

Quantitative extraction and determination of polyhydroxyalkanoate accumulated in *Alcaligenes latus* dry cells

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ABSTRACT: In order to improve polyhydroxyalkanoate (PHA) production, it is necessary to investigate and understand the kinetic growth profile of fermentable bacteria under various fermentation conditions. Modern determination methods can only give the amount and type of PHA monomers without other polymer information such as molecular weight, melting temperature, viscosity, and tensile strength. We therefore isolated and purified PHA to determine the extraction yield and the quality of the PHA. Both the extraction methods used for the isolation and the cell lytic pretreatment prior to the extraction step substantially affect the yield. In the pretreatment step, the oven-dried biomass needs to be treated with short-chain alcohols which disrupt cell walls and dissolve impurities without affecting the PHA. Furthermore, agitations significantly accelerate the pretreatment step and reduce the chance of polymer degradation therefore increasing isolation yield and conserving polymer properties. Continuous Soxhlet extraction showed a significantly higher yield than from direct solvent extraction. Low-boiling point chlorinated partial solvents such as dichloromethane and chloroform were found to give a high PHA quality and a high extraction yield without decomposition over long period of extraction. Short-chain alcohols or hexane gave optimal results in aggregating polymers in the purification step. Purity and properties of isolated PHA were determined by NMR spectroscopy, differential scanning calorimetry, and diluted solution viscometry.

KEYWORDS: PHA, biopolymer, biodegradable plastic, isolation, characterization, assay

INTRODUCTION

Recently, biodegradable plastics have been widely studied in order to replace the conventional fuel based synthetic plastics to minimize environmental effects and global warming. A group of biodegradable polyesters called polyhydroxyalkanoates (PHAs)¹, which can be harvested from the energy storage granules inside the cytoplasm of many fermentable microorganisms², have excellent mechanical and physical properties suitable for various plastics applications especially in medicine³ e.g., medical apparatus, body part implants, and stitching string. In the last century research on biodegradable plastics has mainly focused on screening, fermentation processes, and applications⁴. The isolation process has not been investigated systematically⁵. Our preliminary study of PHA production from the shake flask fermentation of *Alcaligenes latus*^{6,7} fed with various carbon sources⁸ resulted in dry cells with the short-chain-length PHAs, poly-3-hydroxybutyrate, poly(3-hydroxybutyrate-co-

3-hydroxyvalerate), and poly(3-hydroxybutyrate-co-4-hydroxyvalerate-co-3-hydroxyvalerate) as both homopolymer and copolymers with a high cell concentration (6.77 g/l) and high PHA content (73.8% w/w).

In order to improve the production of PHA, knowledge of the kinetic growth profile of various fermentation conditions is essential. Kinetic studies based only on cell growth using cell concentration determined by light scattering at 660 nm ($OD_{660\text{ nm}}$) using spectrometry, or cell dry weight determined by gravimetry do not correspond to the exact PHA accumulation in *A. latus* ATCC 29714 at various growth phases. Unlike *A. eutrophus* and several other bacteria, *A. latus* does not accumulate PHA during the cell growth period. Moreover, the PHA content can change at any stage depending on the C:N ratio, pH, dissolved oxygen, and cell concentrations (unpublished observations).

Recently, several modern analytical techniques have been used to quantify PHA content in bacterial dry cells such as gas chromatography (GC),

high performance liquid chromatography, and NMR spectroscopy^{9,10}. However, PHA determination using these techniques can only give the amount and type of PHA monomers but not other engineering properties such as molecular weight, melting temperature, viscosity, and tensile strength. Hence, the isolation and purification of PHA was investigated to obtain the extraction yield and quality of the PHA.

MATERIALS AND METHODS

Media

One litre of the basal mineral medium broth used contains $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 4.7 g, KH_2PO_4 1.5 g, $(\text{NH}_4)_2\text{SO}_4$ 2.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 10 mg, H_3BO_3 0.3 mg, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 mg, $\text{NaMoO}_4 \cdot 6\text{H}_2\text{O}$ 30 mg, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 20 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 10 mg, ferrous ammonium citrate 72 mg, and 20 g of glucose as the carbon source. The basal mineral medium agar used was obtained by adding 18 g of agar. All media were sterilized at 121 °C under high pressure (15 psi) for 15 min.

Fermentation of *Alcaligenes latus*

A fully grown colony of *A. latus* ATCC 29714 in basal mineral medium agar was inoculated to 50 ml of basal mineral medium broth and shaken at 30 °C using an orbital shaker at 250 rpm for 48 h. The culture was then transferred into a 1-l Erlenmeyer flask containing 300 ml of basal mineral medium broth. After the initial cell concentration was adjusted to an $\text{OD}_{660\text{ nm}}$ of 0.1, the broth was vigorously shaken (250 rpm) at 30 °C for another 24 h. The biomass was then obtained by centrifugation at 4700g at 4 °C for 15 min. After rinsing twice with saline solution (0.89% w/v) followed by a 15-min centrifugation, the precipitate was collected, oven dried at 60 °C for 4–12 h until constant weight, cooled, and kept under high vacuum in a desiccator to maintain dry until further use.

PHA content using GC analysis

To determine the PHA content of the fermented dry cells in each batch before further study, methyl benzoate (40.0 mg) was added as an internal standard to a 10-ml sealed tube containing a suspension of dry cells in 2 ml of methanol and 2 ml of chloroform followed by an addition of 0.5 ml of concentrated sulphuric acid. It was then heated to reflux in a silicone oil bath at 100 °C for 2 h¹⁰. After cooling to room temperature, it was washed with 2 ml of water and a saturated solution of NaHCO_3 , dried over anhydrous Na_2SO_4 , and filtered with a 0.45 µm nylon

syringe filter. GC analysis was performed by injecting 1 µl (splitless mode) of the obtained solution into an Agilent 4890D gas chromatograph equipped with a packed column (10% carbowax 20 M Chromosorb WHP 100/120 mesh) and a flame-ionization detector set at 200 °C. Helium was used as the carrier gas. The oven temperature protocol was to hold at 65 °C for 5 min, increase 5 °C/min for 16 min, hold for 3 min, then increase again for another 3 min, and finally hold at 200 °C for 5 min. The standardization was done under the same conditions, except that analytical grade PHB (Fluka) was used instead of the dry cells.

Study of isolation process

To extract most of the PHA from the biomass, the dry cells need to be ruptured. To avoid hard surfactants, strong bases, or sodium hypochlorite which can cause decomposition of polymers¹¹, we used acetone and short chain alcohols such as methanol, ethanol, n-propanol, isopropanol, or n-butanol with good cell lytic properties. Our choice of solvents was made as a result of their availability, toxicities, and cell lytic properties. In this pretreatment step, 0.40 g of the 12-h oven dried biomass was submerged in 10 ml of methanol, ethanol, isopropanol, or distilled water at room temperature without agitation for 1 h. After a 15-min centrifugation at 4700g, the precipitate was transferred to a cellulose extraction thimble and inserted in the Soxhlet extractor containing 150 ml of chloroform which was used as the extracting solvent. After 5 h of extraction, the solvent was evaporated under vacuum to obtain about 1 ml of residue. Then, 2 ml of cold methanol was added to re-precipitate and to obtain a dull white solid which then was filtered, dried, weighed, and subjected to further analysis. The extraction yields were compared with the determination using gas chromatography to confirm the percentage of recovery and most isolated polymers were characterized by IR and NMR spectroscopy. Their purities and properties were determined by ¹H NMR spectroscopy, differential scanning calorimetry (DSC), and diluted solution viscometry.

The continuous Soxhlet extraction over 5 h was initially used as follows. A suspension of dry cells (0.40 g) in 10 ml of ethanol was subjected to various agitation methods (simmering, shaking, stirring, or sonicating) for 1 h at 30 °C. After a 15-min centrifugation at 4700g, the precipitate was subjected to 5-h Soxhlet extraction and analysed as described previously.

The duration of pretreatment was varied to get the optimal result not only in terms of yield but also of quality of the isolated polymer checked by ¹H NMR

spectroscopy¹². A suspension of dry cells 0.50 g suspended in 10 ml of ethanol was shaken at 130 rpm in an orbital shaker at 30 °C for 1, 2, or 3 h. After a 15-min centrifugation at 4700g, the precipitate was subjected to 5-h Soxhlet extraction and analysed as described above.

After optimization of the pretreatment step, the extraction step was then investigated by varying the extraction solvents, selected by their prices, availabilities, solubilities, and boiling points¹³. To understand the nature of the extracted PHA during the extraction process, a non-polar solvent such as hexane was used and some polar protic solvents such as methanol, ethanol, and propanols were also used in this step. Furthermore, medium polar non protic solvents such as acetone, chloroform, dichloromethane, and ethyl acetate were included in this study to optimize PHA quality and extraction yield. To investigate effect of solvents, a suspension of dry cells (0.40 g) in 10 ml of ethanol was rigorously shaken at 130 rpm in an orbital shaker at 30 °C for an hour. After the sample was centrifuged at 4700g for 15 min, the precipitate was transferred to a cellulose extraction thimble and inserted in the Soxhlet extractor containing 150 ml of various extracting solvents (chloroform, dichloromethane, *N,N*-dimethyl formamide, ethyl acetate, hexane, toluene, methanol, ethanol, propanol, butanol, isopropanol, or *t*-butanol). After 5 h of extraction, the solvent was evaporated under vacuum to obtain about 1 ml of residue. Then 2 ml of cold methanol was added to re-precipitate to obtain a solid which then was filtered, dried, weighed, and subjected to further analysis.

In addition, the effect of extraction period was briefly investigated. The experiment was done as above using chloroform as the extracting solvent, except that the time of extraction was extended to 10 h. To ensure that the amount of PHA did not correlate with extraction yield, the experiment was done as above using chloroform as the extracting solvent except *A. latus* dry cells with 18.87%, 28.49%, 41.10% and 50.64% PHA content were used.

Finally, in order to enhance the isolated PHA purity, the solvent effect on re-crystallization of the crude extracted polymer was investigated as follows. A suspension of dry cells (4.001 g) suspended in 80 ml of ethanol was shaken at 130 rpm in an orbital shaker at 30 °C for 1 h. After a 15-min centrifugation at 4700g, the precipitate was transferred into a cellulose-extraction thimble and inserted in the Soxhlet extractor containing 150 ml of chloroform. After 5 h of extraction, the solvent was evaporated under vacuum to obtain 1.890 g of crude residue containing 80.1%

PHA (as determined by ¹H NMR spectroscopy). The residue was then re-dissolved into 7.00 ml. To each 1.00-ml aliquot was added 2 ml of various cold solvents (methanol, ethanol, propanol, isopropanol, ethyl acetate, hexane, or acetone) to re-precipitate to obtain a solid which then was filtered, dried, weighed, and subjected to further analysis.

In parallel, solid-liquid extraction was investigated in comparison. A suspension of dry cells (0.50 g) in 10 ml of ethanol was shaken (130 rpm) at 30 °C for 1 h. After a 15-min centrifugation at 4700g, the precipitate was transferred into an Erlenmeyer flask containing 150 ml of various extracting solvents (chloroform, dichloromethane, *N,N*-dimethyl formamide, ethyl acetate, hexane, or toluene). After shaking (130 rpm) at 30 °C for 5 h, the mixture was poured into a 250-ml separating funnel and washed with 50 ml of water. The aqueous phase was extracted again with 50 ml of the same organic solvent. The combined organic phase was then washed with 50 ml of brine, dried over anhydrous Na₂SO₄ and evaporated under vacuum to obtain about 1 ml of residue. Then 2 ml of cold methanol was added to re-precipitate to obtain a dull white solid which then was filtered, dried, weighed, and subjected to further analysis. Also, to investigate the effect of the extraction period, 10-h extraction was applied to the same experiment with chloroform as the extracting solvent. The experiment at 40 °C was also studied.

Characterization of the isolated PHA

Most isolated polymers were characterized by NMR spectroscopy and IR spectroscopy. The ¹H NMR spectrum of the isolated and purified PHA was determined using a 3 mg sample in 1 ml of deuterated chloroform (CDCl₃) containing tetramethylsilane as the internal standard in a Bruker Uxnmr 300 MHz spectrometer for 16 scans. The IR spectrum of a thin film was obtained from a Perkin Elmer Spectrum 2000 Fourier-Transform IR spectrophotometer. The isolated PHA (0.1 g) was dissolved in 2 ml of CHCl₃, poured into a Petri dish, air-dried, and then kept in a desiccator under high vacuum to get a thin film. Melting temperatures and glass temperatures of most of the isolated PHAs were determined by a DSC 2910 differential scanning calorimeter scanning from -30 °C to 200 °C at a heating rate of 10 °C/min. The average viscosity molecular weights (M_v) of the isolated PHA were determined using diluted solution viscometry using chloroform as solvent. We obtained the polymer intrinsic viscosity, η , which is equal to kM_v^a , where $k = 1.21 \times 10^{-4}$ and $a = 0.75$ ¹⁴.

Table 1 Percentage of PHA recovery from *A. latus* dry cells with different pretreatments.

	Treatment	% recovery
Pretreatment solvent	none	39 ± 2.1
	water	29 ± 2.6
	methanol	71 ± 1.7
	ethanol	84 ± 1.6
	isopropanol	66 ± 1.7
Pretreatment method	none	39 ± 2.1
	simmering	74 ± 2.6
	shaking	84 ± 1.6
	stirring	100 ± 0.8
	sonicating	83 ± 1.7
Pretreatment period	0 h	39 ± 2.1
	1 h	84 ± 1.6
	2 h	100 ± 0.8
	3 h	98 ± 2.5

RESULTS AND DISCUSSION

In this cell lytic pretreatment, the results in Table 1 indicate that the proper solvents are mostly protic solvents with good solvolytic properties to break or rupture bacterial cell membrane, but are not good solvents for PHA. Ethanol and methanol are preferable. The ¹H NMR spectrum of the ethanolic and methanolic extracts from this step showed only grease and lipid cell debris. 1-propanol, 2-propanol, and larger alcohols require a longer time to penetrate and swell out dry cells. Furthermore, the ¹H NMR spectrum of the alcoholic extracts showed some PHA along with grease and lipid cell debris. This indicates that medium chain alcohols can dissolve some of the PHA from the bacterial cell which is probably good for being used as extracting solvents.

In the case of 1-h pretreatment, vigorous mechanical agitations are necessary. Stirring with magnetic bar for a small scale extraction gives the highest extraction yield. However, shaking and sonicating also give high % recovery (Table 1). As a control agitation, shaking with the orbital shaker at 130 rpm was then used in most experiments unless stated otherwise.

The time for the pretreatment step affects the extraction yield and gives 100% PHA recovery in 2 h (Table 1). Longer pretreatment time decreases the yield possibly as a result of to PHA degradation. Dry conditions are favourable. Moisture-free solvents and cotton-sealed vessels are needed. Moreover, an attempt to increase the alcoholic pretreatment temperature under dry atmosphere results in a significantly a lower extraction yield and more decomposition (unpublished results).

The best solvents for this step tend to be the 'partial solvents' CHCl₃ and CH₂Cl₂ while 'non solvents' give a very low percentage of recovery and polar protic solvents diminish extraction yield (Table 2). Methanol and ethanol give low polymer purity since PHA is quite polar and hygroscopic and tends to decompose in the presence of moisture. With the same extraction process described above using chloroform as extracting solvent for 5, 6, and 10 h give 84, 99, and 100% recovery, respectively. Submission of dry cells with various PHA contents: 18.87%, 28.49%, 41.10%, and 50.64% gave essentially the same % recovery (83%, 85%, 84%, and 86%, respectively).

With the solid-liquid extraction method, the extraction yield tends to be slightly lower than with the continuous Soxhlet extraction method. However, in the case of ethyl acetate, DMF and toluene give better results (Table 2).

An attempt to increase the shaking time from 5 h to 10 h in this solid-liquid extraction method decreases extraction yield from 66 to 59% recovery. Elevation of extracting temperature from room temperature to 40 °C dramatically decreases the extraction yield from 66 to 44% recovery. This may imply that the polymer starts to degrade as it heats over time and also explains the reduction in yield after 2 h of pretreatment.

Characterization of the isolated PHA

In most cases, the obtained ¹H NMR and IR spectra suggested that the isolated PHA from *A. latus* fed with glucose as carbon source is poly(3-hydroxybutyrate) or PHB only with very high purity (> 99%) especially after purification. However, when using alcohols as the extraction solvent, the obtained polymer usually contained lipid impurities. Thermograms of most PHA samples run by the DSC 2910 showed only a sharp peak melting temperature at 169 °C without a glass temperature. The average viscosity molecular weight of the obtained PHA was around 110 kDa.

CONCLUSIONS

It is important to note that short chain alcohols such as ethanol and methanol are preferable in the pretreatment step. Unlike strong bases or hard surfactants, these alcohols rupture only bacterial cell wall, not the polymer granules. Hence, the isolated PHA properties remain the same in most cases. Furthermore, agitations such as stirring, shaking, and sonicating significantly accelerate the cell lytic pretreatment step thus avoiding PHA decomposition and so increasing the PHA isolation yield. In the extraction step, aprotic polar 'partial solvents', especially chlorinated solvents such as dichloromethane and chloroform, were found

Table 2 Effect of extracting solvents on % recovery of the PHA extraction using the continuous Soxhlet extractor and using direct solid-liquid extraction.

Extracting solvents	Chloroform	Dichloro-methane	Ethyl acetate	Dimethyl formamide	Toluene	Hexane
% recovery using Soxhlet	84 ± 2.7	87 ± 2.6	29 ± 2.8	42 ± 3.0	22 ± 3.2	49 ± 3.2
% recovery using solid-liquid	66 ± 3.2	66 ± 2.8	36 ± 3.5	68 ± 3.6	29 ± 2.4	26 ± 2.2

to give high PHA quality and high extraction yield. Chloroform, in particular, tends to extract or dissolve out only PHA with little impurities from the bacterial cells. In addition, low boiling-point chloroform does not cause PHA degradation during prolonged extraction periods up to 10 h. The direct solid-liquid extraction developed in this experiment also gives similar result with high PHA content dry cells (60% or more).

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