easily. Besides, PHAs are commercially valuable biocompatible and biodegradable polymers with numerous potential industrial applications [12,13], which could be considered as the secondary value-added product.

The substrate spectrum of PHAs-producing strains ranges from carboxylic acids and sugars to alcohols, indicating potential capability of using multiple compounds in glycerol ADE. For example, carboxylic acids from acetate, propionate, lactate, and butyrate [14,15] to medium chain fatty acids such as octanic acid [16] and 4-hydroxyhexanoic acid [17], even aromatic acids such as phenylacetic acid [18] were reported as carbon sources for PHAs synthesis. Meanwhile, glycerol was also reported for PHAs production [19–21]. In contrast, 1,3-PDO with a similar chemical structure to glycerol was not widely tested for PHAs synthesis, and few microorganisms such as the isolated *Chromobacterium* sp. were reported to degrade 1,3-PDO with PHAs production [22]. Thus, it was not clear whether 1,3-PDO could be widely used by PHAs-producing strains. Except pure chemicals, ADEs of activated sludge and food waste rich in carboxylic acids were reported as feedstock for PHAs production as well [15,23]. The results showed carboxylic acids with various carbon chain lengths could be effectively converted into PHAs. However, unlike these ADEs, glycerol ADE contains both of high content of alcohol, 1,3-PDO, and carboxylic acids [24]. PHAs-producing strains with mixed carbon sources of carboxylic acids and alcohols is not reported yet. It was not clear whether the presence of high concentration of 1,3-PDO in glycerol ADE would possess inhibition effect over PHAs microorganism in the utilization of carboxylic acids.

Herein, the objective of this study was to achieve microbial removal of carboxylic acids from 1,3-PDO in glycerol ADE by PHAs-producing strains. Cell growth of PHAs-producing strains on single and multiple carbon sources was first conducted, and based on substrate preferences, a selected consortium was applied to glycerol ADE. Subsequently, impacts of ratio of organic carbon concentration to inorganic nitrogen concentration (C:N, w/w) and medium pH on the consortium were further investigated. Finally, kinetics study was carried out in a fermentor to further explore dynamic process of nitrogen and carbon utilization and PHAs synthesis. It showed that the microbial removal of carboxylic acids from 1,3-PDO in glycerol ADE and concurrent PHA synthesis were feasible.

2. Material and methods

2.1. Growth test of PHAs-producing strains in single and multiple carbon sources

Since glycerol ADE was the target substrate, PHAs producing strains should be able to grow in mixed substrate and accumulate high PHAs contents. According to these criteria, seven PHAs-producing strains *Pseudomonas putida* KT2440 ATCC 47054 [25], *Corynebacterium hydrocarbooxydans* ATCC 21767 [26], *Bacillus megaterium* DSM 90 [27,28], *Corynebacterium glutamicum* DSM 20137 [27], *Cupriavidus necator* DSM 13513 [15], *Nocardia lucida* NCIMB 10980 [26], and *Bacillus thuringiensis* DSM 2046 [28] were selected in this study. They were designated as S1 to S7 in this study, respectively. All these strains were purchased from either German Collection of Microorganisms and Cell Culture (DSMZ) or American Typical Culture Center (ATCC) or National Collection of Industrial and Marine Bacteria (NCIMB).

The strains were maintained by monthly subculture on 1.5% (w/v) agar plates containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. The plates were cultivated for 48 h at 30 °C and then kept at 4 °C. The same medium without agar was used for seed cultivation. The seed culture was prepared in 250 mL flask containing 100 mL medium in an incubator for 48 h at 200 rpm and 30 °C. The seed cell was harvested aseptically by twice centrifugation at 10,000 rpm for 5 min followed by sterilized phosphate buffer solution washing and transferred to carbon source medium. Initial optical density at 600 nm (OD_{600}) was set at 0.2 adsorption unit (AU), and 50 mL sterilized tube was filled with 15 mL carbon source medium. The inoculated medium was incubated at 200 rpm and 30 °C for 3 days.

Based on the constituents of 20 g/L glycerol ADE, four single carbon source solutions were designed as 9 g/L 1,3-PDO, 3 g/L acetate, 2 g/L butyrate, and 1 g/L glycerol. Ammonium sulfate was added as the solo nitrogen source and C:N ratio was fixed at 4:1 (w/w). The cultivation medium also contained the following substances per liter: 0.012 g $CaCl_2$, 0.032 g $MgSO_4{\cdot}7H_2O$, 4.410 g K_2HPO_4, 3.460 g KH_2PO_4, 0.12 g NaCl, and 1 mL of a microelement solution that contained (g/L in 1 M HCl): $FeSO_4{\cdot}7H_2O$ 2.78, $MnCl_2{\cdot}4H_2O$ 1.98, $CoSO_4{\cdot}7H_2O$ 2.81, $CaCl_2{\cdot}2H_2O$ 1.67, $CuCl_2{\cdot}2H_2O$ 0.17, and $ZnSO_4{\cdot}7H_2O$ 0.29 was added to 1 L medium [15]. Additionally, three multiple carbon sources solutions comprising acetate plus butyrate, acetate plus butyrate and 1,3-PDO, and acetate plus butyrate, 1,3-PDO, and glycerol were prepared to investigate the cell growth and assess potential inhibition effect of 1,3-PDO. Their concentrations were designed as above specified, and ammonium sulfate was fed as nitrogen source at the ratio of carbon to nitrogen 4:1 (w/w). The other components in the medium was the same to above-mentioned. Medium pH was adjusted to pH 7.0 before autoclave. All substrate tests were conducted in triplicate.

2.2. Consortium of S2 and S3 grown in synthetic and real glycerol ADE

Due to successful growth in multiple carbon sources and no substrate spectrum overlap, S2 and S3 were further selected for the cell growth and substrate utilization tests on synthetic and real glycerol ADE. The synthetic glycerol ADE contained the following substances (in g/L): 1,3-PDO 9.00, acetate 2.40, butyrate 1.60 and glycerol 0.5. The real glycerol ADE was generated by feeding the enriched 1,3-PDO-producing community from mangrove sediment with 20 g/L glycerol under anaerobic condition for 2 days. It comprised the following substance (in g/L): 1,3-PDO 8.96, acetate 2.26, butyrate 1.51, glycerol 0.40 and succinate, lactate, formate less than 0.25. The C:N ratio was fixed at 4:1 but carbon concentration from 1,3-PDO was excluded because 1,3-PDO was not well utilized by S3. The other minerals and micro-minerals were the same to that specified in Section 2.1. Both medium pH was adjusted to 7.00 prior to autoclave. The medium with 50 mL volume was inoculated with cell OD_{600} set at S2:S3 = 0.1:0.1 in a 250 mL shaking flask and incubated at 200 rpm and 30 °C for 2 days. All tests were conducted in triplicate.

2.3. The impacts of initial pH and C:N ratio on consortium of S2 and S3

Since glycerol ADE contained high content of acids, pH of glycerol ADE tended to be lower than 7.0. If the consortium of S2 and S3 could grow well in acidic condition, less base would be required for pH control. Because the real glycerol ADE had an initial pH 5.2, three initial pH values were test as 5.2, 6.0, and 7.0. Apart from initial pH difference, all other cultivation parameters were the same as specified in Section 2.2.

The availability of nitrogen would determine carbon distribution to biomass growth and PHAs synthesis. Under high C:N ratio, insufficient nitrogen supply would result in limited cell growth and correspondingly limited intracellular space for PHAs storage. Meanwhile, high nitrogen supply may stimulate cell growth but

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