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Proceeding Paper

Evaluation of Bacterial Growth Ability and PHA Production Using Various Combinations of Fatty Acids †

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Abstract: The present study explains the growth pattern and polyhydroxyalkanoate (PHA) production capacity of *Bacillus* sp. CYR1 using various fatty acids as carbon sources. Various combinations of fatty acids were used for the growth of strain CYR1. Among them, strain CYR1 showed good growth with the combination of two fatty acids (acetic acid and butyric acid) and the combination of three fatty acids (acetic acid, propionic acid, and caproic acid). Apart from the growth pattern, PHA production was also evaluated. PHA production was in coordination with the growth pattern. Bacteria incubated with the combination of acetic acid-butyric acid produced 0.158 g/L, and acetic acid-propionic acid-caproic acid produced 0.241 g/L of PHA. It is essential to reduce the substrate cost for PHA production by replacing expensive carbon sources with wastewater. The organic compounds in domestic and industrial wastewater contain various fatty acids. By studying different combinations of fatty acids, we gained new insights into utilizing wastewater containing various fatty acids as substrates for PHA production.

Keywords: *Bacillus* sp. CYR1; fatty acids; PHA; reuse; wastewater

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1. Introduction

Plastic has become one of the most used materials in modern society. The primary raw material for the production of plastics is petroleum, and it is estimated that 830 million tons are produced annually in the world [1]. Fossil fuels such as petroleum are exhaustible resources, and in the future, plastic manufacturing industries may face problems. On the other side, the amount of plastic waste is increasing rapidly, and flow into the environment, such as the ocean. Because plastics are persistent, they remain in the ocean for a long time and are eaten by marine organisms such as cetaceans and sea turtles, polluting the entire marine ecosystem [2].

On the other hand, many microorganisms can synthesize and accumulate macromolecular polymers with (*R*)-3-hydroxyalkanoic acid monomers in their cells under certain nutrient (such as nitrogen and phosphorus) deficient conditions [3]. This high-molecular-weight polymer exhibits thermoplasticity similar to that of synthetic plastics and has the property of being degraded in the natural environment. Among macromolecular polymers, polyhydroxyalkanoate (PHA) is biodegradable plastic. The production cost of PHA depends on the productivity of the microorganisms used, substrates such as carbon and nitrogen sources, temperature, pH value, and culture conditions such as aeration, recovery, and purification processes [4]. Among them, the substrate cost accounts for 25% to 45% of the production cost [4], which is one of the significant obstacles in the industrial production of PHA.

Sugar components in various wastewaters are mainly converted into fatty acids after anaerobic digestion. Domestic wastewater and sewage systems mainly contain acetic and propionic acid [5,6]. PHA production using fatty acids has been reported because of its usefulness, but most experiments are limited to using one or two kinds of fatty acids [7–11]. PHA production in the presence of three or more types of fatty acids has not yet been reported. Under such circumstances, experiments were conducted to provide essential data that would serve as scientific evidence for evaluating the utility value of various wastewaters obtained from each region for PHA production. We have not used wastewater in this experiment, but we examined the growth ability of *Bacillus* sp. CYR1 in various combinations (two, three, or four types) of fatty acids by assuming that these fatty acids exist in the wastewater and for the use of real wastewater for PHA production in the future.

2. Methods

2.1. Evaluation of the Growth Ability of CYR1 Strain by a Combination of Various Fatty Acids

Short-chain and medium-chain fatty acids were used as substrates in this experiment. Acetic acid, propionic acid, butyric acid, valeric acid, and caproic acid were used as short-chain fatty acids. Enanthic acid and caprylic acid were used as medium-chain fatty acids. A preculture was performed for 8 h with Nutrient Broth (NB) medium to ensure a sufficient number of cells before the main culture. The mineral salt medium [8,9] was placed in a 300 mL beaker, and two, three, or four types of fatty acids were added at the final concentration of 20 g/L. After that, the pH was adjusted to 7.0 using 5N NaOH, and the 30 mL medium was dispensed into 50 mL vials. After autoclaving the vials, preculture of *Bacillus* sp. CYR1 strain (DNA Data Bank of Japan accession number LC049103) (8% *v*/*v*) was inoculated to the vials aseptically. The vials were capped with a silicon stopper and cultured at 30 °C with shaking at 100 rpm. After that, sampling was performed every 24 h for 96 h. For sampling, 0.7 mL of the culture medium was taken for pH measurement, and 1.0 mL was taken for OD_{600} analysis. Experiments were performed in triplicate.

2.2. PHA Production by CYR1 Strain

Based on growth curve results, we selected the combinations that gave the best growth of CYR1 strain in the combination of two kinds of mixed fatty acids in the combination of three kinds of mixed fatty acids. Wet cell mass, dry cell mass, PHA production, and PHA content were measured. The experiment was performed in duplicate. PHA was extracted according to the method of Reddy et al. [8].

3. Results and Discussion

Figure 1a,b showed the time-dependent changes in OD_{600} and pH of strain CYR1 in the presence of two kinds of mixed fatty acids. From the OD_{600} values, it was found that the combination of acetic acid (A) and butyric acid (C) showed a higher OD_{600} value of 1.7. The combination of acetic acid (A) and valeric acid (D) showed a high OD_{600} value of 1.6 (Figure 1a, upper). According to Watanabe [12], the strain CYR1 showed OD_{600} value, 1.3-1.5 with acetic acid at the concentration of 20 g/L, OD_{600} value, 0.7–0.8 with butyric acid at the concentration of 5 g/L, and valeric acid at the concentration of 5 g/L. The OD_{600} value of 1.3–1.5 was observed with the combination of acetic acid (20 g/L), butyric acid (5 g/L), and valeric acid (20 g/L) (Figure 2a). From this; it is considered that the synergistic effect of OD_{600} can be obtained by mixing simple fatty acids with high proliferation. On the other hand, the change in pH over time indirectly confirms PHA production. In addition, a significant increase in pH was observed in combination with a high growth rate, suggesting that pH increase, growth, and PHA production are inter-linked each other (Figure 1b). The increase in pH was attributed to the formation of PHA monomers or copolymers.

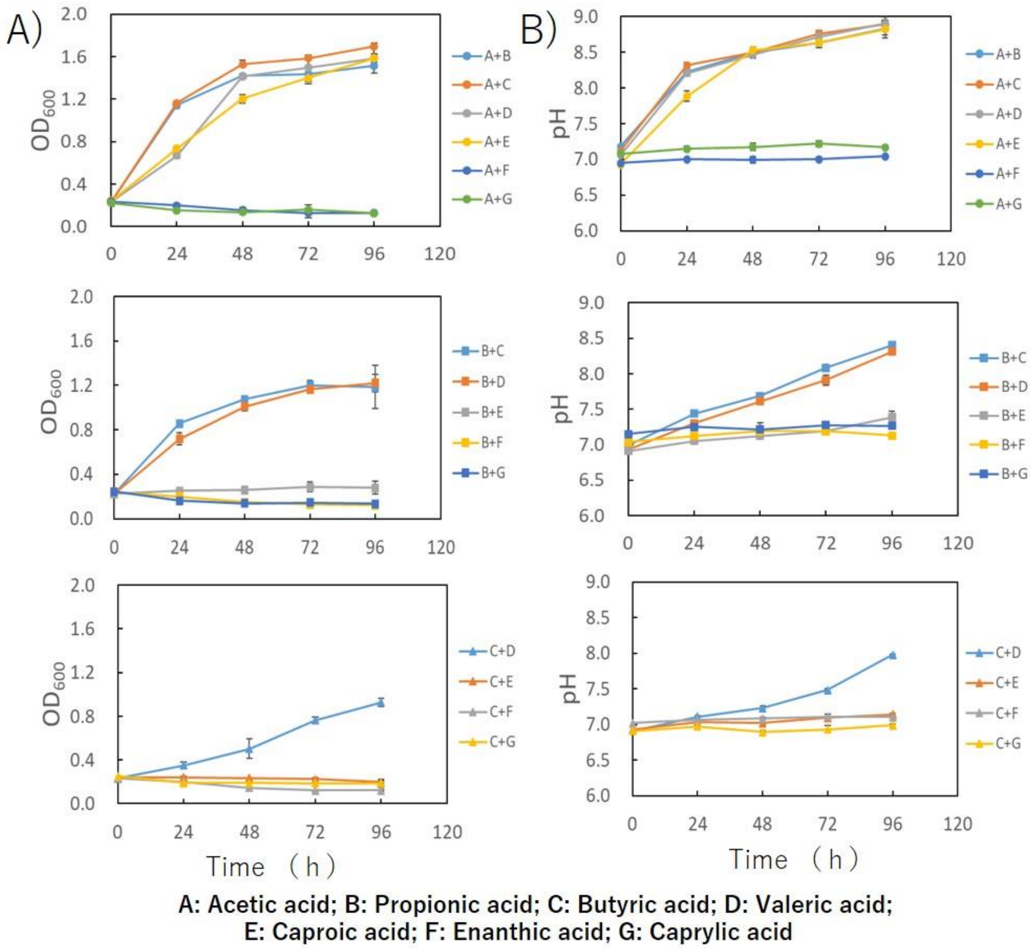

Figure 1. Time-dependent changes in OD_{600} (**A**) and pH (**B**) of medium grown with strain CYR1 in the mixture of two kinds of fatty acids.

Figure 2a,b showed the time-dependent changes in OD_{600} and pH of the medium in the presence of a mixture of three different fatty acids. The growth was high (OD_{600} is 1.6) compared with the combination of acetic acid (A), propionic acid (B), and caproic acid (E), high (OD_{600} is 1.5) with the combination of acetic acid (A), butyric acid (C), and caproic acid (E) (Figure 2a). CYR1 growth was slightly decreased with the mixture of three different fatty acids, compared with the mixture of two different fatty acids. Watanabe reported that the OD_{600} was 0.8–0.9 at a propionic acid concentration of 5 g/L and 0.8–0.9 at a caproic acid concentration of 5 g/L, and the proliferation rate was considerably decreased at concentrations above 5 g/L. When comparing the results of this experiment, the growth of the CYR1 strain was promoted even in the presence of three types of mixed fatty acids, suggesting that the substrate conditions are suitable for PHA production. On the other hand, an increase in pH was observed when the proliferation of cells was observed, but no change in pH was observed under conditions in which a gradual decrease in OD_{600} was observed (Figure 2b).

The time-dependent changes in OD_{600} and pH of medium grown with strain CYR1 in the presence of a mixture of four kinds of fatty acids were tested. The OD_{600} was 1.6, which was almost the same as the optimal growth conditions using the mixture of three fatty acids (Figure 3a). Among the mixture of four fatty acids, acetic acid (A), propionic acid (B), butyric acid (C), and valeric acid (D) mixture showed the highest growth (Figure 3a). The growth with this mixture is even higher than using a single type of fatty acids. On the other hand, no growth was observed in the presence of medium-chain fatty acids under any conditions. Watanabe [12] also reported growth inhibition in the presence of medium-chain fatty acids enanthic acid and caprylic acid, suggesting that enanthic acid and caprylic acid are unsuitable substrates for PHA production using the strain CYR1. In the case of pH, an increase in pH was observed when cell proliferation was started (Figure 3b). In conclusion, the combination of acetic acid and butyric acid showed the best growth in the presence of a mixture of two fatty acids, the combination of acetic acid, propionic acid, and caproic acid showed the best growth in the presence of a mixture of three fatty acids.

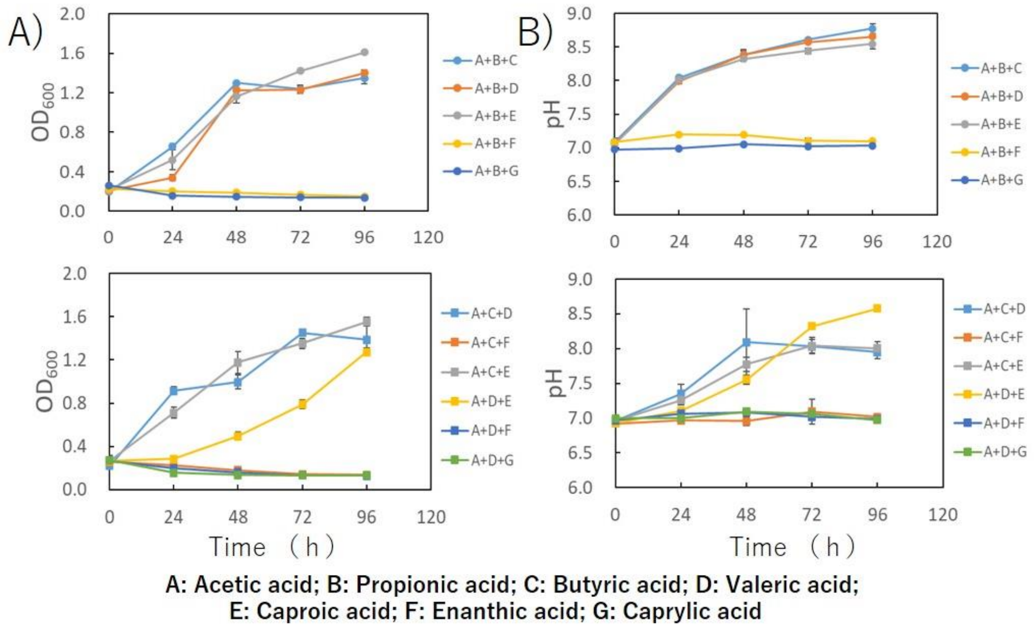

Figure 2. Time-dependent changes in OD_{600} (**A**) and pH (**B**) of medium grown with strain CYR1 in the mixture of three kinds of fatty acids.

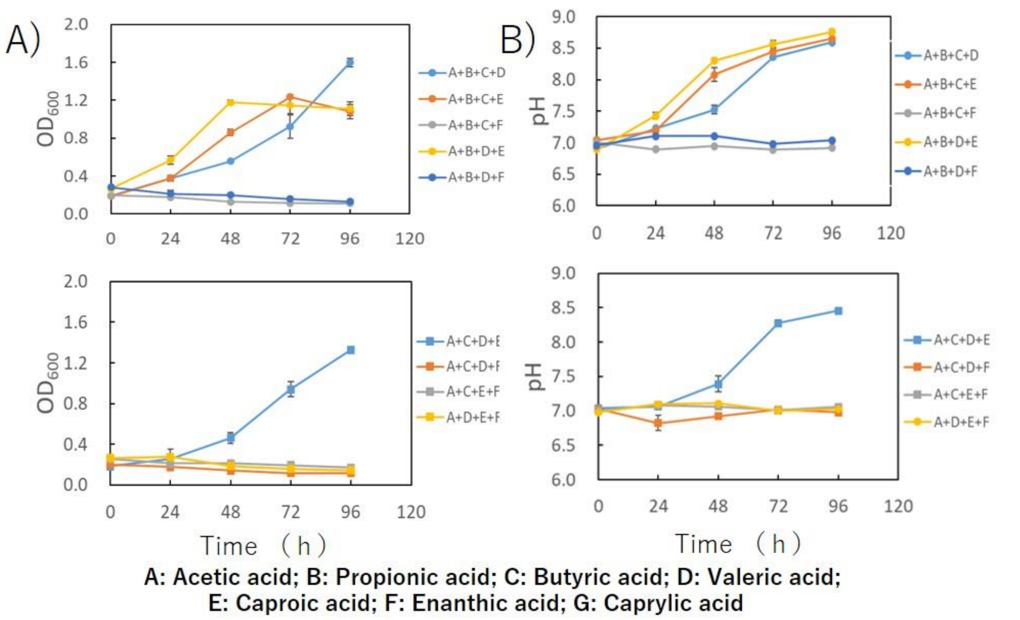

Figure 3. Time-dependent changes in OD_{600} (**A**) and pH (**B**) of medium grown with strain CYR1 in the mixture of four kinds of fatty acids.

As shown in Table 1, the amount of PHA (g/L) and PHA production (% cell dry mass (CDM)) calculated based on the Soxhlet extraction method was 0.158 g/L and 41.8 (%CDM) for the combination of acetic acid and butyric acid, and 0.241 g/L and 40.16 (%CDM) for the combination of acetic acid, propionic acid, and caproic acid. The wet and dry weights were higher in the mixture of three fatty acids, but the % PHA was higher in the mixture of two fatty acids.

Table 1. PHA production with the mixture of two or three types of fatty acids.

Substrates	CWM (g/L)	CDM (g/L)	PHA Production (g/L)	PHA Production (%CDM)
Acetic acid, Butyric acid	2.07	0.38	0.158	41.80
Acetic acid, Propionic acid, Caproic acid	2.51	0.60	0.241	40.16

Data are the means of experiments performed in duplicate. CWM: cell wet mass; CDM: cell dry mass.

4. Conclusions

The mixture of various fatty acids, such as acetic acid, valeric acid, and butyric acid, enhanced the growth and PHA production capacity of the CYR1 strain. Based on the results of this experiment, it is worth investigating PHA production using sewage or sludge, which generally contains a mixture of fatty acids. However, to bring PHA production using fatty acids closer to practical use, it is necessary to evaluate the physical properties, heat resistance, and stretchability of the produced PHA.

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References

1. Geyer, R.; Jambeck, J.R.; Law, K.L. Production, use, and fate of all plastics ever made. *Am. Sci. Adv.* **2017**, *3*, e1700782. https://doi.org/10.1126/sciadv.1700782.
2. Yamashita, R.; Tanaka, A.; Takada, S. Marine Plastic Pollution: Dynamics of plastic debris in marine ecosystem and effect on marine organisms. *Jpn. J. Ecol.* **2016**, *66*, 51–68.
3. Uchino, K.; Shiraki, M.; Saitou, M. Metabolism of poly-3-hydroxy butyric acid. *Sci. J. Kanagawa Univ.* **2009**, *20*, 283–286. (In Japanese).
4. Nath, A.; Dixit, M.; Bandiya, A.; Chavda, S.; Desai, A.J. Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24. *Bioresour. Technol.* **2008**, *99*, 5749–5755.
5. Yun, Z.; Yun, G.H.; Lee, H.S.; Yoo, T.U. The variation of volatile fatty acid compositions in sewer length, and its effect on the process design of biological nutrient removal. *Wat. Sci. Technol.* **2013**, *67*, 2753–2760.
6. Fernado, M.S.; Anton, K.; Peter, J.; Steven, P.; Nico, B.; Paul, L.; Alan, W. Production of polyhydoxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics nitrogen and phosphorus. *Water Res.* **2010**, *44*, 5196–5211.

7. Liliana, M.H.; Bronwyn, L.; Alan, W.; Steven, P. The evolution of polymer composition during PHA accumulation: The significance of reducing equivalents. *Bioengineering* **2017**, *4*, 20. https://doi.org/10.3390/bioengineering4010020.
8. Reddy, M.V.; Mawatari, Y.; Yajima, Y.; Satoh, K.; Mohan, S.V.; Chang, Y.C. Production of poly-3-hydroxybutyrate(P3HB) and poly(3-hydroxbutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV) from synthetic wastewater using *hydrogenophaga palleronii*. *Bioresour. Technol.* **2016**, *215*, 155–162.
9. Reddy, M.V.; Mawatari, Y.; Onodera, R.; Nakamura, Y.; Yajima, Y.; Chang, Y.C. Polyhydroxyalkanoates (PHA) production from synthetic waste using *Pseudomonas pseudoflava*: PHA synthase enzyme activity analysis from *P. pseudoflava and P. palleronii*. *Bioresour. Technol.* **2017**, *234*, 99–105.
10. Jian, Y. Production of PHA from starchy wastewater via organic acid. *J. Biotechnol.* **2001**, *86*, 105–112.
11. Munawar, K.; Munawar, M.; Simarani, K.; Mohamad, S.; Mohamad, A. Bioconversion of mixed free fatty acid to poly-3-hydroxyalkanoates by *Pseudomonas putida* BET001 and modeling of its fermentation in shake flasks. *Electron. J. Biotechnol.* **2016**, *19*, 50–55.
12. Watanabe, A. A Study on PHA Production Using Multiple Fatty Acids by *Bacillus* sp. CYR1 Strain. Graduation Thesis, Muroran Institute of Technology, Muroran, Japan, 2020. (In Japanese).