Application of the Alkaline-Digestion-HPLC Method to the Rapid Determination of Polyhydroxyalkanoate in Activated Sludge

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ABSTRACT
A new method for analyzing polyhydroxyalkanoate (PHA) in activated sludge based on alkaline digestion followed by high-performance liquid chromatography (HPLC) was evaluated. The effects of the concentration of NaOH, reaction time, and reaction temperature were examined using activated-sludge samples containing PHA and commercially purchased PHA. To determine PHA concentrations, 0.5 mL 2 N NaOH was added to 1 mL activated-sludge mixed liquor, and the resulting mixture was heated at 105°C in a drying oven for 1 h to convert 3-hydroxybutyrate units into 2-butenoate and 3-hydroxyvalerate units into 2-pentenoate. The mixture was then acidified with 0.5 mL of 2 N H₂SO₄, and the solids were removed and subsequently analyzed by HPLC. The present method gave results consistent with those obtained by the conventional method of methanolysis followed by gas chromatography. The present method can facilitate investigations of PHA in activated sludge by reducing the time and labor required for analysis and by eliminating the use of organic solvents.

Keywords: polyhydroxyalkanoate, activated sludge, alkaline digestion–HPLC method

INTRODUCTION
Polyhydroxyalkanoates (PHAs), including poly (3-hydroxybutyrate) (PHB), are biopolymers formed in prokaryotic cells. These polymers are also present in activated sludge [1]. Thus far, PHAs in activated sludge have been studied from different viewpoints, including (1) as one of the keys to identifying filamentous bacteria that cause sludge bulking [2], (2) as a temporal carbon storage product formed in activated sludge when activated sludge and sewage are mixed under aerobic conditions [3], (3) as a temporal carbon storage product in the anaerobic stage of enhanced biological phosphorus removal (EBPR) processes [4–6], (4) as a potential source of biodegradable polyester because PHAs are thermoplastic [7–9], and (5) as an intermediate product in the conversion of organic pollutants in sewage into methane as a biomass energy source [10,11]. In these studies, except for (1), where PHA granules in cells are observed under a microscope, a method for determining PHAs in activated-sludge samples is required.

The method developed by Law and Slepecky [12] has long been used in its original protocol or with minor modifications to determine PHB in activated sludge. In these methods, a biomass sample is washed, lysed by hypochlorite, and dried; the dried materials are then heated in concentrated H₂SO₄ to convert the 3-hydroxybutyrate (3HB) units into crotonic acid, which is a strong UV absorber. To simplify the protocol, Karr et al. [13] omitted the pretreatment and determined crotonic acid by high-performance liquid chromatography (HPLC). Yet, these sulfuric acid dehydration methods have not been applied to analyze PHA samples that contain monomer units other than 3HB.

Alcoholysis–gas chromatography (GC) methods are now the most widely used methods to analyze PHA in activated sludge. In these methods, the samples are dehydrated, the PHAs are converted into alkyl esters of PHA monomers, and
the alkyl esters are extracted from the reaction mixture and analyzed by GC. The original protocol was developed by Braunegg et al. [14]; in this method, methanolysis is induced in a mixed solution of chloroform and methanol acidified with H$_2$SO$_4$ to determine PHB. The methanolysis conditions and GC separation have been modified to determine PHA containing monomer units other than 3HB [15–19]. The method has also been modified to reduce the amounts of toxic organic solvents used. The method proposed by Riis and Mai [20] utilizes dichloromethane and HCl-acidified 1-propanol and has been slightly modified and utilized to determine PHA in activated sludge [21–23]. The method proposed by Werker et al. [24] further eliminated the use of any chlorinated solvent. In their method, PHA is butanolized and the butyl esters of the monomers are subsequently extracted with hexane [25,26]. Although alcoholysis–GC methods are used in many studies because of their accuracy in the analysis of PHA with diverse monomer units, they are generally laborious and time-intensive.

Capon et al. [27] reported the formation of 2-butenoate (2BE, also called crotonate) and 2-pentenoate (2PE) from 3HB and 3-hydroxyvalerate (3HV) units, respectively, in PHA by digestion under alkaline conditions. Del Don et al. [28] reported a method to analyze PHA containing 3HB and 3HV based on alkaline digestion in combination with HPLC.

In 2012, Watanabe et al. [29] reported a simple and efficient method to determine PHA in culture cells in microplate wells. In their method, PHA in whole cells is digested under alkaline conditions, and the resulting 2-alkenoic acids are determined by HPLC. Sakamoto and Kudo [30] also applied the method to analyze PHA in activated sludge samples. These methods do not require organic solvents and do not include an extraction step. Thus, the whole analytical procedure is much simpler than the alcoholysis–GC methods. In addition, the alkaline digestion–HPLC method can be used to analyze PHA containing monomer units other than 3HB. Thus, as a method to analyze PHA in activated sludge, the alkaline digestion–HPLC method is highly attractive.

Here, we further improved the alkaline digestion–HPLC method to analyze PHA composed of 3HB and 3HV in activated sludge. Reaction conditions were selected to yield the best conversion of PHA into 2BE and 2PE. The conversion yields into 2BE from 3HB and into 2PE from 3HV were then evaluated using purified PHA. Finally, PHA in activated sludge samples was analyzed by the alkaline digestion–HPLC method and methanolysis–GC method, and the results were compared.

### MATERIALS AND METHODS

#### Chemicals

Pure poly (3HB-co-3HV) (88 wt% 3HB) and poly (3HB) were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. These two standard chemicals are referred to hereafter as “PHAstd” and “PHBstd,” respectively. (E)-2-butenoic acid (2BE), (E)-2-pentenoic acid (2PE), 2-methyl-2-butenoic acid (2M2BE), 2-methyl-2-pentenoic acid (2M2PE), reagent-grade sodium hydroxide, concentrated sulfuric acid, methanol, and chloroform were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Sodium 3-hydroxybutyrate was obtained from Tokyo Chemical Industry Co., Tokyo, Japan. Ultrapure water (Milli-Q Synthesis, Merck Millipore Corp., Darmstadt, Germany) was used. The quaterpolymer of 3HB, 3HV, 3-hydroxy-2-methylbutyrate (3H2MB), and 3-hydroxy-2-methylvalerate (3H2MV) was extracted with chloroform from lyophilized laboratory activated sludge. The purified polymer was recovered as precipitates by addition of methanol to the chloroform under vigorous mixing. The monomer composition was determined by $^1$H nuclear magnetic resonance analysis ($^1$H NMR) at Hitachi Chemical Techno Service Co., Hitach, Ibaraki, Japan. The $^1$H NMR resolves hydrogen atoms based on differences in the electromagnetic conditions in molecules. Further, the integrated peak areas quantitatively reflect the difference in abundance. The determined molecular composition of the quaterpolymer was 3HB:3HV:3H2MB:3H2MV = 1:9:24:8.

#### Activated sludge samples

Activated sludge samples were obtained either from a laboratory activated sludge reactor treating synthetic sewage containing acetate, propionate, peptone, and yeast extract as the main carbon source, or from full-scale wastewater treatment plants in Yokohama City, Japan. These activated sludge samples were obtained as mixed liquors.

#### Alkaline digestion–HPLC experiments

In the present study, the alkaline digestion–HPLC method was performed as follows.

Step 1: A 1 mL sample was collected in a 2-mL plastic centrifuge tube (BM-20, BM Equipment Co., Tokyo, Japan). If the sample was PHAstd or PHBstd, a known volume of its chloroform solution at a known PHA or PHB concentration was collected in a 2-mL tube, the chloroform was removed by evaporation, and 1 mL of water was added.

Step 2: To the tube, 0.5 mL NaOH solution was added; the cap was then closed, and the contents were mixed.
Step 3: The tubes were placed on a plastic rack for 2-mL plastic centrifuge tubes (BIO-BIK 96-well reversible rack, Ina Optica Co., Osaka, Japan) and heated in a drying oven (DVS-602, Yamato Scientific Co., Tokyo, Japan) at 105°C for 1 h. During heating, a weight was put on the tubes to prevent them from opening.

Step 4: The tube was cooled to room temperature, acidified with 0.5 mL 2 N H₂SO₄ solution, and mixed.

Step 5: Solids in the acidified sample were removed by centrifugation and filtration. Centrifugation was typically performed for 5 min at 12,000 rpm (13,000 × g) with a model 3740 centrifuge (Kubota Corp., Tokyo, Japan). For filtration, a DISMIC 25CS020AN 0.20 µm membrane filter (Toyo Roshi Kaisha, Tokyo, Japan) was typically used.

Step 6: Standard solutions of 2BE and 2PE were prepared by dilution with water.

Step 7: The standard solutions and particle-free digested liquids were analyzed by HPLC on a Hitachi 2100 LaChromElite with UV detection at 210 nm and equipped with a SCR-101H column (φ4 mm, 300 mm, Shimadzu, Kyoto, Japan). The eluent was 0.025% H₂SO₄ solutions taken into account. The injection volume was 10 µL.

Step 8: The concentrations of 2BE and 2PE in the digested and acidified samples were calculated.

Step 9: The 3HB and 3HV concentrations in the original samples were calculated on the basis of the conversion yields of 3HB units and 3HV units in PHAs into 2BE and 2PE, respectively, with the dilution by the addition of NaOH and H₂SO₄ solutions taken into account.

Eight experiments were conducted with the following specific objectives:

Experiment 1 was conducted to examine the effect of the NaOH solution concentration added in step 2. Each 1 mL of an activated-sludge sample from the laboratory activated sludge reactor was dispensed into 15 tubes, and processed using steps 1 through 7. Instead of 2 N NaOH, 0.6, 1.2, 1.8, 2.4, and 3.0 N NaOH solutions were used.

Experiment 2 was conducted to examine the effect of heating time in step 3. Each 1 mL of an activated-sludge sample from the laboratory activated sludge reactor was dispensed into 14 tubes and processed according to steps 1 through 7. The heating duration in the oven at 105°C was 10, 20, 30, 45, 60, 90, or 120 min.

Experiment 3 was conducted to examine and determine the conversion yields of 3HB units into 2BE and 3HV units into 2PE with different amounts of PHAstd. Plastic centrifuge tubes containing known and different amounts of PHAstd were prepared, 1 mL water was added, and the resulting samples were subjected to steps 2 through 8.

Experiment 4 was conducted to examine the effect of heating temperature in step 3 on the conversion yield from 3HB units to 2BE and from 3HV units to 2PE. In this experiment, nine 1.5-mL plastic centrifuge tubes (BM-15, BM Equipment Co.) containing the same amounts of PHAstd were prepared, added to 1 mL of water, heated on a heating block (Eppendorf Thermomixer R, Sigma-Aldrich, St. Louis, USA) at 80, 90, or 99°C for 1 h, cooled to room temperature, combined with 0.167 mL 6 N H₂SO₄, and then subjected to steps 6 through 8.

In Exp. 5, the effect of storage of samples before digestion and improvement of stability during storage were studied. Each 1 mL of an activated-sludge sample from the laboratory activated sludge reactor was dispensed into 12 tubes. The 12 tubes were grouped into four groups of three tubes and processed as follows:

-Group 1 (control): Immediately subjected to steps 2 to 3, stored for 5 days at 4°C, and then processed through steps 4 through 7.

-Group 2 (storage with alkaline treatment): Immediately subjected to step 2, stored for 5 days at 4°C, and then processed through steps 3 through 7.

-Group 3 (storage with acid treatment): To each tube, 0.05 mL 6 N H₂SO₄ was added immediately after sampling; the samples were stored for 5 days at 4°C and then processed through steps 2 through 7.

-Group 4 (storage without fixation): Each tube was stored for 5 days at 4°C without the addition of any chemicals and then processed through steps 2 through 7.

In Exp. 6, the same activated sludge samples obtained at the full scale wastewater treatment plants were analyzed by the alkaline digestion–HPLC method and the methanolysis–GC method to compare the results of PHA measurements. The methanolysis–GC analysis was performed as follows: one milliliter of an activated-sludge sample was collected in a 10-mL screw-cap glass tube, 1 mL of methanol was added immediately, then transferred to the laboratory. The mixture was centrifuged, the supernatant liquid was decanted, 2 mL of chloroform and 2 mL of acidified methanol (10 v/v% H₂SO₄) were added, and the tube was tightly closed with a Teflon-lined cap and heated in a drying oven at 90°C for 16 h. After the sample cooled to room temperature, 1 mL 14% ammonia solution was added and mixed. The chloroform phase was separated by centrifugation and transferred to another glass tube, 0.5 mL water was added, and the sample was mixed and then centrifuged; the washed chloroform phase...
was analyzed using a gas chromatograph equipped with an NB-1 column (GL Science, Tokyo, Japan) and a mass detector (QP2010, Shimadzu, Kyoto, Japan). The injection volume was 1 µL at a split ratio of 1:10, and the injector temperature was 180°C. The column temperature was initially maintained at 60°C for 4 min, then increased to 120°C at 12°C/min, and finally increased to 200°C at 30°C/min. Mass spectra were obtained using the electron impact ionization method, and ions with m/z ratios of 57, 71, 74, 88, 103, 105, 117, and 136 were monitored. PHAstd prepared at a known concentration in a 10-mL screw-cap glass tube was used as a standard.

In Exp. 7, alkaline digestion–HPLC method was applied to the following samples: a solution of 20 mM sodium 3-hydroxybutyrate, PHBstd (2.2, 4.4, 13.1, 21.9, 43.8, 131 µg/tube), and quaterpolymer containing 3HB, 3HV, 3H2MB, and 3H2MV (approximately 30 µg/tube). In this experiment, a solution containing reagents 2M2BE and 2M2PE was analyzed by HPLC as per step 7.

**RESULTS**

Figure 1 shows examples of chromatograms obtained by HPLC analyses, where the chromatogram in Fig. 1a is for a mixture of 2BE and 2PE without digestion, the one in Fig. 1b is for pure poly (3HB-co-3HV) after digestion, and the one in Fig. 1c is for an activated sludge sample after digestion. In each chromatogram, distinct peaks corresponding to 2BE (retention time 17.1 min) and 2PE (24.0 min) are observed.

**Figure 1** shows examples of chromatograms for a) standard solution of 2BE and 2PE (0.4 mM/L each), b) PHAstd (34.4 µg/tube), and c) activated sludge.
to the slow heat transfer from air and to the high heat capacity of the tube rack on which the tubes were placed during heating.

On the basis of the results of Exps. 1 and 2, the reaction conditions were fixed as follows: 0.5 mL NaOH solution at 2 N is added to 1 mL of sample to make a NaOH concentration in the reaction mixture of 0.67 N, and the heating is for 60 min at 105°C in a drying oven.

In Exp. 3, different amounts of PHAstd were digested under the aforementioned conditions, and peak areas for 2BE and 2PE were plotted against the amounts of PHAstd in the tubes (Fig. 4). The plots were linear to approximately 500 μgPHA/tube, which is equivalent to 500 mg/L in a sample, and a higher PHA dose resulted in deviation from linearity and lowered reproducibility.

Using the results in Exp. 3, we plotted the conversion yields of 3HB and 3HV into 2BE and 2PE, respectively, against the amount of PHAstd (Fig. 5). When the amount of PHA per tube was between 5.0 and 300 µg, the conversion efficiency was 0.36 ± 0.02 for the 3HB unit to 2BE and 0.32 ± 0.03 for the 3HV unit to 2PE. Here, calibration curves of 2BE and 2PE prepared using the solutions of 2BE and 2PE without digestion were highly linear and reproducible. We therefore speculate that the distribution of the plots in the vertical direction in Fig. 5 originates from errors with respect to the addition of PHAstd to the tubes.

In Exp. 4, the effect of reaction temperature (80, 90, or 99°C) on the conversion yield was examined. The results were 0.372 ± 0.005 at 80°C, 0.382 ± 0.004 at 90°C, and 0.377 ± 0.009 at 99°C for the conversion of 3HB units to 2BE, and 0.296 ± 0.012 at 80°C, 0.317 ± 0.007 at 90°C, and 0.311 ± 0.009 at 99°C (n = 3 in all cases) for the conversion of 3HV units to 2PE. The conversion yield was slightly affected by the reaction temperature, but the effect was very small.

Although the heating device and the temperature were different in Exps. 3 and 4, similar conversion yields were obtained; thus, alkaline digestion was not seriously affected by minor differences in heating conditions.

Because the conversion yield was observed to be stable, hereafter, 2BE and 2PE concentrations detected in the di-
gested samples were converted into the concentrations of 3HB and 3HV units in the original samples using the following equations:

\[ C_{3HB} = \frac{C'_{2BE}}{Y_{3HB}} * D \]  
\[ C_{3HV} = \frac{C'_{2PE}}{Y_{3HV}} * D \]

where \( C_{3HB} \) and \( C_{3HV} \) are the molar concentrations of 3HB and 3HV units in the original samples, \( C'_{2BE} \) and \( C'_{2PE} \) are the molar concentrations of 2BE and 2PE detected in the digested liquids, \( Y_{3HB} \) and \( Y_{3HV} \) are the conversion yields from 3HB to 2BE (0.37 mol/mol) and from 3HV to 2PE (0.32 mol/mol), and D is the dilution of the sample in the whole analytical procedure. When the original sample volume was 1 and 0.5 mL, 2 N NaOH and 0.5 mL 2 N H2SO4 were added to it; thus, \( D = 2 \).

In Exp. 5, the effects of the storage of samples were examined. When the sample was digested immediately after sampling (Group 1, n = 3), 4.22 ± 0.17 mmol/L of 3HB unit and 0.22 ± 0.003 mmol/L of 3HV unit were detected. When the tubes were added with 0.5 mL 2 N NaOH (0.67 N as NaOH final concentration) and stored for 5 days before digestion (Group 2, n = 3), 3.25 ± 0.03 mmol/L of 3HB and 0.19 ± 0.01 mmol/L of 3HV were detected; that is, the detected concentration of 3HB and 3HV were reduced by 22% and 12%, respectively. We expected that the addition of NaOH immediately after sampling would be beneficial to fixing samples by stopping biological reactions. In any event, the detected amount of PHA was substantially reduced by fixation with NaOH and storage under alkaline conditions.

To improve the stability of stored samples, in Group 3, 5 µL 6 N H2SO4 (0.03 N as H2SO4 final concentration) was added to each of the tubes, which were then stored at 4°C for 5 d (n = 3). The detected concentrations of 3HB and 3HV in Group 3 were 3.95 ± 0.08 and 0.21 ± 0.01 mmol/L, respectively. The reduction of 3HB and 3HV concentrations in comparison to those in Group 1 was 6% and 5%, respectively. Thus, the reduction of 3HB and 3HV during storage was substantially improved.

We also stored the samples with no added chemicals at 4°C for 5 d (Group 4). In this case, the detected 3HB and 3HV concentrations were 4.45 ± 0.06 and 0.69 ± 0.07 mmol/L, respectively, and both were higher than those detected in Group 1; that is, the 3HB concentration was increased by 6%, and the 3HV concentration was almost tripled. Most likely, microorganisms fermented glycogen to obtain maintenance energy during storage of the samples, with 3HV as the end product. Such fermentation has been reported in plural studies [16,31–33]. Fixation is necessary if the samples cannot be digested immediately.

In Exp. 6, samples from full-scale wastewater treatment plants were analyzed by both the alkaline digestion–HPLC method and the methanolysis–GC method. The concentrations of 3HB and 3HV detected by the two methods showed linear relationships, as evident in Fig. 6, with \( r^2 \) values of 0.92 and 0.98, respectively. The total PHA concentrations showed an \( r^2 \) value of 0.95, and the alkaline digestion–HPLC method gave approximately 15% higher concentrations than the methanolysis–GC method.

In Exp. 7, a solution of sodium 3-hydroxybutyrate (1.61 mmol/L) was treated using the developed protocol. A small concentration of 2BE (0.13 mmol/L) was detected in the original sample without alkaline digestion, and its concentration was increased by 0.01 mmol/L by alkaline digestion. During alkaline digestion, the formation of 2BE from free 3HB was negligible. When PHBstd was analyzed by the alkaline digestion–HPLC method, the conversion yield from 3HB to 2BE was calculated to be 0.41 ± 0.13, approximately 10% higher than that of the conversion from 3HB to 2BE when PHAstd was analyzed. Fig. 7 shows the chromatogram obtained from the analysis of the quaterpolymer of 3HB, 3HV, 3H2MB, and 3H2MV. In addition to the peaks corresponding to 2BE and 2PE, another peak was observed at 35.2 min. This peak was observed to have the same retention time as 2M2PE. By contrast, the peak for 2M2BE was
observed to have a retention time similar to that of 2PE: 24.6 min for 2M2BE and 24.0 min for 2BE. The PHA for 2M2BE from 3H2MB was not observed as a distinct peak, maybe because it was overwhelmed by the large peak of 2PE.

DISCUSSION

In the protocol of alkaline digestion–HPLC method by Del Don et al. [28] reported in 1994, a lyophilized cell of 1 to 5 mg was placed into a 10-mL screw-cap test tube, 0.5 mL 2 N NaOH was added, and the sample was heated for 30 min in boiling water. The sample was then neutralized with HCl solution, and the solids were removed and subsequently analyzed by HPLC. In spite of the efficiency of the method, the alkaline digestion–HPLC method was not utilized by other researchers until 2012 when Watanabe et al. [29] applied the alkaline digestion method to analyze PHA contents in cells in microplate-wells. In their protocol, alkaline digestion is performed in wells of a 96-well microplate with 0.2 mL of 1 N NaOH, where the samples are heated at 100°C for 3 h. The use of a microplate enables the analysis of numerous samples. They also improved the HPLC conditions.

Sakamoto and Kudo [30] reported applying the alkaline digestion method to activated-sludge samples. In their method, 10 mL of sample was mixed with 5 mL of 2 N NaOH in a 50-mL glass tube, then heated in boiling water, cooled, and acidified with HCl solution. The particles were then removed by filtration and analyzed by HPLC using a UV detector. They proposed using 2BE and 2PE standard solutions for routine analyses. Their report originally in Japanese is outlined as attached as the supplemental material.

The protocol proposed in the present study has only minor differences compared with that of Sakamoto and Kudo [30]. The differences are mainly the reaction size (1.5 mL or 15 mL), material of the tube (plastic or glass), and heating device (drying oven or boiling water). We modified the protocol so that the analysis of PHA can more easily be performed: the plastic 2-mL tubes are economical, occupy less space, and are easy to handle to process numerous samples, and a drying ovens set at around 105°C is usually readily available in water-quality laboratories at wastewater treatment plants. Here, it should be noted that 105°C is the set-temperature of the drying oven which has a lower heat transfer efficiency compared to other heating devices. If a heat block is to be employed for heating, temperature should be reduced to boiling point of water or lower for safety.

On the basis of the results of Exps. 1 and 2, we deduced the following alkaline digestion conditions: 105°C in a drying oven for 1 h in the presence of 0.667 N NaOH in the reaction mixture. The conversion yields of 3HB and 3HV units into 2BE and 2PE were determined from Exps. 3 and 4, and the yields were observed to not be substantially affected by either the reaction temperature or the heating device. The results of Exp. 5 revealed that samples can be more stably stored under acidic conditions when alkaline digestion cannot be done immediately. Sakamoto and Kudo [30] reported the conversion yield of 3HB to 2BE to be 0.38, similar to the value obtained in the present study, and that of 3HV to 2PE to be 0.41, which is approximately 30% larger than that obtained in the present study.

The mechanisms of formation of 2BE and 2PE from 3HB or 3HV units in PHA can be explained on the basis of the E1 elimination reaction, as shown in Fig. 8. That is, when a hydroxide ion removes a proton from the alpha carbon, the excess electron pair is used to form double bonds between alpha and beta carbons, the alcoholic CO bond on the β-carbon.
is cleaved, and 2BE or 2PE are formed. By contrast, the monomer unit on the hydroxyl end (the left-most unit in Fig. 8) is not converted to 2BE or 2PE because a similar reaction would result in the formation of OH$^-$, and OH$^-$ already exists in high concentration in the reaction mixture. Thus, PHAs with lower polymerization are expected to give lower conversion yields because hydroxyl ends are more abundant in PHAs with lower polymerization. Similarly, free 3HB and 3HV are thought to not be converted into 2BE and 2PE, respectively, as was confirmed in Exp. 7.

The formation of 2BE and 2PE via the E1 mechanism is thought to compete with hydrolysis. As a likely consequence, the conversion yields of 3HB and 3HV units in PHA into 2BE and 2PE remains at low level. If PHA is hydrolyzed during sample storage, PHA will be underestimated when analyzed by the alkaline digestion–HPLC method, as was observed in Exp. 5.

In Exp. 7, a PHA quaterpolymer containing 3H2MB and 3H2MV units was analyzed by the alkaline digestion–HPLC method. The HPLC chromatogram shown in Fig. 7 suggests that the alkaline digestion–HPLC method can also be used to analyze PHA containing 3H2MV units. By contrast, the peak corresponding to 2M2BE from 3H2MB is not observed in Fig. 7. This lack of a peak does not necessarily mean that 3H2MB cannot be analyzed by the alkaline digestion–HPLC method. Rather, the peak may not be resolved because of the similar retention times of 2PE and 2M2BE and because of the relatively high and low concentrations of 3HV and 3H2MB in the PHA.

When PHBstd was analyzed by the protocol in Exp. 7, the conversion yield from 3HB to 2BE was higher than that obtained in the analysis of PHAstd. This difference in conversion yields may be originating not only from differences in the degree of polymerization but also from the differences in chemical composition. For rigorous estimation of the reliability of the alkaline digestion–HPLC method, the conversion yields should be examined using PHA with different compositions and molecular weights.

**CONCLUSIONS**

In the present study, the applicability of the alkaline digestion–HPLC method to analyze PHA containing 3HB and 3HV units in activated sludge was examined. A basic protocol was proposed, and the effects of the changes in the reaction conditions were evaluated. The conversion yields from 3HB units into 2BE and 3HV units into 2PE were stable even when the NaOH concentration and reaction time were slightly varied. In cases where samples must be stored before alkaline digestion, they can be fixed with H$_2$SO$_4$.

The possibility of applying the alkaline digestion–HPLC method to analyze PHA with 3H2MB and 3H2MV units was suggested. In addition, the conversion yields were suggested to be affected by the difference in chemical composition of
PHA. Further investigations of these results remain as future work. By contrast, the PHA contents determined by the alkaline digestion–HPLC method were shown to give results consistent with those determined by the methanolysis–GC method.

The method does not require the use of organic solvents. For each sample, the hands-on time is very short (i.e., the addition of chemicals twice, heating, filtration, and transfer to HPLC vials). Given its ease of use, the alkaline digestion–HPLC is far superior to the methanolysis–GC method and was demonstrated here to be applicable to PHA in activated sludge from full-scale treatment plants. The authors hope that the method proposed here accelerates studies related to PHA in activated sludge and mixed culture samples.

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