UNIVERSITI PUTRA MALAYSIA

# *IMPROVED BIOSYNTHESIS AND RECOVERY OF POLYHYDROXYALKANOATES FROM COMAMONAS SP. EB172*

NOR ASMA BINTI AB. RAZAK

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# IMPROVED BIOSYNTHESIS AND RECOVERY OF POLYHYDROXYALKANOATES FROM *COMAMONAS* SP. EB172



**NOR ASMA BINTI AB. RAZAK**

**DOCTOR OF PHILOSOPHY**

**UNIVERSITI PUTRA MALAYSIA**

**2013**

IMPROVED BIOSYNTHESIS AND RECOVERY OF POLYHYDROXYALKANOATES FROM *COMAMONAS* SP. EB172

By

NOR ASMA BINTI AB. RAZAK

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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## DEDICATIONS

PRAISE TO ALLAH

and THANK YOU to;

*Supporting Mum, Norhana Hanim binti Abdullah*

*Understood Dad, Ab. Razak bin Ahmad*

*Beloved Husband, Azhari bin Samsu Baharuddin*

*Loving Sons and Daughter; Muhammad Akmal, Muhammad Azimi, Muhammad Akram, Muhammad Arif and Nor Arissa binti Azhari*

*Encouraged Sisters and Brothers;*

*Mohd Rostam, Nor Aida and Nor Aisah with family*

*Muhammad Reduan bin Ab. Razak*

*Muhammad Raafi bin Ab. Razak*

*and others who contributes officially or unofficially*

*to make this thesis become realm*

ALHAMDULLILAH and THANK YOU

ONLY ALLAH CAN REWARDS THE GOODNESS OF ALL

And Hope The Knowledge Give Benefits To All

...

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

**IMPROVED BIOSYNTHESIS AND RECOVERY OF POLYHYDROXYALKANOATES FROM** *Comamonas* SP. EB172

By

**NOR ASMA BINTI AB. RAZAK**

**September 2013**

**Chairperson: Prof. Mohd Ali Hassan, Ph.D.**

**Institute : Bioscience**

Polyhydroxyalkanoates (PHA) is a potential biodegradable polymer which can be used to replace a petrochemical synthetic polymer. PHA is synthesised when bacteria are exposed to a surplus of carbon and limited nitrogen. Under these conditions, cells are unable to grow but they do accumulate carbon-based polyesters. The objectives of this study were to improve PHA production by *Comamonas* sp. EB172 through fed-batch and repeated fed-batch, to characterise the effect of freeze-drying and oven-drying on cell and PHA and to improve recovery of PHA from *Comamonas* sp. EB172 using chemicals (solvent and sodium hydroxide) and biological (protease) methods. From this studies, *Comamonas* sp. EB172 which is novel locally isolated strain produced 6-9 g/L DCW, 77-86% PHA content, 5-12 mol% 3HV and 172-177 kDa *Mw* from fed-batch and 4-11 g/L DCW, 50-64% PHA content, 9-13 mol% 3HV and 832 kDa *Mw* from repeated fed-batch fermentation.Thus, through repeated fed-batch fermentation, high *Mw* could be obtained which gave new properties for different applications.
The oven-dried changed the chemical structure, developed crystal and changed the thermal properties of P(3HB-*co*-3HV) compared to freeze-dried P(3HB-*co*-3HV). Meanwhile, membrane cell of oven-dried become shrink, agglomerate to each other and become flakes compared to membrane cell of freeze-dried which become etched due to freezing and vacuum freezing. More than 90% purity of P(3HB-*co*-3HV) and P(3HB-*co*-3HHX) were obtained using acetone, chloroform, methanol and *n*-hexane as precipitate solvents. In addition, NaOH and protease from ginger, *Zingiber officinale* Roscoe were chosen as alternative recovery methods as they're environmental friendly, cheap and could be used to reduce chloroform effect to operator health and environment. Thus, the improvement on NaOH method were

done include different NaOH concentrations (0.1, 0.2, 0.3, 0.6, 0.8, 1 and 2 *N*), different residence time of cells (63, 87 and 111 h), initial drying cell conditions (wet broth, wet pellet, freeze-dried and oven-dried) and washing (distilled water, 20% and 100% ethanol). The protease which is highly specific to degrade cell membrane was successfully extracted and precipitated using acetone precipitation gave 256 U/mg with 60% recovery yield. The effect of cell concentration (5,10 and 20 g/L), incubation times and washing (distilled water, 20% and 100% ethanol) were done for P(3HB-*co*-3HV) recovery using protease. The best combination of freeze-drying cells for NaOH and protease are using 1*N* NaOH, incubation 1 hour with 100% cell concentration, centrifuged, washing two times with 20% ethanol and 0.02% cell concentration, incubation with 256 U/mg specific activity enzyme protease for 50 min, centrifuged and washing two times with 100% ethanol. Both methods gaves more than 90% P(3HB-*co*-3HV) purity and are comparable with recovery using chloroform and *n*-hexane with different fold *Mw* composition, thermal and physical properties. The properties of PHA are highly dependent upon their mode of fermentation and recovery techniques; hence, biodegradable polymer having a wide range of properties which can be used in different applications.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENINGKATAN PENGHASILAN DAN PEMULIHAN POLIHIDROKSIALKANOAT DARIPADA *COMAMONAS* SP. EB172**

Oleh

**NOR ASMA BINTI AB. RAZAK**

**September 2013**

**Pengerusi: Prof. Mohd Ali Hassan, Ph.D.**

**Institusi : Biosains**


Polihidroksialkanoat (PHA) adalah polimer boleh urai yang berpotensi sebagai pengganti polimer petro-kimia sintetik. PHA dihasilkan apabila bakteria terdedah kepada lebihan karbon dan kekurangan nitrogen. Dalam keadaan ini, sel tidak lagi membesar tetapi mengumpul poliester berasaskan karbon. Objektif kajian ini adalah untuk membaiki penghasilan PHA oleh *Comamonas* sp. EB172 melalui penggunaan suapan berkelompok dan pengulangan suapan berkelompok, menciri kesan beku kering dan ketuhar kering kepada sel dan PHA dan membaiki pemulihan PHA daripada *Comamonas* sp. EB172 menggunakan kaedah kimia (pelarut dan sodium hidroksida) dan biologi (protease). Daripada kajian ini, *Comamonas* sp. EB172 tempatan baru yang dipencilkan menghasilkan 6-9 g/L DCW, 77-86% kandungan PHA, 5-12 mol% 3HV dan 172-177 kDa *Mw* daripada suapan berkelompok dan 4-11 g/L DCW, 50-64% kandungan PHA, 9-13 mol% 3HV dan 832 kDa *Mw* daripada pengulangan suapan berkelompok. Maka, melalui pengulangan suapan berkelompok, $M_w$ P(3HB-*ko*-3HV) yang tinggi boleh didapati yang memberikan ciri baru bagi aplikasi berlainan.

Sel ketuhar-kering mengalami perubahan dari struktur kimia, penghasilan kristal dan perubahan ciri haba berbanding P(3HB-*ko*-3HV) dalam sel beku-kering P(3HB-*ko*-3HV). Manakala, sel membran ketuhar-kering menjadi kecut, mengumpal antara satu sama lain dan menjadi serpihan berbanding sel beku-kering yang tergores disebabkan beku dan beku vakum. Lebih kurang 90% tulen P(3HB-*ko*-3HV) dan P(3HB-*ko*-3HHX) didapati menggunakan aseton, kloroform, metanol dan *n*-heksana sebagai pelarut pemendak.Tambahan pula, NaOH dan protease daripada halia, *Zingiber officinale* Roscoe telah dipilih kerana ia mesra alam, murah dan

boleh digunakan sebagai mengurangkan kesan kloroform kepada kesihatan pengendali dan persekitaran. Jadi pembaikan pemulihan menggunakan kaedah NaOH telah dijalankan termasuk menggunakan pelbagai kepekatan (0.1, 0.2, 0.3, 0.6, 0.8, 1 and 2 *N*), masa kediaman sel yang berbeza (63, 87 and 111 jam), keadaan awal pengeringan sel (sup basah, pelet basah, beku-kering dan ketuhar-kering) dan cucian (air suling, 20% dan 100% etana). Protease yang sangat spesifik untuk memecahkan membran sel berjaya diekstrak dan ditulenkan menggunakan pemendakan aseton menghasilkan 256 U/mg dan 60% hasil pemulihan. Kesan kepekatan sel (5, 10 dan 20 g/L), masa eraman dan cucian (air suling, 20% ethanol dan 100% etana) dilakukan kepada pemulihan P(3HB-*co*-3HV) menggunakan protease. Kombinasi terbaik menggunakan sel beku-kering untuk NaOH dan protease adalah dengan menggunakan 1 *N* NaOH, inkubasi 1 jam dengan 100% kepekatan sel, emparan, cucian dua kali dengan 0.02% kepekatan sel, eraman dengan 256 U/mg aktiviti enzim protease khas untuk 50 minit, emparan dan cucian dua kali dengan 100% etana. Kedua-dua kaedah menmberi lebih daripada 90% P(3HB-*co*-3HV) tulen dan setara dengan peulihan menggunakan kloroform and *n*-hexane dengan berlainan nisbah kandungan berat jisim, ciri haba dan fizikal. Ciri PHA adalah sangat bergantung kepada mod fermentasi dan kaedah pemulihan; jadi, polimer biourai mempunyai pelbagai ciri yang dapat digunakan untuk aplikasi berbeza.

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**NOR ASMA BT AB. RAZAK, September 2013**

I certify that a Thesis Examination Committee has met on **13 SEPTEMBER 2013** to conduct the final examination of **NOR ASMA BINTI AB. RAZAK** on her thesis entitled "**IMPROVED BIOSYNTHESIS AND RECOVERY OF POLYHYDROXYALKANOATES FROM** *COMAMONAS* SP. EB172." in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the **Degree of Doctor of Philosophy**.

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# DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

**NOR ASMA BT AB. RAZAK**

Date: 13 September 2013

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| *A. eutropha* | *Alcaligenes eutropha* |
| ANOVA | analysis of variance |
| AOT | sodium 1,4-bis(2-ethylhexoxy)-1,4-dioxobutane-2-sulfonate |
| BSA | bovine serum albumin |
| CDW | cell dry weight |
| CRD | complete randomized design |
| Da | Dalton |
| DOT | dissolved oxygen tension |
| delta *Hm* | enthalpy of fusion |
| $dH_2O$ | double distilled water |
| DSC | differential scanning calorimetry |
| DTG | derivative thermogravimetric |
| EFB | empty fruit bunch |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediamine tetraacetic acid |
| EtOH | ethanol |
| FT-IR | Fourier transform infrared spectroscopy |
| *g* | gravity |
| *g* | gram |
| GC | gas chromatography |
| GhG | greenhouse gases |
| GPC | gel permeation chromatography |
| h | hour |
| HDPE | high density polyethylene |
| HPLC | high performance liquid chromatography |
| $H_2SO_4$ | acid sulphuric |
| KOH | kalium hydroxide |
| kDa | kiloDalton |
| L | liter |
| LDPE | low density polyethylene |
| LPS | lipopolysaccharides |
| MOSTI | Ministry of Science Technology and Innovation |
| mg | milligram |
| min | minute |
| ml | milliliter |

| | |
|---|---|
| mcl-PHA | medium-chain-length-polyhydroxyalkanoates |
| $M_n$ | average molecular number |
| $M_v$ | average molecular viscosity |
| $M_w$ | average molecular weight |
| $N$ | normality |
| NaCl | sodium chloride |
| $NaNO_3$ | sodium nitrate |
| NaOCl | sodium hyphochlorite |
| $NH_4Cl$ | ammonium chloride |
| $NH_4OH$ | ammonium hydroxide |
| $NH_4NO_3$ | ammonium nitrate |
| NMR | nuclear magnetic resonance |
| NPCM | non polymer cell materials |
| OD | optical density |
| P | probability |
| PDI | polydispersity index |
| pH | power of hydrogen |
| PLA | polylactic acid |
| PHA | polyhydroxyalkanoates |
| PP | polypropylene |
| P(3HB) | poly(3-hydroxybutyrate) |
| P(3HB-*co*-4HB) | poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) |
| P(3HB-*co*-3HV) | poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) |
| P(3HB-*co*-3HHX) | poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) |
| P(3HB-*co*-3HV-*co*-3HHX) | poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) |
| P(3HB-*co*-3HV-*co*-3HMV) | poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxy-2-methylvalerate) |
| POME | palm oil mill effluent |

| | |
|---|---|
| *R. eutropha* | *Ralstonia eutropha* |
| scl-PHA | short-chain-length polyhydroxyalkanoates |
| SEM | scanning electron microscopy |
| sp. | species |
| spp. | subspecies |
| TEM | transmission electron microscopy |
| TGA | thermogravimetric analysis |
| $T_c$ | crystallinity temperature |
| $T_g$ | glass transition temperature |
| $T_m$ | melting temperature |
| UM | Universiti Malaya |
| UPM | Universiti Putra Malaysia |
| USM | Universiti Sains Malaysia |
| v/v | volume per volume |
| w/v | weight per volume |
| 3HB | 3-hydroxybutyrate |
| 3HV | 3-hydroxyvalerate |
| 3HMV | 3-hydroxy-2-methylvalerate |
| 3HHX | 3-hydroxyhexanoate |
| % | percent |
| % | weight per 100 ml solution |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Study

The carbon and energy sources from wastes are regarded as promising alternatives for the production of PHA on a large scale as they are low cost and at the same time they help to reduce and solve pollution problems. The use of biowaste would help to increase more biodegradable products and clean processes with low-pollution, develop less expensive and novel products not available from petroleum source, use less expensive raw materials and reduce dependence on fossil fuels.

PHA is an excellent bioplastic option; it is a clean material alternative with no emissions of greenhouse gases, which helps in addressing the challenge of global climate changes. PHA has a broad flexibility interest because it can be completely recycled, biodegraded into carbon dioxide and water, present good mechanical resistance, hydrophobic, resistant to liquid and grease and it is a biocompatible thermoplastic which can be melted and molded. Due to its different properties, PHA has commercial potential as the sole structural material or as part of a degradable composite in various areas such as packaging industry, agriculture, medicine, foodstuff industry, chemical industry and others.

Optimisation of PHA process is needed for cost-effective production to improve production, increase the microorganism capabilities to produce PHA, ease downstream processing, reduce wastewater, lower unit production cost per kg PHA, reduce investment of equipments as well as to reduce the laborious process. The high volume and initial PHA content appears to lower the price of PHA, with less utilities and equipment capacity.

Interestingly, the production of P(3HB-*co*-3HV) by using mixed organic acids from anaerobic treated POME can be used chosen as an alternative biodegradable thermoplastic since it is a renewable and inexpensive carbon substrate. In addition P(3HB-*co*-3HV) characteristics are completely biodegradable, possessing high melting point and high tensile strength which can replace the non degradable polypropylene and polystyrene plastics.

New developments in research on biomass in Malaysia emphasised the use of palm oil mill effluent (POME) waste as a renewable resource of carbon substrate to produce the environmental-friendly plastic (Phang et al., 2003; Sim, 2003; Hassan et al., 2002, 1997). The high amount of POME waste in oil palm industry were anaerobically treated to obtain concentrated mixed organic acids for the production of P(3HB-*co*-3HV) (Mumtaz et al., 2008).

*R. eutropha* is an excellent producer of P(3HB), it is slow in growth due to low preference of an organic acids as carbon substrates which limits the commercial purposes. Thus, a new local strain known as *Comamonas* sp. EB172 was successfully isolated from open digester treating palm oil at Serting, Negeri Sembilan and it was capable to grow and accumulate PHA using mixed organic acids from POME (Zakaria et al., 2010a,b; Mumtaz, 2010; Mumtaz et al., 2009; Zakaria et al., 2008). The production of PHA through fed-batch fermentation gave 9.5-10 g/L CDW (cell dry weight) with 59-73% P(3HB-*co*-3HV) and 5-12 mol% 3HV were obtained by *Comamonas* sp. EB172 (Zakaria et al., 2010b; Mumtaz et al., 2010, 2009).

Separation and purification are essential to remove NPCM (non polymer cell materials) to give the high yield and purity of PHA. Thus, the development of a clean, simple and efficient process for PHA recovery with suitable characterisation are needed for end-products uses. The drying methods would effects the PHA recovery as reported that high purity and molecular weight ($M_w$ ) PHA compared to wet and dried biomass of *Alcaligenes eutrophus* (Chen et al., 2001a).

Selection of various types of acids and alkaline were done by Choi and Lee (1999) to degrade recombinant *E. coli* XL 1-Blue containing P(3HB). They found out that NaOH (sodium hydroxide) is the best choice of PHA extraction because it is cheap and environmentally friendly compare to chloroform. Thus, improvement of the P(3HB-*co*-3HV) recovery is necessary to increase yield and purity for further applications.

## 1.2 Problem Statement

i) Improvement of PHA production : The low cost of bioplastic requires the production of high CDW bacteria containing high PHA with mol% 3HV. It can be seen that commercial strain of *R. eutropha* ATCC 17699 is Gram negative bacteria, less resistant cell wall and not acid-tolerant. The undissociated acid will dominant, cross the cell wall, rapidly dissociate, acidify the cytoplasm and kill the cell. Thus, the alternative strain and fermentation methods would helps to increase the PHA.

ii) Characteristic of drying : The drying involved thermal changed on the physical, chemical and structures of PHA inside the cells. The detailed characteristics need to be identified to minimize the loss of PHA through drying prior storage and recovery.

iii) Improvement of recovery methods : Although, solvents are hazardous and expensive, they are the preferred conventional method since they do not destroy the morphology of PHA and eliminates endotoxin. Alternatives solvents used to extract PHA would gave various effect on characteristics of PHA. Thus, an improvement of NaOH treatment was needed for recovering the intracellular PHA.

Mitra studied the effects of low cell concentration and temperature for PHA recovery from similar strain but different method to produce PHA, which affect the initial PHA content,$M_w$ and 3HV content. Meanwhile, the biological treatment such as enzymatic hydrolysis is able to give high purity, recovery yield and mild effect on the PHA. However, an alternative enzymes source is needed as the commercial enzymes are expensive which increases the overall PHA cost.

iii) High polymer quality : The production and recovery processes of PHA need to be optimised, thus giving different characteristics and quality of PHA. The usage of high technology instruments in this research are important and allowed more detailed information regarding PHA as a future bioplastic material.

## 1.3 Strategies

i) Improvement of PHA production : The improvement of the fed-batch fermentation is necessary, to increase the uptake of organic acids in the bioreactor by provided the suitable conditions.*Comamonas* sp. EB172, a new novel locally isolated strain was used in a fed-batch and repeated fed-batch to produce P(3HB-*co*-3HV) utilising synthetic organic acids which mimics the bioacids from POME waste. The *Comamonas* sp. EB172 was chosen instead because it prefer organic acids as carbon sources compared to *R. eutropha* , a commercial producer of PHA.

The usage of synthethic organic acids is necessary to improved the fermentation and to avoid any inhibition of cell growth and less PHA formation due to substrate. Then, the P(3HB-*co*-3HV) obtained from different modes of fermentation were characterised.

ii) Characteristic of drying : Initially, characteristic effect of freeze-drying and oven-drying treatments on the cells and PHA were done. The optimum drying method is needed to reduce the changes on the characteristics of PHA prior to storage or after recovery.

iii) Improvement of recovery methods : An improved, simpler and environmentally friendly way of recovering intracellular PHA is needed. Selection of different type of precipitant solvents and chloroform and characterisation were done to extract P(3HB-*co*-3HV) and P(3HB-*co*-3HHX) from *Comamonas* sp. EB172 and *R. eutropha* PHB-4/pBBREE32d13.

The recovery methods involved the high cell concentration and temperature by using high concentration of NaOH. The improvement on NaOH method include different NaOH concentrations (0.1, 0.2, 0.3, 0.6, 0.8, 1 and 2 *N*), different residence time of cells (63, 87 and 111 h), initial drying cell conditions (wet broth, wet pellet, freeze-dried and oven-dried) and washings (distilled water, 20% and 100% ethanol) were done and characterised. Protease from ginger, *Zingiber offic-*

*inale* were extracted and partial purified using acetone. Then, the effects of cell concentrations, incubation times and washings for the P(3HB-*co*-3HV) recovery using protease were done.

iii) High polymer quality : The quality of PHA obtained after production and recovery processes needs to be characterise for further applications. The instruments used in this study are DTG (Derivative Thermogravimetric) and TGA (Thermogravimetry Analysis), DSC (Differential Scanning Calorimeter), GC (Gas Chromatography), GPC (Gel Permeation Chromatography), HPLC (High Performance Liquid Chromatography), NMR (Nuclear Magnetic Resonance), SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy).

## 1.4 Objectives of Study

The aims of the study are:
1. to improve the PHA production by using *Comamonas* sp. EB172 through fed-batch and repeated fed-batch;

2. to characterise the effect of freeze-drying and oven-drying on cell and PHA; and

3. to improve recovery of PHA from *Comamonas* sp. EB172 using chemicals (solvent and sodium hydroxide) and biological (protease) methods.

## 1.5 Organization of Thesis

To achieve all the objectives in this study, the work presented in this thesis has been divided into eight chapters as follows:

The first chapter includes introduction, problem statement, strategies and objectives related to this study.

The aim to study in greater details about PHA, the role and benefits of PHA have been outlined in the second chapter. Different types of PHA, classification, physiology and biochemistry, comparison of PHA biosynthesis using various kinds of waste as carbon substrate, types of PHA producer and fermentation process. In addition, several extraction and factors influencing the extraction of PHA from the cell are reviewed in this section.

The third chapter includes the experimental materials, design and procedures that were carried out in this research work.

In Chapter 4, description on locally isolated strain, *Comamonas* sp. EB172 was used to produce P(3HB-*co*-3HV) using mixed synthetic organic acids through fed-batch and repeated fed-batch fermentation were demonstrated.

In the fifth chapter, the effect of oven-drying and freeze-drying on the chemical, structural and thermal characteristic of P(3HB-*co*-3HV) were investigated prior PHA recovery.

Chapter 6 describes the extraction methods of P(3HB-*co*-3HV) recovery. The conventional methods using chloroform and effect of different precipitation chemicals were studied. NaOH was used as alternative methods, whereby the effects of NaOH concentrations, residence times, initial drying cell conditions and washings on properties of P(3HB-*co*-3HV) recovery were studied. Protease from ginger, *Zingiber officinale* was extract and partially purified and then, was used for P(3HB-*co*-3HV) recovery.

And finally, in chapter 7 contains the main conclusions as well as recommendations for further works are suggested.

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