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Determination of Poly-β-Hydroxybutyrate and Poly-β-Hydroxyvalerate in Activated Sludge by Gas-Liquid Chromatography

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A convenient gas-liquid chromatography procedure to quantify poly-β-hydroxybutyrate and poly-β-hydroxyvalerate in activated sludge was developed by combining lyophilization of the samples, purification of the chloroform phase by water reextraction, and the use of capillary columns. With a flame ionization detector the sensitivity was estimated at 10^{-5} g/liter.

Poly-β-hydroxybutyrate (PHB) is a common bacterial lipid polymer involved in energy storage (5). It has been found in activated sludge samples from conventional wastewater treatment plants (13, 16) and in samples from biological phosphate removal plants (4, 7, 9, 10). It is considered to play an important role in phosphate removal in those plants (2, 11). However, other poly-β-hydroxyalkanoates (PHAs), such as poly-β-hydroxyvalerate (PHV), are also accumulated by the sludge and might play a similar role (3). In order to test this idea we have developed a simple routine assay for separating and accurately quantifying PHB and PHV by gas chromatography (GC), since the gravimetric (5), turbidimetric (19), spectrophotometric (8, 14, 17), and density gradient separation (12) methods did not distinguish the various forms of PHAs. Two published sensitive GC methods required too many purification steps and were unsuitable for the analysis of a large number of samples (6, 13), while one simpler method (1) was inefficient in the presence of water, caused premature column degradation due to acids and particulate debris in the extract, and could not adequately resolve PHV from neighboring peaks. The improved method presented in this paper eliminated these problems by including sludge lyophilization, purification of the chloroform extract by reextraction with water, and capillary gas-liquid chromatography.

Activated sludge samples (10 to 15 ml) for the development of the technique were taken from a pilot plant treating domestic wastewater at the University of British Columbia (3), but the technique has also been applied successfully to samples from the full-scale treatment plant at Kelowna, British Columbia, Canada. After centrifugation the sludge pellets were frozen and lyophilized. A weighed amount (about 20 mg) of lyophilized sludge was combined with 2 ml of acidified methanol (3% H_2SO_4), containing benzoic acid as the internal standard, and 2 ml of chloroform. DL-3-Hydroxybutyric acid (HB) sodium salt dissolved in acidified methanol was used as the standard. Samples and standards were heated for 3.5 h at 100°C in Pyrex test tubes (volume, 15 ml) with Teflon-lined caps. Further purification was achieved by transferring 1.9 ml of the denser chloroform phase to another Pyrex tube (volume, 10 ml) containing 0.5 ml of distilled water. This reextraction step greatly improved the reliability of the method by removing acids and particulate debris which caused premature degradation of the GC column. After 5 min of vigorous shaking and centrifugation (1,500 × *g* for 3 min), the chloroform phase containing the PHA methyl esters was transferred to a GC vial for split injection of a 1-μl sample into a Hewlett-Packard 5880A GC equipped with a programmable autosampler and a megabore J&W Scientific Co. capillary column (length, 15 m; inside diameter, 0.52 mm; coated with 1.0 μm of DB-Wax). At 200°C the linear velocity was 20 to 30 cm/s with helium as the carrier gas. The temperature of the injection port was set at 210°C, and that of the flame ionization detector port was set at 220°C. The following temperature profile was used: 1 min at 50°C, followed by a temperature increase rate of 8°C/min, 5 min at 160°C, a post-run of 4 min at 200°C, and an equilibration time of 3 min at 50°C.

A typical GC chromatogram is presented in Fig. 1. The identity of the peaks was established by GC-mass spectrometry on a Hewlett-Packard 5985B instrument. The same column and similar operating conditions as those used for PHA separation were used. The instrument was autotuned with perfluorotributylamine and operated at a 70 MeV electron impact ionization energy. A compound match was established by probability base peak search with the U.S. Environmental Protection Agency/National Institutes of Health library file (15). The use of a capillary instead of a packed column (1) removed solvent interference with the PHA peaks and improved the resolution of the various peaks. In particular, the chosen temperature program provided the essential separation of β-hydroxyvaleric acid from the interfering 4-oxovaleric acid which was also present in the samples.

Reproducibility with standards was estimated with six replicates each of 50, 100, and 200 μg of HB per ml. Reproducibility with samples was tested by extracting triplicates of 5, 15, 25, 35, 45, and 55 mg of one large lyophilized sludge sample. The coefficients of variation were calculated as 2.0 and 3.0%, respectively.

When samples were spiked with HB standards and compared with unspiked samples, the recovery was 99.4 ± 2.4% for lyophilized samples weighing 5, 25, and 45 mg (n = 3 for each). To test the effect of the weight of lyophilized sludge on the extraction efficiency, replicate subsamples ranging from 5 to 55 mg (n = 3 for 15, 35, and 55 mg and n = 6 for 5, 25, and 45 mg) were used. In this instance, however, the recovery decreased as larger amounts of sludge were ex-

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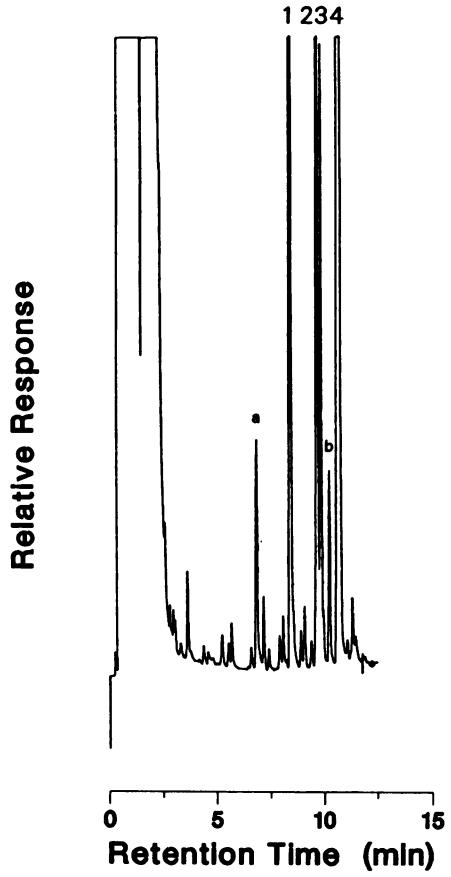

FIG. 1. Gas-liquid chromatogram of methyl ester derivatives extracted from an activated sludge sample. Components identified by GC-mass spectrometry were methyl esters of β-hydroxybutyric acid (peak 1), β-hydroxyvaleric acid (peak 2), 4-oxo-valeric acid (peak 3), and benzoic acid (peak 4) as the internal standard. Peaks a and b were not identified.

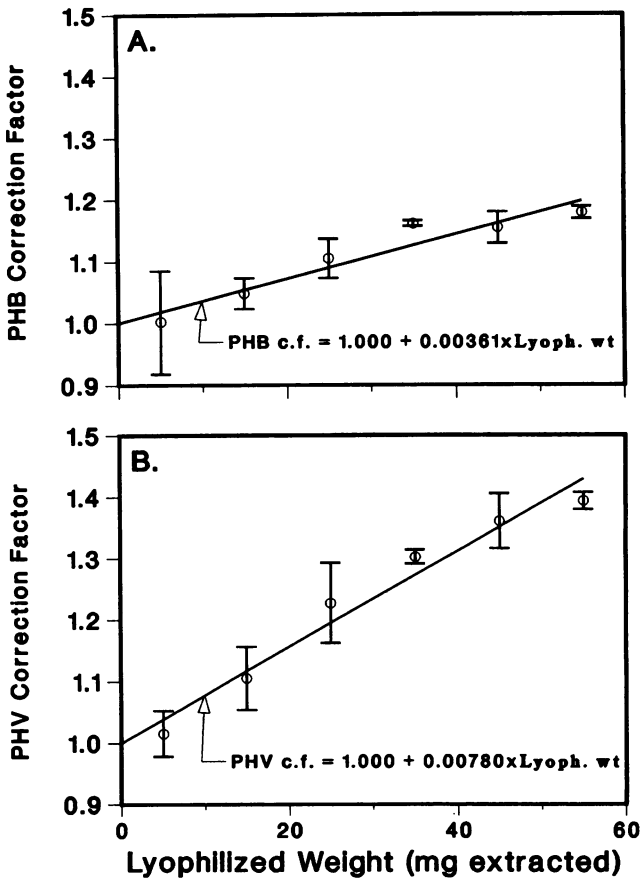

FIG. 2. Correction factors (c.f.'s) for PHB (A) and PHV (B) as a function of the weight of lyophilized sludge extracted (lyoph. wt). Averages and error bars for standard deviations are shown.

tracted. Based on these results, correction factors were calculated for PHB and PHV such that extrapolation to a weight of 0.00 mg gave a factor of 1.000 (Fig. 2): PHB correction factor = 1.000 + 0.00361 × lyophilized weight; PHV correction factor = 1.000 + 0.00780 × lyophilized weight.

The correlation coefficients (r) obtained were 0.95 and 0.98 for PHB and PHV, respectively. For example, the mass of PHAs measured in a 10-mg sample of lyophilized sludge should be increased by 3.6% for PHB and 7.8% for PHV. The fact that the extraction efficiency decreased as the weight of sludge extracted increased might have been due to competing interactions with extraneous chemicals present in the samples. The difference in slopes between the two compounds, however, suggests a lower extraction efficiency of the solvent system for PHV than for PHB, as if PHV had a higher affinity for particulate materials found in activated sludge.

With HB standards, the detection limit was estimated at 10^{-5} g of HB per liter. This limit is comparable to that of the method of Braunegg et al. (1). However, when activated sludge was extracted without lyophilization, the sensitivity of the test was reduced by 1 order of magnitude. Water has been reported to reduce the extraction efficiency of PHAs from aqueous samples (13). The weight of lyophilized sludge correlated directly with the dry weight of suspended solids in a ratio of 1.000 ± 0.019 mg of lyophilized weight per mg of suspended solids (n = 9).

No standards were available to directly calibrate the HV response from the GC. Thus, it was assumed that the relative response factors for the methyl esters of hydroxybutyric and hydroxyvaleric acids would be similar to the relative response factors for the methyl esters of butyric and valeric acids (18). Since valerate contains more carbon atoms per mass of compound than butyrate does, the response factor on the GC flame ionization detector was expected to be higher for the same mass. Valerate and butyrate standards were extracted such that triplicate samples of 46, 92, 185, 369, and 738 ng of valeric acid and 26, 52, 104, 209, and 418 ng of butyric acid were injected in the GC. The chromatograph, flame ionization detector, and operating conditions were the same as those used for PHA, but a lower polarity J&W Scientific Co. capillary column (length, 30 m; inside diameter, 0.32 mm; coated with 1 μm of DB-5) was used. The observed ratio of response factors for valeric acid to butyric acid was calculated as 1.211 ± 0.029 (n = 14). Thus, the relative amount of PHV was estimated from a calibration curve of HB standards and divided by 1.21 to give the corresponding amount of PHV.

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