Polyhydroxyalkanoate quantification in organic wastes and pure cultures using a single-step extraction and \(^1\)H NMR analysis

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**ABSTRACT**

In this study, a proton nuclear magnetic resonance (\(^1\)H NMR) method was developed to quantitatively analyze polyhydroxyalkanoate (PHA) content in *Cupriavidus necator* H16, *Azotobacter vinelandii* AvOP, and mixed microbial cultures from the effluent of an agricultural waste treatment anaerobic digester. In contrast to previous methods, a single-step PHA extractive method using deuterated chloroform was established, thereby facilitating direct \(^1\)H NMR analysis. The accuracy of the method was verified through comparison with well-established gas chromatography (GC) methanolysis techniques. Nile blue fluorescence staining was also carried out to serve as an independent and qualitative indicator of intracellular PHA content. The results indicate that the \(^1\)H NMR method is appropriate for rapid and non-destructive quantification of overall PHA content and determination of PHA copolymer composition in a variety of cultures. Notably, this technique was effective in measuring PHA content in full-strength waste samples where high concentrations of background impurities and organic compounds are present. The straightforward procedures minimize error-introducing steps, require less time and materials, and result in an accurate method suitable for routine analyses.

**Key words** | gas chromatography, Nile blue, NMR, polyhydroxyalkanoate quantification, P(HB-co-HV)

**INTRODUCTION**

Polyhydroxyalkanoates (PHAs) are a class of polyesters that represent a source of non-petrochemically derived biodegradable plastics (Brandl et al. 1990; Suchada 2010). The material properties of PHAs can potentially be tailored for specified applications by adjusting the copolymeric composition. For example, the availability of volatile fatty acids like valerate and propionate lead to the formation of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) copolymers, P(HB-co-HV) (Doi 1990; Oehmen et al. 2005).

Alternative production methods utilizing low-value waste feedstock and mixed microbial cultures may decrease PHA production costs and energy requirements (Serafim et al. 2008). For example, polyphosphate-accumulating organisms (PAOs) are enriched to simultaneously achieve biological phosphorus removal and bioplastic production (Mino et al. 1998). In these complex environmental systems, as well as in traditional fermentative processes, efficient quantification and chemical characterization of PHA is critical for process development, monitoring, and optimization.

Methods for estimation of PHA content include fluorometry, gravimetry, and spectrophotometry (Serafim et al. 2002; Furrer et al. 2007). Of these, Nile blue A sulfate staining is perhaps the most rapid method, and is based on fluorescent detection of dye bound to intracellular PHA (Kitamura & Doi 1994; Page & Tenove 1996; Oshiki et al. 2011). However, Nile blue non-selectively binds to non-PHA materials like waxes and lipids and can result in inaccurate and inconsistent measurements (Spiekermann et al. 1999). Gravimetric and spectrophotometric methods are time intensive and require substantial amounts of PHA-containing sample and are also susceptible to interference by cell materials and lipids (Serafim et al. 2002). These methods do not provide information on PHA composition and are insufficient for microbial systems that are likely to produce mixtures of PHA polymers.


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Gas chromatography (GC) is used for PHA analysis, specifically to determine both PHA content and composition. Sample preparation for GC analysis requires extraction, depolymerization, and derivatization of the PHA. Some disadvantages of GC include: possible incomplete depolymerization, and derivatization of the PHA. Some sample destruction as a result of derivatization, peak overlap by contaminants in complex samples with high organic loads (e.g. samples from activated sludge), and instrument maintenance issues due to the presence of debris, acid, and water in prepared samples (Serafim et al. 2002).

Nuclear magnetic resonance spectroscopy (NMR, e.g. $^1$H NMR and $^{13}$C NMR) is a diagnostic tool that is used for PHA structural analysis and metabolic pathway studies (de Rijk et al. 2005), but has received less attention for routine quantification of PHA polymers, especially in environmental samples. The NMR signal is quantitative, and unique splitting patterns enable structural determination of the compound that could otherwise require mass spectrometric analysis if using GC-based methods (de Rijk et al. 2005). Unlike GC methods that require polymer derivatization, NMR is non-destructive, thereby leaving the polymer intact for other analyses (Doi 1990).

Previous use of NMR for PHA characterization has employed time-consuming, multi-step purification methods (Jan et al. 1996; Furrer et al. 2007; Dai et al. 2008). These methods can increase opportunity for inaccuracies due to material loss and handling. Due to a lack of direct and efficient sample preparation techniques, routine use of $^1$H NMR for PHA quantification has been limited (Furrer et al. 2007).

Quantitative $^1$H NMR-based measurement has primarily been applied to PHB extracted from pure cultures (Jan et al. 1996). A study by Dai et al. (2008) used $^1$H NMR to measure PHA in environmental samples. The $^1$H NMR method developed in this current study was used to evaluate PHA content in both pure cultures and full-strength samples taken directly from the effluent of an anaerobic digester treating agricultural waste. The utility of this method was evaluated through comparison with GC and fluorescence staining methods.

**EXPERIMENTAL**

**Chemicals**

PHB (Product No. 363502) and P(HB-co-HV) (12% HV) (Product No. 403121) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Sulfuric acid (95–98% c. p.) and deuterated chloroform (99.8% atom-D) with 0.03% (v/v) tetramethylsilane (TMS) were used (Acros Organics, Thermo Fisher Scientific, Morris Plains, NJ). Other chemicals, solvents, and reagents were purchased from Fisher Scientific Research (Pittsburgh, PA) and were of analytical grade.

**Microorganisms and culture methods for pure strains**

*Cupriavidus nectator* H16 (ATCC 17699) (previously *Ralstonia eutropha* H16) and *Azotobacter vinelandii* AvOP (ATCC BAA-1303) were used. Both *C. nectator* and *A. vinelandii* are known PHA producers (Suchada 2010). A phosphate-limited base medium with 10 g/L fructose was used for *C. nectator* cultivation and PHB production (Ryu et al. 1997). Burk’s nitrogen-free medium containing 20 g/L sucrose was used for *A. vinelandii* growth (Wilson & Knight 1952). Cells from frozen glycerol stocks (20% v/v) were pre-cultured in 50 mL of corresponding media, and late log-phase cultures were used to inoculate (1:70 dilution) 3 L Kontes® Cytostir® (Kimble Chase Life Science and Research Products, Vineland, NJ) stirred bioreactors containing sterile media. Oxygen was supplied by sparging filtered (0.22 μm) air into the media. Each strain was grown in duplicate vessels. Samples (approximately 50 mL) were periodically analyzed for culture density and PHA content in order to establish applicability of the analytical methods used over a broad concentration range.

**Environmental samples**

Effluent samples from a pilot-scale anaerobic digester (Wade Dairy, Ogden, UT) were used as an environmental source of PHAs. Details of reactor operating conditions and parameters are reported elsewhere (Hansen & Hansen 2002). Digester samples were subjected to periodic cycling between aerobic and anaerobic conditions with the intent of creating a population of phosphorus-accumulating organisms (PAOs) (Mino et al. 1998; Blackall et al. 2002). PAO enrichment experiments were carried out in 3 L stirred tank reactors. Anaerobic-phase cultures (where PHA content is highest) were analyzed after three weeks of cycling, which allowed for enriched, stable populations of PHA-accumulating organisms. Volatile fatty acids were measured in the environmental waste samples by the Utah State University Analytical Labs (USUAL, Logan, UT).

**Sample preparation**

Pure culture samples were centrifuged at 2,000 × g and lyophilized in a Labconco Lyph-Lock 4.5 freeze-drying unit.
(Model 77510, Labconco Corporation, Kansas City, MO) at −40 °C and 33 × 10⁻⁴ mBar for 15 h. Lyophilized biomass was used to determine dry cell weight and construct growth curves. Sample preparation in NMR and GC/FID (extraction and/or derivatization) was carried out in 2 mL crimp-top autosampler vials with PTFE-lined septa (Fisher Scientific, Hanover Park, IL) using 15 (±0.2) mg of dry cells. The samples from each time point were analyzed by ¹H NMR, GC/FID, and fluorescence staining to allow for direct comparison of methods.

¹H NMR sample preparation procedures were developed based on a chloroform–sodium hypochlorite dispersion method for PHA extraction (Hahn et al. 1994), with modifications and CDCl₃ substituted for CHCl₃. Approximately 0.7 mL of a 5% (vol/vol) sodium hypochlorite solution and 1 mL of CDCl₃ (0.03% TMS) were added to each sample of dried cells. These mixtures were vortexed for 10 min, and incubated on a shaker table for 2 h at 30 °C. PHA standards were incubated at a higher temperature (50 °C) to facilitate dissolution. After the 2 h extraction, samples were centrifuged at 1,500 × g for 10 min to induce phase separation. The PHA-containing bottom organic layer of CDCl₃ was removed and transferred to a 5 mm NMR tube. Because CDCl₃ was used for extraction, aliquots from the organic layer were subjected directly to ¹H NMR analysis.

GC sample preparation was carried out in accordance with reported acid methanolysis procedures (Braunegg et al. 1978; Oehmen et al. 2005). Equal volumes (0.7 mL) of acidified methanol (0.03% H₂SO₄) and chloroform were added to each sample. These mixtures were vortexed and incubated at 100 °C for 2 h on a shaker table. After cooling, 0.4 mL of distilled water was added, followed by vortexing for 10 min. Following a phase separation time of 20 min, the organic phase was transferred to a new vial for GC analysis. The total sample preparation times required for ¹H NMR and GC analysis were 2.15 and 2.5 h, respectively.

Environmental samples were prepared by repeated centrifugation and washing of aliquots (1.5 mL). The centrifuged pellet was subsequently extracted and digested in accordance with ¹H NMR and GC sample preparation procedures described above.

Analytical methods

A Jeol ECX-300 spectrometer (Jeol USA, Inc., Peabody, MA) with an Oxford 54-mm-bore magnet was used to obtain 300.53 MHz ¹H NMR spectra in Fourier transform mode. The operating parameters were: 13.43 μs pulse width at 90°, 5636 Hz spectral width, 32 k data points, and 32 scans with a 1 s relaxation delay. TMS at 0.03% was used as an internal standard. Jeol Delta NMR Processing Software was used to analyze spectra.

An HP 6890 Series II GC system (Hewlett Packard, Wilmington, DE) equipped with a flame ionization detector (FID) and an autosampler was used in conjunction with an HP-INNOWax cross-linked polyethylene glycol capillary column with dimensions of 30 m × 0.25 mm ID × 0.25 μm film thickness (Agilent Technologies, Wilmington, DE). A split injection ratio of 1:20 was used and argon (3.8 mL/min) was used as the carrier gas. Chromatography was performed using an initial temperature of 60 °C, held constant for 4 min, followed by a 15 °C/min increase to a final temperature of 250 °C, held constant for 5 min. FID and injection port temperatures were maintained at 250 °C. HP-Chemstation software was used for peak integration and analysis. The lower initial temperature enabled separation between chloroform and PHA peaks. The retention times for HB and HV methyl esters were 9.55 and 10.31 min, respectively. A new calibration curve was constructed with each set of GC analyses to account for variability.

Nile blue A sulfate fluorescent dye solution was prepared at a concentration of 0.5 g/L ethanol (Kitamura & Doi 1994). Approximately 150 μL of the dye solution was added to 50 μL of sample contained in wells of Microfluor 96-well black flat-bottom microtiter plates (Product No. 7805, Thermo Scientific, Hanover Park, IL). Black microtiter plates were used to reduce interference from background signals during fluorescence analyses. Fluorescence measurements were obtained using a Synergy™ 4 Multi-Mode Microplate Reader with Hybrid Technology™ (BioTek, Winooski, Vermont). Respective excitation and emission wavelengths of 490 and 580 nm were used (Kitamura & Doi 1994). The total amount of cells in each sample was correlated to the fluorescence output readings using calculated dry cell weight data. Each relative fluorescence measurement was divided by the product of the sample volume (50 μL) and the dry cell weight (mg/mL) to yield specific fluorescence intensity. Fluorometry measurements of relative PHA content were correlated to quantitative ¹H NMR and GC data.

All experiments and analyses were replicated. Growth experiments were carried out in duplicate. Standard curves were constructed using triplicate, independently prepared standards. A minimum of three replications was used for fluorescence staining measurements.
RESULTS AND DISCUSSION

PHB and PHBV standards

$^1$H NMR spectra were obtained for PHB and PHBV copolymer standards. These peaks match those observed in previous studies (Doi et al. 1986; Jan et al. 1996). The PHB and P(HB-co-HV) $^1$H NMR calibration curves were obtained using commercial standards (data not shown). The area of each PHA-specific signal was linearly proportional to polymer concentration. From the P(HB-co-HV) data, the average copolymer composition was determined as 12.34% ± 0.52% HV by calculating the intensity ratio of the HB and HV methyl signals at 1.28 and 0.90 ppm, respectively. The vendor-specified HV content was 12%. The ratio of methine peak areas at 5.2 and 5.16 ppm also gave similar values for HV content (Doi et al. 1986; Furrer et al. 2007). The CDCl$_3$ peak at 7.25 ppm served as a reference signal, and the TMS peak at 0.00 ppm was used as an internal standard. The commercial standards used for calibration by NMR were also used to generate standard GC calibration curves following polymer derivatization. Both $^1$H NMR and GC/FID methods were used to quantify PHA standards at low concentrations of approximately 10 μg PHA/mL, which is consistent with reported PHA detection limit values for GC (Serafim et al. 2002).

$^1$H NMR correlation with GC and fluorescence measurements in pure cultures

C. necator and A. vinelandii grew to high cell densities when cultured in the presence of simple sugars, and exclusively accumulated PHB. The signals observed in $^1$H NMR spectra for C. necator and A. vinelandii (Figures 1(a) and 1(b) respectively) correspond closely to those obtained from standard PHB samples. Samples were periodically withdrawn and PHB content was measured using both $^1$H NMR and GC/FID methods to compare these techniques for varying levels of polymer accumulation.

A strong correlation was observed between the $^1$H NMR and GC/FID data for all replicate experiments with both strains, as shown in Figure 2. The slope of the linear regression line fitted through these data is 0.992 (forced through the origin) with an $R^2$ value of 0.991. Thus, a nearly 1:1 correspondence of measured PHA content by both methods with only slight data scatter was obtained, demonstrating the precision of the $^1$H NMR method as being similar to the GC-based technique. Overall, the maximum observed PHB content (as measured by $^1$H NMR) was 68.6% (w/w) and 73.3% (w/w) in C. necator and A. vinelandii, respectively, which is consistent with reported values in other studies (Holmes 1985; Brandl et al. 1990).

From Figure 2, $^1$H NMR consistently predicted slightly higher PHB content in C. necator samples, as evidenced by the regression slope of 1.01 ($R^2$ value of 0.998). In A. vinelandii (regression slope of 1.03 and $R^2$ of 0.934), PHB content was slightly under-predicted by $^1$H NMR. This mild discrepancy in $^1$H NMR-GC/FID correlation between different strains is possibly due to polymer
composition. *A. vinelandii* can produce high molecular weight PHB, ranging from 1 to 4 million Daltons (Chen & Page 1994). $^1$H NMR is non-destructive to the polymer, so high molecular weight polymer and increased viscosity of CDCl$_3$ could affect measured values. In this study, the CDCl$_3$ layer became highly viscous with larger concentrations of extracted PHA from *A. vinelandii*. When necessary, this issue was corrected for through using a lower amount of lyophilized cell mass.

Fluorescence staining was used as an independent, qualitative indication of intracellular PHA content. As reported previously, fluorescence output from whole-cell samples stained with Nile blue is proportional to PHA concentrations (Page & Tenove 1996). Figures 3(a) and 3(b) verify the correlation between $^1$H NMR-measured PHA content and relative fluorescence intensity for *C. necator* and *A. vinelandii* cultures, respectively. The Nile blue A sulfate staining method was demonstrated as sufficient for *C. necator* but insufficient for accurate quantitative assessment of PHA in *A. vinelandii*. Although useful as an independent qualitative measurement, it was found that fluorescence procedures are insufficient for PHA characterization, due to variability not only between analyses but also in fluorescence intensities between cell types (Figure 3).

**PHA analysis in environmental samples**

To verify wider applicability of the $^1$H NMR method, PHA analysis was performed for samples that (1) consist of a mixed microbial consortium, (2) contain different types of PHA, and (3) are known to contain high concentrations of background organic compounds that could compromise the integrity of analytical signals. A variety of volatile fatty acids were measured in the waste samples, including acetate (4,474.56 mg/L), propionate (3,196.63 mg/L), iso-butyrate (547.09 mg/L), butyrate (270.35 mg/L), iso-valerate (879.13 mg/L), and valerate (258.19 mg/L).

In these experiments, aqueous PO$_4^{3-}$ concentration in the agricultural waste fluctuated as expected – P uptake was observed when cultures were subjected to aerobic conditions, and P release occurred during anaerobic conditions. Rate of PO$_4^{3-}$ uptake increased with each anaerobic-to-aerobic cycle, indicating enrichment of a PAO population. The PHA content of late-stage anaerobic samples was analyzed in triplicate.

An $^1$H NMR analysis of extracted polymer from the waste samples is presented in Figure 4. The maximum PHA content was determined using $^1$H NMR as 0.299 ± 0.021 mg PHA/mL of effluent, and using GC/FID as 0.278 ± 0.038 mg PHA/mL of effluent. The PHA copolymer structure was determined by $^1$H NMR using the HV- and HB-methyl peaks at 0.90 ppm and 1.28 ppm and the HV- and HB-methine peaks at 5.16 and 5.26 ppm. Approximately 12.98% ± 0.25% of the PHA copolymer was represented by PHV. Copolymer formation was expected due to significant presence of volatile fatty acids.

**Advantages of $^1$H NMR method for PHA quantification**

The developed $^1$H NMR procedure for PHA characterization is advantageous over previous $^1$H NMR methods due
to substantially reduced time and material requirements, improved accuracy through more direct extraction and analysis, and verified application to full-strength waste samples. Previous methods such as Jan et al. (1996) and Doi et al. (1986) used additional extraction steps to first isolate the PHA before $^1$H NMR was carried out. For example Doi et al. used a hot chloroform extraction method with a Soxhlet apparatus, which is an extra step in the analysis process. In comparison to GC/FID, $^1$H NMR allows for non-destructive measurement of an intrinsic property instead of a derived parameter, fewer issues with possible sample loss and incomplete derivatization, and reduced time and material requirements.

For PHA analysis, $^1$H NMR may be considered more reliable than GC, as different structural components have unique chemical shifts that can be evaluated independently. In contrast, GC analyses produce only one assessable peak per monomer type. A lack of separation between HB and HV peaks or overlap with other compounds in a sample can hinder accurate analysis of PHA content and the ratio of monomer units (Serafim et al. 2002). Therefore, $^1$H NMR is more robust against signal overlap from sample contaminants, which is particularly relevant for complex environmental samples.

### CONCLUSIONS

This study has outlined a consistent, simple, and efficient procedure for direct PHA quantification by $^1$H NMR using single-step extraction through direct use of CDCl$_3$ for both extraction and analysis. The results verify the accuracy of the $^1$H NMR method as equivalent to GC-based techniques for routine assessment of PHA content and composition in a range of diverse samples with varied compositions. The $^1$H NMR and GC measurements were highly similar for every PHA type and source investigated in this study. Most significantly, the $^1$H NMR-based method was used to accurately analyze PHA content in full-strength agricultural waste samples, demonstrating the benefit of its use despite high levels of background impurities.

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