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Journal of Chromatography A, 1070 (2005) 131–136
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

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

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Received 21 October 2004; received in revised form 31 January 2005; accepted 9 February 2005
Available online 8 March 2005

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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doi:10.1016/j.chroma.2005.02.020

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