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Optimisation of poly-β-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems

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Abstract

Poly-β-hydroxyalkanoate (PHA) is a polymer commonly used in carbon and energy storage for many different bacterial cells. Polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), store PHA anaerobically through metabolism of carbon substrates such as acetate and propionate. Although poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV) are commonly quantified using a previously developed gas chromatography (GC) method, poly-β-hydroxy-2-methylvalerate (PH2MV) is seldom quantified despite the fact that it has been shown to be a key PHA fraction produced when PAOs or GAOs metabolise propionate. This paper presents two GC-based methods modified for extraction and quantification of PHB, PHV and PH2MV from enhanced biological phosphorus removal (EBPR) systems. For the extraction of PHB and PHV from acetate fed PAO and GAO cultures, a 3% sulfuric acid concentration and a 2–20 h digestion time is recommended, while a 10% sulfuric acid solution digested for 20 h is recommended for PHV and PH2MV analysis from propionate fed EBPR systems.

Keywords: Poly-β-hydroxyalkanoate; Poly-β-hydroxy-2-methylvalerate; Enhanced biological phosphorus removal; Polyphosphate accumulating organisms; Glycogen accumulating organisms; Propionate

1. Introduction

Enhanced biological phosphorus removal (EBPR) is a very commonly used and sustainable method for phosphorus removal from wastewater. A group of bacteria known as the polyphosphate accumulating organisms (PAOs) are primarily responsible for successful EBPR in activated sludge systems. Another group of bacteria known as the glycogen accumulating organisms (GAOs) have been shown to be able to compete with PAOs for the limiting carbon substrates in these systems. PAOs and GAOs are both able to anaerobically take up volatile fatty acids (VFAs) and convert them into intracellular poly-β-hydroxyalkanoates (PHAs). Although the VFA composition in wastewater systems can be diverse, acetate and propionate have been shown to be the primary fractions of VFA present in the influent to EBPR plants [1,2], consequently most research has focused on the utilisation of these two carbon sources for PAO and GAO enrichment. PAOs tend to chiefly produce poly-β-hydroxybutyrate (PHB) from acetate [3], and mainly poly-β-hydroxyvalerate (PHV) and poly-β-hydroxy-2-methylvalerate (PH2MV) from propionate [4,5]. GAOs primarily convert acetate to PHB and PHV [6,7], while PHV and PH2MV are the major PHA fractions produced through propionate uptake [8,9].

Although most prior work in this field has focused on the utilisation of acetate as the sole carbon source, recent findings have suggested that a propionate feed source can provide PAOs an advantage over GAOs [8,10,11], resulting in more reliable EBPR operation. Despite this recent interest in propionate as a carbon source, many researchers do not currently quantify PH2MV production [10,12–15], perhaps due to the lack of a proven method for analysing this particular PHA fraction. PH2MV has been shown to make up approximately half of the total PHA content when propionate is the sole carbon source [4,5], therefore, the total PHA yield by PAOs and

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GAOs has been commonly underestimated in literature. It is clear that the analytical method for PHA analysis should be revisited and expanded to include PH2MV.

The most common method for PHA extraction and quantification in EBPR systems is based on the gas chromatography (GC) method originally proposed by Braunegg et al. [16] and later expanded and modified by Comeau et al. [17]. The extraction method involves hydrolysation of the polymer and conversion to a methyl-ester of the monomeric 3-hydroxyalkanoate (3HA) fraction. An acidified alcohol solution (i.e. sulfuric acid in methanol) and a solvent (i.e. chloroform) are added to the sample, which is digested at 100 °C, cooled and mixed with water to achieve phase separation, and the organic phase is quantified using GC. Riis and Mai [18] have modified this method for PHB quantification through use of a different solvent (dichloroethane) and an alternate acidified alcohol solution (HCl in propanol). A test performed by several European research groups [19] has shown that a high reproducibility of PHB concentration was observed despite variations in the solvent (e.g. chloroform, dichloroethane, dichloromethane) and acidified alcohol solutions (e.g. sulfuric acid in methanol, HCl in propanol). The extraction procedure originally proposed by Braunegg et al. [16] with a chloroform solvent combined with sulfuric acid in methanol, has been frequently used for PHB and PHV analysis [20] and is implemented in this study.

There are currently many variations to the extraction method outlined above using chloroform and acidified methanol, without a clear indication of the advantages or disadvantages of each variation. The sulfuric acid concentration in methanol has been varied from 3% [7,10,16,17,21] to 10% [22] or even 20% [12,23]. Others have varied the extraction time from 3.5 h [17,23] to 6 h [7,10] to 20 h [12,21,22]. Testing of the effects of sulfuric acid concentration and digestion time on PHA extraction is necessary in order to standardise this analytical method, as well as to optimise the extraction of PH2MV.

Another common method for PHA analysis is through the use of nuclear magnetic resonance (NMR). NMR is a very useful technique for the identification of different PHA fractions and their chemical structures. Lemos et al. [24] used this approach for the identification of PH2MV and other PHA fractions in their propionate fed EBPR system. The advantage of using GC analysis, however, is that it is more accurate for quantitative analysis than NMR and more suitable for high-throughput routine analyses. When combined with mass spectrometry (GC–MS), the identity and mass of the PHA monomers can also be measured [20].

This study aims to develop a method for accurate quantification of all relevant PHA fractions. The GC method is chosen for PHA analysis with an extraction procedure that uses a sulfuric acid in methanol solution mixed with a chloroform solvent. GC–MS is used for confirmation of the PHA fractions produced by the activated sludge. The effects of sulfuric acid concentration and digestion time are tested with samples containing varying levels of PHB, PHV and PH2MV, in order to provide a suitable method for the quantification of each of these biopolymers.

1.1. Materials and methods

PHA was analysed through the following procedure. Sludge samples from lab-scale sequencing batch reactors (SBRs) were mixed with formaldehyde at a ratio of approximately 1% formaldehyde per sample volume in order to inhibit biomass activity in the sludge. The samples were centrifuged and the supernatant was removed, then washed with a phosphate buffer solution, re-centrifuged, and the supernatant decanted once more. All samples were then lyophilised through a freeze drying unit (FTS, Queensland, Australia) operated at −54 °C and 0.1 mbar for at least 20 h. Approximately 20 mg of lyophilised sludge was added to 2 mL of chloroform and 2 mL of an acidified methanol solution (containing either 3%, 10% or 20% sulfuric acid by volume, as well as approximately 100 mg/L of sodium benzoate [17], used as an internal standard). Six standard solutions were composed of 0–3 mg of a R-3-hydroxybutyric acid (3HB) and R-3-hydroxyvaleric acid (3HV) copolymer (7:3) (Fluka, Melbourne, Victoria, Australia) and 0–3 mg of 2-hydroxycaproic acid (Sigma–Aldrich, Melbourne, Victoria, Australia). Due to the unavailability of a direct standard for 3-hydroxy-2-methylvaleric acid (3H2MV), it was assumed that the relative response factor for 2-hydroxycaproic acid would be similar to that of 3H2MV for GC quantification purposes, since these two molecules are isomers of each other. The samples and standards were then digested in tightly sealed 10 mL glass vials for either 2, 6 or 20 h at 100 °C, and cooled to room temperature. Distilled water (1 mL) was then added and mixed vigorously with each sample to remove particulate debris from the chloroform phase and prevent degradation of the GC column [17]. After mixing, 1 h of settling time was allowed to achieve phase separation. The chloroform (bottom) phase was then transferred to another vial, dried with approximately 0.5–1 g of granular sodium sulphate pellets, and separated from the solid phase. Three microlitres of the chloroform phase was analysed with a Perkin-Elmer gas chromatograph. The chromatograph was operated with a DB-5 column (30 m length × 0.25 mm I.D. × 0.25 μm film), a split injection ratio of 1:15 and helium as the carrier gas (1.5 mL/min). A flame ionisation detection (FID) unit was operated at 300 °C with an injection port temperature of 250 °C. The oven temperature was set to 80 °C for 1 min, increased at 10 °C/min to 120 °C, and then to 270 °C at 45 °C/min and held for 3 min.

The GC–MS system incorporated a similar column (DB-5MS) coupled with a Shimadzu mass spectrometer GC–MS-QP5050 (Shimadzu, Japan) and an autosampler AOC-1400. The mass spectrometer was run in scan mode at a detector voltage of 1.5 kV in the mass range of 40–600 amu. The scan speed and interval were 2000 amu/s and 0.3 s, respectively. Deconvolution of GC–MS peaks was performed using the automated mass spectral deconvolution and identification system (AMDIS32), and identification of

the compounds was obtained using the National Institute of Standards and Technology (NIST02) database. The background of each component was manually subtracted before searching the database. The closest spectra match in the NIST02 database was recorded (chemical formula, molecular weight and NIST number) along with a match factor (out of 1000, converted to a percentage).

The samples used for these experiments were obtained from enriched cultures of PAOs and GAOs. Samples high in PHB and PHV were obtained from an enriched culture of GAOs fed with acetate as the sole carbon source, while the PHV and PH2MV rich samples were obtained from an enriched PAO culture fed with propionate as the sole carbon source. Further operational details of the acetate–GAO and propionate–PAO enriched cultures may be found in Oehmen et al. [11,5], respectively. Testing of varying sulfuric acid concentrations and digestion times was performed on both the acetate–GAO and propionate–PAO sludges. During each trial, identical samples from either the PAO or GAO enrichments were analysed three to five times each per tested variable.

2. Results and discussion

2.1. Identification of PHA fractions with GC–MS

Gas chromatograms showing the 3HB and 3HV extracted from the acetate enriched GAO culture and the 3HV and 3H2MV extracted from the propionate enriched PAO culture are shown in Figs. 1 and 2, respectively. These samples were also analysed using GC–MS. For 3HB, 3HV and 3H2MV, match factors of 92.5%, 87.3% and 90.3%, respectively, were observed with the mass spectra found in the NIST02 database. The close match between the measured and reference spectra indicate that the measured compounds are indeed highly likely to correspond with 3HB, 3HV and 3H2MV. The large peak shown in between the 3HV and 3H2MV peaks was identified as 4-oxovaleric acid by GC–MS. This peak has also been identified from similar samples by Comeau et al. [17], however, unlike 3HB, 3HV and 3H2MV, 4-oxovaleric acid did not show the cyclic anaerobic increase and aerobic decrease displayed by typical PAO and GAO metabolism.

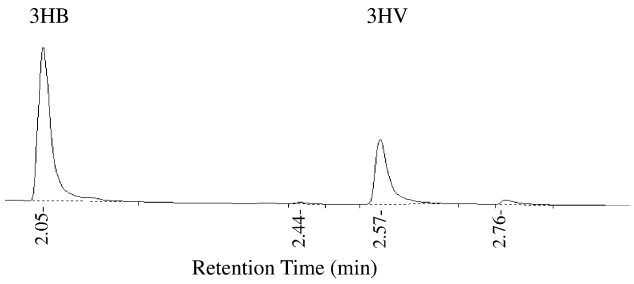

Fig. 1. Gas chromatogram of 3HB and 3HV from an acetate enriched culture of GAOs, extracted with a 3% sulfuric acid solution for a period of 20 h.

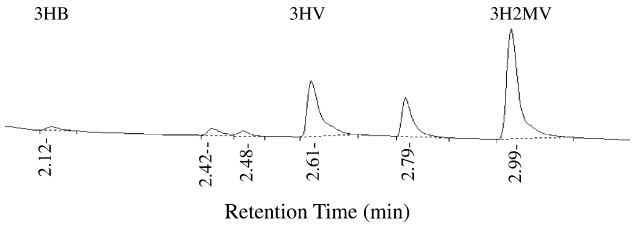

Fig. 2. Gas chromatogram of 3HV and 3H2MV from a propionate enriched culture of PAOs, extracted with a 10% sulfuric acid solution for a period of 20 h. The peak shown in between 3HV and 3H2MV was identified as 4-oxovaleric acid by GC–MS, however, this compound was not shown to exhibit anaerobic–aerobic cycling and did not merit further consideration.

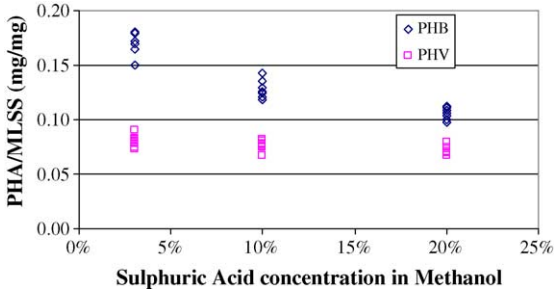

Fig. 3. A comparison of PHB and PHV extraction with varying levels of sulfuric acid concentration. PHB recovery decreased dramatically with an increase in sulfuric acid concentration, while PHV recovery showed only minor variability.

Hence, 4-oxovaleric acid did not merit further consideration as it does not seem to be associated with the intracellular storage and utilisation processes of PAOs and GAOs.

2.2. Optimisation of PHB and PHV extraction

The extraction of PHB and PHV using varying sulfuric acid concentrations is shown in Fig. 3. It can clearly be observed that the extraction of PHB was strongly influenced by the concentration of sulfuric acid in the methanol. The 3% sulfuric acid solution yielded optimal recovery of PHB, which is consistent to the results reported by Braunegg et al. [16]. Observation of the chromatogram in Fig. 4 clearly shows that a separate peak close to the 3HB peak was found

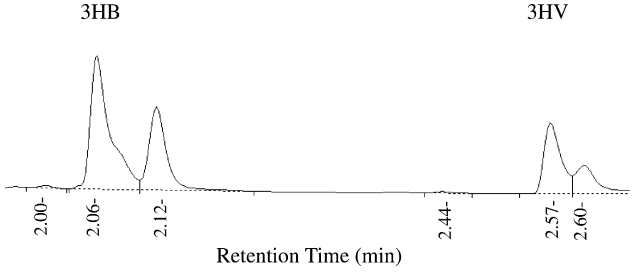

Fig. 4. Gas chromatogram of 3HB and 3HV from an acetate enriched culture of GAOs, extracted with a 20% sulfuric acid solution for a period of 20 h. The higher sulfuric acid concentration led to the observance of ‘double peaks’ for both fractions of PHA.

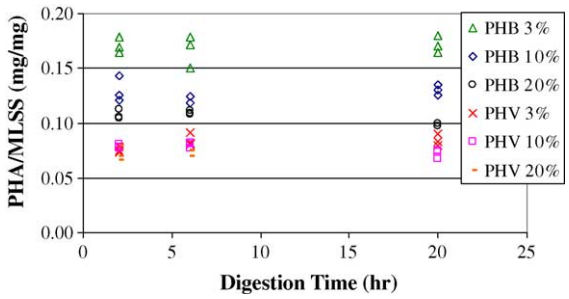

Fig. 5. A comparison of PHB and PHV extraction with varying lengths of digestion time. Only minor variability of PHB and PHV recovery was observed regardless of the length of time tested.

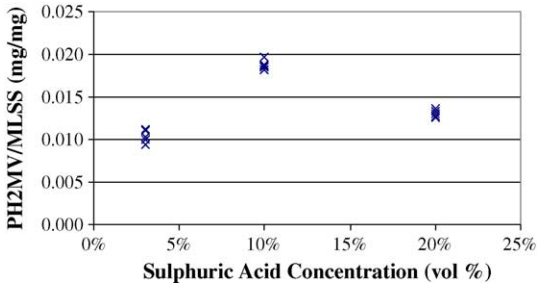

Fig. 6. A comparison of PH2MV extraction with varying levels of sulfuric acid concentration. A 10% sulfuric acid solution yielded optimum PH2MV recovery.

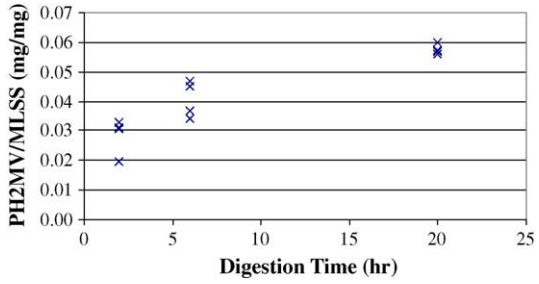

Fig. 7. A comparison of PH2MV extraction with varying lengths of digestion time. A long digestion period produced improved recovery of PH2MV.

at a concentration of 20% (also observed at 10%), but was not present at 3% (see Fig. 1). This 'double peak' is likely caused by degradation of the 3HB fractions contained in these samples. Braunegg et al. [16] proposed that a 3% sulfuric acid concentration was critical for PHB extraction, because of the formation of degradation products at concentrations above 3%, and partial hydrolysis resulting from concentrations below 3%. Due to the strong agreement between this study and Braunegg et al. [16], a 3% sulfuric acid concentration in methanol is recommended for quantification of the PHB content in activated sludge.

In contrast to PHB, the results shown in Fig. 3 suggest that the extraction of PHV is not affected significantly by changes in sulfuric acid concentration. As shown in Fig. 4, however, a 'double peak' was observed for PHV at a sulfuric acid concentration of 20%, suggesting that some degradation of this compound may occur at this concentration. Sulfuric acid concentrations of 3% and 10% yielded much more distinguishable peaks, as can be observed in Figs. 1 and 2, respectively, therefore 3% or 10% sulfuric acid concentrations are recommended for extraction of PHV.

The effects of digestion time on the extraction of PHB and PHV are shown in Fig. 5. There was little variability between the PHB and PHV levels observed at 2, 6 and 20 h of digestion time, regardless of the sulfuric acid concentration used in the experiments. This suggests that even 2 h of digestion was sufficient for extraction of the majority of the PHB and PHV within the cells. Thus, for samples containing mainly PHB and PHV, such as acetate enriched PAO and GAO cultures, a 3% sulfuric acid concentration and a digestion time between 2 and 20 h should yield satisfactory extraction of the total PHA produced by the sludge.

2.3. Optimisation of PH2MV extraction

The extraction of PH2MV under conditions of different sulfuric acid concentrations and digestion times are shown in Figs. 6 and 7, respectively. From Fig. 6, it can be observed that PH2MV was influenced greatly by the sulfuric acid concentration, where a concentration of 10% was observed to yield the highest extraction of PH2MV. The lower level of PH2MV extraction at 20% sulfuric acid concentration could be explained by the observed 'double peak' shown in Fig. 8, which suggests partial degradation of PH2MV at this concentration. In contrast, the peak observed when 10% sulfuric acid was added (shown in Fig. 2) appears to be better defined. From Fig. 7, it was observed that a long digestion time resulted in the highest level of PH2MV extraction. Therefore, a sulfuric acid concentration of 10% and a digestion time of 20 h appear to be optimal conditions for the quantitation of PH2MV through the proposed method. Figs. 3 and 5 suggest that PHV extraction would also be satisfactory under these conditions. Therefore, for propionate enriched PAO and GAO cultures (rich in PHV and PH2MV), it is recommended that a sulfuric acid concentration of 10% and a digestion time of 20 h be used for PHA extraction.

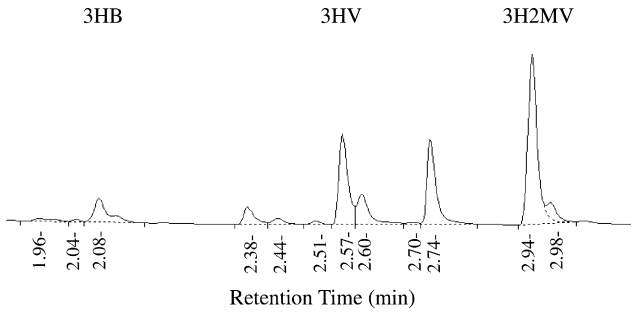

Fig. 8. Gas chromatogram of 3HV and 3H2MV from a propionate enriched culture of PAOs, extracted with a 20% sulfuric acid solution for a period of 20 h. The high sulfuric acid concentration led to the observance of 'double peaks' for all fractions of PHA.

Table 1
Comparison of anaerobic stoichiometry by propionate fed PAOs with PHA measured with different concentrations of sulfuric acid and 20 h of digestion time

Anaerobic stoichiometry	Model predictions [5]	Sulfuric acid concentration			
		3%	10%	20%	Units
PHB/VFA	0	0.06	0.04	0.05	C mol/C mol
PHV/VFA	0.56	0.54	0.55	0.65	C mol/C mol
PH2MV/VFA	0.67	0.30	0.65	0.40	C mol/C mol
PHA/VFA	1.22	0.91	1.23	1.10	C mol/C mol

The method proposed for extraction of PHV and PH2MV was performed by Oehmen et al. [5] in the characterisation of propionate metabolism by PAOs. Table 1 displays a comparison between the theoretical PHA production from propionate uptake by PAOs (based on stoichiometric mass and energy balances) and the PHA extraction observed with the tested sulfuric acid concentrations. It is clear that the anaerobic PHA production observed through extraction with 10% sulfuric acid and 20 h of digestion time was shown to correlate very well with the anaerobic stoichiometry of PAOs fed with propionate. Extraction with 3% and 20% sulfuric acid appeared to significantly underestimate the production of PH2MV, as was suggested by Fig. 6. There was a strong agreement between the model predictions and the PHV production observed for the 3% and 10% extraction methods, suggesting again that either method should yield satisfactory recovery of PHV. The 20% sulfuric acid concentration extraction method appeared to overestimate the production of PHV, perhaps due to the 'double peak' problem shown in Fig. 8. The results presented in Table 1 support the hypothesis that a sulfuric acid concentration of 10% in the acidified methanol solution along with a 20 h digestion time is optimal for analysis of both PHV and PH2MV.

Table 2
Repeatability of the extraction methods at various PHA concentrations

Sulfuric acid concentration (%)	PHA fraction	Average PHA concentration (mg/mg MLSS)	Standard deviation (%)
3	PHB	0.0046	2.8
		0.0430	1.7
		0.0555	2.3
		0.1230	2.8
3	PHV	0.0007	0.6
		0.0164	3.9
		0.0492	1.6
		0.0974	2.2
10	PHV	0.0048	6.3
		0.0225	3.2
		0.0692	1.4
		0.1100	2.8
10	PH2MV	0.0069	1.1
		0.0269	3.0
		0.0735	0.9
		0.1071	4.2

2.4. Repeatability of the extraction methods

The proposed extraction methods were tested with numerous samples repeated in triplicate and containing variable concentrations of PHA. The results are summarised in Table 2, where a 3% sulfuric acid concentration and a 20 h digestion time were used for the PHB and PHV rich samples, and a 10% sulfuric acid concentration digested for 20 h was performed for the samples high in PHV and PH2MV. The relative standard deviation was found to average 2.6% for all samples tested, which correlates closely with the reproducibility value reported by Comeau et al. [17] for PHB, where a similar set of tests resulted in a 3% standard deviation. This level of reproducibility is quite acceptable for many applications, however, when data of the highest accuracy is desired, multiple analyses of the same sample is recommended.

3. Conclusions

PHB, PHV and PH2MV were the identified fractions of PHA from EBPR systems fed with acetate and propionate. Testing of the proposed method for PHA analysis shows that a 3% sulfuric acid concentration was required for optimal PHB extraction, while a 10% sulfuric acid concentration and 20 h of digestion time produced the highest recovery of PH2MV. Only minor differences were observed for PHV extraction under these conditions, and neither PHB nor PHV recovery were significantly affected by variations in digestion time. Thus, a 3% sulfuric acid concentration and a 2–20 h digestion time is recommended for analysis of PHB and PHV from acetate fed PAO and GAO cultures, while a 10% sulfuric acid solution digested for 20 h is recommended for PHV and PH2MV analysis from propionate fed EBPR systems. Reproducibility of these methods was found to be very high, with an average standard deviation of 2.6%. Future research aiming to establish one method for the simultaneous quantification of PHB, PHV, and PH2MV would be beneficial.

Acknowledgements

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