Production and Properties Development of Polyhydroxyalkanoates-Natural Rubber Blends

Mananya Martla

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology
Prince of Songkla University
2019

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Author	Miss Mananya Martla
Major Program	Microbiology

Major Advisor

……..…
(Asst. Prof. Dr. Kamontam Umsakul)

Co-advisor

……..
(Assoc. Prof. Dr. Varaporn Tanrattanakul)

Examining Committee :

……...……..Chairperson
(Prof. Dr. Duangporn Kantachote)

…….…....Committee
(Asst. Prof. Dr. Kamontam Umsakul)

…….…….Committee
(Dr. Pilanee Vaithanomsat)

…….….Committee
(Dr. Wankuson Chanasit)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of philosophy Degree in Microbiology

…..……..
(Prof. Dr.Damrongsak Faroongsarng)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

…… Signature
(Asst. Prof. Dr. Kamontam Umsakul)
Major Advisor

……Signature
(Miss Mananya Martla)
Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

……Signature
(Miss Mananya Martla)
Candidate

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are microbial thermoplastic that are completely degraded in various natural environments. The production of PHAs is limited by the expensive carbon source and recovery processes. PHAs-producing bacteria that can use biodiesel liquid waste (BLW) as a carbon source were studied. *Pseudomonas mendocina* PSU produced 79.7 wt% poly(3-hydroxybutyrate) (PHB) when cultured in mineral salts medium (MSM) containing 2% (v/v) BLW for 72 h. The weight average molecular weight (M_w) and number average molecular weight (M_n) were 8.39 x10^4 and 4.60 x 10^4, respectively. PHAs-producing bacteria SB01 and SB02 were isolated from Sirindhorn peat swamp forest in Thailand. Strain SB01 cultured in MSM containing BLW produced 91.98 wt% PHB at 78 h. When palm oil was used as a carbon source, this strain produced 87.50 wt% PHB at 84 h. Strain SB02 cultured in MSM containing 2% BLW produced 97.95 wt% PHB at 78 h. When palm oil was used as a carbon source, this strain produced 96.60 wt% PHB at 84 h. This study shows the potential use of a cheap substrate to produce a high amount of PHAs. Moreover, these two strains produced high molecular weight PHB which is more flexible than PHB from *P. mendocina* PSU. M_w and M_n of PHB from SB01 were 2.3 x 10^5 and 1.3 x 10^5 when BLW was used as a carbon source. M_w and M_n of of PHB from SB01 were 2.8 x 10^5 and 2.1 x 10^5 when palm oil was used as a carbon source.

M_w and M_n of PHB from SB02 were 2.5 x 10^5 and 2.0 x 10^5 when BLW was used as a carbon source. M_w and M_n of PHB from SB02 were 7.9 x 10^5 and 4.3 x 10^5 when palm oil was used as a carbon source. PHAs-producing bacteria SB01 and SB02 were identified as *Burkholderia seminalis* and *Burkholderia contaminans*, respectively according to the 16s rRNA sequence analysis. Even though these two strains are very promising, but *Burkholderia* spp. have also emerged as important human opportunistic pathogens and the risks associated with their uses remain unclear. Therefore, *P. mendocina* PSU was selected for further study. For the development of the biomaterials, PHB properties of *P. mendocina* PSU were improved by both copolymer production of PHBV and blending methods. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) copolymer was produced. Effects of sodium propionate, which is the precursor for PHBV production, and nitrogen sources were investigated. *P. mendocina* PSU produced 38.6 wt% PHBV with 13 hydroxyvalerate (HV) mol% at 72 h when 0.3% sodium propionate and 0.5 g/L ammonium sulphate were added. The properties of PHBV were better than PHB because of high molecular weight and low melting temperature. PHA recovery processes by biological method using mealworms and chloroform extraction were investigated. M_w and M_n from biological method were higher than those of the chloroform extraction. Melting temperature (T_m) and purity of PHAs were comparable. This study confirmed the ability of mealworms to extract PHAs from various kinds of bacterial cells. In the attempt to improve the properties of PHB, PHB was then blended with modified epoxidized natural rubber 25 (ENR25) by solution blending, melt blending by internal mixture and melt blending by twin screw extrusion. Unfortunately, PHB and modified ENR25 could not mix well together.

Biodegradation of all polymers from this study were determined by soil burial test and treating with the rubber degrading bacteria consortium. PHB and PHBV from *P. mendocina* PSU and rubber glove showed higher degradation rate than other materials when buried in the soil. The rubber degrading bacteria consortium degraded PHB-ENR25 blend films better than PHB and PHBV, NR and ENR25. This study has successfully established a low-cost process to synthesize biodegradable polymer. However, the polymer properties are needed to be improved further.

Key words: polyhydroxyalkanoate (PHA); poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV); *Pseudomonas* sp.; *Burkholderia* sp.; biodiesel liquid waste (BLW).

ชื่อวิทยานิพนธ์ การผลิตและพัฒนาสมบัติของพอลิเมอร์ผสมระหว่างพอลีไฮดรอกซีอัลคาโนเอต และยางธรรมชาติ

ผู้เขียน นางสาว มณัลยา หมาดหล้า

สาขาวิชา จุลชีววิทยา

ปีการศึกษา 2561

บทคัดย่อ

โพลีไฮดรอกซีอัลคาโนเอต (PHAs) เป็นพลาสติกที่ทนต่อความร้อนได้ดีและสามารถย่อยสลายได้อย่างสมบูรณ์ในสิ่งแวดล้อม ในระดับอุตสาหกรรมการผลิตPHAsยังมีข้อจำกัดเนื่องจากแหล่งคาร์บอนที่ใช้ในการผลิตมีราคาแพงและกระบวนการสกัด PHAs มีต้นทุนที่สูง งานวิจัยนี้เป็นการศึกษาแบคทีเรียที่สามารถสังเคราะห์ PHAs ได้โดยใช้ของเหลือใช้จากการผลิตไบโอดีเซลเป็นแหล่งคาร์บอน *Pseudomonas mendocina* PSU สามารถผลิตโพลีไฮดรอกซีบิวทีเรตหรือ PHB ได้ 79.7 wt% เมื่อเลี้ยงในอาหาร mineral salts medium (MSM) ที่มีของเหลือใช้จากการผลิตไบโอดีเซล 2% เป็นแหล่งคาร์บอนโดยใช้เวลาในการเลี้ยง 72 ชั่วโมง น้ำหนักโมเลกุลเฉลี่ยโดยน้ำหนัก (M_w) และน้ำหนักโมเลกุลเฉลี่ยโดยจำนวน (M_n) ของ PHB เท่ากับ 8.39 x10^4 และ 4.60 x 10^4 ในการศึกษานี้ได้แยกเชื้อสายพันธุ์ SB01 และ SB02 จากดินบริเวณป่าพรุโต๊ะแดงประเทศไทย ที่สามารถใช้ของเหลือใช้จากการผลิตไบโอดีเซลเป็นแหล่งคาร์บอนและผลิต PHB ที่มีน้ำหนักโมเลกุลสูง การศึกษาผลของแหล่งคาร์บอนต่อการผลิต PHAs โดยการเลี้ยงเชื้อในอาหาร MSM ที่มีแหล่งคาร์บอนเป็นของเหลือใช้จากการผลิตไบโอดีเซลหรือน้ำมันปาล์มพบว่าเชื้อ SB01 ที่ถูกเลี้ยงในอาหาร MSM ที่มีของเหลือใช้จากการผลิตไบโอดีเซลเป็นแหล่งคาร์บอนสามารถผลิต PHB ได้ 91.98 wt% ที่เวลา 78 ชั่วโมง และเมื่อเลี้ยงในในอาหาร MSM ที่มีน้ำมันปาล์มเป็นแหล่งคาร์บอน

เชื้อสามารถผลิต PHB ได้ 87.50 wt% ที่เวลา 84 ชั่วโมง เชื้อ SB02 ที่ถูกเลี้ยงในอาหาร MSM ที่มีของเหลือใช้จากการผลิตจากไบโอดีเซลเป็นแหล่งคาร์บอนสามารถผลิต PHB ได้ 97.95 wt% ที่เวลา 78 ชั่วโมง และเมื่อเลี้ยงในในอาหาร MSM ที่มีน้ำมันปาล์มเป็นแหล่งคาร์บอนเชื้อสามารถผลิต PHB ได้ 96.60 wt% ที่เวลา 84 ชั่วโมง M_w และ M_n ของ PHB จากเชื้อ SB01 เท่ากับ 2.3 x 10^5 และ 1.3 x 10^5 เมื่อใช้ของเหลือใช้จากการผลิตจากไบโอดีเซลเป็นแหล่งคาร์บอน. M_w และ M_n ของ PHB จากเชื้อ SB01 เท่ากับ 2.8 x 10^5 และ 2.1 x 10^5 เมื่อใช้น้ำมันปาล์มเป็นแหล่งคาร์บอน M_w และ M_n ของ PHB จากเชื้อ SB02 เท่ากับ 2.5 x 10^5 และ 2.0 x 10^5 เมื่อใช้ของเหลือใช้จากการผลิตจากไบโอดีเซลเป็นแหล่งคาร์บอน. M_w และ M_n ของ PHB จากเชื้อ SB02 เท่ากับ 7.9 x 10^5 และ 4.3 x 10^5 เมื่อใช้น้ำมันปาล์มเป็นแหล่งคาร์บอน งานวิจัยนี้แสดงถึงศักยภาพของการใช้ของเหลือใช้จากการผลิตจากกระบวนการผลิตไบโอดีเซลซึ่งเป็นวัตถุดิบที่มีราคาถูกมาผลิต PHAs ในปริมาณที่สูงได้ นอกจากนี้เชื้อทั้งสองสายพันธุ์ที่แยกได้ยังสามารถผลิต PHB ที่มีน้ำหนักโมเลกุลสูงและมีความยืดหยุ่นมากกว่า PHB จากเชื้อ *P. mendocina* PSU อีกด้วย จากการศึกษาลำดับเบสของยีน 16s rRNA พบว่าเชื้อ SB01 และ SB02 เป็นเชื้อ *Burkholderia seminalis* และเชื้อ *Burkholderia contaminans* ดังนั้นถึงแม้ว่าเชื้อสองชนิดนี้มีประสิทธิภาพสูงในการสร้าง PHB แต่เชื้อในกลุ่ม *Burkholderia* spp. จัดเป็นเชื้อฉวยโอกาสก่อโรคที่สำคัญในมนุษย์และอาจมีความเสี่ยงที่เกิดจากการนำเชื้อกลุ่มนี้มาใช้งาน ดังนั้นในการศึกษานี้จึงเลือกใช้ *P. mendocina* PSU ในการผลิต PHAs เพื่อพัฒนาคุณสมบัติของวัสดุทางชีวภาพ โดยการผลิตโคพอลิเมอร์และการทำพอลิเมอร์ผสม โดยการเลี้ยง *P. mendocina* PSU ที่มีการเติมโซเดียม-โพรพิโอเนต พบว่าสามารถผลิตโพลี(3-ไฮดรอกซีบิวทิเรต-โค-3-ไฮดรอกซีวาเลอเรต) หรือ PHBV ได้ 38.6 wt% โดยโคพอลิเมอร์ที่ได้มีโมโนเมอร์ของไฮดรอกซีวาเลอเรตอยู่ 13 mol% เมื่อทำการเลี้ยงเชื้อเป็นเวลา 72 ชั่วโมง ในอาหารเลี้ยงเชื้อที่มี

0.3% โซเดียมโพรพิโอเนตและ 0.5 g/L แอมโมเนียมไนเตรตเป็นองค์ประกอบ คุณสมบัติของ PHBV ที่ได้ดีกว่า PHB เนื่องจากเป็นวัสดุที่มีน้ำหนักโมเลกุลสูงและจุดหลอมเหลวที่ต่ำกว่า จากการเปรียบเทียบกระบวนการสกัด PHAs โดยการสกัดทางชีวภาพที่ใช้หนอนนกและการสกัดโดยใช้คลอโรฟอร์มในทดลองพบว่า M_w และ M_n ของ PHAs ที่ทำการสกัดโดยวิธีทางชีวภาพมีค่าสูงกว่าการสกัดโดยใช้คลอโรฟอร์ม พอลิเมอร์ที่ได้มีความบริสุทธิ์และมีจุดหลอมเหลวที่ใกล้เคียงกัน การทดลองนี้ยืนยันประสิทธิภาพของการใช้หนอนนกในการสกัด PHAs จากเซลล์แบคทีเรียได้หลายชนิด การศึกษานี้ยังได้ทำการปรับปรุงคุณสมบัติของ PHB โดยการผลิตพอลิเมอร์ผสมระหว่างPHBและยางอิพอกซิไดซ์ 25 (ENR25) ที่ผ่านการดัดแปลงแล้ว โดยใช้การผสมโดยใช้ตัวทำละลาย การผสมโดยใช้ความร้อน ของเครื่องผสมแบบระบบปิด และการผสมโดยใช้ความร้อน ของเครื่องอัดรีดสกรูคู่ แต่พบว่า PHB กับยาง ENR25 ที่ผ่านการดัดแปลงแล้วไม่สามารถผสมเป็นเนื้อเดียวกันได้สมบูรณ์ เมื่อทำการทดสอบการย่อยสลายพอลิเมอร์ที่ได้จากการทดลองทุกชนิดโดยการฝังดินและการใช้กลุ่มเชื้อย่อยสลายยาง พบว่า PHB และ PHBV จากเชื้อ *P. mendocina* PSU และถุงมือยางมีอัตราการย่อยสลายที่สูงเมื่อทำการฝังดิน แต่เมื่อทำการย่อยสลายโดยใช้กลุ่มเชื้อย่อยสลายยางพบว่าพอลิเมอร์ผสมระหว่าง PHB และยาง ENR25 มีอัตราการย่อยสลายที่สูงกว่า PHB PHBV ยางธรรมชาติ และยาง ENR25 งานวิจัยนี้สามารถผลิตพอลิเมอร์ย่อยสลายได้ทางชีวภาพที่มีต้นทุนในการผลิตต่ำ แต่อย่างไรก็ตามยังมีความจำเป็นที่ต้องศึกษาและปรับปรุงคุณสมบัติของพอลิเมอร์ให้ดียิ่งขึ้น

คำสำคัญ: โพลีไฮดรอกซีอัลคาโนเอต (PHA) โพลี(3-ไฮดรอกซีบิวทิเรต-โค-3-ไฮดรอกซีวาเลอเรต) (PHBV) *Pseudomonas* sp. *Burkholderia* sp. ของเหลือใช้จากการผลิตไบโอดีเซล

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Mananya Martla

CONTENTS

CONTENTS (continued)

CONTENTS (continued)

CONTENTS (continued)

CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS AND SYMBOLS

BLW = Biodiesel liquid waste

C/N = Ratio of carbon to nitrogen

DCW = Dry cell weight

% DCW = % Dry cell weight

GC = Gas chromatography

PHA = Polyhydroxyalkanoates

PHB = Poly(3-hydroxybutyrate)

PHBV = Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

HV = Hydroxyvalerate

^{1}H-NMR = Proton magnetic resonance

^{13}C-NMR = Carbon magnetic resonance

MSM = Mineral salt medium

SCL-PHA = Short chain length - Polyhydroxyalkanoate

MCL-PHA = Medium chain length - Polyhydroxyalkanoate

MW = Molecular weight

Mn = The number-average molecular weight

Mw = The weight-average molecular weight

mol% = Percentages of mole or molar fraction

T_c = Crystallization temperature

T_g = Glass transition temperature

T_m = Melting temperature

3HB = 3-Hydroxybutyrate

3HD = 3-Hydroxydecanoate

3HHx = 3-Hydroxyhexanoate

3HO = 3-Hydroxyoctanoate

% (v/v) = % volume by volume

% wt = % weight

% (w/w) = % weight by weight

n.d. = Data was not detected

LIST OF PAPERS AND PROCEEDINGS

1. Martla, M., Sudesh, K. and Umsakul, K. 2018. Production and recovery of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from biodiesel liquid waste (BLW). Journal of Basic Microbiology. 58: 977-986.

2. Martla, M. and Umsakul, K. 2018. Isolation and characterization of a polyhydroxyalkanoates producing bacterium using biodiesel liquid waste as a carbon source. In: The 2nd International Conference on Environment, Livelihood, and Services; ICELS 2018 (19th – 22th November 2018, C asean 10 floor, CW Tower Bangkok, Thailand)(Proceeding)

CHAPTER 1

INTRODUCTION

Rational and Background

According to human population and economic activity has grown rapidly, so materials consumption is increased. The over use of conventional plastics such as polypropylene and polyethylene is greatly concerned. These plastics are produced from hydrocarbons sources and take several years to decompose. Toxic compounds are produced during degradation. Plastics accumulate in landfills and polluting the land and natural waters (Cornell, 2007; Rehm, 2010). Alternative materials are required for future economical and ecologically safe biopolymers of natural origin. Biopolymers can be produced from renewable resources obtained from agricultural or biotechnological processes. Polyhydroxyalkanoates (PHAs) are biodegradable and have properties similar to petrochemical-based plastics (Steinbüchel, 2001). They are accumulated in bacterial cells when carbon source is excess and at least one other essential nutrient for growth is depleted. PHAs are water insoluble polyesters accumulated in cell cytoplasm and serve as storage compounds for carbon and energy sources (Asrar and Gruys, 2002). A wide variety of bacteria produce PHAs using different pathways from coenzyme A thioesters of hydroxyalkanoic acids obtained during carbohydrate metabolism or fatty acid metabolism. During the last 20 years, approximately 150 different hydroxyalkanoic acids have been isolated from various

bacteria. PHAs can be used for various applications such as natural fiber composites, binder in paints tissue engineering and drug delivery (Zinn *et al*., 2001; Thakor *et al*., 2006). They are completely degraded by soil and aquatic microorganisms that have specific enzymes called PHA depolymerases. The complete degradation process produced nontoxic products to the environment as carbon dioxide and water under aerobic conditions or methane, carbon dioxide and water under anaerobic conditions (Jendrossek and Handrick, 2002; Kim and Rhee, 2003).

At present, applications of PHAs are limited by their high cost and some poor properties. They exhibit a narrow processing window because they are sensitive to temperature and shear stress. PHAs chains are cleaved under high shear stress and their molecular weight is rapidly reduced. Additives, blends, and composites are the most apparent ways to enhance toughness and flexibility of materials. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) co-polymer can be produced by adding the valerate content. Impact strength increases with increasing hydroxyvalerate (HV) content, while melting temperature (T_m), glass transition temperature (T_g), crystallinity, water permeability and tensile strength decreases in the copolymers (Kotnis *et al*., 1995). Polymer blending is used to improve mechanical, biodegradation, and morphological properties to broaden PHAs applications. Polylactic acid (PLA), poly(butylene adipate-*co*-terephthalate) (PBAT), polycaprolactone (PCL) and starch have been blended with PHAs for this purpose (Reddy *et al*., 2013). Natural rubbers are elastomeric in nature and biodegradable polymer. Several species of bacteria and fungi can degrade natural rubber (Rose and Steinbüchel, 2005). Use of natural rubbers for blending with PHAs may help in

decreasing its melting temperature (T_m) and reducing the overall crystallinity to provide ductility and toughness required for many industrial applications.

1. Biopolymers

Biopolymers are polymers that are synthesized and catabolized by various organisms. They have attracted considerable attention as green materials. Biopolymers do not cause toxic effects in the host and have certain advantages over petroleum-derived plastic (Suriyamongkol *et al*., 2007). Biopolymers show highly diverse characteristics with respect to their shape and their properties such as diverse strength, elasticity, toughness or robustness. They can replace some synthetic plastics in many applications such as photonics, drug delivery, electronic device, textiles, sutures, tissue scaffolds and microfluidic device (Gronau *et al*., 2012).

Biopolymers can be classified into three categories depending on the sources

1. Polymers from biomass such as the agro-polymers.

    1.1 Polysaccharides such as starches (wheat, potatoes, maize), ligno-cellulosic products (wood, straw) and others (pectin, chitosan/chitin, gum).

    1.2 Protein and lipids from animals (casein, whey, collagen/gelatin) and from plants (zein, soya and gluten)

2. Polymers from microbial production such as polyhydroxyalkanoates (PHAs), poly(3-hydroxybutyrate) (PHB), Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), cellulose, dextran, glycogen and poly-Ɣ-glutamate.

3. Polymers from chemical synthesized using monomers obtained from agro-resources such as poly(lactic acid) (PLA) (Vieira *et al*., 2011)

Biopolymers from microbial production

A wide range of biopolymers can be synthesized by bacteria. Biopolymers properties are suitable for various industrial and medical applications (Table 1). Bacteria use different carbon sources to produce a diverse range of polymers with different chemical and material properties. Four major classes of polymers produced by many species of bacteria are polyesters, polysaccharides, polyamides and polyphosphates (Rehm, 2010).

Table 1 Classes of bacterial polymers and their characteristics (Rehm, 2010)

Polymer class	**Polymer localization**	**Primary structure**	**Main components**	**Precursors**	**Polymerizing enzyme**	**Producer**	**Industrial applications**
Polysaccharides							
Glycogen	Intracellular	α-(1,6)-branched α-(1,4)-linked homopolymer	Glucose	ADP–glucose	Glycogen synthase (GlgA)	Bacteria and Archaea	NA
Alginate	Extracellular	β-(1,4)-linked non-repeating heteropolymer	Mannuronic acid and guluronic acid	GDP–mannuronic Acid	Glycosyltransferase (Alg8)	*Pseudomonas* spp. and *Azotobacter* spp.	Biomaterial (Tissue scaffold and drug delivery)
Xanthan	Extracellular	β-(1,4)-linked repeating heteropolymer consisting of pentasaccharide units	Glucose, mannose and glucuronate	UDP–glucose, GDP–mannose and UDP–glucuronate	Xanthan polymerase (GumE)	*Xanthomonas* spp.	Food additive (Thickener and emulsifier)
Dextran	Extracellular	α-(1,2)/α-(1,3)/α-(1,4)-branched α-(1,6)-linked homopolymer	Glucose	Saccharose	Dextransucrase (DsrS)	*Leuconostoc* spp. and *Streptococcus* spp.	Blood plasma extender and chromate-graphy media
Curdlan	Extracellular	β-(1,3)-linked homopolymer	Glucose	UDP–glucose	Curdlan synthase (CrdS)	*Agrobacterium* spp., *Rhizobium* spp. and *Cellulomonas* spp.	Food additive (Thickener and gelling agent)

Table 1 (cont.) Classes of bacterial polymers and their characteristics (Rehm, 2010)

Polymer class	**Polymer localization**	**Primary structure**	**Main components**	**Precursors**	**Polymerizing enzyme**	**Producer**	**Industrial applications**
Gellan	Extracellular	β-(1,3)-linked repeating heteropolymer consisting of tetrasaccharide units	Glucose, rhamnose and glucuronate	UDP–glucose, dTDP–rhamnose and UDP–glucuronate	Gellan synthase (GelG)	*Sphingomonas* spp.	Culture media additive, food additive (Gelling agent) or for encapsulation
Colanic acid	Extracellular	β-(1,4)-linked repeating heteropolymer consisting of hexasaccharide units	Fucose, glucose, glucuronate and galactose	GDP–l-fucose, UDP–d-glucose, UDP–d-galactose and UDP–d-glucuronate	Colanic acid polymerase (WcaD)	*Escherichia coli*, *Enterobacter* spp., *Shigella* spp., and *Salmonella* spp.	NA
K30 antigen	Capsular	β-(1,2)-linked repeating heteropolymer consisting of tetrasaccharide units	Mannose, galactose and glucuronate	UDP–d-glucose, UDP–d-galactose and UDP–d-glucuronate	Polysaccharide polymerase (Wzy)	*Escherichia coli*	NA

Table 1 (cont.) Classes of bacterial polymers and their characteristics (Rehm, 2010)

Polymer class	**Polymer localization**	**Primary structure**	**Main components**	**Precursors**	**Polymerizing enzyme***	**Producer**	**Industrial applications‡**
Cellulose	Extracellular	β-(1,4)-linked homopolymer	d-glucose	UDP–d-glucose	Cellulose synthase (BcsA)	Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Gram-positive bacteria	Diaphragms of acoustic transducers and wound dressing
Hyaluronic acid	Extracellular	β-(1,4)-linked repeating heteropolymer consisting of disaccharide units	Glucuronate and *N*-acetyl glucosamine	UDP–d-glucuronate and UDP–*N*-acetyl glucosamine	Hyaluronan synthase (HasA)	*Streptococcus* spp. and *Pasteurella multocida*	Cosmetics, Viscosupplementation, tissue repair and drug delivery
Polyamides							
Cyanophycin granule peptide	Intracellular	Intracellular Repeating heteropolymer consisting of dipeptide units	Aspartate and arginine	(β-spartatearginine) 3-phosphate, ATP, l-arginine and l-aspartate	Cyanophycin synthetase (CphA)	Cyanobacteria, *Acinetobacter* spp. and *Desulfitobacterium* spp.	Dispersant and water softener (after removal of arginyl residues)
ε-poly-l-lysine	Extracellular	Homopolymer	l-lysine	l-lysine, ATP and l-lysine–AMP	ε-poly-l-lysine synthetase (Pls)	*Streptomyces Albulus* sub sp. *lysinopolymerus*	Feed preservative and, when cross-linked, adsorbent (in medicine)

Table 1 (cont.) Classes of bacterial polymers and their characteristics (Rehm, 2010)

Polymer class	**Polymer localization**	**Primary structure**	**Main components**	**Precursors**	**Polymerizing enzyme**	**Producer**	**Industrial Applications**
Poly-γ-glutamate	Extracellular or capsular	Homopolymer	d-glutamate and/or l-glutamate	(Glutamate)*n*-phosphate, ATP and glutamate	Poly-γ-glutamate synthetase (PgsBC; also known as CapBC)	*Bacillus* spp., *Fusobacterium Nucleatum, Natronococcus occultus* and *Natrialba aegyptiaca*	Replacement of polyacrylate, thickener, humectant, drug delivery and cosmetics
Polyester							
Polyhydroxyalkanoates	Intracellular	Heteropolymer	(*R*)-3-hydroxy fatty acids	(*R*)-3-hydroxyacyl CoA	Polyhydroxyalkanoate synthase (PhaC)	Bacteria and Archaea	Bioplastic, Biomaterial,and matrices for binding proteins
Polyanhydrides							
Polyphosphate	Intracellular	Homopolymer	Phosphate	ATP	Polyphosphate kinase (PPK)	Bacteria and Archaea	Replacement of ATP in enzymatic synthesis and flavour enhancer

2. Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are thermoplastic polyesters of R-hydroxyalkanoic acids which are renewable, biodegradable and biocompatible polymers (Doi, 1990; Reddy *et al*., 2013). PHAs are the class of linear polyester compounds with similar properties to polypropylene and polyethylene (Suriyamongkol *et al*., 2007). PHAs are synthesized by microorganisms from various substrates as carbon sources. They are intracellular carbon and energy storage in bacteria under carbon excess and limitation of nitrogen, phosphorus or oxygen. If limiting nutrient is provided to the cell, PHAs will be degraded and used as carbon source for bacterial growth. PHAs accumulate in cytoplasmic granules with diameter from 0.2 to 0.5 μm. These granules can be divined by phase contrast microscopy or using staining dyes such as Sudan Black B and Nile red (Urtuvia *et al*., 2014). The structure, physico-chemical properties, monomer composition and the number and size of the granules vary depending on the organisms (Ha and Cho, 2002). PHAs are synthesized by a wide variety of bacteria such as *Bacillus megaterium, Ralstonia eutropha, Alcaligenes latus, Azotobacter vinelandii, Pseudomonas oleovorans, Pseudomonas acidophila, Escherishia coli, Acinetobacter* spp. and the family *Halobactericeae* of the *Archaea* (Moita and Lemos, 2011). They have diverse structures and display different properties. Over 150 different types of PHAs can be synthesized by different bacterial species and growth conditions, resulting in an excessive diversity of material properties (Reddy *et al*., 2013). PHAs exhibit a crystallinity ranging from 30% to 70% and a melting temperature from 50 °C to 180 °C; these thermoplastic material properties make PHAs commercially suitable to oil-based plastics. PHAs are accumulated as spherical intracellular inclusions with an

amorphous, hydrophobic PHAs core that enclosed by proteins involved in PHAs metabolism (Figure 1) (Grage *et al*., 2009; Jendrossek, 2009). In addition, PHAs are biocompatible polymer and their breakdown products are 3-hydroxyacids, which are normally found in animals. Because of versatility and biocompatibility, PHAs can be very useful in many medical applications, such as bone implants, sutures, osteosynthetic materials, gauzes, suture filaments, nanoparticles for drug delivery, and biocompatible porous implants (Zinn *et al*., 2001; Sudesh, 2004; Chen, 2005; Chen and Wu, 2005A, B; Sudesh and Doi, 2005; Rehm, 2010; Shrivastav *et al*., 2013 and Urtuvia *et al*., 2014) (Table1). In addition, PHAs can be used in daily-life products including containers, bottles, razors, bags, pencils, plates, glasses and materials for food packaging (Urtuvia *et al*., 2014). PHAs latex can be used to produce a water-resistant layer for film, paper or cardboard (Hocking and Marchessault, 1994).

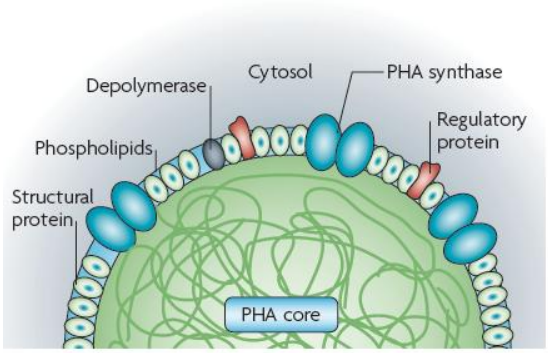


Figure 1 The structure of polyhydroxyalkanoate (PHA) granules in bacteria (Rehm, 2010)

3. Substrates for bacterial PHAs production

One of the most significant barriers for an increase industrial PHAs production is its high cost. PHAs price vary from 2.5 to 5 USD per kg whereas polypropylene price varies from 0.5-2 USD per kg. Production costs of PHAs are currently 5–10 times of the cost of plastic derived from petrochemicals, which is a major drawback for the successful commercialization.

The product price depends up to 50% of raw material cost, mainly the carbon source (Khanna and Srivastava, 2005; Rehm, 2010). A wide range of substrates used for PHAs production by different pathways are shown in Figure 2. Substrates that have been reported for PHAs production are:

1. Renewable sources such as starch, cellulose, sucrose, triglycerides, wheat and hemicelluloses

2. Sub-products such as glycerol, molasses, whey, rice bran and corn steep liquor

3. Organic acids such as 4-hydroxybutyric acid and propionic acid

4. Fossil resources such as mineral oil, methane, hard coal and lignite

5. Wastes such as wastewater, activated sludge and palm oil mill effluents (Urtuvia *et al*., 2014).

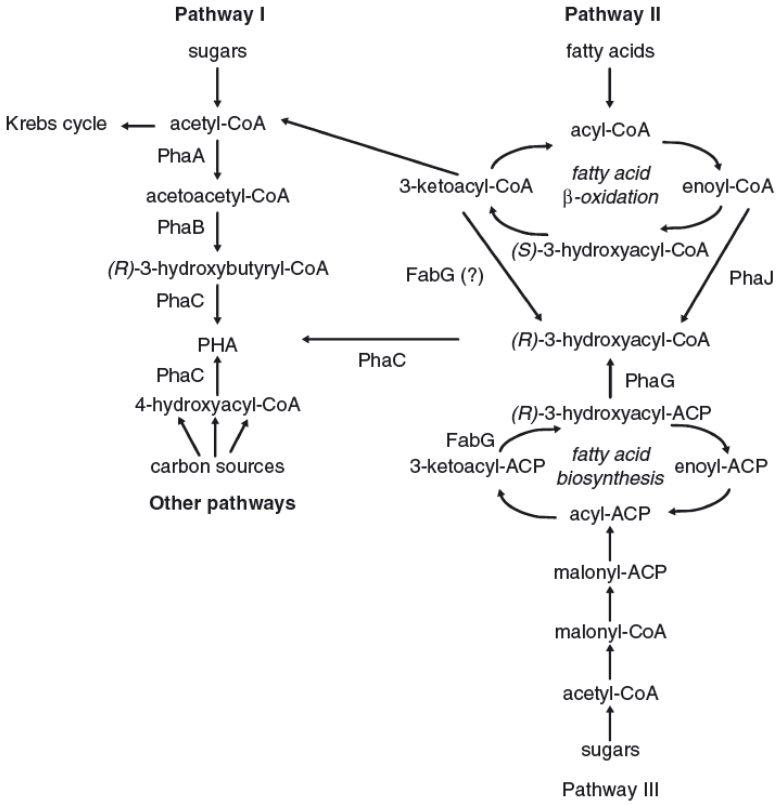


Figure 2 Metabolic pathways of PHAs synthesis by different carbon sources (Tsuge, 2002)

Glucose and sucrose are used in pathway I and III to produce PHB and pathway II to produce copolymers (Figure 2). These two sugars have been used for industrial PHA production. Glucose and sucrose show high impact on PHAs production cost. Therefore, low cost raw materials are interesting for industrial scale (Tsuge, 2002; Keshavarz and Roy, 2010). Low cost carbohydrate waste such as sugar beet molasses and soy molasses have been used for PHB production (Mercan and Beyatli, 2005; Full *et al.*, 2006). Low cost fatty acids such as renewable vegetable oils, waste free frying oil, biodiesel liquid waste and various waste products are metabolized by pathway II to produce PHB and copolymers (Steinbüchel and Lutke-Eversloh, 2003; Fernandez *et al*., 2005; Wong *et al*., 2004, 2005; Chanasit *et al*.,

2014; Chanasit *et al*., 2016). Additionally, cyanobacteria can use carbon dioxide to produce PHAs (Jau *et al*., 2005) (Table 2).

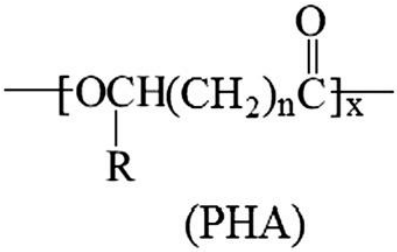
Table 2 Overview of bacterial strains used low cost carbon sources to produce PHAs

Bacterial strain (s)	Carbon source (s)	Polymer (s) produced	Reference
Alcaligenes latus	Malt, soy waste, milk waste,vinegar waste, sesame oil	PHB	Wong *et al*., 2004, 2005
Bacillus cereus	Sugarbeet Molasses	PHB	Yilmaz and Beyatli, 2005
Bacillus spp.	soy molasses	PHB	Full *et al*., 2006
Burkholderia cepacia	Palm olein, palm stearin, crude palm oil, palm kernel oil sugarbeet molasses	PHB, PHBV	Alias and Tan, 2005 Celik *et al*., 2005
Pseudomonas aeruginosa	waste free fatty acids, waste free frying oil	mcl-PHA	Fernandez *et al*., 2005
Rhizobium meliloti, R. viciae, Bradyrhizobium japonicum	sugar beet molasses, whey	PHB	Mercan and Beyatli, 2005
Spirulina platensis (cyanobacterium)	Carbon dioxide	PHB	Jau *et al*., 2005
Staphylococcus epidermidis	Malt, milk waste, soy waste, sesame oil, vinegar waste	PHB	Wong *et al*., 2004, 2005
Bacillus aryabhattai	Biodiesel liquid waste	PHB	Chanasit *et al*., 2014
Pseudomonas mendocina	Biodiesel liquid waste	PHB	Chanasit *et al*., 2016
*Cupriavidus necator*H16	Hydrogen, carbon dioxide, waste glycerol	PHB	Pohlmann *et al*., 2006; Cavalheiro *et al*., 2009

4. PHAs structure

PHAs are composed of 3-hydroxy fatty acid monomers, which form linear, head-to-tail polyesters. PHAs are commonly produced as a polymer of 10^3 to 10^4

monomers, which accumulate as inclusions of 0.2–0.5 μm in diameter. Alkyl groups, which show the R configuration at the C-3, vary from one carbon (C1) to over 14 carbons (C14) in length (Figure 3). PHAs can be separated into three broad classes according to the size of the monomers. PHAs involve up to C5 monomers are classified as short chain length PHAs (scl-PHA). PHAs with C6–C14 are classified as medium chain length (mcl-PHA). PHAs containing more than C14 monomers are classified as long chain length (lcl-PHA) PHAs (Madison and Huisman, 1999). Scl-PHAs have properties close to conventional plastics (Lee, 1996). PHAs made of longer monomers, such as mcl-PHAs and lcl-PHA are regarded as elastomers and sticky materials, which can also be modified to make rubbers. The large diversity of monomers found in PHAs keeps a wide spectrum of polymers with differing physical properties.



(PHA)

x = 100 - 30000

		PHA	
n = 1	R = H	Poly(3-hydroxypropionate)	(P3HP)
	R = methyl	Poly(3-hydroxybutyrate)	(P3HB)
	R = ethyl	Poly(3-hydroxyvalerate)	(P3HV)
	R = propyl	Poly(3-hydroxyhexanoate)	(P3HHx)
	R = pentyl	Poly(3-hydroxyoctanoate)	(P3HO)
	R = nonyl	Poly(3-hydroxydodecanoate)	(P3HDD)
n = 2	R = H	Poly(4-hydroxybutyrate)	(P4HB)
n = 3	R = H	Poly(5-hydroxyvalerate)	(P5HV)

Figure 3 General structure of polyhydroxyalkanoates (Hazer *et al*., 2012)

5. PHAs biosynthesis

Biosynthesis of PHAs basically occurs in three steps (Figure 4).

1. ß-ketothiolase or (PhaA) function to produce acetoacetyl-CoA from acetyl-CoA.

2. Generation of hydroxyacyl-CoAs (HA-CoAs) using diverse metabolites as precursors and NADPH-dependent acetoacetyl-CoA reductase or (PhaB).

3. Polymerization of the HA-CoAs into PHAs which using PHA synthases (PhaC).

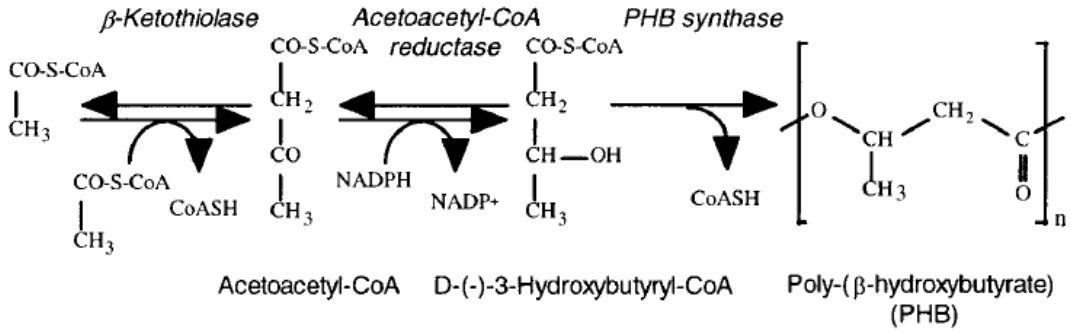


Figure 4 Common three-step biochemical pathway for PHB production (Steinbüchel and Lutke-Eversloh, 2003)

PHA synthases are the key enzymes of PHAs biosynthesis. These enzymes have a broad range of substrate specificities. PHA synthases are generally classified into four groups: class I, II, III, and IV (Table 3) (Park *et al*., 2012).

Class I and II PHA synthases are consist of 1 subunit enzyme, PhaC. Class I PHA synthases from bacteria such as *Alcaligenes latus* and *Ralstonia eutropha* accept SCL-HA-CoAs for polymerization (Schubert *et al*., 1988). Class II PHA synthases mainly from *Pseudomonas* sp. show substrate specificity towards MCL-HA-CoAs (Steinbüchel *et al*., 2010). Some class II PHA synthases from *Pseudomonas* sp. 6–19

and *Pseudomonas* sp. 61–3 accept MCL monomers and weak activity towards SCL-monomers (Matsusaki *et al*., 1998). Class III PHA synthases are consist of 2 different subunits, PhaC and PhaE (Liebergesell and Steinbüchel, 1992). Class III PHA synthases are highly specific for SCL-HA-CoAs. However, it can accept MCL-HA-CoAs when expressed in some *Pseudomonas* sp. (Liebergesell *et al*., 2000). Class IV PHA synthases are consist of 2 different subunits such as PhaC and PhaR. This enzyme is usually found in *Bacillus* strains accumulating PHB (McCool and Cannon, 2001; Satoh *et al*., 2002).

Table 3 Classical PHA synthases in different classes depending on the subunit composition and substrate specificities (Park *et al*., 2012).

PHA synthases	Subunits	Organism	Substrate
Class I	PhaC and PhaC	*Ralsonia eutropha*	C3-C5
Class II	PhaC1 and PhaC2	*Pseudomonas* sp.	C6-C14
Class III	PhaC and PhaE	*Allochromatium vinosum*	C3-C6
Class IV	PhaC and PhaR	*Bacillus cereus*	C3-C5

6. PHA copolymers

Polyhydroxybutyrate (PHB) is one of the best-known biodegradable PHAs. PHB is usual thermoplastic polyester and has many mechanical properties proportional to synthetically-produced degradable polyesters. The homopolymer PHB is a relatively stiff and brittle bioplastic, which has limited use.

PHAs consist of HB with a longer chain monomers fraction such as HV, HO or HH are tougher and more flexible plastics. They can be used in a wide variety of

products including bottles, razors, containers and materials for biomedical applications, food packaging and latex (Hocking and Marchessault, 1994).

Heteropolymers can be produced by polymerization between more than one kind of monomer. Copolymers of PHAs can be produced containing 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (HV), 4-hydroxybutyrate (4HB) or 3-hydroxyhexanoate (HHx) monomers. Most of the microorganisms synthesize either scl-PHAs containing primarily 3HB units or mcl-PHAs containing 3-hydroxydecanoate (HD) and 3-hydroxyoctanoate (HO) as the major monomers (Anderson and Dawes, 1990; Steinbüchel and Schlegel, 1991; Lee, 1996; Steinbüchel, 2001). The final material properties were affected by copolymers composition. Carbon source is one of the factors that determine the type of PHAs constituents. Microorganisms are capable of producing PHAs from various carbon sources. Different metabolic pathways have been shown the various types of monomer incorporated into PHAs (Figure 5) (Park *et al*., 2012). Unusual PHA monomer, except PHB, cannot be produced from simple carbon sources. Precursor substrates are needed to be incorporated into PHA's structure. The position of hydroxyl group of the component is already contained in the structure of carbon source. Hydroxyl group may be replaced by an amino or a keto group but an unusual hydroxyacyl-coenzyme A thioester is synthesized from the structurally related carbon source. Precursor substrates are more expensive than renewable resources and most of them are toxic. Precursor carbon source can be provided at high concentration in the medium with advanced process engineering for efficient PHAs production (Steinbüchel and Eversloh, 2003). PHBV copolymers are produced under nitrogen limited conditions from *Alcaligenes latus, Bacillus cereus, Pseudomonas pseudoflava, Pseudomonas*

cepacia, *Micrococcus halodenitrificans*, and *Ralstonia eutropha* when cells are supplied with glucose (or sucrose in case of *A. latus*) and propionic acid or other propionogenic carbon sources (Ramsay *et al*., 1990). The mixture of the substrates can also produce either PHB homopolymer or PHBV copolymers depending on their concentrations (Koçer *et al*., 2003). When HV monomer is increase, the melting temperature, glass transition temperature, Young's modulus and tensile strength are low and Impact strength is high. So, the material with high HV content is easy to process, flexible and high toughness (Hocking and Marchessault, 1994). *Aeromonas hydrophila* is able to synthesize PHBHHx copolyesters containing about 15 mol% 3HHx when grown in the presence of long chain fatty acids, such as dodecanoate, regardless of growth conditions (Lee *et al*., 2000). When gluconate is used as a cosubstrate together with dodecanoate, the recombinant strain produces PHBHHx containing 3–12 mol% of 3HHx, depending on the gluconate concentration in media (Qiu *et al*., 2004).

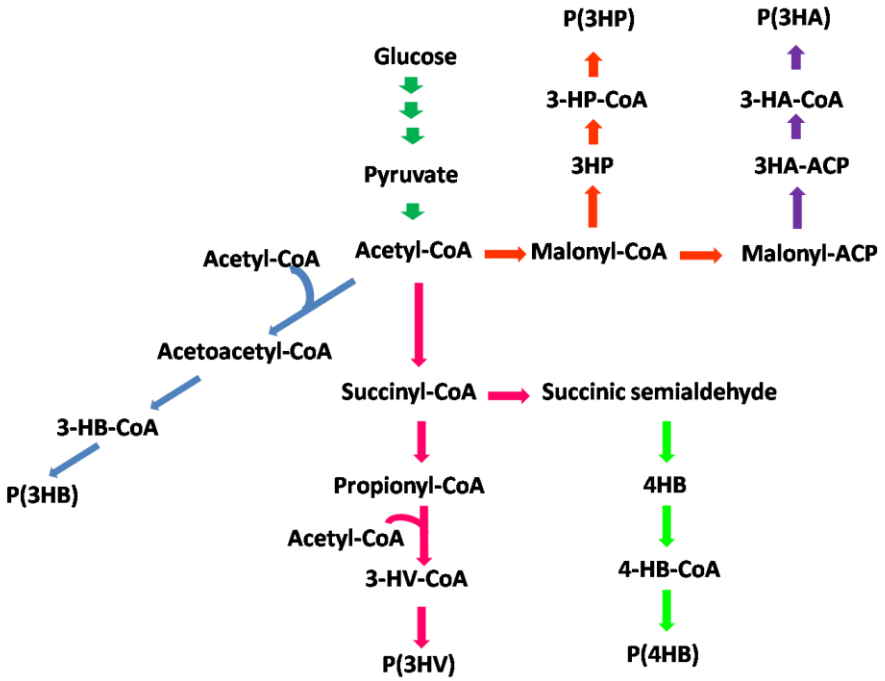


Figure 5 Biosynthesis of PHAs consisting of 3HP, 3HA, 3HB, 3HV and 4HB containing PHA (Park *et al*., 2012)

7. PHAs applications

PHAs have been used in many medical and pharmaceutical applications because they are biodegradable, biocompatible and wide physical properties. Recently, PHAs are used as heart valve scaffolds, pulmonary conduits, sutures, screws, bone plates, repair patches, stents, bone marrow scaffolds and drug delivery, wound dressings, tissue engineered, cardiovascular devices, bone-marrow scaffolds, tendon repair devices, nerve guides, articular cartilage repair devices, guided tissue repair devices, stents, adhesion barriers, orthopedic pins, cardiovascular patches, slings, repair patches, nerve repair devices and sutures (Table 4) (Furrer *et al*., 2008; Hazer *et al*., 2012; Butt *et al*., 2018). For examples, PHB microspheres could release rifampicin 90% in 24 h. So, drug release rate could be controlled by drug loading and particle size (Kassab *et al*., 1997). PHBV was used as an antibiotic-loaded carrier to

treat implant-related and chronic osteomyelitis. The result showed that infection subsided after 15 days and was completely healed after 30 days (Yagmurlu *et al*., 1999). PHBV film that hydrophilized by oxygen plasma treatment was used for growing epithelium cells (Tezcaner *et al*., 2003). PHO and P4HB with porous surface were used as tri-leaflet heart valve scaffold (Sodian *et al*., 2000). PHBHHx was suitable biomaterial for osteoblast attachment, proliferation and differentiation in bone marrow cells (Wang *et al*., 2004). Moreover, PHA *co*-polymers that composed of HB with long chain monomers, such as HV, HHx or HO, are more flexible and less brittle than PHB. They can be used as containers, bottles, razors, and materials for food packaging (Martini *et al*., 1989). PHAs were used as packaging films and disposable items such as shopping bags, cosmetic containers and paper coatings (Clarinval and Halleux, 2005; Mikova and Chodak, 2006).

Table 4 PHAs used in biomedical applications (Hazer *et al*., 2012).

Modified/unmodified PHA	**Physical properties**	**Medical application**
P3(HB)	-	Subcutanous patches
PHB/VA	-	Subcutanous patches
PHB/PHV	-	Suture
PHB/VA	Design of a 3D microfibrous material-formed by the blend and electrospun into fiber materials	Myocardial patch
P3(HB)		Peripheral nerve guide
PHBHHx	Flexible	Vessel stent, hemocompatibility and cytocompatibility; it holds promise as a blood-contact material with less platelet adhesion, reduced erythrocyte contact
PHBHHx	Porous tube form, flexible	Nerve conduit
Fibronectin and alginate covered PHB-fiber		Spinal cord injury
PHBV-PLGA	Turning a porous micropatterned film into a tube wrapping aligned electrospun fibers	Nerve guide
PHB	Asymetric patches	Stomach wall patch
PHBHHx	Scaffold, flexible	Bone regeneration
PHB/VA		Bone regeneration, better performances on attachment, proliferation, and differentiation of osteoblasts
HA reinforced PHB		Bone regeneration
HA reinforced PHB/VA		Bone regeneration

Table 4 (cont.) PHAs used in biomedical applications (Hazer *et al*., 2012)

Modified/unmodified PHA	**Physical properties**	**Medical application**
PHBHHx	Porous three dimensional scaffolds, flexible	Cartilage proliferation
PHB/VA	Chondrocyte seeded	Cartilage proliferation
PHB/PHBHHx	Human adipose tissue embedded scaffolds	Cartilage proliferation
PHB microspheres	Solvent evaporation technique	Drug delivery, chemoembolization
PHBV and P(3HB-4HB)	Semicrystalline	Drug delivery
PHB homopolymers	Unable to entrap the drug because of its high melting temperature and rapid crystallization rate	Not available for drug delivery
PHO	Autologous ovine endothelial cell seeded	Pulmonary valve
PHO	Autologous ovine endothelial cell seeded porous PHO patches	Pulmonary heart valve
PHO-Sy		Subcutaneous patches
PHOU-Au		Subcutaneous patches
PHACOS	Biosynthetic good thermal stability up to 200 °C	

8. Chemical modifications of PHAs

PHB is a potential material substitute in biomedical and packaging fields because of biodegradability, biocompatibility, availability and thermoplasticity. However, the use of PHB is primarily limited in industrial and biomedical applications by their high cost, thermal instability and poor mechanical properties. The major drawbacks of PHB is high melting temperature and low toughened due to secondary crystallization and slow nucleation rate. PHB was improved by drawing and thermal treatment, blending with biomaterials or synthetic polymers, forming reinforced with composites such as natural fibers and inorganic fillers (Yeo *et al*.,

2018). A common way to insert broad applicability properties in PHB and to minimize the unwanted properties is blending with suitable well defined polymers (Bhatt *et al*., 2008).

9. Polymer blends

Polymer blends is a mixing of two or more different polymers together that make it possible to achieve various property combinations of the final material. The cost of specific materials that made from this method are lower than synthesis of new polymers. Five different methods are used for the preparation of polymer blends (Khan *et al*., 2018).

9.1 Melt mixing is the most comprehensive method of polymer blend. The blend components are mixed in the molten state in batch mixers or extruders. Advantages of the method are well-defined components and acceptance of mixing devices. Disadvantages of the method are high energy consumption and possible unfavorable chemical changes of blend components.

9.2 Solution blending is frequently used for preparation of polymer blends on a laboratory scale. The blend components are dissolved in a common solvent and intensively stirred. The blend is separated by precipitation or evaporation of the solvent. Advantages of the process are rapid mixing of the system without large energy consumption and the potential to avoid unfavorable chemical reactions. However, this method is limited by the requirement to find a common solvent for the blend components and to remove large amounts of organic solvent.

9.3 A blend with heterogeneities can be prepared by mixing of latexes without using large energy consumption or organic solvents. Energy is required only for

removing water and finally achievement of finer dispersion by melt mixing. The requirement to have all components in latex form limits the use of the process.

9.4 Block or graft copolymerization can be prepared by forming a copolymer sufficient for achieving good adhesion between immiscible phases of homopolymer. Properties of materials that prepared by this method are better than pure melt mixing. However, this process is complicated and expensive.

9.5 Formation of interpenetrating polymer networks can be prepared by forming network between two polymers. One polymer is swollen with the other monomer or prepolymer. Then, the monomer or prepolymer is crosslinked. Only reactoplastics are prepared by this method.

10. PHA blending

Blending is the easiest, most effective and economical method to improve physical and mechanical properties of materials. Recently, PHB blended with biodegradable polymers have been an interest because of sustainability and eco-friendly (Yeo *et al*., 2018). For example, PHBV was added to PLA fibers to increase the flexibility of PLA. PLA/PHBV blends (95/5 and 90/10) were used as textile fibers for knitting socks (Pivsa-Art *et al*., 2011). PHBV/chitosan fibrillar scaffolds were successfully produced for used in biomedical field. This hybrid fibers show high degradation rates. (Veleirinho *et al*., 2011). Addition of (3-hydroxyoctanoate) (PHO) to PLA was improved the elongation at break and impact properties of PLA (Nerkar *et al*., 2015). PHB blended with starch was lower cost and more thermally stable than PHB. It can be used in paper coating and cardboard packaging field (Godbole *et al*., 2003). PHB/starch ratio of 70:30 reduced the size of PHB spherulites and enhanced toughness of PHB and thermal stability (Zhang *et al*., 2010). Cellulose nanocrystals

(CNCs) was used as bio-fillers for PHB polymer. Tensile modulus and strength of PHB/CNC nanocomposites (6% CNC content) had increased 50% and 35%, respectively. Moreover, elastic modulus was increased 2% and hardness values was 155–165 MPa (Seoane *et al*., 2016). Hydrogen bonding between carbonyl groups of PHB and amine groups of chitin promoted rapid growth of PHB crystals. So, the structure of PHB in PHB/Chitin blends was changed and crystallinity was decreased (Raghunatha *et al*., 2015). PHB that blended with PVAs showed higher toughness. Elongation at break of the material increased from 23 to 257% and impact strength increased from 0.4 to 0.8 ft lb/in with no expense in stiffness (Whitehouse and Lexington, 2016).

11. Natural rubber

Natural rubber is the most important polymers produced by plants. It is a strategically important raw material used in many products in medical devices. Natural rubber is obtained from latex, an aqueous emulsion present in the laticiferous vessels or parenchymal cells of rubber-producing plants. Although more than 2500 plant species are known to produce natural rubber latex. Recently, there is only one important commercial source, *Hevea brasiliensis* (the Brazilian rubber tree). Another important plant that can produce rubber latex is *Parthenium argentatum*, which called guayule. The rubber from this plant is being marketed as non-allergenic natural rubber (Puskas *et al*., 2006).

The natural rubber polymers composed of 100% cis-1,4-polyisoprene with Mw ranging from 1 x 10^6 to 2.5 x 10^6. Natural rubber was added to improve the elasticity properties (ductility and impact resistance) of polymers. The main objective of rubber blend is to improve the mechanical properties (stiffness and hardness)

because the adding natural rubber to a thermoplastic component increases the toughness (impact strength) but unfortunately decreases the stiffness (tensile modulus), strength and hardness of the resulting blend (Bendjaouahdou and Bensaad, 2013). Blending rubbers with PHA may help in decreasing melting temperature (T_m) and reducing crystallinity which provides elasticity and toughness needed for many plastic applications (Bhatt *et al*., 2008). Moreover, rubbers are another class of polymers which are biodegradable. Several species of bacteria and fungi are able to degrad rubber (Rose and Steinbüchel, 2005). Rubber blends attemp interesting possibility of preparing biodegradable materials with useful mechanical properties. However, natural rubber and PHA have different molecular weight and polarity. Natural rubber should be modified by reducing molecular weight of natural rubber or using epoxidized natural rubber (ENR).

12. Epoxidized natural rubber (ENR)

Epoxidized natural rubber (ENR) is a modified natural rubber (NR) which has recently attracted the attention of many researchers. ENR is prepared from NR (*cis*-1,4-isoprene) by treating it with peracetic acid as shown in Figure 6a. Isoprene and epoxidized isoprene are monomer units that randomly distributed along the polymer chain. ENR has some special properties such as low gas permeability, excellent oil resistance, good anti-wet skid resistance and highly damping properties (Hamzah *et al*., 2012). Degree of epoxidation of NR is depended on isoprene units in the polymer chain. ENR-25, ENR-50 and ENR-75 are polymer chain that isoprene units are epoxidized 25%, 50% and 75%, respectively. The toughness of ENR is relative to natural rubber (NR). However, some mechanical and thermal properties slightly decrease because of the presence of epoxy groups on the backbone chains. The epoxy

linkage is expected to be a reactive site for bonding between the PHB and ENR chains (Gelling, 1991; Lee *et al*., 2005; Hamzah *et al*., 2012; Tanjung *et al*., 2015).

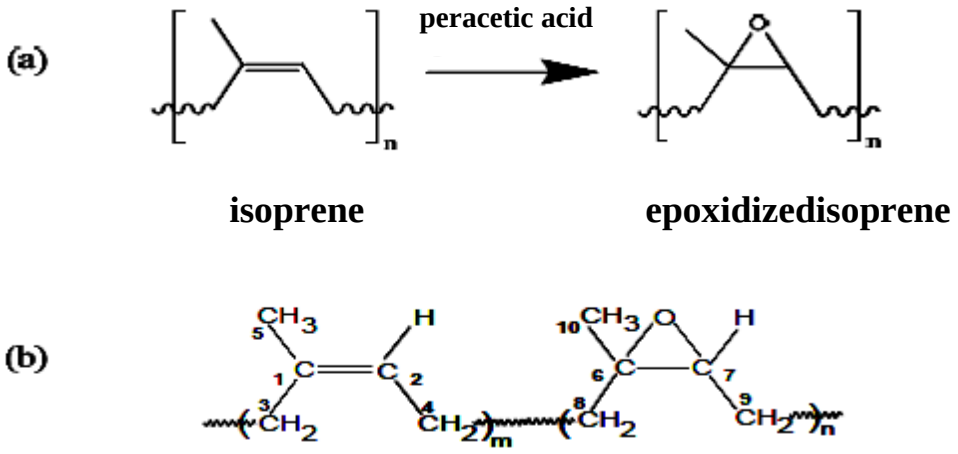


Figure 6 (a) Modification of NR to ENR.

(**b**) The general structure of ENR-50 (Hamzah *et al*., 2012).

ENR particles were added in a plastic matrix to improve the toughness of the material. This method makes them suitable for applicationin automotive, aerospace, thermal, and electrical insulatingsystems (Pire *et al*., 2011). The major products of ENR/plastic blends are mold forms such as sponges, curing tubes, carpet underlays, connectors, curing flaps, bumpers, heavy duty pads, seals, gaskets and wheels (Saito *et al*., 2007). Furthermore, ENR blends are used as pressure-sensitive adhesives, adhesive tapes, packaging tapes, surgical tapes and plasters (Johnson *et al*., 2000).

13. Polyhydroxyalkanoates degradation

PHAs can be degraded completely to form products non-harmful to the environment such as carbon dioxide and water under aerobic conditions or methane and water under anaerobic conditions. Biodegradation rate is depended on environmental factors such as temperature, pH, moisture, nutrient supply, microbial

population and PHAs properties such as crystallinity, additives and surface area (Doi, 1990). PHAs are degraded by soil and aquatic microorganisms that have specific enzymes; PHA depolymerases (Jendrossek and Handrick, 2002; Kim *et al*., 2003). There are two main types of PHA depolymerases which can be classified as intracellular and extracellular depolymerases. Intracellular depolymerases required nutrients to degrade the native PHA. While extracellular depolymerases hydrolyze water insoluble PHA to soluble monomers. Six hundred PHA depolymerases from various microorganisms have been identified by comparison of their amino-acid sequences provided a basis for uniting them in 8 super families including 38 families (Knoll *et al*., 2009). This type of polymer degradation depends on conditions like inorganic nutrient composition and environment temperature. Among PHA biodegraders, various microorganisms such as *Pseudomonas lemoignei, Alcaligenes faecalis* and *Comamonas testosteroni* (Varsha and Savitha, 2011) degraded PHAs in soil, compost, fresh waters, and marine environments (Volova *et al*., 2010). PHBHHx was degraded 80% when buried at a secondary forest for 3 weeks and PHB was degraded 50% when buried at a secondary forest for 8 weeks (Ong and Sudesh, 2016). PHB films was degraded 50% when buried in a tropical mangroved for 4 weeks (Sridewi *et al*., 2006). PHB and PHBV were degraded 42% and 46% when incubated in the marine environment for 160 days (Volova *et al*., 2010).

OBJECTIVES

1. To optimize the condition for polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) production
2. To produce polyhydroxyalkanoates (PHA)-natural rubber blends
3. To characterize PHB, PHBV and PHB– natural rubber blends
4. To study biodegradation of PHB, PHBV and PHB- natural rubber blends

Expected outcomes

1. To achieve the optimum condition for PHB and PHBV production.
2. To obtain the materials that have higher elastic properties and lower melting point than PHB
3. The obtained material that can be naturally degraded in environment

CHAPTER 2

RESEARCH METHODOLOGY

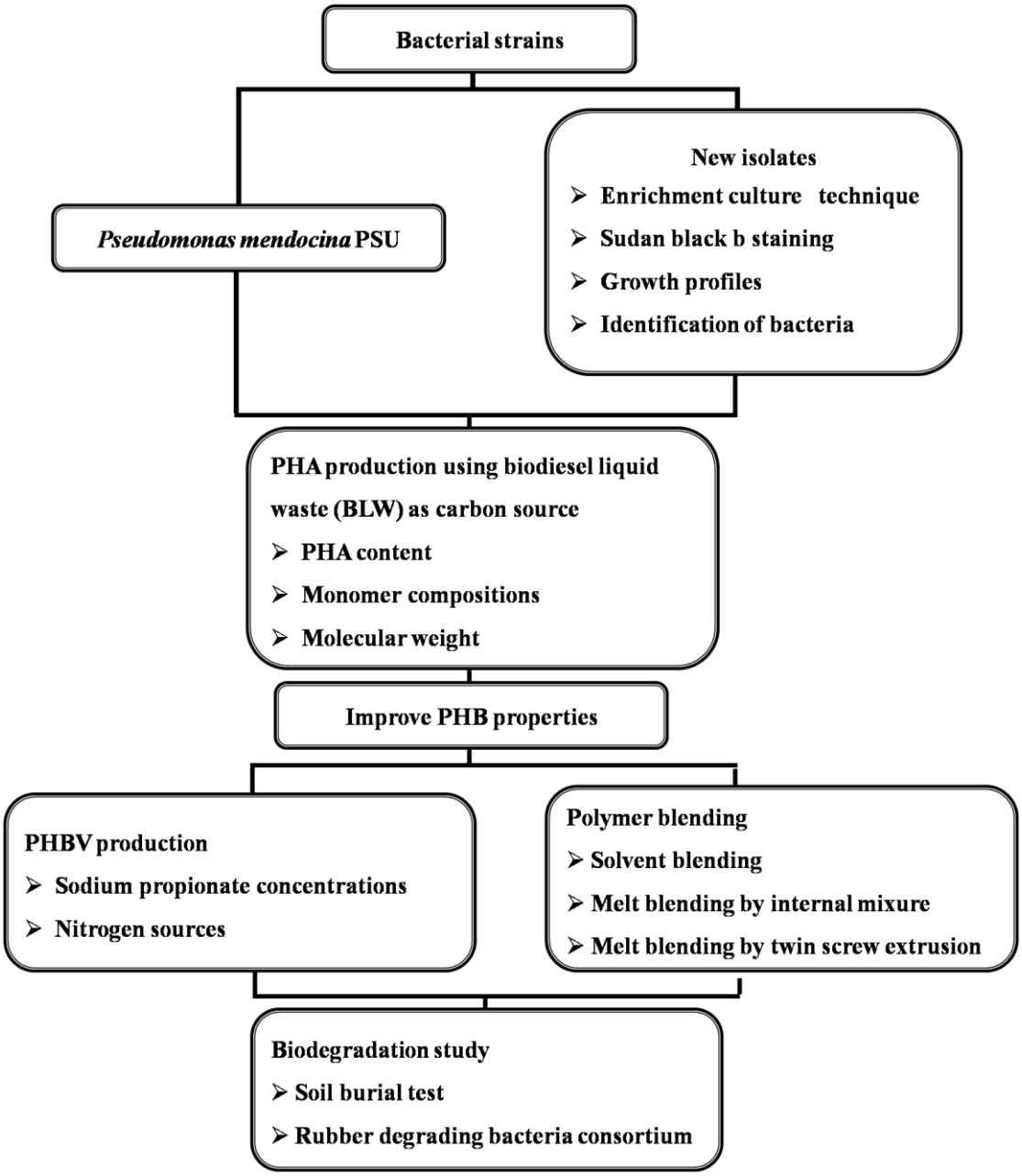


Figure 7 The research outline of this study

1. PHAs production using Biodiesel liquid waste (BLW) as the sole carbon source

1.1 PHAs production by *Pseudomonas mendocina* PSU

Pseudomonas mendosina PSU was successfully isolated and identified by Miss Wankuson Chanasit (Chanasit *et al*., 2016). It was grown on a nutrient rich (NR) agar plate for 24 h. Then, the cells were suspended in 0.85 % (w/v) sodium chloride to an optical density (600 nm) of 0.5. Two milliliter of cell suspension was inoculated into 50 mL of NR medium (Appendix A2) and incubated on a rotary shaker at 35 °C and 150 rpm (Chee *et al*., 2010). The PHAs production was carried out in shake flasks using the biodiesel liquid waste (BLW) as carbon source. After the *P. mendosina* PSU grew in NR broth until it reached mid exponential growth, 4.0 % (v/v) of the pre-grown inoculums was inoculated into a 2 L Erlenmeyer flask containing 1,300 mL of mineral salt medium (MSM) pH 7.0 supplemented with 2.0 % (v/v) of BLW (Appendix A2). Aerobic condition was maintained by shaking at 150 rpm and 35 °C. Samples were taken every 12 h until 72 h of cultivation for the biomass concentration and PHAs content analysis. Dry cell weight (DCW) was measured following centrifugation of 10 mL of culture at 8,000 rpm for 20 min at 4 ºC and the harvested cells were then dried at 60 ºC to a constant weight (Chanasit *et al*., 2016).

1.1.1 Determination of monomer composition and molecular weight of PHA from *P. mendocina* PSU

PHAs monomer composition was determined by Gas Chromatography (GC). Two milliliter of chloroform and acidified methanol (3.0% (v/v) H_2SO_4) were added into 20 mg of dried cells and then heated at 80 °C for 3.5 h. After cooling, 2 mL of

distilled water was added followed by vigorous shaking for 4 min. Allowed the layers to separate for 12 h and the chloroform portion containing the PHA methyl esters was transferred to a vial for analysis by GC (Agilent 7890A, Agilent Technologies, US and Canada) (Sun *et al*., 2007). Benzoic acid was used as the internal standard.

PHAs monomer composition was confirmed by ^{1}H NMR analysis. Approximately 10 mg of the extracted PHAs sample was dissolved in 0.68 ml deuterated chloroform ($CDCl_3$) for ^{1}H NMR analysis. ^{1}H spectra were recorded at 500MHz and room temperature on Fourier Transform NMR Spectrometer 500MHz Unity Inova, Varian (Germany). Chemical shifts were reported in ppm relative to the signal of the $CDCl_3$.

Molecular weight of PHAs from *P. mendocina* PSU was determined by Gel Permeation Chromatography (GPC). PHAs were dissolved in chloroform (1 mg mL^{-1}) and filtered through 0.22 μm PTFE filter. The molecular weights of PHAs were determined using Agilent 1200 Series GPC with Shodex K-806M and Shodex K-802 column at 40 °C. Chloroform was used as the eluent. The injection volume was 50 mL with the flow rate of 1.00 mL min^{-1}.

1.2 Isolation of PHAs-accumulating bacteria and production of PHA using BLW and palm oil as the sole carbon source

1.2.1 Screening of PHAs-accumulating bacteria by the enrichment culture technique

The PHAs-producing bacteria were isolated from Sirindhorn peat swamp forest in Narathiwat, Thailand. Bacteria were isolated by adding 10 g of soil samples into 100 mL of MSM consisting of 2.0 % (v/v) BLW or palm oil as carbon source

(Kulpreecha *et al.,* 2009). The pH of MSM was adjusted to 7.0 before sterilization. Cultures were incubated at 35 °C and 150 rpm for 4 weeks. Every week, 0.1 mL of bacterial culture was plated on MSM agar containing 2.0 % (v/v) BLW or palm oil. Bacterial colonies were stained with Sudan black B for 5 min and Safranin for 1 min to check for possible PHAs deposits (Burdon, 1946). The colonies that have PHAs granules were chosen and further analyzed.

1.2.2 Growth of newly isolated strains in the pre-culture medium

Each selected PHAs-producing bacteria was grown on a NR agar plate for 24 h. Then, cells were suspended in 0.85 % (w/v) sodium chloride to an optical density (600 nm) of 0.5. Four percent of cell suspension was inoculated into1.3 L of NR medium (Lau *et al.,* 2011) and incubated on a rotary shaker at 150 rpm and 35 °C for 76 h. Every 2-6 h, samples were taken and measured optical density (600 nm) by spectrophotometer.

1.2.3 PHAs accumulation of newly isolated strains in MSM medium

The pre-culture was incubated in a rotary incubator shaker at 150 rpm and 35 °C until mid-log phase was reached. Then, 4.0 % (v/v) of the pre-culture was added to the MSM consisting 2.0 % (v/v) BLW or palm oil as a carbon source. The culture was incubated on a rotary shaker at 150 rpm and 35 °C for 96 h. Dried cell weight and PHAs content were analyzed every 6 h. Ten mL of culture samples was centrifuged at 8000 rpm for 20 min at 4 °C and the harvested cells were then dried at 60 °C to a constant weight. DCW was calculated as grams of dry weight per liter. PHAs monomer composition was determined by GC. Molecular weight of PHAs was determined by GPC (Wong *et al.*, 2012).

Identification of the bacterial strain

The 16s rRNA gene sequence analysis was performed to identify and classify the PHAs-producing bacterium. The genomic DNA was extracted from the pellet using a TIANamp genomic DNA extraction kit (TIANamp bacteria DNA kit, Tiangen biotech, China). The primers used were 27F (5'- AGAGTTTGATCCTGGCTCAG –3') and 1492R (5'- GGTTACCTTGTTACGACTT –3') to amplify the full length of the 16s rRNA gene. The following PCR parameters were used: 94 °C for 3 min, 35 thermal cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min; followed by a final extension step at 72 °C for 10 min (Miller *et al.*, 2013). Nucleotide sequences of purified PCR products were determined using the DNA Analyzer and 16s rRNA nucleotide sequences were subsequently analyzed using the data available from the GenBank database of The National Center for Biotechnology Information. The max scores of *Burkholderia* type strains and an out-group strain named *Caballeronia concitans* LMG 29315 were collected for ClustalW alignment. A phylogenetic tree was constructed with Neighbour-joining statistical analysis (Tamura *et al*., 2013).

2. PHBV production

2.1 Effect of sodium propionate concentrations on PHBV production

Sodium propionate was added as 3HV-precursors. PHA biosynthesis was carried out in one-stage cultures in shake flasks. The starter culture was grown in NR medium for 18 h at 35 ºC. Four percent of the culture was transferred into 1,300 mL of MSM supplemented with 2.0 % (v/v) BLW and sodium propionate at 4 different concentrations (0.1, 0.3, 0.5, and 0.7%). The culture was incubated at 35 ºC, 150 rpm in a rotary shaker and samples were taken every 12 h until 72 h of cultivation. DCW was measured and PHA monomer composition was determined by GC.

2.2 Effect of nitrogen sources on PHBV production

The starter culture was pre-cultured in NR medium at 35 °C for 18 h. After that, the culture (4% v/v) was transferred into MSM. To observe the impact of nitrogen contents on PHBV production by *P. mendocina* PSU, concentration of urea and ammonium sulfate in MSM medium were varied. Ammonium sulfate (0.5, 1, and 1.5 g L^{-1}) and equal mole of urea were used as nitrogen sources. These culture flasks were then incubated at 35 °C, 150 rpm. Samples were taken to analyze biomass concentrations and PHBV contents every 12 h until 96 h of cultivation. DCW was calculated as grams of dry weight per liter. PHBV monomer composition was determined by GC. Monomer composition was confirmed by ^{13}C NMR analysis. Approximately 20 mg of the extracted PHBV was dissolved in 0.68 mL deuterated chloroform ($CDCl_3$) for ^{13}C NMR analysis. ^{13}C spectra were recorded at 500MHz and room temperature on Fourier Transform NMR Spectrometer 500MHz Unity Inova, Varian (Germany). Chemical shifts were reported in ppm relative to the signal of the $CDCl_3$. Molecular weight of PHA was determined by GPC.

3. PHA recovery

3.1 Chemical recovery of PHAs by chloroform extraction

Cells were harvested after 72 h of incubation and centrifuged at 8000 rpm for 15 min at 4 ºC, washed twice in distilled water and dried at 60 ºC for 3 days. The biomass (10 g) was extracted with 1 L of chloroform in a glass bottle and stirred for 5 days at room temperature. The chloroform solution was filtered to remove any cell debris and concentrated by rotary evaporation to a final volume of approximately 100 mL. PHAs were purified by precipitation, the PHAs chloroform solution was added into a cold methanol solution (400 mL) while being rapidly stirred by using a

magnetic stirrer. The precipitated white PHA was then filtered through a Whatman No. 1 filtered paper, dried at room temperature and weighed. The polymer content was calculated as the percentage of PHAs in the cell. The purified polymer was kept at room temperature. Molecular weight of PHAs was determined by GPC. The glass transition temperature (T_g) and the melting temperature (T_m) were determined using Differential Scanning Calorimeter, DSC8500, Perkin Elmer (USA). Approximately 5 mg of the samples were encapsulated in standard aluminium DSC pans and heated from -50 °C to 200 °C at heating rate of 20 °C min^{-1}. Then, sample was cooled from 200 °C to -50 °C at heating rate of 20 °C min^{-1} under a nitrogen atmosphere. After that, sample were reheated from -50 °C to 200 °C with heating rate of 20 °C min^{-1}.

3.2 Biological recovery of PHAs by mealworms

PHAs from *P. mendocina* PSU cell pellets was extracted by mealworms (*Tenebrio molitor*). Mealworms obtained from the local shops were cultivated in plastic containers at room temperature. Two hundred grams of mealworms were fed with 10 g of the dried *P. mendocina* PSU cells for 5 days. Fecal pellets of mealworms were collected by sieving. After that, the fecal pellets containing PHAs granules were washed with 1% (w/v) SDS at 50 °C, 250 rpm for 10 h (1 g fecal pellets/10 mL 1% SDS). Every 2 h, the fecal pellets were collected by centrifugation and transferred to a new 1% SDS solution. Finally, cell pellets were dried overnight at 60 °C. The polymer content was calculated as the percentage of PHA in the cell.

The purified polymersfrom both methods were kept at room temperature. Molecular weight of PHA was determined by GPC. T_g and T_m were determined using DSC.

4. Preparation of PHB–rubber blends

Epoxidized natural rubber (ENR) is a modified natural rubber (NR) with epoxide rings on the rubber chain. The epoxy linkage is expected to be a reactive site for bonding between rubber chains and PHB. Moreover, ENR particles were added in a plastic matrix to improve the toughness of the material. Molecular weight of ENR was different from PHB, so molecular weight ENR was reduced by grinding with two roll mill. PHB was blended with modified ENR25 rubber in the ratio of 70:30 (PHB: ENR25) by 3 methods as followed:

4.1 Blending by solution blending

PHB (2.1 g) was dissolved in chloroform for 10 min. Then, ENR25 rubber (0.9 g) was added and stirred at 100 rpm for 12 h. The polymer blend was poured into petri dish. Solvent was removed by vacuum oven at 40 °C, 200 mbar for 3 h.

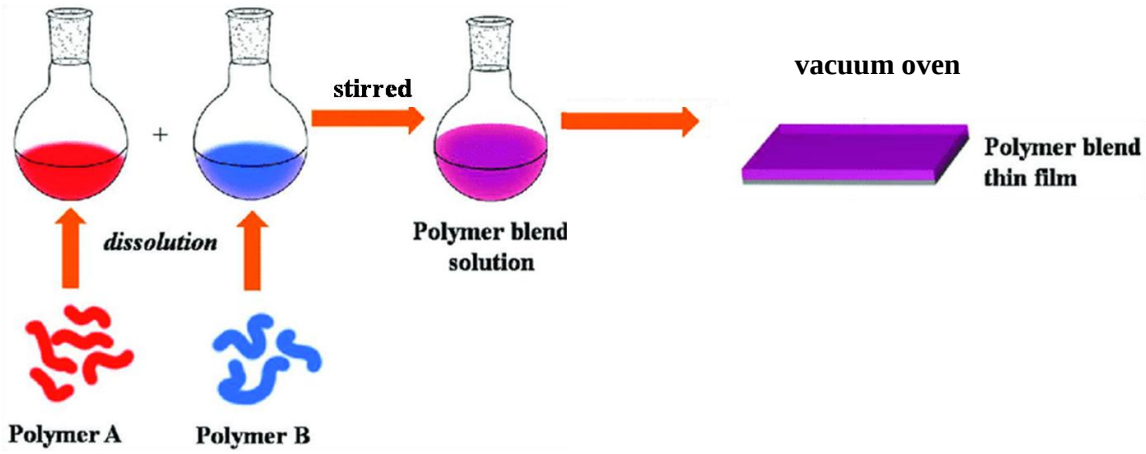


Figure 8 Diagram of solution blending method (Dong *et al.*, 2018)

4.2 Blending by internal mixture

PHB (49 g) was blended with modified ENR25 rubber (21 g) by internal mixture at 170 °C, 60 rpm for 10 min (Mixture 350E, Brabender®, Germany). The

polymer films approximately 0.3 mm thick were prepared by rolling with two roll mill.

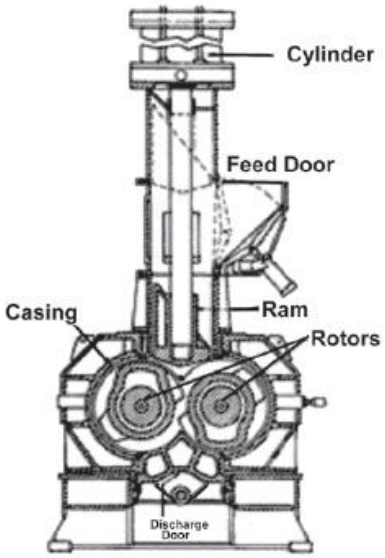


Figure 9 Schematic of internal mixture (Albert and Drobny, 2006)

4.3 Blending by twin screw extrusion

PHA (70 g) was blended with modified ENR25 (30 g) by twin screw extrusion. Melt compounding of PHB with modified ENR25 rubber by twin screw extrusion was achieved in a twin screw extruder with injection molder system (TSE 16 TC, Prism Co., LTD, Latvia). The extruder is supplied with co-rotating screws having lengths of 23 cm, with L/D ratio of 15:1. PHB and modified ENR25 were mixed together and fed to the extruder barrel. The materials were melted at 175 $^{\circ}$C and 150 rpm.

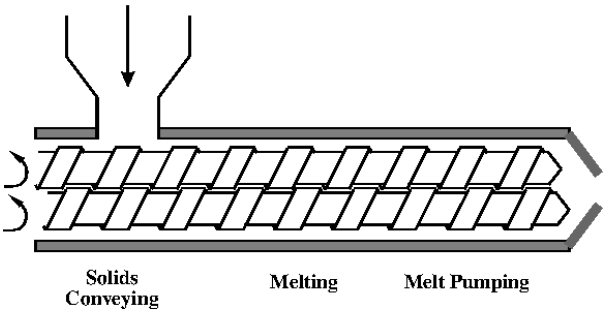


Figure 10 Schematic of twin screw extrusion (White, 1991)

Characteristics and properties of mixed polymer films from the 3 methods were observed and analysed.

5. Biodegradation studies of PHAs and the blend films

5.1 Biodegradation studies of PHAs and the blend films by the soil burial test

The PHB-rubber blend films from solvent blending and melt blending, PHBV, PHB from *P. mendocina,* PHB from *B. Contaminans,* NR, rubber glove and ENR25, were cut into small pieces with the dimension of 1 cm x 1 cm. A plastic net was sewed to keep the films. Each square film was weighed and placed into the net. Samples were buried in the soil at a depth of 15 cm (duplicates for each sample) for 4 weeks. Every 2 weeks, polymer films were removed from the pouch and rinsed with distilled water to remove the soil. The samples were dried at room temperature and placed in a desiccator until constant weights were reached. The weight of each film was reported. The degradation of the films was calculated in terms of percentage of weight loss by using the following formula:

$$\frac{\text{(Weight of initial film - Weight of film after degradation)}}{\text{Weight of initial film}} \times 100$$

The photos of the polymer films were taken to record the physical changes of the films.

Figure 11 Biodegradation studies of PHAs and the blend films by the soil burial test

5.2 Biodegradation studies of PHAs and the blend films by rubber degrading bacteria consortium

The consortium was obtained from Miss Sirimaporn Watcharakul (Watcharakul *et al.*, 2016). The microbial consortium was priory enriched in MSM with rubber pieces for a period of 7 days, and was then used as an inoculum. Each tested film was grown with the rubber degrading consortium. The investigations of materials degradation were established using two replicates. In addition, an abiotic control experiment was always done in parallel in the same conditions. All cultures were incubated 30 °C, 150 rpm in Mineral Salt Medium (MSM, 9 g/L $Na_2HPO_4.12\ H_2O$, 1.5 g/L KH_2PO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4.7\ H_2O$, 0.02 g/L $CaCl_2.2\ H_2O$ and 1.2 mg/L Fe(III) ammonium citrate) (Jendrossek *et al.*, 1997) supplemented with 1x1 cm pieces of sterilized material as a carbon source. Every 2 weeks, polymer films were removed from the pouch and rinsed with distilled water to remove the soil. The samples were dried at room temperature and placed in a desiccator until constant weights were reached. The

weight of each film was reported. The degradation of the films was calculated in terms of percentage of weight loss by using the following formula:

$$\frac{\text{(Weight of initial film - Weight of film after degradation)}}{\text{Weight of initial film}} \times 100$$

The photo of the polymer films were taken to record the physical changes of the films.

CHAPTER 3

RESULTS AND DISCUSSION

1. PHAs production using Biodiesel liquid waste (BLW) as the sole carbon source

1.1 Biodiesel liquid waste (BLW) and PHAs production using BLW

Biodiesel liquid waste (BLW) is generated at approximately 10% (w/w) as the main by-product of biodiesel production (Yang *et al*., 2012). BLW contained about 40% of carbon and it was used as a carbon source for microbial growth. BLW used in this study had 40% carbon elements and 0% nitrogen elements. This carbon source contained high carbon content but low nitrogen content that was suitable for PHAs production. The major component of BLW is glycerol. In addition, BLW also contains free fatty acid (FFA) and fatty acid methyl esters (FAME) that can be used as carbon sources to enhance bacterial growth and PHA biosynthesis (Chanasit *et al*., 2016). Since, BLW is cheap and classified as a renewable resource, it becomes very attractive as a carbon source for PHAs production. *Bacillus sonorensis* and *Halomonas hydrothermalis* isolated from soil and marine sources were able to utilize biodiesel by-product to produce 71.8 and 75 wt% PHB, respectively (Shrivastava *et al*., 2010). *Bacillus aryabhattai* used 2% BLW as a carbon source to produce 72.3 wt% PHB (Chanasit *et al*., 2014). *Burkholderia cepacia* ATCC 17759 was successfully cultivated in a 200 L fermenter and yielded DCW and PHB concentration of 23.6 g/L and 7.4 g/L, respectively when using BLW as a carbon source (Zhu *et al*., 2010).

1.2 PHAs production by *Pseudomonas mendocina* PSU

Pseudomonas mendocina PSU that used BLW as a sole carbon source was isolated by Miss Wankuson Chanasit (Chanasit *et al*., 2016) from biodiesel-contaminated sediment derived from the biodiesel ethyl ester pilot plant, the

specialized R & D center for alternative energy from palm oil and oil crops, Faculty of Engineering, Prince of Songkla University, Thailand. *P. mendocina* PSU's colony forms were flat, smooth, and brownish-yellow color on NR agar. This strain is an aerobic gram-negative rod shaped bacterium. *P. mendocina* PSU started to produce PHAs from the mid-log phase and reached the maximum value when the culture was in the stationary phase (Figure 12). This result is similar to *Brevibacillus invocatus* MTCC 9039 and *Actinobacillus* sp. EL-9 that the maximum accumulation of PHAs was observed at the stationary phase (Yu *et al*., 2002; Berezina, 2012). *P. mendocina* PSU produced 79.6 wt% PHB with DCW 3.77 g/L at 72 h when grown in MSM medium with 2% (v/v) BLW (Table 5). Many *Pseudomonas* species were reported as potential PHAs-producing bacteria. *P. putida* GPo1 previously known as *P. oleovorans* produced PHAs from different carbon sources such as alkanes and alkenes (De Smet *et al*., 1983; Preusting *et al*., 1993), carboxylic acids (Brandl *et al*., 1988), alcohols (Haywood *et al*., 1989) and plant oils (Silva-Queiroz *et al*., 2009). *P. extorquens* and *P.aeruginosa* used alcohol and fatty acids to produce PHAs (Haywood *et al*., 1989). *Pseudomonas* sp. strain NCIMB 40135 used carbohydrates as carbon sources (Haywood *et al*., 1990). In this study, *P. mendocina* PSU used BLW which is a cheap carbon source to produce high PHB content. This strain used glycerol, free fatty acids and fatty acid methyl ester in BLW to support its growth and PHAs production (Chanasit *et al*., 2016). When BLW was used as a carbon source, PHAs production cost was reduced and the waste disposal problem was minimized.

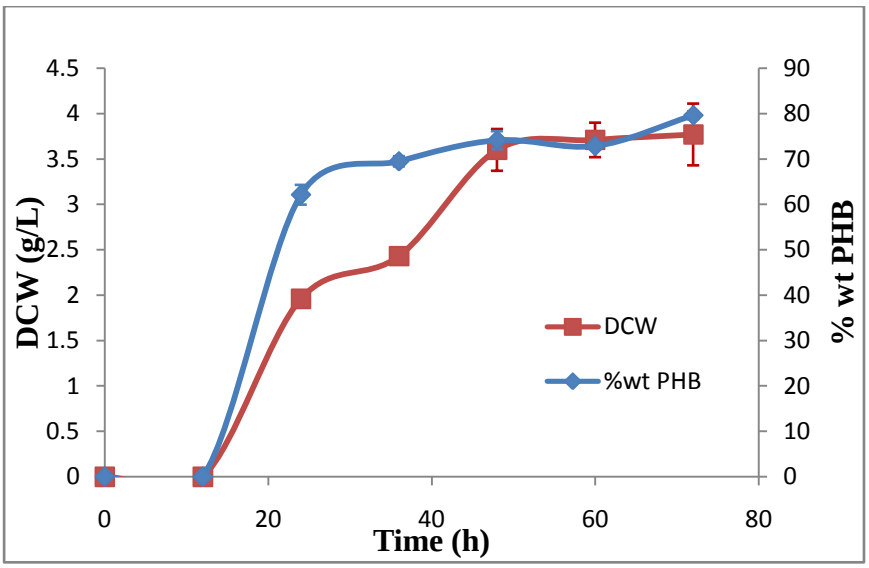


Figure 12 Biosynthesis of PHB in *P. mendocina* PSU when grown in MSM with 2% BLW

Table 5 Biosynthesis of PHB in *P. mendocina* PSU when grown in MSM with 2% BLW

Time (h)	%PHB/DCW	DCW (g/L)
0	nd*	Nd
12	nd	Nd
24	62.12 ± 2.17	1.96 ± 0.04
36	69.49 ± 1.13	2.43 ± 0.09
48	74.14 ± 1.98	3.60 ± 0.23
60	72.88 ± 0.18	3.71 ± 0.19
72	79.63 ± 0.89	3.77 ± 0.34

*Not detected; MSM, Mineral Salts Medium; PHB, poly(3-hydroxybutyrate)

1.2.1 Monomer composition of PHAs from *P. mendocina* PSU

The monomer composition of PHAs produced from *P. mendocina* PSU when grown with BLW was identified using gas chromatography (GC) analysis and the obtained chromatogram was compared with the PHB standard. Only the

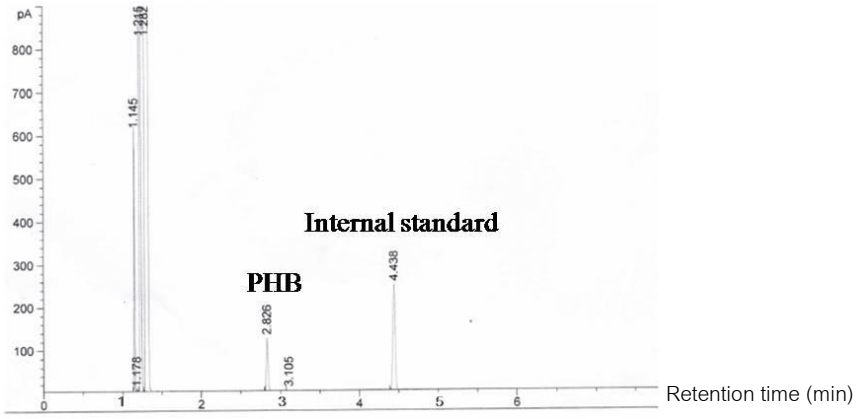
homopolymer PHB was accumulated and detected at retention time 2.82 by this strain (Figure 13). This material was confirmed by ^{1}H NMR. ^{1}H NMR spectrum demonstrated that the sample contained pure PHB. Three groups of signal characteristics of PHB were observed (Figure 14). The resonance observed at 1.22 and 1.24 ppm were responsible for methyl proton of HB side group, at 2.41-2.88 ppm were methylene protons of HB bulk structure and at 5.19-5.25 ppm were methine protons attached to an ester function. Monomer composition is based on different substrates that are specific to PHA polymerase. This enzyme is responsible for the assembly of PHA monomeric precursors [(*R*)-3-hydroxyacyl-CoAs] and the specialization of metabolic and regulatory networks in each species (Prieto *et al*., 2015). In this study, *P. mendocina* PSU utilized glycerol which was most preferred by this strain. Glycerol was converted to glyceroldehyde-3-phosphate followed by pyruvate and acetyl-CoA, a precursor for PHB biosynthesis through glycolysis and the pyruvate decarboxylation process (Figure 15). PHB biosynthesis presumably was catalyzed by enzyme *β*-ketothiolase (*phaA*), acetoacetyl-CoA reductase (*phaB*), and SCL-PHA synthase (*phbCPs*), respectively (Solaiman *et al*. 2000; Zhu *et al*. 2013).

The molecular weights of PHB samples were determined by gel permeation chromatography. The weight-average molecular weight (M_w) was 8.39 x 10^4 and the number-average molecular weight (M_n) was 4.60 x 10^4. Molecular weight of PHB from *P. mendocina* PSU was similar to PHB from *Pseudomonas* sp. strain NCIMB 40135 which produced low molecular weight PHB (M_w = 1.43 x 10^5 and M_n = 6.19 x 10^4) when glucose was used as a carbon source (Haywood *et al*., 1990) and *Burkholderia cepacia* ATCC 17759 which produced low molecular weight PHB (M_w = 1.73 x 10^3 and M_n = 3.04 x 10^3) when xylose and glycerol were used as carbon

sources (Zhu *et al*., 2010). PHB produced from this strain has low molecular weight and is brittle. When the glycerol was used as carbon source, PHB chain was reduced. The polymerization step for PHB chain propagation was terminated by glycerol-end capping through the covalent esterification of glycerol to PHB in a chain terminating position. When glycerol concentration was increased, chain termination rate was high and resulted in short chain PHA (Ashby *et al*., 2005).

In this study, we also isolated other bacteria that used BLW to produce high molecular weight PHA to compare the production of PHA with *P. mendocina* PSU.

(A)



(B)

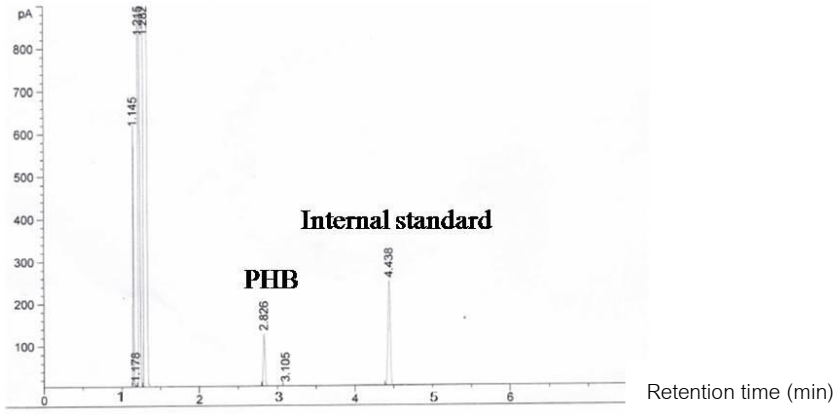


Figure 13 GC analysis of the PHAs produced by *P. mendocina* PSU (A) standard PHB (B) PHB produced by *P. mendocina* PSU (pA: peak area)

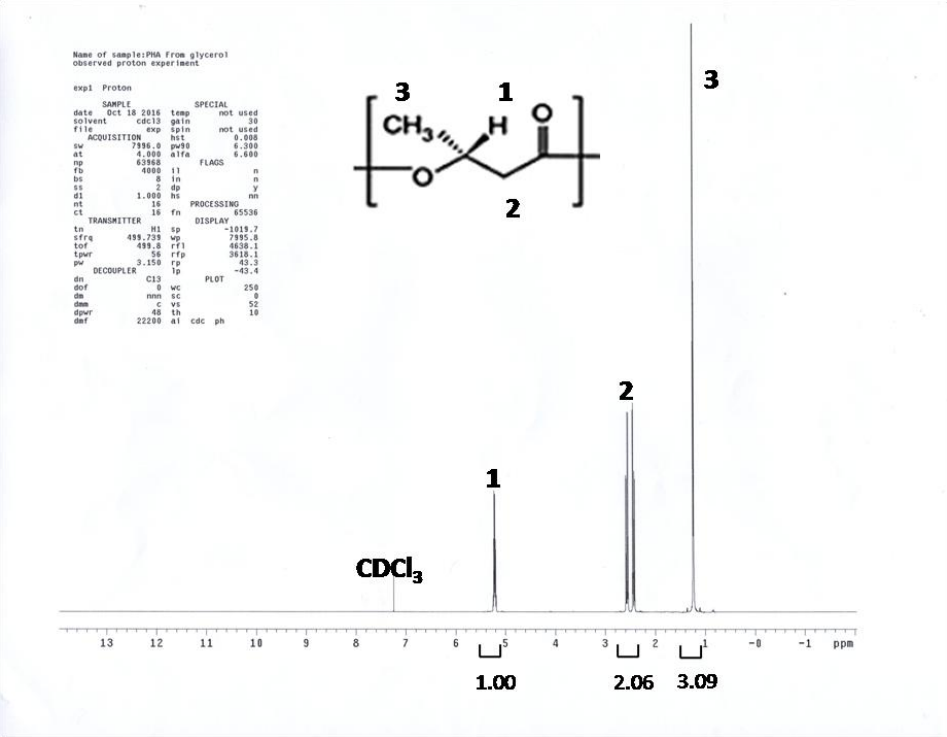


Figure 14 ^{1}H NMR spectrum in $CDCl_3$ of PHB from *P. mendocina* PSU

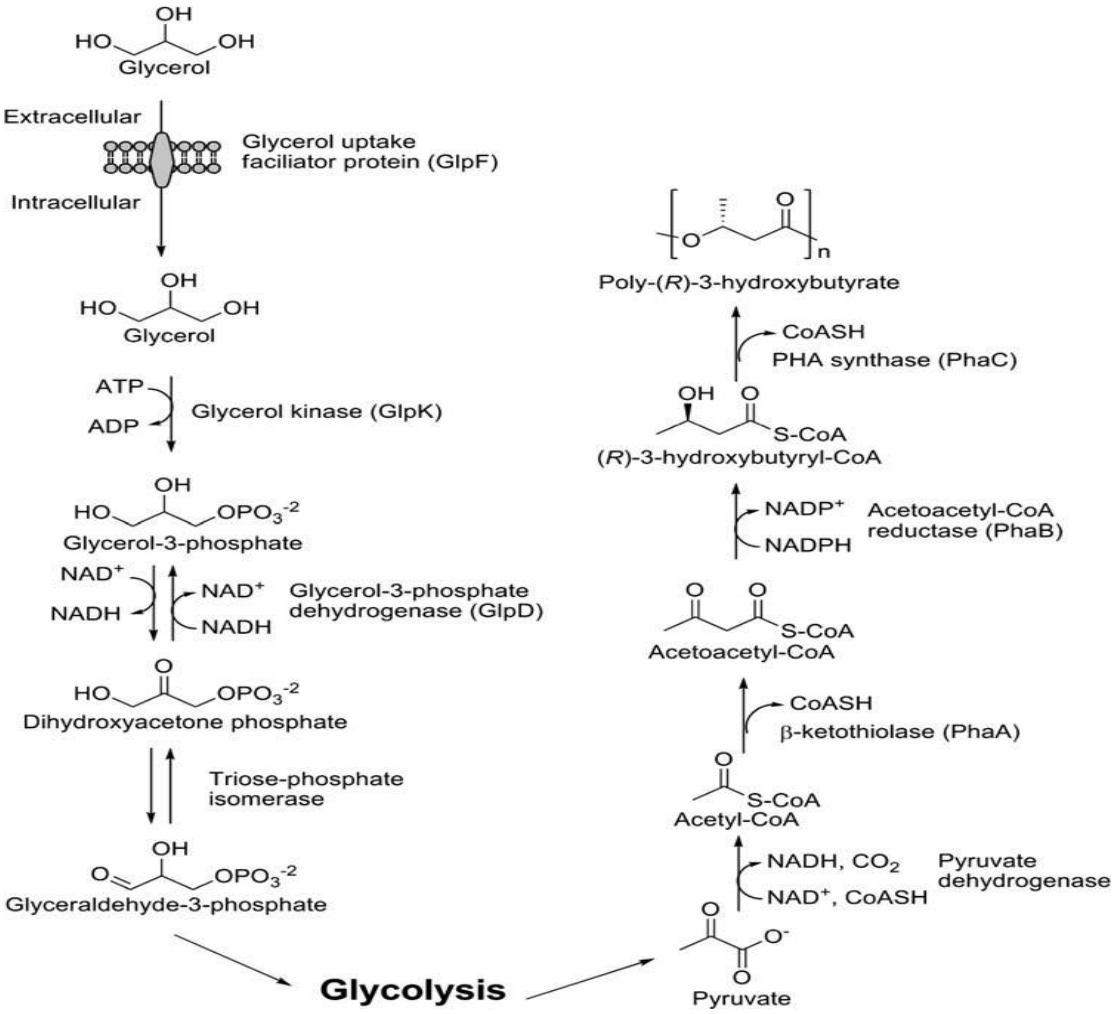


Figure 15 Biosynthesis of PHAs from Glycerol (Zhu *et al*., 2013)

1.3 Isolation of PHAs-accumulating bacteria and production of PHAs using BLW and palm oil as sole carbon source

1.3.1 Screening of PHAs-accumulating bacteria by the enrichment culture technique

PHAs accumulating bacteria were obtained by the enrichment culture technique. Soil sample from Sirindhorn peat swamp forest was added to MSM containing 2% BLW or palm oil as the sole carbon source. Isolated colonies were primarily screened for the presence of granule inside the cell. The selected bacteria were further confirmed by checking for the accumulation of PHAs using GC analysis.

Approximately 95 isolates were obtained from the soil samples and only 12 isolates showed black spots inside the cells indicating the presence of PHAs granules. Strain SB01 and SB02 which showed big black spots inside the cells were chosen for further work (Figure 16B and 16D). SB01's colony was flat, smooth and white in color. SB02's colony was raised, smooth and yellow in color. Both strains were aerobic gram-negative rod-shaped bacteria (Figure 16A and 16C) and were able to use palm oils and BLW as carbon sources for growth.

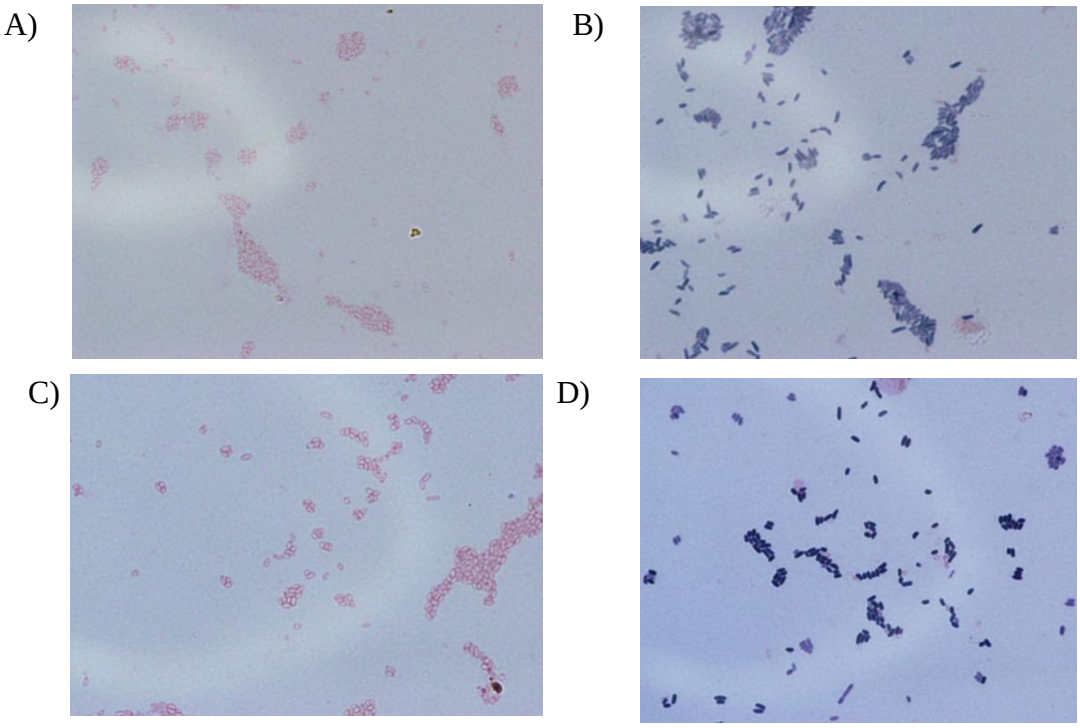


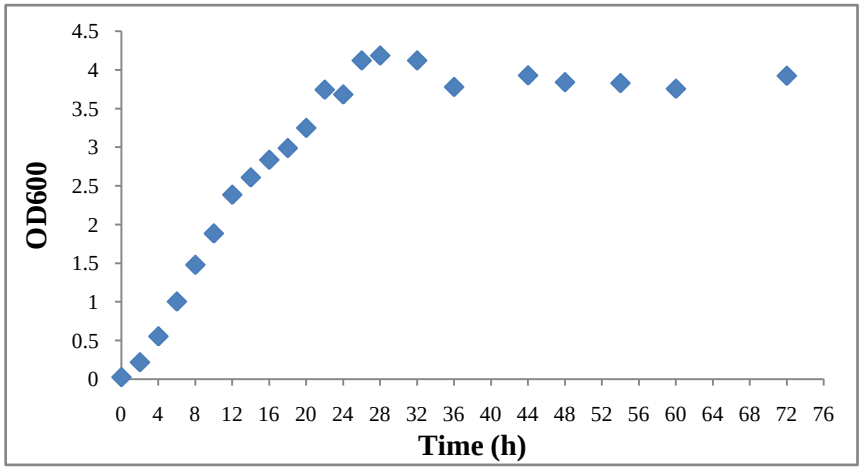
Figure 16 Micrographs of PHB producing bacteria: (A) Gram staining of SB01 (B) Sudan black B staining of SB01 (C) Gram staining of SB02 (D) Sudan black B staining of SB02 when cultured in MSM with 2% BLW for 60 h

1.3.2 Growth of newly isolated SB01 and SB02 in the pre-culture medium

The growth profiles of the SB01 and SB02 were studied in pre-culture medium (Figure 17). The exponential growth phase of both strains were 2-24 h of

cultivation. The mid log phase presenting the highest growth rate were calculated. The mid log phase of SB01 and SB02 were 12 h and 14 h of cultivation, respectively.

A)



B)

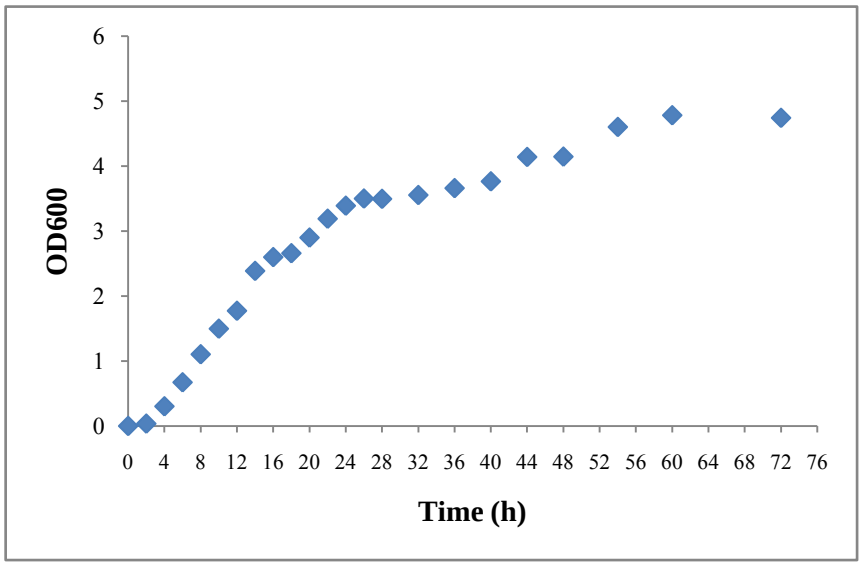
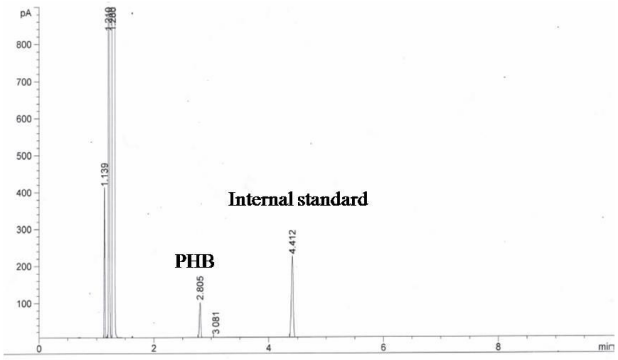


Figure 17 Growth profiles of A) strain SB01 and B) strain SB02 when cultivated in NR medium at 35 °C and 150 rpm for 76 h

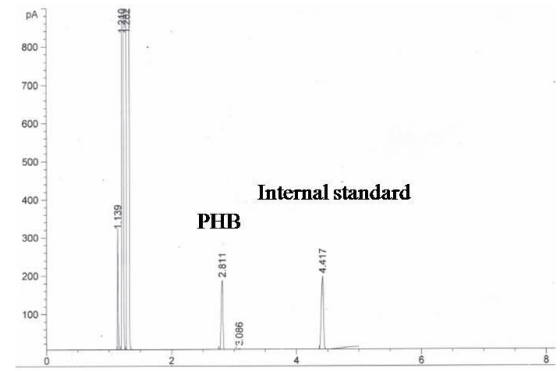
1.3.3 PHAs accumulation of SB01 and SB02 in MSM medium

The monomer composition and the PHAs produced from SB01 and SB02 when grown in MSM containing 2% BLW and 2% palm oil were identified using gas chromatography (GC) analysis and the obtained chromatogram was compared with the PHB standard. Only the homopolymer PHB was accumulated and detected by these 2 strains (Figure 18).

A)



B)



C)

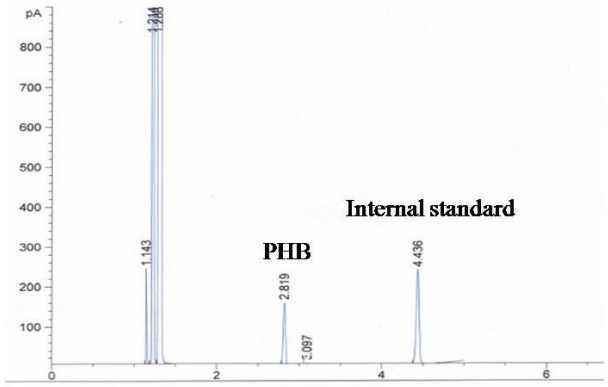


Figure 18 GC analysis of the PHAs produced by SB01 and SB02 (A) standard PHB (B) PHB produced by SB01 (C) PHB produced by SB02 (pA: peak area)

PHAs accumulation reached the maximum value during the exponential growth phase in MSM containing 2% BLW or 2% palm oil. SB01 accumulated 91.98 wt% PHB at 78 h when grown in MSM medium with 2 % (v/v) BLW. This strain accumulated 87.50 wt% PHB at 84 h when grown in MSM medium with 2 % palm oil. SB02 accumulated 94.41 wt% PHB at 60 h when grown in MSM medium with 2 % (v/v) BLW. This strain accumulated 96.60 wt% PHB at 84 h when grown in MSM medium with 2 % palm oil (Table 6). The PHB productivity from different carbon source was not much different. BLW which is a cheap carbon source was selected for futhur studied. Two strains produced higher PHB contents when compared with *Bacillus aryabhattai* (72.31 wt% PHB) (Chanasit *et al*., 2014) and *P. mendocina* PSU (77 wt% PHB) (Chanasit *et al*., 2016) using BLW as carbon source. SB01 started to produce PHB during the period of the exponential growth phase. This strain was similar to *Burkholderia* sp. USM that the accumulation of PHB increased during the exponential growth phase when cultured in MSM containing crude palm kernel oil (Chee *et al.*, 2010). These results clearly showed that BLW, a cheap renewable source and a biodiesel by-product, is a very attractive and an economically feasible carbon source for the production of PHB. In addition, molecular weight of PHB from 2 strains that produced at different times was measured. These two strains produced high molecular weight PHB. M_w and M_n of SB01 were 2.3 x 10^5 and 1.3 x 10^5, respectively when cultured in MSM with BLW at 60 h. M_w and M_n of SB01 were 2.8 x 10^5 and 2.1 x 10^5 , respectively when cultured in MSM with palm oil at 78 h. M_w and M_n of SB02 were 2.5 x 10^5 and 2.0 x 10^5 , respectively when cultured in MSM with BLW at 60 h. M_w and M_n of SB02 were 7.9 x 10^5 and 4.3 x 10^5 , respectively when cultured in MSM with palm oil at 54 h. Molecular weights of PHB produced from

palm oil were higher than those obtained from BLW. Two strains utilized glycerol in BLW as major carbon source. Glycerol can enter to the active site of the PHA synthase and terminates the extention of the PHA polymer resulting in production of low molecular weight PHAs. These 2 strains produced PHB with high molecular weight using simple and low-cost carbon sources leading to a possible cheaper production of a more flexible biopolymer.

TABLE 6 Biosynthesis of PHB by SB01 and SB02 grown in MSM medium with BLW or palm oil, incubated at 35 °C, 150 rpm for 96 h

Time (h)	SB01						SB02					
	%PHB/ DCW (BLW)	DCW (g/L)	Productivity (g/L/h)	%PHB/ DCW (Palm oil)	DCW (g/L)	Productivity (g/L/h)	%PHB/ DCW (BLW)	DCW (g/L)	Productivity (g/L/h)	%PHB/DCW (Palm oil)	DCW (g/L)	Productivity (g/L/h)
6	-	0.21 ± 0.02	-	16.76 ± 1.02	0.75 ± 0.01	0.02	21.58 ± 1.16	0.55 ± 0.03	0.02	-	0.30 ± 0.01	
12	-	0.33 ± 0.04	-	18.39 ± 3.77	1.02 ± 0.04	0.02	45.52 ± 4.43	0.98 ± 0.19	0.04	45.74 ± 2.11	0.77 ± 0.13	0.03
18	28.50 ± 2.97	1.11 ± 0.01	0.02	26.94 ± 1.63	1.35 ± 0.06	0.02	64.85 ± 2.79	1.12 ± 0.08	0.04	62.25 ± 9.97	1.23 ± 0.09	0.04
24	30.33 ± 3.23	1.48 ± 0.07	0.02	37.15 ± 0.38	1.52 ± 0.12	0.02	65.78 ± 4.19	1.46 ± 0.14	0.04	65.03 ± 4.12	1.55 ± 0.17	0.04
30	42.18 ± 1.30	1.63 ± 0.08	0.02	36.95 ± 2.78	1.61 ± 0.05	0.02	70.80 ± 3.70	1.60 ± 0.12	0.04	57.40 ± 3.56	1.74 ± 0.04	0.03
36	42.55 ± 5.77	1.78 ± 0.11	0.02	64.90 ± 6.06	1.63 ± 0.19	0.03	76.05 ± 1.59	1.84 ± 0.39	0.04	76.05 ± 6.04	1.92 ± 0.11	0.04
42	52.58 ± 3.37	1.95 ± 0.20	0.02	38.71 ± 3.33	2.03 ± 0.06	0.02	65.13 ± 2.53	1.81 ± 0.17	0.03	68.87 ± 2.44	2.01 ± 0.34	0.03
48	66.23 ± 6.06	2.31 ± 0.15	0.03	32.68 ± 0.36	2.23 ± 0.12	0.02	66.98 ± 3.57	2.56 ± 0.12	0.04	71.43 ± 4.76	2.67 ± 0.23	0.04

TABLE 6 Biosynthesis of PHB by SB01 and SB02 grown in MSM medium with BLW or palm oil, incubated at 35 °C, 150 rpm for 96 h (cont.)

Time (h)	SB01						SB02					
	%PHB/ DCW (BLW)	DCW (g/L)	Productivity (g/L/h)	%PHB/ DCW (Palm oil)	DCW (g/L)	Productivity (g/L/h)	%PHB/ DCW (BLW)	DCW (g/L)	Productivity (g/L/h)	%PHB/DCW (Palm oil)	DCW (g/L)	Productivity (g/L/h)
54	77.59 ± 4.77	2.61 ± 0.19	0.04	44.65 ± 1.42	2.75 ± 0.29	0.02	75.06 ± 6.70	2.22 ± 0.14	0.03	83.03 ± 6.55	3.11 ± 0.39	0.05
60	84.88 ± 2.62	3.45 ± 0.14	0.05	48.93 ± 2.95	2.99 ± 0.18	0.02	94.41 ± 2.49	2.44 ± 0.29	0.04	63.61 ± 5.79	3.54 ± 0.07	0.04
66	81.11 ± 0.43	3.97 ± 0.22	0.05	55.81 ± 3.14	3.68 ± 0.34	0.03	70.13 ± 2.03	2.77 ± 0.15	0.03	65.89 ± 2.45	4.03 ± 0.13	0.04
72	72.22 ± 1.68	4.43 ± 0.27	0.04	62.38 ± 1.92	4.76 ± 0.17	0.04	66.10 ± 4.91	4.12 ± 0.04	0.04	69.07 ± 4.95	4.25 ± 0.47	0.04
78	91.98 ± 1.01	4.89 ± 0.35	0.06	86.44 ± 2.55	5.02 ± 0.42	0.06	97.85 ± 5.71	4.02 ± 0.09	0.05	72.74 ± 7.24	4.78 ± 0.04	0.04
84	90.17 ± 1.16	5.21 ± 0.24	0.06	87.50 ± 1.27	5.42 ± 0.31	0.06	89.25 ± 3.37	4.65 ± 0.10	0.05	96.60 ±0.92	5.01 ± 0.29	0.06
90	86.80 ± 6.05	7.89 ± 0.19	0.08	82.11 ± 2.28	7.82 ± 0.25	0.07	75.13 ± 3.35	5.55 ± 0.59	0.05	84.76 ± 3.12	6.11 ± 0.13	0.06
96	90.40 ± 4.07	8.34 ± 0.44	0.08	83.44 ± 3.21	8.90 ± 0.38	0.08	80.01 ± 8.82	6.55 ± 0.30	0.05	81.19 ± 4.97	6.58 ± 0.35	0.06

1.3.4 Identification of the SB01 and SB02 strains

16s rRNA gene sequence analysis was performed to identify and classify the PHAs-producing bacteria, SB01 and SB02. Genomic DNA was extracted and amplified the full length of the 16S rRNA gene. The 1500 bp PCR product of the 16s rRNA gene was identified (Miller *et al.*, 2013). Nucleotide sequences were analyzed using NCBI database and phylogenetic tree analysis. SB01 and SB02 were identified as *Burkholderia seminalis* and *Burkholderia contaninans*, respectively (Figure 19). Other study showed that *Burkholderia contaminans* has a class I PHA synthase that produces polyhydroxybutyrate-*co*-hydroxydodecanoate copolymer when sucrose or glucose was used as a carbon source. This strain produced high molecular weight copolymer which has elastomeric properties (Matias *et al.*, 2017). Class I PHA synthases are composed of one subunit enzyme, PhaC. It is the key enzymes of PHA biosynthesis. It catalyze the polymerization of hydroxyalkanoic acids into PHA. Class I PHA synthases accept SCL-HA-CoAs for polymerization (Schubert *et al*., 1988). Class I PHA synthases was found in *Ralstonia eutropha*, *Alcaligenes latus*, *Burkholderia cepacia*, *Burkholderia pseudomallei* and *Rhodopseudomonas palustris* (Rehm and Steinbüchel, 2005). Other *Burkholderia* sp. that produced PHA such as *Burkholderia cepacia* ATCC 17759 produced 81.9 wt% PHB when glycerol was used as the carbon substrate (Zhu *et al.*, 2010). *Burkholderia sacchari* IPT101 produced 68 wt% PHB when sucrose was used as the carbon source and produced 65 wt% PHBV content when glucose and propionic acid were provided as carbon sources (Brämer *et al.*, 2001). However, *Burkholderia seminalis* and *Burkholderia contaninans* are members of the *Burkholderia cepacia* complex (BCC) that are human and animal pathogens (Vanlaere *et al.*, 2008; Vanlaere *et al.*, 2009). BCC organisms are opportunistic pathogenic bacteria that spread to patients with cystic fibrosis and cause serious epidemic outbreaks. The risks associated with agricultural uses of BCC strains remain unclear. The pathogenic mechanisms are needed to study before using *Burkholderia* genus in the large-scale (Jones *et al.*, 2004; Drevinek *et al.*, 2010; Sousa *et al.*, 2011). So, *Pseudomonas mendocina* PSU was therefore selected for further study.

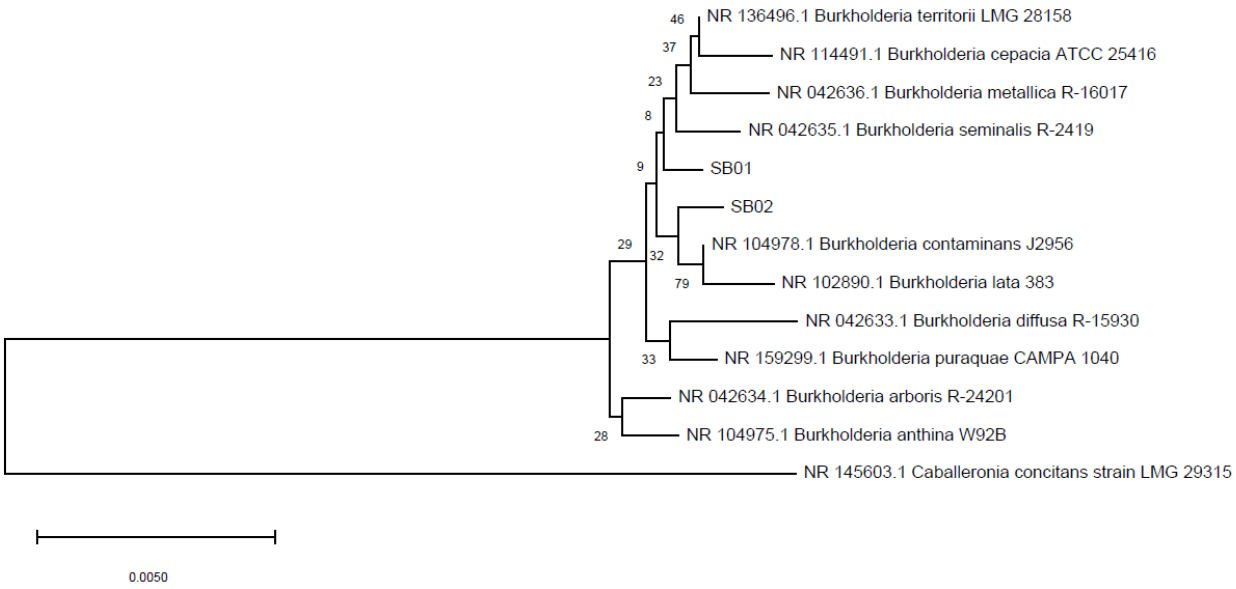


FIGURE 19 A phylogenetic tree of SB01 and SB02 related to other species of *Burkholderia* based on aligned sequences of the 16s rRNA gene. *Caballeronia concitans* LMG 29315 was used as an out-group

3. PHBV production by *P. mendocina* PSU

PHAs are thermoplastic and elastomeric materials. About 150 different monomers have been identified as PHAs constituents. Since mechanical and thermal properties of different monomer compositions, PHAs could be used for different applications (Silva-Queiroz *et al*., 2009). Since hydroxyvalerate (HV) monomer has a longer side chain than HB monomer, increasing amount of HV content of the PHA copolymers showed higher molecular weight, higher toughness and higher flexibility than PHB resulting in better morphology and properties (Aramvash *et al*., 2016; Jost *et al*., 2017). Impact resistance, toughness and biopolymer flexibility are increased when HV content is increased. Increasing of HV which has longer side chain reduces PHB crystallinity, the biopolymer is more like the conventional plastics. Decreasing of HV content causes stiffness and brittleness to the biopolymer like polystyrene (Antipov *et al*., 2006). Sodium propionate acts as a precursor for the incorporation of 3HV units into PHB backbone for PHBV production. BLW with 0.3% (w/v) sodium propionate was used for PHBV production. The monomer composition and PHAs content produced from *P. mendocina* PSU when grown in MSM containing 2% BLW and 0.3% (w/v) sodium propionate was identified using GC analysis and the obtained

chromatogram was compared with the PHBV standard. Both PHB and PHV were detected (Figure 20). The maximum PHBV accumulation was reached at stationary phase of culture. This strain accumulated 43.5 wt% PHBV with 8.6 mol% HV at 60 h (Table 7). Propionic acid is the most widely used precursor substrate for PHBV biosynthesis. This substrat is converted to propionyl-CoA. Moreover, propionic acid is catabolized to pyruvic acid and succinyl-CoA, which are intermediates for TCA cycle (Steinbüchel and Lutke-Eversloh, 2003). This study showed relatively high PHBV production when compared with other *Pseudomonas* sp. Haywood *et al*. (1989) found that *Pseudomonas* sp. 28D used methanol and valerate to produce 11 wt% PHBV . *P. oleovorans* used glucose and valerate to produce 2 wt% PHBV. *P. extorquens* used methanol and valerate to produce 5 wt% PHBV. *P. extorquens* used methanol and propionate to produce 26 wt% PHBV (Haywood *et al*., 1989). Kang *et al*. (2001) found that *Pseudomonas* sp. DSY-82 produced 42 wt% PHBV with 2 mol% HV when nonanoate and valerate were used as carbon sources. *Pseudomonas* sp. DSY-82 produced PHBV 29 % PHBV content with 3 mol% HV when 10-undecanoate and valerate were used as carbon sources.

A)

B)

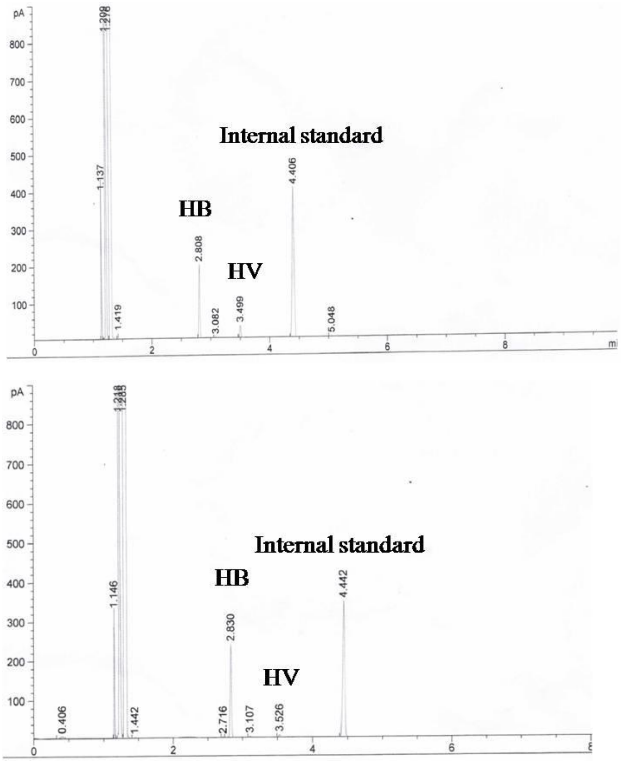


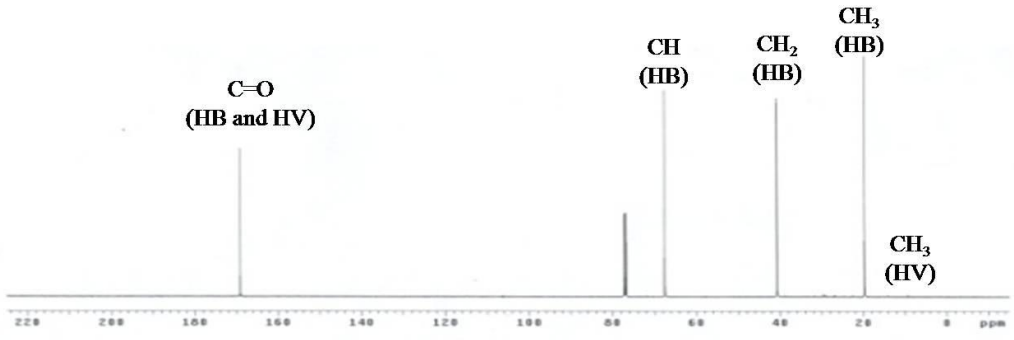
Figure 20 GC analysis of the PHA produced by *P. mendocina* PSU (A) standard PHBV (B) PHBV produced by *P. mendocina* PSU (pA: peak area)

Table 7 Biosynthesis of PHBV of *P. mendocina* PSU grown in MSM with 2% BLW and 0.3% sodium propionate

Time (h)	MSM with 2% biodiesel liquid waste and 0.3% sodium propionate		DCW (g/L)
	% PHBV/DCW	3HV mol %	
0	nd*	nd	Nd
12	nd	nd	Nd
24	2.45 ± 0.03	nd	1.47 ± 0.12
36	2.91 ± 0.11	nd	1.48 ± 0.09
48	11.16 ± 0.35	7.52 ± 0.14	2.13 ± 0.14
60	43.58 ± 0.44	8.62 ± 0.59	2.17 ± 0.21
72	27.69 ± 0.07	8.36 ± 0.71	2.16 ± 0.11

*Not detected; MSM, Mineral Salts Medium; PHB, poly(3-hydroxybutyrate)

A)



B)

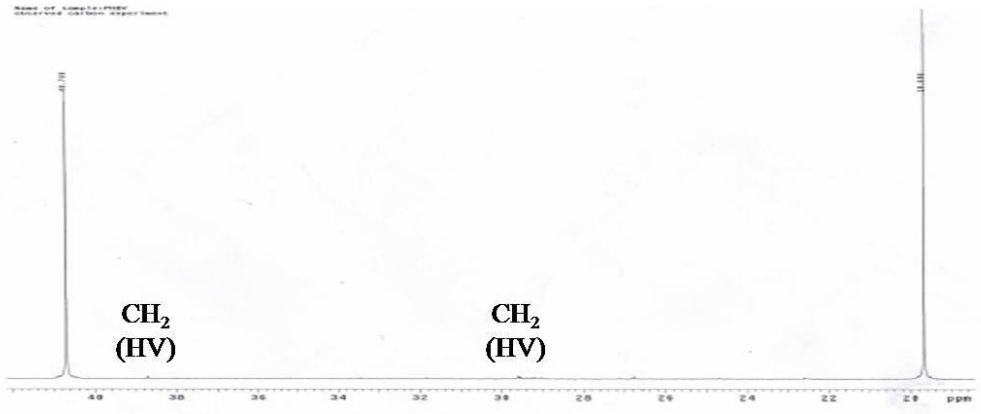


Figure 21 ^{13}C NMR spectra in $CDCl_3$ of PHBV extracted by chloroform extraction

Figure 21 shows the ^{13}C NMR spectra of PHA confirming PHBV production. These spectra demonstrated that the sample contains copolymer with two monomeric units of HB and HV, P(HB-*co*-HV). The chemical shifts were assigned for each carbon resonance signals according to data reported for the PHBV obtained from mixed microbial cultures grown in MSM with propionate. C_1 (169.07 ppm), C_2 (67.52 ppm), C_3 (40.69 ppm), C_4 (19.68 ppm), C_5 (9 ppm), C_6 (38 ppm) and C_7 (27 ppm)

chemical shifts were the same chemical shift signals for PHBV monomers that showed in Table 8.

Table 8 Chemical shift signals (in ppm) from ^{13}C NMR spectra of PHBV copolymer (Kemavongse *et al*., 2008).

Position	**Carbon atom**	**Chemical shift signals (in ppm)**
C_1	C=O (HB) C=O (HB)	169
C_2	CH (HB)	72
C_3	CH_2 (HB)	40
C_4	CH_3 (HB)	19.66
C_5	CH_3 (HV)	9
C_6	CH_2 (HV)	38
C_7	CH_2 (HV)	27

3.1 Effect of sodium propionate concentrations on PHBV production by *Pseudomonas* sp. PSU

The effect of sodium propionate concentrations with 2% BLW on PHBV production was determined. Sodium propionate at 4 different concentrations (0.1, 0.3, 0.5, and 0.7%) was investigated. Figure 22 showed that at 96 h, cell biomass (3.88 g/L) and PHBV content (48.1%) were the highest when 0.1% propionate was added (Table 9). However, HV mol% in this condition was only 4%. 0.3% propionate was considered as the suitable concentration because cell biomass was 3.04 g/L, PHBV

content was 25.6 % and HV mol% was 10 at 72 h. This result might be due to the toxic of sodium propionate that decreased microbial cells growth but supported HV monomer production (Annuar *et al*., 2008). This result showed that *P. mendocina* PSU can use sodium propionate as a precursor to produce PHBV. However, PHBV production was still low therefore the effect of nitrogen sources was also investigated to improve the production.

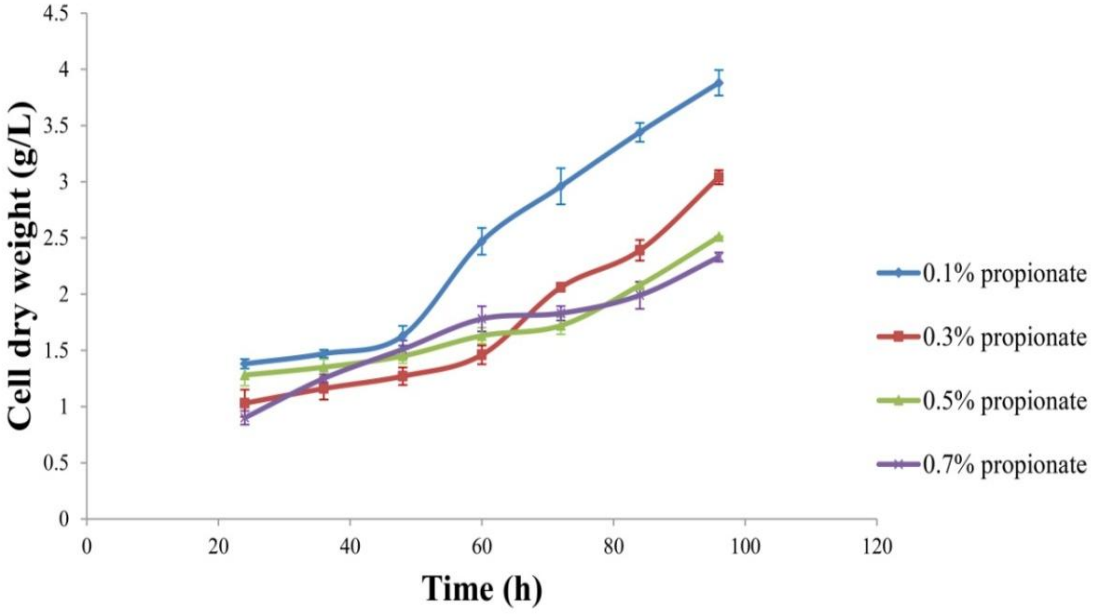


Figure 22 Growth of *P. mendocina* PSU when cultivated in MSM with 2% biodiesel liquid waste and various concentrations of sodium propionate, incubated at 35 ºC, 150 rpm for 96 h

Table 9 Biosynthesis of PHBV by *P. mendocina* PSU grown in MSM medium with 2% BLW and various sodium propionate concentrations, incubated at 35 ºC, 150 rpm for 96 h

Time (h)	0.1% sodium propionate		0.3% sodium propionate		0.5% sodium propionate		0.7% sodium propionate	
	%PHBV/DCW	3HV mol%	%PHBV/DCW	3HV mol%	%PHBV/DCW	3HV mol%	%PHBV/DCW	3HV mol%
0	*nd	nd	Nd	nd	nd	nd	nd	nd
12	Nd	nd	Nd	nd	nd	nd	nd	nd
24	11.56±1.14	0	2.88 ± 0.12	0	1.76 ± 0.09	0	1.63± 0.12	0
36	18.61±0.98	0	5.12 ± 0.07	0	1.12 ± 0.07	0	1.22 ± 0.22	0
48	27.24±1.17	6.16 ± 0.12	9.06 ± 0.44	11.74 ± 0.31	4.28 ± 0.35	0	1.54 ± 0.87	0
60	34.04±0.83	5.86 ± 0.11	17.06 ± 0.04	9.69 ± 0.23	7.65 ± 0.42	12.64 ± 1.71	6.24 ± 1.81	0
72	35.61±0.52	5.88 ± 1.16	25.63 ± 0.57	10.09 ± 0.12	14.66 ± 0.77	11.93 ± 0.29	9.18 ± 1.18	0
84	35.11±1.95	5.14 ± 0.27	28.58 ± 0.09	9.71 ± 0.23	18.73 ± 0.42	12.55 ± 0.56	22.19 ± 1.38	11.24 ± 1.24
96	48.14±1.02	4.64 ± 0.06	36.23 ± 0.06	8.95 ± 0.19	24.43 ± 0.01	12.96 ± 0.09	22.51 ± 1.71	11.03±1.10

*nd = Not detected

Table 10 Biosynthesis of PHBV by *P. mendocina* PSU grown in MSM media supplemented with 2% BLW and 0.3 % sodium propionate with different nitrogen sources, incubated at 35 ºC for 96 h

Time (h)	Ammonium sulphate 0.5 g/L (C/N = 76.19)		Ammonium sulphate 1.0 g/L (C/N = 38.09)		Ammonium sulphate 1.5 g/L (C/N = 25.40)		Urea 0.23 g/L (C/N = 80)		Urea 0.45 g/L (C/N = 40)		Urea 0.68 g/L (C/N = 26.67)	
	%PHBV/DCW	3HV mol%	%PHBV/DCW	3HV mol%	%PHBV/DCW	3HV mol%	%PHBV/DCW	3HV mol%	%PHBV/DCW	3HV mol%	%PHBV/DCW	3HV mol%
0	*nd	Nd	nd	nd	Nd	nd	nd	Nd	nd	nd	nd	nd
12	nd	Nd	nd	nd	Nd	nd	nd	Nd	nd	nd	nd	nd
24	7.12±0.54	0	7.59±0.66	0	7.20±1.18	0	11.20±0.63	0	11.78±0.07	0	13.22±0.76	0
36	12.80±0.45	9.68±0.56	13.80±0.95	6.96±0.34	8.78±0.99	0	16.17±0.63	5.43±1.84	15.78±1.49	0	16.28±0.78	2.06±0.01
48	19.25±0.53	9.87±0.62	28.08±1.12	6.59±0.45	22.42±0.96	5.66±1.22	26.54±1.10	5.60±1.02	20.33±1.01	0	26.48±1.05	4.13±0.12
60	34.51±1.77	11.80± 0.85	33.32±1.88	6.86±0.52	35.95±1.54	7.14±0.27	33.43±2.14	8.40±1.10	27.48±1.33	2.49±1.70	30.62±2.01	3.61±0.56
72	38.55±1.41	12.58±0.37	41.97±1.56	7.30±0.11	36.91±2.62	7.18±0.19	33.55±0.98	8.91±0.02	36.61±2.29	4.71±0.27	48.72±1.88	3.40±0.32
84	47.28±1.81	9.79±0.78	34.72±1.02	7.19±0.42	33.34±0.69	6.57±0.10	36.69±0.75	6.38±2.93	37.76±0.69	6.28±1.67	52.18±2.11	3.38±0.17
96	38.53±0.93	8.21±0.45	28.57±0.88	6.89±0.62	32.77±1.54	6.32±0.08	36.61±1.65	5.14±0.03	54.55±2.13	6.50±0.10	59.55±0.98	3.21±0.95

*nd = Not detected

2.2 Effect of nitrogen content on PHBV production by *P. mendocina* PSU

One of the main factors that affects the production of PHAs is nitrogen source. Nitrogen limitation boosted PHB accumulation in bacteria (Bluhm *et al*., 1986; Gunel *et al*., 2013; Kosseva *et al*., 2018). The effect of urea and ammonium sulfate concentration in MSM medium were investigated. Table 10 shows the effect of nitrogen contents on PHBV production. The highest PHBV production was obtained when *P. mendocina* PSU was cultured in MSM medium with 0.5 g/L ammonium sulphate. At 72 h, this strain produced 38.6 wt% PHBV with 12.6 mol% HV. PHBV content and mol% HV increased when nitrogen content was optimized. Increasing nitrogen concentration would benefit the growth but inhibit PHB accumulation therefore the lowest concentration of ammonium sulfate used in this study (0.5 g/L) was the optimum concentration for PHBV production. High PHBV production when using ammonium sulfate as nitrogen source may be due to the uptake of ammonium at a high rate in the presence of sulfate anion (Belal *et al*., 2013; Nunes *et al*., 2017). Moreover, the presence of inorganic chemicals such as ammonia supports the growth phase of bacteria (Gomaa, 2014). Another important factor that effects the biosynthesis of PHAs is the ratio of carbon to nitrogen (C/N) because the accumulation of acetyl-CoA is determined by the amount of nitrogen present in the medium (Du *et al*., 2001). A C/N ratio 20–40 has been widely used for the biosynthesis of PHAs. *P. mendocina* strain 0806 was grownin media containing 20 g/L glucose and various concentrations of ammonium sulfate. The highest biomass and PHA content were synthesized when C/N ratio was 40 after 48 h of cultivation (Tian *et al*., 2000; Amirul *et al*., 2008; Lee *et al*., 2008). In this study, C/N ratio affected PHBV production in term of increasing mol% HV when C/N ratio was increased to 76. The optimum condition that used to produce PHBV is 0.3% sodium propionate with 0.5 g/L ammonium sulphate. *P. mendocina* PSU produced 38.6 wt%PHBV with 12.6 mol% HV.

3. PHA recovery

3.1 Chemical recovery of PHAs by chloroform extraction

Normally, PHA extraction processes are performed by using organic solvents such as chloroform. Ten grams of biomass from *P. mendocina* PSU that cultured in MSM containing 2% BLW producing PHB and MSM containing 2% BLW and 0.3% sodium propionate producing PHBV were extracted with chloroform. Cell debris were removed and PHAs solution was concentrated by rotary evaporation to a final volume of approximately 100 mL. PHAs were purified by precipitation with a cold methanol solution. PHA which extracted from chloroform extraction has white color (Figure 23). The purity of PHB from *P. mendocina* PSU was 90.89% with % yield of 66.46. M_w was 8.39 x 10^4 and M_n was 4.60 x 10^4. The purity of PHBV from *P. mendocina* PSU was 90.5% with % yield of 55.83. M_w was 1.07 x 10^5 and M_n was 6.68 x 10^4. The roles of solvents in extracting PHAs granules from the biomass are to change the permeability of cell membrane and to dissolve the polymer inside the cells. Chloroform is the best organic solvents for PHA extraction because this method provides the highest solubility of PHAs. However, chloroform is quite harmful to handle, and has serious impacts on human health and highly toxic to the environment (Yabueng and Napathorn, 2018). Moreover, the high amount of solvent used in this method leads to high cost of PHAs production. The recovery and purification of PHAs are known to contribute significantly to the costs of the polymers. Various studies have been performed to improve the process such as surfactant-based extraction (Yazdi, 2010) and low frequency sonic waves extraction (Murugesan and Iyyaswami, 2017). Murugan *et al.* (2016) used the biological extraction method by using mealworm to extract PHAs from *Cupriavidus necator* and found that purity and

properties of PHAs were similar to chloroform extraction. Biological extraction method was therefore used in this study to reduce the cost and save the environment.

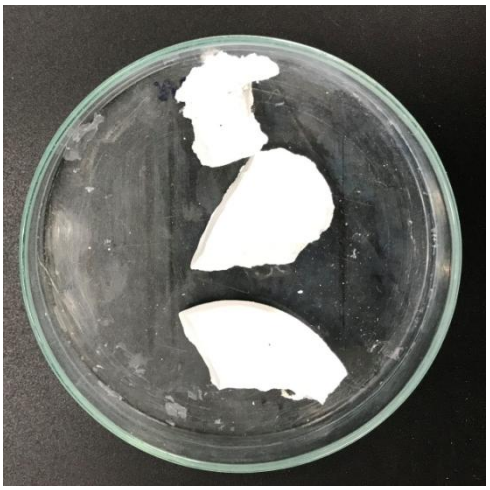


Figure 23 PHB recovery from *P. mendocina* PSU cells using chloroform extraction

3.2 Biological recovery of PHAs by mealworms

P. mendocina PSU cells cultivated in MSM medium containing 2% BLW were dried at 60 ºC. Ten grams of dried *P. mendocina* PSU cells were consumed by 200 g of mealworms in 5 days. Mealworms consumed about 1 wt% cells pellet of their body weight every day and excreted brown fecal pellets that contained PHA granules. After that, fecal pellets were washed with 1% SDS solution. The purity of PHB from biological extraction was 88.2% with %yield of 43.20. M_w was 7.30 x 10^4 and M_n was 1.46 x 10^5. The purity of PHBV from biological extraction was 89.4% with %yield 37.0. M_w was 1.60 x 10^5 and M_n was 1.33 x 10^5. However, the color of PHAs granules from biological method in this study was light brown with odor because small amounts of protein were still present (Figure 24). Major proteins that contained in PHAs sample were acetyl-CoA acetyltransferase and phasin from bacterial cells and alpha amylase and larvalcuticle protein from mealworm (Murugan *et al*., 2016). Proteins content in PHAs that extracted by chloroform exteaction was

lower than biological extraction because this material not obtained protein from mealworm. In addition, PHAs were futher purified by precipitate with cold methanol. (Yabueng and Napathorn, 2018). The purity of those two extraction method was not much different. Percent yield of biologically extracted PHAs was lower than that of chloroform extraction. This might be due to more losses of fecal pellets during the PHAs pellets recovery process. The M_w and M_n of PHB from biological extraction were much higher than those from chloroform extraction (Table 11). However, polydispersity index of the biologically extracted PHBV and PHB was less than those of chloroform extraction. In addition, the melting temperature (T_m) of PHAs from the biological extraction and chloroform extraction were similar. However, this biological method uses much less chemicals and solvents. Recently, biological extraction has been used as the alternative efficient method and the M_w and M_n of extracted PHAs were higher. Mealworm has been recently reported for their ability to degrade polystyrene (PS) and two PS-degrading bacterial strains were isolated from the mealworm gut (Jung *et al*., 2014). The remarkable findings of the ability of mealworm in extracting PHAs granules has led to this study. It is interesting to note that biological recovery can extract many kinds of PHAs from many kinds of microorganisms and does not affect the properties of the polymers (Murugan *et al*., 2016; Ong *et al*., 2018). This study confirmed the ability of using mealworm in PHAs extraction from *Pseudomonas* sp. and supported the results of Murugan (Murugan *et al*., 2016) that the mealworm does not cause any molecular degradation to PHAs polymers. PHAs purity was also high (89%) and thermal property (T_m) did not change when compared with chemical-extracted PHAs. Moreover, this method does not require high amount of organic solvents and heat.

Figure 24 Biological recovery process of PHAs from *P. mendocina* PSU cells using mealworms. (A) Mealworms feeding on dried *P. mendocina* PSU cells (B) Mealworms and fecal pellets after almost complete feeding on cells (C) Fecal pellets before washing and further purification (D) Fecal pellets containing PHB after being purified by 1% SDS

Table 11 Properties of PHAs from *P. mendocina* PSU that extracted by biological recovery and chloroform extraction methods.

Sample	M_n	M_w	Polydispersity	% yield	%purity	T_m (°C)
PHB (chloroform extraction)	4.60×10^4	8.39×10^4	1.83 ± 0.01	66.46 ± 2.11	90.89 ± 0.91	172.00
PHB (Biological extraction)	7.30×10^4	1.46×10^5	1.72 ± 0.09	55.83 ± 1.53	88.23 ± 0.47	173.52
PHBV (chloroform extraction)	6.68×10^4	1.07×10^5	1.61 ± 0.08	43.20 ± 1.15	90.53 ± 0.50	170.64
PHBV (Biological extraction)	1.33×10^5	1.60×10^5	1.20 ± 0.03	37.00 ± 1.81	89.43 ± 0.88	170.35

The biological extraction method was also performed with *Burkholderia* sp. SB01 and *Burkholderia* sp. SB02 which can produce high molecular weight PHB. The molecular weight of PHB that extracted by biological extraction and chloroform extraction were also not much different. The highest M_w and M_n of *Burkholderia* sp. SB01 were 2.9 x 10^5 and 2.6 x 10^5 when cultured in MSM with BLW at 60 h and extracted by biological extraction. The highest M_w and M_n of *Burkholderia* sp. SB02 were 4.6 x 10^5 and 9.7 x 10^5 when cultured in MSM with palm oil at 54 h and extracted by biological extraction (Table 12). M_w and M_n of PHB that produce with different culture time were different. When culture time is increased, the bacteria are stressed under carbon starvation conditions leading to intracellular degradation of PHAs. PHA granules are internally hydrolyzed by intracellular PHA depolymerases that are produced by the bacteria themselves. The degraded products can be directly utilized as a carbon or energy source for cell regeneration and cell maintenance (Khanna and Srivastava, 2005). It is interesting that mealworm can extract many kinds of PHAs from many kinds of microorganisms and does not affect the properties of the polymers.

Table 12 Properties of PHAs obtained from biological recovery and chloroform extraction methods.

Strains	Time (h)	Carbon source	Extraction Method	M_n	M_w	D	Productivity (g/L/h)	wt% PHB	DCW (g/L)
Burkholderia sp. SB01	48	BLW	Chloroform extraction	2.2×10^5	3.1×10^5	1.39	0.03	66.23	2.31
		BLW	Biological extraction	2.7×10^5	4.6×10^5	1.74			
	60	BLW	Chloroform extraction	1.3×10^5	2.3×10^5	1.64	0.05	84.44	3.45
		BLW	Biological extraction	2.6×10^5	2.9×10^5	1.63			
	36	Palm oil	Chloroform extraction	2.3×10^5	3.1×10^5	1.38	0.03	64.90	1.63
		Palm oil	Biological extraction	2.6×10^5	2.1×10^5	2.40			
	78	Palm oil	Chloroform extraction	2.1×10^5	2.8×10^5	1.33	0.06	86.44	5.02
		Palm oil	Biological extraction	2.9×10^5	5.4×10^5	1.85			
Burkholderia sp. SB02	30	BLW	Chloroform extraction	2.6×10^5	3.6×10^5	1.39	0.04	70.8	1.60
		BLW	Biological extraction	2.1×10^5	3.2×10^5	1.52			
	60	BLW	Chloroform extraction	2.0×10^5	2.5×10^5	1.51	0.04	94.41	2.44
		BLW	Biological extraction	2.0×10^5	2.6×10^5	1.34			
	36	Palm oil	Chloroform extraction	6.0×10^5	1.5×10^6	2.47	0.04	76.05	1.92
		Palm oil	Biological extraction	4.6×10^5	9.3×10^5	2.03			
	54	Palm oil	Chloroform extraction	4.3×10^5	7.9×10^5	1.83	0.05	82.03	3.11
		Palm oil	Biological extraction	4.6×10^5	9.7×10^5	2.12			
P. mendocina PSU	72	BLW	Chloroform extraction	4.60×10^4	8.39×10^4	1.83	0.04	79.6	3.77
			Biological extraction	7.30×10^4	1.46×10^5	1.72			
	72	BLW+ sodium propionate	Chloroform extraction	6.68×10^4	1.07×10^5	1.61	0.02	38.6	3.04
			Biological extraction	1.33×10^5	1.60×10^5	1.20			

M_w = weight average molecular weight, M_n = number average molecular weight, D = polydispersity index

4. Preparation of PHB–rubber blends

Polymers blending is another possible method to prepare biodegradable materials with useful mechanical properties (Hazer and Steinbüchel, 2007). The compatibility of two polymers depended on viscosity, molecular weight, and molecular structure (Niaounakis, 2015). Natural rubbers are another type of polymers which are elastomeric in nature and biodegradable. Several species of bacteria and fungi can degrade rubber (Rose and Steinbüchel, 2005). The incorporation of rubber particles into a brittle thermoplastic matrix has been known to improve the toughness and impact properties of the polymer (Paruleka *et al.,* 2005). Different rubbers (NR, ENR25 and ENR50) were blended with PHB from *P. mendocina* PSU to reduce crystallinity of PHB (Bhatt *et al.,* 2008). PHB produced from *P. mendocina* PSU was improved by blending with natural rubber. However, it was almost impossible to produce PHB-natural rubber blend because viscosity, molecular weight and molecular structure of natural rubber were much different from PHB (Paruleka *et al.,* 2005). The molecular weight and viscosity of rubbers were reduced by grinding with two roll mill and ENR which have epoxy groups on the backbone chains were used in this study. The most common techniques to blend polymers are solution blending and melt blending (Bhatt *et al.,* 2008). Three different methods were used to blend PHB and natural rubber as followes:

4.1 Blending by solution blending

Solution blending is a rapid mixing process without large energy consumption and chemical reactions. For this method, it is necessary to find a simple solvent for the blend components and to remove organic solvent. Three kinds of rubbers including natural rubber (NR), epoxidized natural rubber 50 (ENR50) and

epoxidized natural rubber 25 (ENR25) were chosen to mix with PHB by solution blending. PHB was dissolved in chloroform. Then, each kind of rubbers was added and stirred. ENR50 could not dissolve in chloroform, thus it could not mix with PHB. PHB was immiscible with modified NR. PHB mixed well with modified ENR25. Therefore, PHB was blended with modified ENR25 rubber in the ratio of 70:30. After stirring PHB and ENR25 for 12 h, the polymers blend in chloroform solution was poured into a petri dish. Solvent was removed by vacuum oven. The polymers blend obtained from this method is shown in Figure 25. This polymers blend was brittle and surface of the polymer was rough. PHB was still partially miscible with modified ENR25. This result was different from Bhatt *et al*. (2008) that used solution blending and successfully produced polymer blend of mcL-PHA produced by *Comamonas testosteroni* and natural rubber. It might be due to PHA structure that produced from different strain and carbon source. Molecular weight and viscosity of mcl-PHA is higher than scl-PHAs in this studied. So, mcl-PHA was mix well with natural rubber (Bhatt *et al.,* 2008).

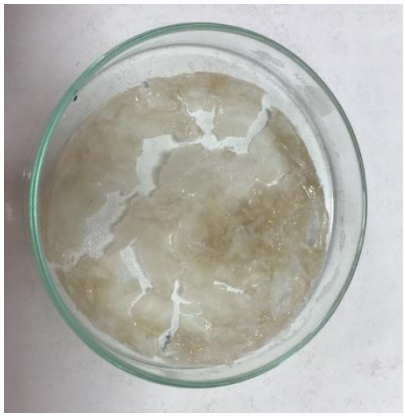


Figure 25 PHB was blended with modified ENR25 rubber (70:30) by solution Blending (chloroform)

4.2 Blending by melt mixing (internal mixture)

Melt mixing is the most comprehensive method of polymer blend. The blend components are mixed in the molten state in batch mixers or extruders. Advantages of the method are well-defined components and acceptance of mixing devices. Disadvantages of this method are high energy consumption and possible unfavorable chemical changes of blend components. Internal mixers are general purpose machines which is able to mix a wide range of rubber compounds without any change to the machine geometry needed. PHB was blended with modified ENR25 rubber in the ratio 70:30 by internal mixture at 170 $^{\circ}$C, 60 rpm for 10 min. The maximum temperature of this machine was 170 $^{\circ}$C but the melting temperature of PHB was 173 $^{\circ}$C. Eventhough, PHB could mix with ENR25, some part of PHB did not melt as shown in Figure 26.

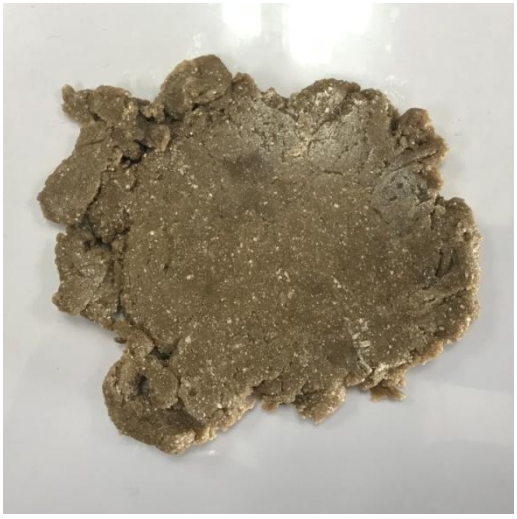


Figure 26 PHB was blended with modified ENR25 rubber (70:30) by internal mixture

4.3 Blending by melt mixing (twin screw extrusion)

Twin screw extrusion is one of the melt mixing equipments. It is used extensively for mixing, compounding, or reacting polymeric materials. The flexibility of twin screw extrusion equipment allows this operation to be designed specifically for the formulation being processed. For example, the two screws may rotate in the same direction (co-rotating) or opposite direction (counter-rotating) and may be fully intermeshing, non intermeshing or partially intermeshing. In addition, the configurations of the screws themselves may be varied using forward conveying elements, reverse conveying elements, kneading blocks, and other designs in order to achieve particular mixing characteristics (White, 1991). PHB was blended with modified ENR25 rubber in the ratio 70: 30 by twin screw extrusion at 175°C, 150 rpm. PHB and ENR25 did not mix well (Figure 27), and the two phases of polymers were observed after compressed to polymers film (Figure 28).

Figure 27 PHB was blended with modified ENR25 rubber (70:30) by twin screw extrusion

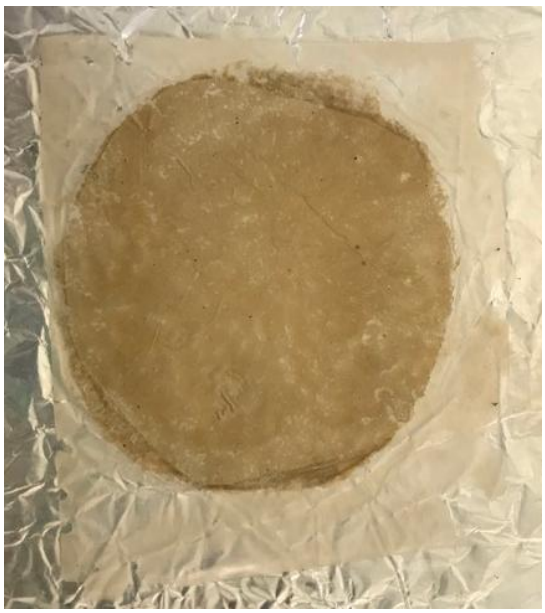


Figure 28 Polymer blended film of PHB and modified ENR25 by twin screw extrusion

The incorporation of rubber particles into a PHB matrix is the way to improve the impact properties and the toughness of PHB. Unfortunately, PHB and ENR25 did not mix homogeneously by blending method so the properties of PHB could not be improved. Blending mechanism highly depends on the mechanical properties of two polymers, the dispersion of the modifier, the elastomeric modifier and the interfacial adhesion among the different phases. Molecular weight, mechanical properties and polarity of PHB and ENR25 were different. So, it was hard to make homogeneous polymers blend. The blend system can be recognized as binary immiscible blend of an elastic polymer and a brittle polymer. The effective properties of polymers blend are precepted by the continuous phase and properties of the continuous phase. The the elastomeric and plastic phases are different in their rheological properties. Hence, for the elastomeric phase to be continuous, a compatibilizer needs to be added to increase interfacial adhesion. The compatibilizer reduces the interfacial tension that is

important for phase separation (Thompson and Matsen, 2000). So, these two polymers should be modified before blending or being compatibilized, which could improve the interfacial adhesion (Parulekar and Amar, 2006). In this study, solvent blending seems to be the suitable method for blending PHB and ENR25 since the two polymers mixed well. In addition, this method is rapid mixing process without large energy consumption and chemical reactions (Khan *et al*., 2018). However, the polymer ratio could be changed to improve polymer properties.

5. Biodegradation studies of PHAs and the blend films

5.1 Biodegradation studies of PHAs and the blend films by the soil burial test

PHB from *P. mendocina* PSU (Figure 29a), PHBV from *P. mendocina* PSU (Figure 29b), PHB from *B. contaminans* (Figure 29c), NR (Figure 29d), ENR25 (Figure 29e), rubber glove (Figure 29f), PHB-ENR25 blend films from solvent blending (Figure 29g) and PHB-ENR25 blend films from melt blending (Figure 29h) were cut into small pieces with the dimension of 1 cm x 1 cm. A plastic net was sewed to keep the films. Each square film was weighed and placed into the net. Samples were buried in the soil at the rubber plantation area (15 cm depth). The soil type at the buried location was clay loam, pH was 4.5-5 and the moisture content was 20-30% (This might be due to equipment errors). The biodegradation study was performed for 4 weeks during mid of May to mid of June 2019. Figure 29 shows physical changes on the PHAs and the blend films during the degradation period. The surface morphology of all films clearly showed the formation of edges and color changes. After 4 weeks, the degradation of all samples were measured based on the average weight loss of the films (Table 13). The weight loss of the PHB from *P. mendocina* PSU, PHBV from *P. mendocina* PSU and rubber glove were 34.1%,

29.12% and 30.47%, respectively. PHAs films and rubber glove also serve as a carbon source for soil microbes which results in a rapid degradation of the films (Reddy *et al*., 2003). Weight loss of PHB-ENR25 blend films from solvent blending and PHB-ENR25 blend films from melt blending were 5.20% and 3.93%. Blend film had lower degradation rate than PHAs film. The result was similar to PHAs-NR blend from other studies that used *Pseudomonas* sp. 202 to degrade the blend film (Bhatt *et al*., 2006). In comparison, the degradation rate of thicker film such as NR and ENR25 was low. It was harder for the microbes to attack the thicker films resulting in low rubber degradation rates. Rubber degradation rate was lower than PHAs so % weight loss of PHB-ENR25 blend films was lower than PHB (Rose and Steinbüchel, 2005). However, by mixing PHAs into rubber, the degradation of PHAs might enhance the degradation rate of rubber in PHB-ENR25 blend film (Bhatt *et al*., 2006). Weight loss of PHB from *B. contaminans* SB02 which has high molecular weight was 9.28%. Different biodegradation rates of PHAs were due to the various depolymerase enzymes secreted by the microbial population in environment (Ohura *et al*., 1999). Other environmental factors that could affect the degradation rate of PHAs films are type of soil, pH, moisture, temperature, nutrient supply and microbial activity. Moreover, the properties of PHAs such as composition, crystallinity, the presence of additives and surface area could also affect the degradation of PHAs (Jendrossek *et al*., 2002).

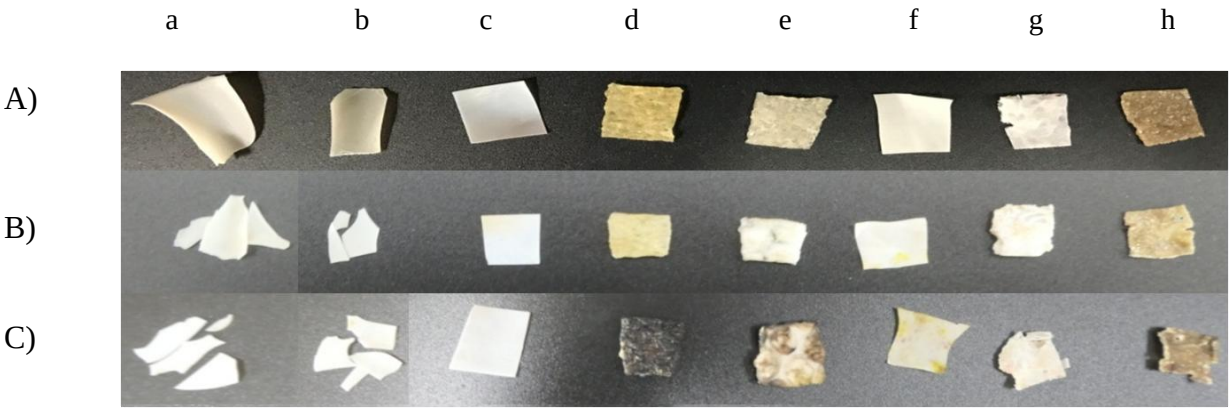


Figure 29 Physical changes on the PHB from *P. mendocina* PSU (a), PHBV from *P. mendocina* PSU (b), PHB from *B. contaminans* SB02 (c), NR (d), ENR25 (e), rubber glove (f), PHB-ENR25 blend films from solvent blending (g) and PHB-ENR25 blend films from melt blending (h) after buried in the soil for 0 week (A), 2 weeks (B) and 4 weeks (C)

Table 13 Weight loss of PHAs and the blend films from the soil burial test

Sample	Time (weeks)	Weight loss (%)
PHB from *P. mendocina* PSU	2	11.24 ± 0.08
	4	34.10 ± 4.37
PHBV from *P. mendocina* PSU	2	13.20 ± 3.45
	4	29.12 ± 2.61
PHB from *B. contaminans* SB02	2	6.45 ± 0.03
	4	9.28 ± 0.10
NR	2	2.45 ± 0.23
	4	2.78 ± 0.08
ENR25	2	0.77 ± 0.18
	4	4.68 ± 0.08
Rubber glove	2	16.37 ± 2.04
	4	30.47 ± 3.20
PHB-ENR25 blend films from solvent blending	2	2.71 ± 0.28
	4	5.20 ± 0.39
PHB-ENR25 blend films from melt blending	2	2.31± 0.06
	4	3.93 ± 0.83

5.2 Biodegradation studies of PHAs and the blend films by the rubber degrading bacterial consortium

PHB from *P. mendocina* (Figure 30a), PHBV from *P. mendocina* (Figure 30b), PHB from *B. contaminans* SB02 (Figure 30c), NR (Figure 30d), ENR25 (Figure 30e), rubber glove (Figure 30f), PHB-ENR25 blend films from solvent blending (Figure 30g) and PHB-ENR25 blend films from melt blending (Figure 30h) were cut into small pieces with the dimension of 1 cm x 1 cm. Each square film was weighed and used as a carbon source for the rubber degrading bacteria consortium. This bacterial consortium was grown in medium containing tested PHA, rubber and blend film. They presented evidence of adhesive growth by colonization on a surface and merging into PHB from *B. contaminans* SB02, NR, ENR25, rubber glove, PHB-ENR25 blend films from solvent blending and PHB-ENR25 blend films from melt blending (Figure 30). Rubber degrading bacteria were enriched by using rubber glove as a carbon source. After 4 weeks, the weight loss of rubber glove was 75.92%. The degradation rate of thicker film such as NR and ENR25 was low. It was harder for the microbes to attack the thicker films resulting in low rubber degradation rates. The weight loss of ENR25 was 1.80%. The low degradation might be due to the structure of ENR25 or the release of toxic metabolites during the degradation. The weight loss of the PHB from *P. mendocina* PSU was 4.57%. The weight loss of PHB-ENR25 blend films from solvent blending and PHB-ENR25 blend films from melt blending were 9.24 and 12.96%, respectively. The weight losses of all films in abiotic control were lower than 1% (Data not shown). The weight loss of blend film was higher than PHB and ENR25. PHB-ENR25 blend proved their degradation by the rubber degrading bacteria. The higher growth of degrading bacteria was obtained in the

flasks containing PHB-ENR25 blend as can be observed by the heavy colonization on a surface of PHB-ENR25 blend. Microbial adhesion is a fascinating and imperative step in the process of microbial biodegradation of polymers. The hydrophobic interactions between materials and microorganisms are the extensive factors in the early stages of microbial adhesion (Klotz, 1990). The weight losses of the PHBV from *P. mendocina* PSU and PHB from *B. contaminans* SB02M were 10.27% and 5.91%, respectively (Table 14). Different biodegradation rates of PHA were due to the various depolymerase enzymes secreted by the microbial population (Ohura *et al*., 1999) and the properties of PHA such as crystallinity and composition (Jendrossek *et al*., 2002).

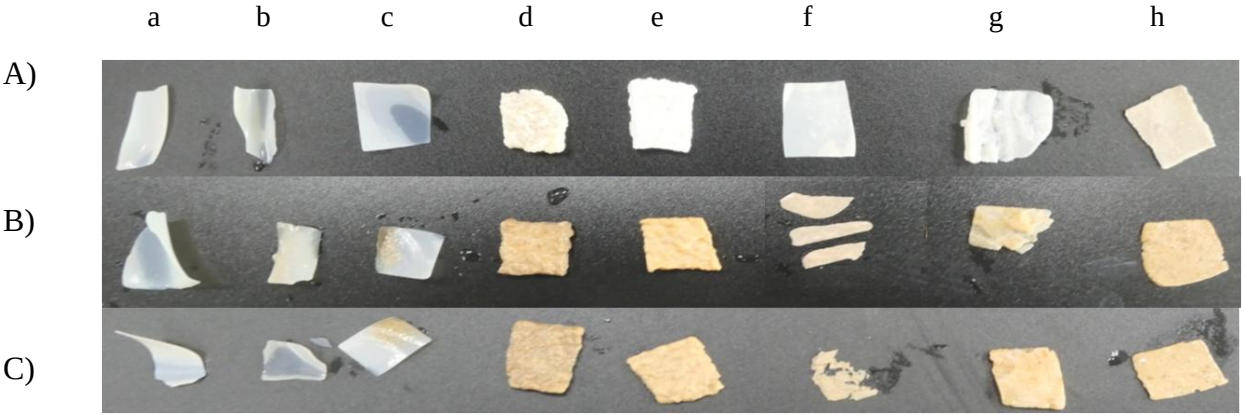


Figure 30 Physical changes of PHB from *P. mendocina* PSU (a), PHBV from *P. mendocina* PSU (b), PHB from *B. contaminans* SB02 (c), NR (d), ENR25 (e), rubber glove (f), PHB-ENR25 blend films from solvent blending (g) and PHB-ENR25 blend films from melt blending (h) after degraded by rubber degrading bacteria for abiotic control (A), 2 weeks (B) 4 weeks (C) and abiotic control

Table 14 Weight loss of PHAs and the blend films after degraded by the rubber degrading bacterial consortium

Sample	Time (weeks)	Weight loss (%)
PHB from *P. mendocina* PSU	2	2.80 ± 0.24
	4	4.57 ± 0.12
PHBV from *P. mendocina* PSU	2	6.27 ± 0.01
	4	10.27 ± 0.97
PHB from *B. contaminans* SB02	2	4.25 ± 0.72
	4	5.58 ± 0.05
NR	2	3.87 ± 0.06
	4	5.91 ± 0.95
ENR25	2	1.34 ± 0.01
	4	1.80 ± 0.05
Rubber glove	2	55.98 ± 5.18
	4	75.92 ± 3.06
PHB-ENR25 blend films from solvent blending	2	1.84 ± 0.32
	4	9.24 ± 0.81
PHB-ENR25 blend films from melt blending	2	7.12 ± 0.92
	4	12.96 ± 2.50

PHB and PHBV from *P. mendocina* PSU is a good biomaterial because it is a biodegradable polymer that can be produced from low cost carbon sources and extraction method. Molecular weight, toughness and flexibility of PHB were low. The properties of this material should be improved for using in a wide application range. On the other hand, PHBV is thermoplastic polyester. This material is thermostable and can be melted and molded to form various shapes. The mechanical properties of PHBV are better than PHB. In addition, PHBV can act as bioplastic material for conductive bioplastics that used in high-tech electronic devices (Chanprateep, 2010). Incorporation of rubber particles into a brittle thermoplastic matrix is known to improve the toughness and the impact properties of the polymer. However, phase separation between PHAs and rubber should be improved. A compatibilizer might be needed to change chemical or physical interactions between two polymers. The mechanical properties of polymer blend may be improved.

CHAPTER 4

CONCLUSIONS

Three PHAs-producing bacteria were successfully used BLW, by-product from biodiesel production, as carbon source for PHB production. *P. mendocina* PSU was previously isolated from biodiesel-contaminated sediment. *P.mendocina* PSU produced 79.6 wt% PHB with DCW 3.77 g/L at 72 h when grown in MSM medium with 2% (v/v) BLW. PHB produced from this strain had low molecular weight and was brittle. Two other bacteria SB01 and SB02 that used BLW to produce high molecular weight PHB were also isolated. SB01 accumulated 91.98 wt% PHB at 78 h and SB02 accumulated 94.41 wt% PHB at 60 h when grown in MSM medium with 2 % (v/v) BLW. The two strains produced higher PHB contents and higher molecular weight PHB when compared with *P. mendocina* PSU. All results clearly showed that BLW, a cheap renewable source and a biodiesel by-product, was a very attractive and an economically feasible carbon source for the production of PHB. SB01 and SB02 were identified as *Burkholderia seminalis* and *Burkholderia contaninans*, respectively. These two strains are members of the *Burkholderia cepacia* complex (BCC) that are human and animal pathogens. Since the pathogenic mechanisms of these environmental isolates are still unclear, therefore, *P. mendocina* PSU was selected for further study.

P. mendocina PSU produced PHBV when sodium propionate, a precursor for PHBV production, was added. The maximum PHBV accumulation was 38.6 wt%

PHBV with 13 mol% HV when *P. mendocina* PSU was cultured in MSM containing 2% BLW, 0.3% (w/v) sodium propionate and 0.5 g/L ammonium sulphate for 72 h. This study showed higher PHBV production when compared with other *Pseudomonas* spp. The properties of PHBV were better than PHB because of higher molecular weight and lower melting temperature. Recovery processes of PHAs including the biological method using mealworms and the chemical extraction method using chloroform were investigated. M_w and M_n of PHAs from the biological method were higher than those from the chloroform extraction. T_m and the purity of PHAs were comparable. This study confirmed the ability of mealworms to extract PHAs from of bacterial cells. This method does not require high amount of organic solvents and heat.

In the attempt to improve the biodegradable materials with better mechanical properties, blending PHB with other polymers is a promising option. Natural rubbers are polymers which are elastomeric and biodegradable. However, viscosity, molecular weight, and molecular structure of natural rubber were much different from PHB. The molecular weight and viscosity of rubbers were reduced by grinding with two roll mill and ENR which contains epoxy groups on the backbone chains was used in this study. PHB and ENR25 were blended by solution blending and melt blending. Unfortunately, PHB and ENR25 did not mix homogeneously so the properties could not be improved. Blending mechanism highly depends on the mechanical properties of two polymers, the dispersion of the modifier, the elastomeric modifier and the interfacial adhesion among the different phases. A compatibilizer needs to be added to increase interfacial adhesion.

Biodegradation of all polymers from this study were investigated by the soil burial test and treating them with the cultures of rubber degrading bacteria. PHB and PHBV from *Pseudomonas mendocina* PSU and rubber glove had higher degradation rate than other materials when buried in the soil. Treating with the rubber degrading bacteria culture, PHB-ENR25 blend films showed higher degradation rate than PHB and PHBV, NR and ENR25. This study has successfully established a low-cost process to synthesize biodegradable polymer. However, PHB and PHBV properties are needed to be studied further.

Suggestion for further study

1. Bacterial strains SB01 and SB02 have potential to produce high content and high molecular weight PHB by using simple and low-cost carbon sources. However, the pathogenicity of these two strains is needed to be examined before use.
2. PHAs extracted by the biological method in this study still contained small amounts of protein, the purity of PHAs should be improved by using other surfactant or heat to remove the protein.
3. PHB and ENR25 did not mix homogeneously by blendings. Blending mechanism highly depends on the mechanical properties of two polymers, the elastomeric modifier, the dispersion of the modifier, and the interfacial adhesion among the different phases. Since molecular weight, mechanical properties and polarity of PHB and ENR25 were different, this can be modified by adding a compatibilizer to increase interfacial adhesion of two polymers or modifying the chemical and physical structures of two polymers before blending. The ratio of two polymers might also be altered to determine the suitable mixture.

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APPENDIX

A. Media and Reagents

Media

1. Nutrient rich medium (NR) (Chee *et al*., 2010b)

NR consisted of 10 g/L peptone, 10 g/L meat extract and 2.0 g/L yeast extract.

2. Mineral Salt Medium (MSM) for PHA production (Kulpreecha *et al*., 2009)

MSM consisted of the following components in g/L: $(NH_4)_2SO_4$ 1.0, KH_2PO_4 2.0, Na_2HPO_4 0.6, $MgSO_4 \cdot 7H_2O$ 1.0, citric acid 0.75, 20 mL BLW, and trace element solution 1 mL (in g/L 20 $CaCl_2 \cdot 2H_2O$, 1.3 $ZnSO_4 \cdot 7H_2O$, 0.2 $FeSO_4 \cdot 7H_2O$, 0.6 $(NH_4)_2MoO_4$, and 0.6 boric acid. Media are sterilized in an autoclave at 121°C, 15 psi for 20 min.

3. Mineral Salt Medium (MSM) used in the biodegradation analysis by the rubber degrading bacteria (Jendrossek *et al*., 1997)

MSM consists of the following components in g/L: MSM, 9 g/l $Na_2HPO_4.12\ H_2O$, 1.5 g/L KH_2PO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4.7\ H_2O$, 0.02 g/L $CaCl_2.2\ H_2O$, 1.2 mg/L Fe(III) ammonium citrate. Carbon source e.g. rubber gloves piece (1x1 cm) PHB (1x1 cm), PHBV (1x1 cm), NR (1x1 cm), ENR25 (1x1 cm) or PHA-ENR25 blend (1x1 cm) is sterilized together with medium in an autoclave at 121°C, 15 psi for 20 min.

Reagent

1. Sudan black B

3% sudan black B was dissolve in ethanol and filter with filter paper.

2. Methanolysis solution containing benzoic acid as an internal standard

The methanolysis solution is prepared by adding 15 mL of concentrated sulphuric acid into 85 mL chilled methanol. The solution has to be chilled as the mixing is exothermic. The addition of sulphuric acid is done drop-wise slowly in a fume hood while the chilled methanol is continuously stirred gently using a magnetic stirrer. The solution is then kept in the chiller at 4˚C until used. The internal standard benzoic acid is prepared by dissolving 1 g of benzoic acid into 500 mL of the methanolysis solution in a volumetric flask (Sun *et al.*,2007).

B. Report of microbial identification by 16s rRNA sequence analysis

LOCUS NR_104978 1485 bprRNA linear BCT 12-MAR-2019

DEFINITION *Burkholderia contaminans* strain J2956 16S ribosomal RNA, partial sequence.

ACCESSION NR_104978

VERSION NR_104978.1

DBLINK Project: 33175

BioProject: PRJNA33175

KEYWORDS Ref Seq.

SOURCE *Burkholderia contaminans*

ORGANISM *Burkholderia contaminans*

Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia; Burkholderiacepacia complex.

REFERENCE 1 (bases 1 to 1485)

AUTHORS Yarza, P., Sproer, C., Swiderski, J., Mrotzek, N., Spring, S., Tindall, B.J., Gronow, S., Pukall, R., Klenk, H.P., Lang, E.,

Verbarg, S., Crouch, A., Lilburn, T., Beck, B., Unosson, C., Cardew, S., Moore, E.R., Gomila, M., Nakagawa, Y., Janssens, D., De Vos, P., Peiren, J., Suttels, T., Clermont, D., Bizet, C., Sakamoto, M., Iida, T.,Kudo, T., Kosako, Y., Oshida, Y., Ohkuma, M., Arahal, D., Spieck, E., Roeser, A., Figge, M., Park, D., Buchanan, P., Cifuentes, A., Munoz, R., Euzeby, J.P., Schleifer, K.H., Ludwig, W., Amann, R., Glockner, F.O. and Rossello-Mora, R.

TITLE Sequencing orphan species initiative (SOS): Filling the gaps in the 16S rRNA gene sequence database for all species with validly published names

JOURNAL Syst. Appl. Microbiol. 36 (1), 69-73 (2013)

PUBMED 23410935

REFERENCE 2 (bases 1 to 1485)

CONSRTM NCBI RefSeq Targeted Loci Project

TITLE Direct Submission

JOURNAL Submitted (25-NOV-2013) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

REFERENCE 3 (bases 1 to 1485)

AUTHORS Peiren, J. and Suttels, T.

TITLE Direct Submission

JOURNAL Submitted (19-OCT-2012) Microbiology and Biochemistry, BCCM/LMG Bacteria Collection, K.L. Ledeganckstraat 35, Ghent, East-Flanders 9000, Belgium

COMMENT REVIEWED REFSEQ: This record has been curated by NCBI staff. The reference sequence is identical to JX986975:1-1485.

FEATURES Location/Qualifiers

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/type_material="type strain of Burkholderiacontaminans"

/db_xref="taxon:488447"

rRNA<1..>1485

/product="16S ribosomal RNA"

ORIGIN

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121 gcccggcgaaagccggattaataccgcatacgatctacggatgaaagcgggggaccttcg

181 ggcctcgcgctatagggttggccgatggctgattagctagttggtggggtaaaggcctac

241 caaggcgacgatcagtagctggtctgagaggacgaccagccacactgggactgagacacg

301 gcccagactcctacgggaggcagcagtggggaattttggacaatgggcgaaagcctgatc

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1441 ttcatgactggggtgaagtcgtaacaaggtagccgtatcggaagg//

LOCUS NR_042635 1498 bprRNA linear BCT 12-MAR-2019

DEFINITION *Burkholderia seminalis* strain R-2419 16S ribosomal RNA, partial sequence.

ACCESSION NR_042635

VERSION NR_042635.1

DBLINK Project: 33175

BioProject: PRJNA33175

KEYWORDS Ref Seq.

SOURCE *Burkholderia seminalis*

ORGANISM *Burkholderia seminalis*

Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia; Burkholderiacepacia complex.

REFERENCE 1

AUTHORS Vanlaere, E., Lipuma, J.J., Baldwin, A., Henry, D., De Brandt, E., Mahenthiralingam, E., Speert, D., Dowson, C. and Vandamme, P.

TITLE *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. nov., novel species within the *Burkholderia cepacia* complex

JOURNAL Int. J. Syst. Evol. Microbiol. 58 (PT 7), 1580-1590 (2008)

PUBMED 18599699

REFERENCE 2 (bases 1 to 1498)

CONSRTM NCBI Ref Seq Targeted Loci Project

TITLE Direct Submission

JOURNAL Submitted (08-AUG-2011) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

REFERENCE 3 (bases 1 to 1498)

AUTHORS Vanlaere, E.

TITLE Direct Submission

JOURNAL Submitted (08-JUN-2007) Vanlaere E., Laboratory of Microbiology, Ghent University, K.L. Ledeganckstraat 35, Gent 9000, Belgium

COMMENT REVIEWED REFSEQ: This record has been curated by NCBI staff. The reference sequence is identical to AM747631.

FEATURES Location/Qualifiers

source 1..1498

/organism="Burkholderiaseminalis"

/mol_type="rRNA"

/strain="R-2419; DSM 23518"

/isolation_source="cystic fibrosis patient"

/culture_collection="DSM:23518"

/type_material="type strain of Burkholderiaseminalis"

/db_xref="taxon:488731"

/country="USA"

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/product="16S ribosomal RNA"

ORIGIN

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181 accttcgggcctcgcgctatagggttggccgatggctgattagctagttggtggggtaaa

241 ggcctaccaaggcgacgatcagtagctggtctgagaggacgaccagccacactgggactg

301 agacacggcccagactcctacgggaggcagcagtggggaattttggacaatgggcgaaag

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1441 ggtaggattcatgactggggtgaagtcgtaacaaggtagccgtatcggaaggtgcggc//

C1. Formula for PHA production

PHA concentration:

$$\frac{\text{PHA concentration in reaction tube x total volume of chloroform x DCW}}{\text{Total cell in reaction tube}}$$

$$\%\text{wt PHA} = \frac{\text{PHA concentration x 100}}{\text{DCW}}$$

C2. Formula for Biodegradation

$$\%\ \text{Weight loss} = \frac{\text{(Weight of initial film - Weight of film after degradation)}}{\text{Weight of initial film}} \times 100$$

VITAE

Name Miss Mananya Martla

Student ID 5610230013

Educationnal Attainment

Degree	Name of instution	Years of graduation
Bachelor of Science (Chemistry)	Mahidol University	2008
Master of Science (Biochemistry)	Chulalongkorn University	2011

Scholarship Awards during Enrolment

- PSU-Ph.D. Scholarship, Grant number: PSU2556-021
- Research Grant for Thesis

List of Publication and Proceeding

1. Martla, M., Sudesh, K. and Umsakul, K. 2018. Production and recovery of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from biodiesel liquid waste (BLW). Journal of Basic Microbiology. 58: 977-986.
2. Martla, M. and Umsakul, K. 2018. Isolation and characterization of a polyhydroxyalkanoates producing bacterium using biodiesel liquid waste as a carbon source. In: The 2nd International Conference on Environment, Livelihood, and Services; ICELS 2018 (19th – 22th November 2018, C asean 10 floor, CW Tower Bangkok, Thailand) (Proceeding)