Conference paper

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Direct determination of poly(3-hydroxybutyrate) accumulated in bacteria by thermally assisted hydrolysis and methylation-gas chromatography in the presence of organic alkali<https://doi.org/10.1515/pac-2017-0905>

Abstract: There is considerable interest in the development of simple methods for quantifying production of the biodegradable polyester poly(3-hydroxybutyrate) [P(3HB)] by bacteria. Cells of *Cupriavidus necator* were grown on agar medium containing different concentrations of glucose (10–25 g/L) as a sole carbon source. Trace amounts ($100 \pm 5 \mu\text{g}$) of dried *C. necator* cells were directly subjected to thermally assisted hydrolysis and methylation-gas chromatography (THM-GC) in the presence of tetramethylammonium hydroxide (TMAH). On the resulting chromatograms, a series of characteristic peaks, attributed to the THM products from poly(3-hydroxybutyrate) accumulated in the bacterial cells, were clearly observed without any interfering component. Based on the peak intensities, the contents of P(3HB) in *C. necator* were determined precisely and rapidly without using any cumbersome sample pretreatment. Furthermore, the values of the P(3HB) contents coincided overall with those obtained by the conventional method involving solvent extraction followed by gravimetric determination.

Keywords: *Cupriavidus necator*; POLYCHAR-25; poly(3-hydroxybutyrate); tetramethylammonium hydroxide (TMAH); thermally assisted hydrolysis and methylation-gas chromatography (THM-GC).

Introduction

Poly(3-hydroxybutyrate) [P(3HB)], and its copolymers are biodegradable polyesters, widely used as a compost bag and packaging materials due to its excellent physical properties, non-toxic behavior and biocompatibility [1, 2]. P(3HB) is known to be produced by various bacteria as an energy storage compounds in their cells [3, 4]. It has been reported that the amount of P(3HB) accumulated in the cells changes drastically depending on the species of bacteria and their culture conditions [4]. *Cupriavidus necator* is among the bacteria that have been extensively studied for the production of P(3HB) granules [4]. *Cupriavidus necator* has been known to accumulate P(3HB) significantly when the carbon source in growth media is rich, but the nitrogen source is limited [5]. Therefore, in order to determine the species of bacteria and the culture conditions suited for the effective production of P(3HB), it is necessary to develop a practical and highly sensitive method to analyze the content of the accumulated P(3HB) in the cells.

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In general, the content of P(3HB) in the bacterial cells has been analyzed mainly by the gravimetric determination or gas chromatography (GC) following various sample pretreatments, such as solvent extraction, purification and trans-methylation [6–8]. For example, Betancourt et al. [7] determined the P(3HB) components accumulated in *Aspergillus latus* by means of GC following solvent extraction using chloroform and methanol, and trans-methylation with H_2SO_4 in methanol. Furthermore, Jiang et al. analyzed poly-3-hydroxyalkanoates (PHAs) in the cells of *Pseudomonas putida* by the gravimetric determination and GC measurement with sample pretreatments. They also reported that the consecutive use of acetone and methanol as the extraction media was effective for quantitative recovery of PHA including their medium-chain-length components. However, these methods are not always easily adapted to routine analysis because of relatively long time required for sample pretreatments including polymer extraction, purification and trans-methylation.

Recently, a thermally assisted hydrolysis and methylation-gas chromatography (THM-GC) method in the presence of strong organic alkali such as tetramethylammonium hydroxide (TMAH) has become a useful technique for structural and compositional analyses of various condensation polymers [3, 9–13]. This method often provides extremely simplified and quantitative chromatograms mainly consisting of methyl derivatives of the constituent monomers in the polymer chains. This method has been also successfully used for compositional analysis of copolymer-type PHAs. For example, Sato et al. [3] reported the compositional analysis of commercially available poly(3-hydroxybutyrate-co-hydroxyvalerate) [P(3HB-co-3HV)] by the THM-GC method. In this report, they revealed the reaction mechanism of P(3HB-co-3HV) with TMAH in detail, and analyzed the copolymer compositions precisely and accurately based on the peak intensities of the THM products on the chromatograms [3]. Furthermore, the authors applied this technique to the direct compositional analysis of P(3HB-co-3HV) and poly(3-hydroxybutyrate-co-hydroxyhexanoate) [P(3HB-co-3HHx)] in whole bacterial cell [12, 13]. Here, trace amounts (0.1 mg) of the bacterial cells containing these copolymers were directly subjected to THM-GC in the presence of TMAH. The obtained chromatograms clearly showed a series of characteristics peaks, attributed to the THM products from each monomer unit in the polymer chains without any appreciable interference by the bacterial matrix components. The copolymer compositions were determined rapidly without using any cumbersome sample pretreatments such as solvent extraction and trans-methylation [12, 13]. However, there has been no report concerning direct quantitative analysis of PHAs in bacterial cells by THM-GC.

In this work, the rapid and highly sensitive determination of P(3HB) accumulated in bacteria was tried by using the THM-GC technique in the presence of TMAH. First, the reaction efficiency of P(3HB) with TMAH were examined in detail by focusing on the reaction temperature and the amount of the TMAH solution. Then, THM-GC under the thus-optimized conditions were applied to the direct determination of P(3HB) accumulated in bacterial cells. Furthermore, the obtained data by the THM-GC technique were compared with those by the conventional method involving solvent extraction and gravimetric determination.

Experimental

Materials

Stock cultures of *C. necator* (NBRC 102504) were purchased from Biological Resource Center, National Institute of Technology and Evaluation, Japan. An industry available P(3HB) was supplied by Mitsubishi Gas Chemical Company. A methanol solution of tetramethylammonium hydroxide (TMAH) (2.2 M) purchased from Aldrich (Milwaukee, WI, USA) was used as an organic alkali reagent.

Culture conditions of *C. necator*

Cupriavidus necator colonies were cultured at 30 °C for 5–7 days on the agar medium containing glucose and ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ as sole carbon and nitrogen sources, respectively. The composition of the agar

Table 1: Composition of agar medium used for P(3HB) production [2].

Element	Amount (g/L)
Agar	20.0
Glucose	10–25
Ammonium sulfate	0.5
K_2HPO_4	5.8
KH_2PO_4	3.7
$MgSO_4 \cdot 7H_2O$	0.1
1 mL of trace elements solution:	
$CaCl_2 \cdot 2H_2O$	1.67
$ZnSO_4 \cdot 7H_2O$	2.78
$FeSO_4 \cdot 7H_2O$	0.29
$MnCl_2 \cdot 4H_2O$	1.98
$CuCl_2 \cdot 2H_2O$	0.17

medium used for P(3HB) production is shown in Table 1. As shown in this table, the *C. necator* colonies were cultured with different concentrations of glucose (10–25 g/L) in order to control the P(3HB) content in the bacterial cells. In addition, the colonies were also grown on the agar medium containing propanoic acid instead of glucose as a carbon source to produce the cells without P(3HB).

Approximately 600 mg of the growth colony on the agar plate were carefully collected by using a micro spatula-spoon and transferred into 1.5 mL sample tube. The cells were washed with 1 mL of acetone and dried at 40 °C overnight. The obtained dry powder samples for each colony were homogenized using an agate mortar before analyzed by the THM-GC method and conventional solvent extraction method.

THM-GC measurement

The THM-GC system in this study was basically the same as that described in our previous papers [10–13]. A vertical microfurnance pyrolyzer (Frontier Laboratories, PY-2010D) was attached to a GC (Agilent, HP 4890) equipped with a flame ionization detector (FID). The small platinum cup (2 mm i.d × 4 mm height) containing 100 ± 5 µg of the dry powder sample of bacterial colonies and the TMAH solution was dropped in the center of a pyrolyzer heated at an elevated temperature between 350 and 500 °C under a helium carrier gas flow (50 mL/min). A part of the flow (1 mL/min) reduced by a splitter was introduced into a metal capillary separation column (Frontier Laboratories, Ultra ALLOY-5 (MS/HT); 30 m long × 0.25 mm i.d) coated with immobilized 5 % diphenyl-95 % dimethylpolysiloxane (1.0 µm film thickness). The column temperature was programmed from 35 °C to 230 °C at a rate of 5 °C/min.

Conventional solvent extraction method

Polymer extraction from the *C. necator* colonies was performed according to the procedure reported by Suzuki et al. [6]. Twenty milligram of the dried *C. necator* powders were subjected to solvent extraction using 2 mL of chloroform with applying sonification at 40 °C for 1 h. After the chloroform extract was filtered with a syringe filter (0.20 µm Minisart RC 15) to remove residual cell materials. Then, 6 mL of n-hexane was added in order to precipitate P(3HB). The mixture was left in refrigerator for 30 min and, after centrifugation, the supernatant was discarded. This precipitation process was repeated twice to recover P(3HB) quantitatively. The precipitate was washed with dimethyl ether, dried at 40 °C overnight and weighed to determine the P(3HB) content in the original bacterial cells.

Fig. 1: Chromatograms of (a) P(3HB) and *C. necator*, cultured in agar medium containing (b) propanoic acid and (c) glucose as a carbon source, obtained by THM-GC at 400 °C in the presence of TMAH. See Table 2 for peak assignment.

Results and discussion

Figure 1 shows typical chromatograms of (a) an industrially available P(3HB) sample and intact *C. necator* cells, cultured by adding (b) propanoic acid and (c) glucose (20 g/L) as a carbon source, obtained by THM-GC in the presence of TMAH at 400 °C. As was expected from former papers [3, 12, 13], on the chromatogram of P(3HB) (a), four peaks derived from 3HB units in the polymer chains were clearly observed after the elution of

Table 2: Identification of the characteristic peaks on the chromatograms of P(3HB) obtained by THM in the presence of TMAH.

Peak number	Compound name	Structure	MW
1	Methyl 3-butenolate		100
2	Methyl cis-2-butenolate		100
3	Methyl trans-2-butenolate		100
4	Methyl 3-methoxybutanoate		132

the reagent-related peaks. Among these products, peaks 1–3 have been assigned to the possible three isomers, methyl 3-butenate (1), methyl *cis*-2-butenate (2), and methyl *trans*-2-butenate (3), generated from 3HB units through *cis*-elimination followed by the THM reaction, while peak 4 to methyl 3-methoxybutanoate formed by the ideal THM reaction [3]. The identification of these peaks was summarized in Table 2 together with their molecular structures. Next, on the chromatogram of the *C. necator* sample cultured by adding propanoic acid (b), almost no peak was observed, suggesting that the interference from the bacterial matrix components (lipids and proteins) was not appreciable under the THM conditions used in this measurement. On the other hand, the chromatogram of the *C. necator* sample cultured with glucose (c) clearly showed the characteristic four peaks attributed to the THM-products from P(3HB) in the bacterial cells similarly to the case for the P(3HB) sample.

Then, the optimization of the THM conditions, such as reaction temperature and the amount of the TMAH solution, was carried out by using an intact *C. necator* sample cultured with glucose (20 g/L). Figure 2 shows typical chromatograms of the *C. necator* sample obtained by adding 2 μ L of the TMAH solution at different temperatures: (a) 350 °C, (b) 400 °C, and (c) 500 °C. On the chromatogram obtained at 350 °C (a), the

Fig. 2: Chromatograms of *C. necator* obtained by THM-GC in the presence of 2 μ L TMAH at different reaction temperatures; (a) 350 °C, (b) 400 °C, and (c) 500 °C. See Table 2 for peak assignment. ※ These peaks might be derived from bacterial matrix components such as proteins and lipids.

Fig. 3: Relationship between the amount of TMAH solution and the total intensity of four peaks derived from P(3HB) in *C. necator* normalized by the sample weight.

peak intensities of the THM products from P(3HB) were relatively small due to insufficient thermal energy to promote the THM reaction of the polymer chains quantitatively. Furthermore, the chromatogram obtained at 500 °C (c) showed several interference peaks from the bacterial matrix components, one of which was overlapped with peak 4 to some extent. In the chromatogram obtained under 400 °C (b), the strong four peaks characteristic to the THM products from P(3HB) were clearly observed without no interference from the matrix component.

Next, Fig. 3 shows the relationship between the amount of the TMAH solution and the total intensities of the four peaks from 3HB units, normalized by the sample weight, observed in the chromatograms of the *C. necator* sample. As shown in this figure, the peak intensity of P(3HB) content became almost constant when more than 2 μL of the TMAH solution was added. In addition, stoichiometry tells us that 0.10 mg of the *C. necator* sample containing 30 wt% of P(3HB) requires only ca. 0.14 μL of the TMAH solution to methylate all the 3HB units in the bacteria sample. The above result and fact suggest that; (1) the excess amount of TMAH (more than 2 μL) is required to promote the THM reaction of P(3HB) in *C. necator* quantitatively, and (2) the added amounts of the TMAH solution more than 2 μL do not cause any differences in the determination of P(3HB) in the bacteria samples because the additional TMAH solution would be consumed by the self-degradation and/or decomposition of the bacterial matrix components. Thus, the following measurements were carried out with the addition of 2 μL of the TMAH solution at 400 °C as the THM temperature.

Using the chloroform solution samples of P(3HB) with three different concentrations (6.25 mg/mL, 12.5 mg/mL, and 25.0 mg/mL), the calibration curve for P(3HB) contents was acquired on the basis of the total intensities of the four peaks derived from 3HB units on the chromatograms obtained under the thus-optimized conditions. Here, the relative standard deviation of the total intensities was less than 7 % for three repeated runs. Furthermore, a good linear relationship (correlation coefficient, $r = 0.9993$) was observed between the total intensities and the amount of P(3HB) introduced into the sample cup, suggesting that the P(3HB) content was able to be determined by using this calibration curve.

Finally, the contents (wt%) of P(3HB) in the *C. necator* samples, cultured on the agar medium containing different concentrations of glucose (10–25 g/L), were determined from the total intensities of the four characteristic peaks by using the calibration curve as follows:

$$C_{\text{P(3HB)}} (\text{wt}\%) = \frac{W_{\text{P(3HB)}}}{W_{\text{sample}}} \times 100, \quad (1)$$

where $W_{\text{P(3HB)}}$ and W_{sample} are the amounts of P(3HB) determined by the calibration curve and that of the bacterial cells introduced into the pyrolyzer ($100 \pm 5 \mu\text{g}$), respectively. Figure 4 shows the relationship between

Fig. 4: Comparison of P(3HB) contents (wt%) accumulated in *C. necator* determined by THM-GC and conventional extraction method. Error bars: standard deviations for the P(3HB) contents by THM-GC and conventional method.

the contents of P(3HB) in *C. necator* samples determined by the THM-GC technique and the conventional gravimetric method following solvent extraction and purification. This figure showed a good linear relationship between the values obtained by the two method ($r^2 = 0.9972$), although a discrepancy was observed in a manner that the data for THM-GC were somewhat higher than those for the conventional method. This higher estimation of the P(3HB) contents by THM-GC might be owing to the detection not only of the 3HB units in the polymer chains but also of these units present as monomeric and oligomeric components by this method. Moreover, the relative standard deviation for the contents estimated by THM-GC were less than 8 % based on three repeated runs. These results indicate that THM-GC can be used as a practical method for the screening of bacteria suited for the production of PHAs with high performance.

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