# Study of extraction process and characterization of poly-3-hydroxyalkanoate produced from *Alcaligenes latus*

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## Abstract

In order to obtain proper quality and high extraction yield of biodegradable polymer, poly-3-hydroxyalkanoate (PHA), from *Alcaligenes latus* each of 3 extraction steps; pretreatment step, extraction step and purification step was systematically investigated. In pretreatment step, the oven dried biomass has been submerged in various organic solvents with and without agitation. Among common over-the-shelf solvents, short chain alcohols (such as methanol, ethanol and isopropanol) gave high yield and high PHA purity without further purification. Furthermore, agitations (such as stirring, shaking and sonicating) significantly accelerate pretreatment step and increase PHA isolated yield. As a study model for various extracting solvents, the continuous Soxhlet extraction over 5 hours has been initially used. The chlorinated solvents such as dichloromethane and chloroform were found to give high PHA quality and high extraction yield. From these preliminary results, our direct solvent extraction method has been developed. First, the dried cell was shaken in ethanol for an hour followed by an addition of chloroform and 5-hour shaking at room temperature. Then the solution was washed with water and extracted twice with chloroform using separatory funnel. After drying, the organic layer was evaporated to obtain a crude PHA. In the purification step, alcohols and hexanes gave optimal results in aggregating polymers. Most extracted PHA characterization by Infrared Spectroscopy and Nuclear Magnetic Resonance spectroscopy corresponds to the structure of poly-3-hydroxybutyrate with $T_m$ = 169 ºC determined by Differential Scanning Calorimeter and its average viscosity molecular weight ($M_v$) of $1.1\times10^5$ Dalton.

***Keywords:*** *poly-3-hydroxyalkanoate, extraction process, characterization*

## Introduction

Recently biodegradable plastics have been widely studied in order to replace the conventional fuel based synthetic plastics to minimize environment effects and global warming. Besides starches and other polysaccharide biopolymers, a group of biodegradable polyesters called poly-3-hydroxyalkanoates (PHAs) [1] which can be harvested from the energy storage granules inside the cytoplasm of many fermentable microorganisms [2] provide several excellent mechanical and physical properties in various plastic applications especially in medical applications [3] e.g. medical apparatus, body part implants and stitching string. In the last century most research topic focuses mainly on screening and fermentation processes as well as

biodegradable plastic applications. [4] However, the isolation process is yet a bit under investigated systematically. [5] Our preliminary study of PHA production from the shaked-flask fermentation of *Alcaligenes latus* [6] fed with various carbon sources [7] obtained dry cells with various short-chain-length PHAs as both homopolymer and copolymers: poly-3-hydroxybutyrate, poly(3- hydroxybutyrate-*co*-3-hydroxyvalerate) and poly (3-hydroxybutyrate-*co*-4-hydroxyvaler-ate-*co*-3-hydroxyvalerate) with high cell concentration (6.77 g/l) and high PHA content (73.8%w/w).

In order to obtain proper quality and high extraction yield of biodegradable poly (3-hydroxyalkanoates), *Alcaligenes latus* ATCC 29714 fed with glucose was used in this systematic investigation in each isolation step; i) pretreatment step ii) extraction step and iii) purification step. In pretreatment step, the 12-hour oven dried biomass has been submerged in various organic solvents with and without agitation. Moreover, the pretreatment and time period of pretreatment were varied to get the optimal result not only in term of yield but also the quality of the isolated polymer checked by $^{1}$H NMR spectroscopy.[9] Our choice of solvents was made from their availabilities, toxicities and cell lytic properties. Besides, in order to avoid hard surfactants, strong bases, and sodium hypochlorite that can cause decomposition of polymers[10], among common over-the-shelf solvents, acetone and short chain alcohols such as methanol, ethanol n-propanol, isopropanol and n-butanol with good cell lytic property were used in this study. Furthermore, agitations (such as stirring, shaking and sonicating) were applied to optimize the pretreatment step and increase PHA isolated yield. As a study model for various extracting solvents, the continuous Soxhlet extraction over 5 hours has been initially used. After optimization of the pretreatment step, the extraction step was then investigated varying solvent for extraction, time of extraction, method of extraction and PHA content in *Alcaligenes latus* dry cells. The extraction solvents in the study were normally chosen by their prices and availabilities, PHA and impurity, solubilities [11] and boiling points. Also to understand the nature of the extracted PHA during the extraction process, a non-polar sovent such as hexanes 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. Finally, in order to enhance the isolated PHA purity, the solvent effect of recrystallization of the crude extracted polymer was investigated.

## Materials and Methods

### *Mediums:*

In 1 liter, basal mineral medium broth used contains $Na_2HPO_4.7H_2O$ 4.7 g, $KH_2PO_4$ 1.5 g, $(NH_4)_2SO_4$ 2.0 g, $MgSO_4.7H_2O$ 0.2 g, $CaCl_2.2H_2O$ 10 mg, $H_3BO_3$ 0.3 mg, $CoCl_2.6H_2O$ 0.2 mg, $ZnSO_4.7H_2O$ 0.1 mg, $NaMoO_4.6H_2O$ 30 mg, $NiCl_2.6H_2O$, 20 mg, $CuSO_4.5H_2O$ 10 mg, ferrous ammonium citrate 72 mg, and glucose 20 g as carbon source. An addition of agar 18 g gives basal mineral medium agar used. All mediums were sterilized at 121 $^{o}$C under high pressure (15 psi) for 15 minutes.

### *Fermentation of Alcaligenes latus*

A fully grown culture of *Alcaligenes latus* ATCC 29714 in basal mineral medium agar was inoculated to 50 ml basal mineral medium broth and incubated at 30 $^{o}$C well-shaken using the Orbital shaker at 250 rpm for 48 hours. The culture was then transferred

into a 1-liter Erlenmeyer flask containing 300 ml of basal mineral medium broth. After the initial cell concentration was adjusted to $OD_{660nm}$ at 0.1, the broth was vigorously shaken (250 rpm) at 30 $^{o}$C for another 24 hours. Biomass was then centrifuged at 6,500 rpm under 4 $^{o}$C for 15 minutes. After rinsing twice with saline solution 0.89 %w/v and a 15-minute centrifuge, the precipitate was then collected, oven dried at 60 $^{o}$C for 4-12 hours until constant weight, cooled and kept under high vacuum in a desiccator to maintain dry until further use.

***GC analysis of PHA in dry cells***

To determine the PHA content [13] of the fermented dry cells each batch before further study, methyl benzoate 40.0 mg was added as the internal standard to a 10-ml sealed tube containing a suspension of dry cell 40.0 mg in 2 ml of methanol and 2 ml of chloroform followed by an addition of 0.5 ml of concentrated sulfuric acid. It was then heated to reflux in a silicone oil bath at 100 $^{o}$C for 2 hours. 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 0.45 µm nylon syringe filter. GC analysis was performed by injecting 1 µl of the obtained solution into an Agilent 4890D gas chromatograph equipped with a packed column (10% carbowax 20M Chromosorb WHP 100/120 mesh) and a flame ionization detector set to 200 $^{o}$C. Helium was used as the carrier gas. The oven temperature starts at 65$^{o}$C and follows the temperature program showed in Fig 1.

**Study of isolation process**

***Pretreatment step***

***Effect of solvents:*** Dry cells 0.40 g was submerged in 10 ml of methanol, ethanol, isopropanol or distilled water at room temperature for an hour. After a 15-minute centrifugation at 6,500 rpm, the precipitate was transferred into a cellulose extraction thimble and inserted in the Soxhlet extractor containing 150 ml of chloroform used as the extracting solvent. After 5 hours of extraction, the solvent was evaporated under vacuum to obtain about 1 ml of residue. Then it was added 2 ml of cold methanol to re-precipitate to obtain a dull white solid which then was filtered, dried, weighed and subjected to further analysis.

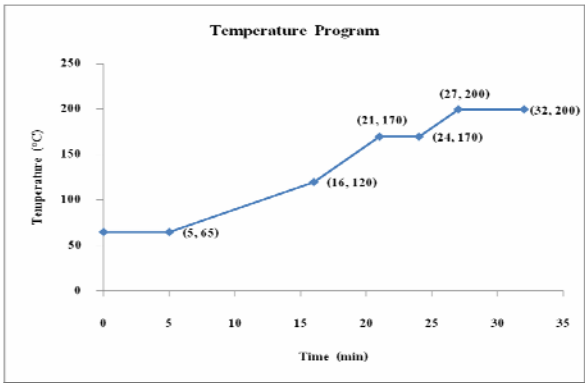


**Figure 1 The oven temperature program used for GC analysis**

***Effect of mechanical agitations:*** A suspension of dry cells 0.40 g in 10 ml of ethanol was subjected to various agitation methods (simmering, shaking or sonicating) for an hour at 30 $^{o}$C. After a 15-minute centrifuge at 6,500 rpm, the precipitate was subjected to 5-hour Soxhlet extraction and analyzed as described previously.

***Effect of pretreatment time:*** A suspension of dry cells 0.50 g suspended in 10 ml of ethanol was shaken at 130 rpm using the Orbital shaker at 30 $^{o}$C for 1, 2 or 3 hours. After a 15-minute centrifuge at 6,500 rpm, the precipitate was subjected to 5-hour Soxhlet extraction and analyzed as described previously.

**Extraction step**

***Effect of Solvents:*** A suspension of dry cells 0.40 g in 10 ml of ethanol was rigorously shaken at 130 rpm using the Orbital shaker at 30 $^{o}$C for an hour. After a 15-minute centrifuge at 6,500 rpm, the precipitate was transferred into a cellulose extraction thimble and inserted in the Soxhlet extractor containing 150 ml of various extracting solvents (chloroform, dichloro- methane, *N*,*N*-dimethyl formamide, ethyl acetate, hexanes, toluene, methanol, ethanol, propanol, butanol, isopropanol or *t*-butanol). After 5 hours of extraction, the solvent was evaporated under vacuum to obtain about 1 ml of residue. Then it was added 2 ml of cold methanol to re-precipitate to obtain a solid which then was filtered, dried, weighed and subjected to further analysis.

***Effect of extraction time:*** The experiment was done same as above using chloroform as the extracting solvent except time of extraction was extended to 10 hours.

***Effect of PHA content:*** The experiment was done same as above using chloroform as the extracting solvent except dry cells used in the study contains PHA 18.87%, 28.49%, 41.10% or 50.64%.

**Purification**

***Effect of solvents:*** Dry cells 4.001 g suspended in 80 ml of ethanol was shaken at 130 rpm using the Orbital shaker at 30 $^{o}$C for an hour. After a 15-minute centrifuge at 6,500 rpm, the precipitate was transferred into a cellulose extraction thimble and inserted in the Soxhlet extractor containing 150 ml of chloroform. After 5 hours of extraction, the solvent was evaporated under vacuum to obtain 1.890 g of crude residue containing PHA 80.1% determined by $^{1}$H NMR spectroscopy. Then it was re-dissolved into 7.00 ml. Each 1-ml aliquot was added 2 ml of various cold solvents (methanol, ethanol, propanol, isopropanol, ethyl acetate, hexanes or acetone) to re-precipitate to obtain a solid which then was filtered, dried, weighed and subjected to further analysis.

**Solid-liquid extraction**

***Effect of Solvents:*** A suspension of dry cells 0.50 g in 10 ml of ethanol was shaken (130rpm) at 30 $^{o}$C for an hour. After a 15-minute centrifugation (6,500 rpm), the precipitate was then transferred into an Erlenmeyer flask containing 150 ml of various extracting solvents (chloroform, dichloromethane, *N*,*N*-dimethyl formamide, ethyl acetate, hexanes or

toluene). After shaking (130 rpm) at 30 $^{o}$C for 5 hours, the mixture was poured into a 250-ml separatory funnel and washed with 50ml of water. The aqueous phase was extracted again with 50ml of the same organic solvent. The combined organic phase was then washed with 50 ml of brine, dried over anhydrous $Na_2SO_4$ and evaporated under vacuum to obtain about 1 ml of residue. Then it was added 2 ml of cold methanol to re-precipitate to obtain a dull white solid which then was filtered, dried, weighed and subjected to further analysis.

***Effect of shaking time:*** The experiment was done same as above using chloroform as the extracting solvent except time of extraction was extended to 10 hours.

***Effect of simmering temperature:*** The experiment was done same as above using chloroform as the extracting solvent except the extracting temperature was done at 40 $^{o}$C.

**Charterization of PHA**

***NMR spectroscopy:*** $^{1}$H NMR spectra of the isolated and purified PHA about 3 mg in 1 ml of $CDCl_3$ containing tetramethylsilane (TMS) as the internal standard was taken by a Bruker UXNMR 300 MHz spectrometer for 16 scans.

***FT-IR spectroscopy:*** The isolated PHA 0.1 g was dissolved in 2 ml of $CHCl_3$, poured into a petridisc, air-dried, and then kept in a desiccator under high vacuum to get a thin film. FTIR spectrum was obtained from a Perkin Elmer Spectrum 2000 Fourier-Transform Infrared Spectrophotometer.

***Differential Scanning Calorimetry:*** Most melting temperatures ($T_m$) and glass temperatures ($T_g$) of most isolated PHA were determined by a DSC 2910 **Differential Scanning Calorimetry** scanning from -30 $^{o}$C to 200 $_{o}$C at the rate of 10 $^{o}$C/min.

***Molecular weight determination:*** Most average viscosity molecular weights ($M_v$) of the isolated PHA determined using Diluted Solution Viscometry (DSV) using chloroform as solvent to obtain polymer intrinsic viscosity [$\eta$] which equals to $kM_v^a$ where $k = 1.21 \times 10^{-4}$ and $a = 0.75$. [12]

**Results**

**Pretreatment step**

***Effect of Solvents:*** In this cell lytic pretreatment, the result in Fig. 2 indicates that the proper solvent are mostly protic solvents with good solvolytic properties to break or rupture bacterial cell wall, but not-so-good solvent or “partial solvent” for PHA. Ethanol and methanol are preferable. Propanols and larger alcohols require longer time to penetrate and swell out dry cells.

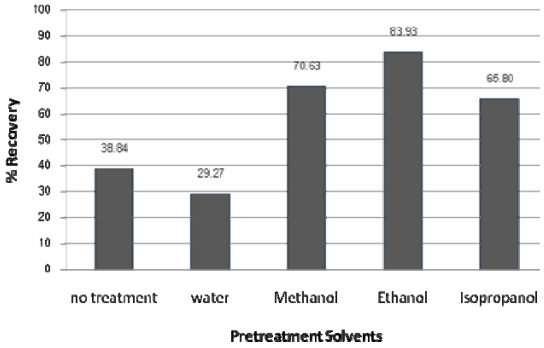


**Figure 2 Pretreament solvent effect on %recovery of the PHA isolation from *A. latus* dry cells**

***Effect of mechanical agitations:*** In 1 hour period of pretreatment, vigorous mechanical agitations are required. Stirring with magnetic bar for a small scale extraction gives quantitative extraction yield. However, shaking and sonicating also give high % recovery as shown in Fig. 3. To set a control agitation, shaking with the Orbital shaker at 130 rpm was then used in most experiment unless stated otherwise.

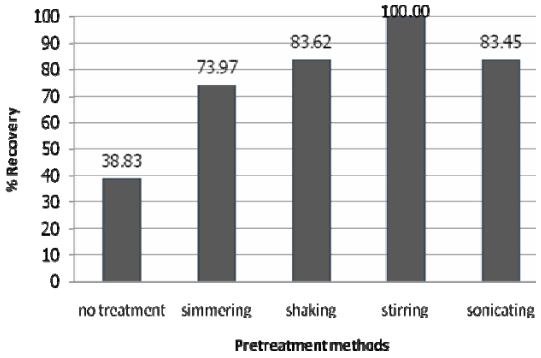


**Figure 3 Pretreatment agitation methods and their effects on % recovery of the PHA isolation from *A. latus* dry cells**

***Effect of pretreatment time:*** The time period used in pretreatment step tends to increase extraction yield and gives 100% PHA recovery in 2 hour. However, longer time of pretreatment can cause the loss of yield possibly due to PHA degradation. An attempt to increase pretreatment temperature shows low yield and high decomposition.

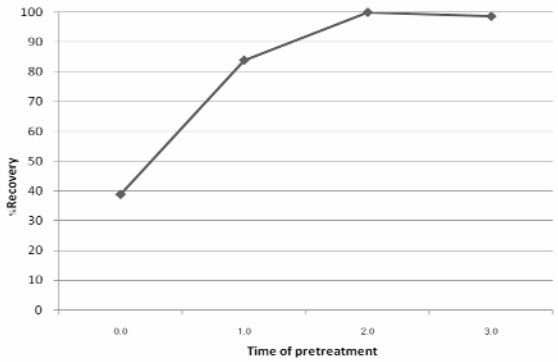


**Figure 4 The % recovery of PHA isolation at various time period in pretreatment**

**Extraction step**

***Effect of Solvents:*** The proper solvents for this step tend to be those "partial solvents": $CHCl_3$ and $CH_2Cl_2$ while "non solvents" give very low % recovery and polar protic solvents diminish extraction yield. Methanol and ethanol give low polymer purity since PHA is quite polar and hydroscopic and tends to decompose by moisture over time.

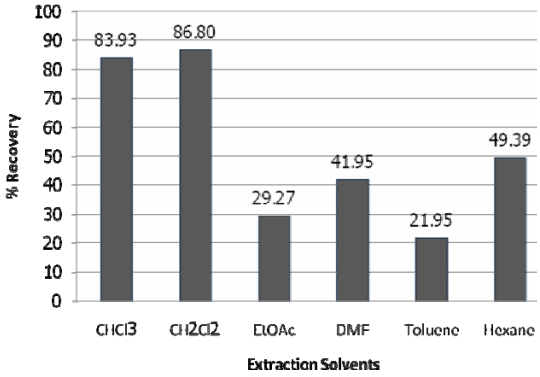


**Figure 5 Extraction solvents and %Recovery of the PHA extraction from *A. latus* using the continuous Soxhlet extractor.**

***Effect of extraction time:*** With the same extraction process described above using chloroform as extracting solvent for 5, 6, and 10 hours gives 84, 99 and 100% recovery, respectively.

***Effect of PHA content:*** Submission of dry cells with various PHA contents: 18.87%, 28.49%, 41.00% and 50.64% gives essentially the same %recovery as shown in Fig 6.

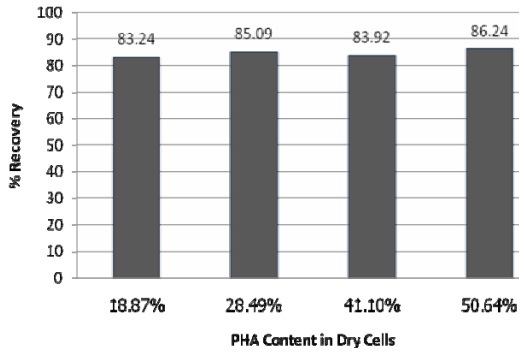


**Figure 6 % Recovery of PHA isolation with various PHA content samples**

**Solid-liquid extraction**

***Effect of Solvents:*** By this method, the extraction yield tends to be slightly lower than the continuous Soxhlet extraction method. However, in case of ethyl acetate, DMF and toluene give better result shown in Fig 7. A single study showed the slightly loss of yield became less when the PHA content is high above 60%.

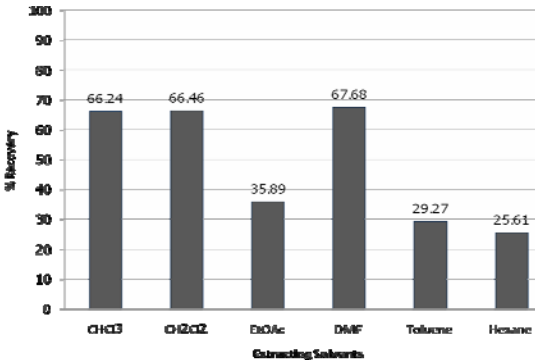


**Figure 7 Extraction solvents and %Recovery of the PHA extraction from *A. latus* using the direct solid-liquid extraction.**

***Effect of extraction time:*** An attempt to increase the shaking time from 5 hours to 10 hours in this case decreases extraction yield from 66 to 59% recovery.

***Effect of simmering temperature:*** By elevating the extracting temperature from RT or 30 $^{o}$C to 40 $^{o}$C dramatically decreasing the extraction yield from 66 to 44% recovery. This result may imply that the polymer starts to degrade as it heats over time and also explains loss of yield after 2 hours of pretreatment.

**Characterization of PHA**

In most cases, the obtained $^{1}$H NMR and FTIR spectra suggested that the isolated PHA from *Alcaligenes latus* fed with glucose as carbon source is poly(3-hydroxybutyrate) or PHB only with very high purity ($\geq$ 99%) especially after purification. However, when using alcohols as the extraction solvent, the obtained polymer usually contains lipid impurity. Thermograms of most PHA sample run by DSC 2910 showed only a sharp peak $T_m$ at 169 $^{o}$C without $T_g$. The average viscosity molecular weight of the obtained PHA fit in the range around 1.1 x $10^5$ Dalton.

**Discussion and Conclusion**

From this study, several extraction parameters can be used to obtain % recovery of PHA from *Alcaligenes latus* dry cells. In pretreatment step, short chain alcohols such as ethanol and methanol are preferable. Furthermore, agitations (such as stirring, shaking and sonicating) significantly accelerate pretreatment step to avoid PHA decomposition, hence increase PHA isolated yield. In extraction step, partial solvents especially chlorinated solvents such as dichloromethane and chloroform were found to give high PHA quality and high extraction yield. The direct solid-liquid extraction developed also give similar result with high PHA content dry cells 60% or more.

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