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Butyric Acid and Caproic Acids Production Using Single and Mixed Bacterial Cultures †

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Abstract: In this study, we explored different bacterial strains (*Clostridium beijerinckii*, *C. acetobutylicum*, *C. oryzae*, and *C. kainantoi*) belonging to the Clostridium group and produced butyric acid (C4) using acetate as a carbon source. All the strains produced significant amounts of C4, but *C. beijerinckii* produced 1.54 g/L of C4, which is almost equivalent to the production capacity (1.63 g/L) of *C. kluyveri*. Further experiments were performed using diluted raw cheese whey (CW) by inoculating mixed bacterial cultures containing Clostridia, Bacillus, and Desulfobacteraceae groups. *Clostridium kluyveri* was added to the mixed culture, it stimulated the caproic acid (C6) production. Mixed bacterial culture produced 13.97 g/L, 10.83 g/L and 6.81 g/L of C6 when incubated with two times, five times and ten times diluted CW, respectively, within a 20-day incubation period. Compared with our previous study, the C6 production was higher and faster. These results indicated the dilution ratio of CW is an important factor in facilitating the C6 production, and higher fatty acids are produced with mixed culture than that of a single culture, i.e., *C. kluyveri*. Results have depicted the potentiality of employing the bio-augmentation strategy for the valorization of bioresources into valuable products like butyric acid and caproic acid.

Keywords: butyric acid; caproic acid; *C. kluyveri*; mixed culture; PHB; biomass

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1. Introduction

Products derived from fossil fuels have brought stability and convenience to humans. However, fossil fuels are limited resources and depleting every year because of their consumption. Therefore, biomass-based renewable processes have been developed as an alternative to fossil fuel-derived processes, and bioethanol is the most known. However, it requires a distillation process with high energy consumption. As a process to solve this problem, researchers are producing volatile fatty acids (VFAs) using anaerobic mixed culture. VFAs can be used in various fields as precursors in dyes, pharmaceuticals, food additives, bioplastic production, etc. Butyric acid is a precursor of polyhydroxybutyric acid (PHB), which is a main component of bioplastics [1], and caproic acid is a precursor in the production of biodiesel and is now drawing attention [2].

Recently Amabile et al. [3,4] used methane and VFAs from organic substrate digestion, produced the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with mechanical and thermal properties comparable with fossil fuel-based plastics. They showed that VFAs can be used as building blocks for several biopolymers. Among the VFAs studied, valeric acid induced the highest accumulation of 3-hydroxyvalerate units. Cai et al. have demonstrated that especially, even carbon VFAs (acetate and butyrate) synthesized only poly(3-hydroxybutyrate) (PHB), while the addition of odd carbon VFAs (propionate and

valerate) resulted in PHBV production [5]. Tian et al. also have mentioned that *Photobacterium* sp. TLY01 could effectively utilize plant oils or corn starch to synthesize poly-3-hydroxybutyrate (PHB). When propionate or valerate was added as secondary carbon source, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was produced [6].

Clostridium kluyveri is the most known bacteria producing fatty acids [7]. In previous studies, *Clostridium kluyveri* produced various fatty acids [8,9]. However, it is unknown whether *Clostridium kluyveri* is superior to other Clostridium group strains in the production of fatty acids. Hence, in this study, we explored different bacterial strains (*C. beijerinckill*, *C. acetobutylicum*, *C. oryzae*, and *C. kainantoi*) belonging to the Clostridium group and produced butyric acid (C4) using acetate as a carbon source. Further experiments were performed using diluted raw cheese whey (CW) by inoculating mixed bacterial cultures containing Clostridia, Bacillus, and Desulfobacteraceae groups. *Clostridium kluyveri* was added to the mixed culture to stimulate the production of caproic acid (C6).

Many efforts were made in the last years to lower the market price of PHB, and the possibility of using low-cost carbon sources was also explored [3,10–13]. Reports are also available for the valorization of CW into PHB [11,13]. CW is the liquid part of milk that is separated from the curd at the beginning of cheese manufacture, is available in large amounts as a by-product, and contains fermentable nutrients, such as lactose, lipids, and soluble proteins [11]. Its disposal as waste causes serious pollution problems. Therefore, the conversion of CW into a useful product, fatty acid, is a good option to reduce the environmental burden [13].

Numerous studies have been reported to produce VFAs as precursors in bioplastic production [8]. However, only a few studies succeeded in caproic acid production using CW [14–17]. Meanwhile, we reported that the dilution ratio of CW affected the PHB production ability [13]. Optimum substrate concentration may increase the productivity of valuable material. Therefore, this experiment seeks to improve the caproic acid content by diluting CW. The overview of fatty acid production by anaerobic mixed culture is shown in Figure 1.

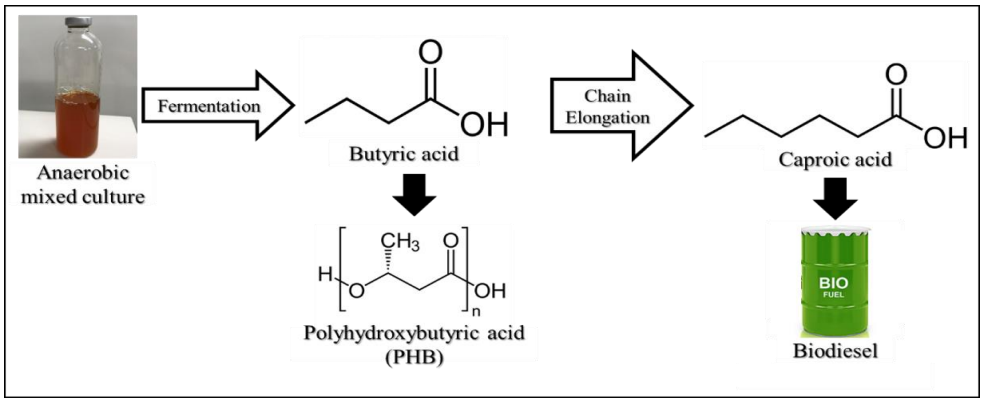

Figure 1. The overview of fatty acid production by anaerobic mixed culture.

2. Methods

2.1. Single Culture

The strains (*Clostridium kluyveri* NBRC 12016, *C. beijerinckii* NBRC 109359, *C. acetobutylicum* NBRC 13948, *C. oryzae* NBRC 110163, and *C. kainantoi* NBRC 3353) were used to investigate the C4 production. The medium contained (per liter) the following: 7.5 g acetate, 10 g yeast extract, 2.6 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4{\cdot}7H_2O$, 1.0 mL $FeSO_4$ (0.02 mmol/L), 1.0 mL Na_2MoO_4 (0.01 mmol/L), and 1.0 mL/L $MnSO_4$ (0.01 mmol/L). The medium (70 mL) was poured into 100-mL vials, sterilized by autoclave (121 °C, 20 min) and added with 4 mL/L ethanol and 0.25 g/L $CaCl_2{\cdot}2H_2O$. The headspace was flushed with nitrogen for 10 min and capped with an aluminum cap and butyl stopper. Finally, the medium was inoculated at a 10% (*v*/*v*) concentration using preculture of each strain, which was then

incubated anaerobically under orbital shaking at 80 rpm for 40 days at 37 °C for C4 production. All the conditions were tested as biological triplicates (three bottles, $n = 3$) within a single experiment. The data are presented as the mean ± standard deviation.

2.2. Mixed Culture

CW was collected from a cheese-producing company in Hokkaido, Japan, and stored in the dark at 4 °C. CW was further diluted (two times, five times, and ten times) and underwent the process of anaerobic digestion by adding enrichment culture containing *Anaerobaculum mobile*, *Actinomyces naturae*, *Bacterium*, *Bacillus cereus*, *Bacteroides fragilis*, *Clostridium glycolicum*, *Clostridium thiosulfatireducens*, *Clostridium* sp. LZLJ009, *Clostridium* sp. E034, *Desulfobacteraceae*, *Lactobacillus helveticus*, *Oscillibacter ruminantium*, *Sphingobacteriales* (10% (*v*/*v*)), and *Clostridium kluyveri* (10% (*v*/*v*)). Ethanol (15 mL/L) was added as an electron donor to stimulate C6 generation. The vials containing CW (70 mL) and bacteria were incubated under anaerobic conditions for 20 days. The bottles were closed and capped tightly with butyl rubber stoppers and aluminum caps, and the headspace was purged with nitrogen gas for 10 min to maintain strict anaerobic conditions. The bottles were kept at 37 °C in a rotating shaker (80 rpm). All the conditions were tested as biological triplicates (three bottles, $n = 3$) within a single experiment. The data are presented as the mean ± standard deviation.

2.3. Analysis

The concentrations of C4 were analyzed using high-performance liquid chromatography (HPLC, Shimadzu) with a refractive index (RI) detector and a Shim-pack SCR-102 (H) column (Shimadzu, Kyoto, Japan) [9]. Filtered and degassed perchloric acid was used as the mobile phase at a flow rate of 0.6 mL/min. The column was maintained at a temperature of 40 °C in a thermostat chamber. C6 was estimated by a transesterification method. For the transesterification of C6, boron trifluoride (BF_3) in methanol (14% *w*/*v*, GL series Inc. Japan) was used. C6 was analyzed by gas chromatography (GC, GC-2014, Shimadzu Co., Japan) equipped with a flame ionized detector (FID) and J&W DB-5 ms capillary column at a split rate of 10 and eluted with nitrogen as the carrier gas. Prepared samples were directly injected into the column at 40 °C. The column temperature was maintained at 40 °C for 2 min and then raised linearly at 10 °C /min to 150 °C. The detector and injector temperatures were 300 °C and 275 °C.

3. Results and Discussion

3.1. C4 Production by Single Culture

In the first experiment, all the strains produced significant amounts of C4. At 40th day, *C. beijerinckii* produced 1.54 g/L of C4, which is almost equivalent to the production capacity (1.63 g/L) of *C. kluyveri* (Figure 2). However, the maximum C4 production was accomplished within 5 days when *C. kluyveri* was used. This result indicated that *C. kluyveri* is the most useful bacterium among the tested Clostridium group.

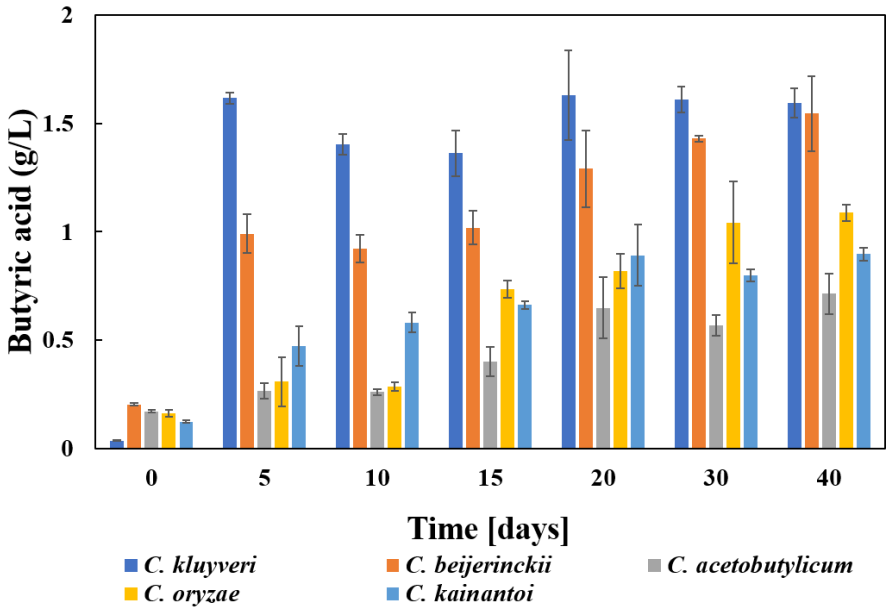

Figure 2. The time course of C4 production by each species of the genus, Clostridium.

The data are presented as the mean ± standard deviation. The variation range of each condition on butyric acid production was 9–20%.

3.2. C6 Production by Mixed Culture

It has been reported that *C. kluyveri* can grow within pH range of 6.0–7.5, and the optimum pH is 6.4 [18]. Therefore, pH measurement was performed to examine the change in pH. When the pH was below 6.0, pH adjustments were made with 5M NaOH. Figure 3 shows the changes in pH values over the time in diluted cheese whey. Although the pH was 6.4 after the start of the culture, it decreased to pH 5.0 on the second day, so the pH was adjusted. After that, the pH was 6.0 or higher until the 6th day, so the pH was not adjusted. On the 8th day, the pH decreased to 6.0 or less, so the pH was adjusted again, no pH adjustment was done afterwards. Interestingly, the difference in pH fluctuations pattern was insignificant at any dilution rate.

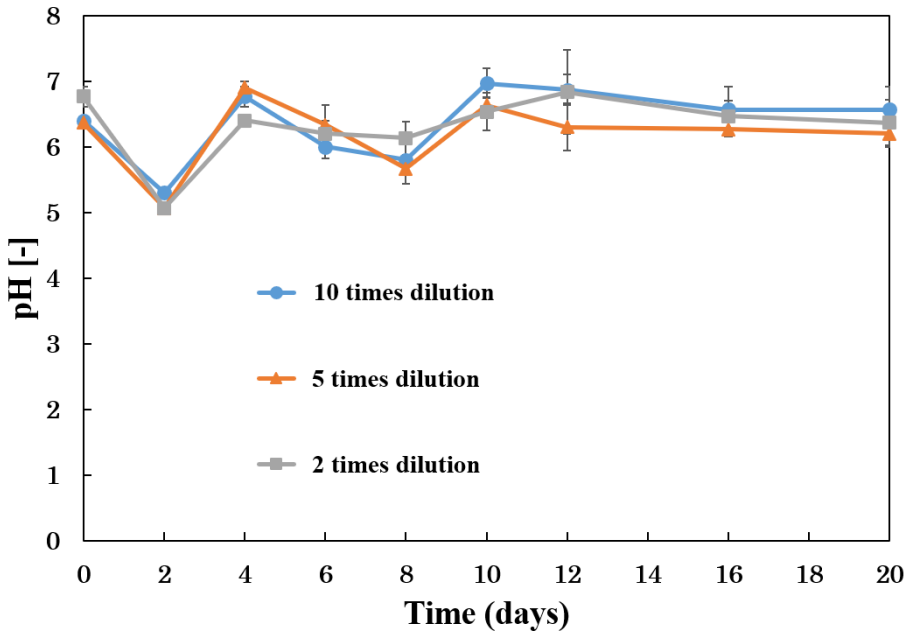

Figure 3. The time course of pH change in each CW dilution conditions.

The data are presented as the mean ± standard deviation. The pH variation range of each condition was 0.5–5.9%.

On the other hand, C6 was not observed when raw CW was used (data not shown). However, mixed bacterial culture produced 13.97 g/L, 10.83 g/L, and 6.81 g/L of C6 when incubated with two times, five times, and ten times diluted CW, respectively, within a 20-day incubation period (Figure 4). Compared with our previous study (4.8 g/L at 53rd day), the C6 production was higher and faster [9]. These results indicated the dilution ratio of CW is an important factor in facilitating C6 production, and higher fatty acids are produced in mixed culture than that in a single culture, i.e., *C. kluyveri* at 3.2 g/L with acetate [9]. Previous research reported on C6 production using various substrates. Production rates can be achieved from the organic fraction of municipal solid waste by applying a two-stage conversion, such as an acidification step followed by a chain elongation step. They reported the production of C6 (12.6 g/L) [19]. Domingos et al. reported the generation of C6 (4.0 g/L) using CW and mixed bacterial cultures [15]. Sarkar et al. produced C6 (13.02 g COD/L) by co-fermenting brewery spent grains and CW [14]. Therefore, C6 production in this study (13.97 g/L) is superior to those of previous studies.

Substrate concentration is important to VFA composition during anaerobic fermentation [20]. Wang et al. [21] reported that chain elongation from acetate to *n*-butyrate played an important role with a substrate concentration of 200 g/L in the acidogenesis process. With the substrate concentration of 100 g/L, the obtained VFA production was low, with the substrate concentration of 200 g/L, total VFA production increased obviously. When substrate concentration further increased to 300 and 400 g/L, the VFA production was inhibited, which might be attributed to the accumulation of lactate because of the rapid and easy lactate fermentation [22]. The experiment was performed under non-methanogenesis. Methanogenesis was not observed in our study. Substrate concentration as chemical oxygen demand (COD) at each dilution was 107 g/L (raw CW), 53.5 g/L (two times diluted CW), 21.4 g/L (five times diluted CW), 10.7 g/L (ten times diluted CW). When CW concentration increased from 21.4 g/L to 53.5 g/L, C6 production increased obviously (Figure 4). The C/N ratio is also an important factor in the production of VFAs [23]. However, in this study, all experiments were performed with the same C/N ratio, even though the dilution ratio of CW was different. Nitrogen concentration also affects VFA production [24]. In this study, nitrogen concentration as total nitrogen at each dilution was 1.4 g/L (raw CW), 0.7 g/L (two times diluted CW), 0.28 g/L (five times diluted CW), 0.14 g/L (ten times diluted CW). The highest C6 production was observed at 0.7 g/L (Figure 4). Kamzolova et al. [24] reported that nitrogen source concentrations limit the growth of *Y. lipolytica,* and nitrogen deficiency is the main cause of citric acid excretion. Citric acid was accumulated in meaningful quantities only in media containing 3–10 g/L $(NH_4)_2SO_4$ with the maximum concentration of citric acid at 4 g/L ammonium sulfate.

On the other hand, Gildemyn et al. reported that a lower ethanol/substrate (acetic acid) ratio (3:1 instead of 10:1) enabled faster and more efficient *n*-caproic acid production [2]. A similar result was obtained in this study. The total concentration of substrates in raw CW is shown in Table 1. Ethanol/substrates ratio of two times, five times, and ten times diluted CW are calculated as (4.9:1.0), (12.3:1.0), and (24.7:1.0). Results have depicted the potentiality of employing the bio-augmentation strategy for the valorization of bioresources into valuable products like butyric acid and caproic acid.

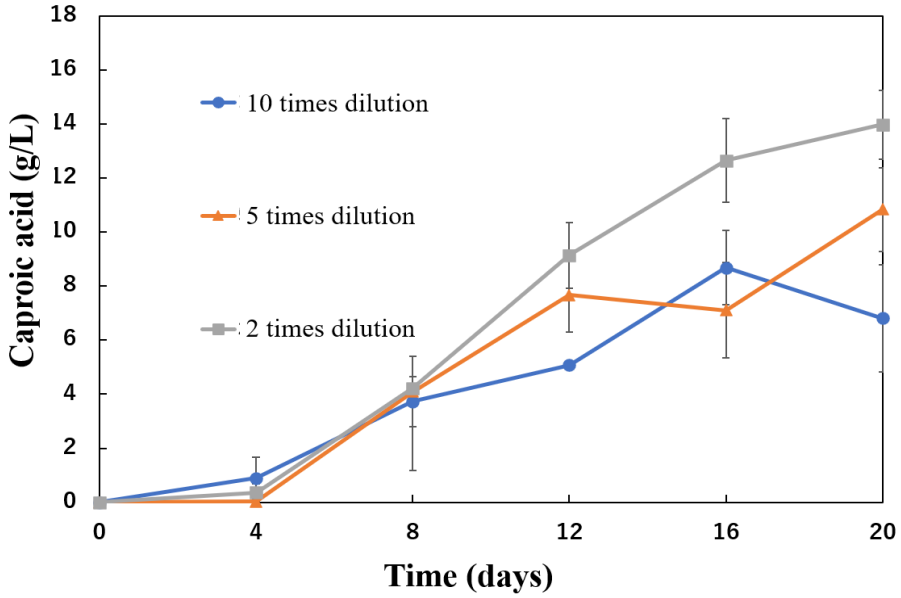

Figure 4. The time course of C6 production by a mixed culture.

The data are presented as the mean ± standard deviation. The variation range of each condition on caproic acid production was 9.2–60%.

Table 1. Calculated concentration of each substrate in raw CW.

Substrates	Concentration (g/L)	Concentration (mmol/L)
Lactose	30.1	87.9
Lactic acid	3.1	34.4
Acetic acid	0.7	11.7
Propionic acid	0.8	10.8
Butyric acid	0.0	0.0
Total amount	-	144.8
Ethanol (in CW)	1.4	30.4
Total amount (with addition of Ethanol)	16.4	357

The data are presented as the mean of two samples. The variation range of each concentration was 0.5–5.8%.

4. Conclusions

In this study, C4 production experiments were carried out using *C. kluyveri*, which are well-known as single- and medium-chain fatty acid-producing strains, and the productibility of C4 was compared with other Clostridium strains. *C. kluyveri* produced higher C4 than other Clostridium group strains. Based on the above results, we conducted further experiments and produced C6 from cheese whey using mixed bacteria containing *C. kluyveri*. Cheese whey was diluted, and the ratio of ethanol to the substrate was controlled to examine the effect on C6 production. As a result, no C6 production was observed from the undiluted cheese whey, but it was found that the 2-fold diluted conditions showed higher production than the 5-fold and 10-fold diluted conditions. These results indicated that the ratio of ethanol to substrate is an essential factor for the elongation of fatty acids.

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