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Note

Gas chromatographic determination of poly-β-hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis

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Many bacterial strains synthesize poly-β-hydroxybutyric acid (PHB) as carbon reserve material. This polyester constitutes up to 80% of the dry cell mass and can be isolated by extraction. There is increasing interest in PHB as a biopolymer. The optimization of the fermentative production for maximum yield requires a simple and reliable method for the determination of the PHB content of bacteria.

The first method used for the determination of PHB was described by Lemoigne[1]. It is based on the extraction of the dry bacterial mass with chloroform, subsequent precipitation and gravimetric determination. Novikova[2] determined PHB also gravimetrically after the destruction of the other cell material with sodium hypochlorite. Williamson and Wilkinson[3] used a combination of hypochlorite treatment and nephelometry. The spectrophotometric determination according to Law and Slepecky[4] has found wide application. After hypochlorite disintegration the remaining PHB is depolymerized with concentrated sulphuric acid to crotonic acid, the concentration of which is measured by UV absorption at 235 nm. Juettner *et al.*[5] determined PHB after extraction with chloroform directly in the resulting solution by infrared spectroscopy. Interfering lipids are determined separately after chromatographic separation from the PHB. Braunegg *et al.*[6] developed a gas chromatographic (GC) method on the basis of methanolysis of the cells in the presence of sulphuric acid and chloroform.

In this paper an improved method is presented, based on the hydrolysis and transesterification of PHB with propanol and hydrochloric acid.

EXPERIMENTAL

Instrumentation and materials

The gas chromatographs used were a GCHF 18.3-4 (VEB Chromatron, Berlin) with a packed column and an HP 5880 (Hewlett-Packard, Avondale, PA, U.S.A.) with a capillary column. The columns were of steel (2 m × 4 mm), packed with 10% Silar 10 C or 10% Reoplex 400 on Porolith (80–100 mesh), or fused-silica capillary (25 m × 0.2 mm), coated with SE-54.

Standard PHB was made in-house using the bacterial strain Methylobacterium spec. MB 126 (collection of our institute) cultivated on methanol. The product isolat-

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ed initially was purified by a three-fold reprecipitation applying different solvents for dissolution, precipitation and washing. The identity and purity of the product was verified by comprehensive analytical investigations [mass spectrometry (MS), NMR, GC].

1,2-Dichloroethane (DCE) and trichloroethylene (TCE) were used as solvents and extractives (VEB Laborchemie, Apolda, G.D.R.). *n*-Propanol (VEB Laborchemie) was applied for esterification, and benzoic acid (Merck, Darmstadt, F.R.G.) as an internal standard. All chemicals were of analytical grade. Benzoic acid ethyl ester was prepared in the laboratory and was chromatographically pure.

Procedure

About 40 mg of dry bacterial mass were weighed in a tightly sealable vial (volume 10 ml). A 2-ml volume of DCE, 2 ml propanol containing hydrochloric acid (1 volume concentrated hydrochloric acid + 4 volume propanol) and 200 μl of internal standard solution (2.0 g benzoic acid in 50 ml propanol) were added and the whole kept for 2 h in an incubator at 100°C. The mixture was shaken at the beginning and also during the incubation from time to time. After cooling to room temperature, 4 ml of water were added, and the mixture shaken for 20–30 s. The heavier phase (DCE–propanol) was injected directly into the gas chromatograph. The quantitative evaluation was effected by means of the quotient, Q, of the peak areas of hydroxybutyric acid and benzoic acid.

Gas chromatographic conditions

Packed column (Reoplex 400): 170°C isothermal; carrier gas, nitrogen 90 ml/min; flame ionization detection (FID); injection volume 0.2 μl. Capillary: 100°C isothermal; carrier gas, nitrogen, 2 ml/min; splitting ratio 1:100; FID; injection volume <0.1 μl.

Samples which are rich in PHB cause no complications due to fatty acid esters in isothermal GC. Only a daily heating of the columns is necessary. If this method is applied to the determination of PHB contents $<5\%$, temperature-programmed operation is required because of the necessarily higher content of lipids. The hydrolysis and esterification can also be effected by boiling under reflux. In this case the reaction time has to be prolonged to 3.5 h, and a larger scale is necessary.

Calibration

Approximately 200 mg of PHB were dissolved by heating in DCE (or TCE) using a calibrated 10-ml flask. After cooling to room temperature, the mixture was made up to 10 ml. Volumes of 200, 400, 600 and 800 μl of this solution were treated as outlined previously. The relationship between the peak-area quotient and the quantity of PHB was observed to be linear. As high-molecular-weight PHB is not soluble in the given concentration, the volume may need to be increased proportionately.

RESULTS AND DISCUSSION

Sulphuric acidic methanolysis has two disadvantages: (1) besides the depolymerization and transesterification of PHB, sulphuric acid effects a further decom-

position of the hydroxybutyric acid, which results in a narrow range for an optimum reaction time; (2) the methyl ester formed is water-soluble and does not go completely into the chloroform phase. This effect diminishes the sensitivity of the method.

We found that the alcoholysis of PHB in hydrochloric acidic medium proceeds with the same efficiency as with sulphuric acid, but without the decomposition of hydroxybutyric acid. The second disadvantage is eliminated by the use of propanol or butanol. Instead of chloroform, higher-boiling solvents such as 1,2-dichloroethane (b.p. 83.5°C) or trichloroethylene (b.p. 86.9°C) are used. In contrast to Braunegg's method, it is thus possible to work also in an open system. The higher temperatures ensure a sufficient reaction rate.

In Fig. 1 the reaction course is shown for 80 and 100°C using pure PHB. To exclude a falsification of the values at short reaction times owing to an incomplete esterification of the benzoic acid (internal standard), benzoic acid ethyl ester was added after the reaction. The figure shows that at 80°C a reaction time of 3.5 h is necessary, at 100°C, 2 h are sufficient. Analogous curves result for PHB-containing biomasses. Even after 6 h no decrease in intensity by degradation of the monomeric hydroxybutyric acid ester was observed. Also, secondary reaction products could not be detected in the capillary gas chromatogram.

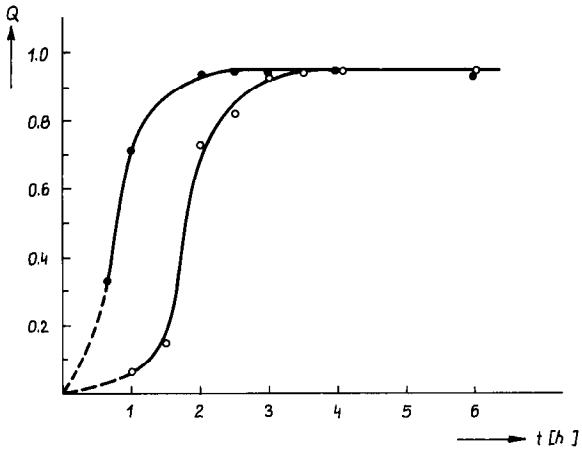

Fig. 1. Intensity of the hydroxybutyric acid propyl ester peak (HBA) as a function of the reaction time at 80 (○) and 100°C (●). Q = area HBA/area internal standard.

The calibration functions for the given columns are identical. There is a good reproducibility over a working period of several months. Three measurement series demonstrate the error of the method, using pure PHB and cell material (Table I). Each solution to be analyzed was injected three times, and the quantity of PHB was calculated from the averaged Q values.

A typical chromatogram is shown in Fig. 2. The method is characterized by an high sensitivity. A 20-μg amount of PHB can be determined reproducibly even on the packed column by raising the injection volume to 1 μl and decreasing the reaction volume by a quarter. This corresponds to an hydroxybutyric acid quantity of 20 ng per injection.

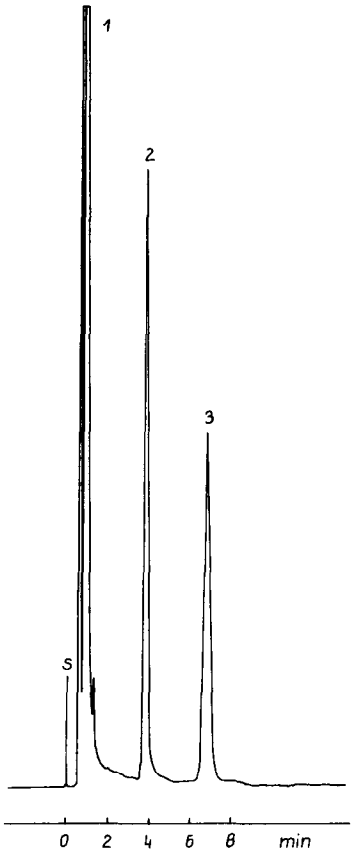

Fig. 2. Typical chromatogram of the reaction solution (organic phase) on the packed column (10% Reoplex 400 on Porolith). For conditions see text, S = Starting impulse. Peaks: 1 = solvents (DCE and propanol); 2 = hydroxybutyric acid propyl ester; 3 = benzoic acid propyl ester.

The PHB content of several bacterial samples was determined both by the method outlined and gravimetrically. The results are given in Table II. There is a satisfactory agreement between the two methods considering the larger error of the gravimetric method. The main error of the latter results from the isolation of the

TABLE I

REPRODUCIBILITY OF THE METHOD DETERMINED BY TEN PARALLEL ANALYSES

	Pure PHB in solution (theoretical value: 12.20 mg)	*Bacterium samples*	
		Open system	*Sealed vessel*
Average	12.14 mg	50.0%	49.8%
Standard deviation	0.26	1.95	1.53
Coefficient of variation (%)	2.2	3.9	3.1

TABLE II

RESULTS OF ANALYSES FOR DIFFERENT BACTERIAL BIOMASSES

Sample	*Content (%, w/w)*	
	GC	*Gravimetry*
A	25.3	23.6
B	20.2	19.1
C	42.7	40.8
D	48.4	50.4
E	46.2	48.3
F	44.4	41.6
G	61.4	59.6
H	50.6	49.2

PHB by filtration or centrifugation. The GC method stands out by its simplicity[6]. In the case of a closed system, one works with minimum quantities and the few manipulations are made in one and the same vessel. This reduces possible errors. The method is well suited for routine analyses and allows an high sample frequency.

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