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GAS-CHROMATOGRAPHIC ANALYSIS OF POLY(3-HYDROXYALKANOATES) IN BACTERIA

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SUMMARY

The accuracy and reproducibility of the gas-chromatographic method for the analysis of PHB and PHA in whole cells of *Alcaligenes eutrophus* H16 and *Pseudomonas putida* KT2442 were determined. It was found that for analysis of PHA the methanolysis time in the assay had to be increased to 4 h. Accuracy of the PHB and PHA assay were 0.018 mg and 0.304 mg respectively, based on estimation of the measurement error.

INTRODUCTION

Microbial production of poly(3-hydroxyalkanoates) (PHA) has gained considerable interest because of the potential applications of these polyesters as biodegradable plastics. A co-polyester of 3-hydroxybutyrate and 3-hydroxyvalerate is traded by Zeneca (formerly ICI) in England and the commercial production and application of many other types of PHAs are currently being examined. The development and optimization of a process for production of these polymers necessitates a PHA detection method which is both reliable and accurate. A PHA assay that meets these requirements was originally developed by Braunegg et al (1978). In this method PHB containing cells are hydrolyzed in an acidic methanol (3% v/v H_2SO_4)/chloroform mixture (ratio 1:1) for 3.5 hours at 100 °C. The resulting methyl-3-hydroxybutyric acid can easily be analysed by gas-chromatography (Braunegg et al 1978). Lageveen et al have adapted this method for analysis of medium chain

length PHA synthesized by *Pseudomonas oleovorans*. In this method which is now widely used whole cells containing PHA are hydrolyzed in an acidic methanol (15% v/v H_2SO_4)/chloroform mixture (ratio 1:1) at 100°C for 140 min (Lageveen et al. 1988).

We have examined in more detail the reproducibility and accuracy of this assay for the determination PHB as well as medium chain length PHAs in whole cells.

MATERIAL AND METHODS

Bacterial strains *Alcaligenes eutrophus* H16 and *Pseudomonas putida* KT2442 were used throughout this study.

Media and growth conditions The cells were cultivated in a 15 l batch fermenter (Applikon, Schiedam the Netherlands) with temperature and pH control. Temperature was kept at 30°C and the pH was maintained at 7.0 by addition of 2 M NaOH. Stirrer speed was 1250 rpm and the culture was aerated at 120 l/h. The growth medium was 0.5*E2 medium which is a mineral salts medium. (Huijberts et al 1992). Oleic acid (Merck, Darmstadt F.R.G.) was used as carbon and energy source. The fermenter was inoculated with 50 ml of an exponentially growing culture and after 72 h growth the cells were harvested, washed and lyophilized.

Gas-chromatographic analysis of PHAs Analysis of the methyl-3-hydroxy acids was performed on a Carlo Erba GC6000 gas-chromatograph (Carlo Erba, Milan Italy) equipped with a Cp-Sil 5 CB column (25 m by 0.32 mm) (Chrompack, Middelburg the Netherlands). 1 µl samples were injected by split injection (split ratio 1:40). Temperature programming for PHB analysis was 2 min 70 °C, temperature ramp 10 °C/min, 6 min 280°C. Under these conditions the retention time for methyl-3-hydroxybutyric acid is 2.2 min, methyl-3-hydroxyvaleric acid is 3.3 min. For analysis of medium chain length PHAs temperature programming was 2 min 80°C, temperature ramp 10°C/min, 6 min 280°C. The retention times for the methyl esters of 3-hydroxy fatty acids present in PHA derived from oleic acid are 3-hydroxyhexanoate, 3.7 min; 3-hydroxyoctanoate, 6.7 min; 3-hydroxydecanoate, 9.7 min; 3-hydroxydodecanoate, 12.5 min; 3-hydroxy-*cis*-5-tetradecenoate, 14.6 min. Hydrogen was used as the carrier gas, injector temperature was 260 °C and detector temperature was 300°C. methyl-3-hydroxy fatty acid concentrations were calculated from peak areas as determined by an integrator (Carlo Erba, Milan Italy).

PHA determination in whole cells 20 mg samples of freeze-dried cells were added to a mixture of 2 ml 15% sulphuric acid in methanol and 2 ml chloroform, containing methylbenzoate as internal standard, in a closed screw cap tube (Pierce, Rockford Illinois) with a magnetic stirrer bar. The tubes were incubated at 100 °C for various time intervals. After the reaction the tubes were cooled on ice for 5 min, 1.0 ml distilled water was added and the tubes were vortexed for 1 min. After phase separation by centrifugation for 5 min at 3500 rpm in a bench top centrifuge the organic phase was collected, dried over Na_2SO_4 and analyzed by gas-chromatography.

Isolation of PHA PHA and PHB were isolated by Soxhlet extraction of lyophilized cells with hot chloroform and precipitated with methanol (Huijberts et al 1992). By GC and NMR analysis it was shown that the resulting PHA is free of lipid contaminants.

Preparation of methyl-3-hydroxy fatty acids 1 g of PHA isolated from *P. putida* KT2442 cultivated on oleic acid was dissolved in 100 ml chloroform. 100 ml methanol and 35 ml 97% sulphuric acid were added and the mixture was refluxed for 48 h. 50 ml distilled water was added and phases were separated in a separation funnel. The organic phase was collected and the chloroform was removed by film evaporation. The resulting methyl-3-hydroxy fatty acids were analyzed by GC to check for purity and by ^{13}C-NMR to check for complete methanolysis of the PHA.

Statistical methods Statisical analysis was carried out on samples with a range of weights (5 to 40 lyophilized *P. putida* KT2442 cells and 5 to 25 mg *A. eutrophus* H16 cells) and a methanolysis time of 15 hours. Duplicate measurements were made for each sample. The linearity of the relationship between the detected amount of PHA and the amount of PHA or PHB present in the sample was investigated using simple linear regression with the detected PHA (PHB) as the dependent variable. Similarly, simple linear regression was used to investigate the amount of methyl-3-hydroxy fatty acids detected and the amount present in the assay.

The accuracy of the assay is defined as 2*standard error of measurement. For a given assay the measured PHA ± 2*standard error of measurement gives a 95% confidence interval for the 'true' PHA amount. The accuracy of the assays for PHA and PHB was estimated using an analysis of variance to determine within-sample variance.

RESULTS AND DISCUSSION

Methanolysis time To determine the influence of the methanolysis time on the detected amount of PHA we hydrolyzed and methanolized samples of lyophilized *A. eutrophus* H16 and *P. putida* KT2442 cells during increasing time intervals. The result of these experiments are shown in Fig 1 and 2. It can be concluded that there is an increased amount of methyl-3-hydroxy fatty acids detected with increasing methanolysis time with a maximum after 2 h in the case of *A. eutrophus* H16 and 4 h in the case of *P. putida* KT2442. Prolonged methanolysis apparently has no detrimental effects on the amount of methyl-3-hydroxy fatty acids: even after 24 h the same amount methyl-3-hydroxy fatty acids is detected. This does not correspond with the results presented by Lageveen *et al* (1988) who observed a decrease in the amount of methyl-3-hydroxy fatty acids during prolonged methanolysis. An explanation for this decrease could be the formation of crotonic

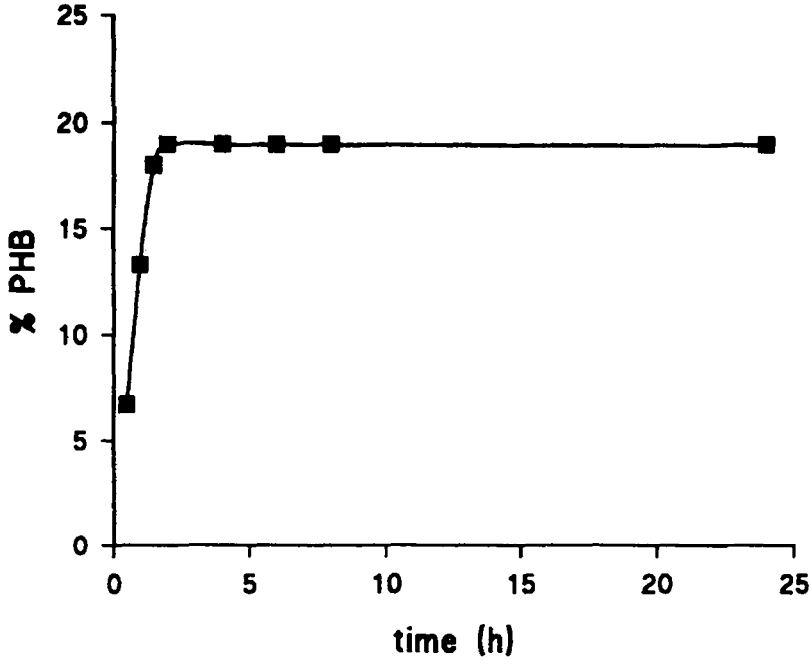

Fig. 1 Influence of methanolysis time on the amount of PHB detected in A. eutrophus H16 cells.

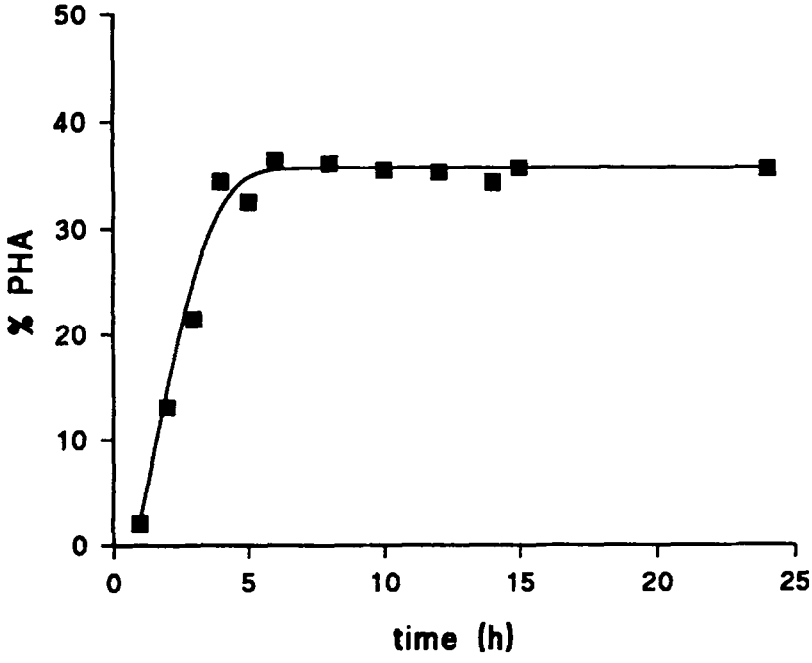

Fig. 2 Influence of methanolysis time on the amount of PHA detected in P.putida KT2442 cells.

acid methylester as a result of the acid catalyzed elimination reaction on the 3-hydroxyl group. ^{13}C-NMR analysis of the reaction products, however, shows that this reaction does not occur in our assay. (^{13}C-NMR spectrum not shown).

From these experiments it can be concluded that 140 min. methanolysis time is sufficient for complete conversion of PHB to methyl-3-hydroxybutyric acid. In the case of PHA as synthesized by *P. putida* KT2442 the 140 min. methanolysis time results in an incomplete reaction and consequently in underestimated PHA concentrations. The relative composition of the PHA is not influenced by the methanolysis time (data not shown).

Recovery To determine whether the extraction step in the assay results in a complete recovery of PHB or PHA we assayed different amounts of purified PHB, purified PHA and purified methyl-3-hydroxy fatty acids derived from PHA, respectively. Purified methyl-3-hydroxy fatty acids were used instead of PHA for reasons of convenience, because PHA isolated from oleic acid grown cells is difficult to handle due to its sticky nature.

It was found that in the case of PHB approximately 30% of the amount of PHB present in the assay was detected. This may be ascribed to partitioning of the methyl-3-hydroxybutyric acid in the water/organic 2-phase system. In the case of methyl-3-hydroxy fatty acids or PHA approximately 56% was detected. In order to control whether the recovery of PHB or methyl-3-hydroxy fatty acids was influenced by the presence of biomass, we determined the percentage recovery with increasing amounts of biomass in the assay. It was found that the percentage recovery was not affected by the presence of biomass.

Linearity In order to check the linearity of the assay we have analyzed samples PHB or methyl-3-hydroxy fatty acids for 15 hours. Regression analysis revealed linear relations between the amount of PHB or methyl-3-hydroxy fatty acids present in the sample and the detected amount of PHB or methyl-3-hydroxy fatty acids, with R^2 respectively of 99.8% (n=8) and 97.0% (n=12). Therefore it is possible to use purified PHB and methyl-3-hydroxy fatty acids to corrrect for the incomplete recovery. To determine the linearity of the assay with whole cells, we assayed samples of various weight. Regression analysis shows a linear relation between the amount of methyl-3-hydroxy fatty acids containing biomass and the detected amount of PHB or PHA (R^2=99.9%, n=8 and 97.9%, n=12). This shows that

bacterial samples of various weight and PHB or PHA content can be analyzed and also that for estimation of the "true" amount of PHA in the cells purified PHB or methyl-3-hydroxy fatty acids can be used to correct for incomplete recovery.

Accuracy It was found that PHB concentrations can be determined with an accuracy of 0.018 mg. PHA concentration determination has an accuracy of 0.304 mg. The decrease in accuracy is most likely a result of the increased number of different monomers present in the PHA.

CONCLUSIONS

The assay as modified by Lageveen et al (1988) when applied in the analysis of PHB in whole cells is both rapid and reliable. In our hands, PHB concentrations can be determined with an accuracy of 0.018 mg. It is also shown that the methanolysis time can be reduced to 2 hours. For determination of medium and long chain PHAs, however, the methanolysis time in the assay has to be increased to at least 4 hours in order to achieve complete conversion of the polyester to methyl-3-hydroxy fatty acids. According to our method, PHA concentrations can be determined with an accuracy of 0.304 mg. For estimation of PHB or PHA concentrations in bacterial cells purified PHB or methyl-3-hydroxy fatty acids can be used to correct for incomplete recovery.

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