

Thin layer chromatographic detection of poly- β -hydroxybutyrate (PHB) and poly- β -hydroxyvalerate (PHV) in cyanobacteria

Bhabatarini Panda, Laxuman Sharma, Akhilesh Kumar Singh and Nirupama Mallick*

Agricultural and Food Engineering Department, Indian Institute of Technology, Kharagpur 721 302, India

Received 22 January 2007; revised 18 June 2007; accepted 25 September 2007

A simple method for the detection of poly- β -hydroxybutyrate (PHB) and poly- β -hydroxyvalerate (PHV) by thin layer chromatography was developed. Using this method, PHB and PHV were detected in four cyanobacterial species, viz. *Nostoc muscorum*, *Spirulina platensis*, *Calothrix* sp. and *Synechocystis* sp. PCC 6803. Presence of hydroxybutyrate (HB) units and hydroxyvalerate (HV) units was confirmed by UV-spectrophotometric and gas chromatographic analyses.

Keywords: Cyanobacteria, gas chromatography, β -hydroxybutyrate, β -hydroxyvalerate, propanolysis, thin layer chromatography

Introduction

Polyhydroxyalkanoates (PHAs) are the most appropriate materials for alternative plastics owing to their complete biodegradable nature. Poly (β -hydroxybutyrate-co- β -hydroxyvalerate) [P(HB-co-PV)], a co-polymer developed by ZENECA Bio Products, can be used to make various products, including films, coated paper and board, compost bags, disposable food service-ware, and molded products such as bottles and razors. PHAs are found intracellularly as storage compounds in many prokaryotes¹ and their accumulation is triggered under the condition of nutrient limitations, particularly nitrogen and phosphorus in the presence of excessive carbon sources^{2,3}. In an attempt of cost reduction and sustainable production of PHAs, cyanobacteria are thought to be of potential candidates because of their prokaryotic nature, photoautotrophic growth and need of some simple inorganic nutrients for their growth and multiplication. Presence of PHAs has been reported in certain cyanobacterial species⁴⁻¹¹. As PHAs are inclusion bodies, appropriate analytical methods are required for detection and quantification of different types of PHAs.

PHAs are detected by a number of methods, e.g. microscopic observation¹², UV-spectrophotometry¹³ and high-pressure liquid chromatography¹⁴. Of these,

the latter two require higher purification steps and are able to detect poly- β -hydroxybutyrate (PHB), the common component of PHAs. Through microscopy, there is a chance of mistaking other inclusion bodies as PHAs. However, these three methods failed to detect poly- β -hydroxyvalerate (PHV), another vital component of PHAs. HB and HV units can be detected by infrared spectroscopy¹⁵, gas chromatography^{16,17}, ion-exchange chromatography¹⁸ and enzymatic determination¹⁸. Though these methods are precise and reliable but they are not simple and economic. In this context, thin layer chromatography (TLC) is a simple tool, which could be applied for rapid screening. Detection of PHB through TLC is based on sulphuric acid methanolysis of microbial samples¹⁹. Sulphuric acid methanolysis is prone to depolymerization and trans-esterification, and sulphuric acid also accelerates the decomposition of PHB. Further, methylester is water-soluble, so complete phase separation to the organic phase cannot be ascertained. Therefore, a new approach for the detection of HB and HV units in cyanobacterial species by TLC is presented.

Materials and Methods

Organisms and Growth Conditions

Cyanobacterial species, viz. *Nostoc muscorum*, *Spirulina platensis*, *Calothrix* sp., and *Synechocystis* sp. PCC 6803, maintained in the laboratory were taken for this study. Axenic cultures of *N. muscorum* and *Calothrix* sp. were grown in 150 mL Erlenmeyer

*Author for correspondence:

Tel: 91-3222-283166; Fax: 91-3222-282244

E-mail: nm@iitkgp.ac.in

flasks containing 50 mL of nitrogen free BG-11 medium²⁰. *S. platensis* and *Synechocystis* sp. PCC 6803 were, however, grown in complete CFTRI²¹ and BG-11 media with NaNO₃, respectively. pH of the culture media was maintained at 8.0 for *N. muscorum*, *Calothrix* sp. and *Synechocystis* sp., and 9.5 for *S. platensis* with Tris-buffer (4.0 mM) as these pHs were reported suitable for PHB accumulation^{6,7,22}. The cultures were incubated in a temperature-controlled incubator at 25±1°C under 14 h light (75 µmol photon m⁻²s⁻¹ PAR):10 h dark cycles. Cultures at stationary phase were taken for the detection of PHAs.

Extraction of PHAs

A known amount of cultures after centrifugation was suspended in methanol at 4°C (overnight) for removal of pigments. The pellet obtained by centrifugation was dried at 60°C. The PHAs were extracted in hot chloroform followed by precipitation with cold diethyl ether. The precipitate was centrifuged at 11,000 g for 20 min. The pellet was washed with acetone and then dissolved in hot chloroform following Yellore and Desia²³.

Propanolysis of PHAs

The samples containing the polymer in chloroform as well as the standard PHB and P(HB-co-HV) (Aldrich, USA) were taken in crimp top vials and evaporated. The polymer was esterified with propanol containing hydrochloric acid (4:1). Trichloroethylene was used as solvent and the reaction was allowed to continue in tightly sealed crimp top vials at 100°C for 2, 3 and 4 h. After cooling to room temperature, the esterified samples were supplemented with 1 mL of water for phase separation. The separated ester (organic) phases of the sample as well as the standard (40 µL) were spotted onto TLC plate.

TLC

TLC was carried out on glass plates (20 × 10 cm²) coated with silica (6 g/30 mL chloroform) prepared using a spreader. 40 µL of the propanolysed organic phase of the samples as well as the standard were spotted on the TLC plate and allowed to dry at room temperature. The TLC run was carried out in different proportions (1:1, 2:1, 4:1, 9:1, 19:1, 29:1, 39:1, 49:1, 99:1) of benzene and ethyl acetate, and the plate was left to dry after the run. The spots were visualized with iodine vapours and the R_f values were calculated.

Confirmation of Presence of PHB and PHV by Gas Chromatography Study and UV-Spectrophotometry

Scraps of the observed spots on TLC plates were eluted in hot trichloroethylene and centrifuged at 11,000 g for 20 min. The supernatant, free of silica fraction, was collected, filtered through PTFE membrane (11806-25-N, 0.45 µm, Sartorius, Germany) and was analyzed using GC with FID detector (Perkin-Elmer's Elite-1 Dimethyl-polysiloxane capillary column: 30 m × 0.25 mm × 0.25 µm, injector temp: 280°C, detector temp: 300°C, oven temp: 280°C, gas flow: N₂ 1 mL, H₂ 40 mL and air 400 mL min⁻¹).

The spectrophotometric assay was performed as per Law and Slepecky¹³. The sample containing the polymer was transferred to a clean test tube. Trichloroethylene was evaporated followed by addition of 10 mL of concentrated H₂SO₄ and then the solution was heated in a boiling water bath for 10 min. After cooling and thorough mixing, the absorption spectra of the solution was measured against H₂SO₄ blank in Specord S 100 Spectrophotometer (Analytic Jena, Germany) and was compared with the H₂SO₄ digested standard PHB (Aldrich, USA). Final matching of the spectra was done with crotonic acid (Sigma Chemical Co, USA).

Results and Discussion

Maximum accumulation of PHAs at the stationary phase of cyanobacteria^{6,7,22} made the basis for selecting the stationary phase cultures to detect PHB and PHV. Initial treatment of the samples with 90% methanol for overnight at 4°C, not only removed the major pigments completely but also the non-PHA lipid compounds to certain extent²⁴. The chloroform extraction followed by precipitation with cold diethyl ether and washing with acetone removed all the non-PHA materials and assured purified PHAs²³. Amongst various combinations of solvents, best resolution of propylesters was achieved in benzene:ethyl acetate (49:1) on TLC plates. The propylesters of PHAs extracted from all the four cyanobacterial species grown under photoautotrophic condition depicted one distinct spot at R_f 0.44, resembling the R_f of standard PHB after propanolysis for 3 or 4 h (Figs 1A & B). On the other hand, lowering the propanolysis time to 2 h resulted into two distinct spots, at R_f 0.44 and 0.79 (data not shown). Methyl esters of homopolymer of poly-β-hydroxybutyric acid is reported to exhibit two spots¹⁹. This could also be true for the propylesters.

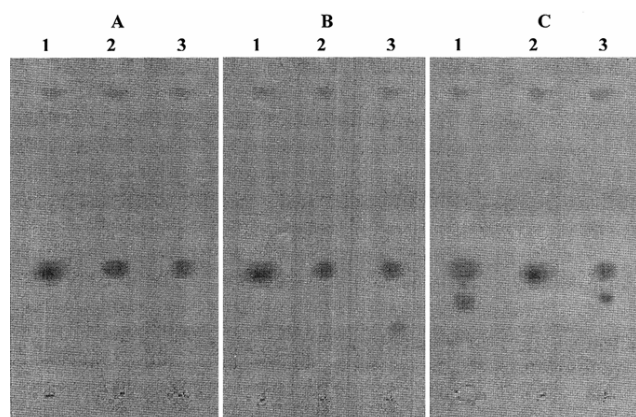


Fig. 1—Thin layer chromatograms of propylesters of standards and cyanobacterial samples with benzene:ethyl acetate solvent system at a ratio of 49:1. A: 1- Standard PHB, 2- *Synechocystis* sp. PCC 6803, and 3- *Spirulina platensis* grown under photoautotrophic condition. B: 1- Standard PHB, 2- *Nostoc muscorum* and 3- *Calothrix* sp. grown under photoautotrophic condition. C: 1- Standard P(HB-co-HV), 2- Standard PHB, 3- *Synechocystis* sp. PCC 6803 grown in presence of propionate.

Thus, the spot at R_f 0.79, as reported by Rawte and Mavinkurve¹⁹ could be of dimeric hydroxybutyrate, which is more non-polar and, therefore, separated first. Depiction of a single spot at R_f 0.44 in the samples propanalysed for 3 or 4 h could be ascribed to the fact that increasing propanalysis time caused conversion of the dimeric hydroxybutyrate to monomeric units.

Fig. 1C presents the TLC profile of the standard P(HB-co-HV), standard PHB and *Synechocystis* sp. PCC 6803 grown in presence of 20 mM propionate. Appearance of an additional spot at R_f 0.36 corresponding to the second spot of the standard P(HB-co-HV) indicates the possible synthesis of HV units by *Synechocystis* sp. PCC 6803 in the presence of propionate. Similar spots were detected in *N. muscorum*, *S. platensis* and *Calothrix* sp. when grown in the presence of propionate (data not shown), thus demonstrating the potential of cyanobacteria in synthesizing copolymer of P(HB-co-HV) when grown in propionate-supplemented media, as reported in case of *Anabaena variabilis* 10 C by Lama *et al.*⁵. This demonstrates that presence of HV units can also be detected using TLC.

Fig. 2A presents the spectrophotometric analysis of the scraps of the TLC of the spots of R_f 0.44 after sulphuric acid digestion. All the four cyanobacterial samples along with the standard PHB depicted similar spectra with a peak at 235 nm. Hydroxybutyric acid when digested in sulphuric acid is converted into

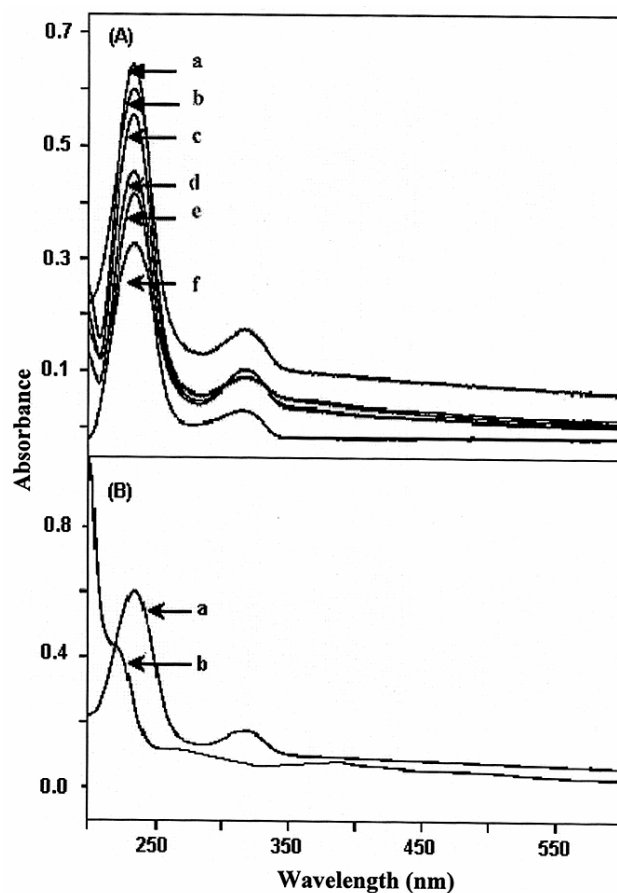


Fig. 2—A. Comparison of the spectrum of crotonic acid with the scraps of TLC of the spots at R_f 0.44 after sulphuric acid digestion: (a) Crotonic acid, (b) Standard PHB, (c) *Nostoc muscorum*, (d) *Spirulina platensis*, (e) *Synechocystis* sp. PCC 6803, and (f) *Calothrix* sp. B. (a) crotonic acid, and (b) scraps of TLC of the spot at R_f 0.36 after sulphuric acid digestion.

crotonic acid, which has absorption maxima at 235 nm¹³. Thus, the spots of the acid-digested samples as well as the standard at R_f 0.44 match with the spectrum of crotonic acid (Fig. 2A), confirming the presence of PHB in all the cyanobacterial species used in the experiments. Further, the acid-digested scraps of R_f 0.79 also depicted similar spectra with a peak at 235 nm (data not shown), supporting the view of Rawte and Mavinkurve¹⁹ that the spot of R_f 0.79 also belongs to hydroxybutyrate group. However, the acid-digested scrap of R_f 0.36 did not match with the spectrum of crotonic acid after acid digestion (Fig. 2B).

The presence of β -hydroxybutyrate, was confirmed by analyzing the chromatogram of the spot at R_f 0.44 of *N. muscorum* in a GC (Fig. 3C), which depicted a single peak at retention time of 4.8 min, matching

with the peak of the standard PHB (Fig. 3A). Similar results were also observed for the spot with R_f 0.44 of *S. platensis*, *Calothrix* sp., and *Synechocystis* sp. PCC 6803 (data not shown). The spot at R_f 0.36 depicted a peak at the retention time of 6.28 min (Fig. 3D), corresponding to the second peak of the standard P(HB-co-HV, Fig. 3B), thus confirming the synthesis of HV units in the *Synechocystis* sample grown in presence of propionate.

To check the efficiency of propanolysis over methanolysis, the standard PHB was subjected to alcoholysis by both the methods, i.e. with methanol + H_2SO_4 for methanolysis and propanol + HCl for propanolysis. Samples were spotted on the TLC plates as described earlier and subjected to run. Analysis of spots from both the runs demonstrated only 3-7% loss of sample in propanolysis (data not shown), whereas 24-32% loss was observed in the samples undergone

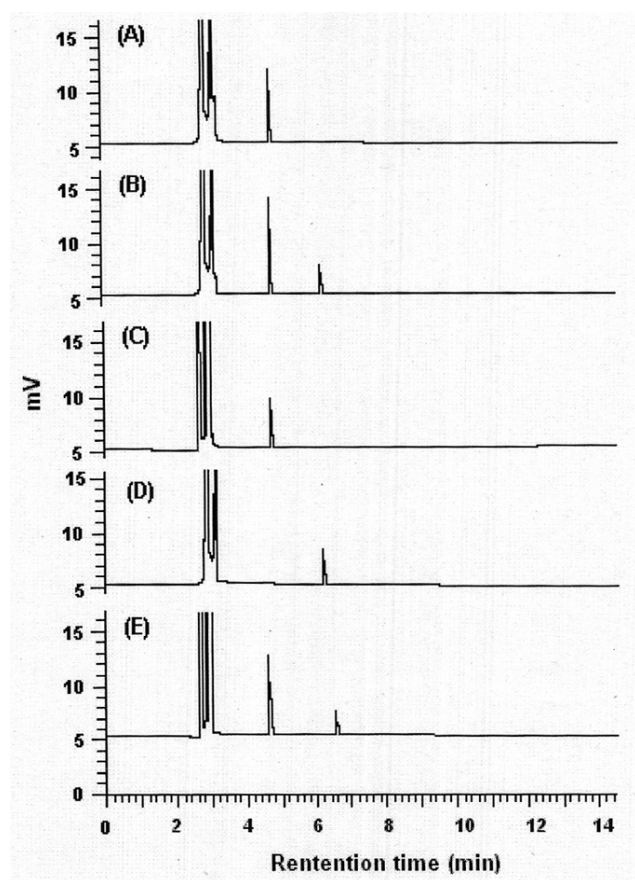


Fig. 3—Gas chromatograms of (A) propylester of standard 3-hydroxybutyric acid, (B) propylester of standard P(3HB-co-3HV), (C) eluted scrap at R_f 0.44 from TLC of *N. muscorum*, (D) eluted scrap at R_f 0.36 from TLC of *Synechocystis* sp. PCC 6803, and (E) propylester of the whole extract of the co-polymer produced by *Synechocystis* sp. PCC 6803.

methanolysis. This could be due to the fact that methanolysis is prone to depolymerization and transesterification. Sulphuric acid used in this method was also reported to degrade PHB¹⁷. Further, methylesters are water soluble, so complete phase separation might not be achieved. This proves the superiority of propanolysis over methanolysis.

Acknowledgement

Financial support from Council of Scientific and Industrial Research (CSIR), New Delhi, India is thankfully acknowledged.

References

- Liebergessell M, Sonomot K, Madkour M, Mayer F & Steinbuechel A, Purification and characterization of the polyhydroxyalkanoic acid synthase from *Chromatium vinosum* and localization of the enzyme at the surface of polyhydroxyalkanoic acid granules, *Eur J Biochem*, 226 (1994) 71-80.
- Anderson A J & Dawes E A, Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates, *Microbiol Rev*, 54 (1990) 450-472.
- Lee S Y, Bacterial polyhydroxyalkanoates, *Biotechnol Bioeng*, 49 (1996) 1-14.
- Carr N G, The occurrence of poly- β -hydroxybutyrate in the blue-green algae *Chlorogloea fritschii*, *Biochim Biophys Acta*, 120 (1966) 308-310.
- Lama L, Nicholaus B, Calandrelli V, Manca M C, Romano I *et al.*, Effect of growth condition on endo and exopolymer biosynthesis in *Anabaena cylindrica*, *Phytochemistry*, 42 (1996) 655-659.
- Panda B, Jain P, Sharma L & Mallick N, Optimization of cultural and nutritional conditions for accumulation of poly- β -hydroxybutyrate in *Synechocystis* sp. PCC 6803, *Bioresour Technol*, 97 (2006) 1296-1301.
- Sharma L & Mallick N, Accumulation of poly- β -hydroxybutyrate in *Nostoc muscorum*: Regulation by pH, light-dark cycle, N and P status and carbon sources, *Bioresour Technol*, 96 (2005) 1304-1310.
- Sharma L & Mallick N, Enhancement of poly- β -hydroxybutyrate accumulation in *Nostoc muscorum* under mixotrophy, chemoheterotrophy and limitations of gas-exchange, *Biotechnol Lett*, 27 (2005) 59-62.
- Stal L J, Poly(hydroxyalkanoates) in cyanobacteria: A review, *FEMS Microbiol Rev*, 103 (1992) 169-180.
- Sudesh K, Taguchi K & Doi Y, Effect of increased PHA synthase activity on polyhydroxyalkanoates biosynthesis in *Synechocystis* sp. PCC 6803, *Int J Biol Macromol*, 30 (2002) 97-104.
- Vincenzini M, Sili C, De Philippis R, Ena A & Materassi R, Occurrence of poly- β -hydroxybutyrate in *Spirulina* species, *J Bacteriol*, 172 (1990) 2791-2792.
- Jensen T E & Sicko L M, Fine structure of poly- β -hydroxybutyric acid granules in a blue-green alga, *Chlorogloea fritschii*, *J Bacteriol*, 106 (1971) 683-686.
- Law J H & Slepecky R A, Assay of poly- β -hydroxybutyric acid, *J Bacteriol*, 82 (1961) 33-36.

- 14 Karr D B, Waters J K & Emerich D W, Analysis of poly- β -hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high pressure liquid chromatography and UV detection, *Appl Environ Microbiol*, 46 (1983) 1339-1344.
- 15 Juttner R R, Lafferty R M & Knackmuss H J, A simple method for the determination of poly- β -hydroxybutyric acid in microbial biomass, *Eur J Appl Microbiol*, 1 (1975) 233-237.
- 16 Braunegg G, Somnleitner B & Lafferty R M, A rapid gas chromatographic method for the detection of poly- β -hydroxybutyric acid in microbial biomass, *Eur J Appl Microbiol Biotechnol*, 6 (1978) 29-37.
- 17 Riis V & Mai W, Gas chromatographic determination of poly- β -hydroxybutyric acid in microbial biomass, *J Chromatogra*, 445 (1988) 285-289.
- 18 Hesselmann R P X, Fleischmann T, Hany R & Zehnde A J B, Determination of polyhydroxyalkanoates in activated sludge by ion chromatographic and enzymatic methods, *J Microbiol Methods*, 35 (1999) 111-119.
- 19 Rawte T & Mavinkurve S, Characterization of polyhydroxyalkanoates-biodegradable plastics from marine bacteria, *Cur Sci*, 83 (2002) 562-564.
- 20 Rippka R, Deruelles J, Waterbury J B, Herdman M & Stanier R Y, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *J Gen Microbiol*, 111 (1979) 1-61.
- 21 Singh P K, Dhar D W, Pabbi S, Prasanna R & Arora A, *BGA-Azolla biofertilizers—A manual for their production, evaluation and utilization* (Venus Printers and Publishers, New Delhi, India) 2002, 74.
- 22 Jain P, *Screening and optimization of poly- β -hydroxybutyrate biosynthesis in certain cyanobacterial species*. M Tech Thesis, Indian Institute of Technology, Kharagpur, 2004.
- 23 Yellore V & Desia A, Production of poly- β -hydroxybutyrate from lactose and whey by *Methylobacterium* sp. ZP24, *Lett Appl Microbiol*, 26 (1998) 391-394.
- 24 Caballero K P, Karel S F & Register R A, Biosynthesis and characterization of hydroxybutyrate-hydroxycaproate copolymers, *Int J Biol Macromol*, 17 (1995) 86-92.