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**Highlights**

PHAs-producing strains showed distinct substrate preferences.

Selected consortium had higher cell density and faster substrate utilization rate.

Microbial consortium scraped carboxylic acids but preserved 1,3-PDO in glycerol ADE.
Microbial removal of carboxylic acids from 1,3-propanediol in glycerol anaerobic digestion effluent by PHAs-producing consortium

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Abstract

Microbial conversion of glycerol to 1,3-propanediol (1,3-PDO) is conceived as one of the most promising applications for crude glycerol produced from biodiesel industry. However, glycerol anaerobic digestion effluent (ADE) consists of carboxylic acids and 1,3-POD, imposing difficulties for further 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. This study first conducted growth tests of PHAs-producing microorganisms on single and multiple carbon sources and then investigated performances of selected microbial consortium on glycerol ADE. Results showed that Corynebacterium hydrocarbooxydans had preference for butyrate while Bacillus megaterium for acetate and glycerol. Compared to single strain, their consortium had a higher cell density and a faster substrate utilization rate when fed with glycerol ADE. The kinetic study further revealed that after the consortium depleted carboxylic acids, over 80% of fed 1,3-PDO was preserved, resulting in TOC contribution from 1,3-PDO rose from initial 55.8% to 84% after removal. The produced PHAs comprised 3-hydroxybutyrate (3-HB) units, which are regarded as commercially-valuable biocompatible and biodegradable plastics. The results showed that this study as the first attempt provided 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.

Keywords: Glycerol anaerobic digestion effluent; Polyhydroxyalkanoates; 1,3-Propanediol; Microbial removal; Microbial consortium
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. Due to the highly reduced property of glycerol, the microbial conversion of glycerol to 1,3-PDO is considered as a promising value-added uses for crude glycerol because of high process 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 as high as 0.72 mol/mol [5]. 1,3-PDO is a monomer for commercial polyesters such as D Dupont’s Sorona® and CDP Natureworks® or Shell Chemical’s CorterraTM, and annual consumption of 1,3-PDO is more than 100 million pounds [6]. However, it is noted that carboxylic acids such as acetic and butyric acids were accompanied with 1,3-PDO production to achieve redox balance in microbial anaerobic process [7]. Although 1,3-PDO is accumulated at high concentration, the separation of 1,3-PDO from carboxylic acids is necessary for downstream applications. Some chemical processes based on ion exchange have 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 their distinct properties is also 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. Alcohols consist of 1,3-PDO and occasionally residual glycerol while carboxylic acids mainly encompass
acetic and butyric acids. If some bacteria with higher preferences for carboxylic acids than alcohols, it is possible for them to consume carboxylic acids first but retain 1,3-PDO.

As some strains of polyhydroxyalkanoates (PHAs)-producing bacteria have been reported to take carboxylic acids as substrates [9, 10], PHAs-producing bacteria could be good candidates for microbial removal of carboxylic acids from 1,3-PDO in glycerol ADE. Furthermore, aerobic PHAs-producing bacteria differ from other aerobes by the capability to synthesize and accumulate intracellular PHAs as stored carbon and energy material [11], which makes further cell separation from liquid easily. Beside, 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 range from carboxylic acids and sugars to alcohols, indicating potential capability of using multiple compounds in glycerol ADE. For example, carboxylic acids from short carbon chain 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 test for PHAs synthesis and few microorganisms such as the isolated Chromobacterium sp. were reported to have the capability 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, the mixture of anaerobic digestion effluent 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 ADE, 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. In order to achieve this goal, cell growth of PHAs-producing strains on single and multiple carbon sources was conducted, and based on substrate preferences, a selected consortium was applied to glycerol ADE. Subsequently, impacts of C:N ratio and medium pH on the consortium were further investigated. Finally, kinetics study was done 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 was feasible.

2. Material and methods

2.1 PHAs-producing strains selection and growth tests on single and multiple carbon sources.

Since glycerol ADE was the target substrate, the PHAs producing microorganism should have the properties of acclimation to mixed substrates and high PHAs accumulation contents. According to these criteria, seven PHAs-producing strains \textit{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 hours 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 hours at 200 rpm and 30 °C. The seed cell was harvested aseptically by twice centrifugation at 10,000 rpm for 5 min followed by phosphate buffer solution washing and transferred to carbon source medium. Initial optical density at 600 nm (OD<sub>600</sub>) was set at 0.2, 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 carbon composition 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 the ratio of carbon to nitrogen was fixed at 4:1 (w/w). The cultivation medium also contained the following substances per liter: 0.012 g CaCl<sub>2</sub>, 0.032 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 4.410 g K<sub>2</sub>HPO<sub>4</sub>, 3.460 g KH<sub>2</sub>PO<sub>4</sub>, 0.12 g NaCl, and 1 mL of a microelement solution that contained (g/L in 1 M
HCl): FeSO₄·7H₂O 2.78, MnCl₂·4H₂O 1.98, CoSO₄·7H₂O 2.81, CaCl₂·2H₂O 1.67, CuCl₂·2H₂O 0.17, and ZnSO₄·7H₂O 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 on synthetic and real glycerol ADE

Due to successful growth on 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 ratio of C:N 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

The 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. On the other hand, overly high nitrogen supply may stimulate cell growth but channel less carbon for PHAs synthesis. Since cell molecules could be simplified as C$_5$H$_7$NO$_2$ [29], C:N ratio of 4:1 would represent the maximum theoretical ratio for only biomass synthesis. To investigate the optimal C:N ratio for the consortium of S2 with S3 to balance cell growth and PHA accumulation, the C:N ratios in real glycerol ADE was set at 4:1, 8:1 and 16:1 but the carbon contribution from 1,3-PDO was excluded. All of other cultivation parameters were the same as specified in Section 2.2.

2.4 Kinetics study in a 10 L fermentor

In order to further explore the dynamic process of cell growth, nitrogen and carbon source consumption and PHAs synthesis, the kinetics study was conduct in a 10 L fermentor filled with 3.5 L medium. The medium was real 20 g/L glycerol ADE generated as detailed in
Section 2.2. Initial pH value was adjusted to 7.00 while C:N ratio was fixed at 8:1 excluding carbon from the 1,3-PDO. Cell cultivation and harvest of S2 and S3 was the same as described in Section 2.1. The inoculation cell density OD$_{600}$ of S2 and S3 was set at 0.1 and 0.1, which was equivalent to 25.9 mg cell/L and 27.8 mg cell/L. The pH, dissolved oxygen (DO) saturation and stirring were online monitored. The pH was controlled at 7.00 ± 0.20 by addition of 1 M HCl or 1 M NaOH. Air was sterilized by the filtration through a sterilized Nylon membrane filter with pore size of 0.25 µm and the aeration flowrate was control by a flowmeter. The initial aeration flowrate and stirring speed were set at 1.0 L/min and 50 rpm, respectively. Manual adjustments of aeration flowrate and stirring speed would be applied to sustain DO at the range of 20% to 50%.

2.5 Analytical methods

Cell density was measured at 600 nm by a photometer (Cary 50, Varian, US) and biomass concentration was calculated based on the linear relationship between cell density and OD$_{600}$ below 1.2. The pH value was measured by a pH probe (G20, Mettler Toledo, Switzerland). The acids and alcohols components in medium were measured in the high performance liquid chromatography (HPLC) equipped with a size-exclusion column (Rezex ROA-organic acid, Phenomenex, US) and diode array detector (Ultimate 3000, Dionex, Germany). The filtrated sample via 0.45 µm polyvinylidene fluoride (PVDF) membrane was acidified with 50 mM sulfuric acid at the ratio of 9:1 (v/v). The acidified sample with 10 µL volume was injected and the flowrate of mobile phase consisted of 5 mM sulfuric acid in water was set at 0.7 mL/min while the column temperature was constant at 42±0.5 °C. Alcohols were detected at wavelength of 190 nm while acids at
210 nm. The ammonium nitrogen in medium was detected by the kits following the vendor’s instruction (TNT 832, Hach, US). Total organic carbon (TOC) concentration in the medium was detected by TOC instrument (TOC-L, Shimadzu, Japan). PHAs was analyzed by the GC-MS method detailed in [30] with the modification of using 3% (v/v) sulfuric acid acidified methanol solution instead of 15%. The copolymer of poly-3-hydroxybutyrate with poly-3-hydroxyvalerate (88:12, w/w, Sigma-Aldrich, USA) was used as the standard for calibration curve.

3. Results and discussion

3.1 Cell growth in single and multiple carbon sources

Since the glycerol ADE is a mixture with carboxylic acids and alcohols, the desired properties of microorganism candidates should include the capability to grow in the mixture and take carboxylic acids as preferred substrate. Based on the criteria of high accumulation of PHAs contents and wide substrate spectrum, seven PHAs-producing strains as detailed in Section 2.1 was selected for growth test in single and multiple carbon sources. As shown in Table 1, different PHAs-producing strains grown on single and multiple carbon sources showed distinct substrate preference and cell growth patterns at 72 h. S1 showed cell growth on four test single carbon sources, and it was the only test strain had cell growth on 1,3-PDO. Compared to single carbon source, S1 exhibited relatively poor adaption to multiple carbon sources. For S2, it had growth on acids but not on alcohols, and its particular preference for butyrate was probably responsible for the cell growth in the multiple carbon sources. In contrast, S3 had higher preference for acetate and glycerol, and cell growth on multiple carbons were comparable to that on single carbon sources of acetate
and glycerol. S4 seemed to have growth preference for acetate, but the corresponding OD$_{600}$ were slightly higher than initial 0.2. S5 could grow well on single carbon sources of acetate and glycerol, but its growth on multiple carbon source was minor. Surprisingly, S6 and S7 almost showed no cell growth in all test substrate groups and these performances were contradictory to reported results [26, 28]. Probably, the lack of key growth factor resulted in the growth failure since trace thiamin was added in their medium but not in the medium of this study [26].

[Insert Table 1. here]

Specific to all three test multiple carbon sources, only S2 and S3 showed significant growth but it was not clear which carbon source was consumed for cell growth and the order of substrate preference. As shown in Fig. 1.a-c, it demonstrated that cell growth of S2 was closely associated with consumption of butyrate while the other carbon sources almost kept at the fed concentration. The presence of glycerol and 1,3-PDO did not possess inhibition effects on cell growth and butyrate utilization rate. For S3, it had distinct substrate preferences. When acetate was co-fed with butyrate for S3, 3 g/L acetate was almost used up while 2.17 g/L butyrate just dropped slightly to 1.92 g/L within 72 hours, indicating higher preference for acetate over butyrate. The addition of 1,3-PDO into acetate and butyrate as illustrated in Fig. 1.e retarded the acetate utilization and cell growth. Meanwhile, it was noted that 1,3-PDO concentration slightly decreased as cell grew, indicating S3 had potential capability to utilize 1,3-PDO but at a slower rate and with less preference. Further addition of glycerol would stimulate the cell growth in the first 24 hours, but the concentration of acetate at 24 h did not show significant difference to another two
groups, indicating S3 had high preference for glycerol for cell growth. Previous study showed S3 could take acetate and 3-hydrobutyrate as carbon sources for cell growth and PHA synthesis [28]. Like S2 and S3, *Ralstonia eutropha* (Currently renamed as *Cupriavidus necator*) showed preferences for certain carboxylic acids when fed with mixed volatile fatty acids [14]. The observation was aligned with the results from the study of Shahid, Mosrati, Ledauphin, Amiel, Fontaine, Gaillard and Corroler [27]. However, it did observe that 1,3-PDO was consumed more in Fig. 1.f than that in Fig. 1.e. Perhaps due to similar chemical structure of glycerol and 1,3-PDO, the addition of glycerol might induce the consumption of 1,3-PDO. On the other hand, the degradation rate of 1,3-PDO was not comparable with that of acetate and glycerol, and the proportion of degraded 1,3-PDO merely accounted for less than 10% of fed 1,3-PDO.

[Insert Fig. 1. here]

Therefore, it clearly shows that S2 had high preference for butyrate while S3 on glycerol and acetate. Since the main components in glycerol ADE were acetate, butyrate and 1,3-PDO, occasionally with glycerol residue, it was expected that consortium of S2 and S3 would enhance the rate of acids removal without severe substrate competition and, to large extent, preserve 1,3-PDO.

3.2 *Consortium of S2 with S3 grown on synthetic and real glycerol ADE*

After single strain growth test, the consortium of S2 and S3 were further selected for growth test in glycerol ADE. Fig. 2 shows that the consortium of S2 with S3 grown on synthetic and real glycerol ADE had a higher cell density and a faster substrate utilization rate than that of single strain as shown in Fig. 1. The higher cell density might be attributed
to the minor substrate competition as illustrated in Section 3.1. However, those two glycerol ADE differed from each other in the growth pattern and substrate consumption. At 24 h, cell OD$_{600}$ in real glycerol ADE had a 16-fold increase while synthetic ADE had a 7-fold increase. The consumption of acetate and butyrate were concurrent with cell growth while the concentration of 1,3-PDO remained unchanged in both groups. However, at 48 h, the cell density in synthetic glycerol ADE underwent another 4-fold growth from 1.54 to 5.98 while merely minor increase by 0.369 was observed in real glycerol ADE. The 1,3-PDO in synthetic glycerol ADE was consumed by 3.33 g/L during the second day, and corresponding carbon concentration was almost equivalent to that from fed acetate and butyrate. In contrast, 1,3-PDO consumption in real glycerol ADE was less than 0.25 g/L, accounting for less than 3% of the fed 1,3-PDO. Consequently, the cell density in synthetic glycerol was almost double as that in real glycerol ADE due to double carbon utilization. The higher cell density found in synthetic glycerol ADE was not only dependent on the consumptions of acetate, butyrate, and glycerol but also on 1,3-PDO consumption. S3 probably be responsible for the 1,3-PDO degradation because it exhibited the capability to utilize 1,3-PDO even at the lower rate and less preference as shown in Section 3.1. The high consumption of 1,3-PDO was suspected to be associated with the induction of glycerol degradation because glycerol and 1,3-PDO had a similar chemical structure.

[Insert Fig. 2. here]

3.3 The impact of initial pHs values over cell growth and substrate utilization

Since the consumption of carboxylic acids by PHA-producing bacteria brought about pH increase while the glycerol ADE tended to be acidified, if the consortium of S2 and S3
could grow in acidic medium, then no base would be required for medium pH neutralization. As shown in Fig. 3, no cell growth was found in the acidic pH while at pH 7.0 cell density was comparable to that in Fig. 2.b. Concurrent consumption of carboxylic acids with cell growth resulted in pH increment from 6.90 to 8.68. In contrast, solution pH almost sustained at the initial values at pH 5.20 and 6.0 because no cell growth and negligible substrate consumption were observed. The inhibition of acidic pH over cell growth and substrate utilization rate was also reported by Yu and Si [31] in which *Ralstonia eutropha* consumed less than 10% of organic acids at pH 4.2 but over 95% at pH 7.0. After 40 hours in the medium, the consortium cell was streaked on the LB agar plates and cultivated for two days. Based on the pure strain growth on LB agar plates, S2 colonies featured with orange color while S3 colonies showed white color. In Fig. S1, it clearly demonstrates that as pH increased from 5.2 to 7.0, the white colonies representing S3 occupied nearly every streaks. The overlaid region by white and bright yellow colonies without clear boundary revealed S2 and S3 could co-culture with each other without significant competition for substrate. At pH 5.2, the plate was solely occupied by the orange colonies represented S2. This indicated that S2 was more tolerant to acidic solution than S3. On the other hand, it was noticed that neither of them could grow in acidic medium at pH 5.2 and 6.0. For the consortium of S2 and S3, the initial medium pH should be set around the neutral pH.

[Insert Fig. 3 here]
3.4 The impact of C:N ratios on cell growth and substrate utilization

Nitrogen source availability would regulate the carbon flux to biomass synthesis, PHAs production and others. Without nitrogen source for cell growth, there is no space for the intracellular PHAs storage. In other extreme conditions, over sufficient nitrogen supply would stimulate biomass synthesis but not PHAs synthesis. In order to balance the cell growth and PHAs synthesis, the impact of C:N ratios on the consortium performance was investigated. As shown in Fig. 4.5, C:N ratios of 4:1 and 8:1 could support cell growth well and ensure depletion of acetate and butyrate within 49 hours while C:N 16:1 could not support sufficient cell growth and retarded carbon source utilization. Although the cell density and substrate utilization rate in C:N 4:1 were comparable to that in C:N 8:1, after depletion of acetate and butyrate almost half of fed 488 mg N-NH$_4^+$/L was left in C:N 4:1 while only about 25 mg N-NH$_4^+$/L was still present in C:N 8:1. This meant the surplus nitrogen supply in C:N 4:1. In contrast, the nitrogen source in C:N 16:1 was used up within the first 24 hours, and insufficient nitrogen supply limited cell growth in subsequent 48 hours, resulting in a cell density plateau at the half as that in C:N 4:1 And C:N 8:1. The lower cell density brought about half of fed acetate left at 74 h. Compared to the utilization of acetate, butyrate was used much faster than acetate particularly in first 24 hours in C:N 16:1. Since S2 had preference for butyrate to acetate in Section 3.1, S2 probably have competitive advantages in cell growth when nitrogen was abundant. Meanwhile, it was noticed that high nitrogen also enhanced the 1,3-PDO degradation in C:N 4:1 and C:N 8:1, but insufficient nitrogen supply in C:N 16:1 resulted in a minor concentration drop of 1,3-PDO by less than 0.4 g/L. S3 was found to have the potential to utilize 1,3-PDO as illustrated in Section 3.2, and probably S3 at higher cell density utilized 1,3-PDO after
depletion of acetate and butyrate. On the other hand, it was interesting that only 3-hydroxylvalerate (3HV) PHA unit was detected in biomass from C:N 16:1, indicating 1,3-PDO was possibly a structure relevant substrate. However, 3HV unit was not detected in the biomass from C:N 4:1 and C:N 8:1 even if more 1,3-PDO was consumed in those two groups (data not shown). It seemed that abundant nitrogen would direct 1,3-PDO for biomass synthesize while scarcity of nitrogen source would drive 1,3-PDO for PHA synthesis. With regards to cell growth, carboxylic acids utilization rate and 1,3-PDO degradation, the C:N ratio of 8:1 provided sufficient nitrogen for cell growth and minimized the 1,3-PDO degradation. In contrast, most of other studies adapt either separated two stages strategy or high initial inoculated biomass concentration. In two stages strategy, cell growth occurred in the nitrogen abundant medium first, and then grown cell was transferred to nitrogen free or limited medium for PHA accumulation. The C:N ratio in the nitrogen abundant medium could approach to 3 (w/w) while no or low nitrogen was present in the nitrogen free medium [14, 27]. Alternatively, inoculated biomass concentration as high as 1.5 g/L was applied to test medium after growth in the nutrient rich medium [10]. Both methods could well highlight the important role of nitrogen source in PHAs accumulation, but it was not practical for the removal process.

[Insert Fig. 4. here]

3.5 Kinetics study of S2 and S3 consortium fed with real glycerol ADE in a fermentor

In order to further explore the dynamic processes of carbon and nitrogen utilization, a kinetic study in a fermentor was conducted. As shown in Fig. 5.a, the consortium of S2 and S3 had very short lag phase and exponential growth occurred at 4 h after inoculation in the
fermentor. After 32 hours, due to depletion of acetate and butyrate, the biomass concentration stabilized at a maximum concentration of 1.12 g/L. Compared with the study of single strain fed with mixed carboxylic acids sources [32], the consortium in this study used up the acetate and butyrate in a shorter time. Accompanied with cell growth, PHAs content comprising only 3-HB unit exhibited a linear increase from 0.066 g/L at 16 h up to 0.227 g/L at 28 h. Corresponding PHAs contents in dry cell weight (DCW) were 11.9% and 21.4%, respectively. Subsequently, the PHAs concentration decreased possibly as a result of PHAs degradation after depletion of acetate and butyrate. Although 1,3-PDO was present at a concentration higher than 5.5 g/L, there seemed no tend to intake 1,3-PDO for cell growth and PHA synthesis. The accumulation of PHA initiated when nitrogen was abundant. Chen, König and Lafferty [28] also reported PHAs accumulation in *B. megaterium* when it was grown in nitrogen rich LB medium. The PHAs tests revealed that S3 grown in nitrogen rich LB medium could accumulate 4.8% to 10.0% PHAs in DCW while no PHAs was detected in S2 grown in LB medium (data not shown). This meant the PHA accumulation occurring in the condition of nitrogen rich probably be attributed to S3. Alone with exponential cell growth, nitrogen source in the form of ammonium nitrogen (AN) was consumed at a high rate, dropping from 230 mg/L at 8 h to less than 50 mg/L at 28 h. The residual AN after depletion of acetate and butyrate indicated that there was space to further raise C:N ratio. Moreover, it was noticed that the oxygen consumption peaked at 28 h (data not shown). Even if the aeration flowrate and stirring speed were adjusted four-fold as the initial settings, DO saturation was resultantly sustained around 10%. Since oxygen is taken up for ATP generation [33], the coincidence of exponential cell growth and PHAs accumulation with high oxygen consumption indicated that both processes were energy-intensive.
From Fig. 5.b further illustrated the concentration of carbon sources versus time. Initially, 1,3-PDO, acetate, and butyrate contributed to 55.80%, 10.37%, and 15.76% of TOC, respectively. The apparent carbon source consumption began after 8 h when the cell density reached to 0.096 g/L. Subsequently, acetate, butyrate, 1,3-PDO, and TOC showed a similar decline as cell density further increased. After 32 h, acetate and butyrate was used up, but 1,3-PDO was still present at 6.75 g/L. Extending the cultivation to 48.5 h resulted in additional loss of 0.92 g/L 1,3-PDO, and this loss was possibly associated with high density of S3. Meanwhile, although 1,3-PDO and TOC decreased with time, the percentage of TOC from 1,3-PDO surged from initial 55.8% to 84% at 48 h, exhibiting effectiveness of removing carboxylic acids and preserving 1,3-PDO. The enhanced proportion of 1,3-PDO in TOC was attributed to the depletion of acetate and butyrate, which accounted for 26% of initial TOC. Further extension of cultivation resulted in more 1,3-PDO degradation, but the percentage of TOC from 1,3-PDO was not further improved. This highlighted the optimal time to terminate experiment would maximally preserve 1,3-PDO and remove carboxylic acids. If the non-PHAs biomass was simplified as $\text{C}_5\text{H}_7\text{NO}_2$ [29], 19.2 % of consumed organic carbon was converted to dry biomass and 3.2 % was stored as PHAs at the depletion of acetate and butyrate. The high conversion of acetate and butyrate was comparable to other study taking ADE rich in carboxylic acids, but the PHAs content in dry cell weight was not high [31]. Since the main objective of study was to polish carboxylic acids from 1,3-PDO, PHA production was perceived as a secondary target. Probably another growth factor, phosphate, in this study was too high to enhance PHA synthesis.
Phosphate was added to buffer the pH drops in the glycerol anaerobic fermentation, and its concentration was up to 1.6 g PO$_4^{3-}$-P/L. Some reports have showed phosphate limiting was a triggering factor for PHA synthesis. For example, *Halofex mediterranei* had a PHA content up to 75.4% of DCW when phosphate concentration was purposely controlled [34]. Moreover, some phosphate accumulating bacteria was reported to intake phosphate at the sacrifice of PHAs in aerobic condition [35]. It was not clear whether PHA-producing bacteria had a similar mechanism to store phosphate when phosphate was abundant. Nevertheless, further performance optimization of the consortium might require nutrient control in upstream glycerol ADE production.

4. Conclusion

PHAs producing strains of *C. hydrocarbooxydans* and *B. megaterium* had preferences for butyrate and glycerol plus acetate, respectively. Their consortium grown in glycerol ADE showed a higher cell density and a faster utilization rate of carboxylic than single culture. Initial acidic pH severely inhibited cell growth, and C:N 8:1 provided sufficient nitrogen for cell growth and ensured fast carboxylic acids consumption rate. In kinetics study, at the depletion of carboxylic acids, over 80% of fed 1,3-PDO was preserved, and the TOC contribution from 1,3-PDO rose from 55.80% to 84%, indicating effectiveness of this bioprocess. Further optimization over nutrient control especially phosphate probabaly reduce consumption of 1,3-PDO. Therefore, this study provided a win-win solution to remove carboxylic acids from 1,3-PDO present in glycerol ADE and produce PHAs as a secondary value-added product for the first time.
Acknowledgements

The authors want to thank Nanyang Technological University (NTU) for providing NTU Research Scholarship to support this study.

Reference


Lists of table and figures

Table 1. Cell OD_{600} at 72 h grown on single and multiple carbon sources with initial inoculation 0.20.

Fig. 1. Consumption of carbon sources and cell growth of single strain of S2 and S3 on multiple carbon sources. (a) S2 growth on acetate and butyrate. (b) S2 growth on acetate, butyrate and 1,3-PDO. (c) S2 growth on acetate, butyrate, 1,3-PDO and glycerol. (d) S3 growth on acetate and butyrate. (e) S3 growth on acetate, butyrate and 1,3-PDO. (f) S3 growth on acetate, butyrate, 1,3-PDO and glycerol.

Fig. 2. Profiles of carbon concentrations and cell density of consortium of S2 with S3 grown on synthetic and real glycerol ADE. (a) S2 and S3 grown on synthetic glycerol ADE with four carbon sources. (b) S2 and S3 grown on real glycerol ADE.

Fig. 3. Cell growth under different initial pH values and pH with time in real glycerol ADE. Full line presents the pH and dashed line presents OD_{600}.

Fig. 4. The profiles of cell density, ammonium nitrogen and carbon source concentration of consortium of S2 and S3 grown on real glycerol ADE at different C:N ratios. (a) Overview of cell density and ammonium nitrogen concentration at different C:N ratios. (b) Carbon sources concentration at C:N ratio of 4:1. (c) Carbon sources concentration at C:N ratio of 8:1. (d) Carbon sources concentration at C:N ratio of 4:1.

Fig. 5. Cell growth and PHAs synthesis with carbon consumptions in a fermentor. (a) Profile of ammonium nitrogen, biomass and PHAs content with time. (b) Profiles of carbon source and TOC concentration, and TOC from 1,3-PDO with time.
Table 1.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ace</th>
<th>But</th>
<th>Gly</th>
<th>1,3-PDO</th>
<th>Ace &amp; But</th>
<th>Ace, But &amp; 1,3-PDO</th>
<th>Ace, But, Gly &amp; 1,3-PDO</th>
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<tr>
<td>S1</td>
<td>0.73±0.29</td>
<td>1.26±0.12</td>
<td>0.87±0.01</td>
<td>0.58±0.04</td>
<td>0.31±0.02</td>
<td>0.31±0.01</td>
<td>0.30±0.02</td>
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<td>S2</td>
<td>0.47±0.03</td>
<td>1.53±0.08</td>
<td>0.16±0.01</td>
<td>0.03±0.01</td>
<td>1.13±0.15</td>
<td>1.19±0.04</td>
<td>1.02±0.15</td>
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<tr>
<td>S3</td>
<td>1.73±0.07</td>
<td>0.65±0.04</td>
<td>1.43±0.20</td>
<td>0.08±0.01</td>
<td>1.67±0.03</td>
<td>1.56±0.05</td>
<td>1.74±0.04</td>
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<tr>
<td>S4</td>
<td>0.38±0.02</td>
<td>0.11±0.01</td>
<td>0.20±0.02</td>
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<td>S5</td>
<td>1.44±0.01</td>
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<td>S7</td>
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<td>0.14±0.00</td>
<td>0.11±0.00</td>
<td>0.16±0.01</td>
</tr>
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</table>

Note: Ace: acetate; But: butyrate; Gly: glycerol.
Fig. 1.
**Fig. 1**

(a) Acetate, Butyrate, Glycerol, 1,3-PDO, OD<sub>600</sub> vs. Duration (Day)

(b) Lactate, Acetate, Butyrate, Glycerol, 1,3-PDO, OD<sub>600</sub> vs. Duration (Day)
Fig. 3.
Fig. 4.
Fig. 5.
**Fig. S1.** Consortium of S2 with S3 grown on LB agar plates after 40 hours in pH 5.2 (left), 6.0 (middle) and 7.0 (right).