UNIVERSITI PUTRA MALAYSIA

IMPROVED BIOSYNTHESIS AND RECOVERY OF POLYHYDROXYALKANOATES FROM COMAMONAS SP. EB172

NOR ASMA BINTI AB. RAZAK

IB 2013 37
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

SEPTEMBER 2013
COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of other material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright  Universiti Putra Malaysia
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 $M_w$ from fed-batch and 4-11 g/L DCW, 50-64% PHA content, 9-13 mol% 3HV and 832 kDa $M_w$ from repeated fed-batch fermentation. Thus, through repeated fed-batch fermentation, high $M_w$ 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 1N 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 gives 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.
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 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, Mw 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. Tambah 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 pemandakan aseton menghasilkan 256 U/mg dan 60% hasil pemulihan. Kesep 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 menberi lebih daripada 90% P(3HB-co-3HV) tulen dan setara dengan pemulihan 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.
ACKNOWLEDGEMENTS

Alhamdullilah.
Thanks to Almighty ALLAH and his kindness, I can do and completed the Degree of Doctor of Philosophy entitled, "IMPROVED BIOSYNTHESIS AND RECOVERY OF POLYHYDROXYALKANOATES FROM COMAMONAS SP. EB172."

I would like to thank Prof. Dr. Mohd Ali bin Hassan from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Science, UPM as main supervisor, Prof Yoshihito Shirai from Department of Biological Functions and Engineering, Kyushu Institute of Technology, Japan, Prof. Dato’ Dr Wan Md. Zin bin Wan Yumus, Department of Chemistry, Faculty of Science, UPM and Dr. Phang Lai Yee from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Science, UPM as committee members for pleasant collaboration, motivation, useful discussions, help and advice in this research.

Thanks and gratitude again to EB groups especially Prof Dr. Suraini Abd. Aziz, Associate Prof. Dr. Nor’ Aini Abd Rahman, Dr. Tabassum Mumtaz, Dr. Norjan Yusof, Dr. Hidayah Arrifin, Dr. Kuppurcmammy, Dr. Mohd Rafein Zakaria, Dr. Mitra Mohamadi, Dr. Alawi Sulaiman, Mr. Noor Azman bin Mohd Johar, Dr. Yee Liang Ngit, Dr. Mior Ahmad Khusairi Mohd Zahari, Miss Asma Hashim, Mrs. Elmy Nahida binti Othman, Mr. Zulkhairi bin Mohd Yusoff, Mr. Mohd Ridzuan bin Othman, Mrs. Zuraidah Zanirun, Dr. Saleha Shamsuddin, Miss Nazlina bt Mohd Yasin, Miss Syahinaz Shahrzadi and others.

My special thanks to contribution of knowledge by Prof. Dato’ Dr. Tengku Azmi Tengku Ibrahim from Department of Veterinary Preclinical Sciences, Faculty of Veterinary, UPM in microscopy, Dr. Ahmad Selamat from Department of Biometric, Faculty of Agriculture, UPM and Dr. Mohd Bakri Adam and his colleagues, Department of Mathematics, Faculty of Science, UPM for SAS, Excel and LaTeX software knowledge and Dr. Suraya Abdul Rashid from Department of Chemical and Environmental Engineering, Faculty Engineering, UPM.

Instrumentation and technical supports from Mr. Rosli Aslim, Mrs. Aluyah Marzuki, Mrs Renuga a/p Panjamurti and Mr. Khairul Basyar Baharudin from Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Science, UPM, Mrs. Rusnani Amirudin, Mrs. Zaidina Mohd Daud and Mr. Mohamad Johadi Iskandar Che Janil from Department Chemistry, Faculty Science, UPM, Mr. Zahiruddin Daud and Mr. Amer Syaifuddin from Department of Food Process and Engineering, Faculty Engineering, UPM, Mr. Rafiuz Zaman Haroun, Mrs. Zahidah Muhamed, Mrs. Fairadh Akmal Ismail and Mrs. Aminah Jusoh from Microscopy Unit, Institute Bioscience, UPM, Mrs. Liyana Ithinin, Mr. Mohd Rizal Kapri and Mr. Md Sabri Mohd Yusoff from Fermentation Technology Unit,
Institute Bioscience, UPM and Mr. Ishak Mohd Yusuff from SIRIM Berhad.

Acknowledge to Institute Biosciences, SIRIM Berhad and Faculty of Biotechnology and Biomolecular Science, UPM for their conducive places, Ministry of Science Technology and Innovation (MOSTI), FELDA and National Science Foundation (NSF) for the projects funds and financial scholarships. And special thanks to Universiti Sains Malaysia (USM), SIRIM and Institute of Massachusetts (MIT) contribution of knowledge.

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**.

Members of the Thesis Examination Committee were as follows:

**Prof. Dr. Arbakariya b. Ariff, Ph.D.**  
Professor  
Faculty of Biotechnology and Science Biomolecule  
Universiti Putra Malaysia  
(Chairperson)

**Dr. Rosfarizan binti Mohamad, Ph.D.**  
Associate Professor  
Faculty of Biotechnology and Science Biomolecule  
Universiti Putra Malaysia  
(Internal Examiner)

**Dr. Mohd Yunus bin Abd. Shukor, Ph.D.**  
Associate Professor  
Faculty of Biotechnology and Science Biomolecule  
Universiti Putra Malaysia  
(Internal Examiner)

**Prof. Dr. Virendra Swarup Bisaria, Ph.D.**  
Professor  
Department of Biochemistry Engineering and Biotechnology  
Indian Institute of Technology-Delhi Hauz Khas  
110016 New Delhi, India  
(External Examiner)

---

**NORITAH OMAR, Ph.D.**  
Associate Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

**Date:** 13 February 2014
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of DOCTOR OF PHILOSOPHY.

The members of the Supervisory Committee were as follows:

Mohd Ali Hassan, Ph.D.
Professor
Faculty of Biotechnology and Science Biomolecule
Universiti Putra Malaysia
(Chairperson)

Wan Md. Zin Wan Yunus, Ph.D.
Professor
Director
Center of Publication
Universiti Pertahanan Nasional Malaysia
(Member)

Yoshihito Shirai, Ph.D.
Professor
Department of Biological Functions and Engineering
Graduate School of Life Science and Systems Engineering (LSSE)
Kyushu Institute of Technology, Japan
(Member)

Phang Lai Yee, Ph.D.
Senior Lecturer
Faculty of Biotechnology and Science Biomolecule
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM NUAT, Ph.D.
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:
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
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>DEDICATIONS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>viii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td>xviii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xx</td>
</tr>
</tbody>
</table>

## CHAPTER

1 INTRODUCTION
   1.1 Background of Study 1
   1.2 Problem Statement 2
   1.3 Strategies 3
   1.4 Objectives of Study 4
   1.5 Organization of Thesis 4

2 LITERATURE REVIEW
   2.1 Polymer 6
   2.2 Polyhydroxyalkanoates (PHA) as an Alternative Bioplastic 8
   2.3 Commercialization, Future Prospects and related Patent of PHA 10
   2.4 Classification of PHA 13
   2.5 Physiology and Biochemistry of PHA Synthesis 16
   2.6 Biosynthesis of PHA 18
      2.6.1 Substrate 18
      2.6.2 Microorganisms 19
      2.6.3 Fermentation Technique for PHA Production 22
      2.6.4 Batch Fermentation 22
      2.6.5 Fed-Batch Fermentation 22
      2.6.6 Repeated-Fed Batch Fermentation 24
   2.7 Cells Disruption Methods for extraction of PHA 27
      2.7.1 Factor that Influences the Selection of Cell Disruption Methods 27
      2.7.2 Solvent Extraction Methods 30
      2.7.3 Chemical Extraction Methods 33
      2.7.4 Enzyme Hydrolysis Methods 33
      2.7.5 Combination Methods of Cell Disruption 34
5 EFFECT OF DRYING METHODS ON STRUCTURAL, MORPHOLOGY AND THERMAL PROPERTIES OF POLY(3-HYDROXY BUTYRATE-CO-3-HYDROXYVALERATE) FROM COMAMONAS SP.

EB 172 65
5.1 Introduction 65
5.2 Materials 66
5.3 Methods 66
5.3.1 Drying 66
5.3.2 Characterisation of P(3HB-co-3HV) 66
5.4 Results and Discussion 67
5.4.1 Effects of Oven-dried Temperature on CDW and PHA Content 67
5.4.2 Effects of Drying on PHA Content and mol% 3HV 67
5.4.3 Effects of Drying on Chemical Characteristics 71
5.4.4 Effects of Drying on Thermal Characteristics 72
5.4.5 Effects of Drying on Structural Characteristics 75
5.5 Summary 78

6 IMPROVED RECOVERY AND CHARACTERISATION OF POLYHYDROXYALKANOATES (PHA) USING CHLOROFORM, SODIUM HYDROXIDE AND PROTEASE FROM GINGER, ZINGIBER OFFICINALE ROSCOE 80

6.1 Introduction 80
6.2 Materials 81
6.2.1 Chemicals 81
6.3 Methods 82
6.3.1 Recovery of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) and Poly(3-Hydroxybutyrate-co-3-Hydroxyhexanoate) from Comamonas sp. EB172 and W. eutropha PHB-4/pBBREE32d13 using Solvent Extraction 82
6.3.2 Recovery of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) using Sodium Hydroxide (NaOH) from Comamonas sp. EB172 83
6.3.3 Recovery of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) from Comamonas sp. EB172 using Protease Extracted from Ginger, Zingiber officinale 84
6.3.4 Mass Balances for Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) Recovery 85
6.3.5 Analytical Procedures 85
6.3.6 Characterisation of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) 86
6.4 Results and Discussion 86
6.4.1 Recovery of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) and Poly(3-Hydroxybutyrate-co-3-Hydroxyhexanoate) from Comamonas sp. EB172 and W. eutropha PHB-4/pBBREE32d13 using Solvent Extraction 86
6.4.2 Recovery of Poly(3-Hydroxybutyrate- co-3-Hydroxyvalerate) using Sodium Hydroxide (NaOH) from Comamonas sp. EB172 94
6.4.3 Recovery of Poly(3-Hydroxybutyrate- co-3-Hydroxyvalerate) from Comamonas sp. EB172 using Protease Extracted from Ginger, Zingiber officinale 101
6.4.4 Mass Balances for PHA Recovery 106
6.5 Summary 110

7 CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDIES 112
7.1 Conclusions 112
7.2 Recommendations 113

REFERENCES/BIBLIOGRAPHY 114

APPENDICES 127
A.1 Calibration of dissolved oxygen and pH probe 128
A.2 Sterilization of 2 L and 10 L bioreactors 128
A.3 Sterilization of 50 L bioreactors 128
A.4 Setting up bioreactors prior fermentation 129
C.1 Method, standard and sample preparation of PHA content (g/L) 132
C.2 Method, standard and sample preparation of organic acid content (g/L) 132
D.1 Method and sample preparation of methylene blue for light microscopy 136
D.2 Method and sample preparation of SEM 136
D.3 Method and sample preparation of TEM 137
E.1 Method, standard and sample preparation of Bradford 139
F.1 Method and sample preparation of GC (wt%) 141
F.2 Calculation K value for GC 145
F.3 Calculation PHA content (wt %) 145
F.4 Calculation for P(3HB) (mol %) 146
F.5 Calculation P(3HV) (mol %) 146
G.1 Calculation of PHA purity 147
G.2 Calculation of PHA recovery 147
H.1 Method and calculation of C/N 148

Biodata of Student 150
List of Seminars/Workshops 151
List of Publication and Patent 152

xiv
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Energy requirement for the production of materials used in packaging</td>
<td>7</td>
</tr>
<tr>
<td>2.2</td>
<td>Market *prices for biodegradable and non-biodegradable materials</td>
<td>11</td>
</tr>
<tr>
<td>2.3</td>
<td>Application of PHA</td>
<td>12</td>
</tr>
<tr>
<td>2.4</td>
<td>Some patents of PHA</td>
<td>14</td>
</tr>
<tr>
<td>2.5</td>
<td>Biosynthesise of PHA by different microorganisms using various carbon sources</td>
<td>20</td>
</tr>
<tr>
<td>2.6</td>
<td>The effects of organic acids on P(3HB-co-3HV) production through batch fermentation by using <em>Ralstonia</em> sp.</td>
<td>23</td>
</tr>
<tr>
<td>4.1</td>
<td>Medium composition for growth medium (GM) by using <em>Comamonas</em> sp. EB172</td>
<td>51</td>
</tr>
<tr>
<td>4.2</td>
<td>Medium composition for production of PHA by using <em>Comamonas</em> sp. EB172</td>
<td>52</td>
</tr>
<tr>
<td>4.3</td>
<td>Trace elements* of PHA by using <em>Comamonas</em> sp. EB172</td>
<td>52</td>
</tr>
<tr>
<td>4.4</td>
<td>Improvement of biosynthesis of P(3HB-co-3HV) by using <em>Comamonas</em> sp. EB172 in fed-batch and repeated fed-batch fermentation</td>
<td>54</td>
</tr>
<tr>
<td>4.5</td>
<td>Characteristic of P(3HB-co-3HV) obtained from different run of fed-batch fermentation by using <em>Comamonas</em> sp. EB172</td>
<td>57</td>
</tr>
<tr>
<td>4.6</td>
<td>Characteristic of P(3HB-co-3HV) obtained from repeated fed-batch fermentation by using <em>Comamonas</em> sp. EB172</td>
<td>61</td>
</tr>
<tr>
<td>4.7</td>
<td>The distribution particle size and specific surface area of the <em>Comamonas</em> sp. EB 172 obtained from repeated fed-batch fermentation</td>
<td>61</td>
</tr>
<tr>
<td>4.8</td>
<td>The comparison of P(3HB-co-3HV) production by using <em>Comamonas</em> sp. EB172 utilises mixed organic acids</td>
<td>62</td>
</tr>
<tr>
<td>5.1</td>
<td>Effect of oven-dried temperature on CDW and PHA content of <em>Comamonas</em> sp. EB 172</td>
<td>67</td>
</tr>
</tbody>
</table>
5.2 Effect of oven-dried and freeze-dried cell containing P(3HB-co-3HV) of *Comamonas* sp. EB 172

5.3 The chemical shift (theta) of $^1$H NMR of drying cell containing P(3HB-co-3HV)

5.4 Effects of oven-dried and freeze-dried on the thermal properties of P(3HB-co-3HV) of *Comamonas* sp. EB 172

5.5 The effect of drying on distribution particle size and specific surface area of the *Comamonas* sp. EB 172

6.1 Solubility of PHA

6.2 Effect of different precipitant on P(3HB-co-3HV) recovery and morphology

6.3 Effect of different precipitate chemicals to molecular composition and thermal properties of P(3HB-co-3HV) recovery from *Comamonas* sp. EB172

6.4 Effect of different residence times on P(3HB-co-3HV) recovery from *Comamonas* sp. EB172 using 1 N NaOH

6.5 Effect of different initial cell conditions to molecular composition and thermal properties on P(3HB-co-3HV) recovery from *Comamonas* sp. EB172 using 1 N NaOH

6.6 Effect of different washings on P(3HB-co-3HV) recovery from *Comamonas* sp. EB172 using 1 N NaOH

6.7 Purification table protease from ginger, *Zingiber officinale*

6.8 Effect of different washings on P(3HB-co-3HV) recovery from *Comamonas* sp. EB172 using protease

6.9 Summary table P(3HB-co-3HV) from *Comamonas* sp. EB172 using the improvement methods of solvent, NaOH and protease
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Some of the natural and synthetic polymer available in the market</td>
</tr>
<tr>
<td>2.2</td>
<td>A balance ecosystem with PHA</td>
</tr>
<tr>
<td>2.3</td>
<td>General chemical structure of PHA monomer</td>
</tr>
<tr>
<td>2.4</td>
<td>Biosynthesis pathway of P(3HB-co-3HV) in <em>R. eutropha</em> using acetic, butyric and propionic acid</td>
</tr>
<tr>
<td>2.5</td>
<td>General method for extraction and purification</td>
</tr>
<tr>
<td>2.6</td>
<td>Method of PHA recovery using (a) mechanical and non-solvent and (b) solvent</td>
</tr>
<tr>
<td>2.7</td>
<td>Some of the methods used for PHA characterisation</td>
</tr>
<tr>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td><em>Comamonas</em> sp. EB172 growth profile fermentation. Data obtained from 5 experiments with standard error bars</td>
</tr>
<tr>
<td>4.2</td>
<td>P(3HB-co-3HV) profile in repeated fed-batch fermentation of <em>Comamonas</em> sp. EB 172</td>
</tr>
<tr>
<td>5.1</td>
<td>The $^1$H NMR spectrum P(3HB-co-3HV) of a) freeze-dried and b) oven-dried <em>Comamonas</em> sp. EB 172</td>
</tr>
<tr>
<td>5.2</td>
<td>The TGA and DTG of <em>Comamonas</em> sp. EB 172 a) freeze-dried and b) oven-dried</td>
</tr>
<tr>
<td>5.3</td>
<td>The DSC thermogram of <em>Comamonas</em> sp. EB 172 a) freeze-dried and b) oven-dried</td>
</tr>
<tr>
<td>6.1</td>
<td>TGA of P(3HB-co-3HV) in (a) SIGMA, (b) <em>Comamonas</em> sp. EB172 and after precipitate using (c) acetone, (d) chloroform, (e)$n$-hexane and (f) methanol</td>
</tr>
<tr>
<td>6.2</td>
<td>DSC of P(3HB-co-3HV) from (a) SIGMA, (b) <em>Comamonas</em> sp. EB172, after precipitate using (c) acetone, (d) chloroform, (e) methanol and (f) $n$-hexane</td>
</tr>
</tbody>
</table>
6.3 Effect of NaOH concentrations on soluble protein content released recovery

6.4 Effects of cell concentrations and incubation times on soluble protein release using protease extracted from Ginger, *Zingiber officinale*

6.5 Flow chart of the mass balances for PHA recovery using a) chloroform and *n*-hexane, b) NaOH and c) *protease from Zingiber officinale*

C.1 Chromatogram for standard P(3HB) content showed peak at 5.508 min indicated the 4mM sulphuric acid as mobile phase and peak at 23.825 min indicated the P(3HB) at 6 g/L.

C.2 Chromatogram for standard mixed organic acid at 6 g/L where peak at 10.045 min indicated the 4mM sulphuric acid as mobile phase, peak at 12.655 min indicated the formic acid, peak at 15.453 min indicated the acetic acid, peak at 18.203 min indicated the propionic acid, peak at 20.055 min indicated the butyric acid and peak at 22.258 min indicated the *n*-butyric acid.

E.1 Standard curve for protease content using Bradford

F.1 Chromatogram for standard PHB content using GC showed peak at 2.515 min indicated the chloroform as volatile mobile phase, peak at 4.429-4.746 min indicated the P3HB and 11.836 min indicated the internal benzoic acid standard.

F.2 Chromatogram for standard PHBV content using GC showed peak at 2.515 min indicated the chloroform as volatile mobile phase, peak at 4.451-4.751 min indicated the P3HB, 6.399 indicated the P3HV and 11.617 min indicated the internal benzoic acid standard
LIST OF PLATES

3.1 Instruments used (a) close up of sample location and (b) machine of DTG and TGA 45
3.2 Instruments used (a) sample placed in aluminium pan, (b) aluminium pan sealer to tight the pan, and (c) machine of DSC 46
3.3 Instrument GC 47
3.4 Instrument HPLC 48
3.5 Instrument Particle Size 49

4.1 Comamonas sp. EB172 in a) 50 L stirred tank bioreactor, (b) after 48 h fermentation; under light microscope at magnification X40 during (c) growth and (d) production; micrograph of SEM at magnification X10000 during (e) growth and (f) production of P(3HB-co-3HV) 56
4.2 Repeated fed-batch fermentation (a) in 2 L stirred tank bioreactor; (b) micrograph of TEM at magnification X8000 during production shown the black colour inside the cell is PHA, and (c) produced foams 59

5.1 Instruments (a) freeze-dried and (b) oven 66
5.2 Statistical analysis of different oven-dried temperature of Comamonas sp. EB 172 on a) CDW and b) PHA content 68
5.3 Effect of different oven-temperatures on cell containing P(3HB-co-3HV). From left 50, 60 and 105 °C 69
5.4 Statistical analysis effect of oven-dried and freeze-dried cell of Comamonas sp. EB 172 on a) PHA content and b) mol% 3HV 70
5.5 Micrograph of SEM Comamonas sp. EB 172 at magnification of a) X8 000 freeze-dried cell, b) X20 000 enlarge freeze-dried, c) X10 000 oven-dried and d) X2 000 crystal growth on the surface of oven-dried 76
5.6 Micrograph of TEM at magnification of a) X20 000 shrinkage cell after freeze at −80 °C and b) X30 000 of freeze-dried cell contained P(3HB- 77
5.7 Statistical analysis effect of Comamonas sp. EB 172 on a) different cell conditions and b) drying on particle size 79

6.1 Solubility test which shows PHA in (a) solubilise and (b) not solubilise conditions 82
6.2 Methods of PHA recovery using solvent 83
6.3 White precipitate of P(3HB-co-3HV) from Comamonas sp. EB172 after adding precipitate with (a) methanol and (b) n-hexane 87
6.4 Micrograph of TEM at magnification X30 000 of (a) *W. eutropha* PHB-4/pBBREE32d13 containing P(3HB-co-3HHX) and (b) *Comamonas* sp. EB172 containing P(3HB-co-3HV). Micrograph of SEM at magnification X10000 using chloroform and *n*-hexane of extracted (c) P(3HB-co-3HHX) and (d) P(3HB-co-3HV), and standard from SIGMA of (f) P(3HB) and (g) P(3HB-co-3HV)

6.5 P(3HB-co-3HV) recovered from *Comamonas* sp. EB172 after precipitate using (a) acetone, (b) chloroform, (c) methanol and (d) *n*-hexane

6.6 Effect of centrifugation cycle (a) first and (b) second

6.7 Micrograph of SEM at magnification X8 000 and X60 000 of (a) (b) cell wall breakage with P(3HB-co-3HV) and (c) NPCM obtained after separation from P(3HB-co-3HV) using 1 N NaOH

6.8 P(3HB-co-3HV) recovered using 1 N NaOH in (a) freeze-dried and (b) oven-dried. Micrograph of TEM at magnification X200 000 of (c) freeze-dried P(3HB-co-3HV) and (d) oven-dried P(3HB-co-3HV). Micrograph of SEM at magnification X10 000 of (e) P(3HB) and (f) P(3HB-co-3HV) from SIGMA

6.9 The (a) rhizome of ginger, *Zingiber officinale*, (b) blended ginger with acetone, (c) mixed acetone and ginger were filter with Whatman No. 1 and (d) acetone-ginger powder after air-dried

6.10 A (a) mixture of cell with protease from ginger, (b) water removal using rotary-evaporator and (c) white PHA obtained after evaporate

B.1 Spectrophotometer HACH DR 2800

F.1 Instrument (a) heat block, (b) samples in test tube contains 2 layer after heat and add distilled water and (c) sample in GC tubes prior analyzed

Plates Page
# 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** — enthalpy of fusion
- **dH₂O** — 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
- **H₂SO₄** — 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 hypochlorite
NH$_4$Cl  ammonium chloride
NH$_4$OH  ammonium hydroxide
NH$_4$NO$_3$  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
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. eutropha</em></td>
<td><em>Ralstonia eutropha</em></td>
</tr>
<tr>
<td>scl-PHA</td>
<td>short-chain-length polyhydroxyalkanoates</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>spp.</td>
<td>subspecies</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TGA</td>
<td>thermogravimetric analysis</td>
</tr>
<tr>
<td>$T_c$</td>
<td>crystallinity temperature</td>
</tr>
<tr>
<td>$T_g$</td>
<td>glass transition temperature</td>
</tr>
<tr>
<td>$T_m$</td>
<td>melting temperature</td>
</tr>
<tr>
<td>UM</td>
<td>Universiti Malaya</td>
</tr>
<tr>
<td>UPM</td>
<td>Universiti Putra Malaysia</td>
</tr>
<tr>
<td>USM</td>
<td>Universiti Sains Malaysia</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>3HB</td>
<td>3-hydroxybutyrate</td>
</tr>
<tr>
<td>3HV</td>
<td>3-hydroxyvalerate</td>
</tr>
<tr>
<td>3HMV</td>
<td>3-hydroxy-2-methylvalerate</td>
</tr>
<tr>
<td>3HHX</td>
<td>3-hydroxyhexanoate</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>%</td>
<td>weight per 100 ml solution</td>
</tr>
</tbody>
</table>
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 environment-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 synthetic 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 offici-*
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.
BIBLIOGRAPHY


