



**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 fulfillment 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 gave more than 90% P(3HB-*co*-3HV) purity and are comparable with recovery using chloroform and *n*-hexane with different fold *M<sub>w</sub>* 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, mencari kesan beku kering dan ketuhar kering kepada sel dan PHA dan membaiki pemuliharaan 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  $M_w$  daripada suapan berkelompok dan 4-11 g/L DCW, 50-64% kandungan PHA, 9-13 mol% 3HV dan 832 kDa  $M_w$  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, mengumpul 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 mengurangi 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 memberi 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

Alhamdulillah.

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 Yunus, 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. Kuppurchammy, Dr. Mohd Rafein Zakaria, Dr. Mitra Mohammadi, Dr. Alawi Sulaiman, Mr. Noor Azman bin Mohd Johar, Dr. Yee Liang Ngit, Dr. Mior Ahmad Khushairi 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 Shahrazi 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 Jamil 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. Faridah 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 Massachuetes (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 HUAT, 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

	<b>Page</b>
<b>DEDICATIONS</b>	i
<b>ABSTRACT</b>	ii
<b>ABSTRAK</b>	iv
<b>ACKNOWLEDGMENTS</b>	vi
<b>APPROVAL</b>	viii
<b>DECLARATION</b>	x
<b>LIST OF TABLES</b>	xv
<b>LIST OF FIGURES</b>	xvii
<b>LIST OF PLATES</b>	xviii
<b>LIST OF ABBREVIATIONS</b>	xx
<b>CHAPTER</b>	
<b>1 INTRODUCTION</b>	1
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
<b>2 LITERATURE REVIEW</b>	6
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
2.6.7 Research and Development related to PHA in Malaysia	25
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

2.7.6	Mass Balances of PHA Recovery	35
2.8	PHA Characterisation	35
2.8.1	Chemical Characteristic	37
2.8.2	Structural Characteristic	38
2.8.3	Thermal Characteristic	39
2.9	Summary	40
<b>3</b>	<b>GENERAL MATERIALS AND METHODS</b>	<b>43</b>
3.1	Material	43
3.1.1	Microorganisms	43
3.2	General Methods	43
3.2.1	Overall Experimental Design	43
3.2.2	Setting Up and Sterilization of Bioreactor	43
3.2.3	Biosynthesis of PHA	43
3.2.4	Harvest and Storage of Cell	43
3.2.5	Analytical Procedures	43
3.3	PHA Characterisation Methods	45
3.3.1	Derivative thermogravimetric (DTG) and Thermogravimetric analysis (TGA)	45
3.3.2	Differential Scanning Calorimeter (DSC)	45
3.3.3	Gas Chromatography (GC)	45
3.3.4	Gel Permeation Chromatography (GPC)	47
3.3.5	High Performance Liquid Chromatography (HPLC)	47
3.3.6	Microscopy	47
3.3.7	Nuclear Magnetic Resonance (NMR)	48
3.3.8	Particle size	48
3.4	Statistical Analysis	49
<b>4</b>	<b>IMPROVED BIOSYNTHESIS AND CHARACTERISATION OF POLY(3-HYDROXY BUTYRATE-<i>CO</i>-3-HYDROXYVALERATE) FROM <i>COMAMONAS SP. EB172</i></b>	<b>50</b>
4.1	Introduction	50
4.2	Materials	51
4.2.1	Chemicals	51
4.2.2	Media	51
4.2.3	Microorganism	51
4.3	Methods	51
4.3.1	Pre-culture of P(3HB- <i>co</i> -3HV) from <i>Comamonas sp. EB172</i>	51
4.3.2	Biosynthesis of P(3HB- <i>co</i> -3HV) from <i>Comamonas sp. EB172</i> in Fed-batch and Repeated Fed-batch Fermentation	53
4.3.3	Analytical Procedures	53
4.4	Results and Discussion	53
4.5	Summary	64



5	<b>EFFECT OF DRYING METHODS ON STRUCTURAL, MORPHOLOGY AND THERMAL PROPERTIES OF POLY(3-HYDROXY BUTYRATE-<i>CO</i>-3-HYDROXYVALERATE) FROM <i>COMAMONAS</i> SP.</b>	
	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- <i>co</i> -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	<b>IMPROVED RECOVERY AND CHARACTERISATION OF POLY-HYDROXYALKANOATES (PHA) USING CHLOROFORM, SODIUM HYDROXIDE AND PROTEASE FROM GINGER, <i>ZINGIBER OFFICINALE ROSCOE</i></b>	
		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- <i>co</i> -3-Hydroxyvalerate) and Poly(3-Hydroxybutyrate- <i>co</i> -3-Hydroxyhexanoate) from <i>Comamonas</i> sp. EB172 and <i>W. eutropha</i> PHB-4/pBBREE32d13 using Solvent Extraction	82
6.3.2	Recovery of Poly(3-Hydroxybutyrate- <i>co</i> - <b>3-Hydroxyvalerate</b> ) using Sodium Hydroxide (NaOH) from <i>Comamonas</i> sp. <b>EB172</b>	83
6.3.3	Recovery of Poly(3-Hydroxybutyrate- <i>co</i> - <b>3-Hydroxyvalerate</b> ) from <i>Comamonas</i> sp. <b>EB172</b> using Protease Extracted from Ginger, <i>Zingiber officinale</i>	84
6.3.4	Mass Balances for Poly(3-Hydroxybutyrate- <i>co</i> - <b>3-Hydroxyvalerate</b> ) Recovery	85
6.3.5	Analytical Procedures	85
6.3.6	Characterisation of Poly(3-Hydroxybutyrate- <i>co</i> - <b>3-Hydroxyvalerate</b> )	86
6.4	Results and Discussion	86
6.4.1	Recovery of Poly(3-Hydroxybutyrate- <i>co</i> - <b>3-Hydroxyvalerate</b> ) and Poly( <b>3-Hydroxybutyrate-<i>co</i>-3-Hydroxyhexanoate</b> ) from <i>Comamonas</i> sp. <b>EB172</b> and <i>W. eutropha</i> PHB-4/pBBREE32d13 using Solvent Extraction	86

6.4.2	Recovery of Poly(3-Hydroxybutyrate- <i>co</i> -3-Hydroxyvalerate) using Sodium Hydroxide (NaOH) from <i>Comamonas</i> sp. EB172	94
6.4.3	Recovery of Poly(3-Hydroxybutyrate- <i>co</i> - <b>3-Hydroxyvalerate</b> ) from <i>Comamonas</i> sp. <b>EB172</b> using Protease Extracted from <b>Ginger</b> , <i>Zingiber officinale</i>	101
6.4.4	Mass Balances for PHA Recovery	106
6.5	Summary	110
<b>7</b>	<b>CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDIES</b>	<b>112</b>
7.1	Conclusions	112
7.2	Recommendations	113
	<b>REFERENCES/BIBLIOGRAPHY</b>	<b>114</b>
	<b>APPENDICES</b>	<b>127</b>
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
	<b>BIODATA OF STUDENT</b>	<b>150</b>
	<b>LIST OF SEMINARS/WORKSHOPS</b>	<b>151</b>
	<b>LIST OF PUBLICATION AND PATENT</b>	<b>152</b>

## LIST OF TABLES

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

5.2	Effect of oven-dried and freeze-dried cell containing P(3HB- <i>co</i> -3HV) of <i>Comamonas</i> sp. <b>EB 172</b>	69
5.3	The chemical shift (theta) of <sup>1</sup> H NMR of drying cell containing P(3HB- <i>co</i> -3HV)	72
5.4	Effects of oven-dried and freeze-dried on the thermal properties of P(3HB- <i>co</i> -3HV) of <i>Comamonas</i> sp. <b>EB 172</b>	75
5.5	The effect of drying on distribution particle size and specific surface area of the <i>Comamonas</i> sp. <b>EB 172</b>	78
6.1	Solubility of PHA	86
6.2	Effect of different precipitant on P(3HB- <i>co</i> -3HV) <b>recovery and morphology</b>	88
6.3	Effect of different precipitate chemicals to molecular composition and thermal properties of P(3HB- <i>co</i> -3HV) <b>recovery from <i>Comamonas</i> sp. EB172</b>	91
6.4	Effect of different residence times on P(3HB- <i>co</i> -3HV) <b>recovery from <i>Comamonas</i> sp. EB172 using 1 N NaOH</b>	97
6.5	Effect of different initial cell conditions to molecular composition and thermal properties on P(3HB- <i>co</i> -3HV) <b>recovery from <i>Comamonas</i> sp. EB172 using 1 N NaOH</b>	98
6.6	Effect of different washings on P(3HB- <i>co</i> -3HV) <b>recovery from <i>Comamonas</i> sp. EB172 using 1 N NaOH</b>	100
6.7	Purification table protease from ginger, <i>Zingiber officinale</i>	104
6.8	Effect of different washings on P(3HB- <i>co</i> -3HV) <b>recovery from <i>Comamonas</i> sp. EB172 using protease</b>	108
6.9	Summary table P(3HB- <i>co</i> -3HV) <b>from <i>Comamonas</i> sp. EB172 using the improvement methods of solvent, NaOH and protease</b>	109

## LIST OF FIGURES

Figure	Page
2.1 Some of the natural and synthetic polymer available in the market	7
2.2 A balance ecosystem with PHA	9
2.3 General chemical structure of PHA monomer	15
2.4 Biosynthesis pathway of P(3HB- <i>co</i> -3HV) in <i>R. eutropha</i> using acetic, butyric and propionic acid	17
2.5 General method for extraction and purification	28
2.6 Method of PHA recovery using (a) mechanical and non-solvent and (b) solvent	31
2.7 Some of the methods used for PHA characterisation	36
3.1	44
4.1 <i>Comamonas</i> sp. EB172 growth profile fermentation. Data obtained from 5 experiments with standard error bars	55
4.2 P(3HB- <i>co</i> -3HV) profile in repeated fed-batch fermentation of <i>Comamonas</i> sp. EB 172	60
5.1 The <sup>1</sup> H NMR spectrum P(3HB- <i>co</i> -3HV) of a) freeze-dried and b) oven-dried <i>Comamonas</i> sp. EB 172	71
5.2 The TGA and DTG of <i>Comamonas</i> sp. EB 172 a) freeze-dried and b) oven-dried	73
5.3 The DSC thermogram of <i>Comamonas</i> sp. EB 172 a) freeze-dried and b) oven-dried	74
6.1 TGA of P(3HB- <i>co</i> -3HV) in (a) SIGMA, (b) <i>Comamonas</i> sp. EB172 and after precipitate using (c) acetone, (d) chloroform, (e) <i>n</i> -hexane and (f) methanol	92
6.2 DSC of P(3HB- <i>co</i> -3HV) from (a) SIGMA, (b) <i>Comamonas</i> sp. EB172, after precipitate using (c) acetone, (d) chloroform, (e) methanol and (f) <i>n</i> -hexane	93

6.3	Effect of NaOH concentrations on soluble protein content released recovery	95
6.4	Effects of cell concentrations and incubation times on soluble protein release using protease extracted from Ginger, <i>Zingiber officinale</i>	107
6.5	Flow chart of the mass balances for PHA recovery using a) chloroform and <i>n</i> -hexane, b) NaOH and c) protease from <i>Zingiber officinale</i>	111
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.	133
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 <i>n</i> -butyric acid.	135
E.1	Standard curve for protease content using Bradford	140
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.	143
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	144

## 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	<b><i>Comamonas</i> 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-<i>co</i>-3HV)</b>	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 <i>Comamonas</i> sp. EB 172 on a) CDW and b) PHA content	68
5.3	Effect of different oven-temperatures on cell containing P(3HB- <i>co</i> -3HV). From left 50, 60 and 105 °C	69
5.4	Statistical analysis effect of oven-dried and freeze-dried cell of <i>Comamonas</i> sp. EB 172 on a) PHA content and b) mol% 3HV	70
5.5	Micrograph of SEM <i>Comamonas</i> 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 <i>Comamonas</i> 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- <i>co</i> -3HV) from <i>Comamonas</i> sp. EB172 after adding precipitate with (a) methanol and (b) <i>n</i> -hexane	87

6.4	Micrograph of TEM at magnification X30 000 of (a) <i>W. eutropha</i> PHB-4/pBBREE32d13 containing P(3HB-co-3HHX) and (b) <i>Comamonas</i> sp. EB172 containing P(3HB-co-3HV). Micrograph of SEM at magnification X10000 using chloroform and <i>n</i> -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)	89
6.5	P(3HB-co-3HV) recovered from <i>Comamonas</i> sp. EB172 after precipitate using (a) acetone, (b) chloroform, (c) methanol and (d) <i>n</i> -hexane	90
6.6	Effect of centrifugation cycle (a) first and (b) second	96
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	99
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	102
6.9	The (a) rhizome of ginger, <i>Zingiber officinale</i> , (b) blended ginger with acetone, (c) mixed acetone and ginger were filter with Whatman No. 1 and (d) acetone-ginger powder after air-dried	103
6.10	A (a) mixture of cell with protease from ginger, (b) water removal using rotary-evaporator and (c) white PHA obtained after evaporate	105
B.1	Spectrophotometer HACH DR 2800	131
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	142

**Plates Page**



## LIST OF ABBREVIATIONS

<i>A. eutropha</i>	<i>Alcaligenes eutropha</i>
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 <i>H<sub>m</sub></i>	enthalpy of fusion
dH <sub>2</sub> O	double distilled water
DSC	differential scanning calorimetry
DTG	derivative thermogravimetric
EFB	empty fruit bunch
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
EtOH	ethanol
FT-IR	Fourier transform infrared spectroscopy
<i>g</i>	gravity
<i>g</i>	gram
GC	gas chromatography
GhG	greenhouse gases
GPC	gel permeation chromatography
h	hour
HDPE	high density polyethylene
HPLC	high performance liquid chromatography
H <sub>2</sub> SO <sub>4</sub>	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 <sub>3</sub>	sodium nitrate
NaOCl	sodium hypochlorite
NH <sub>4</sub> Cl	ammonium chloride
NH <sub>4</sub> OH	ammonium hydroxide
NH <sub>4</sub> NO <sub>3</sub>	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- <i>co</i> -4HB)	poly(3-hydroxybutyrate- <i>co</i> -4-hydroxybutyrate)
P(3HB- <i>co</i> -3HV)	poly(3-hydroxybutyrate- <i>co</i> -3-hydroxyvalerate)
P(3HB- <i>co</i> -3HHX)	poly(3-hydroxybutyrate- <i>co</i> -3-hydroxyhexanoate)
P(3HB- <i>co</i> -3HV- <i>co</i> -3HHX)	poly(3-hydroxybutyrate- <i>co</i> -3-hydroxyvalerate- <i>co</i> -3-hydroxyhexanoate)
P(3HB- <i>co</i> -3HV- <i>co</i> -3HMV)	poly(3-hydroxybutyrate- <i>co</i> -3-hydroxyvalerate- <i>co</i> -3-hydroxy-2-methylvalerate)
POME	palm oil mill effluent

<i>R. eutropha</i>	<i>Ralstonia eutropha</i>
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 Seriting, 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 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.



## BIBLIOGRAPHY

- Abdelwahed, W., Degobert, G., Stainmesse, S. and Fessi, H. 2006. Freeze-drying of nanoparticles: Formulation, process and storage considerations. *Advanced Drug Delivery Reviews* 58: 1688–1713.
- Akiyama, M., Tsuge, T. and Doi, Y. 2003. Environmental life cycle comparison of Polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation. *Polymer Degradation and Stability* 80: 183–194.
- Alias, Z. and Tan, I. K. P. 2005. Isolation of palm oil-utilising, Polyhydroxyalkanoate (PHA)-producing bacteria by an enrichment technique. *Bioresource Technology* 96: 1229–1234.
- Allen, A. D., Anderson, W. A., Ayorinde, F. O. and Eribo, B. E. 2010. Biosynthesis and characterization of copolymer Poly(3HB-co-3HV) from saponified *Jatropha curcas* oil by *Pseudomonas oleovorans*. *Journal Industrial Microbiology Biotechnology* 37: 849–856.
- Arun, A., Murrugappan, R., Ravindran, A. D. D., Veeramanikandan, V. and Balaji, S. 2006. Utilization of various industrial wastes for the production of Polyhydroxybutyrate (PHB) by *Alcaligenes eutrophus*. *Journal of Biotechnology* 5 (17): 1524–1527.
- Avella, M., Malinconico, M. and Orlando, P. 2004. Chemistry and biology of Polymer Degradation. In *Low Environmental Impact Polymers* (eds. T. Nick and J. Mark). Rapra Technology.
- Barnard, G. N. and Sanders, J. K. M. 1989. The Poly-beta-hydroxybutyrate granule *in vivo* : A new insight based on NMR spectroscopy of whole cells. *Journal of Biological Chemistry* 264 (6): 3286–3291.
- Bassetti, L. and Johannes, T. 1994. Organic solvent toxicity in *Morinda citrifolia* cell suspensions. *Enzyme and Microbial Technology* 16: 642–648.
- Bengtsson, S., W. A. C. M. and Welander, T. 2008. Production of Polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. *Bioresource Technology* 99: 509–516.
- Bhubalan, K., Lee, W.-H., Loo, C.-Y., Yamamoto, T., Tsuge, T., Doi, Y. and Sudesh, K. 2008. Controlled biosynthesis and characterization of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) from mixtures of palm kernel oil and 3-HV precursors. *Polymer Degradation and Stability* 93: 17–23.
- Bormann, E. J., Leibner, M., Roth, M., Beer, B. and Metzner, K. 1998. Production of Polyhydroxybutyrate by *Ralstonia eutropha* from protein hydrolysate. *Applied Microbiology and Biotechnology* 50: 604–607.

- Braunegg, G., Sonnleitner, B. and Lafferty, R. M. 1978. A rapid gas chromatographic method for the determination of Poly-3-hydroxybutyric acid in microbial biomass. *European Journal Applied Microbiology and Biotechnology* 6: 29–37.
- Carraher, C. E. J. 2008. *Seymour/Carraher's polymer chemistry*. 7th edn. USA: CRC Press Taylor and Francis Group.
- Castilho, L. R., Mitchell, D. A. and Freire, D. M. G. 2009. Production of Polyhydroxyalkanoates (PHA) from waste materials and by-products by submerged and solid-state fermentation. *Bioresource Technology* 100: 5996–6009.
- Chakraborty, P., Gibbons, W. and Muthukumarappan, K. 2009. Conversion of volatile fatty acids into Polyhydroxyalkanoate by *Ralstonia eutropha*. *Journal of Applied Microbiology* 106: 1996–2005.
- Chen, Y., Chen, J., Yu, C., Du, G. and Lun, S. 1999. Recovery of Poly-3-hydroxybutyrate from *Alcaligenes eutrophus* by surfactant-chelate aqueous system. *Process Biochemistry* 34: 153–157.
- Chen, Y., Xu, Q., Yang, H. and Gu, G. 2001a. Effects of cell fermentation time and biomass drying strategies on the recovery of Poly-3-hydroxyalkanoates from *Alcaligenes eutrophus* using a surfactant-chelate aqueous system. *Process Biochemistry* 36: 773–779.
- Chen, Y., Yang, H., Zhou, Q., Chen, J. and Gu, G. 2001b. Cleaner recovery of Poly(3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus*. *Process Biochemistry* 36: 501–506.
- Choi, G. G., Kim, H. W., Kim, Y. B. and Rhee, Y. H. 2005. Biocompatibility of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolyesters produced by *Alcaligenes* sp. *Biotechnology and Bioprocess Engineering* 10: 540–545.
- Choi, G. G., Kim, M. W., Kim, J.-Y. and Rhee, Y. H. 2003. Production of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with high molar fractions of 3-hydroxyvalerate by a threonine-overproducing mutant of *Alcaligenes* sp. SH-69. *Biotechnology Letters* 25: 665–670.
- Choi, J.-i. and Lee, S. Y. 1999. Efficient and economical recovery of Poly(3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. *Biotechnology and Bioengineering* 62 (5): 546–553.
- Chung, Y. J., Cha, H. J., Yeo, J. S. and Yoo, Y. J. 1997. Production of Poly(3-hydroxybutyric-co-3-hydroxyvaleric) acid using propionic acid by pH regulation. *Journal of Fermentation and Bioengineering* 83 (5): 492–495.
- Dai, Y., Yuan, Z., Jack, K. and Keller, J. 2007. Production of targeted Poly(3-hydroxyalkanoates) copolymers by glycogen accumulating organisms using acetate as sole carbon source. *Journal of Biotechnology* 129 (129): 489–497.

- de Koning, G. J. M., Kellerhals, M., van Meurs, C. and Witholt, B. 1997. A process for the recovery of Polyhydroxyalkanoates) from *Pseudomonads*. Part 2 : Process development and economic evaluation. *Bioprocess Engineering* 17: 15–21.
- Dhariwal, A., Mohrdieck, M., Leitermann, F., Arjol, I. M., Manresa, A., Syldatk, C., Janke, H. D. and Chmiel, H. 2008. Effect of emulsified feeding of oily substrate via submerged ceramic membranes on surfactant production in *Pseudomonas aeruginosa* fermentation. *Bioprocess Biosystem Engineering* 31: 401–409.
- Doi, Y. 1990. Microbial polyester: Economic aspects of biopolymer production. In *Biotechnology of Biopolymers. From synthesis to patents* (eds. S. A and D. Y), 1107–1136. New York: EWiley-Vch. Verlag GmbH and Co. KGaA, Weinheim,.
- Du, G., Chen, L. X. L. and Jian, Y. J. 2004. High-efficiency production of bioplastics from biodegradable organic solids. *Journals of Polymers and the Environment* 12 (2): 89–94.
- Furrer, P., Sven, P. and Manfred, Z. 2007. Efficient recovery of low endotoxin medium-chain-length Poly([R]-3-hydroxyalkanoate) from bacterial biomass. *Journal of Microbiological Methods* 69: 206–213.
- Galaev, I. and Mattiasson, B. 2008. Smart polymer : Applications in Biotechnology and Biomedicine. In *Microgels from smart polymers* (eds. N. Kausar, B. Z. Chowdry, and M. J. Snowden), 1–472. Taylor and Francis Group.
- Galan, B., Dinjaski, N., Maestro, B., de Eugenio, L. I., Escapa, I. F., Sanz, J. M., Garcia, J. L. and Prieto, M. A. 2011. Nucleoid-associated PhaF Phasin drivers intracellular location and segregation of Polyhydroxyalkanoate granules in *Pseudomonas putida* KT2442. *Molecular Microbiology* 79 (2): 402–418.
- Gayet, J. C. and Masaro, L. 2004. Polyhydroxyalkanoates : The next generation of bioplastics. In *Chemistry and biology of polymer degradation* (eds. T. Nick and J. Mark), *Low Environmental Impact Polymers* , 239–248. Rapra Technology.
- Gerald, S. 1999. *Polymers and the environment*. Cambridge : Royal Society of Chemistry.
- Goncalves, S. P. C., Martins-Franchetti, S. M. and Chinaglia, D. L. 2009. Biodegradation of the films of PP, PHBV and its blends in soil. *Journal Polymer Environment* 17: 280–285.
- Gursel, I., Balcik, C., Arica, Y., Akkus, O., Akkas, N. and Hasirci, V. 1998. Synthesis and mechanical properties of inter-penetrating networks of Polyhydroxybutyrate-co-hydroxyvalerate and Polyhydroxyethyl methacrylate. *Biomaterials* 19: 1137–1143.

- Ha, C.-S. and Cho, W.-J. 2002. Miscibility, properties and biodegradability of microbial polyester containing blends. *Progress in Polymer Science* 27: 759–809.
- Hahn, S. K., Chang, Y. K., Kim, B. S. and Chang, H. N. 1994. Optimization of microbial Poly(3-hydroxybutyrate) recover using dispersions of sodium hypochlorite solution and chloroform. *Biotechnology and Bioengineering* 44 (2): 256–261.
- Hahn, S. K., Chang, Y. K. and Lee, S. Y. 1995. Recovery and characterisation of Poly (3-hydroxybutyrate) synthesized in *Alcaligenes eutrophus* and recombinant *E. coli* . *Applied and Environmental Microbiology* 61: 34–39.
- Hashim, M. M., Mingsheng, D., Iqbal, M. F. and Xiaohong, C. 2011. Ginger rhizome as potential source of milk coagulating cysteine protease. *Phytochemistry* 72: 458–464.
- Hassan, M. A., Nawata, O., Yoshihito, S., Nor Aini, A. R., Phang, L. Y., Ariff, A. B. and Abdul Karim, M. I. 2002. A proposal for zero emission from palm oil industry incorporating the production of Polyhydroxyalkanoates from palm oil mill effluent. *Journal Chemistry Engineering Japan* 35: 9–14.
- Hassan, M. A., Shirai, Y., Kusubayashi, N., Abdul Karim, M. I., Nakanishi, K. and Hashimoto, K. 1996. Effect of organic acid profiles during anaerobic treatment of palm oil mill effluent on the production of Polyhydroxyalkanoates by *Rhodobacter sphaeroides*. *Journal of Fermentation and Bioengineering* 82 (2): 151–156.
- Hassan, M. A., Yoshihito, S., Umeki, H., Yamazumi, H., Jin, S., Yamamoto, S., Abdul Karim, M. I., Nakanishi, K. and Hashimoto, K. 1997. Acetic acid separation from anaerobically treated palm oil mill effluent by ion exchange resins for the production of Polyhydroxyalkanoate by *Alcaligenes eutrophus*.. *Bioscience Biotechnology Biochemistry* 61 (9): 1465–1468.
- Huang, T.-Y., Duan, K.-J., Huang, S.-Y. and Chen, C. W. 2006a. Production of Polyhydroxyalkanoates from inexpensive extruded rice bran and starch by *Haloferaz mediterranei*. *Journal Indian Microbiology Biotechnology* 33: 701–706.
- Jacquel, N., Lo, C. W., Wei, Y. H., Wu, H. S. and Wang, S. S. 2007. Solubility of Polyhydroxyalkanoates by experiment and thermodynamic correlations. *AIChE Journal* 53: 2704–2714.
- Jacquel, N., Lo, C. W., Wei, Y.-H., Wu, H.-S. and Wang, S. S. 2008. Isolation and purification of bacterial Poly(3-Hydroxyalkanoates). *Biochemical Engineering Journal* 39: 15–27.
- Jiang, X., Ramsay, J. A. and Ramsay, B. A. 2006. Acetone extraction of Mcl-PHA from *Pseudomonas putida* KT2440. *Journal of Microbiological Methods* 67: 212–219.

- Jung, K., Hany, R., Rentsch, D., Storni, T., Egli, T. and Witholt, B. 2000. Characterization of new bacterial copolyesters containing 3-hydroxyalkanoates and Acetoxy-3-hydroxyalkanoates. *Macromolecules* 33: 8571–8575.
- Kai, Z., Ying, D. and Guo-Qiang, C. 2003. Effects of surface morphology on the biocompatibility of Polyhydroxyalkanoates. *Biochemical Engineering Journal* 16: 115–123.
- Kasuya, K.-I., Inoue, Y., Tanaka, T., Akehata, T., Iwata, T., Fukui, T. and Doi, Y. 1997. Biochemical and molecular characterization of the Polyhydroxybutyrate depolymerase of *Comamonas acidovorans* YM1609, isolated from fresh water. *Applied and Environmental Microbiology*. 63 (12): 4844–4852.
- Kathiraser, Y., Aroua, M. K., Ramachandran, K. B. and Tan, I. K. P. 2007. Chemical characterization of medium-chain-length Polyhydroxyalkanoates (PHA) recovered by enzymatic treatment and ultrafiltration. *Journal of Chemical Technology and Biotechnology* 82: 847–855.
- Keenan, T. M., Nakas, J. P. and Tanenbaum, S. 2006. Polyhydroxyalkanoate copolymers from forest biomass. *Journal of Industrial Microbiology and Biotechnology* 33: 616–626.
- Kek, Y.-K., Lee, W.-H. and Sudesh, K. 2008. Efficient bioconversion of palm acid oil and palm kernel acid oil to Poly(3-hydroxybutyrate) by *Cupriavidus necator*. *Canadian Journal Chemistry* 86: 535–539.
- Khanna, S. and Srivastava, A. K. 2005. A simple structured mathematical model for Biopolymer (PHB) production. *Biotechnology Progress* 21: 830–838.
- Khosravi-Darani, K., Vasheghani-Farahani, E., Shojaosadati, S. A. and Yamini, Y. 2004. Effect of process variables on supercritical fluid disruption of *Ralstonia eutropha* cells for Poly(R-hydroxybutyrate) recovery. *Biotechnology Progress* 20: 1757–1765.
- Kim, H.-Y., Park, J.-S., Shin, H.-D. and Lee, Y.-H. 1995. Isolation of glucose utilizing mutant of *Alcaligenes eutrophus*, its substrate selectivity, and accumulation of Poly-b-hydroxybutyrate. *Journal of Microbiology* 51–58.
- Kim, S. and Dale, B. E. 2005. Life cycle assessment study of biopolymers (Polyhydroxyalkanoates) derived from no-tilled corn. *International Journal Life Cycle Assessment* 10 (3): 200–210.
- Koller, M., Bona, R., Braunegg, G., Hermann, C., Horvat, P., Kroutil, M., Martinz, J., Neto, J., Pereira, L. and Varila, P. 2005. Production of Polyhydroxyalkanoates from agricultural waste and surplus materials. *Biomacromolecules* 6: 561–565.
- Kumar, S., Mudliar, S. N., Reddy, K. M. K. and Chakrabarti, T. 2004. Production of biodegradable plastics from activated sludge generated from a food processing industrial wastewater treatment plant. *Bioresource Technology* 95: 327–330.

- Kuusipalo, J. 2000a. PHB/V in extrusion coating of paper and paperboard : Study of functional properties. Part 1. *Journal of Polymers and the Environment* 8 (1): 39–47.
- Kuusipalo, J. 2000b. PHB/V in extrusion coating of paper and paperboard : Study of functional properties. Part 2. *Journal of Polymers and the Environment* 8 (2): 49–57.
- Lageveen, R. G., Huisman, G. W., Preusting, H., Ketelaar, P., Eggink, G. and Witholt, B. 1988. Formation of polyesters by *Pseudomonas oleovorans* : Effect of substrates on formation and composition of Poly-R-3-hydroxyalkanoates and Poly-3-hydroxyalkanoates. *Applied and Environmental Microbiology* 54 (12): 2924–2932.
- Lakshman and Shamala. 2006. Extraction of Polyhydroxyalkanoate from *Sinorhizobium meliloti* cells using *Microbispora* sp. culture and its enzyme. *Enzyme and Microbial Technology* 39 (39): 1471–1475.
- Lawrence, A. G., Schoenheit, J., He, A., Tian, J., Liu, P., Stubbe, J. and Sinskey, A. J. 2005. Transcriptional analysis of *Ralstonia eutropha* genes related to Polyhydroxybutyrate homeostasis during batch fermentation. *Applied Microbiology and Biotechnology* 68: 663–672.
- Lee, K.-M. and Gilmore, D. F. 2005. Formulation and process modeling of biopolymer (Polyhydroxyalkanoates : PHA) production from industrial wastes by novel crossed experimental design. *Process Biochemistry* 40: 229–246.
- Lee, S. and Yu, J. 1997. Production of biodegradable thermoplastics from municipal by a two-stage bioprocess. *Resources, Conservation and Recycling* 19: 151–164.
- Lee, S. Y. and Chang, H. N. 1995. Production of Poly (hydroxyalkanoic acid). *Advanced Biochemistry Engineering Biotechnology* 52: 27–58.
- Lee, S. Y., Park, S. J., Park, J. P., Lee, Y. and Lee, S. H. 2005. Metabolic flux analysis on the production of Poly(3-hydroxybutyrate). In *Biotechnology of biopolymers. From synthesis to patents* (eds. A. Steinbuchel and Y. Doi), 193–206. EWiley-Vch. Verlag GmbH and Co. KGaA, Weinheim.
- Lee, W.-H., Loo, C.-Y., Nomura, Christopher, T. and Sudesh, K. 2008. Biosynthesis of Polyhydroxyalkanoates copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. *Bioresource Technology* 99: 6844–6851.
- Lee, W.-K., Ryou, J.-h. and Ha, C.-S. 2003. Retardation of enzymatic degradation of microbial polyesters using surface chemistry : Effect of addition of non-degradable polymers. *Surface Science* 542: 235–243.
- Leonard, Y. M. 2004. Synthesis of polymers from sustainable resource origin raw materials. In *Low Environmental Impact Polymers* (eds. T. Nick and J. Mark), *Low Environmental Impact Polymers* , 2. Rapra Technology.

- Leung, W. W. F. 2007. *Centrifugal separations in Biotechnology*. Academic Press.
- Liu, W.-T., Hanada, S., Marsh, Terrence, L., Kamagata, Y. and Nakamura, K. 2002. *Kineosphaera limosa* gen. nov., a novel Gram-positive Polyhydroxyalkanoate accumulating coccus isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology* 52: 1845–1849.
- Lo, C. W., Wu, H. S. and We, Y. H. 2011. High throughput study of separation of Poly(3-hydroxybutyrate) from recombinant *Escherichia coli* XL1 blue. *Journal of the Taiwan Institute of Chemical Engineers* 42: 240–246.
- Loo, C.-Y., Lee, Wing-Hin, T. T., Doi, Y. and Sudesh, K. 2005. Biosynthesis and characterization of Poly3-hydroxybutyrate-co-3-hydroxyhexanoate from palm oil products in a *Wautersia eutropha* mutant. *Biotechnology Letters* 27: 1405–1410.
- Loo, C. Y. and Sudesh, K. 2007. Polyhydroxyalkanoates: Bio-based microbial plastics and their properties. *Malaysian Polymer Journal* 2: 31–57.
- Luengo, J. M., Garcia, B., Sandoval, A., Naharro, G. and Olivera, E. R. 2003. Bioplastics from microorganisms. *Current Opinion in Microbiology* 6: 251–260.
- Luo, R., Chen, J., Zhang, L. and Chen, G. 2006. Polyhydroxyalkanoate copolyesters produced by *Ralstonia eutropha* PHB<sup>-4</sup> harboring a low-substrate-specificity PHA synthase PhaC2ps from *Pseudomonas stutzeri* 1317. *Biochemical Engineering Journal* 32 (32): 218–225.
- Madison, L. L. and Huisman, G. W. 1999. Metabolic engineering of Poly(3-Hydroxyalkanoates) : From DNA to plastic. *Microbiology and Molecular Biology Review* 63 (1): 21–53.
- Mengmeng, C., Hong, C., Qingliang, Z., Shirley, S.-N. and Jie, R. 2009. Optimal production of Polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation. *Biore-source Technology* 100: 1399–1405.
- Misra, S. K., Valappil, S. P., Roy, I. and Boccaccini, A. R. 2006. Polyhydroxyalkanoates (PHA)/inorganic phase composites for tissue engineering applications. *Biomacromolecules* 7 (8): 2249–2257.
- Mitra, M. 2011. *Alternative recovery methods of intracellular Polyhydroxyalkanoates from local isolate Comamonas* sp. EB172. PhD thesis, Faculty of Biotechnology and Biomolecular Science. Universiti Putra Malaysia.
- Mittendorf, V., Robertson, Elizabeth, J., Leech, R. M., Kruger, N., Steinbuchel, A. and Poirier, Y. 1998. Synthesis of medium-chain length Polyhydroxyalkanoates in *Arabidopsis thaliana* using intermediates of peroxisomal fatty acid beta-oxidation. In *Proceedings National Academy of Science*, 13397–13402. File1-27,6-40.

- Mostert, M. E., Botha, B. M., Du Plessis, L. M. and Duodu, K. G. 2007. Effect of fruit ripeness and method of fruit drying on the extractability of Avocado oil with hexane and supercritical carbon dioxide. *Journal of the Science of Food and Agriculture* 87: 2880–2885.
- Mumtaz, T. 2010. *Production of P(3-hydroxybutyrate-co-3-hydroxyvalerate) using Comamonas sp. EB172 from organic acids derived from anaerobic treatment of palm oil mill effluent*. PhD thesis, Faculty of Biotechnology and Biomolecular Sciences. Universiti Putra Malaysia.
- Mumtaz, T., Abd-Aziz, S., Abdul Rahman, N. A., Phang, L. Y., Shirai, Y. and Hassan, M. A. 2008. Pilot-scale recovery of low molecular weight organic acids from anaerobically treated palm oil mill effluent (POME) with energy integrated system. *African Journal of Biotechnology* 7 (21): 3900–3905.
- Mumtaz, T., Abd-Aziz, S., Abdul Rahman, N. A., Phang, L. Y., Shirai, Y. and Hassan, M. A. 2009. Fed-batch production of P(3HB-co-3HV) copolymer by *Comamonas sp. EB172* using mixed organic acids under dual nutrient limitation. *European Journal of Scientific Research* 33 (3): 374–384.
- Mumtaz, T., Abd-Aziz, S., Phang, L. Y., Wan Yunus, W. Z., Shirai, Y. and Hassan, M. A. 2010. Synthesis, characterization and structural properties of intracellular copolyester Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) produced by *Comamonas sp. EB172* from renewable resource. *International Journal of Polymer and Analytical Characteristics* 15: 329–340.
- Nafi, A., Foo, H. L., Jamilah, B. and Ghazali, H. M. 2013. Properties of proteolytic enzyme from ginger (*Zingiber officinale* Roscoe). *International Food Research Journal* 20 (1): 363–368.
- Nath, A., Dixit, M., Bandiya, A., Chavda, S. and Desai, A. J. 2008. Enhanced PHB production and scale-up studies using cheese whey in fed-batch culture of *Methylobacterium sp. ZP24*. *Bioresource Technology* 99: 5749–5755.
- Neumann, L., Spinozzi, F., Sinibaldi, R., Rustichelli, F., Potter, M. and Steinbuechel, A. 2008. Binding of the major Phasin, PhaP1 from *Ralstonia eutropha* H16 to Poly(3Hydroxybutyrate) granules. *Journal of Bacteriology* 190 (8): 2911–2919.
- Nomura, C. T., Taguchi, K., Gan, Z., Kuwabara, K., Tanaka, T., Takase, K. and Doi, Y. 2005. Expression of 3-Ketoacyl carrier protein reductase (fabG) genes enhances production of Polyhydroxyalkanoate copolymer from glucose in recombinant *Escheria coli* JM109. *Applied and Environmental Microbiology* 71 (8): 4297–4306.
- Nubia, M., Ivonne, G., Dionisio, M., Victoria, G., Dolly, R., Diego, S., Juan, G., Fabio, A., Armando, E. and Dolly, M. 2007. Bioprospecting and characterization of Polyhydroxyalkanoate (PHAs) producing bacteria isolated from Colombian sugarcane producing areas. *African Journal of Biotechnology* 6 (13): 1536–1543.



- Ojumu, T. V., Yu, J. and Solomon, B. O. 2004. A review: Production of Polyhydroxyalkanoates, A bacterial biodegradable polymer. *Journal of Biotechnology* 3 (1): 18–24.
- Oliveira, F. C., Dias, Macros, L., Castilho, Leda, R. and Freire, D. M. G. 2007. Characterization of Poly(3-hydroxybutyrate) produced by *Cupriavidus necator* in solid-state fermentation. *Bioresource Technology* 98: 633–638.
- Ong, L., Dagastine, R. R., Kentish, S. E. and Gras, S. L. 2011. Microstructure of milk gel and cheese curd observed using Cryo Scanning Electron Microscopy and confocal microscopy. *LWT - Food Science and Technology* 44: 1291–1302.
- Osborne, S. J., Leaver, J. and Turner, M. K. and Dunnill, P. 1990. Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. *Enzyme and Microbial Technology* 12: 281–291.
- Page, W. J., Manchak, J. and Rudy, B. 1992. Formation of Poly(hydroxybutyrate-co-hydroxyvalerate) by *Azobacter vinelandii* UWD. *Applied and Environmental Microbiology* 58 (9): 2866–2873.
- Patel, M., Gapes, D. J., Newman, R. H. and Dare, P. H. 2009. Physico-chemical properties of Polyhydroxyalkanoate produced by mixed-culture nitrogen-fixing bacteria. *Applied Microbiology and Biotechnology* 82: 545–555.
- Peters, M. S. and Timmerhaus, Klaus, D. 2002. *Plant design and economics for chemical engineers*. 4th edn. McGraw-Hill International, New York.
- Phang, L. Y., Hassan, M. A., Yoshihito, S., Wakisaka, M. and Abdul Karim, M. I. 2003. Continuous production of organic acids from palm oil mill effluent with sludge recycle by the freezing-thawing method. *Journal Chemistry Engineering. Japan* 36: 707–710.
- Philip, S., Sengupta, S., Keshavarz, T. and Roy, I. 2009. Effect of impeller speed and pH on the production of Poly(3-hydroxybutyrate) using *Bacillus cereus* SPV. *Biomacromolecules* 10: 691–699.
- Platt, D. K. 2006. *Biodegradable polymer : Market report*. Smithers Rapra Limited, United Kingdom.
- Poirier, Y., Nawrath, C. and Somerville, C. 1995. Production of Polyhydroxyalkanoates. A Family of biodegradable plastics and elastomers in bacteria and plants. *Biotechnology* 13: 142–150.
- Pouton, C. W. and Akhtar, S. 1996. Biosynthetic Polyhydroxyalkanoates and their potential in drug delivery. *Advanced Drug Delivery Reviews* 18: 133–162.
- Ramsay, B. A., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P. and Ramsay, J. A. 1990. Production of Poly(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. *Applied and Environmental Microbiology* 56 (7): 2093–2098.

- Ramsay, B. A., Znoj, G. M. and Cooper, D. G. 1986. Use of a nylon manufacturing waste as an industrial fermentation substrate. *Applied and Environmental Microbiology* 52 (1): 152–156.
- Ramsay, J. A., Berger, E., Voyer, R., Chavarie, C. and Ramsay, B. A. 1994. Extraction of Poly-3-hydroxybutyrate using chlorinated solvents. *Biotechnology Technology* 8: 589–594.
- Reddy, S. V., Thirumala, M. and Mahmood, S. K. 2009. A novel *Bacillus* sp. accumulating Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from a single carbon substrate. *Journal Indian Microbiology and Biotechnology* 36: 837–843.
- Rhim, J. W., Koh, S. and Kim, J. M. 2011. Effect of freezing temperature on rehydration and water vapor adsorption characteristics of freeze-dried rice porridge. *Journal of Food Engineering* 104: 484–491.
- Ribera, R. G., Monteoliva-Sanchez, M. and Ramos-Cormenzana, A. 2001. Production of Polyhydroxyalkanoates by *Pseudomonas putida* KT2442 harboring pSK2665 in wastewater from olive oil mills (Alpechin). *Journal of Biotechnology* 4 (2): 116–119.
- Riis, V. and Mai, W. 1988. Gas chromatographic determination of Poly-Beta Hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. *Journal of Chromatography* 445: 285–289.
- Rossi, S., Azghani, A. O. and Omri, A. 2004. Antimicrobial efficacy of a new antibiotic-loaded Poly(hydroxybutyric-co-hydroxyvaleric acid) controlled release system. *Journal of Antimicrobial Chemotherapy* 54: 1013–1018.
- Rothermich, M. M., Guerrero, R., Lenz, Robert, W. and Goodwin, S. 2000. Characterization, seasonal occurrence, and diel fluctuation of Polyhydroxyalkanoate in photosynthetic microbial mats. *Applied and Environmental Microbiology* 66 (10): 4279–4291.
- Salmiati, Z., Ujang, M. R., Salim, M. F. M. D. and Ahmad, M. A. 2007. Intracellular biopolymer productions using mixed microbial cultures from fermented POME. *Water Science and Technology* 56 (8): 179–185.
- Santhanam, A. and Sasidharan, S. 2010. Microbial production of Polyhydroxyalkanoates (PHA) from *Alcaligenes* spp. and *Pseudomonas oleovorans* using different carbon sources. *African Journal of Biotechnology* 9 (21): 3144–3150.
- Savenkova, L., Gercberga, Z., Bibers, I. and Kalnin, M. 2000. Effect of 3-hydroxy content on some physical and mechanical properties of Polyhydroxyalkanoates produced by *Azobacter chroococcum*. *Process Biochemistry* 36: 445–450.
- Shamala, T. R., Chandrashekar, A., Vijayendra, S. V. N. and Kshama, L. 2003. Identification of Polyhydroxyalkanoate (PHA)-producing *Bacillus* spp. using the Polymerase Chain Reaction (PCR). *Journal of Applied Microbiology* 94: 369–374.

- Shi, F., Ashby, R. D. and Gross, R. A. 1997. Fractionation and characterization of microbial polyesters containing 3-Hydroxybutyrate and 4-Hydroxybutyrate repeat units. *Macromolecules* 30: 2521–2523.
- Sim, K. H. 2003. *Utilisation of organic acids from anaerobically treated palm oil mill effluent with or without sludge recycle for Polyhydroxyalkanoate production*. PhD thesis, Faculty of Biotechnology and Biomolecular Sciences. Universiti Putra Malaysia.
- Solaiman, D. K. Y., Ashby, Richard, D., Foglia, T. A. and Marmer, W. N. 2006. Conversion of agricultural feedstock and coproducts into Polyhydroxyalkanoates. *Applied Microbiology and Biotechnology* 71: 783–789.
- Song, J. J., Yoon, S. C., Yu, S. M. and Lenz, R. W. 1998. Differential scanning calorimetric study of Poly(3-hydroxyoctanoate) inclusions in bacterial cells. *International Journal of Biological Macromolecules* 23: 165–173.
- Steinbuchel, A. 1992. Biodegradable plastics. *Current Opinion in Biotechnology* 3: 291–297.
- Sudesh, K., Bhubalan, K., Chuah, J.-A., Kek, Y.-K., Kamilah, H., Sridewi, N. and Lee, Y.-F. 2011. Synthesis of Polyhydroxyalkanoates from palm oil and some new applications. *Applied Microbiology and Biotechnology* 89: 1373–1386.
- Sujatha, K., Mahalakshmi, A. and Shenbagarathai, R. 2007. Molecular characterization of *Pseudomonas sp.* LDC-5 involved in accumulation of Poly 3-hydroxybutyrate and medium-chain-length Poly 3-hydroxyalkanoates. *Archives of Microbiology* 188 (5): 451–462.
- Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M. and Shah, S. 2007. Biotechnological approaches for the production of Polyhydroxyalkanoates in microorganisms and plants - A review. *Biotechnology Advances* 25: 148–175.
- Suzuki, T., Yamane, T. and Shimizu, S. 1986. Mass production of Poly-3-hydroxybutyric acid by fully automatic fed-batch culture of *Methyltroph*. *Applied Microbiology and Biotechnology* 23: 322–329.
- Taguchi, K., Taguchi, S., Sudesh, K., Maehara, A., Tsuge, T. and Doi, Y. 2005. Metabolic pathways and engineering of PHA biosynthesis. In *Biotechnology of biopolymers. From synthesis to patents* (eds. A. Steinbuchel and Y. Doi), 163. EWiley-Vch. Verlag GmbH and Co. KGaA, Weinheim.
- Tajima, K., Igari, T., Nishimura, D., Nakamura, M., Satoh, Y. and Munekata, M. 2003. Isolation and characterization of *Bacillus sp.* INT005 accumulating Polyhydroxyalkanoate (PHA) from gas field soil. *Journal of Bioscience and Bioengineering* 95 (1): 77–81.
- Takabatake, H., Satoh, H., Mino, T. and Matsuo, T. 2002. PHA (Polyhydroxyalkanoate) production potential of activated sludge treating wastewater. *Water Science and Technology* 45 (12): 119–126.

- Tamer, I. M., Moo-Young, M. and Chisti, Y. 1998. Disruption of *Alcaligenes latus* for recovery of Poly(beta-hydroxybutyric acid) : Comparison of high-pressure homogenization, bead milling and chemically induced lysis. *Indian Engineering Chemical Resource* 37: 1807–1814.
- Valappil, Sabeel, P., Boccaccini, A. R., Bucke, C. and Roy, I. 2007. Polyhydroxyalkanoates in Gram-positive bacteria : Insight from the genera *Bacillus* and *Streptomyces*. *Antonie van Leeuwenhoek* 91: 1–17.
- Van-Thuoc, D., Quillaguaman, J., Mamo, G. and Mattiasson, B. 2007. Utilization of agricultural residues for Poly(3-hydroxybutyrate) production by *Halomonas boliviensis* LC1. *Journal of Applied Microbiology* 104: 420–428.
- van Wyk, J. P. H. 2001. Biotechnology and the utilization of biowaste as a resource for bioproduct development. *Trends in Biotechnology* 19 (15): 172–177.
- Voon, P. T. 2005. *Environmental friendly alternative methods for the recovery of intracellular Polyhydroxyalkanoates (PHA)*. PhD thesis, Faculty of Biotechnology and Biomolecular Sciences. Universiti Putra Malaysia.
- Wang, J., Fang, F. and Yu, H.-Q. 2007. Substrate consumptions and biomass growth of *Ralstonia eutropha* at various *So/X0* levels in batch culture. *Biore-source Technology* 98: 2599–2604.
- Wang, J. and Yu, J. 2001. Kinetic analysis on formation of Poly(3-hydroxybutyrate) from acetic acid by *Ralstonia eutropha* under chemically defined conditions. *Journal of Industrial Microbiology and Biotechnology* 26: 121–126.
- Wang, J., Yue, Z.-B., Sheng, G.-P. and Yu, H.-Q. 2010a. Kinetic analysis on the production of Polyhydroxyalkanoates from volatile fatty acids by *Cuprividus necator* with a consideration of substrate inhibition, cell growth, maintenance and product formation. *Biochemical Engineering Journal* 49: 422–428.
- Wang, X., Chen, Z., Chen, X., Pan, J. and Xu, K. 2010b. Miscibility, crystallization kinetics and mechanical properties of Poly(3-hydroxyvalerate-co-3-hydroxybutyrate) (PHBV)/ Poly(3-hydroxyvalerate-co-3-hydroxybutyrate-co-4-hydroxybutyrate) (P3/4HB) blends. *Journal of Applied Polymer Science* 117: 838–848.
- Weusthuis, R. A., Kessler, B., Dielissen, M. P. M., Witholt, B. and Eggink, G. 2005. Fermentative production of medium chain-length Poly(3hydroxyalkanoates). In *Biotechnology of biopolymers. From synthesis to patents* (eds. A. Steinbuchel and Y. Doi), 235. EWiley-Vch.Verlag GmbH and Co. KGaA, Weinheim.
- Witholt, B. and Kessler, B. 1999. Perspectives of medium chain length Poly(hydroxyalkanoates), a versatile set of bacterial bioplastics. *Current Opinion in Biotechnology* 10: 279–285.

- Wrobe, M., Zebrowski, J. and Szopa, J. 2004. Polyhydroxybutyrate synthesis in transgenic flax. *Journal of Biotechnology* 107 (107): 41–54.
- Wu, S.-T., Lin, Y.-C. and Too, J.-R. 2009. Continuous production of Poly(3-hydroxybutyrate-3-hydroxyvalerate) : Effects of C/N ratio and dilution/rate on HB/HV. *Korean Journal Chemical Engineering* 26 (2): 411–416.
- Xu, Y., Wang, R.-H., Koutinas, A. A. and Webb, C. 2010. Microbial biodegradable plastic production from a wheat-based biorefining strategy. *Process Biochemistry* 45: 153–163.
- Yamane, T., Chen, X.-F. and Ueda, S. 1996. Polyhydroxyalkanoate synthesis from alcohol during the growth of *Paracoccus denitrificans*. *Federation of European Microbiological Societies Microbiology Letters* 135: 207–211.
- Yan, Q., Du, G. and Chen, J. 2003. Biosynthesis of Polyhydroxyalkanoates (PHAs) with continuous feeding of mixed organic acids as carbon sources by *Ralstonia eutropha*. *Process Biochemistry* 39: 387–391.
- Yan, Q., Sun, Y., Ruan, L. and Chen, J. Y. 2005. Biosynthesis of short-chain-length Polyhydroxyalkanoates during the dual-nutrient-limited zone by *Ralstonia eutropha*. *World Journal of Microbiology and Biotechnology* 21: 17–21.
- Yang, Y.-H., Brigham, C. J., Budde, C. F., Boccazzi, P., Willis, L. B., Hassan, M. A., Mohd Yusof, Z. A., Rha, C. and Sinskey, A. J. 2010. Optimization of growth media components for Polyhydroxyalkanoates (PHA) production from organic acids by *Ralstonia eutropha*. *Applied Microbiology and Biotechnology* .
- Yasotha, K., Aroua, M. K., Ramachandran, K. B. and Tan, I. K. P. 2006. Recovery of medium-chain-length Polyhydroxyalkanoates (PHA) through enzymatic digestion treatments and ultrafiltration. *Biochemical Engineering Journal* 30 (3): 260–268.
- Yoon, J.-S., Chin, I.-J., Kim, M.-N. and Kim, C. 1996. Degradation of microbial polyesters: A theoretical prediction of molecular weight and polydispersity. *Macromolecules* 29 (29): 3303–3307.
- You, J.-W., Chiu, H.-J., Shu, W.-J. and Don, T.-M. 2003. Influence of hydroxyvalerate content on the crystallization kinetics of Poly(hydroxybutyrate-co-hydroxyvalerate). *Journal of Polymer Research* 10: 47–54.
- Yu, J., Si, Y. and Wong, W. K. R. 2002. Kinetics modeling of inhibition and utilisation of mixed volatile fatty acids in the formation of Polyhydroxyalkanoates by *Ralstonia eutropha*. *Process Biochemistry* 37: 731–738.
- Yu, S. T., Lin, C. C. and Too, J. R. 2005. PHBV production by *Ralstonia eutropha* in a continuous stirred tank reactor. *Process Biochemistry* 40: 2729–2734.

- Zakaria, M. R., Abd-Aziz, S., Arrifin, H., Abdul Rahman, N. A., Phang, L. Y. and Hassan, M. A. 2008. *Comamonas* sp. EB172 isolated from digester treating palm oil mill effluent as potential Polyhydroxyalkanoate (PHA) producer. *African Journal of Biotechnology* 7 (22): 4118–4121.
- Zakaria, M. R., Ariffin, H., Mohd Johar, N. A., Abd-Aziz, S., Nishida, H., Shirai, Y. and Hassan, M. A. 2010b. Biosynthesis and characterization of Poly(hydroxybutyric-*co*-hydroxyvaleric acid) copolymer from wild-type *Comamonas* sp. EB172. *Polymer Degradation and Stability* 95: 1382–1386.
- Zakaria, M. R., Tabatabaei, M., Mohamad Ghazali, F., Abd-Aziz, S., Shirai, Y. and Hassan, M. A. 2010a. Polyhydroxyalkanoates production from anaerobically treated palm oil mill effluent by new bacterial strain *Comamonas* sp. EB172. *World Journal Microbiology and Biotechnology* 26 (5): 767–774.