# การศึกษากระบวนการสกัดและหาคุณสมบัติเฉพาะของพอลิ-3-ไฮดรอกซีอัลคาโนเอตที่ผลิตจากเชื้อ *Alcaligenes latus*

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

*สริญญา ชวพันธ์ (Sarinya Shawaphun)*[1,2]*
*ธารา มานะงาน (Thara Manangan)*[1,2]

## บทคัดย่อ

เพื่อให้การสกัดพลาสติกชีวภาพชนิดพอลิ-3-ไฮดรอกซีอัลคาโนเอต (PHA) จากเชื้อ *Alcaligenes latus* ให้ได้ปริมาณมากและพอลิเมอร์มีคุณภาพดี จึงได้ทำการศึกษากระบวนการสกัดอย่างเป็นระบบ ทั้ง 3 ขั้นตอนย่อย คือ ขั้นตอนการเตรียมเซลล์ ขั้นตอนการสกัด และขั้นตอนการทำพอลิเมอร์ให้บริสุทธิ์ อาทิ ในขั้นตอนการเตรียมเซลล์ ทำให้เซลล์แตกด้วยตัวทำละลายอินทรีย์ ร่วมกับการใช้แรงเชิงกลหลายๆรูปแบบ การเปรียบเทียบวิธีและตัวแปรทางการสกัดด้วยตัวทำละลายต่างๆ ทั้งวิธีการสกัดแบบต่อเนื่องและการสกัดโดยตรง ส่วนการทำให้บริสุทธิ์นั้นเน้นการตกผลึกด้วยตัวทำละลายอินทรีย์ที่เหมาะสม จากนั้น นำพอลิเมอร์ที่สกัดได้มาวิเคราะห์เอกลักษณ์ด้วยเทคนิคอินฟราเรดและนิวเคลียร์แมกเนติกเรโซแนนซ์ สเปกโตรสโคปี และวิเคราะห์สมบัติเฉพาะทางความร้อนและน้ำหนักโมเลกุลของพอลิเมอร์ที่ได้ด้วยเทคนิคดิฟเฟอเรนเชียลสแกนนิงแคลอริเมทรี และการวัดค่าความหนืดของสารเจือจาง ตามลำดับ

## Abstract

In order to obtain high extraction yield and proper quality of 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, cell lytic pretreatment using various organic solvents and mechanical agitations were studied. Comparison of various extraction techniques and solvent extraction parameters including continuous extraction and direct extraction was made. The purification step mainly focused on recrystallization of the extracted polymer using proper solvents. Finally, most extracted polymers then were identified by FTIR and NMR spectroscopy and also characterized by differential scanning calorimetry (DSC) and dilute solution viscometry (DSV) for thermal property and molecular weight, respectively.

**คำสำคัญ:** พลาสติกที่ย่อยสลายได้ทางชีวภาพ, พอลิไฮดรอกซีอัลคาโนเอต, การสกัด

**Keywords:** Biodegradable plastic, Poly-3-hydroxyalkanoate, Extraction

[1] *Lecturer, Department of Industrial Chemistry, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand*
[2] *Researcher, Research Center of Nano-Industries and Bio-plastics, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand*
** Corresponding author, e-mail: sarinya73@yahoo.com*

## 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 (Smith, 2005; Fletcher, 1993). Besides starches and other polysaccharide biopolymers, a group of biodegradable polyesters called poly-3-hydroxyalkanoates (PHAs) which can be harvested from the energy storage granules inside the cytoplasm of many fermentable microorganisms (Braunegg et al., 2009) provide several excellent mechanical and physical properties in various plastic applications especially in medical applications e.g. medical apparatus, body part implants and surgical suture (Khanna and Srivastava, 2005; Pouton and Akhtar, 1996). In the last century most research topic focuses mainly on bacterial screening and fermentation processes as well as biodegradable plastic applications (Sudesh et al., 2000). However, the isolation process is yet a bit under investigated systematically (Lakshman and Shamala, 2006). Our preliminary study of PHA production from shake-flask fermentation of *Alcaligenes latus* (Wang and Lee, 1997; Wang and Inoue, 2001) fed with various carbon sources (Salehizadeh and Van Loosdrecht, 2004) 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-hydroxy butyrate-*co*-4-hydroxyvalerate-*co*-3-hydroxy valerate) 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 (TISTR) 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 (Jacquel et al., 2008). Moreover, the pretreatment solvents 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 (Marchessault and Yu, 2002). Our choice of solvents was made from their availabilities, toxicities and cell lytic properties. To avoid the decomposition of polymers by using strong surfactants, bases and oxidizing agents (Hahn et al., 1994), short chain alcohols with cell lytic property such as methanol, ethanol, n-propanol, isopropanol and n-butanol were used in this study (Jiang et al., 2006). Furthermore, agitations (such as stirring, shaking and sonicating) were applied to optimize the pretreatment step and increase PHA isolated yield (Harrison, 1991). As a study model for various extracting solvents, the Soxhlet extraction over 5 hours has been initially used. After optimization of the pretreatment step, the extraction step was then investigated by varying extraction solvents, extraction periods, extraction methods and PHA contents in *Alcaligenes latus* dry cells used (Hesselmann et al., 1999). The extraction solvents in the study were normally chosen by their prices and availabilities, PHA solubilities and boiling points (Terada and Marchessault, 1999). Also to understand the nature of the extracted PHA during the extraction process, non-polar solvents such as hexane and some polar protic solvents such as methanol, ethanol and propanols were also used in this step. Furthermore, medium polar aprotic solvents such as acetone, chloroform, dichloromethane, and ethyl acetate were included in this study to optimize PHA quality and extraction yield (Jiang et al., 2006). Finally, in order to enhance the isolated PHA purity, the solvent effect of recrystallization of the crude extracted polymer was investigated (Marchessault and Yu, 2002).

## Research Methodology

**Medium:**

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, $C_6H_8O_7FeNH_3$ 72 mg, and glucose 20 g as carbon source. 18 g of agar was added to the broth to give basal mineral medium agar used in this study. All mediums were sterilized at 121 °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 °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 °C for another 24 hours. Biomass was then centrifuged at 6,500 rpm under 4 °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 °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 contents of the fermented dry cells of 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 (Valappil et al., 2007; Seo et al., 1998). It was then heated to reflux in a silicone oil bath at 100 °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 °C. Helium was used as the carrier gas.

**Study of isolation process**

***Pretreatment step***

*Effect of solvent pretreatment:* 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 2 ml of cold methanol was added to re-precipitate PHA, and the dull white solid was obtained, filtered, dried, weighed and subjected to further analysis.

*Effect of mechanical agitation:* A suspension of dry cells (0.40 g) in 10 ml of ethanol was subjected to various agitation methods for an hour at 30 °C such as shaking at 130 rpm using Orbital shaker, stirring using magnetic stirrer or sonicating using ultrasonic bath and without agitation by just simmering in ethanol (Hwang et al., 2006). After a 15-minute centrifugation at 6,500 rpm, the obtained precipitate was subjected to 5-hour Soxhlet extraction and analyzed as described previously.

*Effect of pretreatment period:* A suspension of dry cells (0.50 g) in 10 ml of ethanol was shaken at 130 rpm using the Orbital shaker at 30 °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 extraction solvent:* 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 °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 such as chloroform, dichloromethane, *N,N*-dimethyl formamide, ethyl acetate, hexane, toluene, methanol, ethanol, propanol, butanol, isopropanol or *t*-butanol (Noda, 1998; Kurdikar et al., 2000; Gorenflo et al., 2001). After 5 hours 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 the polymer, and the obtained solid which then was filtered, dried, weighed and subjected to further analysis.

*Effect of extraction period:* The experiment was done the same as above using chloroform as the extraction solvent, however the extraction period was extended to 10 hours.

*Effect of PHA contents in dried cell:* The experiment was done the same as above using chloroform as the extraction solvent except the dry cells used in the study contained different amount of PHA accumulations including 18.87% w/w, 28.49% w/w, 41.10% w/w and 50.64% w/w.

***Purification Step***

*Effect of solvent precipitation:* Dry cells (4.00 g) suspended in 80 ml of ethanol was shaken at 130 rpm using the Orbital shaker at 30 °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 of chloroform. Each 1-ml aliquot of this solution was added to 2 ml of various cold solvents e.g. methanol, ethanol, propanol, isopropanol, ethyl acetate, hexane or acetone to re-precipitate. The obtained solid then was filtered, dried, weighed and subjected to further analysis.

**Solid-liquid extraction method**

*Effect of extraction solvent:* A suspension of dry cells (0.50 g) in 10 ml of ethanol was shaken at 130 rpm in Orbital shaker at 30 °C for an hour. After a 15-minute centrifugation at 6,500 rpm, the precipitate was then transferred into an Erlenmeyer flask containing 150 ml of various extraction solvents (chloroform, dichloromethane, *N,N*-dimethyl formamide, ethyl acetate, hexane or toluene). After shaking at 130 rpm in Orbital shaker at 30 °C for 5 hours, the mixture was poured into a 250-ml separatory 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 saturated NaCl solution, dried over anhydrous $Na_2SO_4$ and evaporated under vacuum to obtain about 1 ml of residue. Then 2 ml of cold methanol was added to re-precipitate polymer. The obtained white solid then was filtered, dried, weighed and subjected to further analysis.

*Effect of extraction period:* The experiment was done the same as above using chloroform as the extraction solvent except extraction period was extended to 6 and 10 hours.

*Effect of extraction temperature:* The experiment was done the same as above except the extraction temperature used was at 40 $^{o}$C.

**Characterization of PHA**

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

*FTIR spectroscopy:* The isolated PHA (0.1 g) was dissolved in 2 ml of chloroform, 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 Dilute 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 $a = 0.75$ (Higan, 1996).

## Results and Discussion

**Pretreatment step**

*Effect of pretreatment solvent:* In this cell lytic pretreatment, the result in Figure 1 indicates that the proper solvents 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.

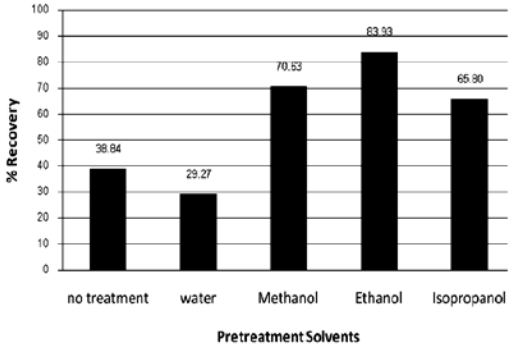


**Figure 1.** Cell lytic solvent effect on% recovery of the PHA isolation from *A. latus* dry cells.

*Effect of mechanical agitation:* In 1 hour period of pretreatment, vigorous mechanical agitation is required. Stirring with magnetic bar at 30 $^{o}$C for a small scale extraction gives quantitative extraction yield. Moreover, shaking and ultra-sonicating also give high % recovery as shown in Figure 2. Due to its practicality and controllability, shaking at 30 $^{o}$C with the Orbital shaker at 130 rpm was then used in most experiments unless stated otherwise.

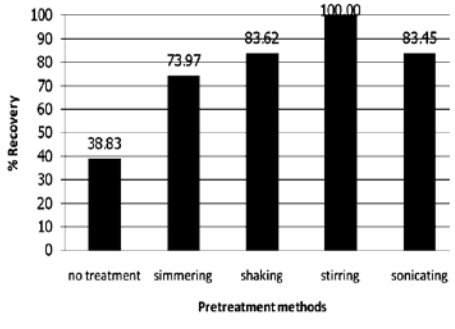


**Figure 2.** Pretreatment agitation methods and their effects on % recovery of the PHA isolation from *A. latus* dry cells.

*Effect of pretreatment period:* The time period used in pretreatment step tends to increase extraction yield and gives 100% PHA recovery in 2 hour (Figure 3). However, longer time of pretreatment can cause the loss of yield possibly due to PHA degradation. $^{1}$H NMR spectrum of ethanolic residue from the pretreatment step after evaporation *in vacuum* showed mostly lipid and also some PHA.

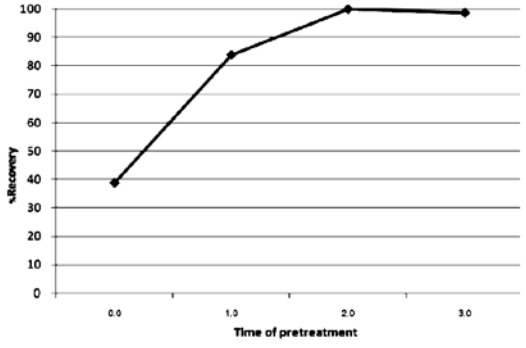


**Figure 3.** (Percent) Recovery of PHA isolation at various time periods in pertreatment.

*Effect of extraction solvent:* 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 (Figure 4). Methanol and ethanol give low polymer purity since PHA is quite polar and hydroscopic and tends to decompose by moisture over time.

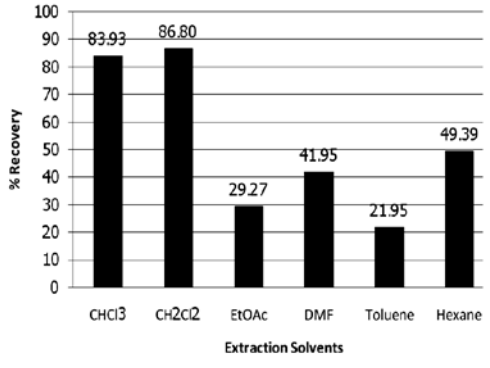


**Figure 4.** Extraction solvents and % recovery of the PHA extraction from *A. latus* using the continuous Soxhlet extractor.

*Effect of extraction period:* 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. When extending the extraction period, % PHA recovery was increased.

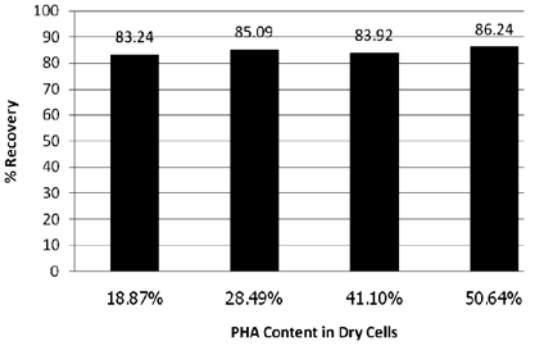


**Figure 5.** (Percent) Recovery of PHA isolation with various PHA content samples.

*Effect of PHA content:* Submission of dry cells with various PHA contents: 18.87%, 28.49%, 41.00% and 50.64% give essentially the same % recovery as shown in Figure 5. This method is effective for all PHA contents.

**Solid-liquid extraction**

*Effect of extraction solvent:* 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 Figure 6. A single study showed the loss of yield became less when the PHA content is above 60%.

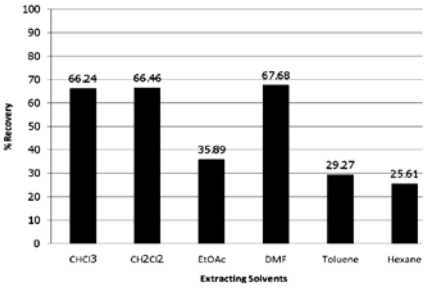


**Figure 6.** Extraction solvents and % recovery of the PHA extraction from *A. latus* using the direct solid-liquid extraction.

*Effect of extraction period:* An attempt to increase the extraction period from 5 hours to 10 hours in this case decreases extraction yield from 66 to 59% recovery (data not shown here).

*Effect of simmering temperature:* By elevating the extracting temperature from room temperature or 30 $^{o}$C to 40 $^{o}$C dramatically decreasing the extraction yield from 66 to 44% recovery (data not shown here). 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 solely poly (3-hydroxybutyrate) or PHB 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 samples 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.

## Recommendations

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) for an hour at 30 $^{o}$C significantly accelerate the cell lytic pretreatment step to avoid PHA decomposition, hence increase PHA isolated yield. In the extraction step, partial solvents especially chlorinated solvents such as dichloromethane and chloroform were found to give high PHA quality and high extraction yield. The solid-liquid extraction developed also give similar result with high PHA content dry cells 60% or more.

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