การนำกลับพอลิ3-ไฮดรอกซีบิวทิเรตจาก *Cupriavidus necator* สายพันธุ์ A-04
โดยใช้ตัวทำละลายที่เป็นมิตรต่อสิ่งแวดล้อม

นางสาวณัตติพร ยะบึง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

RECOVERY OF POLY(3-HYDROXYBUTYRATE) PRODUCED FROM *Cupriavidus necator* strain A-04 USING ENVIRONMENTAL FRIENDLY SOLVENTS

Miss Nuttipon Yabueng

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การใช้ตัวทำละลายที่เป็นมิตรต่อสิ่งแวดล้อมเพื่อนำกลับพอลิไฮดรอกซีแอลคาโนเอต (polyhydroxyalkanoates, PHAs) จากเซลล์จุลินทรีย์เป็นหนึ่งในปัจจัยที่สำคัญสำหรับการพัฒนาเทคโนโลยีที่มีประสิทธิภาพและยั่งยืนสำหรับกระบวนการผลิตระดับอุตสาหกรรม ในงานวิจัยนี้ตัวทำละลายที่เป็นมิตรต่อสิ่งแวดล้อมที่ถูกคัดเลือกคือ 2-เมทิล เตตราไฮโดรฟูแรน 1,3-โพรเพนไดออล 1,3-ไดออกโซแลน และเอทิล แลกเตท ถูกนำมาทดสอบการนำกลับพอลิไฮดรอกซีบิวทิเรต (polyhydroxybutyrate, PHB) จาก *Cupriavidus necator* สายพันธุ์ A-04 และเปรียบเทียบกับการใช้คลอโรฟอร์ม การนำกลับ PHB ถูกทดสอบกับเซลล์แห้งและเซลล์เปียกที่ปริมาตรในช่วง 2 มิลลิลิตร และ 40 มิลลิลิตร โดยใช้อัตราส่วนของ PHB ต่อตัวทำละลายเท่ากับ 5% (w/v) เซลล์ซึ่งมี PHB อยู่ภายในเซลล์ถูกเตรียมขึ้นโดยการเพาะเลี้ยงแบบแบต์ชในถังหมักขนาด 5 ลิตร ผลการสกัด PHB จากเซลล์แห้งที่ 80 องศาเซลเซียส นาน 6 ชั่วโมงพบว่า 1,3-ไดออกโซแลนให้ PHB ที่มีความบริสุทธิ์ 84.3±5.4% และมีประสิทธิภาพการนำกลับ 86.3±4.0% จากนั้นนำน้ำมาใช้เป็นพลาสติกไซเซอร์ที่สามารถก่อให้เกิดการแยกเฟสของฟิล์ม PHB ออกจากของผสมตัวทำละลายและทำให้เพิ่มความบริสุทธิ์สูงถึง 99.2±1.4% เพื่อลดขั้นตอนการทำแห้งของเซลล์จึงนำภาวะการนำกลับเดียวกันมาประยุกต์ใช้ในการสกัด PHB ออกจากเซลล์เปียกและได้ PHB ที่มีความบริสุทธิ์ 98.5±8.6% และมีประสิทธิภาพการนำกลับ 63.4±3.9% เพื่อประเมินคุณภาพของ PHB บริสุทธิ์จึงนำแผ่นฟิล์ม PHB ไปทดสอบสมบัติเชิงความร้อน สมบัติเชิงกล และการกระจายตัวของน้ำหนักโมเลกุล พบว่าวิธีการนำกลับที่ได้รับการพัฒนาจากงานวิจัยนี้ให้ผลการทดสอบเชิงคุณภาพของ PHB ที่ใกล้เคียงกันกับผลของ PHB ที่นำกลับโดยการใช้คลอโรฟอร์มและ PHB ทางการค้า

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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5671958923 : MAJOR MICROBIOLOGY AND MICROBIAL TECHNOLOGY
KEYWORDS: 1,3-DIOXOLANE / SOLVENT EXTRACTION / POLYHYDROXYBUTYRATE / PHA RECOVERY / ENVIRONMENTAL FRIENDLY SOLVENT

NUTTIPON YABUENG: RECOVERY OF POLY(3-HYDROXYBUTYRATE) PRODUCED FROM *Cupriavidus necator* strain A-04 USING ENVIRONMENTAL FRIENDLY SOLVENTS. ADVISOR: ASST. PROF. SUCHADA CHANPRATEEP NAPATHORN, Ph.D., 117 pp.

The use of environmental friendly solvents to recover polyhydroxyalkanoates (PHAs) from microbial cells is one of important factors for the development of efficient and sustainable technology for industrial production process. In this study, selected environmental friendly solvents: 2-methyl tetrahydrofuran, 1,3-propanediol, 1,3-dioxolane and ethyl lactate, were investigated to recover polyhydroxybutyrate (PHB) from *Cupriavidus necator* strain A-04 and compared that with the use of chloroform. The PHB recovery was investigated with dried cells and wet cells at volumes ranging from 2 mL to 40 mL using a PHB to solvent ratio of 5% (w/v). Cells contained intracellular PHB was prepared by batch cultivation in 5 L fermentor. Based on PHB extraction from dried cells at 80 °C for 6 h, 1,3-dioxolane gave 84.3±5.4% of purity with 86.3±4.0% of recovery. Next, water was used as plasticizers that can cause phase separation of PHB film from the solvent mixture and increase the purity up to 99.2±1.4%. To eliminate step of cell drying, the same recovery conditions were applied to extract PHB from wet cells and 98.5±8.6% of purity with 63.4±3.9% of recovery were obtained. To evaluate the quality of purified PHB, thermal properties, mechanical properties and molecular weight distribution were tested. It was found that our developed recovery method provide similar quality of PHB film compared with those of PHB from chloroform extraction and commercial PHB.

Department:	Microbiology	Student's Signature	.....
Field of Study:	Microbiology and Microbial Technology	Advisor's Signature	.....
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LIST OF ABBREVIATIONS

%	=	Percent
°C	=	Degree Celsius
g	=	Gram
g/L	=	Gram per liter
h	=	Hour
min	=	Minute
mL	=	Milliliter
mm	=	Millimeter
ref.	=	Reference
v/v	=	Volume by volume
vvm	=	Volume per volume per minute
w/v	=	Weight by volume

CHAPTER I
INTRODUCTION

1.1 Statement of the problem

Plastics are used in everyday life products, for instance, plastic bags, packaging materials, vehicles, construction equipments, electronic devices, medical materials and agricultural equipments. Advances in material science and technology offer a number of advantages for plastics such as their light weight, durability, low cost and manufacturing easiness. Hence, their properties are suitable for their usage in a variety of applications. These factors have increased the popularity of plastic usage. At the same time, plastic waste accumulation has been increased in environment as well. It has been well known that plastics are durable in the environment for over 100 years as they are difficult to be degraded naturally. Plastic waste accumulation appears in both terrestrial ecosystems and aquatic ecosystems which are harmful to wild animals. Furthermore, commercial plastics are petroleum based products derived from crude oil that is non-renewable source. This problem has been realized and solved in various ways such as invention of bioplastics to replace the use of petroleum based plastics [1, 2]. Biodegradable plastics are considered as alternative natural materials to achieve sustainability because they are derived from renewable resources. Biotechnological process are employed to produce biodegradable polymers such as polyhydroxyalkanoates (PHAs), a large group of linear polyesters accumulated in granules of microbial cells served as carbon and energy storage compound. The mode of PHAs formation in term of the relationship with the growth of microorganisms has been reported as both growth and non-growth associated product and can be found in all three classes of Gaden's classification: (1) growth associated products in which they are produced directly from the energy metabolism of primary

carbohydrates supplied, (2) product that partially related to growth in which they are produced indirectly from carbohydrate metabolism and (3) products that are not related to bacterial growth in which they are apparently unrelated to carbohydrate oxidation [3]. Their excellent characteristics are biodegradability and biocompatibility. In addition, they are thermoplastic polyesters similar to polypropylene (PP) and polyethylene (PE) that are petroleum based plastics. Several developed countries have realized the importance of bioplastic industries and have been supporting the use of biodegradable polymers instead of petrochemical based plastics. However, PHAs are still not successful in commercial scale because of two major factors. The first factor is high cost of raw material used. More recently, the feasibility of agricultural wastes have been examined to be used as carbons sources including other wastes such as glycerol and waste water derived from food industries to reduce the overall cost. The second factor is recovery process cost including environmental cost which are a main problem to apply in the industry scale.

In this study, we focused on organic solvent extraction which is the first method to extract the polymer from bacteria cells [4]. This method allows high yield of product recovery, high purity and also does not degrade polymer. Moreover, solvent extraction method can reduce endotoxin content in PHAs produced from Gram negative bacteria [5]. Some chlorinated hydrocarbon solvents such as chloroform, 1,2-dichloroethane and methylene chloride perform well for extraction of PHAs from biomass. However, they are toxic and harmful to worker health and environment [6, 7]. Thus, we focused on environmentally friendly solvent to extract PHA from bacteria cells instead of using the toxic solvents. The alternative solvents; 2-methyl tetrahydrofuran, 1,3-propanediol, 1,3-dioxolane and ethyl lactate were selected to extract PHB produced from *Cupriavidus necator* strain A-04 in this study. Data of the environmental friendly solvents are; 2-methyltetrahydrofuran is produced from renewable resources. It has been used as an

alternative for tetrahydrofuran and dichloromethane [8]. 1,3-dioxolane is nontoxic, odorless and environmentally friendly that is prepared by acetalization of aldehydes and ketalization of ketones with ethylene glycol. It can be used as an alternative to dichloromethane and methyl ethyl ketone [9]. 1,3-propanediol is a product obtained from a renewable source such as corn which is non-flammable, non-explosive, no effects on the health of animals and not persistent in environment [10]. The last one, ethyl lactate is generated from biomass raw materials through fermentation [11]. It has been used for long time as ingredients in industrial uses as a solvent in synthetic polymer [12].

1.2 Objective

The aim of this study was to recover PHB produced from *C. necator* strain A-04 using environmentally friendly solvents.

1.3 Scope of the research

- Dried cells and wet cells of *C. necator* strain A-04 containing PHB granules were prepared to be the starter cells for PHB extraction by using environmental friendly solvents.
- Solubility and precipitation of purified PHB in various environmental friendly solvents were investigated in various ratio of purified PHB in solvent solution (2.5, 5, 7.5 and 10% w/v).
- Dried cells containing PHB were extracted in 2 mL and 40 mL by the efficiently solvents and suitable ratio of purified PHB in solvent solution the obtained from previous step. In addition, precipitation was also tested to find proportional a precipitant.

- PHB extraction from wet cells were investigated in 2 mL and 40 mL to reduce energy and time consumption during cell separation and dehydration process.
- PHB was evaluated the quality of purified PHB, thermal properties, mechanical properties and molecular weight distribution by comparison with those of PHB from chloroform extraction and commercial PHB.

CHAPTER II
LITERATURE REVIEW

2.1 Plastics

Plastics are the group of polymers that have been used more than half of century. The root word of plastic is derived from Greek word “plastikos” meaning meltable to different shapes [13].

Plastics are used familiarly in a huge and growing range of applications such as packaging, construction, medicine, transportation, agriculture and electrical equipments etc. Their history begins in early 1863, John Wesley Hyatt, an American carpenter who created celluloid from camphor and cellulose nitrate. This material has a suitable property for a billiard ball stead of an ivory. Likewise, it is popular for making accessories, collars, buttons, toys and filmstrips because of its properties such as oil and water resistant, colorable and inexpensive. In the other words, celluloid is a first semi-synthesis plastic that is produced form modified cellulose. This is a starting point of plastic industries. However, celluloid is flammable and explosive when nitro groups (NO_2) are excessive [14]. Therefore, celluloid is not popular anymore. In 1907, bakelite was the first synthetic plastic material synthesized by Leo Baekeland. Bakelite produces from reaction between formaldehyde and phenol to be the small organic molecules. This material is a hard plastic, heat resistant, easy formability, colorable and inexpensive. In the earlier, bakelite was used to produce electric insulator, household products and accessories as lastly it became to the material of numerous applications [14, 15]. Currently, plastics are composed of organic element (carbon) and inorganic elements (hydrogen, oxygen, chlorine, silicon and nitrogen) [16]. Most conventional plastics are derived from by-products of petroleum industries [17]. The production of plastic starts with fractional

distillation process which separates crude oil or natural gas components. The hydrocarbon compounds, naphtha from previous distillation process is a crucial substrate for the production of plastic [18].

2.2 Characteristics of plastics

Plastics can be classified by thermal properties into two categories.

2.2.1 Thermoplastics

Thermoplastics are a group of linear polymer that have covalent bonds between monomer molecules and van der Waal bonds between polymer chains. They can be melted and formed many times without extreme damage of properties. Therefore, thermoplastics can be recycled into new products. However, their limitation is unstable in high temperature because they will be twisted or misshaped. This examples are polyamide, polymethyl methacrylate, polypropylene, polystyrene, low density polyethylene (LDPE) and high density polyethylene (HDPE).

2.2.2 Thermosetting plastics

Thermosetting plastics are complex network structures which are resistance to higher temperatures. This makes them are suited for permanent materials production. However, thermosetting plastics are incapable of recycling because there are covalent bonds are broken and the material properties cannot be remained. Their examples, epoxy resin, melamine formaldehyde, polyester resin and urea formaldehyde.

2.3 Plastic pollution

Recently, worldwide plastics production and consumption have increased continuously and 140 million tons per annum of synthetic plastics have been produced in the world [19]. Physical and chemical properties of plastics are attractive qualities that are suitable for using in the wide range of applications such as strength, lightness, flexible, water resistance and relatively inexpensive. At the same time, the rapid rise of plastics and the lack of ability in degradation in the environment are the causes of plastic wastes accumulation in ecosystems. Currently, plastic wastes are disposed by landfilling, incineration and recycling. However, these methods are not the most sufficient solution because they are also connected with various problems linked to the environment. For instance, persistent organic pollutants (POPs) (furans and dioxins one the most well-known) are generated by combustion of polyvinylchloride (PVC) plastics [20]. Hence, plastic alternatives are fascinated to study. Especially, bioplastics which are a good choice for replacement commercial plastics and troubleshooting of plastic wastes.

2.4 Polyhydroxyalkanoates (PHAs)

Increasing amounts of plastic wastes accumulation in environment results in increasing interest in biopolymers. In this study, we focused on polyhydroxyalkanoates that are a group of biopolymers. Polyhydroxyalkanoates (PHAs) are biological polyesters of hydroxyalkanoic acids which are synthesized by several microorganisms, particularly bacteria in family Halobacteriaceace [21, 22]. In 1888, transparent granules of PHA were first time noticed by Beijerinck [23]. Figure 1 shows PHA accumulation in granules of *C. necator* DSM 545 [24] and *Ralstonia eutropha* A-04 that were observed by using transmission electron microscope [25]. Then, in 1923, unknown material was extracted from granules of *Bacillus megaterium* using chloroform as an extraction solvent [21]. After that, the material was analyzed and described by Lemoige as a homopolyester of

3-hydroxybutyric acids, namely polyhydroxybutyrate (PHB) [26, 27]. Next, PHB was described as a rapid biodegradable material by microorganisms in nature [22]. PHAs have been dramatically interested as an alternative to petroleum based plastics because of environmental concern trend and climate change.

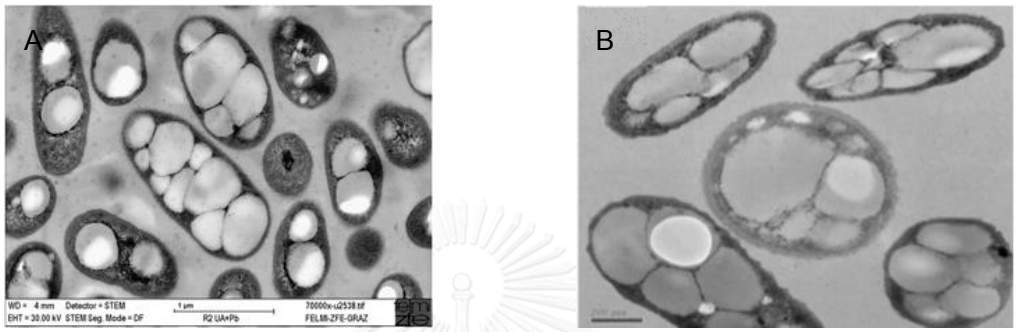


Figure 1. PHA granules (A) *C. necator* DSM 545 [24] and (B) *R. eutropha* A-04 [25] that were observed by using transmission electron microscopy.

PHAs are synthesized by bacteria fermentation. The main carbon sources are biomass-derived feedstocks such as sugar and oil which are renewable substrates. After the end of use, PHAs will be degraded by enzyme depolymerase of microorganisms in nature (Figure 2). Moreover, PHA is biocompatible and can be inserted into the mammals without removing again. For instance, PHA polymer was implanted in the mouse, the result showed that mass of the polymer lost less than 1.6% (w/w) after 6 months [28]. In various tested on animal tissues had shown PHAs group to be compatible with a range of tissues [29].

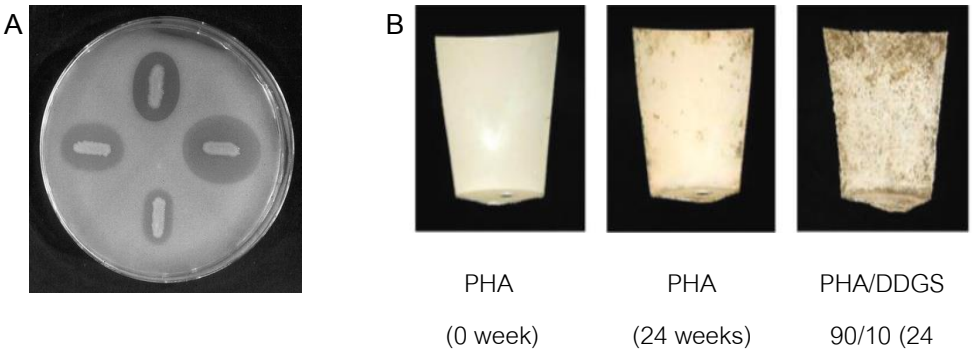


Figure 2. PHAs are degraded by enzyme depolymerase secreted from microorganisms. (A) Growth of recombinant *Escherichia coli* harboring the PHO depolymerase gene of *Pseudomonas fluorescens* on agar medium that contain the latex of PHO. (B) PHA and PHA/distill's dried grains with soluble (DDGS) 90/10 before and after 24 weeks of degradation time in soil medium [30].

2.5 Chemical structure of PHAs

PHAs are consisted of 3-hydroxy fatty acid monomers which are liner and head-to-tail linkages (Figure 3) [31]. Normally, PHAs contain 10^3-10^4 monomers, that are accumulated as inclusion of 0.2-0.5 μm in diameter [32]. However, structures, physio-chemical properties, monomer composition and number and size of granules depend on types of microorganisms and also carbon sources [33].

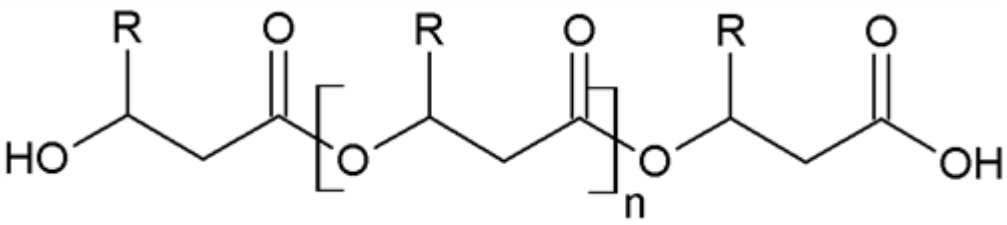


Figure 3. A head to tail configuration of the general structure of PHAs.

PHAs are classified by an alkyl group on β position that can be varied from methyl to tridecyl and divided into two main groups, as follows [34].

1. Short-chain-length PHAs (scl-PHAs) are contained of 3-hydroxy fatty acid monomer units with 3 to 5 carbon atoms. They have properties similar to polypropylene (PP) and polyethylene (PE).

2. Medium-chain-length PHAs (mcl-PHAs) are contained of monomer units ranging from 6 to 14 carbon atoms. Their unique characteristics are elastomer and rubber [35].

PHAs have wide properties depending on the monomer composition that includes homopolymer, copolymer or terpolymer. More than 150 different PHA monomers can be copolymerized within this group to provide materials with different properties [36, 37].

2.6 Polyhydroxybutyrate (PHB)

PHB is a member of PHA family that accumulated by bacteria in nature and has been widely studies as a model in the development of production [38]. PHB is scl-PHA that consists with CH_3 as functional group (Table 1). Its properties are water insoluble, oxygen resistant and thermoplastic. On the other hand, drawbacks of PHB are fragile and brittle due to highly crystalline. Table 2 shows comparison between physical properties of PHAs and petroleum based polymers [38-40].

Table 1. Functional group of PHAs [38-40].

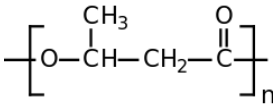
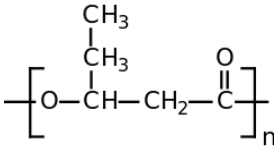
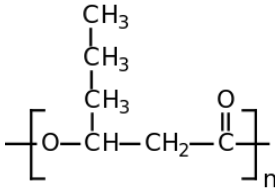
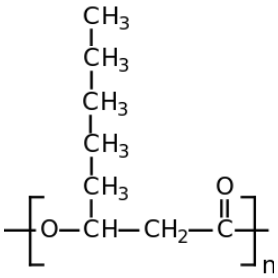
Name	Functional group	Structure
scl-PHA		
poly(3-hydroxybutyrate) (PHB)	methyl	
poly(3-hydroxyvalerate) (PHV)	ethyl	
mcl-PHA		
poly(3-hydroxyhexanoate) (PHH)	propyl	
poly(3-hydroxyoctanoate) (PHO)	pentyl	

Table 2. Physical properties of PHAs [41].

Polymer	Melting temperature (°C)	Glass transition temperature (°C)	Young's modulus (GPa)	Elongation to break (%)	Tensile strength (MPa)
P(3HB)	180	4	3.5	5	40
P(4HB)	53	-48	149	1000	104
P(3HB-co-20%3HV)	145	-1	1.2	50	20
P(3HB-co-16%4HB)	150	-7	-	444	26
P(3HB-co-10%3HHx)	127	-1	-	400	21
P(3HB-co-6%3HD)	130	-8	-	680	17
Polypropylene (PP)	176	-10	1.7	400	34.5
Polystyrene (PS)	240	100	3.1	-	50

2.7 Pathway of PHAs synthesis

The PHAs synthesis genes and other related PHAs synthesis genes are often clustered in bacterial genomes (Figure 4) [42, 43].

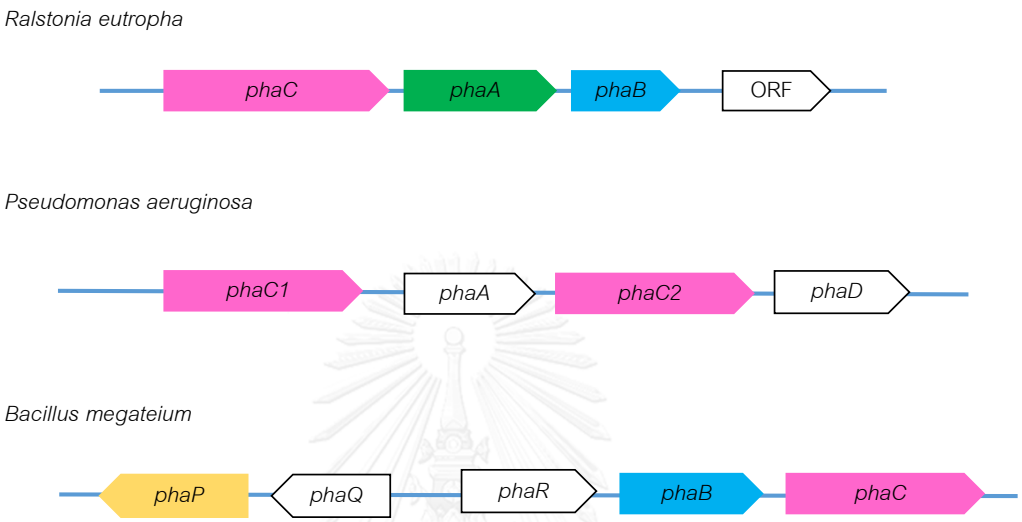


Figure 4. Genetic organization of representative polyester synthase genes encoding the various enzymes. *PhaC*, gene encoding PHA synthase; *phaE*, gene encoding subunit of PHA systhase; *phaA*, gene encoding β-ketothiolase; *phaB*, gene encoding acetoacetyl-CoA reduclase; *phaR*, gene encoding regulator protein; ORF, open reading frame with unknown function; *phaZ*, gene encoding PHA depolymerase; *phaD*, open reading frames with unknown function; *phaP*, gene encoding polyhydroxyalkanoic acid inclusion protein; *phaQ*, poly-beta-hydroxybutyrate-responsive repressor. (modified from [42])

PHAs biosynthesis had been considered to be the simplest synthesis process, with the best- studied in *R. eutropha.* In this case, the genes for PHA synthase (*phaC*), β-ketoacetyl-CoA reductase (*phaA*) and NADP-dependent acetoacetyl-CoA reductase (*phaB*) encoded on the *phaCAB* operon and its size around 4 kbp downstream of this operon (Figure 5) [44]. Process of PHB synthesis begins with β-ketothiolase (*phaA*) links two acetyl-CoA molecules to form acetoacetyl-CoA. Then, acetyl-CoA reductase (*phaB*) reduces acetoacetyl-CoA by NADH to (R)-3-hydroxybutyryl-CoA. Finally, (R)-3-hydroxybutyryl-CoA is polymerized to PHB and coenzyme-A being released by PHA synthase (*phaC*) (Figure 5) [41, 43, 45, 46].

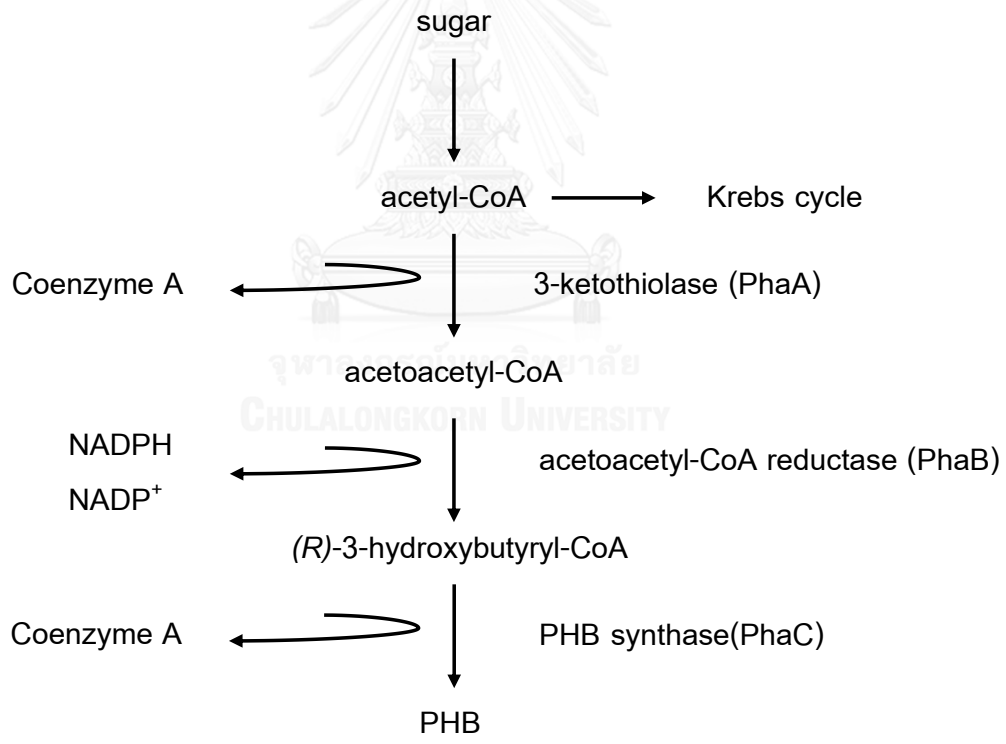


Figure 5. Bioplastic pathway of PHB form sugars (modified from [41])

2.8 Applications of PHAs

PHAs have been considered to replace petroleum-based plastics because they are becoming more expensive as petroleum prices continue to increase. Currently, PHA products have been used in a wide range of applications as follows.

2.8.1 Packaging

Biodegradable polymers are developed to be an alternative to conventional food packaging polymers. Especially, PHAs provide high quality applications for food preservation. Levkane *et al*. (2008) investigated quality of pasteurization system for salad with meat and mayonnaise. The samples were packed in vacuum using conventional packaging films (PE and PP) as well as bio-based packaging (polylactic acid (PLA) and PHB) [47]. Likewise, orange juice that was contained in bioplastic packages (PLA and PHB) had high quality product as high-density polyethylene (HDPE) [48]. In addition, fat rich food (mayonnaise, margarine and cream cheese) are possible to pack in PHB film instead of PP [1]. Therefore, PHB is probably can be applied in food packaging such as acid foods, acidic beverages and other fatty foods [1, 47, 48].

2.8.2 Medical applications

PHAs are applied in several medical applications. The benefits of PHAs in the medical field are biodegradability and biocompatibility [41]. In fact, 3-hydroxybutyric acid is a regular component of blood at concentrations between 0.3 to 1.3 mmol/L [49]. Yang *et al*. (2002) had demonstrated the biocompatibility of PHB and P(HB-*co*-HHx) *in vitro*. Mouse fibroblast cell line L929 was inoculated on the PHB and P(HB-*co*-HHx) films, mixture of PHB and P(HB-*co*-HHx) with PLA as a control experiment. The results showed that the viable cell number on PLA film and PHB film were $1.8\times10^{2}/cm^{2}$ and $8.8\times10^{2}/cm^{2}$, respectively. Meanwhile, the viable cell number on blending PHB and P(HB-*co*-HHx) film

was increased from $9.7\times10^{2}/cm^{2}$ to $1.9\times10^{5}/cm^{2}$ on PHB/ P(HB-*co*-HHx) blended film in ratios of 0.9/0.1 and 0.1/0.9, respectively [50]. Moreover, PHB, P(3HB-*co*-3HV), P(4HB) P(3HO) and P(3HB-*co*-3HHx) are regularly applied in tissue engineering such as bone cements, ostesynthetic materials and surgical sutures [51]. In addition, copolymer of methyl methacrylate and PHB for total joint replacement surgery is produced in commercial scale under acrylic bone cement brand, Antibiotic Simplex® (AKZ, Hownedica New Zealand, Div.of Pfizer Labs) [52].

2.8.3 Agriculture applications

Procter and Gamble have invented a copolymer that is consisted of mainly 3(HB) and mcl monomers. The copolymer can be used as biodegradable agriculture film and a capsule for releasing fertilizers, herbicides and insecticides under trademark of Nodex™ [51].

2.9 PHA recovery

Isolation and the purification of polymers are a key factors for PHA production system. This is a challenge in field of PHAs study for the industrial scale production of PHAs [53]. The principle of PHAs recovery process should lead to maintain high purity and recovery yield including polymer properties at a low overall cost [54]. There are several methods for the extraction and recovery of PHAs from bacterial cells depending on the purpose of PHA applications. The methods can be classified as follows.

2.9.1 Solvent extraction method

Organic solvent extraction is the oldest method. This method was first reported by Lemoigne (1923) on *B. megaterium* by using chlorinated hydrocarbon solvents such as chloroform, 1,2-dichloroethane, methylenechloride, propylene and ethylene carbonates [55]. Then, Baptist (1967) had used solvent mixtures such as chloroform/methanol and dichloromethane/ethanol for PHAs extraction [55]. After that, there are several other solvents that are reported in PHAs extraction from bacterial cell. For example, liquid halogenated solvents such as chloroethane and chloropropane were used for PHAs extraction from *R. eutropha*. The best results found that they do not affect the functional group of PHAs [56]. In addition, the use of 1,2-propandiol for PHA extraction at 140 ºC gave 79% of recovery and 99.1% of purity, diethyl succinate for PHA extraction at 110 ºC gave 90% of recovery and 100% of purity and butyrolactone for PHA extraction 110 ºC gave 90% of recovery and 99.5% of purity [57]. The principle of solvent extraction method can be divided into two main steps. First step is the adjustment of cell membrane permeability. Second step is dissolution and release of PHAs from the cells [58]. The non-solvent precipitations are required after extraction step so that PHAs solid can be separated from the solvents. Typically, low-molecular alcohols (mainly ethanol or methanol), hexane, ether, acetone and water are often used to precipitate PHAs [59]. Moreover, precipitation process is always employed in the purification process so that non PHA polymer material cell (NPMC) can be reduced and purity can be increased [60]. This method is the most widely selected method to extract PHAs from the cells and also used routinely in the laboratory scale because of a simple and rapid process [58, 61]. The strong points of this method provide high purity of PHA polymer with high molecular weights. In addition lipopolysaccharides (LPS), a major component of the outer membrane of Gram negative bacteria was dissolved by solvent. Therefore, this method is possible to provided high pure polymer and high molecular weight [53, 58]. However,

solvent extraction does not success in industrial scale because of its high operation costs and high toxicity from volatile solvents. Furthermore, high viscosity of the extracted polymer solution is a serious problem because the cell derbies are difficult to be separated. The previous studies have suggested that PHA concentration in the solvent extraction process should not be exceeded 5% (w/v) [58, 62-64].

2.9.2 Digestion method

The development of fermentation process had provided high PHA content more than 80% of cell dry mass (CDM) that are high proportion of PHA yield per NPMC [65]. Therefore, a digestion methods were applied to remove the NPMC in order to obtain free PHA granules [59]. Figure 6 shows PHA granules which are surrounded by the cell membrane. This cell membrane protects PHAs from chemical exposure so that it remains the high molecular weight. The hydrophilic components of the NPMC are converted to water-soluble substances by the reaction of chemicals or enzymes. Lastly, PHA granules can be released and isolated by filtration, floatation or centrifugation. This method can be divided into 2 types as follows.

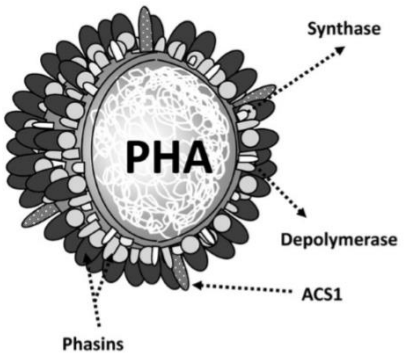


Figure 6. Schematic view of a PHA granule [66].

2.9.2.1 Chemical digestion

Several studies have focused on PHA recovery by chemical digestion method because this method is uncomplicated and fast. Sodium hypochlorite is the most popular chemical for PHA extraction from bacterial cell because it provides high purity with low cost applicable to industrial scale production and suitable for environmental impact [4, 67]. Cell components are oxidized by sodium hypochlorite at high pH value and become water soluble [68]. However, sodium hypochlorite damages the polymer that leads to a decrease of molecular weight of polymer by 50-70% [67, 69]. In addition, sodium hypochlorite treatment has other disadvantages such as the risk of formatting toxic halogenated compounds and difficulty of polymer separation [70].

Surfactants such as sodium dodecyl sulfate (SDS), triton X-100, palmitoyl carnitine and betaine can extract PHA granules from microbial cell. Surfactant monomers interfere cell membrane by integrating itself into the lipid bilayer membrane at low concentrations. At the critical micelle concentration, the membrane is disrupted to develop micelles of surfactant and membrane phospholipids which leads to the release of PHA into the solution [70]. Another function of the surfactant is solubilization of protein and other NPMC [7]. For example, 5% (w/v) SDS were examined to extract PHA from *R. eutropha* and recombinant *E. coli* for 3 to 6 h. These conditions gave 95% of recovery yield while high purity was increased when incubation time was increased [71]. In addition, the recovery PHB directly from high cell density culture broth had been investigated in *R. eutropha* using SDS. The results showed that over 95% of purity and 90% of recovery was obtained under various cell concentrations of 50 - 300 g dry cell/L [72]. Therefore, the advantages of surfactant are able to recover PHA polymer without polymer degradation and also recover the polymer directly from high cell densities. However, only surfactant digestion

provides the low degree of purity, other chemical agents such as sodium hypochlorite and sodium hydroxide are required [53].

2.9.2.2 Enzymatic digestion

The enzymatic digestion method was developed to replace solvent extraction under the patent of Imperial Chemical Industries (London, UK). This method is called as "Zeneca process" [53, 73]. In last decade, enzymatic digestion to recover PHB had been investigated in *R. eutropha* DSM 545. The result showed that 88.8% of PHB purity was obtained using 2% of bromelain (enzyme mass per biomass) at 50 ºC and pH 9. However, pancreatin was used for reducing the high cost of bromelain. It gave 90% of PHB purity and reduced the cost in 3 times of bromelain cost [74]. Likewise, combination of alcalase, SDS and ethylenediaminetetraacetic acid (EDTA) gave around 90% of purity and recovery [75]. In fact, the strong points of this technique are specificity and mild operational condition that make a high quality of PHAs. In other words, the disadvantages of this technique are high cost of enzymes and complicated process [58, 59, 74].

2.9.3 Mechanical disruption method

Mechanical disruption technique is used typically to recover intracellular proteins but it is not popular in recovery of PHAs from bacterial cell [64, 76]. Several mechanical methods have been employed to disrupt cells such as bead milling, high-pressure homogenization and ultra-sonication [64]. Especially, high-pressure homogenizers and bead mills are the most popular method to be used in large-scale production [77]. However, the major drawbacks are high capital investment cost, long processing time and difficulty in scaling up [78, 79].

1. Bead mill

The activity of bead mills occur in the contact zone by the shearing and energy transfer from beads to cells [58]. The considerable parameters are the bead loading and bead diameter [80]. Tamer *et al*. (1998) reported that disruption of *Alcaligenes latus* cells by 512 μm of bead diameter and 2,800 rpm of agitation speed could be completely ruptured within eight passes. [64]. The range of cell disruption depends on various parameters such as residence time distribution (RTD), shear forces, type of microorganisms, cell concentration, feed rate of the suspension, agitation speed, geometry of the grinding chamber and design of the stirrer [81]. However, this method operation generates the heat from shear forces that requires cooling system to remove the heat [53].

2. High pressure homogenization (HPH)

A high-pressure homogenizer disrupts cell suspension under high pressure through an alterable, confined opening discharge valve [82]. The high pressure homogenization is non solvent-based method which is a well-known and widely used for fragmentation and homogenization of microbial cells. The mechanism of high-pressure homogenization in consisted of a pump that crushes the culture broth through two parallel apertures under high pressure. The obtained fluid streams punch on a plate and participate again. Finally, the homogenized solution is squeezed out of a hole. Cell separation takes place at room temperature. The use of this mechanical disruption should consider the biomass concentration and operation [59].

2.10 Environmentally friendly solvents

Environmental problems play a significant role in large area around the world for long time. In 1990, the United States formally submitted the Pollution Prevention Act (PPA) for pollution prevention. This law had encouraged the research about technology and process of industrial and educational sectors for the reduction of pollution before creation [83, 84]. This is the first period of green chemistry [85].

2.10.1 Definition and principle

Green chemistry is a design of chemical products and manufacture that are more environmentally friendly and reduce negative effects to human health and the environment [86]. This definition has been explained into 12 principles below.

Twelve principles of green chemistry

1. It is better to prevent waste generation than to treat waste after it is occurred.
2. Synthetic processes should be designed to maximize the combination of all materials used in the process into the final product.
3. Synthetic processes should be designed to minimize the use and generation of toxic substances.
4. Chemical products should be designed to conserve potency of function while minimizing toxicity.
5. The use of auxiliary substances (such as solvents or separation agents) should be avoided as much as possible. If required, there should be safe.
6. Energy requirements in chemical processes should be minimized. Synthetic processes should be conducted at ambient temperature and pressure.
7. A raw material or feedstock should be renewable rather than non-renewable.

8. Unnecessary derivatization should be avoided whenever possible because it can produce waste.
9. Catalytic reagents should be selected instead of stoichiometric reagent if possible.
10. Chemical products should be designed so that at the end of their function they do not remain in the environment and degrade into innocuous degradation products.
11. Analytical methodologies require a real-time monitoring and control before the generation of hazardous substances.
12. Processes and substances should be selected to minimize the risk of chemical accidents such as releases, explosions and fires.

2.10.2 The candidate environmentally friendly solvents

The design of environmentally friendly solvents and processes has been an important principle of green chemistry over the past decade. Solvents are highly controlled and applied in large quantities. Particularly, organic solvents affect a consideration to the chemical industry because of the large volume used in synthesis, processing, and separations [87]. In this study, 2-methyltetrahydrofuran (2-MTHF), 1,3-propanediol, 1,3-dioxolane and ethyl lactate are used for solvent extraction of PHB and their properties are shown as below.

2-Methyltetrahydrofuran (2-MTHF)

2-MTHF has developed as an alternative to tetrahydrofuran (THF) and dichloromethane [8]. It is derived from non-edible renewable resources by two-steps of hydrogenation mechanism for conversion of 2-furaldehyde to 2-methylfuran and 2-MTHF, respectively [88]. 2-furaldehyde is derived from agricultural waste such as corncob and bagasse [89]. Recently, a company in United Kingdom reported that dichloromethane (DCM) was replaced by MTHF for the first industrial application [90].

1,3-Propanediol

1,3-Propanediol is a product obtained from a renewable source such as corn. Its characteristic and performance comparable to petroleum derivative solvents [91]. Their advantage is very low-level of inhalation hazard [92]. It is used to produce a thermoplastic like polypropylene terephthalate which is a composition in textile and automobile industries [93].

1,3-Dioxolane

1,3-Dioxolane is non-toxic and environmentally friendly solvent that is prepared by acetalization of aldehydes and ketalization of ketone with ethylene glycol. Their properties are considered as an alternative to several solvents such as dichloromethane, dichloroethane and methyl ethyl ketone, tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO) [9, 94]. Therefore, it is required to use in the polymer industries and paint industries [95].

Ethyl lactate

Ethyl lactate produces from nature-based feedstocks through esterification reaction between ethanol and lactic acid and form as ethyl lactate [11]. It is truly biodegradable, easy to recycle, non-corrosive, non-carcinogenic, non-toxic (U.S. Food and Drug Administration approved its use in food products) and non-ozone depleting [96, 97]. It is potentially alternative solvent to halogenated and toxic solvents including ozone depleting chlorofluorocarbons, carcinogenic methylene chloride, ethylene glycol ethers and chloroform [97].

Table 3. The properties of chemicals that were used in this study and others.

Reagents	Boiling point (ºC)	Density (g/cm^3)	Viscosity (cP)	Solubility in water (%)	PEL[a] (ppm)	FDA class[b]	Price ($US/lb)[c]
chemicals in this study							
water	100	1.03	0.91	N.A.	N.A.	Safe	<0.01[d]
chloroform	61	1.48	0.54	0.80	50	2	0.26-0.47
2-methyl tetrahydrofuran	80	0.85	0.6	15	N.A.	N.A.	0.91-22.67
1,3-propanediol	214	1.05	0.52	∞	N.A.	N.A.	0.45-0.68
1,3-dioxolane	76	1.06	0.6	∞	N.A.	N.A.	0.45-4.54
ethyl lactate	154	1.03	0.42	8.30	400	N.A.	1.60-2.10
n-heptane	98	0.68	0.39	0.00	500	3	1.21-1.64
n-hexane	69	0.66	0.3	0.01	500	2	1.15-1.16
methanol	64	0.79	0.54	∞	200	2	0.94-2.03
ethanol	78	0.78	1.04	∞	1000	3	3.10-3.70
iso-propanol	82	0.78	1.96	∞	400	3	0.59-0.68
other chemicals							
ethyl acetate	77	0.89	0.42	8.70	400	3	0.48-0.59
butyl acetate	126	0.88	0.68	0.68	150	3	0.67-0.72
methyl isobutyl ketone	117	0.80	0.6	1.70	100	3	0.74-0.79
methyl ethyl ketone	80	0.80	0.4	26.3	200	3	0.32-0.34
isoamyl alcohol	131	0.81	3.69	2.8	100	3	0.59-0.68
dimethyl carbonate	90	1.07	0.62	13.9	200	N.A.	0.29-0.32
diethyl carbonate	127	0.98	0.75	0.00	N.A.	N.A.	0.41-0.54

[a]PEL is the permissible exposure limit established.

[b]The FDA rate chemicals for use in manufacturing of biomedical, where 1 is most toxic and 3 is least toxic.

[c]Price informations from www.icis.com (2007-2008).

N.A. indicates no data was available.

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments and analytical equipments

3.1.1 5 L Bioreactor model and controller MDL500, (B.E.Marubishi Co., Ltd., Tokyo, Japan)

3.1.2 Analytical balance model AG 204 and AG 285 (Mettler Toledo Co., Ltd., Zurich, Switzerland)

3.1.3 Autoclave model SS-325 and ES-215 (Tomy Seiko Ltd., Tokyo, Japan)

3.1.4 Capillary column (size 30 m length × 0.25 mm ID × 0.25 μm df) model CP-Wax 52 CB (Agilent Technologies, Inc., Middelburg, Netherlands)

3.1.5 Centrifuge model H-103N (Kokusan Ensinki Co., Ltd., Tokyo, Japan)

3.1.6 Deep freezer (-20˚C) (SANYO Commercial Solutions (Thailand) Co., Ltd., Chachoengsao, Thailand)

3.1.7 Fume hood model FH150DC (Official Equipment manufacturing Co., Ltd., Nakorn Pathom, Thailand)

3.1.8 Gas chromatography model 3400C (Varian Inc., Walnut Creek, CA, USA)

3.1.9 Hot air oven model 115 (BINDER GmbH, Tuttlingen, Germany)

3.1.10 Hot plate model KP 1056 A (Severin Elektrogeräte GmbH, Sundern, Germany)

3.1.11 Incubator model INE 500 (Memmert Co., Ltd., Schwabach, Germany)

3.1.12 Laboratory balance model PG 2002-S and PG 6002-S (Mettler Toledo Co., Ltd., Zurich, Switzerland)

3.1.13 Laboratory glass bottle (DURAN®, Germany)

3.1.14 Laminar flow clean bench model LV125U (Official Equipment manufacturing Co., Ltd., Nakorn Pathom, Thailand)

3.1.15 Magnetic stirrer model Ceraplate (Nickel-eletro Ltd., Weston-super-Mare, UK)

3.1.16 Microscope model CH30RF200 (Olympus optical Co., Ltd., Tokyo, Japan)

3.1.17 Nitrocellulose membrane size 0.45 µm (Sartorius Stedim Biotech GmbH, Goettingen, Germany)

3.1.18 pH meter model SevenEasy (Mettler Toledo Co., Ltd., Zurich, Switzerland)

3.1.19 Refrigerated centrifuge model 6500 (Kubota Co., Tokyo, Japan)

3.1.20 Screw cap test tube (PYREX® and KIMAX®, USA)

3.1.21 Shaker model Innova 4330 (New Brunwick Scientific Co., Inc., Edison, N.J., USA)

3.1.22 UV-visible spectrophotometer model Biomate 3S (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA)

3.1.23 Vortex mixer model G-560E (Scientific Industries, Inc., Bohemia, NY, USA)

3.2 Chemicals and reagents

3.2.1 1,3-Dioxolane (Merck KGaA, Darmstadt, Germany)

3.2.2 1,3-Propanedoil (Merck KGaA, Darmstadt, Germany)

3.2.3 2-Methyl tetrahydrofuran (Merck KGaA, Darmstadt, Germany)

3.2.4 Ammonium iron (III) citrate (17% of iron) (Merck KGaA, Darmstadt, Germany)

3.2.5 Ammonium sulfate (Merck KGaA, Darmstadt, Germany)

3.2.6 Benzoic acid (Nacalai tesque, Inc., Kyoto, Japan)

3.2.7 Calcium chloride dehydrate (Merck KGaA, Darmstadt, Germany)

3.2.8 Chloroform (RCI Labscan Ltd., Bangkok, Thailand)

3.2.9 Disodium hydrogen phosphate dehydrate (Merck KGaA, Darmstadt, Germany)

3.2.10 Ethanol (Merck KGaA, Darmstadt, Germany)

3.2.11 Ethyl lactate (Merck KGaA, Darmstadt, Germany)

3.2.12 Glycerol (Merck KGaA, Darmstadt, Germany)

3.2.13 Hydrogen chloride (Merck KGaA, Darmstadt, Germany)

3.2.14 iso-Propanol (Merck KGaA, Darmstadt, Germany)

3.2.15 Magnesium sulfate heptahydrate (Merck KGaA, Darmstadt, Germany)

3.2.16 Methanol (Merck KGaA, Darmstadt, Germany)

3.2.17 *n*-Heptane (Fisher Scientific UK Ltd., Leicestershire, England)

3.2.18 *n*-Hexane (Merck KGaA, Darmstadt, Germany)

3.2.19 Peptone (Difco Laboratories Inc., NJ, USA)

3.2.20 Potassium dihydrogen phosphate (Merck KGaA, Darmstadt, Germany)

3.2.21 Refined sugar (Mitr Phol group, Thailand)

3.2.22 Sodium chloride (Merck KGaA, Darmstadt, Germany)

3.2.23 Sodium hydroxide (Merck KGaA, Darmstadt, Germany)

3.2.24 Hydrogen sulfate (Merck KGaA, Darmstadt, Germany)

3.2.25 Yeast extract (HiMedia Laboratories Pvt. Ltd., Mumbai, India)

3.2.26 Zinc sulphate heptahydrate (Farmitalia Carlo Erba S.p.A, Milan, Italy)

3.3 Bacterial strain

C. necator strain A-04 (formerly known as *R. eutropha* strain A-04 or *Alcaligenes* sp. A-04) was isolated from soil in Thailand and used as a PHB-producing strain in this study [25, 98, 99]. This strain was Gram negative bacterial, aerobic, motile, rod-shaped, peritrichously flagellated, non-endospore-forming and oxidase-positive. It was capable of producing PHB from several carbon sources [98]. Moreover, various types of PHAs such as homopolymer (PHB and P(4HB)), copolymer (P(3HB-*co*-3HV) and P(3HB-*co*-4HB)) and terpolymer (P(3HB-*co*-3HV-*co*-4HB)) were also produced by this strain [25, 98, 99].

3.4 Culture preservation

3.4.1 Short term preservation of bacterial culture

A single colony was streaked on preculture medium (Appendix A) and incubated at 30 ºC for 24 h. Then, cell culture was stored at 4 ºC until use and re-streaked every month.

3.4.2 Long term preservation of bacterial culture

Bacterial cells were cultured in 50 ml of preculture medium (Appendix A) in a 250 mL Erlenmeyer flask and incubated at 30 ºC with shaking at 200 rpm for 24 h. The cells in culture were harvested by centrifugation (10160× *g*, 20 min., 4 ºC), washed twice with 0.85% (w/v) NaCl and re-suspended in 10% (w/v) glycerol. Cell concentration was controlled at 10^9 CFU/mL. Bacterial stock cultures were frozen at -80 ºC for up to 6 months.

3.5 Preculture preparation

A 1 mL of bacterial stock culture in 10% (w/v) glycerol was inoculated in 500 mL Erlenmeyer flask containing 100 mL of preculture medium (Appendix A) and incubated on a rotary shaker at 200 rpm, 30 ºC for 24 h. Next, cells were harvested by centrifugation (10160× *g*, 20 min., 4 ºC), washed twice with 0.85% (w/v) NaCl to remove residual nitrogen. The cells were re-suspended and inoculated to the production medium.

3.6 Culture conditions for PHB production in 5 L bioreactor

Wet cells and dried cells containing intracellular PHB granules were obtained by performing batch cultivation in a 5-L bioreactor (MDL500, B.E. Marubishi Co., Ltd., Tokyo, Japan). A 500 mL of preculture was prepared in flasks and grown on a rotary shaker at 30 ºC at a 200-rpm shaking speed for 24 h. The cells were harvested by centrifugation, washed to remove the nitrogen source and re-suspended in 100 mL of 0.85% (w/v) NaCl. The cells then were inoculated into a production medium (Appendix A) in a 5-L bioreactor. The working volume of the batch cultures was 3 L. The fermentation temperature was 30 ºC, and the pH value was maintained at 7.0 throughout the experiment. An agitation speed was set at 500 rpm and air flow rate was set at 0.5 vvm. After 72 h of cultivation, cells were harvested by centrifugation (10160× *g*, 20 min., 4 ºC), washed twice with 0.85% (w/v) NaCl. Next, aliquots of cell suspension were used to prepare wet cells and dried cells. Wet cells were used as aliquots of fresh cells obtained directly from fermentation. Dried cells were aliquots of fresh cells which were dried in oven at 80 ºC for 24 h (70.3 % of moisture content). The PHB content in microbial cells was measured by Gas chromatography analysis.

3.7 Investigation of solubility and precipitation of PHB in various solvents

3.7.1 Test of solvents for PHB dissolution

Purified PHB from C. *necator* strain A-04 was used as a testing polymer. The solubility test of purified PHB in various environmental friendly solvents; 2-methyltetrahydrofuran, 1,3-propanediol, 1,3-dioxolane and ethyl lactate, were performed in PYREX® screw thread, borosilicate glass test tubes with PTFE-faced rubber line cap. This rubber can protect solvent evaporation. Chloroform was used as a positive control experiment because it is a well-established reference standard [54]. Water was used as a negative control experiment. One mL of each solvent was added in the screw cap test tubes to obtain 2.5%, 5%, 7.5% and 10% (w/v) PHB/solvent solution. The PHB was dissolved by heating in a water bath at 100 ºC for 4 h (modified from [100]). The undissolved solid residue is then separated from the PHB/solvent solution. Next, PHB/solvent solutions were evaporated overnight to recover PHB polymer. The recovery of PHB polymer was measured as dried PHB. The solvent that gave the highest dried PHB mass was chosen as the best solvent for PHB dissolution. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

3.7.2 Test of solvents for PHB precipitation

A 5% (w/v) PHB in 1,3-dioxolane and chloroform were prepared in screw cap borosilicate glass test tube. For precipitant tested, 1 mL of 5% (w/v) PHB solution was transferred into screw cap borosilicate glass test tubes. Next, PHB was precipitated by addition of 1-3 volumes of each precipitants (*n*-heptane, *n*-hexane, methanol, ethanol, isopropanol and water) at room temperature. The mixtures were vortexed and incubated at room temperature for 1 h. The mixtures were centrifuged at 4620× *g* for 10 min. at room

temperature. The supernatant was discarded and PHB pellet was dried overnight in oven at 80 ºC. Then, the recovery of PHB polymer was measured as dried PHB. The solvent that gave the highest dried PHB mass was chosen as the best solvent for PHB precipitation (modified from [100]). The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

3.8 Extraction of PHB from dried cells

3.8.1 Extraction of PHB from dried cells in 2 mL of solvent (small scale extraction)

The 5% (w/v) of dried cells in 2 mL of 1,3-dioxolane was prepared in screw cap borosilicate glass test tubes. Chloroform was used as control experiment. The PHB was extracted from dried cells by incubating samples at 30, 60, 80 and 100 ºC for 4, 6, 12, 24 and 36 h. Samples were mixed by shaking tubes by hand every 30 min. Next, samples were cooled down to room temperature and centrifuged at 4620× *g* for 10 min. at room temperature. Then, PHB solutions were transferred to another screw cap test tube. The PHB solutions were precipitated using 3 volumes of *n*-heptane (as the selection result was obtained from the previous experiment), briefly mixed at room temperature, centrifuged at 4620× *g* for 10 min. at room temperature and washed twice with *n*-heptane. The washed polymers were dried overnight in oven at 80 ºC. The PHB polymers were analyzed percent of purity and recovery as described below. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

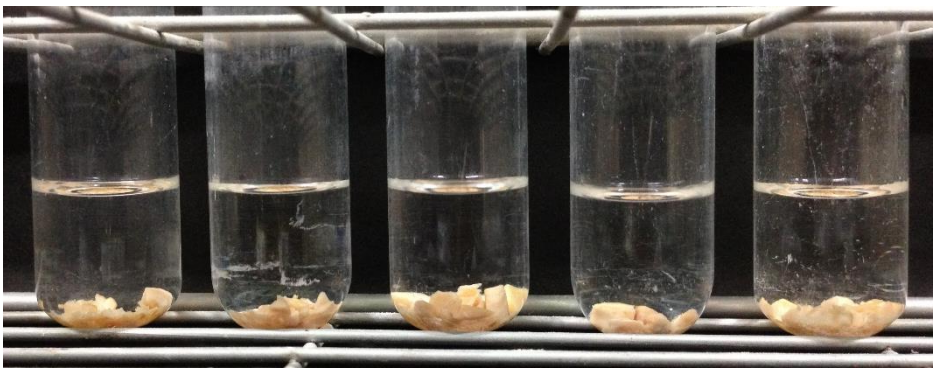


Figure 7. Preparation 0.1 g of dried cells in 2 mL of solvents

3.8.2 Test of PHB precipitation with water and *n*-heptane

To replace the use of *n*-heptane with water, a comparison study was performed to investigate the feasibility of using water as precipitant. The 5% (w/v) of dried cells in 1,3-dioxolane was prepared and PHB was extracted from dried cells by incubating at 80 ºC for 6 h. Samples were mixed by shaking tubes by hand every 30 min. Chloroform was used as a control experiment. Next, the PHB solutions were cooled down to room temperature and centrifuged at 4620× *g* for 10 min. at room temperature. Then, PHB was precipitated from the solutions with 1, 2, 3, 4 and 5 volumes of water and *n*-heptane individually. The samples were briefly mixed at room temperature, centrifuged at 4620× *g* for 10 min. at room temperature and washed twice with water or *n*-heptane. The obtained polymers were dried overnight in oven at 80 ºC. The PHB polymers were analyzed percent of purity and recovery by Gas chromatography. The results were represented of three independent experiments and are presented as the mean value ± standard deviation (S.D.).

3.8.3 Extraction of PHB from dried cells in 40 mL of solvent (large scale extraction)

The extraction conditions were carried out based on the optimal conditions obtained from 3.8.1. The 5% (w/v) of dried cells in 1,3-dioxolane was prepared in 250 mL DURAN® Erlenmeyer flasks. The PHB was extracted from dried cells by incubating at 80 ºC for 6 h, samples were mixed by shaking tubes by hand every 30 min. and cool down to room temperature. The samples were precipitated with 3 volumes of water. The samples were briefly mixed at room temperature, stand for 1 h at room temperature, discard water and washed twice with water. The obtained polymers were dried overnight in oven at 80 ºC. The PHB polymers were analyzed percent of purity and recovery by Gas chromatography. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

3.9 Extraction of PHB from wet cells

3.9.1 Extraction of PHB from wet cells in 2 mL of solvent (small scale extraction)

Equal volumes (50 mL; equivalent of dried cells 0.1 g) of culture broth (containing fresh cells harboring PHB granules) were centrifuged at 10160× *g* for 10 min. at 4 ºC. The supernatant was discarded and the wet cell pellet was transferred into the screw cap borosilicate glass test tube. Then, 2 mL of 1,3-dioxolane were added to obtain the solvent mixtures. Chloroform was used as a control experiment (Figure 8). The PHB was extracted from wet cells by incubating samples at 80 ºC for 6 h, samples were mixed by shaking tubes by hand every 30 min. and cool down to room temperature. After extraction, 2 different fractions were formed as cell debris fraction (upper part) and solvent fraction (bottom part). The mixtures were centrifuged at 4620× *g* for 10 min. at room temperature. The solvent fraction was carefully transferred to another screw cap borosilicate glass test tube. Next, PHB polymer was precipitated with 3 volumes of water, briefly mixed at room

temperature, centrifuged at 4620× *g* for 10 min. at room temperature and washed twice with water. The washed polymers were dried overnight in oven at 80 ºC. The PHB polymers were analyzed percent of purity and recovery by Gas chromatography. The results were represented of three independent experiments and are presented as the mean value ± standard deviation (S.D.).

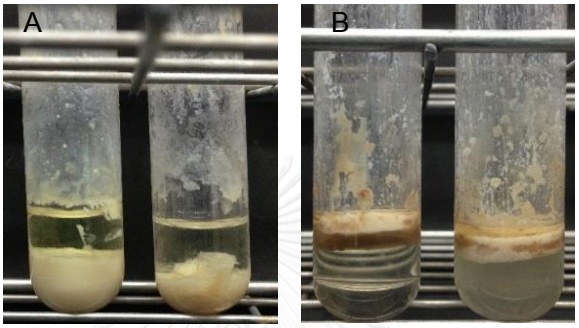


Figure 8. Preparation 50 mL(equivalent of dried cells 0.1 g) of culture broth. A: Wet cells in 2 mL of 1,3-dioxolane. B: Wet cells in 2 mL of chloroform.

3.9.2 Extraction of PHB from wet cells in 40 mL of solvent (large scale extraction)

Equal volumes (700-800 mL; equivalent to dried cells 2 g) of culture broth were centrifuged at 10160× *g* for 10 min. at 4 ºC. The supernatant was discarded and the wet cell pellet was transferred into the 250 mL DURAN® Erlenmeyer flasks. Then, 40 mL of 1,3-dioxolane was added and incubated at 80 ºC for 6 h, samples were mixed by shaking flasks by hand every 30 min. and cool down to room temperature. Chloroform was used as the control experiment. After extraction, 2 different fractions were formed as cell debris fraction (upper part) and solvent fraction (bottom part). The mixtures were filtered by fine sieve. The solvent fraction was carefully transferred to another DURAN® Erlenmeyer flask. The PHB was precipitated with 3 volumes of water, stand for 1 h, discarded water and dried overnight in oven at 80 ºC. Purified PHB was analyzed purity of the polymer, percent

of recovery and physical and mechanical properties. The PHB polymers were analyzed percent of purity and recovery by Gas chromatography. The results were represented of three independent experiments and are presented as the mean value ± standard deviation (S.D.).

3.9.3 Extraction of PHB from wet cells in 2 mL of solvent at room temperature (30 ºC)

Equal volumes (50 mL; equivalent of dried cells 0.1 g) of culture broth were centrifuged at 10160× *g* for 10 min. at 4 ºC. The supernatant was discarded and the wet cell pellet was transferred into the screw cap borosilicate glass test tube. Then, 2 mL of 1,3-dioxolane were added to obtain the solvent mixtures. Chloroform was used as a control experiment. The PHB was extracted from wet cells by incubating samples at room temperature for 4, 6, 12, 24 and 36 h. Next, the mixtures were centrifuged at 4620× *g* for 10 min. at room temperature. The PHB solution was transferred to another screw cap borosilicate glass test tube. The PHB solution was precipitated with 3 volumes of water, briefly mixed at room temperature, centrifuged at 4620× *g* for 10 min. at room temperature and washed twice with water. The washed polymers were dried overnight in oven at 80 ºC. The PHB polymers were analyzed percent of purity and recovery by Gas chromatography. The results were represented of three independent experiments and are presented as the mean value ± standard deviation (S.D.).

3.10 Analytical methods

3.10.1 Cell growth

Growth was monitored by dry substance estimation, which was determined by filtering 5 mL of the culture broth through pre-weighed cellulose nitrate membrane filters (pore size of 0.45 μm, Sartorius, Goettingen, Germany) and drying the filters at 80 ºC for 2 days. The net biomass was defined as the residual biomass concentration, which was calculated by subtracting the amount of PHA from the total biomass.

3.10.2 PHB content in microbial cells

The PHB in the dried cells were methyl-esterified using a mixture of chloroform and 3% methanol-sulphuric acid (1:1 v/v), as described by Braunegg (1978) [101]. The resulting monomeric methyl esters were quantified using gas chromatography (Model CP3800, Varian Inc., Walnut Creek, CA, USA) with a Carbowax-PEG capillary column (0.25 μm df, 0.25 mm ID, 60 m length, Varian Inc., Walnut Creek, CA, USA). The internal standard was benzoic acid, and the external standard was PHB (Sigma-Aldrich Corp., St. Louis, MO, USA).

3.10.3 PHB Purity (%)

The polymer was analyzed percent of purity and recovery by Gas chromatography. The PHB purity (%) of the purified polymer was defined as [100]:

$$\text{Purity (\%)} = \frac{\text{mass of PHB analyzed by GC (g)} \times 100}{\text{mass of PHB recovery (g)}}$$

3.10.4 PHB recovery yield (%)

The PHB recovery yield (%) of the purified polymer was defined as [100]:

$$\text{Recovery yield (\%)} = \frac{\text{mass of recovery PHB (g)} \times \text{purity (\%)}}{\text{mass of PHB in dried cells analyzed by GC}}$$

The results were represented of three independent experiments and are presented as the mean value ± standard deviation (S.D.).

3.10.5 Test of physical and mechanical properties of purified PHB

3.10.5.1 Preparation of PHB films (modified from [102])

The PHA films used for testing the mechanical properties were prepared according to the ASTM: D882 protocol. The PHA films were prepared from chloroform solutions of the polyesters using conventional solvent-casting techniques, using a glass tray (Pyrex, Corning Incorporated, NY, USA) as the casting surface [102]. The thickness of the polyester thin films was regulated by controlling the concentration of the polymer in chloroform (1% w/v) and the volume of the polymer solution. The thickness of the PHA films was 0.05 mm, which was confirmed using a caliper. A minimum of 3 films per each samples of 50 × 100 mm were cut and then aged for one month in a desiccator under ambient temperature to allow them to reach crystallization equilibrium prior to the analyses.

3.10.5.2 Analysis of mechanical properties of PHB films [99]

The mechanical tests were conducted using a Universal Testing Machine (H10KM, Wuhan Huatian Electric Power Automation Co., Ltd., Wuhan, China) with a crosshead speed of 10 mm/min at Scientific and Technological Research Equipment Center, Chulalongkorn University. The measured variables included the elongation at the break point (%), the stress at maximal load (MPa) and the Young's modulus (MPa). Commercial PHB (Sigma-Aldrich Corp., St. Louis, MO, USA) also was tested under the same conditions for comparison. The data represent the mean values for fifteen samples tested under the same conditions.

3.10.5.3 Thermal analysis by differential scanning calorimetry (DSC) [99]

A 10-mg sample of PHB was encapsulated in an aluminium sample vessel and placed in the sample holding chamber of the DSC apparatus (DSC7, PerkinElmer, Inc., MA, USA). The STAR^e software (version SW 10.00; Mettler-Toledo International Inc., OH, USA) was used to operate the DSC apparatus at the Petroleum and Petrochemical College, Chulalongkorn University. The previous thermal history of the sample was removed prior to the thermal analysis by heating the sample from the ambient temperature to 180 ºC at 10 ºC/min. Next, the sample was maintained at 180 ºC for 5 min, before cooling it at the same rate to 30 ºC. The sample was then thermally cycled at 10 ºC/min to 180 ºC. The melting peak temperature is denoted by T_m and is given by the intersection of the tangent to the furthest point of an endothermic peak and the extrapolated sample baseline. The glass transition temperature is denoted by T_g and can be estimated by extrapolating the midpoint of heat capacity difference between glassy and viscous states after heating of the quenched sample.

3.10.5.4 Analysis of molecular weight of polymer [99]

The molecular weight and polydispersity were determined by Gel Permeation Chromatography (GPC) (Shodex GPC®, SHOKO SCIENTIFIC Co., Ltd., Yokohama, Japan) with a GPC K-804L column (8.0 mmID × 300 mmL, Showa Denko K.K., Tokyo, Japan) at the Petroleum and Petrochemical College, Chulalongkorn University. Polymer was dissolved in 1% of chloroform and filtered with 0.45 microns of diameter of membrane filter. A standard curve was determined by polystyrene in the same condition and a molecular weight in the range of 5.00×10^2 to 7.06×10^5.

CHAPTER IV
RESULTS

4.1 Preparation of bacterial cells containing PHB granules

C. necator strain A-04, an efficient PHAs producing bacteria screened from soil in Thailand, was chosen as a representative for Gram-negative bacteria harboring PHAs granules. In the previous study, *C. necator* strain A-04 was able to produce various types of PHAs depending on selected carbon sources [25, 98, 99].

In this study, *C. necator* strain A-04 was grown on 30 g/L of sucrose as a sole carbon source and C/N ratio was 200. Aerobic batch cultivation was performed in 5 L fermentor. The highest PHB content of 63.9±2.8 (% wt) was produced and total cell mass 7.3±1.2 g/L was obtained at 72 h. Figure 9 shows experimental time courses of biomass (g/L), PHB (g/L), residual biomass (g/L), sucrose concentration (g/L) and PHB content (% wt) when *C. necator* strain A-04 was grown on 30 g/L of sucrose and C/N ratio of 200. Wet cells and dried cells were prepared according to material and methods.

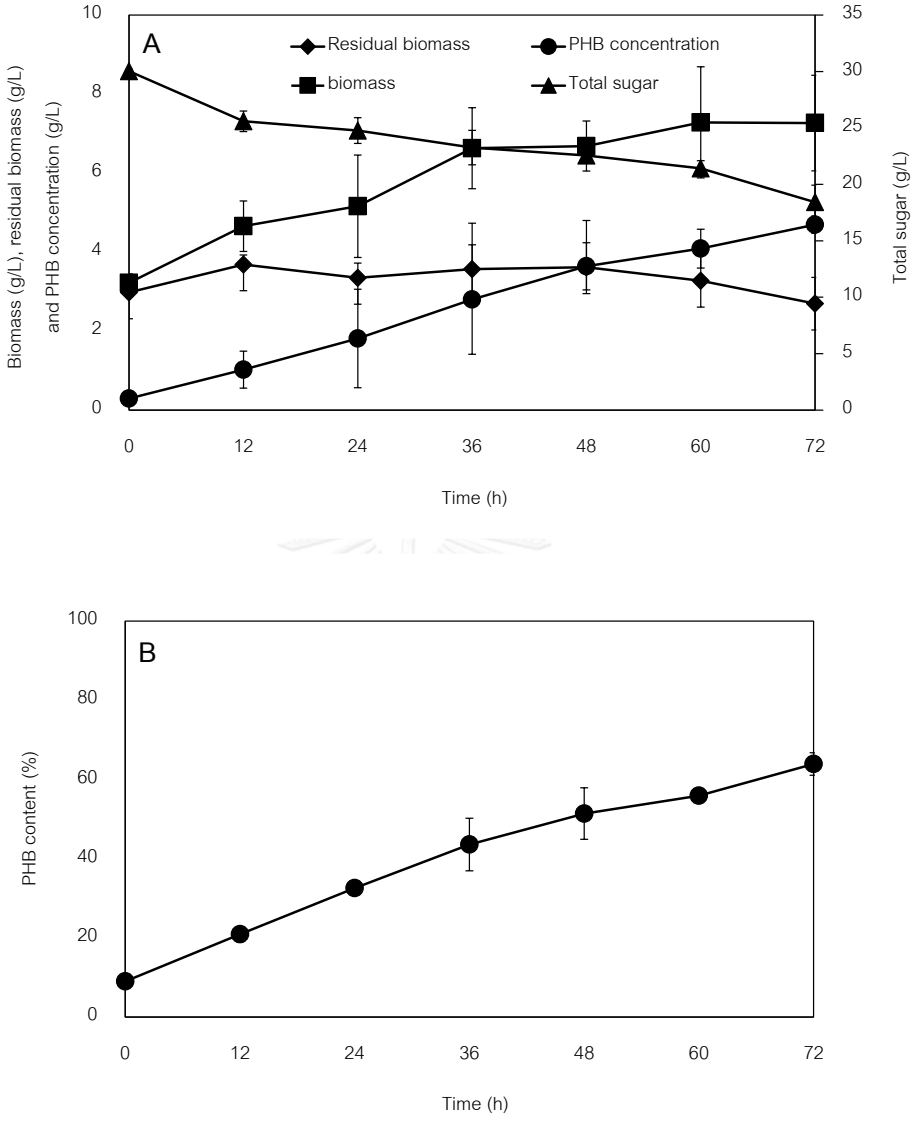


Figure 9. A: Experimental time courses of biomass (g/L), PHB (g/L), residual biomass (g/L), PHB concentration (g/L) and sucrose concentration (g/L), and B: PHB content (wt%) when *C. necator* strain A-04 was grown on 30 g/L of sucrose and C/N ratio of 200 for 72 h. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

4.2 Investigation of solubility and precipitation of PHB in various solvents

The sustainable competitive advantages of PHAs over petroleum based plastic has been hindered by the total cost of production; such as cost of substrate, energy consumption, toxic and non-recyclable solvents used for polymer extraction and purification. Focusing on the extraction and purification of PHAs, the extraction of PHAs from microbial biomass can be performed by (i) PHAs dissolution with organic solvent or (ii) whole cell lysis with surfactants or enzymes (iii) mechanical cell disruption. For the first method, chloroform and dichloromethane are the best organic solvents providing the highest solubility of PHAs, but they are seriously impacts human heaths, highly toxic to the environments, dangerous to handle that hampering their use in industrial processes. In the past few years, investigation on the use of non-halogen solvents and green solvents as an alternative solvents to chlorinated solvents has been reported, for example, propylene carbonate [103], ethyl acetate [100, 104], methyl isobutyl ketone [100], ionic liquids [105], dimethyl carbonate [106]. Although, these chemical reagents listed above could provide excellent purity and recovery yields, they also come with several disadvantages, for instance, cyclic carbonates gave a good PHA dissolubility, but they required high extraction temperature that affected the molecular weight of PHAs. The aim of this study was to explore green solvents; 2-methyltetrahydrofuran (2-MTHF), 1,3-dioxolane, ethyl lactate and 1,3-propanediol, to replace the use of chlorinated solvents. The selection was based on their physical properties and safety characteristics as shown in Table 3.

4.2.1 Test of solvents for PHB dissolution

Several researches have attempted to search for alternative solvents instead of chlorinated hydrocarbon for PHA extraction and purification [100, 103, 104, 106]. In this study, we focused on environmentally friendly solvents consisting of 2-methyltetrahydrofuran (2-MTHF), 1,3-dioxolane, ethyl lactate and 1,3-propanediol as for PHB extraction solvents. Physical properties and safety characteristics of the chemical solvents in this experiment had exhibited in Table 3.

Solvents listed above were tested for PHB dissolution. In this study, purified PHB produced by *C. necator* strain A-04 was used as testing polymer. Chloroform was used as extraction solvent for positive control experiment and water was used as extraction solvent for negative control experiment. Various concentrations of PHB solution were tested and dissolution test was performed at 100 ºC for 4 h. The results are summarized in Figure 10. It was found that chloroform gave the highest PHB solubility up to 91.6±1.7 wt% with 5% (w/v) PHB solution. Among various solvent tested, 1,3-dioxolan give the highest solubility of 83.9±0.7 wt% with 5% (w/v) PHB solution followed by 2-MTHF and ethyl lactate. It was found that water and 1,3-propanediol could not dissolve PHB at all. The concentration of 2.5% and 5% (w/v) PHB solution showed similar percentage of solubility and were higher than those of 7.5% and 10% (w/v) PHB solution. Thus, concentration of 5% (w/v) was chosen for further studies.

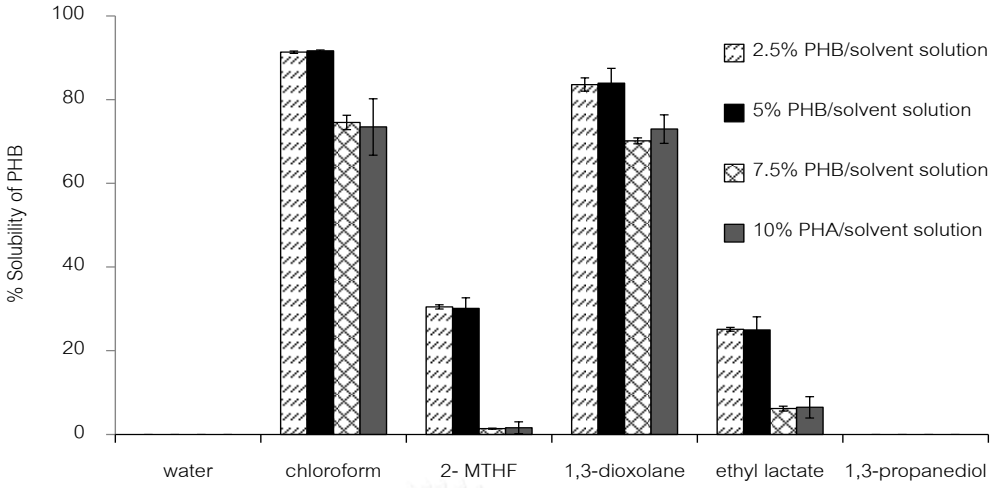


Figure 10. Solubility of PHB in various environmentally friendly solvents. Various concentrations of PHB solution at 2.5% , 5%, 7.5% and 10% (w/v) were tested with water (negative control), chloroform (positive control), 2-MTHF, 1,3-dioxolane, ethyl lactate and 1,3-propanediol. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

4.2.2 Test of solvents for PHB precipitation

In this study, 5% (w/v) PHB solution in 1,3-dioxolane was tested with various precipitants; *n*-hexane, *n*-heptane, water, iso-propanol, methanol and ethanol for their ability to precipitate PHB.

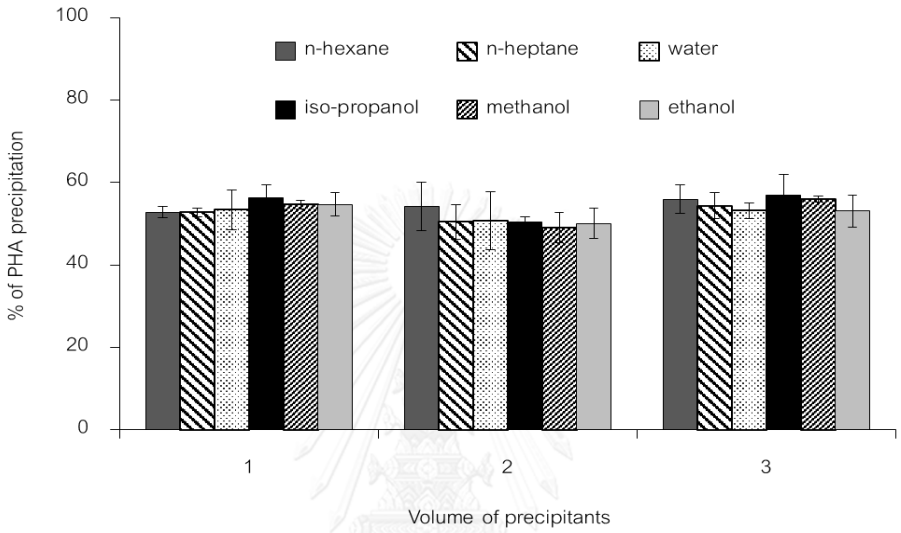


Figure 11. Determination of precipitants for PHB recovery. 5% (w/v) PHB solution in 1,3-dioxolane was tested. The precipitants; *n*-hexane, *n*-heptane, water, iso-propanol, methanol and ethanol were tested for PHB precipitation. A ratio of 1:1, 1:2 and 1:3 (vol/vol) of PHB solution to precipitants were tested and precipitation time was set at room temperature for 1 h. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

All precipitants tested were able to precipitate PHB from 5% (w/v) PHB solution in 1,3-dioxolane at room temperature for 1 h and gave similar results. Thus, these precipitants were tested again with PHB solution obtained from 1,3-dioxolane extraction in comparison with chloroform extraction from dried cells of *C. necator* strain A-04.

4.3 Extraction of PHB from dried cells

4.3.1 Extraction of PHB from dried cells in 2 mL of solvent (small scale extraction)

Based on the results obtain from 4.2.1, 1,3-dioxolane was chosen for solvent extraction of PHB from dried cells containing approximately 63.9±4.5 wt% of PHB. The 5% (w/v) of dried cells in 2 mL of 1,3-dioxolane was prepared in screw cap borosilicate glass test tubes. Chloroform was used as the control experiment. The PHB was extracted from dried cells by incubating samples at 30, 60, 80 and 100 ºC for 4, 6, 12, 24 and 36 h. Among various extraction temperature tested, PHB was not extracted from dried cell with 30 and 60 ºC. The results of 1,3-dioxolane extractions under 80 and 100 ºC for 4, 6, 12, 24 and 36 h are shown in Figure 12. For comparison, the results of chloroform extraction under 80 and 100 ºC for 4, 6, 12, 24 and 36 h are shown in Figure 13.

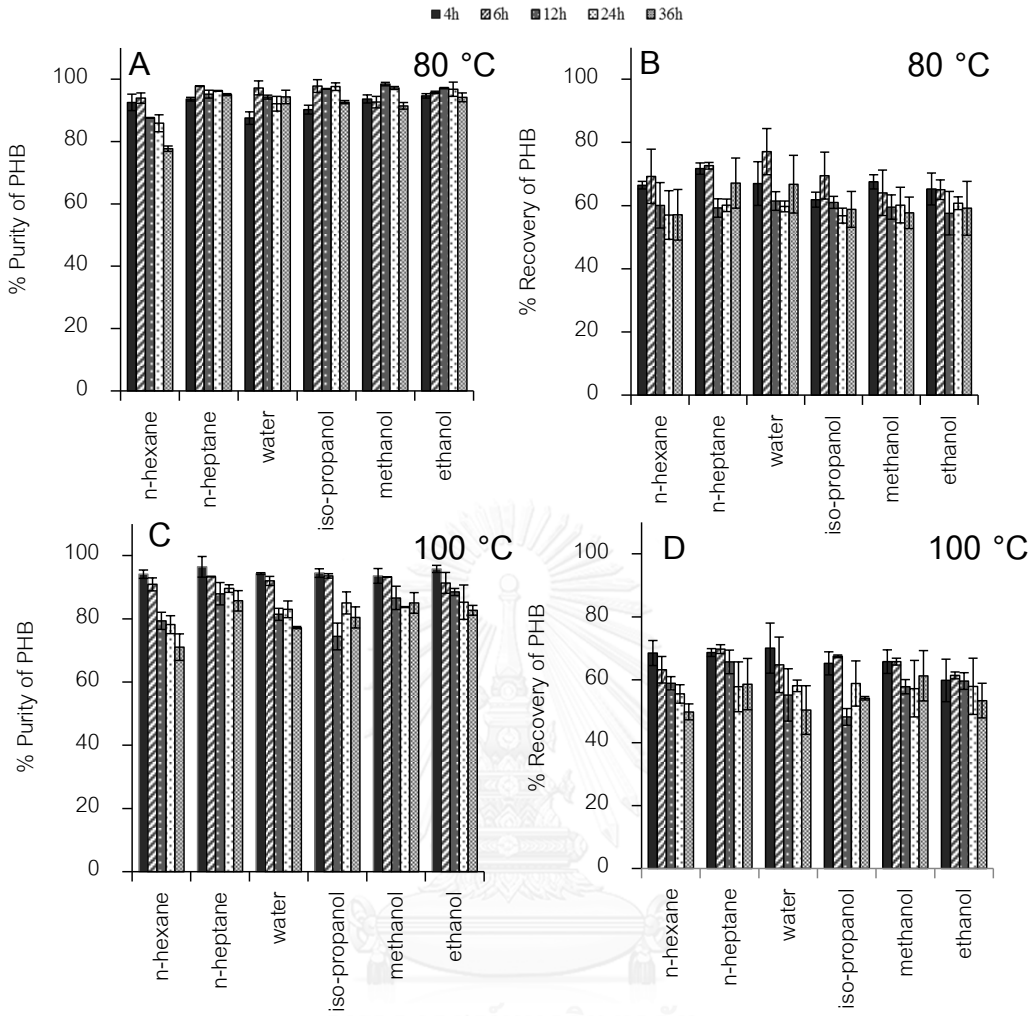


Figure 12. Extraction efficiency of PHB from dried cells with 2 mL of 1,3-dioxolane at 80 and 100 ºC for 4, 6, 12, 24 and 36 h expressed as (%) recovery and (%) purity. In all cases, the extracted PHB was precipitated with 3 volumes of various precipitants; *n*-hexane, *n*-heptane, water, isopropanol, methanol and ethanol, at room temperature for 1 h. A: PHB purity (%) obtained from 1,3-dioxolane extraction at 80 ºC. B: PHB recovery (%) obtained from 1,3-dioxolane extraction at 80 ºC. C: PHB purity (%) obtained from 1,3-dioxolane extraction at 100 ºC. D: PHB recovery (%) obtained from 1,3-dioxolane extraction at 100 ºC. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

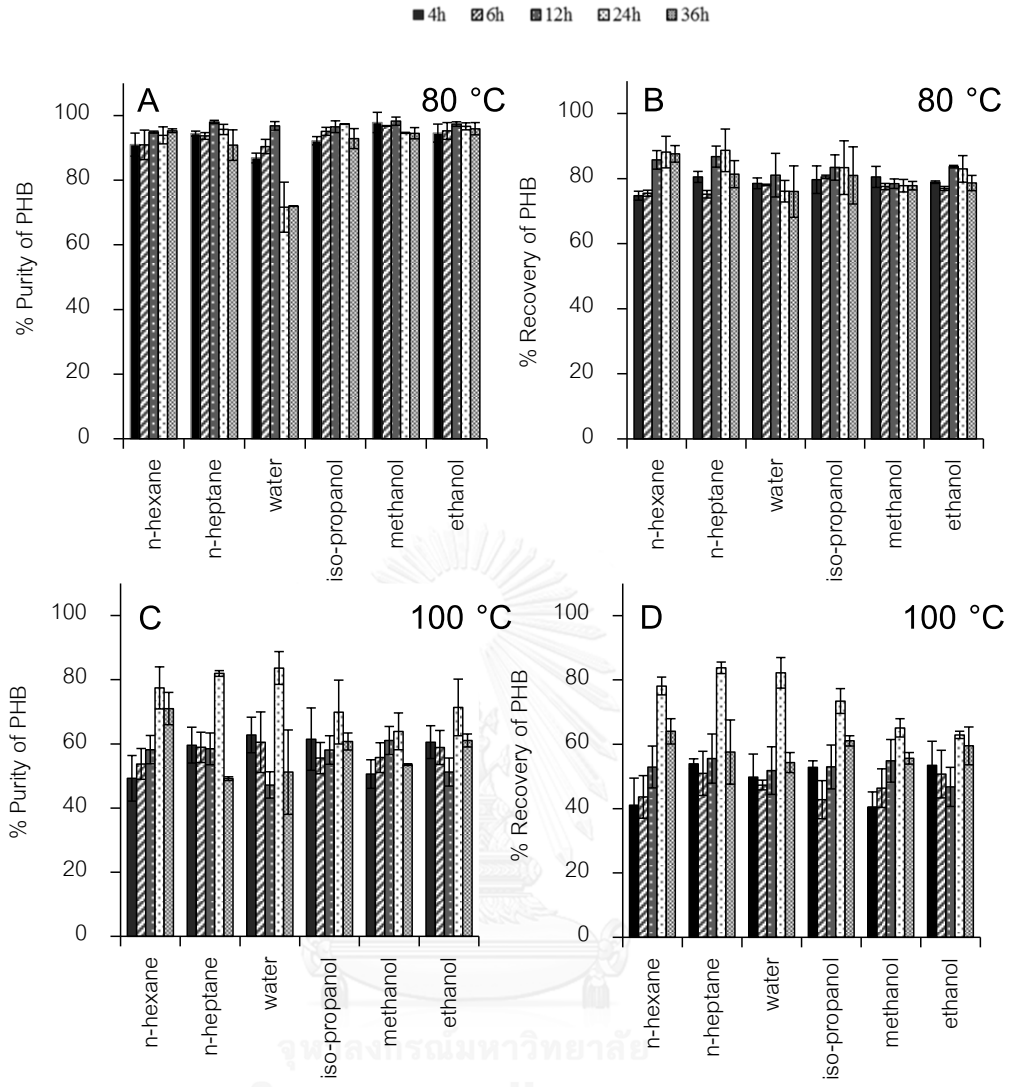


Figure 13. Extraction efficiency of PHB from dried cells with 2 mL of chloroform at 80 and 100 ºC for 4, 6, 12, 24 and 36 h expressed as (%) recovery and (%) purity. In all cases, the extracted PHB was precipitated with 3 volumes of various precipitants; *n*-hexane, *n*-heptane, water, isopropanol, methanol and ethanol, at room temperature for 1 h. A: PHB purity (%) obtained from chloroform extraction at 80 ºC. B: PHB recovery (%) obtained from 1,3-dioxolane extraction at 80 ºC. C: PHB purity (%) obtained from chloroform extraction at 100 ºC. D: PHB recovery (%) obtained from chloroform extraction at 100 ºC. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.)

Table 4. The series of 1,3-dioxolane extraction experiments

Conditions	*n*-hexane		*n*-heptane		water		iso-propanol		methanol		ethanol	
	purity (%)	recovery (%)	purity (%)	recovery (%)	purity (%)	recovery (%)	purity (%)	recovery (%)	purity (%)	recovery (%)	purity (%)	recovery (%)
80°C												
4h	92.5±2.6	66.4±1.2	93.6±0.6	71.7±1.8	87.5±2.0	67.0±6.9	90.2±1.4	61.7±2.4	93.6±1.3	67.5±2.3	94.7±0.7	65.2±5.1
6h	93.9±1.7	69.2±8.6	97.8±0.1	72.6±1.1	97.2±2.3	77.1±7.3	97.8±2.1	69.5±7.5	92.7±1.8	64.0±7.2	95.8±0.4	65.0±3.1
12h	87.6±0.1	60.1±7.2	95.2±1.3	59.2±2.9	94.2±0.7	61.4±3.0	96.9±0.1	61.0±1.9	98.4±0.6	59.5±3.9	97.1±0.2	57.6±6.9
24h	85.9±2.7	57.0±7.7	96.3±0.1	60.1±1.0	92.1±2.3	59.7±1.7	97.6±1.2	56.8±2.4	97.2±0.5	60.2±5.6	96.8±2.3	60.7±2.0
36h	77.7±0.8	57.1±8.0	95.0±0.3	67.1±7.9	94.3±2.2	66.8±9.1	92.6±0.5	58.8±5.7	91.4±1.1	57.7±5.0	94.1±1.4	59.2±8.5
100°C												
4h	94.1±1.3	68.5±4.0	96.4±3.3	68.7±1.3	94.3±0.3	70.1±8.0	94.5±1.4	65.2±3.7	93.6±2.4	65.8±3.7	95.8±1.1	59.8±6.8
6h	90.9±2.1	63.2±4.2	93.4±0.0	69.7±1.4	92.0±1.4	64.7±8.8	93.6±0.7	67.4±0.4	93.2±0.0	65.8±1.1	91.3±3.3	61.4±1.1
12h	79.3±2.7	58.9±2.1	87.9±3.5	65.7±3.7	81.4±1.9	55.2±8.3	74.4±4.2	48.2±2.7	86.6±3.7	57.8±2.3	88.5±1.1	59.6±2.7
24h	78.2±2.8	55.5±2.9	89.6±1.2	57.8±7.9	83.0±2.6	58.1±1.9	85.0±3.5	58.8±7.2	83.7±0.2	57.2±9.0	85.3±5.5	57.9±9.0
36h	71.0±4.2	49.8±2.5	85.7±3.2	58.6±8.2	77.2±0.3	50.4±7.7	80.4±3.3	54.1±0.5	85.0±3.3	61.2±8.0	83.2±1.5	56.5±5.5

Table 5. The series of chloroform extraction experiments

Conditions	*n* -hexane		*n* -heptane		water		iso-propanol		methanol		ethanol	
	purity (%)	recovery (%)	purity (%)	recovery (%)	purity (%)	recovery (%)	purity (%)	recovery (%)	purity (%)	recovery (%)	purity (%)	recovery (%)
80°C												
4h	91.0±3.6	74.7±1.4	94.4±0.8	80.5±1.7	86.9±1.5	78.5±1.7	92.2±1.2	79.7±4.2	97.9±3.1	80.5±3.2	94.6±2.8	78.9±0.4
6h	90.9±4.6	75.5±0.9	93.7±1.0	75.2±1.2	90.4±2.2	78.0±0.2	95.1±1.2	80.5±0.6	96.8±0.1	77.6±1.0	95.2±2.6	77.0±0.6
12h	94.9±0.3	85.8±2.9	98.0±0.5	86.8±3.2	96.8±1.3	81.0±6.7	96.5±1.8	83.4±3.9	98.3±1.3	78.4±1.5	97.3±0.7	83.7±0.4
24h	93.9±2.6	88.2±4.9	95.7±1.6	88.7±6.5	71.6±7.7	76.1±3.3	97.4±0.1	83.4±8.2	94.7±0.2	77.8±1.9	96.7±1.1	82.9±4.1
36h	95.3±0.6	87.6±2.6	90.8±4.7	81.4±4.2	72.0±0.1	76.0±7.9	92.8±3.1	81.0±8.8	94.5±1.7	77.8±1.3	95.9±1.9	78.6±2.4
100°C												
4h	49.2±7.1	41.0±8.4	59.6±5.6	54.0±1.5	62.8±5.5	49.8±7.2	61.5±9.7	52.8±12.0	50.6±4.5	40.5±4.7	60.6±5.1	53.5±7.5
6h	53.7±4.8	43.6±6.6	58.9±4.8	51.0±6.9	60.5±9.4	47.3±1.5	55.6±4.9	42.8±5.9	55.7±4.7	46.4±6.0	58.9±5.3	50.7±7.4
12h	58.2±4.4	52.9±6.5	58.5±4.9	55.6±7.6	47.2±4.1	51.8±7.4	58.1±4.5	53.0±6.8	61.1±4.4	54.9±6.6	51.2±4.3	46.7±6.1
24h	77.4±6.6	78.1±2.8	82.0±0.9	83.8±1.8	83.6±5.1	82.2±4.8	69.9±9.9	73.4±3.9	63.9±5.7	65.1±2.8	71.4±8.8	62.9±1.1
36h	71.0±5.0	64.0±3.9	49.2±0.6	57.6±1.0	51.2±13.1	54.3±3.1	60.8±2.7	61.1±1.6	53.5±0.2	55.6±1.8	61.1±2.0	59.5±5.9

As shown in Table 4, the series of 1,3-dioxolane extraction experiments were performed directly on dried cells to verify the feasibility of 1,3-dioxolane as alternative extraction solvent and test of various precipitants. Based on the results shown on Figure 10 and Table 4, 1,3-dioxolane extraction at 80 ºC for 6 h with water precipitation gave the highest recovery of 77.1±7.3% with the purity of 97.2±2.3 %. For comparison, as shown in Figure 11 and Table 5, chloroform extraction at 80 ºC for 24 h with *n*-heptane precipitation gave the highest recovery of 88.7±6.5 % with the purity of 95.7±1.6 %, followed by *n*-hexane that have the recovery of 88.2±4.9 % with the purity of 93.9±2.6 %. The highest purity of 98.3±1.3 % with the recovery of 78.4±1.5 % were obtained when using chloroform extraction at 80 ºC for 12 h with methanol precipitation. Interestingly, it was clearly observed that the purity of PHB decreased as the extraction temperature and time increased over than optimal time and temperature for solvent extraction.

4.3.2 Test of PHB precipitation with *n*-heptane and water

In the previous experiment, 2 precipitants, *n*-heptane and water, gave similar percentage of recovery and purity. Thus, *n*-heptane water were then tested their precipitation ability with various ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 (vol/vol) of PHB solution to precipitants and precipitation temperature and time was set at room temperature for 1 h. The 5% (w/v) of dried cells in 1,3-dioxolane was prepared and PHB was extracted from dried cells by incubating at 80 ºC for 6 h in comparison with 5% (w/v) of dried cells in chloroform. As shown in Figure 14, with 5% (w/v) of dried cells in 1,3-dioxolane, it was found that the ratio of 1:3 of PHB solution to water gave the highest recovery of 67.6±9.2 % with purity of 91.3±1.2 %. For comparison, with 5% (w/v) of dried cells in chloroform, the ratio of 1:2 of PHB solution to *n*-heptane gave the highest recovery of 74.5±7.0 % with 95.8±2.8 %. However, concerning with environmental friendly solvents,

the ratio of 1:3 of PHB solution to water with 5% (w/v) of dried cells in 1,3-dioxolane was chosen for further studies.

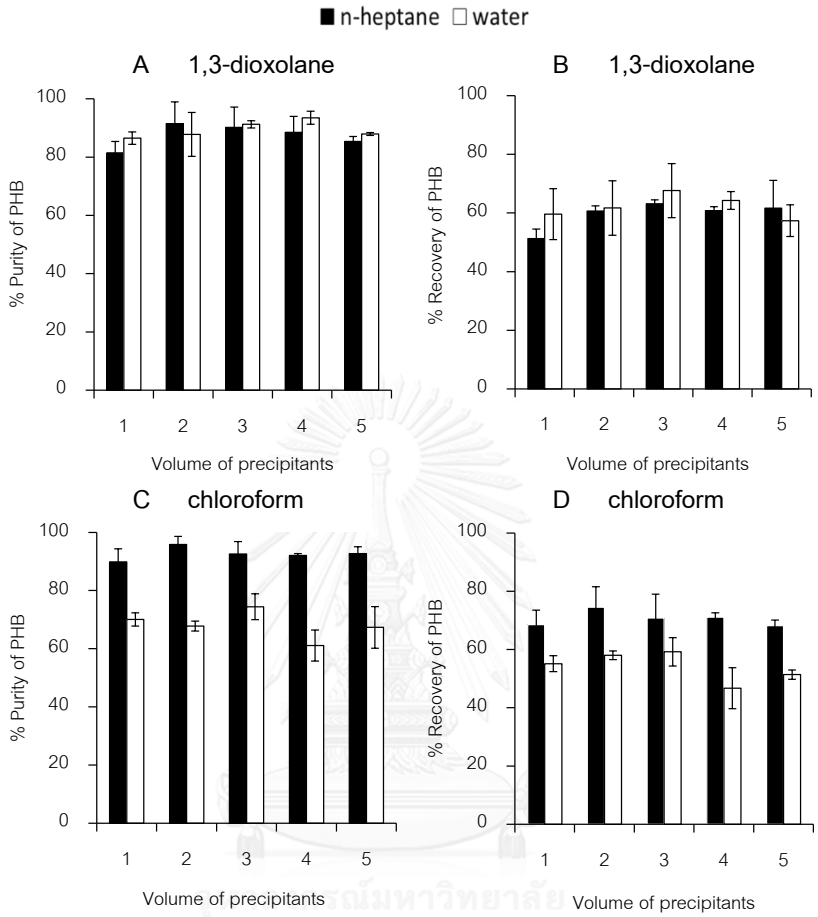


Figure 14. Precipitation efficiency of PHB in 2 mL of 5% (w/v) of dried cells in 1,3-dioxolane and precipitated with water comparing with *n*-heptane. The results are expressed as (%) recovery and (%) purity. The PHB solution was precipitated with various ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 (vol/vol) of PHB solution to precipitants; *n*-heptane and water, at room temperature for 1 h. A: PHB purity (%) obtained from 1,3-dioxolane extraction. B: PHB recovery (%) obtained from 1,3-dioxolane extraction. C: PHB purity (%) obtained from chloroform extraction. D: PHB recovery (%) obtained from chloroform extraction. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

4.3.3 Extraction of PHB from dried cells in 40 mL of solvent (large scale extraction)

Figure 15 shows percentage of recovery and purity when PHB was extracted from dried cells with 5% (w/v) of dried cells in 1,3-dioxolane and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water. It was observed that PHB was extracted from dried cells with 5% (w/v) of dried cells in 1,3-dioxolane and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water gave 74.7±6.4 % of recovery and 97.9±1.8 % of purity. In comparison with chloroform, 76.3±0.7 % of recovery and 95.5±3.0 % of purity were obtained.

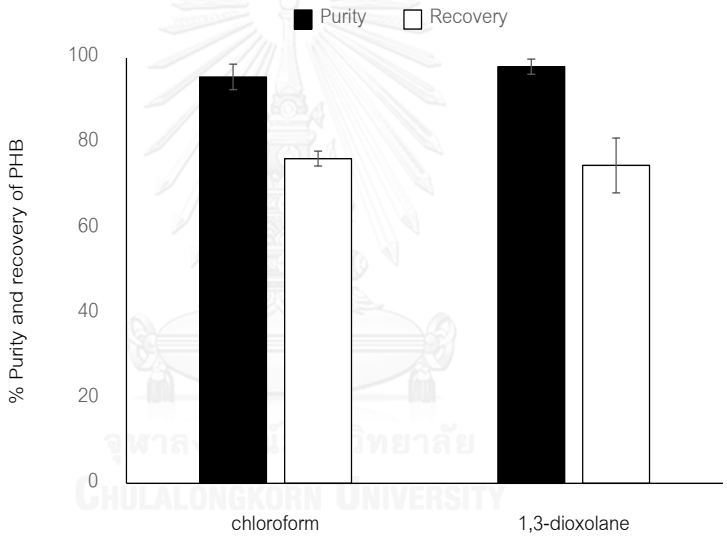


Figure 15. Extraction efficiency of PHB from dried cells in 40 mL of 5% (w/v) of dried cells in 1,3-dioxolane and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water in comparison with 5% (w/v) of dried cells in chloroform and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

4.4 Extraction of PHB from wet cells

Considering the cost related to energy consumption during cell separation and dehydration process, experiments of direct PHB extraction were performed on wet cells to investigate the feasibility of by-passing cell drying step.

4.4.1 Extraction of PHB from wet cells in 2 mL of solvent (small scale extraction)

Based on the results obtain from 4.3.1, wet cells 0.3 g, equivalent to dried cells 0.1 g, was extracted with 1,3-dioxolane at 80 ºC for 6 h. After solvent extraction, cells were lysed and became slurry. As shown in Figure 16A, it was observed that cell debris fraction was settled in the bottom part whereas solvent fraction was the upper part. In comparison with chloroform, as shown in Figure 16B, chloroform was also used as the control experiment. It was clearly observed that 3 phases were formed; the upper part was water phase that released from wet cell, the middle part was cell debris and the bottom part was chloroform phase. The mixtures were centrifuged at 4620× *g* for 10 min. at room temperature. The 1,3-dioxolane and chloroform fractions were carefully transferred to another screw cap borosilicate glass test tube. Next, PHB polymer was precipitated with 3 volumes of water, as shown in Figure 16C for 1,3-dioxolane and Figure 16D for chloroform. The extraction efficiency results were summarized in Figure 17. For wet cell extraction, 1,3-dioxolane gave 84.7±7.4 % of recovery whereas chloroform gave only 43.9±4.0 % of recovery because the difficulty to transfer the chloroform phase (bottom part) to precipitate PHB with water. For chloroform extraction, low percentage of recovery was observed because some amounts of PHB were remained in the middle phase as the water phase was occurred. Interestingly, 1,3-dioxolane gave 94.3±4.4 % of purity as same as chloroform gave 93.7±5.2 % of purity.

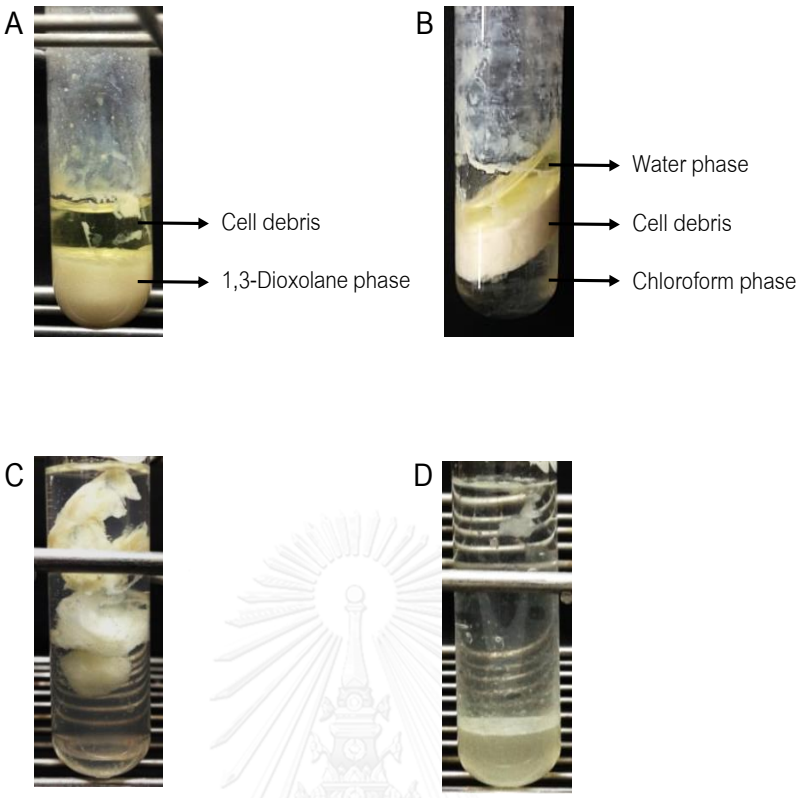


Figure 16. Extraction of PHB from wet cells in 2 mL of solvent at 80 ºC for 6 h and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water. A: Extraction with 1,3-dioxolane, B: Extraction with chloroform. C: Precipitation with 3 volumes of water after 1,3-dioxolane extraction. D: Precipitation with 3 volumes of water after chloroform extraction.

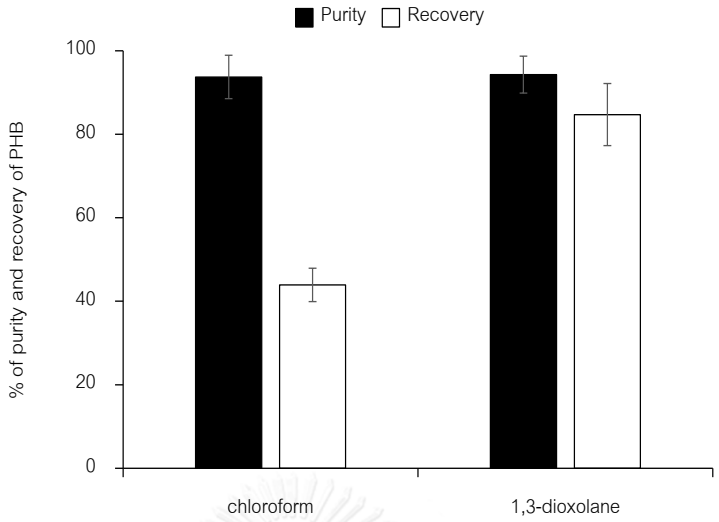


Figure 17. Extraction efficiency of PHB from wet cells in 2 mL of 1,3-dioxolane and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water in comparison with chloroform extraction and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

4.4.2 Extraction of PHB from wet cells in 40 mL of solvent (large scale extraction)

Based on the results obtain from 4.4.1, large scale extraction in 40 mL of solvent was investigated to explore the feasibility of scaling up the application of the environmentally safe extraction method. The extraction efficiency results in 40 mL of solvent were summarized in Figure 18. For wet cell extraction, 1,3-dioxolane gave 78.1±8.1 % of recovery and 91.6±3.0 % of purity comparing with using chloroform that gave 64.3±8.5% of recovery and 89.4±6.1 % of purity.

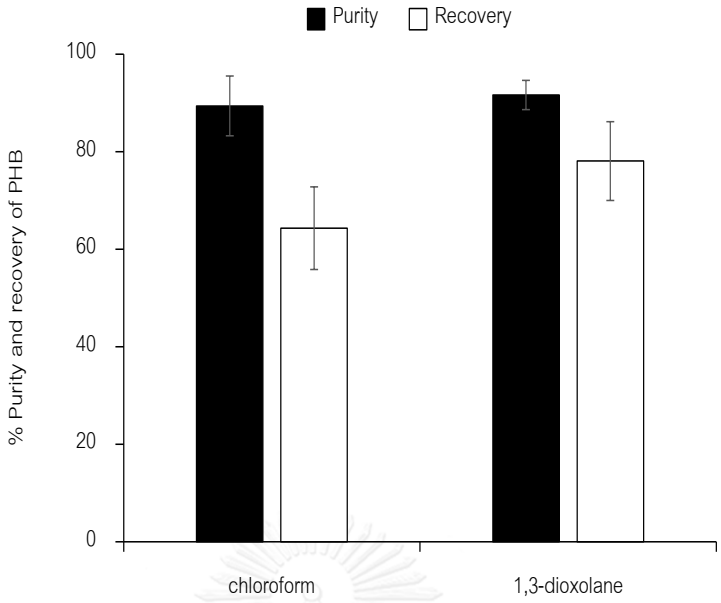


Figure 18. Extraction efficiency of PHB from wet cells in 40 mL of 1,3-dioxolane and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water in comparison with chloroform extraction and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

4.4.3 PHB recovery from wet cells at room temperature in 2 mL of solvent

With the aim of reducing the energy consumption during the solvent extraction process at 80 ºC, the extraction efficiency of PHB from wet cells in 2 mL of 1,3-dioxolane at room temperature for 4, 6, 12, 24 and 36 h was investigated. The results were shown in Figure 19 and it was observed that PHB extraction with 1,3-dioxolane at room temperature was possible and the percentage of recovery increased as the extraction time increased. For the extraction time at 36 h, 77.3±3.9 % of recovery was obtained with 91.3±7.3 % of purity. For comparison, chloroform gave lower percentage of recovery than those of 1,3-dioxolane and only 38.6± 0.7 % of recovery with 97.3±2.6 % of purity was obtained when the extraction time was 36 h.

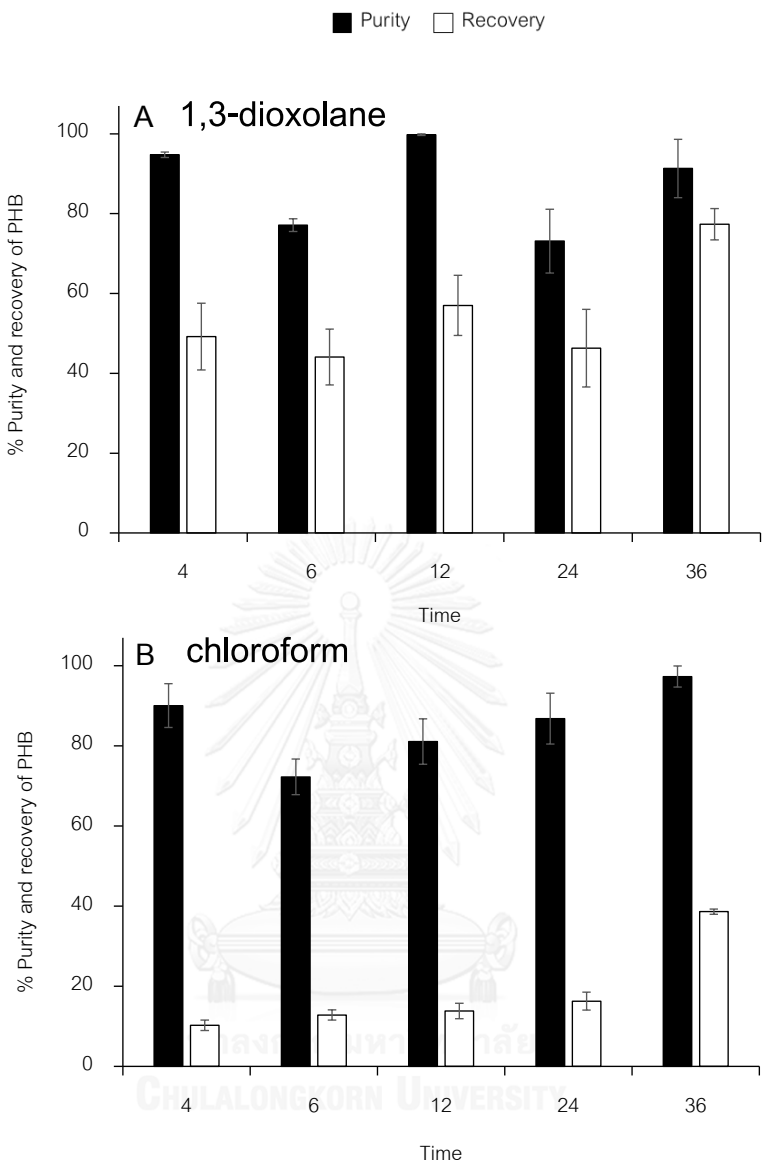


Figure 19. Extraction efficiency of PHB from wet cells in 2 mL of solvent at room temperature for 4, 6, 12, 24 and 36 h and precipitated with the ratio of 1:3 (vol/vol) of PHB solution to water. A: 1,3-dioxolane extraction. B: chloroform extraction. The results were represented of three independent experiments and are presented as the mean value±standard deviation (S.D.).

4.5 Test of physical and mechanical properties of purified PHB

The efficiency of 1,3-dioxolane extraction protocols can evaluate from the characterization of PHB products. The purity and recovery of the PHB polymers were carried out by GC. The average molecular weight (M_w), the number molecular weight (M_n) and the polydispersity index (PDI) were determined by gel permeation chromatography (GPC). The thermal analysis was indicated by differential scanning calorimetry (DSC). The mechanical properties of the film samples were analyzed by universal testing machine (UTM). All parameters were also evaluated in commercial PHB (Sigma-Aldrich Corp., St. Louis, MO, USA) as a comparison in this study (Table 6).

The results showed that purified PHB from chloroform extraction had highest molecular weight of 6.51×10^5 followed by purified PHB from wet cells extraction and commercial PHB. At the same time, the highest molecular weight of purified PHB from 1,3-dioxolane extraction was only 4.86×10^5 that was observed in wet cells extraction.

Concerning the mechanical properties of purified PHB from 1,3-dioxolane extraction was similar with commercial PHB except dried cells extraction without water precipitation and wet cells extraction at room temperature. While, high molecular weight PHB from chloroform extraction had not shown the excellent mechanical value in comparison with purified PHB from 1,3-dioxolane extraction. However, all purified PHB samples had a similar melting temperature (T_m) and slightly higher than commercial PHB.

Table 6. Characterization of PHB produced by *C. necator* strain A-04

Conditions	Purity (%)	Recovery (%)	M_w ($\times 10^5$)	M_n ($\times 10^5$)	PDI	T_m (°C)	Young's modulus (MPa)	Stress at max load (MPa)	Elongation break (%)
commercial PHB CAS 26063-00-3 Batch # 02925CB			5.33	2.27	2.35	172.0	3187.3	40.3	1.4
chloroform (dry cells) with water precipitation	99.7	70.2	6.51	3.61	1.80	178.2	2354.9	24.0	1.8
chloroform (wet cells) with water precipitation	95.9	61.5	5.93	5.78	1.03	n.d.	2212.7	23.6	1.3
1,3-dioxolane (dry cells) solvent evaporation	84.3	86.3	2.25	0.38	5.99	n.d.	1999.0	21.2	3.6
1,3-dioxolane (dry cells) with water precipitation	99.2	74.8	3.14	1.96	1.60	174.7	3273.5	33.9	1.3
1,3-dioxolane (wet cell) solvent evaporation	78.8	69.4	4.03	2.66	1.51	n.d.	3236.3	34.2	1.2
1,3-dioxolane (wet cells) with water precipitation	98.5	63.4	4.86	3.74	1.30	173.1	3628.4	38.3	1.4
1,3-dioxolane (wet cells) room temperature extraction and water precipitation	90.0	89.0	1.82	1.21	1.50	177.3	2392.2	17.0	0.6

n.d. is not determine

CHAPTER V
DISCUSSION

5.1 Preparation of bacterial cells containing PHB granules

C. necator strain A-04, isolated from soil in Thailand [25, 98, 99], was capable of using various carbon sources (Table 7). This strain was preliminarily investigated for the accumulation of PHAs using fructose, butyric acid, valeric acid, γ-hydroxybutyric acid, γ-hydroxybutyric acid lactone and 1,4-butanediol. The highest biomass of 6.8 g/L with PHB content of 78 wt% were produced by fructose as a carbon source. However, these carbon sources are pure grade chemicals that are expensive and unfeasible for industrial scale production [25]. Then, locally-made natural sugars such as refined sugarcane, brown sugarcane, rock sugar, toddy palm sugar and coconut palm sugar were used for PHA production. The results were found that the ability of *C. necator* strain A-04 to utilize these natural sugar for growth and PHA accumulation were poor [99]. In this study, we used 30 g/L of sucrose as a sole carbon source with 200 of C/N ratio for PHB production. The results found that a total cell mass of 7.3±1.2 g/L accumulated up to 63.9±2.8 (wt%) of PHB content. This results were higher that other carbon sources but PHB content was slightly lower than the use of 20 g/L fructose.

Table 7. Comparison with biomass and PHA content of PHB produced by *C. necator* strain A-04 when grown on various carbon sources.

Carbon source	PHA	Biomass (g/L)	PHA content (wt%)	Ref.
20 g/L refined sugarcane	PHB	2.9	21	[99]
20 g/L brown sugarcane	PHB	3.1	17	[99]
20 g/L rock sugar	PHB	2.9	5	[99]
20 g/L toddy palm sugar	PHB	3.4	9	[99]
20 g/L coconut palm sugar	PHB	3.6	7	[99]
20 g/L fructose	PHB	6.8	78	[25]
20 g/L butyric acid	PHB	4.32	52	[25]
30 g/L sucrose	PHB	7.3	63	This study

5.2 Investigation of solubility and precipitation of PHB in various solvents

There are several parameters that may affect the efficiency of PHAs production process. First of all, a total of PHAs content required, available carbon sources such as palm oil, waste water, crude glycerol [100, 107, 108] and efficient fermentation process. Furthermore, good recovery process is needed to achieve high purity of PHAs with worthiness [53]. Using chlorinated hydrocarbon extraction solvents such as chloroform, 1,2-dichloroethane, methylenechloride, propylene and ethylene carbonates achieve high quality of isolated PHAs [55]. However, a major drawbacks of these extraction solvents are health and environment problems [64]. The use of environmentally friendly solvents is an alternative to solve these problems and provide more possibility to produce PHAs the in industrial scale.

5.2.1 Test of solvents for PHB dissolution

According to the result, the PHB dissolution may effected by the boiling point to the solvent. The best solvent for PHB dissolution was 1,3-dioxolane and followed by 2-MTHF and ethyl lactate, respectively. In fact, temperature is a factor that influence the rate of reaction. The rate of reaction doubles for every 10 ºC rise in temperature. Therefore, at the same temperature, low boiling point solvents might be activated easier than high boiling point. Since, the boiling point of 1,3-diolane and 2-MTHF are lower than 100 ºC (76 and 80 ºC, respectively), while ethyl lactate and 1,3-propanediol are higher than 100 ºC (154 ºC and 214 ºC, respectively) (Table 3). 1,3-dioxolane and 2-MTHF showed better result compared to ethyl lactate and 1,3- propanediol. Moreover, 1,3-dioxolane extraction had slightly lower solubility compare to chloroform extraction, which was the positive control experiment. This could be explanted by 2 factors: (1) the boiling point of chloroform is lower than 1,3-dioxolane and, above all, (2) chloroform has lower polarity than 1,3-dioxolane. The polarity of solvents can be indicated by dielectric constant that non-polar solvents have lower dielectric constant than polar-solvents. Dielectric constant of chloroform and 1,3-dioxolane are 5 and 7.13, respectively [109]. In addition, the dielectric constant of PHB was reported as 2.8 [110] that was closer to the dielectric constant of chloroform. So, chloroform is more suitable for PHB solubility than 1,3-dioxolane.

Another parameter that effect the solubility of PHB is PHB content. Lower concentration of PHB tends to dissolve easier than higher concentration of PHB. High viscosity of the solution leads difficulty of removal cell debris which may result in inefficient separation process. From the study, concentrations of 2.5% (w/v) and 5% (w/v) PHB solution showed similar percentage of solubility and also were higher than 7.5(w/v) and 10% (w/v) PHB solution. Several researches were suggested that the high viscosity of the

extracted polymer solution was observed in concentration exceeds 5% (w/v) PHAs solution. Reidel *et al*. (2013) described that at 5% (w/v) P(HB-co-HHx) solution gave better solubility than 10% (w/v) P(HB-co-HHx) solution in various non-halogenated solvents. Hence, we designed to apply the concentration of 5% (w/v) throughout the experiment because of cost saving by reducing amount of solvent in production and treatment processes.

5.2.2 Test of solvents for PHB precipitation

Precipitation step is always required for PHAs solvent extraction method, instead of evaporating the solvent because it will separate PHAs solid from the solvent. Riedel *et al*. (2013) reported that the difficult problem of evaporation in batch operations is the polymer will coat on the vessel after the solvent is discarded [100]. In addition, precipitation step can reduce non PHAs polymer material cell (NPMC) and enhance PHA purity [60]. In this experiment, All precipitants gave similar potential to precipitate purified PHB from 5% (w/v) PHB solution in 1,3-dioxolane at room temperature. However, the results of this experiment was lower recovery yield than other studies such as Riedel *et al*. (2013) had tested efficiency of precipitants; *n*-hexane and *n*-heptane in 5% (w/v) P(HB-*co*-HHx) in methyl isobutyl ketone (MIBK) and butyl acetate (BA) at room temperature. The results showed that a threefold volume of either precipitant gave nearly 100% of P(HB-*co*-HHx) [100]. Moreover, low molecular weight alcohol such as methanol gave 83% of recovery when poly(hydroxyoctanoate-*co*-hydroxyhexanaote) (P(HO-*co*-HH) was extracted in methylene chlorine at room temperature for 24 h and precipitated with tenfold volume of cold methanol [111]. Therefore, these precipitants were applied with 5% (w/v) PHB solution obtained from dried cells extraction with 1,3-dioxolane extraction in 2 mL again. Comparison with chloroform extraction from dried cells of *C. necator* strain A-04.

In all cases, the extracted PHB solution was precipitated with 3 volumes of the various precipitants.

5.3 Extraction of PHB from dried cells

Based on the results of previous experiment (4.2.1), 1,3-dioxolane was selected to extract PHB from dried cells of *C. necator* strain A-04 comparison with chloroform extraction at different scales (2 mL. and 40 mL.).

5.3.1 Extraction of PHB from dried cells in 2 mL of solvent (small scale extraction)

The results showed that 1,3-dioxolane extraction at 80 ºC for 6 h with 3 volume of water precipitation gave the highest recovery of 77.1±7.3% with the purity of 97.2±2.3%, followed by precipitation with 3 volume of *n*-heptane in the same conditions that gave the recovery and purity of 72.6±1.1% and 97.8±0.1 %, respectively. The results had been improved up to 10 % of PHB precipitation in 3 volume of n-hexane, n-heptane, water, iso-propanol, methanol and ethanol at room temperature for 1h in comparison with previous experiment (4.2.2). This might due to PHB solution form dried cells extraction had lower PHB concentration 5% (w/v) solution that was prepared form purified PHB. However, precipitation with other precipitants; *n*-hexane, iso-propanol, methanol and ethanol performed high purity but slightly low recovery in PHB polymer. Concerning the texture of purified PHB after precipitated with *n*-hexane, iso-propanol, methanol and ethanol, gel texture was formed and it was difficult to separate. PHB recovery might be lost in this process. For comparison, with 1,3-dioxolane extraction at different temperatures, efficiency of 1,3-dioxolane extraction was observed at 80 ºC to 100 ºC while at 30 ºC and 60 ºC were ineffective. Likewise, Riedel *et al*. (2013) demonstrated the recovery of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(HB-*co*-HHx)) from freeze-dried cells containing 76% (wt%) of PHA with chloroform and non-halogenated solvents at 50 ºC, 75

ºC and 100 ºC. Their results showed that the solvents could extract almost all PHA present in cells (≥98%) at 75 ºC and 100 ºC [100]. In addition, Samori *et al*. (2015) described a poor recovery of dimethyl carbonate (DMC) extraction at 50 ºC that gave only 11±1% instead of 88±6% at 90 ºC [106]. Hejazi *et al.* (2003) and Aramvash *et al.* (2015) suggested that a higher extraction temperature was more suitable for cell disruption and the solubility of PHB in the solvents than low extraction temperature [112, 113]. The results of extraction at different temperatures were similar trends with chloroform extraction.

5.3.2 Test of PHB precipitation with water and *n*-heptane

After PHAs extraction, the purified PHAs are separated form solvents by using a precipitant such as low-molecular alcohols, *n*-hexane, *n*-heptane, ether, acetone and water [55, 56, 100, 107, 114]. In addition, any NPMC that are also present in the solution will be accompanied with purified PHAs [100, 115]. In this experiment, high PHB recovery and purity were achieved with ratio of 1:3 (vol/vol) of PHB solution to water after extraction with 1,3-dioxolane. The obtained PHB after added water was shown in figure 20 that was different from other precipitants because the PHB was separated and floated on surface of solvent mixture. Moreover, an advantage of water separation was easy to filter. In fact, water is the most environmentally benign and cost effective than *n*-heptane (Table 3). Therefore, water was the best choice to use for next experiments.

5.3.3 Extraction of PHB from dried cells in 40 mL of solvent (large scale extraction)

Large scale extraction was demonstrated to confirm the results of 1,3-dioxolanne extraction at 80 ºC for 6 h with water separation in 2 mL (experiment 4.3.3). Percentage of recovery and purity results of large scale extraction had consistency with the results of small scale. Moreover, characteristic of purified PHB from 1,3-dioxolane extraction with water separation was difference from chloroform extraction. Figure 20 A, PHB separation

with water of chloroform extraction was separated into 2 phases; the upper phase was water phase and the bottom phase was chloroform phase. There are 2 reasons that support this character. First, chloroform do not mix with water because it is low solubility in water. Second, chloroform is the bottom phase and water is upper phase because chloroform has a higher density than water. These properties are shown on Table 3. For comparison, 1,3-dioxolane gave the PHB in solid state after separated with water that showed in Figure 20B. Concerning the solubility in water property of 1,3-dioxolane was miscible solvent. Therefore, 1,3-dioxolane was able to mix with water and PHB was separated out. It could be separated easily by filtration instead of centrifugation. Shift of centrifugation process help to reduce energy consumption and also time consumption.

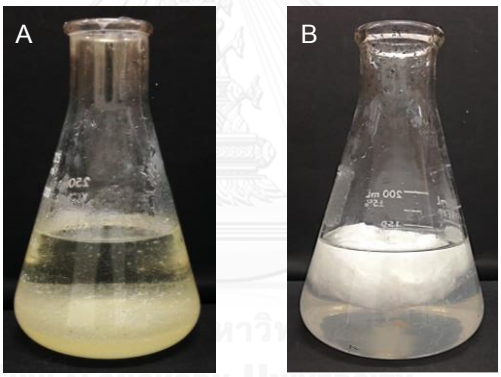


Figure 20. Characteristic of PHB separation with water. A: chloroform extraction. B: 1,3-dioxolane extraction.

5.4 Extraction of PHB from wet cells

High recovery cost is a key factor that lead to unsuccessful in industry scale. The cost and time of process can be reduced by direct recovery from wet cells because a drying process of the cells is removed. Choi and Lee (1999) had improved a simple PHB recovery process by using wet cells of recombinant *E.coli*. They examined various compounds such as surfactants, acids and bases. The achievement result was observed

on digestion of 5% (w/v) of wet cells with 0.2 *N* sodium hydroxide (NaOH) at 30 ºC for 1 h that gave 98.5% of purity and 91.3% of recovery. However, alkali digestion was negligible degradation of PHB during digestion process [5]. Moreover, Riedel *et al*. (2013) demonstrated the recovery of P(HB-*co*-HHx) from 2% (w/v) of wet cells in methyl isobutyl ketone (MIBK) and ethyl acetate at 100 ºC with stirring under reflux conditions for 4 h and they were precipitated by addition of threefold volumes of *n*-hexane. MIBK extraction was presented to be the best solvent for P(HB-*co*-HHx) recovery. The purified copolymer had a purity of up to 99% and recovery yield of up to 84% [100]. Therefore, extraction of PHB from wet cells was investigated in this experiment.

5.4.1 Extraction of PHB from wet cells in 2 mL of solvent (small scale extraction)

The best conditions from dried cells extraction were examined on wet cells extraction. The results found that wet cells could be extracted efficiently by using 1,3-dioxolane. High purity and recovery yield were observed in 1,3-dioxolane extraction while low recovery yield was offered in chloroform extraction. This might be due to water in wet cells had an effect on chloroform extraction because wet cells extraction with chloroform was observed that 3 phases; the upper part was water phase, the middle part was cell debris and the bottom part was chloroform phase (Figure 16B). This stratification had decreased surface area between wet cells and chloroform that had affected to decrease recovery yield. At the same time, 1,3-dioxolane could not be observed the separation of water. This might be due to a concurrent that wet cells and the solvent were enhanced mixing by water in the mixture solvent.

5.4.2 Extraction of PHB from wet cells in 40 mL of solvent (large scale extraction)

Extraction of PHB from wet cells in large scale was performed as same as extraction of PHB from dried cells. The results confirmed that 1,3-dioxolane could extract PHB from wet cells and also related with small scale extraction. Moreover, PHB recovery result obtained with chloroform was lower than that of 1,3-dioxolane extraction.

5.4.3 PHB recovery from wet cells at room temperature in 2 mL of solvent

Generally, mild conditions are preferred in industrial process because it is easy to operate and save cost from energy consumption. At ambient temperature, typical halogenated extraction solvent such as chloroform and methylene chloride were reported that they could dissolved PHAs from biomass [56, 116]. Hence, 1,3-dioxolane was tested for extraction of PHB in comparison with chloroform in this experiment. Wet cells were observed PHB extraction at room temperature (30 ºC) with various extraction times (4, 6, 12, 24 and 36 h) although dried cells extractions in the solvents at low temperatures (30 ºC and 60 ºC) were incapable in previous experiment. Interestingly, wet cells extraction in 1,3-dioxolane at 80 ºC for 6 h gave higher PHB recovery than dried cells extraction at the same conditions in previous experiment. Likewise, Samori *et al*. (2015) reported that recovery of the P(3HB-co-3HV) and P(3HB-co-4HB) from wet cells extraction at 90 ºC with DMC were 87±1% and 95±8%, respectively. At the same conditions, the recovery of the copolymers from freeze-dried cells extraction were 84±5% and 88±1%, respectively. These might be indicated that wet cells were able to extract easier than dried cells. However, the PHB recovery in the solvents extraction at room temperature were increased when time was increased. Completed PHB extraction might be obtained by long extraction time or an increase temperature.

Table 8. Various PHA recovery methods that had been reported.

Conditions	PHA	Bacterial	Purity (%)	Recovery (%)	Ref.
Solvent extraction method					
Chloroform, stirring at 37 °C for 48h	PHB	*B. cereus* SPV	92	31	[117]
chloroform, stirring at 60 °C for 2 h	PHB	*C. necator* DSM 545	95	96	[118]
1,2-propylene carbonate, stirring at 130 °C for 30 min	PHB	*C. necator* DSM 545	84	95	[118]
ethyl acetate, at 100 °c for 4 h	P(HB-*co*-HHx)	*R. eutropha*	97	94	[100]
methyl isobutyl ketone, at 100 °c for 4 h	P(HB-*co*-HHx)	*R. eutropha*	96	84	[100]
dimethyl carbonate, at 90 °c for 4 h	PHB	*C. necator*	95	88	[106]
(13% v/v) of dichloromethane sodium hypochlorite solution, at 37 °c for 4 h	PHB	*C. necator* H16	99	90	[54]
Digestion method					
SDS and sodium hypochlorite	PHA	*Azotobacter chroococcum* G-3	98	87	[119]
EDTA and Triton X-100	PHA	*Sinorhizobium meliloti*	68	16	[120]
0.1 M sodium hydroxide, at 22 °C for 2h	P(HHp-*co*-HO)	*P. putida*	44	92	[121]
1 M sodium hydroxide, at 60 °C for 3h	P(HB-*co*-HV)	*R. eutropha* H 16	78	45	[71]
SDS, at 60 °C for 3h	P(HB-*co*-HV)	*R. eutropha* H 16	90	81	[71]
sodium polyoxoethylene sufate, at 60 °C for 3h	P(HB-*co*-HV)	*R. eutropha* H 16	90	85	[71]

Conditions	PHA	Bacterial	Purity (%)	Recovery (%)	Ref.
sodium alpha olefin sulfonate, at 60 °C for 3h	P(HB-*co*-HV)	*R. eutropha* H 16	91	87	[71]
Brij®58, 5% (w/v) of lyophilized cells at 60 °C for 3h	P(HB-*co*-HV)	*R. eutropha* H 16	83	99	[71]
13% (v/v) of sodium hypochlorite pH 12.3 at room temperature for 1 h	PHB	*R. eutropha* H 16	93	87	[67]
0.5 N sodium hydroxide solution, at 37 °C for 4 h	PHB	*C. necator* H16	93	79	[54]
(13% v/v) sodium hypochlorite solution, at 37 °C for 4 h	PHB	*C. necator* H16	97-98	82	[54]
3.5% (v/v) of hydrosulfuric acid solution, at 80 °C for 6 h	PHB	*C. necator* H16	99	90	[54]
Mechanical disruption method					
SDS-high pressure homogenization	PHB	*Metylobacterium* sp. V49	95	98	[122]
sonication	PHA	*B. flexus*	92	20	[123]

5.5 Test of physical and mechanical properties of purified PHB

The characteristics of purified PHB were comparable to that of the commercial PHB and purified PHB from chloroform extraction. Molecular weight of purified PHB from wet cells extraction with 1,3-dioxolane at 80 ºC for 6 h and water precipitation was slightly higher than dried cells extraction at the same conditions. However, both of them were quite lower molecular weight than commercial PHB and purified PHB from chloroform extraction. Interestingly, their mechanical properties were similar to commercial PHB. Concerning the purified PHB from wet cells with 1,3-dioxolane at room temperature and water precipitation had the highest percentage of recovery while molecular weight and mechanical properties were definitely lower than others. Their properties had been related with obtained PHB film that was quite brittle and fragile. In addition, precipitation step was reported that it was able to increase high purity of PHAs by elimination of contaminate in solvent [100, 114]. The efficient of precipitation was evaluated by comparison with solvent evaporation. In this study, purity of purified PHB gave the different results between purified PHB from water precipitation and purified PHB from solvent evaporation. Adding water precipitation had higher PHB purity than without precipitation. The higher purity led to decrease recovery yield because NPMC could be removed [60]. These led to increase values of molecular weight and also improved mechanical properties. However, purified PHB from in study was remained in similar melting temperature (T_m) up to 170 ºC.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

In this study, 1,3-dioxolane, an environmental solvent, was used to extract PHB from *C. necator* strain A-04. This solvent was able to apply both dried cells and wet cells extraction at 80 ºC for 6 h with water separation. High purity up to 97.9±1.8% with recovery of 74.7±6.4% were observed in dried cells extraction. For the results of wet cells extraction showed 94.3±4.4% of purity with 84.7±7.4% of recovery. Mechanical properties of the obtained PHB were high quality but their molecular weight were a slightly decease in comparison to commercial PHB and purified PHB from chloroform extraction. Moreover, our preliminary study on 1,3-dioxolane extraction of PHB from *Bacillus* sp. MSC-5 was performed according to the method described in the literature [124]. The result of PHB extraction from dried cells of *Bacillus* sp. MSC-5 showed 94.5±3.6% of purity with 90.9±2.2% of recovery. These results showed that 1,3-dioxolane was capable to extract PHB from Gram negative and Gram positive bacteria. For comparison with other methods in Table 8 found that 1,3-dioxolane extraction gave high purity as well as other methods but the recovery was slightly lower than some methods. There were several factor that might affect to PHA recovery: (1) the microorganism production strain (different strain display different fragilities of the cell envelop), (2) the type of PHA polymer regarding the composition on the molecular level (scl-PHA or mcl-PHA), (3) the intracellular content of PHA, (4) the type of biomass (lyophilized cells, dried cells or wet cells) and (5) the conditions for PHA recovery (time, temperature, shake or ratio of biomass load).

The results of this study lead to recommendations for the reduce of production cost from PHB extraction in 1,3-dioxolane. 1,3-dioxolane had a potential to extract PHB from wet cells at room temperature but the obtained PHB was low quality polymer such as low molecular weight, low mechanical properties. This might effect from activity of

depolymerase from this wide type strain. Therefore, we suggested that this process should be developed by use high temperature in the beginning of process and follow with room temperature. In addition, shaking might be required because it might support cells and solvent mix as well. These might be reduce time consumption and improve the quality of purified PHB.

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APPENDIX

APPENDIX A

MEDIUM PREPARATION

1. Preculture medium agar

Composition (per liter):

Yeast extract	2	grams
Peptone	10	grams
Magnesium sulfate heptahydrate	1	grams
Agar	15	grams

All of the ingredients were dissolved in distilled water, and the volume was adjusted with distilled water to be 1 liter. Then the medium was autoclaved at 121 °C under 15 psi pressures for 15 minutes.

2. Preculture medium broth

Composition (per liter):

Yeast extract	2	grams
Peptone	10	grams
Magnesium sulfate heptahydrate	1	grams

All of the ingredients were dissolved in distilled water, and the volume was adjusted with distilled water to be 1 liter. Then, the medium was autoclaved at 121 °C under 15 psi pressures for 15 minutes.

3. Production medium

Composition (per liter)

Ammonium sulfate	Vary with 200 of C/N ratio	
Potassium dihydrogen phosphate	1.5	grams
Disodium hydrogen phosphate dehydrate	4.5	grams
Magnesium sulfate heptahydrate	0.2	grams
Calcium chloride dehydrate	0.02	grams
Ammonium iron (III) citrate (17% of iron)	0.05	grams
Trace element solution	2	milliliters

All of the ingredients were dissolved in distilled water and pH was adjusted with hydrogen chloride (HCl) to be pH 7. Then, the volume was adjusted with distilled water to be 1 liter and autoclaved at 121 °C under 15 psi pressures for 15 minutes.

4. Trace element in 1M HCl

Composition (per liter)

Calcium chloride	1.67	grams
Zinc sulphate heptahydrate	2.78	grams
Manganese dichloride tetrahydrate	1.98	grams
Copper dichloride dihydrate	0.17	grams
Ferrous sulfate heptahydrate	0.2	grams

Magnesium sulfate was separated to dissolve with trace element. All of ingredients were mixed together after magnesium sulfate was dissolve completely. After that pH was adjusted with hydrogen chloride (HCl) to be pH 7. Then, the volume was adjusted with distilled water to be 1 liter and autoclaved at 121 °C under 15 psi pressures for 15 minutes.

5. 30 g/L of sucrose

150 grams of sucrose was dissolved in distilled water, and the volume was adjusted with distilled water to be 1 liter. Then the solution was autoclaved at 110 °C under 15 psi pressures for 15 minutes.

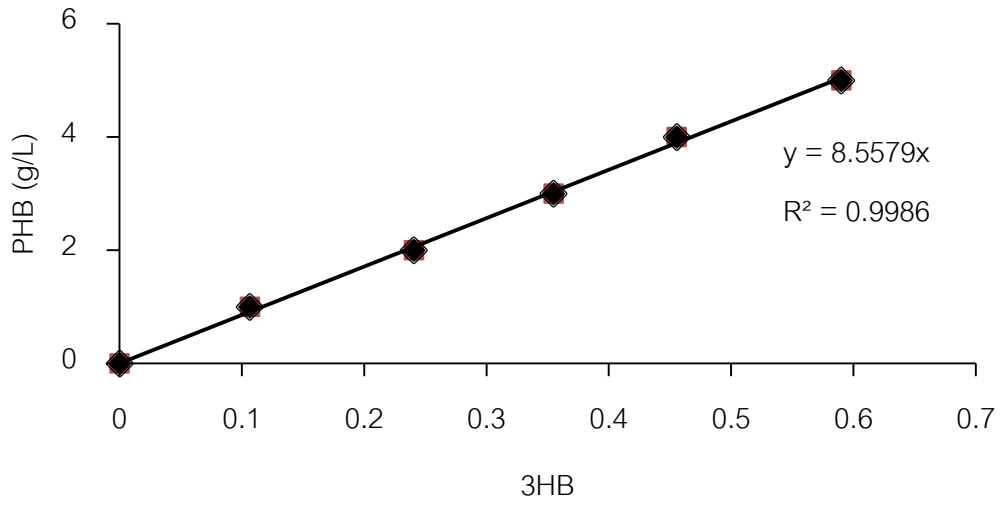
6. 0.85% (w/v) of sodium chloride

8.5 grams of sodium chloride was dissolved in distilled water, and the volume was adjusted with distilled water to be 1 liter. Then the solution was autoclaved at 121 °C under 15 psi pressures for 15 minutes.

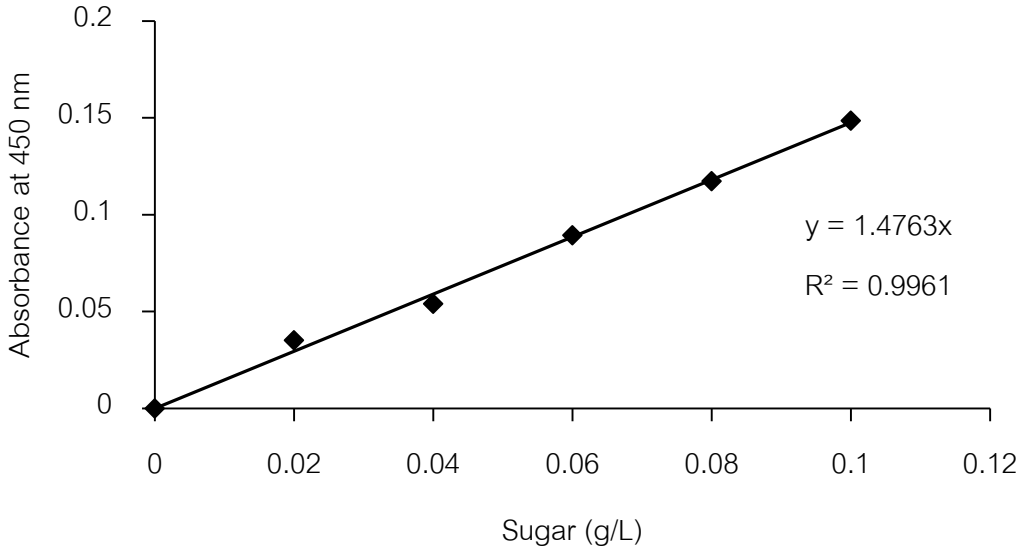
APPENDIX B

STABDARD CURVE

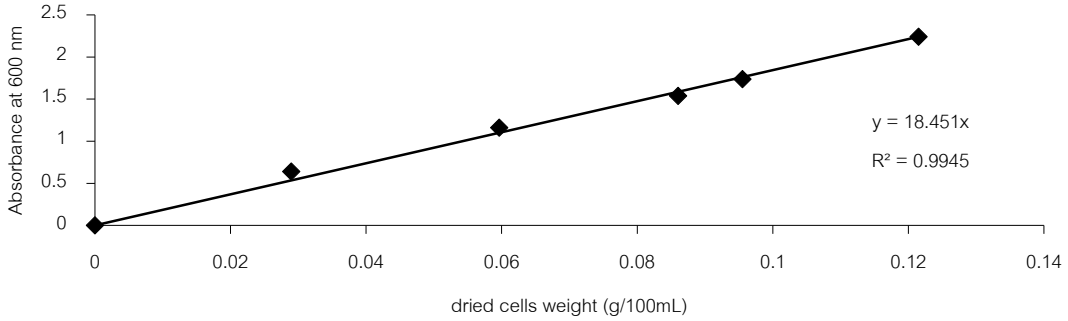
1. PHB standard curve



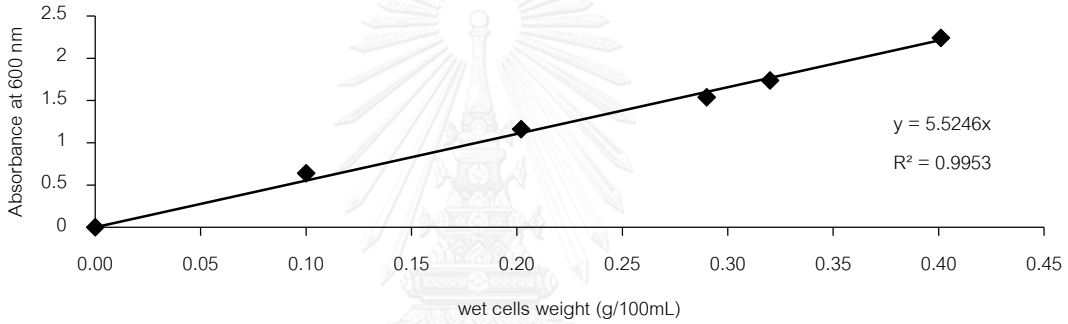
2. Total sugar standard curve



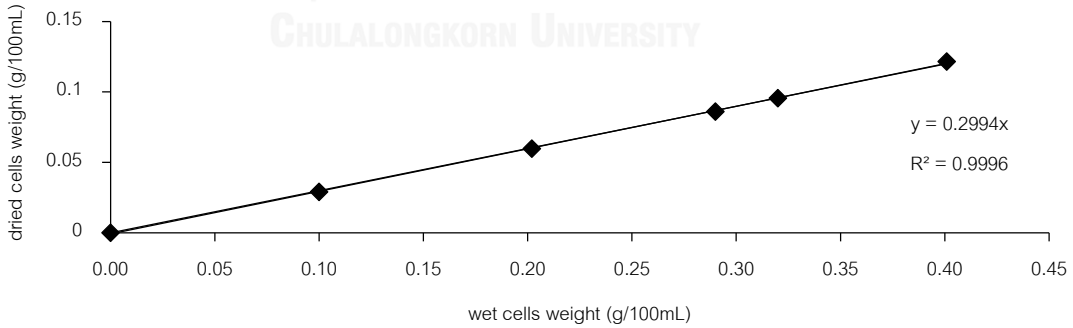
3. Correlation between absorbance at 600 nm and dried cells



4. Correlation between absorbance at 600 nm and wet cells



5. Correlation between wet cells and dried cells



APPENDIX C

TABLE

1. PHB precipitation with *n*-heptane and water

Volume of precipitant (vol/vol)	1,3-dioxolane		chloroform	
	Purity (%)	Recovery (%)	Purity (%)	Recovery (%)
n-heptane				
1:1	81.5±3.9	51.3±3.3	89.8±4.5	68.6±5.0
1:2	91.5±7.5	60.6±1.8	95.8±2.8	74.5±7.0
1:3	90.2±6.9	63.2±1.4	92.6±4.3	70.8±8.2
1:4	88.5±5.4	60.8±1.4	92.1±0.6	71.1±1.5
1:5	85.4±1.7	61.7±9.4	92.7±2.3	68.2±1.9
water				
1:1	86.5±2.1	59.6±8.7	70.1±2.3	55.1±2.7
1:2	87.8±7.5	61.7±9.3	67.8±1.7	58.0±1.6
1:3	91.3±1.2	67.6±9.2	74.4±4.5	59.2±4.9
1:4	93.5±2.2	64.3±3.0	61.1±5.3	46.7±7.0
1:5	87.9±0.5	57.4±5.4	67.3±7.2	51.4±1.6

2. Extraction of PHB from dried cells in 40 mL of solvent (large scale extraction)

Solvent	Purity (%)	Recovery (%)
chloroform	95.5±3.0	76.3±1.8
1,3-dioxolane	97.9±1.8	74.7±6.4

3. Extraction of PHB from wet cells in 2 mL of solvent (small scale extraction)

Solvent	Purity (%)	Recovery (%)
chloroform	93.7±5.2	43.9±4.0
1,3-dioxolane	94.3±4.4	84.7±7.4

4. Extraction of PHB from wet cells in 40 mL of solvent (large scale extraction)

Solvent	Purity	Recovery
chloroform	89.4±6.1	64.3±8.5
1,3-dioxolane	91.6±3.0	78.1±8.1

5. Extraction of PHB from wet cells in 2 mL of solvent at room temperature (30 ºC)

Solvent	Time (h)	Purity (%)	Recovery (%)
Chloroform			
	4	90.1±5.5	10.3±1.3
	6	72.3±4.5	12.8±1.3
	12	81.1±5.7	13.8±1.9
	24	86.8±6.3	16.3±2.3
	36	97.3±2.6	38.6±0.7
1,3-diolane			
	4	94.7±0.7	49.2±8.4
	6	77.1±1.6	44.1±7.0
	12	99.8±0.2	57.0±7.5
	24	73.1±8.0	46.3±9.7
	36	91.3±7.3	77.3±3.9

APPENDIX D

ENVIRONMENTALLY FRIENDLY SOLVENTS

1. 2-Methyltetrahydrofuran (2-MTHF)

Regulatory information

Safety, health and environmental regulations/legislation specific for the substance or mixture

EU regulations

Major Accident Hazard	96/82/EC
Legislation	Highly flammable 7b Quantity 1: 5.000 t Quantity 2: 50.000 t
Occupational restrictions	Take note of Dir 94/33/EC on the protection of young people at work. Observe work restrictions regarding maternity protection in accordance to Dir 92/85/EEC or stricter national regulations where applicable.

Regulation (EC) No 1005/2009 on substances that deplete the ozone layer	not regulated
Regulation (EC) No 850/2004 of the European Parliament and of the Council of 29 April 2004 on persistent organic pollutants and amending Directive 79/117/EEC	not regulated
Regulation (EC) No 689/2008 concerning the export and import of dangerous chemicals	not regulated
Substances of very high concern (SVHC)	This product does not contain substances of very high concern according to Regulation (EC) No 1907/2006 (REACH), Article 57 above the respective regulatory concentration limit of ≥ 0.1 % (w/w).

National legislation

Storage class	3

Chemical Safety Assessment

For this product a chemical safety assessment was not carried out.

2. 1,3-Propanediol

Regulatory information

Safety, health and environmental regulations/legislation specific for the substance or mixture

EU regulations

Major Accident Hazard	96/82/EC
Legislation	Directive 96/82/EC does not apply

National legislation

Storage class	10

Chemical Safety Assessment

For this product a chemical safety assessment was not carried out.

3. 1,3-Dioxolane

Regulatory information

Safety, health and environmental regulations/legislation specific for the substance or mixture

EU regulations

Major Accident Hazard	96/82/EC
Legislation	Highly flammable 7b Quantity 1: 5.000 t Quantity 2: 50.000 t
Occupational restrictions	Take note of Dir 94/33/EC on the protection of young people at work.
Regulation (EC) No 1005/2009 on substances that deplete the ozone layer	not regulated
Regulation (EC) No 850/2004 of the European Parliament and of the Council of 29 April 2004 on persistent organic pollutants and amending Directive 79/117/EEC	not regulated
Regulation (EC) No 689/2008 concerning the export and import of dangerous chemicals	not regulated
Substances of very high concern (SVHC)	This product does not contain substances of very high concern according to Regulation (EC) No 1907/2006 (REACH), Article 57 above the respective regulatory concentration limit of ≥ 0.1 % (w/w).

National legislation

Storage class	3

Chemical Safety Assessment

For this product a chemical safety assessment was not carried out.

4. Ethyl lactate

Regulatory information

Safety, health and environmental regulations/legislation specific for the substance or mixture

EU regulations

Major Accident Hazard Legislation	96/82/EC Flammable. 6 Quantity 1: 5.000 t Quantity 2: 50.000 t
Occupational restrictions	Take note of Dir 94/33/EC on the protection of young people at work.

Regulation (EC) No 1005/2009 on substances that deplete the ozone layer	not regulated
Regulation (EC) No 850/2004 of the European Parliament and of the Council of 29 April 2004 on persistent organic pollutants and amending Directive 79/117/EEC	not regulated
Regulation (EC) No 689/2008 concerning the export and import of dangerous chemicals	not regulated
Substances of very high concern (SVHC)	This product does not contain substances of very high concern according to Regulation (EC) No 1907/2006 (REACH), Article 57 above the respective regulatory concentration limit of ≥ 0.1 % (w/w).

National legislation

Storage class	3

Chemical Safety Assessment

For this product a chemical safety assessment was not carried out.

VITA

Miss Nuttipon Yabueng was born on March 13, 1991 in Lamphun, Thailand. She graduated from Chiang Mai University with a Bachelor of Science degree in Biology in 2013. After a successful undergraduate, she continued studying a Master's degree in Microbiology and Microbial Technology Program at Department of Microbiology, Faculty of Science, Chulalongkorn University. Some part of this work was published in proceeding of The 27th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB), Bangkok, Thailand (November 17-20, 2015): 228-232.