Indian Journal of Experimental Biology
Vol. 40, August 2002, pp. 924-929

A rapid hypochlorite method for extraction of polyhydroxy alkanoates from bacterial cells

T Rawte & S Mavinkurve*

Department of Microbiology, Goa University, Goa 403206, India

Received 19 August 2001; revised 7 March 2002

A new method has been standardized for extraction of polyhydroxy alkanoates from the bacteria, using sodium hypochlorite. This method is simple and quick as compared to the existing methods. Statistical analysis has proved the method to be reliable and reproducible.

Polyhydroxy alkanoates (PHA) are polyesters of hydroxy alkanoates, synthesized and accumulated intracellularly as granules by numerous bacteria[1-3]. PHA have various mechanical properties resembling the recalcitrant thermoplastics and hence have been considered as their potential substitute[1,4-8].

The method of extraction is a very important step in the assay and also for characterization of PHA. In a survey, when a large number of organisms are being screened for their ability to produce PHA, the method of extraction needs to be simple, quick, reproducible and reliable. Various methods have been employed for extracting PHA from the cells using hypochlorite as in Williamson & Wilkinson's method (*WW*)[9] and in Disk assay method (*DA*)[10], or solvents like chloroform (*Chlm*)[11], while some researchers have used both these in a combination like in the dispersion method (*Disp*)[12-14]. No comparative study has however been reported with respect to the relative efficacy, reproducibility, reliability and/or practical advantages of these different methods.

Mangroves constitute a nutrient rich ecosystem, which makes its flora diverse in nature. PHA have been reported in marine and estuarine sediments and from the microorganisms inhabiting these[15-18]. The mid-west coast of India was surveyed and a large number of organisms were found to accumulate PHA[19]. The present paper describes the development of a quick new quantitative extraction method (*Hyp*), using hypochlorite and comparison of various methods of extraction of PHA from five different cultures from marine sediments.

*Correspondent author:
Telefax: 091-832 – 451569.
E-mail : suneela@unigoa.ernet.in

Materials and Methods

Isolation and selection of organisms—Ten-fold dilutions of marine and mangrove sediments collected along the mid-west coast of India, were surface plated on Luria Bertani agar medium in aged sea water. The bacterial isolates were purified by surface streaking and screened microscopically for PHA accumulation by Ostle and Holt's method[20]. Heat fixed smears of the isolates were flooded with 1% (w/v) aqueous nile blue A, heated for 10 min followed by treatment with 8% (w/v) acetic acid. The slides were washed and observed under fluorescence microscope (Olympus) using green filter. Cells exhibiting orange fluorescence were scored positive for PHA accumulation. Amongst the isolates accumulating PHA, five isolates selected at random for the present study were 61/4, 64/4 and 82/4 being Gram positive rod shaped organisms, and 12/BL and 85/6 were Gram negative short rods.

Cultivation—Each culture was grown in 250 ml Erlenmeyer flasks containing 50 ml E2 mineral medium[21], consisting of $NaNH_4HPO_4$. $4H_2O$, 3.5g; K_2HPO_4. $3H_2O$, 7.5g and KH_2PO_4, 3.7g; $MgSO_4.7H_2O$, 10 ml of 100 mM and MT microelements stock solution, 1 ml; containing $FeSO_4.7H_2O$, 2.78 mg; $MnCl_2$. $4H_2O$, 1.98 mg; Co $SO_4.7H_2O$, 2.81 mg, $CaCl_2.2H_2O$, 1.47 mg; $CuCl_2.2H_2O$, 0.17 mg and $ZnSO_4.7H_2O$, 0.29 mg; per litre supplemented with yeast extract, 0.04% (w/v)[19]; glucose was used as carbon source 2% (w/v). The cultures were incubated in an environmental shaker at 28°C for 48 hr at 150 rpm.

All the chemicals used in this study were of analytical grade; solvents were distilled and dried before use.

Methods of extraction of PHA from bacterial cells—Figure 1 shows the flow diagram of the step wise pro-

tocols followed for each method of PHA extraction, namely Williamson and Wilkinson's (WW)[9], Disk assay (DA)[10], Chloroform extraction (Chlm)[11], Chloroform-hypochlorite dispersion (Disp)[12,13] and the new hypochlorite (Hyp) method, developed and reported herein.

Assay of PHA—PHA extracted by above methods were assayed by Slepecky and Law's method[22]. PHA sample (0.5ml) was treated with 4.5 ml concentrated H_2SO_4 and placed in a boiling water bath for10min. On cooling, absorbance was noted at 235 nm on UV-1601 Shimadzu spectrophotometer. Standard was run using DL-β-hydroxy butyric acid in the concentration range 50-500 nM. Each extraction method was repeated five times for each culture so as to check the reproducibility and the reliability.

Results and Discussion

There are various solvent and non-solvent based methods used for extraction of PHA from cells[9-14]. Data regarding comparative study of these extraction methods however are meager. Amongst all the solvents, hypochlorite[9,10,12-14] and chloroform[11-14] are the extractants of choice for PHA. Hypochlorite dissolves the non-PHA cellular material, leaving the insoluble polymer precipitated in solutions, however prolonged incubation over 60 min with hypochlorite is known to degrade the PHA[7]. Hot chloroform dissolves and extracts PHA leaving other cell material in aqueous.

A simple hypochlorite method (*Hyp*) was devised during the present work in which PHA from the cells was extracted with hypochlorite followed by washing it free of lipids with diethyl ether, using two isolates 61/4, Gram positive rods and 12/BL, pigmented Gram negative rods. The optimum time and temperature of incubation in hypochlorite solution and the concentration of the hypochlorite solution were standardized (Fig. 2). The incubation of the cells in hypochlorite solution with 2% (w/v) active chlorine at 37°C, recovered the polymer optimally (Fig. 2 a,b). It was noted that the cells immediately on treatment with hypochlorite were not lysed completely and were pelleted and the assay mixture of these samples exhibited high absorbance at 235 nm and also at other wavelengths. After 5 min of incubation in hypochlorite, PHA were separated from the cellular material par-

WW [9]	*DA* [10]	*Chlm* [11]	*Disp* [12]	*Hyp*
Suspend *pellet in 10 ml hypcl (37°C/2h)	Suspend *pellet in 1 ml D/W	*pellet, acidify	Suspend*pellet in 5 ml hypcl + 5 ml hot $CHCl_3$	Suspend *pellet in 5 ml hypcl
↓	↓	↓	↓	↓
Dialyse D/W/24h	0.2 ml on disk dry at 60°C	Dry at 60°C	Stir at 37°C/10′	Incubate 37°C/10′ on shaker
↓	↓	↓	↓	↓
pH 8.0 with 0.1N NaOH	0.2 ml hypcl (till colourless)	Extract with 10ml hot $CHCl_3$ (x6)	Extract with hot $CHCl_3$ (x2)	©8000rpm/ 20′
↓	↓	↓	↓	↓
©8000rpm/20′	Dry at 80-100°C	ppt with cold DEE	Pool organic phases	Wash pellet with cold DEE
↓	↓	↓	↓	↓
Pellet in minimum D/W + $Na_2S_2O_3$ (0.1 N) 2-3 drops	Hot $CHCl_3$ (0.2 ml x 5)	©8000/20′	ppt with cold DEE	©pellet
↓	↓	↓	↓	↓
Dialyse D/W/24h	Dry disks	Pellet wash with acetone	©8000rpm/10′	Assay
↓	↓	↓	↓	
Lyophilise	Immerse in hypcl 37°C/1h	Dissolve in hot $CHCl_3$	Pellet wash with acetone	
↓	↓	↓	↓	
Extract with DEE, Evaporate DEE	Wash consecutively in D/W, ethanol, acetone, DEE	Evaporate→ film	Dissolve in hot $CHCl_3$	
↓	↓	↓	↓	
Dissolve in hot $CHCl_3$	Dry disks	Assay	Evaporate→film	
↓	↓		↓	
Evaporate→film	Assay		Assay	
↓				
Assay				

Fig. 1—Extraction of PHA from bacterial cells by different methods: *WW*= Williamson and Wilkinson's; *DA*= Disk Assay; *Chlm*= Chloroform; *Disp*= Dispersion; *Hyp*= Hypochlorite; ©= Centrifugation; ppt= precipitate; DEE= Diethyl ether; D/W= Distilled water; Hypcl= sodium hypochlorite (2% w/v active chlorine)*, 10ml bacterial cell suspension was centrifuged at 6000rpm × 10^1, the pellet washed with 10 ml saline, cells were again centrifuged to get the pellet.

tially. The scan of samples incubated for 10 min exhibited a single peak at 235 nm, indicating the purity of the polymer free from cellular material. Incubation for 10 min in hypochlorite recovered the polymer almost fully in isolate 61/4 and completely in isolate 12/BL (Fig. 2c). On further incubation in hypochlorite for 40 min the yield of PHA increased marginally in isolate 61/4, whereas it decreased in 12/BL a Gram negative culture. Prolonged contact with hypochlorite during extraction of PHA is reported to affect the polymer, leading to decreased molecular weight[7]. Considering almost complete extraction of PHA in 10 min with marginal increase, if any, after 40 min and the risk of polymer degradation with increasing incubation period, the treatment of cells with hypochlorite limited for 10 min was adopted for extraction of PHA from the bacterial cultures.

Accordingly, the *Hyp* was standardized as follows. The bacterial cell pellet obtained from 10 ml of stationary phase culture washed once in saline was suspended in 10ml hypochlorite solution with 2% (w/v) active chlorine and incubated at 37°C for 10 min on shaker to allow separation of PHA from cellular material. The PHA pelleted by centrifugation at 8000 rpm for 20 min were then washed with diethyl ether to remove any contaminating lipids and assayed with sulphuric acid[22]. Table 1 gives the recovery of PHA from the selected bacterial cultures using *Hyp* in terms of percent dry weight of cell mass.

Hyp is found to be simple and quick method to extract PHA from bacterial cells and was compared with the four routinely used methods to confirm its efficiency and reproducibility. The relative quantities of PHA obtained by the five extraction methods from five different organisms are presented in Table 2.

Table 1 — Yield of PHA accumulated by the selected cultures

[Values are mean ± SD]

Yield	Isolates				
	61/4	64/4	82/4	12/BL	85/6
Dry weight of cell mass (g/l)	4.52±0.02	4.40±0.06	2.20±0.03	3.28±0.02	2.50±0.05
Wet weight of cell mass (g/l)	45.6±0.012	43.6±0.023	28.47±0.048	36.01±0.091	31.16±0.021
*PHA content (% cellular dry weight)	32.02	33.18	27.18	51.13	53.71

*Calculated using the PHA values obtained on extraction by the *Hyp*

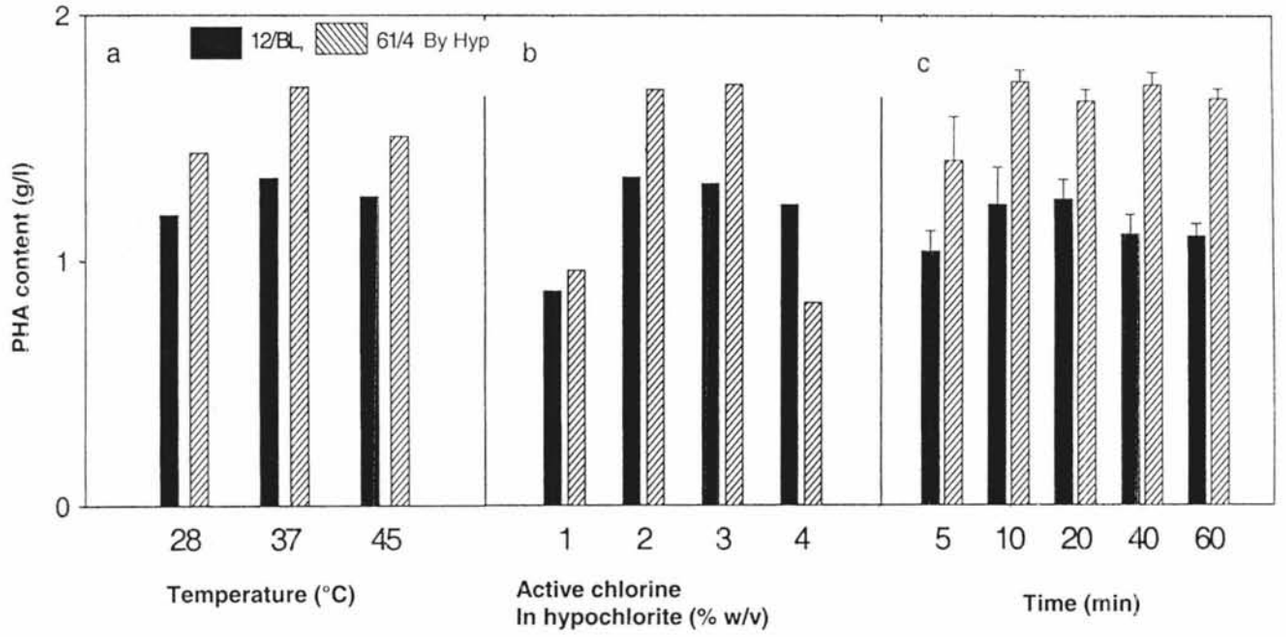

Fig. 2 — Effect of temperature (a); active chlorine in hypochlorite solution (b) and time of incubation (c) during extraction of PHA from the bacterial cells

DA is devised specially for screening and quantitating the polymer content from a large number of organisms [10]. The efficiency of *DA* to extract the polymer varied from culture to culture (Table 2), possibly due to the prolonged 1 hr treatment with hypochlorite[7] and varying amount of lipids and proteins adhering to PHA, interfering during extraction of the dense mass of cells (25mg) applied on glass fiber disks.

The *Disp* involves the use of sodium hypochlorite and chloroform, simultaneously[12], giving marginally higher yield of PHA (Table 2), than by the other extraction methods in all the cultures, except for 82/4, a Gram positive rod which consistently gave lower values of PHA.

The *Chlm* does not involve the use of hypochlorite and is now the most widely accepted method for extraction of PHA especially for the purpose of their characterization[14]. The precipitation of the polymer with dry diethyl ether and acetone in this method rules out the possibilities of any contaminating lipids and proteins and therefore possibly yields lower values than other methods as observed for culture numbers 61/4, 64/4, 85/6 (Table 2). The method is not very convenient for screening large number of isolates due to time consuming steps of repetitive extraction and extensive use of solvents[23].

WW is one of the most popular method[24,25]. It involves the use of solvents like dry diethyl ether and acetone, which precipitate the polymer, making it free of phospholipids, free fatty acids and triglycerides. Incubation with hypochlorite for more than 30 min[6], possibly increases the risk of polymer degradation accounting for the slightly lower content of the polymer extracted from cells (Table 2). Also the method is time consuming because of the two steps of dialysis.

The *Hyp* is a quick and simple method, modified by elimination of dialysis steps. Incubation of the cells with hypochlorite only for a short period of 10 min prevents the polymer degradation and diethyl ether treatment makes the extracted polymer free of any contaminating material such as lipids. In all the cultures, the method gives marginally higher yield, than the other methods. Because of its simplicity, this method could be used for routine extraction and quantification of the polymer from the large number of cultures isolated from mangroves and marine sediment samples[19].

Table 2—Estimation of PHA (g/l) extracted from bacterial isolates by different methods

[Values are mean ± SD]

Methods	Isolates				
	61/4	64/4	82/4	12/BL	85/6
Williamson & Wilkinson (*WW*)[9]	1.0668 ± 0.174	1.1426 ± 0.037	0.4698 ± 0.026	1.3821 ± 0.050	1.0332 ± 0.021
Disk Assay (*DA*)[10]	0.9107 ± 0.068	1.2791 ± 0.010	0.3358 ± 0.073	0.8838 ± 0.045	1.0748 ± 0.011
Chloroform (*Chlm*)[11]	0.8260 ± 0.036	0.8371 ± 0.017	0.5436 ± 0.014	1.4748 ± 0.1486	0.6302 ± 0.0003
Dispersion (*Disp*)[12-13]	1.4070 ± 0.154	1.3659 ± 0.009	0.5256 ± 0.017	1.4863 ± 0.159	1.1204 ± 0.009
Hypochlorite (*Hyp*)	1.4473 ± 0.065	1.460 ± 0.017	0.5980 ± 0.054	1.6772 ± 0.097	1.3428 ± 0.019

Table 3—F ratio 4,16 of variance between isolates for different methods of extraction

Cultures	Isolates				
	61/4	64/4	82/4	12/BL	85/6
For readings within a method	1.386	1.277	1.092	1.053	1.380
For readings of different methods	316.00	203.00	67.56	219.00	3251.00

$F_{4,16}$ value at 5% level of significance is 3.01

Table 4—Efficacy of hypochlorite method compared to other methods of PHA extraction

Method	Isolates				
	61/4	64/4	82/4	12/BL	85/6
Hyp: WW	1: 0.73	1: 0.78	1: 0.78	1: 0.82	1: 0.76
Hyp: DA	1: 0.63	1: 0.87	1: 0.56	1: 0.52	1: 0.80
Hyp: Chlm	1: 0.57	1: 0.57	1: 0.90	1: 0.87	1: 0.46
Hyp: Disp	1: 0.97	1: 0.93	1: 0.87	1: 0.88	1: 0.83

The F test of variance when applied to the data in Table 2, the $F_{(4,16)}$ ratio of variance (Table 3) was within the permissible level of 3.01 at 5% level of significance, for the readings within each method of extraction for any single given culture signifying the reproducibility and reliability of the methods of extraction, for all cultures. But the $F_{(4,16)}$ ratio of variance when applied within a culture for various methods of extraction, the values obtained were much higher than the permissible level, indicating the significant variation in extractability of PHA within the methods. Such variation may be attributed to the various steps involved in the different methods and also the composition of the bacterial cultures, especially the cell envelop. Culturewise variation in the extractability of periplasmic proteins by various methods has been demonstrated earlier[26]. Nevertheless, the efficacy of extraction of *Hyp* was compared with other methods. Table 4 indicates that the efficacy of extraction of PHA varied with culture and the method of extraction. For cultures 64/4 and 85/6 the extractability for all the methods followed the same order while for 12/BL, except for *DA*, the other three methods exhibited almost the same efficacy. The *Hyp*, standardized in the present study, consistently gave a higher value with each of the culture, indicating its efficacy of extraction. From all the five cultures used in the present study PHA could be extracted optimally by this method.

Therefore it can be concluded that the hypochlorite method (*Hyp*) presented herein is a reproducible simple and rapid method, which extracts the polymer fully, from bacterial cells.

Acknowledgement

Financial support from the Department of Ocean Development, New Delhi, especially for the fellowship granted to TR under the Manpower Development Scheme and the Ocean Science Technology Cell (Marine Microbiology) is gratefully acknowledged. The authors thank Mr. J. A. Menezes and Dr. G. R. Bhat for the critical discussions and valuable advice on statistical analysis of the data.

References

1 Anderson A J & Dawes E A, Occurrence, metabolism metabolic role and industrial uses of bacterial PHAs, *Microbiol Revs*, 54 (1990) 450.

2 Dawes E A & Senior P J, The role and regulation of energy reserve polymers in microorganisms, *Advs Microb Physiol*, 10 (1973) 135.

3 Rawte T & Mavinkurve S, Biodegradable Plastics- Bacterial Polyhydroxyalkanoates, *Indian J Microbiol*, 41(2001) 233.

4 Abe H, Matsubara I, Doi Y, Hori Y & Yamaguchi A, Physical properties and enzymatic degradability of PHB stereoisomers with different stereoregularities, *Macromolecules*, 27 (1994) 6018.

5 Barham P J, Keller A, Otun E L & Holmes P A, Crystallization and morphology of a bacterial thermoplastic: Poly-3-hydroxybutyrate, *J Mater Sci*, 19 (1984) 2781.

6 Byrom D, Production of poly-β-hydroxy butyrate: Poly-β-hydroxyvalerate copolymers, *FEMS Microbiol Rev*, 103 (1992) 247.

7 Doi Y, *Microbial polyesters* (VCH Publishers, New York) 1990.

8 Scandola M, Pizzali M, Ceccorulli G, Cesaro A & Padetti S, Viscoelastic and thermal properties of bacterial poly (D-(-)-β-hydroxybutyrate), *Int J Biol Macromol*, 10 (1988) 373.

9 Williamson D H & Wilkinson J F, The isolation and estimation of poly-β-hydroxy butyrate inclusions of *Bacillus* sps, *J Gen Microbiol*, 19 (1958) 198.

10 Ward A C & Dawes E A, A disk assay for poly-β-hydroxy butyrates. *Anal Biochem*, 52 (1973) 607.

11 Yellore V & Desai A, Production of polyhydroxybutyrate from lactose and whey by *Methylobacterium* sp. ZP24, *Lett Appl Microbiol*, 26 (1998) 391.

12 Hann S K, Chang Y K, Kim B S, Lee K M & Chang H N, The recovery of poly (3-hydroxy butyrate) by using dispersion of sodium hypochlorite solution and chloroform. *Biotechnol Techs*, 7 (1993) 209.

13 Hann S K, Chang Y K, Kim B S & Chang H N, Optimization of microbial (3-hydroxy butyrate) recovery using dispersion of sodium hypochlorite solution and chloroform, *Biotechnol Bioengg*, 44, (1994) 256.

14 Lee S Y, Bacterial polyhydroxyalkanoates, *Biotechnol Bioengg*, 49 (1996) 1.

15 Findlay R H & White D C, Polymeric β-hydroxyalkanoate from environmental samples and *B. megaterium*, *Appl Environ Microbiol*, 45 (1983) 71.

16 Herron J S, King J D & White D C, Recovery of poly-β-hydroxybutyrate from estuarine microflora. *Appl Environ Microbiol*, 35 (1978) 251.

17 Nickles J S, King J D & White D C, Polyhydroxybutyrate accumulation as a measure of unbalanced growth of the estuarine detrital microflora. *Appl Environ Microbiol*, 37 (1979) 459.

18 Odham G, Tunlid A, Westerdahl G & Marden P, Combined determination of Poly-β-hydroxyalkanoic and cellular fatty acids in starved marine bacteria and sewage sludge by Gas Chromatography with flame ionization or mass spectrometry detection. *Appl Environ Microbiol*, 52 (1986) 905.

19 Rawte T, Padte M & Mavinkurve S, Incidence of bacteria accumulating Polyhydroxy alkanoates in the mid-west coast of India. *World J Microbiol Biotechnol*, (2002) (*in press*).

20 Ostle A G & Holt J G, Nile Blue A as a fluorescent stain for poly-β-hydroxybutyrate. *Appl Microbiol Microbiol*, 44 (1982) 238.

21 Lageveen R G, Huisman G W, Preusting H, Ketelaar P, Eggink G & Witholt B, Formation of polyesters by *Pseudo-*

monas oleovorans: Effect of substrates on formation and composition of poly-(*R*) -3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates, *Appl Environ Microbiol*, 54 (1988) 2924.

22 Slepecky R A & Law J H, A rapid spectrophotometric assay of alpha, beta unsaturated acids and beta-hydroxy acids. *Anal Chem*, 32 (1961) 1697.

23 Byrom D, Polyhydroxyalkanoates in *Plastics from microbes, microbial synthesis of polymers and polymer precursors* edited by D P Mobley (Hanser, Munich) 1994, 5.

24 Kominek L A & Halvorson H O, Metabolism of poly-β-hydroxybutyrate and acetoin in *Bacillus cereus*, *J Bact*, 90 (1965) 1251.

25 Sabat S, Deshpande M K & Khandwekar P V, Microbial production of poly-β-hydroxybutyrate-A biopolymer, *J Sci Ind Res*, 57 (1998) 654.

26 Lall S D, Eribo B E & Jay J M, Comparison of four methods for extracting periplasmic protein. *J Microbiol Methods*, 9 (1989) 195.