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Production of polyhydroxyalkanoates using sewage and cheese whey

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ABSTRACT

Recently, polyhydroxyalkanoates (PHAs) have been produced using raw sewage in our laboratory; however, the production concentrations are low. Therefore, this study aimed to enhance PHA production by applying different strategies. PHA production was higher in sewage-containing medium than in mineral salt medium and was enhanced 22-fold after glucose supplementation. A relatively high degree of glucose consumption (83.6 ± 1.59 %) was also achieved. Bacteria incubated with cheese whey diluted with sewage showed higher PHA production than bacteria incubated with cheese whey diluted with distilled water did. The expression of the PHA synthase gene (*phaC*) was evaluated via real-time polymerase chain reaction using low- and high-carbon-containing sewage. Relatively higher *phaC* expression levels were observed in high-carbon-containing sewage but at lower nitrogen concentrations. The characteristics of the produced PHA were comparable to those of standard PHA. Therefore, this study revealed that the bacterium *Bacillus* sp. CYR1 can produce PHA from low- or high-carbon-containing wastewater.

1. Introduction

Water scarcity is a global issue that has been exacerbated by the increasing volume of wastewater generated because of the increasing population and economic growth. According to Jones et al. [1], global wastewater production is estimated at 359.4×10^9 m^3 yr^{-1}. Water reuse has emerged as one of the most viable strategies for addressing this challenge [2–4]. Greywater, which comprises used water from bathroom sinks, showers, tubs, and kitchen sewage (excluding toilet and food wastes from garbage grinders) [5], holds potential for reuse. In many European countries, approximately 75 % of treated wastewater is utilized for agricultural purposes [6]. Furthermore, the possibility of reacclimating wastewater from industrial sources is being actively explored [7,8]. Extensive efforts are being directed toward harnessing the practical applications of greywater and treated wastewater.

Although water reuse is a commendable goal, many challenges impede the use of blackwater, which comprises human waste, typically from toilets and similar sources [4,9]. Blackwater is often highly contaminated and requires appropriate treatment to remove pathogens and pollutants before it can be safely discharged into the environment or reused. It is distinct from greywater, which

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comprises wastewater from non-toilet sources such as sinks, showers, and laundry. The utilization of blackwater in chemical production is complicated by the risk of bacterial, viral, and pathogenic contamination. Including sewage, blackwater must be treated before being used in applications that impact human health, such as food production, agriculture, and drinking water supply.

Sewage treatment plants are significant energy consumers that contribute to 0.5 % of Japan's total CO_2 emissions, accounting for 0.7 % of the annual energy consumption [10]. Consequently, the development of technology that enables sewage utilization for chemical production prior to treatment could play a vital role in reducing energy consumption and advancing decarbonisation efforts in the economy. The key advantage of using sewage for chemical production lies in its wide availability, which obviates the need for transportation. Sewage treatment plants are a pervasive, sustainable, and readily accessible resource. Therefore, under rigorous sanitary controls, the potential reuse of blackwater including sewage for chemical production can be explored to mitigate environmental impact and reduce wastewater treatment costs.

Bioplastics play a pivotal role in advancing the sustainability of the circular economy [11], with polyhydroxyalkanoates (PHAs) being a notable example. PHAs produced by various bacteria and archaea using different carbon sources have garnered attention for their eco-friendliness [12]. The utilization of raw sewage presents an innovative upcycling approach that holds the promise of reducing wastewater treatment expenses. In our laboratory, we recently produced PHAs using raw sewage; however, we encountered challenges such as low yields, primarily due to the limited carbon content of sewage [13]. Although previous research has explored PHA production from various biomass sources, including industrial, agricultural, municipal, oil-based, and food-based wastes such as whey, glycerol, molasses, leftover coffee grounds, fruit wastes, and lignin-rich materials [14,15], the market price of PHA polymers remains significantly higher, typically 3–6 times higher, compared to petroleum-based alternatives [16,17]. Hence, leveraging sewage for PHA production not only enhances economic viability but also addresses the daily challenge of managing sewage while alleviating the burden on wastewater treatment facilities.

In an effort to reduce PHA production costs, we explored a novel approach involving the use of sewage. Additionally, when producing PHA from high-carbon wastewater, such as cheese whey, it is necessary to dilute the wastewater to a concentration suitable for microbial utilization [15]. Therefore, we assessed the potential of sewage as a dilution medium for PHA production.

This study was aimed at investigating PHA productivity using sewage instead of deionised water by employing different strategies, including supplementation of additional carbon sources to sewage and dilution of high-carbon wastewater (cheese whey) with sewage. For biocatalysis, we utilized the bacterium *Bacillus* sp. CYR1 previously isolated in our laboratory, which is characterized by its ability to produce various forms of PHA [18]. Strain CYR1 efficiently produces poly (3-hydroxybutyrate) (P3HB) and poly (3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) from fatty acids [13]. Additionally, it exhibits high P3HB production capability when utilizing diverse carbon sources, including glucose, sewage, cheese whey, and various aromatic organic compounds such as phenol, naphthalene, 4-chlorophenol, and 4-nonylphenol [15,19–21]. Furthermore, we evaluated the expression of the PHA synthase gene (*phaC*), a pivotal component in PHA synthesis, in sewage with varying carbon contents. Lastly, we conducted a comprehensive analysis of the PHA produced from sewage-diluted cheese whey using Fourier-transform infrared (FT-IR) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

2. Materials and methods

2.1. Sewage and cheese whey

Two types of wastewaters (raw sewage and raw cheese whey [CW]) were used in this study. Raw sewage was collected from the influent of the Rantoh wastewater treatment plant in Muroran, Japan (Fig. S1), which possessed a chemical oxygen demand (COD) of 102 ± 7.77 mg/L and total nitrogen (TN) content of 27 ± 2.82 mg/L. CW was collected from a cheese-producing company in Noboribetsu City, Japan. The COD and TN of this CW were 107,250 ± 480.83 mg/L and 1375 ± 31.11 mg/L, respectively. After collection, both wastewaters were stored in a dark room at 4 °C for three months. Large-sized suspended solids in the sewage water were crushed using a homogeniser (AHG-160D; AS ONE, Osaka) for 5 min at 1300 rpm. To remove inorganic solids, sewage water was then filtered using a metallic sieve (wire diameter 0.430 mm, Mesh 20; Kansai Wire Netting Co., Ltd., Osaka, Japan).

2.2. Bacteria

Bacillus sp. CYR1, is Gram-positive bacteria, which was previously isolated in our laboratory, was used to produce PHA. Using Gram-positive bacterial strains for PHA production offers several benefits, making them attractive choices for executing this process. For example, (i) it is easier to optimize the metabolic pathways of Gram-positive bacterial strains for PHA production by introducing or modifying genes involved in the PHA biosynthesis process, (ii) endotoxin formation is absent in Gram-positive bacterial strains, which is advantageous for biomedical applications of produced PHAs, (iii) Gram-positive bacterial strains are more robust and less susceptible to contamination compared to some Gram-negative strains, and (iv) Gram-positive bacterial strains typically grow in relatively simple and cost-effective media (including agricultural and industrial waste) and require fewer nutrients and simpler conditions for cultivation. This can result in reduced production costs and easier scale-up. A pre-culture was prepared by inoculating a loop of *Bacillus* sp. CYR1 in 100 mL of nutrient broth in Erlenmeyer flasks. The flasks were subjected to shaking at 120 rpm at 30 °C for 12 h.

2.3. PHA production

PHA production was performed using sewage, mineral salt (MS) medium, and CW, with or without glucose supplementation.

2.3.1. PHA production using sewage

We inoculated pre-cultured *Bacillus* sp. CYR1 at 4 % (v/v) into flasks containing sewage. The cultures were then incubated aerobically in a shaking incubator at 30 °C and 120 rpm for 96 h. PHA production was evaluated using sewage or MS medium as the substrate under three conditions. i) In the first condition, raw sewage was used as the carbon source and as the primary culture. The supernatant obtained after centrifuging the primary culture was used as the secondary culture, and the supernatant obtained after centrifuging the secondary culture was used as the tertiary culture. ii) In the second condition, MS medium supplemented with glucose was used as the carbon source. However, glucose (20 g/L) was supplied at the primary culture stage only, after which it was not added in the secondary and tertiary cultures. iii) In the third condition, sewage supplemented with glucose was used in the primary, secondary, and tertiary cultures. Glucose (20 g/L) was added at each stage of culture (primary, secondary, and tertiary). iv) In the fourth condition, sewage supplemented with glucose was used as the carbon source. However, glucose (20 g/L) was supplied initially at the primary culture stage, after which it was not added in the secondary and tertiary cultures. The above operating conditions are shown in the process flow diagram (Fig. S2).

After adjusting the pH to 7 using 1 N HCl or 1 N NaOH, 500 mL of sewage was added to a 2-L Erlenmeyer flask and then sterilised in an autoclave (121 °C, 15 min). The pre-cultured *Bacillus* sp. CYR1 4 % (v/v) was inoculated into sewage-containing flasks and incubated in a shaking incubator at 30 °C at 120 rpm for 96 h. Sampling was performed at 0, 24, 48, 72, and 96 h for optical density at 600 nm (OD_{600}) and pH measurements. Cell growth was measured as described previously [20]. Protein concentration was determined using the TaKaRa Bradford Protein Assay Kit (Kusatsu, Japan) using the supernatant obtained after centrifugation. Each sewage sample without added bacteria was used as a blank for OD_{600} measurements. PHA extraction was performed 96 h after the culture was grown using raw sewage and defined as the primary culture. Centrifugation was performed to isolate the culture from the sewage. The resulting pellets were used for PHA extraction. After adjusting the pH to 7, the collected supernatant was sterilised, cooled, and incubated with the same bacteria for 96 h to produce PHA and was denoted as the secondary culture. After extracting PHA from the secondary culture, the supernatant was again used to produce PHA and referred to as the tertiary culture. Similar culture conditions were maintained for all experiments.

2.3.2. PHA production using MS medium

PHA production was evaluated using the MS medium. The composition of the MS medium was as described previously [15,19]. After adjusting the pH to 7, 500 mL of MS medium was added to a 2-L Erlenmeyer flask and then sterilised in an autoclave (121 °C, 15 min). The pre-cultured *Bacillus* sp. CYR1 4 % (v/v) was inoculated into MS medium in the flasks and incubated in a shaking incubator at 30 °C and 120 rpm for 96 h. Sterilised glucose solution (20 g/L) was used as a carbon source and supplemented with MS medium. Primary, secondary, and tertiary cultures were prepared; however, the carbon source (20 g/L glucose) was supplied initially at the primary culture stage, and afterwards, it was not provided in the secondary and tertiary cultures. Sampling was performed at different time points for OD_{600} and pH measurements. Cell growth was measured as previously described. PHA extraction was performed at 96 h.

2.3.3. PHA production using CW

Dilutions were performed using either sewage or deionised water. PHA production experiments were performed after removing excess protein from the CW. CW was acidified to reach pH 4.0 using 1.0 M H_2SO_4 to remove excess protein [22]. The solution was autoclaved and centrifuged (8000×*g*) using himac CF16RX (Hitachi, Tokyo, Japan) at 4 °C for 10 min to remove aggregates [23]. The supernatant is filtered. The pH of each diluted sample was adjusted to 6.6. A bacterial pellet obtained from the pre-grown CYR1 cultures (100 mL) was dissolved in 10 mL of diluted CW, from which 2.5 mL was added to 2-L flasks that contained 500 mL of diluted CW. Culture conditions were maintained for PHA production as described in Section 2.3.1. The control experiments were performed without inoculation. All the experiments were performed in duplicates.

2.4. PHA extraction and property evaluation

PHA extraction from cultures was done as described previously [24], as well as by using the Soxhlet extraction method. Centrifugation (8000×*g*) was performed to separate the cells. The obtained biomass pellet was placed in an extraction thimble, chloroform was added to a round-bottom flask, and Soxhlet extraction was performed at 80 °C for 24 h. After Soxhlet extraction, $CHCl_3$ was passed through a filter paper to remove the cell debris and evaporated using a rotary evaporator (Eyela N-1000; Tokyo). Cold ethanol was added to a round-bottomed flask to obtain PHA. PHA was analysed using high-performance liquid chromatography (HPLC) according to the method described by Saito et al. [18].

PHA extracted from bacterium CYR1 that had been incubated with sewage-diluted CW was used to determine the structure and physical properties. FT-IR spectra were measured using a IRSpirit spectrophotometer equipped with QATR-S (Shimadzu, Kyoto, Japan). ^{1}H and ^{13}C NMR analysis, thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC) were carried out according to the methods described by Reddy et al. [19].

2.5. phaC gene expression analysis

Bacillus sp. CYR1 was grown in different flasks and incubated with the sewage. Two types of sewage, namely, raw sewage and concentrated sewage, were used. Concentrated sewage was prepared by concentrating raw sewage (3-fold, 5-fold, and 15-fold) after sterilisation using a rotavapor. Experiments that were performed using unconcentrated sterilised raw sewage were termed as control

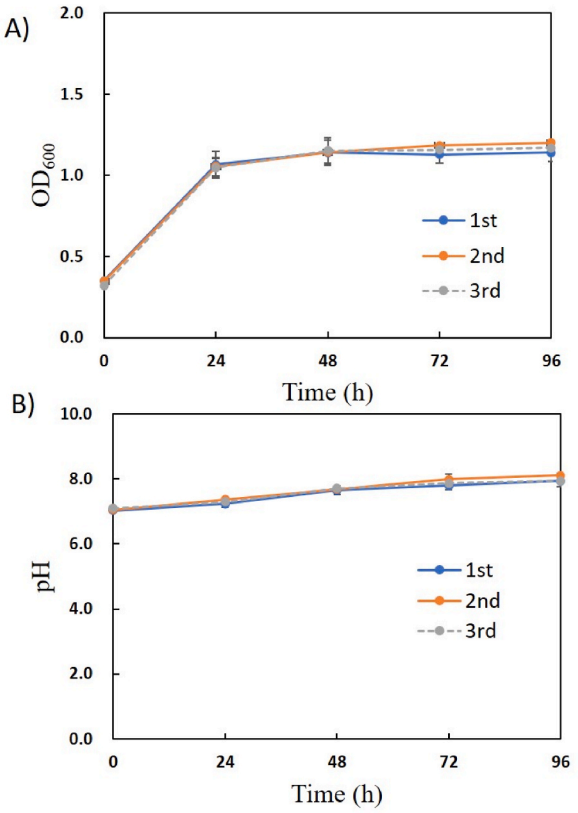

Fig. 1. Changes in (A) OD_{600} and (B) pH at different time intervals using raw sewage. Additional carbon source (glucose) was not supplemented in these experiments. All the conditions were tested as biological duplicates (two Erlenmeyer flasks, $n = 2$) within a single experiment. The data are presented as the mean ± standard deviation. OD_{600}, optical density at 600 nm.

experiments. The cells were incubated at 30 °C for 72 h to induce *phaC* expression and harvested via centrifugation. RNA extraction and real-time polymerase chain reaction was performed as described by Reddy et al. [25].

2.6. Glucose, COD, and TN analysis

The COD_{Mn} and TN were measured using the permanganate method with a COD-60 A meter, DRB 200 dual block, and DR 900 Multiparameter Portable Colorimeter. Glucose concentrations at different time intervals were analysed using HPLC (Shimadzu, Kyoto, Japan) with a refractive index (RI) detector and Shim-pack SCR-102 (H) column (Shimadzu, Kyoto, Japan). The samples collected for HPLC analysis were acidified using phosphoric acid and centrifuged (8000×*g*) using Sigma 1–14 (Osterode am Harz, Germany) at 4 °C. The resulting supernatant was filtered and analysed using HPLC. Filtered and degassed perchloric acid was used as the mobile phase at a flow rate of 1.0 mL/min. The column was maintained at 40 °C in a thermostat chamber.

3. Results and discussion

3.1. Bacterial growth resulted in pH variation

3.1.1. In the first condition

The strain CYR1 was used because our previous studies have shown that it can utilise diverse types of sugars, fatty acids, and aliphatic and aromatic toxic compounds for growth [19–21]. The bacteria incubated with raw sewage showed the highest OD_{600} (1.17 ± 0.02) at 96 h. There was no significant difference in growth among the primary, secondary, and tertiary cultures (Fig. 1). The initial pH of the sewage was adjusted to 7.0, and after 96 h, the pH increased to 8.0 ± 0.16 in all experiments. The protein concentration of the supernatant after centrifugation at each stage was determined to be 297 ± 7.77 mg/L (raw sewage), 253 ± 10.60 mg/L (after primary culture), 189 ± 7.07 mg/L (after secondary culture), and 122 ± 2.12 mg/L (after tertiary culture) (data not shown). During proliferation, the CYR1 strain degrades proteins present in sewage and uses them for PHA production. The initial protein concentration was 297 ± 7.77 mg/L and the final protein concentration in the tertiary culture was 122 ± 2.12 mg/L; hence, 58.9 ± 0.50 % of the protein was removed. Raw sewage and the culture liquid obtained after tertiary culture are shown in Fig. S3. The bacteria incubated

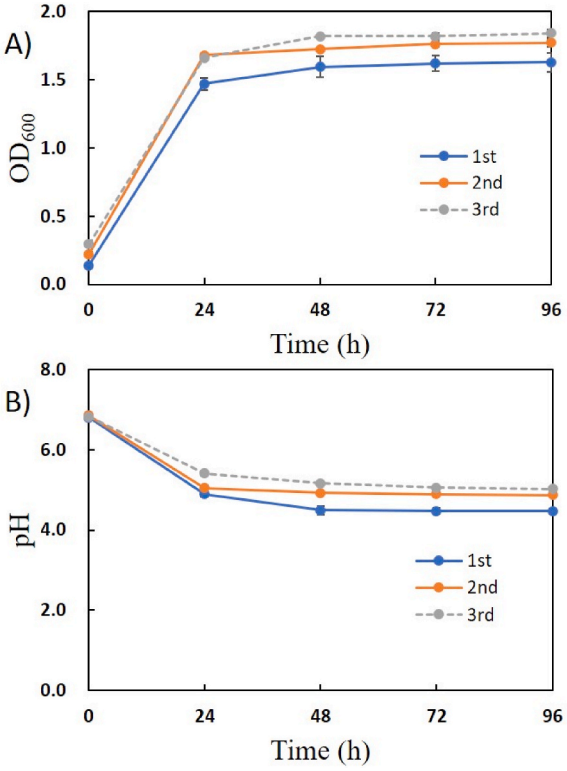

Fig. 2. Changes in (A) OD_{600} and (B) pH at different time intervals using MS medium. Glucose (20 g/L) was supplemented as a carbon source in MS medium at the primary culture stage. Secondary and tertiary cultures were prepared without adding glucose; consequently, the remaining glucose from the primary culture stage was used as a carbon source in these cultures. All the conditions were tested as biological duplicates (two Erlenmeyer flasks, $n = 2$) within a single experiment. The data are presented as the mean ± standard deviation.

with raw sewage showed relatively lower growth and PHA production; hence, subsequent experiments were performed by supplementing the sewage with glucose. Changes in OD_{600} and pH over time were observed in the primary, secondary, and tertiary cultures (Fig. S4). Glucose (20 g/L) was added at each stage, *i.e.,* in the primary, secondary, and tertiary cultures. All cultures showed similar growth patterns. However, the tertiary culture showed the highest growth after 48 h, after which there was no enhancement (Fig. S4).

3.1.2. *In the second condition*

Bacteria cultured in MS medium showed the highest OD_{600} of 1.85 ± 0.02 at 96 h (Fig. 2). Glucose (20 g/L) was supplemented as a carbon source in MS medium only during the primary culture experiment. Secondary and tertiary culture experiments were performed without the addition of glucose; thus, the bacteria utilized the remaining glucose from the primary culture. There was no significant difference in growth between the primary, secondary, and tertiary cultures; however, proliferation in the secondary and tertiary cultures was slightly higher than in the primary culture. The initial pH of the MS medium was adjusted to 7, and after 96 h, the pH decreased to 4.47 ± 0.07 in the primary culture, 4.88 ± 0.01 in the secondary culture, and 5.02 ± 0.04 in the tertiary culture.

After glucose supplementation, CYR1 showed higher growth when incubated with sewage compared with that achieved using MS medium (Fig. S4). Sewage contains organic matter, which is used to produce PHA; however, a fed-batch supply of the carbon source (glucose) enhanced the PHA-accumulating ability of CYR1. The pH decreased over time; a higher pH decrement was observed for the primary (from 7 to 4.51 ± 0.01) and tertiary cultures (from 7 to 5.13 ± 0.03) at 96 h (Fig. S4). The time-dependent changes in OD_{600} and pH of the primary, secondary, and tertiary cultures in sewage containing glucose were observed. Glucose was dissolved in sewage and supplied once at the primary culture stage. A similar growth pattern was observed for all stages (Fig. S5). After 96 h, the pH decreased from 7 to 4.5 ± 0.04 in the primary culture, 7 to 4.85 ± 0.07 in the secondary culture, and 7 to 5.28 ± 0.03 in the tertiary culture.

3.2. *PHA production*

Table 1 shows the wet cell mass, dry cell mass, and PHA production in the primary, secondary, and tertiary stages of CYR1 cultures incubated in sewage and MS medium. CYR1 strain produced 27.5 ± 2.82 mg/L, 55.7 ± 2.95 mg/L, and 41.9 ± 2.94 mg/L of PHA in the primary, secondary, and tertiary cultures, respectively. The main reason for low PHA concentration is the presence of small amounts of sand particles in raw sewage which may inhibit the growth of CYR1. However, sewage supplementation alone does not facilitate

Table 1
PHA production using sewage waste or MS medium with or without addition carbon source (glucose).

Conditions	Stages	CWM (g/L)	CDM (g/L)	PHA production (mg/L)	PHA production (% CDM)
Sewage	Primary	1.81 ± 0.03	0.29 ± 0.01	27.5 ± 2.82	9.48 ± 1.36
	Secondary	1.67 ± 0.03	0.18 ± 0.01	55.7 ± 2.96	30.94 ± 0.78
	Tertiary	1.62 ± 0.01	0.16 ± 0.00	41.9 ± 2.94	26.19 ± 2.30
MS medium + Glucose (supplemented only at the primary stage)	Primary	2.10 ± 0.16	0.78 ± 0.02	372 ± 16.97	47.7 ± 5.44
	Secondary	2.94 ± 0.16	1.08 ± 0.00	396 ± 28.28	36.7 ± 5.44
	Tertiary	2.32 ± 0.40	0.91 ± 0.12	478 ± 5.65	52.5 ± 7.63
Sewage + Glucose (supplemented three times at each stage)	Primary	2.53 ± 0.14	1.05 ± 0.00	435 ± 26.8	41.4 ± 2.82
	Secondary	2.30 ± 0.12	0.97 ± 0.01	567 ± 11.31	58.5 ± 1.82
	Tertiary	3.17 ± 0.07	1.38 ± 0.07	904 ± 29.69	65.5 ± 1.06
Sewage + Glucose (supplemented only at the primary stage)	Primary	3.06 ± 0.16	1.15 ± 0.03	480 ± 12.0	41.7 ± 1.39
	Secondary	2.13 ± 0.12	0.95 ± 0.01	474 ± 24.0	49.9 ± 5.32
	Tertiary	3.11 ± 0.12	1.19 ± 0.14	564 ± 8.00	47.4 ± 4.28

Data are presented as the mean ± standard deviation of *experiments* performed in *duplicate*. CWM: cell wet mass; CDM: cell dry mass.

Table 2
PHA production using cheese whey at different conditions.

Conditions	Measurement items	
Cheese whey diluted with deionised water	CDM (mg) in 1 L	625.2 ± 7.06
	PHA production (mg/L)	147.0 ± 1.68
	PHA production (%CDM)	23.5 ± 0.77
Cheese whey diluted with sewage water	CDM (mg) in 1 L	692.4 ± 7.62
	PHA production (mg/L)	167.0 ± 2.82
	PHA production (%CDM)	24.1 ± 0.70

Cheese whey was diluted 10-fold with deionised water or sewage water. All media were sterilised; Data are presented as the mean ± standard deviation of *experiments* performed in *duplicate*.

sufficient PHA production owing to its low organic content. Therefore, using sewage with a high concentration of carbon sources or mixing sewage with other high-organic-carbon-containing wastewaters may help enhance PHA production. It has also been reported that the total cost of PHA production can be reduced by 30–50 % using wastewater with high COD to produce large amounts of PHA [26,27].

Bacteria incubated with raw sewage showed relatively lower PHA production; hence, subsequent experiments were performed by supplementing the sewage with glucose only at the primary culture stage. Transmission electron micrograph (TEM) images of thin sections of bacteria from Rantoh sewage are shown in Fig. S6. The observation of bacterial cells containing PHA granules in the sewage supported the finding of PHA production from the sewage. PHA production is enhanced by glucose supplementation. PHA concentrations of 480 ± 12.0 mg/L, 474 ± 24.0 mg/L, and 564 ± 8.00 mg/L were obtained in the primary, secondary, and tertiary cultures, respectively. Further experiments were conducted by supplementing the sewage with glucose at each culture stage. PHA production was enhanced after supplementation with 435 ± 26.8 mg/L, 567 ± 11.31 mg/L, and 904 ± 29.69 mg/L glucose in the primary, secondary, and tertiary cultures, respectively (Table 1). PHA production was also evaluated by supplementing MS medium with glucose at the primary culture stage. PHA concentrations of 372 ± 16.97 mg/L, 396 ± 28.28 mg/L, and 478 ± 5.65 mg/L were obtained in the primary, secondary, and tertiary cultures, respectively (Table 1). PHA production was evaluated using CW diluted with deionised water or sewage. Based on the relatively higher nitrogen concentration in CW, CYR1 has been found to produce relatively lower amounts of PHA [12]. Therefore, PHA production experiments were performed using 10-fold diluted CW. Notably, higher PHA production was achieved using CW diluted with sewage water (167 ± 2.82 mg/L) compared with that achieved using CW diluted with deionised water (147 ± 1.68 mg/L) (Table 2).

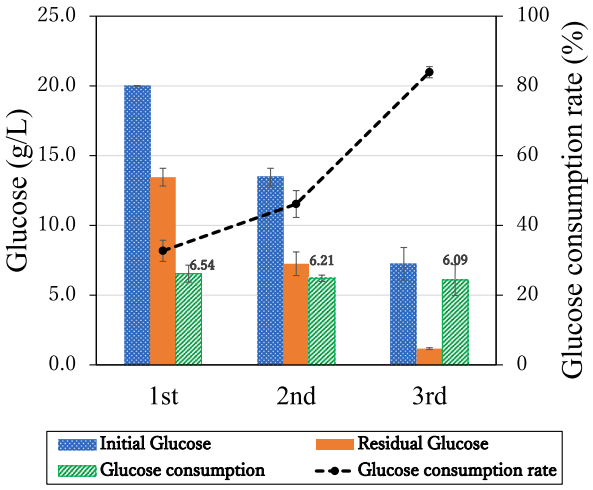

Fig. 3. Degree of glucose consumption in 1st, 2nd, and 3rd cultures. Glucose (20 g/L) was supplemented as a carbon source in MS medium at the primary culture stage. Secondary and tertiary cultures were prepared using the remaining glucose from primary culture stage without adding more glucose. Each culture incubation was performed for 96 h with shaking at 120 rpm. 1st, primary culture; 2nd, secondary culture; 3rd, tertiary culture.

3.3. Glucose consumption

3.3.1. In the second condition

The glucose consumption rates in the primary, secondary, and tertiary cultures are shown in Fig. 3. Glucose (20 g/L) was supplemented as a carbon source in MS medium during the primary culture stage. Secondary and tertiary cultures were prepared without adding glucose; instead, the remaining glucose from the primary culture stage was used as the carbon source. Glucose consumption of 6.54 ± 0.63 g/L, 6.21 ± 0.84 g/L, and 6.09 ± 0.06 g/L was observed for the primary, secondary, and tertiary cultures, respectively. Although the secondary culture showed a slightly lower consumption, there was no significant difference in PHA production. Initial glucose concentration decreased from 20 g/L to 13.46 g/L in the primary culture stage, from 13.46 g/L to 7.25 g/L in the secondary culture stage, and from 7.25 g/L to 1.17 g/L in the tertiary culture stage. The highest degrees of glucose consumption in the primary, secondary, and tertiary cultures were 83.9 ± 3.04 %, 46.1 ± 3.85 %, and 32.7 ± 1.59 %, respectively. This result can be explained by the mechanism underlying carbon source decomposition in microorganisms [28,29]. Bacteria initially convert available glucose into biomass, and the excess glucose is then converted into storage products such as PHA. A study speculated that a high concentration of carbon sources reduces cell growth owing to osmotic pressure [30]. However, a relatively high PHA production has previously been observed under high concentrations of carbon sources, such as CW [31]. Since the optimal conditions differ between these PHA-producing bacteria, the production efficiency may be independent of the carbon source concentration.

3.3.2. In the third condition

The degree of glucose consumption in the primary, secondary, and tertiary fed-batch cultures using sewage was determined by adding 20 g/L of glucose at each stage. The primary culture showed 5.05 g/L of glucose consumption with a glucose consumption degree of 25.3 ± 1.06 %, and the tertiary culture showed 21.9 ± 1.72 g/L of glucose consumption with a consumption degree of 37.94 ± 2.09 % (Fig. S7).

3.3.3. In the fourth condition

The degrees of glucose consumption in the primary, secondary, and tertiary fed-batch cultures using sewage were determined by adding 20 g/L of glucose only at the primary culture stage. The primary culture showed 6.17 g/L of glucose consumption with a glucose consumption degree of 30.86 ± 0.27 %, and the tertiary culture showed 6.71 g/L of glucose consumption with a consumption degree of 78.1 ± 3.47 % (Fig. S8). A higher glucose consumption was observed when glucose was added only to the primary culture. Therefore, the fed-batch culture with one-time addition of glucose at primary culture stage allows reduction of PHA production costs.

Adding glucose exclusively in the first phase proved to be significantly more effective than adding glucose in each phase of the process. This approach not only achieves superior economic results but also enhances overall production yields. Specifically, when glucose was introduced solely in the first phase, the PHA production rate to substrate ratio reached 0.80 PHA g/g glucose (Table 1 and Fig. 3). In contrast, when glucose was supplemented in each phase, the PHA production rate to substrate ratio was 0.62 PHA g/g glucose (Table 1 and Fig. S7).

3.4. phaC expression

PHA was produced in this study using sewage water which exhibited low COD; however, sewage from large cities may exhibit

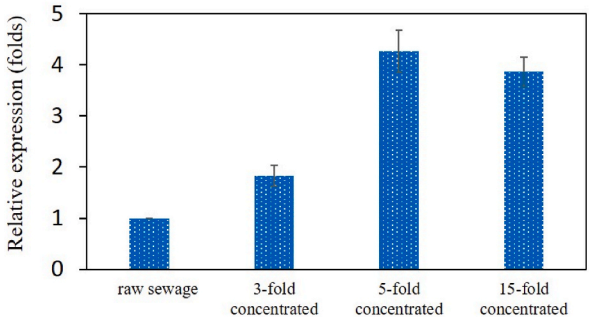

Fig. 4. Results of real-time polymerase chain reaction assay of *phaC* expression in *Bacillus* sp. CYR1 during PHA production. Gene expression is induced at various carbon source concentrations (3–15-fold) in sewage. All the conditions were tested as biological triplicates within a single experiment. The data are presented as the mean ± standard deviation. PHA, polyhydroxyalkanoate.

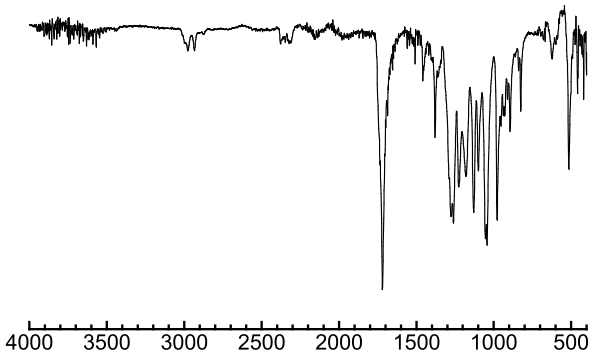

Fig. 5. Fourier-transform infrared spectrum of pure poly (3-hydroxybutyrate) extracted from cheese whey diluted with sewage.

higher COD. Therefore, to extend the feasibility of PHA production using high-COD sewage by CYR1, *phaC* expression was evaluated using low- and high-COD sewage water. *phaC*, which encodes PHA synthase, is mostly synthesized in PHA-producing bacteria. The *phaC* expression level was measured in *Bacillus* sp. CYR1 during PHA production using real-time polymerase chain reaction (Fig. 4). CYR1 was grown in media supplemented with raw sewage, 3-fold-concentrated sewage, 5-fold-concentrated sewage, and 15-fold-concentrated sewage with a COD of 98 ± 2.12 mg/L, 132 ± 5.65 mg/L, 176 ± 4.24 mg/L, and 518 ± 2.82 mg/L, respectively. Relatively higher *phaC* expression was observed in 5-fold-concentrated sewage (4.27-fold) followed by 15-fold-concentrated sewage (3.87-fold), 3-fold-concentrated sewage (1.83-fold), and raw sewage (1.0-fold).

Gene expression was higher in cultures exposed to high COD concentrations, indicating that wastewater with high COD can be used for PHA production. However, the gene expression levels decreased at very high COD concentrations (15-fold-concentrated sewage) owing to relatively higher nitrogen concentration (458 ± 14.14 mg/L). Efficient PHA production was observed at a nitrogen concentration of 150 ± 9.89 mg/L (5-fold-concentrated). This phenomenon was also observed in our previous experiments [25,32]. *phaC* in *Cupriavidus* sp. CY-1 under nitrogen stress has been reported to be upregulated and downregulated by varying nitrogen concentrations [25]. Nitrogen limitation leads to PHA production. Our previous study on PHA production by strain CYR1 cultured with CW-derived fermented acetic acid indicated that the highest PHA production is obtained at a nitrogen concentration of 234 mg/L, rather than at 509 mg/L and 1077 mg/L [19]. Nitrogen limitation leads to PHA accumulation in few bacteria; owing to stress conditions, they try to retain nitrogen by accumulating PHA as a storage substance [33,34]. Therefore, high PHA production was observed at a nitrogen concentration of 150 ± 9.89 mg/L. In contrast, besides nitrogen concentration, the osmotic pressure of the culture solution and phosphorus concentration may affect cell growth and PHA production [35,36].

3.5. PHA characterisation

3.5.1. Functional groups and structure determination

The FT-IR spectrum of PHA produced from the sewage-diluted cheese whey are shown in Fig. 5. The band appearing in the FT-IR spectrum at 1453 cm^{-1} matches the asymmetrical deformation of the C–H bond within CH_2 groups and CH_3 groups at 1379 cm^{-1}. The band at 1724 cm^{-1} corresponds to the stretching of the C═O bond, whereas a series of intense bands located at 1000–1300 cm^{-1} corresponds to the extension of the C–O bond of the ester group. All bands in the sample were identical to those of standard P3HB. A methylene C–H vibration near 2933 cm^{-1} was also observed.

Based on their peak positions, each peak was assigned to the protons on methine (5.26 ppm), methylene (2.62–2.43 ppm), and

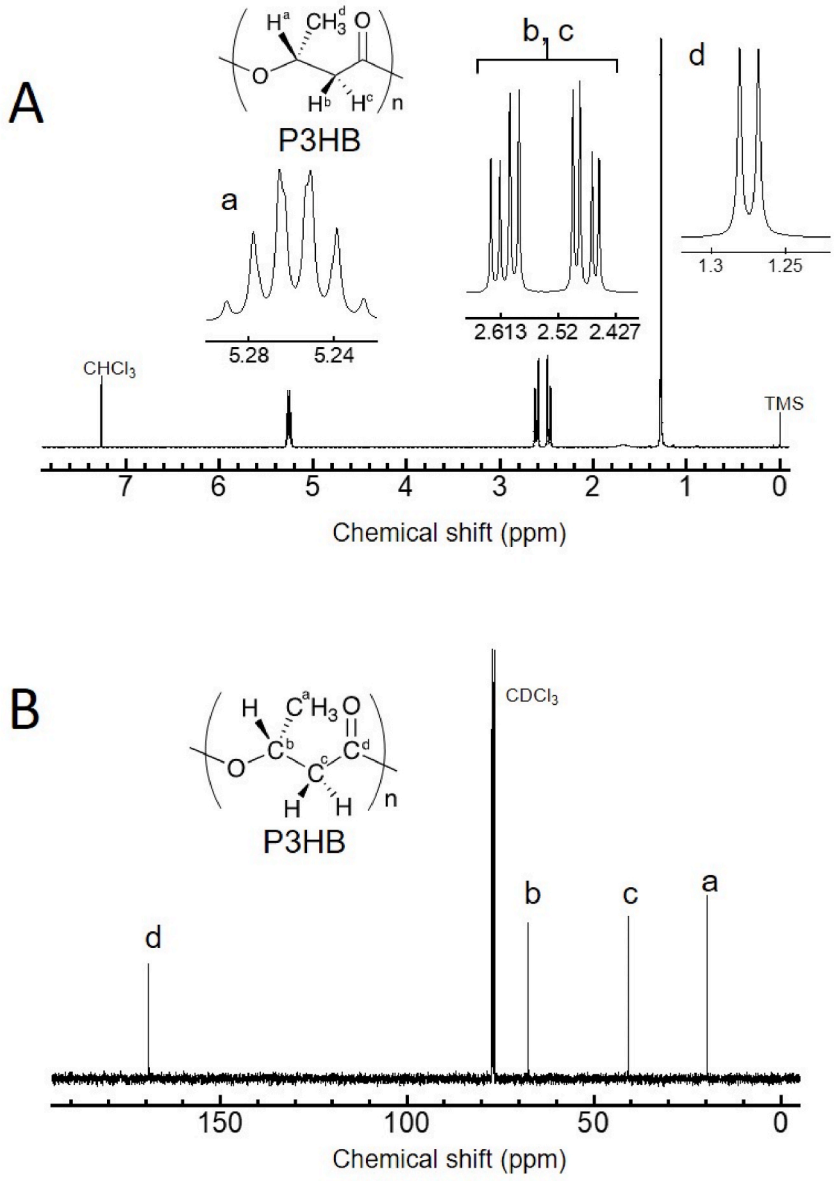

Fig. 6. (A) ^{1}H and (B) ^{13}C NMR spectra of poly (3-hydroxybutyrate) extracted from *Bacillus* sp. CYR1, which was cultured in cheese whey diluted with sewage.

methyl (1.27 ppm) groups in the ^{1}H NMR spectrum (Fig. 6A). The peaks in the ^{13}C NMR spectrum (Fig. 6B) were assigned to the carbon of the carbonyl (169.4 ppm), methine (67.8 ppm), methylene (41.9 ppm), and methyl (20.9 ppm) groups. The chemical shifts of these peaks were nearly identical to those of standard P3HB previously reported [13,32].

3.5.2. Evaluation of thermal properties

TGA was used to evaluate the thermal stability of the polymers, that is, the T_d, with a particular focus on the temperature of 5 % weight loss (T_{d5}). Approximately 2 mg of the sample was folded into an aluminium pan and subjected to a heating rate of 10 °C/min from ambient temperature to 400 °C (Fig. 7A). The weight loss of the sample started at approximately 230 °C, T_{d5} was at 274 °C, and complete decomposition occurred at 310 °C. This T_{d5} was similar to that of standard P3HB [21]. The sample was completely decomposed at 290 °C, indicating that the P3HB extracted from *Bacillus* sp. CYR1 is composed of organic material which may have originated from dry bacterial mass. DSC was used to determine the T_m of the polymers extracted from *Bacillus* sp. CYR1, which was compared with that of standard P3HB. The endothermic peaks in each DSC trace indicated that the polymer extracted from *Bacillus* sp. CYR1 had a T_m of 168 °C, which was consistent with that of standard P3HB (T_m, 178 °C) (Fig. 7B) [19].

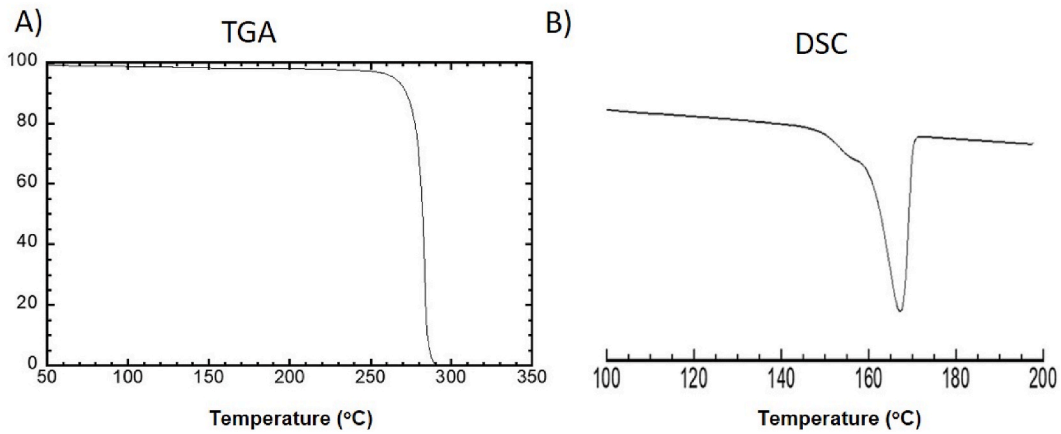

Fig. 7. (A) TGA analysis and (B) DSC of poly (3-hydroxybutyrate) extracted from *Bacillus* sp. CYR1 cultivated in cheese whey diluted with sewage. TGA, thermogravimetric analysis; DSC, differential scanning calorimetry.

3.6. Significance of the study

Considering the current environmental concerns and a growing global population, the pressing need for renewable chemical production is evident [37]. Our study underscores the fact that sewage represents a practical alternative to distilled water for diluting high-COD wastewater, making it suitable for PHA production. Beyond PHA, sewage shows the potential to produce various other chemicals, thus offering a promising avenue to reduce environmental impact and material production costs. However, one significant obstacle to utilizing raw sewage directly in production processes relates to concerns about hygiene, specifically the presence of pathogens in sewage. Recent years have seen research efforts directed toward the extraction of valuable substances, including bioplastics, from wastewater [13,27,38–42] and activated sludge [27,43–47]. It is worth noting that wastewater and sludge harbour diverse bacteria and pathogens. Crutchik et al. [46] have reported studies exploring the valorisation of sewage sludge through PHA production, demonstrating potential cost reductions of up to 19.0 % by utilizing a fraction of secondary sludge for PHA accumulation. Thus, the existing literature highlights the prospect of fully leveraging sewage for PHA production through rigorous sanitary management, akin to the practices applied in deriving valuable substances from sewage sludge. In essence, we must shift our perception of sewage from being merely 'dirty' to recognizing it as a valuable resource with significant untapped potential.

4. Conclusions

The constant generation of sewage in our daily life imposes a significant environmental burden, with extensive energy and cost requirements for sewage treatment. Global efforts to reduce ecological impact and energy consumption emphasize the imperative of sewage reuse. This study has revealed the potential of using raw sewage to both manufacture and dilute water for PHA production when processing CW. Remarkably, the PHA production capacity of the CYR1 strain increased 22-fold with the supplementation of additional carbon sources, accompanied by a notable glucose consumption degree of 83.6 ± 1.59 %. Bacterial cultures incubated using CW diluted with sewage exhibited higher PHA production than those diluted with distilled water. Furthermore, we observed elevated expression levels of the *phaC* gene in sewage with high carbon content, albeit at lower nitrogen concentrations. Analytical findings confirmed that the PHA produced in this study is P3HB, with characteristics comparable to standard P3HB. Consequently, the CYR1 strain shows promise for P3HB production using both low- and high-carbon-containing wastewater sources.

Data availability

No data was used for the research described in the article.

CRediT authorship contribution statement

Young-Cheol Chang: Writing – original draft, Investigation, Conceptualization. **M. Venkateswar Reddy:** Writing – review & editing. **Yusei Tsukiori:** Investigation. **Yasuteru Mawatari:** Investigation. **DuBok Choi:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23130.

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