APPLICATION OF POLY (3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE) IN CONTROLLED RELEASE FERTILIZER FOR OIL PALM SEEDLINGS

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By

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TABLE OF CONTENTS

ACKNOWLEDGEMENT		**ii**
TABLE OF CONTENTS		**iii**
LIST OF TABLES		**x**
LIST OF FIGURES		**xi**
LIST OF SYMBOL AND ABBREVIATIONS		**xiii**
ABSTRAK		**xviii**
ABSTRACT		**xx**
CHAPTER 1: INTRODUCTION		1
1.0	Introduction	1
1.1	Objectives of the study	4
CHAPTER 2: LITERATURE REVIEW		5
2.1	Fertilizers and environmental problems	5
2.2	Polyhydroxyalkanoates (PHAs)	5
2.3	Biosynthesis of PHA	8
2.4	Poly(3-hydroxybutyrate) [P(3HB)]	12
2.5	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)]	13
2.6	*Cupriavidus necator*	14
	2.6.1 Recombinant *Cupriavidus necator* Re2058/pCB113	15
2.7	Thermal properties of PHA	17
2.8	Melt viscoelastic properties of PHA	19
2.9	Single cell protein (SCP)	20
	2.9.1 *Cupriavidus necator* H16 as a source of SCP	22

2.10 Use of PHA in animal feed 23

2.11 Recovery of PHA from bacterial cells and purification methods 24

2.11.1 Pre-treatment processes 26

2.11.2 Dissolution of PHA using organic solvents 28

2.11.3 Extraction of PHA from cells using chemicals 29

2.11.4 Disruption of cells using detergents 29

2.11.5 Disruption of cells using sodium hypochlorite 30

2.11.6 Disruption of cells using enzymes 32

2.11.7 Mechanical disruption of cells 33

2.11.8 Extraction of PHA using supercritical fluids 33

2.11.9 Separation of PHA by air classification 34

2.11.10 Separation of PHA by dissolved-air flotation 35

2.12 Oil palm biomass waste in Malaysia 35

2.12.1 Oil palm trunks 35

2.12.2 Features of oil palm trunk 36

2.13 Controlled release fertilizer (CRF) 38

2.13.1 History of slow release fertilizers and controlled release fertilizers 39

2.13.2 Types of slow release fertilizers and controlled release fertilizers 41

2.13.3 Controlled release fertilizers coated with biodegradable polymer 42

2.14 Advantages of controlled release fertilizers 45

2.14.1 Efficient use of nitrogen source 45

2.14.2 Volatilization of ammonia and emission of nitrous oxide 45

2.14.3 Less toxicity 46

2.15 Disadvantages of controlled release fertilizers 46

2.15.1 Lack of standard methods 46

2.15.2 Accumulation of salts 48

2.16 Factors affecting the use of controlled release fertilizers release 48

2.16.1 High cost 48

2.16.2 Temperature 49

2.16.3 Other factors 50

2.17 Mechanism of nutrient release 51

CHAPTER 3: MATERIALS AND METHODS 52

3.1 Bacterial strain and maintenance 52

3.2 Carbon sources 53

3.3 Cultivation and PHA biosynthesis in shake flask 53

3.3.1 Cultivation and PHA biosynthesis in 13L fermenter 54

3.4 Sample preparation for gas chromatography (GC) analysis 55

3.4.1 Preparation of methanolysis solution 55

3.4.2 Preparation of caprylate methyl ester (CME) solution 56

3.4.3 Gas chromatography (GC) settings for analysis 56

3.4.4 Method of calculation for PHA content and monomer composition 57

3.5 Polymer extraction 57

3.6 Isolation of native PHA granules 58

3.7 Film casting 58

3.8 Feeding studies: Animals and experimental procedures 58

3.9 Purification of the fecal pellets 59

3.10 Characterization 59

3.10.1 Molecular weight analysis of PHA 59

3.10.2 Thermal analysis of PHA (DSC) 60

3.10.3 Thermogravimetric analysis of PHA (TGA) 60

3.10.4 Particle size measurement and scanning electron microscope (SEM) 61

3.10.5 Rheological properties 61

3.10.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 62

3.11 PHA based controlled release fertilizer 62

3.11.1 Preparation and treatments of oil palm seedlings 63

3.11.1(a) Control 63

3.11.1(b) Purified PHA with raw vascular bundles (PRVB) 63

3.11.1(c) Purified PHA with raw vascular bundles and fertilizer blend (PRVBF) 64

3.11.1(d) Purified PHA with grounded vascular bundles and fertilizer blend (PGVFB) 64

3.11.1(e) Immediate release fertilizer (IRF) 66

3.11.1(f) Controlled release fertilizer (CRF) 66

3.12 Plant care and maintenance 66

3.13 Water absorption test 67

3.14 Fertilizer release study in water 67

3.15 Physical measurements of plant growth 67

3.15.1 Plant height and number of leaves 67

3.15.2 Chlorophyll measurements 68

3.15.3 Stem thickness 68

3.15.4 Soil sampling for analyses 68

3.15.5 Fresh biomass 68

3.15.6 PHA degradation 70

3.15.7 Electrical conductivity 70

3.15.8 pH measurement 70

3.15.9 Total organic carbon analysis 71

3.16 Analysis of changes in microbial community profile by the fertilizer enriched soil 71

3.17 Extraction of total DNA from soil sample 71

3.18 Metagenomics analysis 71

3.18.1 Cluster generation and sequencing 73

3.18.2 Bioinformatics analysis 73

3.19 Statistical analysis 74

CHAPTER 4: RESULTS 75

4.1 Biosynthesis of PHA 75

4.1.1 Biosynthesis of P(3HB-*co*-3HHx) in shake flask 75

4.1.2 Biosynthesis of P(3HB-*co*-3HHx) using fermenter (Fed-Batch fermentation) 77

4.2 PHA Recovery and purification 79

4.3 Biological recovery of PHA by using mealworms 79

4.4 Purification of biologically recovered PHA granules 81

4.5 Characterization of biologically recovered P(3HB-*co*-3HHx) granules 83

4.5.1 PHA Purity (GC) and thermal analysis (DSC) 83

4.5.2 Molecular mass and rheology property of purified PHA 85

4.5.3 Thermogravimetric analysis of purified PHA (TGA) 88

4.6 Formulation of PHA-based controlled release fertilizer 90

4.7 Water absorption and fertilizer release in water for PHA-CRF 91

4.8 Evaluation of PHA-CRF on oil palm plants (nursery stage) 91

4.8.1 Effect of different fertilizer formulations on plant height 93

4.8.2 Effect of different fertilizer formulations on number of leaves 95

4.8.3 Effect of different fertilizer formulations on chlorophyll content 97

4.8.4 Effect of different fertilizer formulations on stem thickness 99

4.8.5 Effect of different fertilizer formulations on fresh biomass 99

4.8.6 Effect of different fertilizer formulations on soil pH 101

4.8.7 Effect of different fertilizer formulations on soil conductivity 103

4.8.8 PHA degradation in soil 105

4.8.9 Effect of different fertilizer formulations on total organic carbon content in soil 108

4.9 Effect of different fertilizer formulation on soil microbial community 109

4.9.1 16S metagenomics analysis (Bacterial community) 109

4.9.2 ITS metagenomics analysis (Fungal community) 111

4.10 Appearance of oil palm plants 113

CHAPTER 5: DISCUSSION

5.1 Biosynthesis of PHA 114

5.2 Biological Recovery of PHA and characterization 116

5.3 PHA-CRF formulation 121

5.4 Water absorption and fertilizer release study 122

5.5 Application of PHA-CRF and plant growth measurements 123

5.6 Soil properties 127

5.7 PHA degradation in soil 129

5.8 Soil microbial community analysis 130

CHAPTER 6: CONCLUSIONS 134

6.1 Conclusions 134

REFERENCES 136

APPENDICES 155

LIST OF PUBLICATIONS, CONFERENCES AND PATENTS 162

LIST OF TABLES

		Page
Table 2.1	Classes of PHA synthase	9
Table 3.3	Primers used in metagenomics study	72
Table 4.1	Effect of different concentrations of palm olein (PO) on biosynthesis of P(3HB-*co*-3HHx) by recombinant *C. necator* Re2058/pCB113	76
Table 4.2	Purity and melting temperature of P(3HB-*co*-3HHx) obtained from biological and solvent extraction methods	84
Table 4.3	Molecular Weight (Sampled after purification)	86
Table 4.4	Effect of PHA based controlled release fertilizer on total organic carbon in soil compared to the commercial fertilizer	108

LIST OF FIGURES

		Page
Figure 2.1	The general PHA structures	7
Figure 2.2	Proposed metabolic pathway for the biosynthesis of P(3HB-*co*-3HHx) from the fatty acids consisting of even carbon number	11
Figure 2.3	P(3HB) synthesis by wild type *C. necator* using plant oil (A), Construction of recombinant *C. necator* Re2058/pCB113 (B), P(3HB-*co*-3HHx) synthesis by recombinant *C. necator* Re2058/pCB113 using plant oil (C)	16
Figure 2.4	PHA recovery methods	27
Figure 2.5	Vascular bundles with vessels of the core oil palm trunk	37
Figure 2.6	The nitrogen cycle	47
Figure 3.1	Instruments and process involved during the formulation of PHA based controlled release fertilizer	65
Figure 3.2	Plant growth measurements	69
Figure 4.1	Fed-batch fermentations for P(3HB-*co*-3HHx) production by *C. necator* Re2058/pCB113 using PO as carbon source and urea as nitrogen source.	78
Figure 4.2	Process of biological recovery of PHA from *C. necator* cells using mealworms (A) and fecal pellets (inset), SEM micrographs of fecal pellet morphology (B), P(3HB-*co*-3HHx) granules on the surface of fecal pellet (C), SDS purified fecal pellets (D), DLS results of native granules (E) and SDS purified fecal pellets (F)	80
Figure 4.3	SDS-PAGE-Monitoring of protein removal from purified biologically recovered granules. Biologically recovered granules purified using water (A), 0.1% SDS (B), 0.5% SDS (C), 1% SDS (D), 1% SDS at 50 ℃ (E), 1% sodium hypochlorite (F), 1% Triton X-100 (G), 1% Urea (H) and 50 mM Tris-HCl (I).	82
Figure 4.4	Dynamic viscosity (A and B) of biologically recovered and chloroform extracted P(3HB-*co*-3HHx) samples as a function of time at 190 °C	87

Figure 4.5	TGA chromatograms (A and B) of biologically recovered, purified and chloroform extracted P(3HB-*co*-3HHx) copolymer.	89
Figure 4.6	Different formulations of PHA based controlled release fertilizers	90
Figure 4.7	Water absorption test using PHA based controlled release fertilizer formulations	92
Figure 4.8	Release of fertilizer in water from PHA based controlled release fertilizer formulations	92
Figure 4.9	Effect of different fertilizer formulations on plant height	94
Figure 4.10	Effect of different fertilizer formulations on number of leaves	96
Figure 4.11	Effect of different fertilizer formulations on leaf chlorophyll content	98
Figure 4.12	Effect of different fertilizer formulations on stem thickness	100
Figure 4.13	Effect of different fertilizer formulations on fresh biomass	100
Figure 4.14	Effect of different fertilizer formulations on soil pH	102
Figure 4.15	Effect of different fertilizer formulations on soil conductivity	104
Figure 4.16	Degradation of PHA in different fertilizer formulations	106
Figure 4.17	Appearance of PHA based controlled release fertilizer after 3 months of application	106
Figure 4.18	Scanning electron micrographs (SEM) of PHA based controlled release fertilizers before and after three months of application	107
Figure 4.19	Bacterial community present in the soil treated with PHA based controlled release fertilizer compared with commercial fertilizer	110
Figure 4.20	Fungal community present in the soil treated with PHA based controlled release fertilizer compared with commercial fertilizer	112
Figure 4.21	Appearance of representative oil palm plants	113

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
% DM	Percentage of dry matter
v/v	Volume per volume
w/v	Weight per volume
℃	Degree Celsius
3HA	3-hydroxyalkanoates
3HD	3-hydroxydecanoate
3HDD	3-hydroxydodecanoate
3HO	3-hydroxyoctanoate
4HB	4-hydroxybutyrate
5HV	5-hydroxyvalerate
AAPFCO	Association of American Plant Food Control Officials
AOAC	Association of official analytical chemists
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CID	Collision-induced dissociation
cm	Centimetre
CME	Caprylic methyl ester
CoA	Coenzyme A
CRF	Controlled release fertilizer
CTAB	Cetyltrimethylammonium bromide
DCW	Dry cell weight
DGGE	Denaturing gradient gel electrophoresis

DTT	Dithiothreitol
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimeter
dsDNA	Double stranded DNA
DTA	Differential thermal analysis
DTNB	Dithio-bis(2-nitrobenzoic acid)
EC	Electrical conductivity
EDTA	Ethylene diamine tetra acetic acid
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FTIR	Fourier transform infra-red
G	Gram
GC	Gas chromatography
GCMS	Gas chromatograph mass spectrometer
gDNA	Genomic DNA
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
IRF	Immediate release fertilizer
ITMS	Ion trap mass spectrometry
ITS	Internal transcribed spacer
kDa	Kilo Dalton
Kg	Kilo gram
L	Litre
LAS	Linear alkyl benzene sulfonic acid

LDPE	Low density polyethylene
M	Molarity
mcl	Medium chain length
mg	Magnesium
mHz	Megahertz
min	Minutes
mL	Millilitre
mm	Millimetre
MM	Mineral medium
M_n	Number average molecular weight
mol%	Mole percentage
MPa	Mega Pascal
MRFA	Methionine-Arginine-Phenyl-Alanine
M_w	Weight average molecular weight
N	Normality
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NMR	Nuclear magnetic resonance
NPK	Nitrogen Phosphorous Potassium
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PAGE	Poly acrylamide gel electrophoresis
PC-CRFs	Polymer coated controlled release fertilizers

PCR	Polymerase chain reaction
PDI	Polydispersity index
PEO	Poly(ethylene oxide)
PF	PHA with fertilizer
PGVFB	PHA grounded vascular bundles fertilizer blend
PHAs	Polyhydroxyalkanoates
PLA	Poly (lactic acid)
PO	Palm olein
PRVB	PHA raw vascular bundles
PRVBF	PHA raw vascular bundles fertilizer
PTFE	Poly(tetrafluoroethylene)
PTM	Post-translational modification
PTS	Poly(thioesters)
rDNA	Ribosomal deoxyribonucleic acid
rpm	Rotation per minute
rRNA	Ribosomal ribonucleic acid
$ScCO_2$	Supercritical carbon dioxide
scl	Short chain length
SCP	Single cell protein
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SRF	Slow release fertilizer
T_d	Degradation temperature
TGA	Thermo gravimetric analysis
T_m	Melting temperature

TOC	Total organic carbon
UV	Ultraviolet
WAXRD	Wide angle X-ray diffraction spectroscopy
wt%	Weight percentage
XRF	X-ray fluorescence spectrometry
µm	Micrometre
µmol	Micromolar

APLIKASI POLI(3-HIDROKSIBUTIRAT-*ko*-3-HIDROKSIHEKSANOAT) DALAM BAJA PELEPASAN TERKAWAL UNTUK ANAK KELAPA SAWIT

ABSTRAK

Polihidroksialkanoat (PHA), sejenis biopolimer yang disintesiskan oleh pelbagai jenis bakteria di bawah kekangan sumber nitrogen tetapi dalam kehadiran sumber karbon yang berlebihan. PHA adalah polimer bio-bolehurai dan boleh menjadi bahan yang sesuai untuk menggantikan polimer tidak bio-bolehurai yang digunakan dalam aplikasi baja pelepasan terkawal (CRF). Objektif kajian ini adalah untuk menghasilkan poli(3-hidroksibutirat-*ko*-3-hidroksiheksanoat) [P(3HB-*ko*-3HHx)] bersama dengan pembangunan pengolehan-semula PHA secara biologi. Ia turut melibatkan langkah penulenan dan akhirnya mengaplikasikan bahan untuk membangunkan CRF yang berasaskan PHA. Biosintesis P(3HB-*ko*-3HHx) telah dikaji menggunakan bahan mentah yang mengandungi minyak sawit (PO) oleh rekombinan *Cupriavidus necator* Re2058/pCB113 menggunakan kelalang goncangan. Kemudian, fermentasi secara "fed-batch" telah dijalankan menggunakan PO sebagai sumber karbon tunggal di dalam fermenter 13 L untuk mengkaji pergumpulan pecahan monomer 3HHx dalam sel bakteria pada waktu berbeza. Selain itu, proses pengolehan-semula PHA secara biologi telah dibangunkan menggunakan "mealworm" *Tenebrio molitor*. "Mealworm" didapati boleh memakan sel *C. necator* yang disejuk-beku kering dan mengeluarkan granul PHA dalam bentuk najis berwarna keputihan. Penulenan selanjutnya menggunakan air, detergen dan haba memberikan keputusan hampir 100% PHA tulen apabila diuji

menggunakan kromatografi gas. Perbandingan dengan pengekstrakan menggunakan kloroform menunjukkan tiada tanda pengurangan berat molekul. Granul PHA kemudian dikaji lanjut dengan beberapa siri ujian bagi menentukan ketulenan dan sifatnya. CRF berasaskan PHA telah dibangunkan untuk anak kelapa sawit menggunakan P(3HB-*ko*-25% mol 3HHx) yang dioleh semula secara biologi dengan baja komersial NPK. Prestasi PHA-CRF ditentukan berdasarkan pertumbuhan pokok. Penggabungan berkas vaskular batang pokok kelapa sawit bersama komposit PHA-CRF menunjukkan pertumbuhan yang lebih baik. PHA-CRF telah mengurangkan penggunaan baja kimia. Populasi dan kepelbagaian mikrobial tanah juga meningkat dengan adanya PHA-CRF. Kajian ini telah menunjukkan buat kali pertama kebolehgunaan PHA yang dioleh-semula secara biologi dalam membangunkan CRF untuk anak benih kelapa sawit.

APPLICATION OF POLY (3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE) IN CONTROLLED RELEASE FERTILIZER FOR OIL PALM SEEDLINGS

ABSTRACT

Polyhydroxyalkanoates (PHAs), a type of biopolymer, are synthesized by various types of bacteria under limited nitrogen source but in the presence of excess carbon source. PHAs are biodegradable and would be a suitable material to replace those non-biodegradable polymers used in controlled release fertilizer (CRF) application. The objectives of this study were to produce poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] coupled with development of biological recovery and purification steps and finally applying the material to develop PHA-based CRF. The biosynthesis of P(3HB-*co*-3HHx) was studied using feedstock consisting of palm olein (PO) by recombinant *C. necator* Re2058/pCB113 using shake flask. Then, fed-batch fermentation was carried out using PO as sole carbon source in 13 L fermenter to study the accumulation of 3HHx monomer fraction in the bacterial cells at different time point. Besides that, a biological recovery process of PHA was developed using mealworms, the larva of mealworm beetle (*Tenebrio molitor*). Mealworms readily consumed the freeze-dried *C. necator* cells and excreted PHA granules in the form of whitish feces. Further purification using water, detergent and heat resulted in almost 100% pure PHA granules when tested using gas chromatography. Comparison with chloroform extraction showed no signs of reduction in the molecular weight. The PHA granules were further subjected to a battery of tests to determine their purity and properties. The results obtained further

confirmed that the biological recovery and purification steps can be applied to extract PHA granules from bacterial cells. PHA-based CRF was developed for oil palm seedlings using the biologically recovered P(3HB-*co*-25 mol% 3HHx) with commercial NPK fertilizer. The performance of PHA-CRF was determined based on plant growth. The incorporation of oil palm tree trunk vascular bundles into the PHA-CRF composite showed improved growth. The PHA-CRF reduced the usage of chemical fertilizer. The soil microbial population and diversity were also shown to increase in the presence of PHA-CRF. This study has demonstrated for the first time the applicability of the biologically recovered PHA in developing CRF for oil palm seedlings.

CHAPTER 1

INTRODUCTION

1.0 Introduction

The demand for food is constantly increasing, in parallel with the ever growing world population. Currently, the agriculture industry consumes high amounts of fertilizer. However, nutrients from the conventional uncoated fertilizer are not completely available for uptake by the plants due to leaching, runoff and volatilization. Fertilizer loss increases the cost of agriculture production while causing more environmental pollution. Therefore, an innovation was introduced where fertilizers are coated to have them released in a controlled manner and to supply enough nutrients to meet the plants' demands at different growth stages. The use of non-biodegradable synthetic polymers such as polyolefin coatings and polyurethane-like resins lead to serious environmental problems because such polymers do not decompose naturally in the soil. Moreover it generates substantial amount of undesirable plastic residues accumulation in the soil. The polymer residues become smaller fragments than soil particles and hence reduce the quality of soil. Therefore, the use of non-biodegradable polymers for the polymer coated controlled release fertilizer (PC-CRFs) has raised various concerns. These issues are the motivating factors for researchers to look for natural biodegradable polymers to replace the synthetic non-biodegradable polymer. Therefore, CRF was developed using natural biodegradable polymer such as HPMC, chitosan and sodium alginate. However, these polymers have poor resistance to microorganisms. A plausible solution for this problem is the use of biodegradable polymers such as polyhydroxyalkanoates (PHAs). Polyhydroxyalkanoates (PHAs) are biological polyesters produced by certain bacteria as cell inclusion bodies when there is a

limitation of essential nutrients but in the presence of excessive carbon source (Anderson and Dawes, 1990; Pohlmann et al., 2006). PHAs are known to be degradable by soil bacteria and fungi (Boyandin et al., 2013) thus making them environmentally friendly plastics. Therefore, one can expect the PHA-based microplastics to be completely mineralized in the environment. Among the various types of PHAs that can be synthesized by bacteria, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] is a thermoplastic that has similar mechanical and physical properties comparable to some commodity plastics of petrochemical origin (Doi et al., 1995a). However, the cost of PHAs is more than petrochemical plastics. The production and use of PHAs at industrial level is still at its infancy due to the high cost (Li et al., 2016). One of the reasons for the high cost of PHAs is the recovery process. Since PHAs are accumulated in the bacterial cell cytoplasm, it is necessary to lyse the cells in order to recover the PHA granules. In addition, the PHA granules are closely associated with several types of proteins which are located on the surface of the granules (Sudesh et al., 2004; Bresan et al., 2016). Most of the PHA extraction processes are developed using organic solvents such as chloroform and dichloromethane (Choi and Lee, 1999b). The limitation of solvent based recovery method is such that it requires large amount of solvents which needs a recycling process. Reducing the amount of solvent for recovery leads to high viscosity of the polymer which makes the process difficult and affects the purity of the polymer. Many alternative methods were developed for solvent based recovery method such as chemical disruption by sodium hydroxide (NaOH) and sodium dodecyl sulphate (SDS), enzymatic digestion with the help of thermal pre-treatment and digestion using sodium hypochlorite (Choi and Lee, 1999b; Thakor et al., 2005). The detergent based recovery method is very efficient in disrupting the

cell components and there is no negative effect to the PHA, which is very important for the recovery process (Choi and Lee, 1999b). The drawback of detergent based recovery method is low purity of the resulting PHA and high detergent cost. Moreover it needs large amount of detergent to recover a small quantity of PHA and a large amount of water to remove the cell debris and detergent from the PHA (Jiang et al., 2006). Proteolytic enzymes such as alcalase can digest the proteins completely (De Koning et al., 1997). Dissolution of protein, lipids and other cell components can be achieved by micelles formation using anionic detergents such as SDS (Womack et al., 1983). The combination of alcalase and SDS is effective at the optimum pH and temperature. The reaction time can be reduced with a combination of enzymes and detergents (De Koning et al., 1997). In addition to these methods, many innovative methods are being investigated such as killer bug (Martínez et al., 2016) and secretion of the PHA (Rahman et al., 2013). The present study presents an alternative approach whereby the use of solvents and chemicals can be minimized. More importantly, in the process of recovering the PHA from the bacterial cells, the method described here makes use of the non-PHA cellular materials. As a proof of concept, we have recently demonstrated the use of bacterial cells with PHA as animal feed and subsequent recovery of the PHA from the animal's feces (Kunasundari et al., 2013). Here, we report a further improvement of the biological recovery process by using mealworms, the larvae of mealworm beetle, *Tenebrio molitor*. Mealworm can be grown in high densities, require less water and space, breed prolifically and can consume up to 10 wt% feed of their body weight per day. We have found that mealworm can be used to recover the PHA granules while the non-PHA cellular materials are digested by the mealworm. In contrast to a recent report on the degradation of polystyrene by mealworm (Yang et al., 2015), No

reduction in the molecular weight was observed from the recovered PHA granules. The PHA granules can be further purified using water and SDS. The purified PHA granules were characterized by gas chromatography, gel permeation chromatography, differential scanning calorimetry, thermogravimetric analysis and rheology. This study builds on and contributes to the concept of biological recovery process that we proposed recently (Kunasundari et al., 2013).

In this present study, production of P(3HB-*co*-3HHx) from palm olein and focused on the recovery of the synthesized P(3HB-*co*-3HHx) using a new biological recovery process utilizing mealworm instead of environmentally detrimental organic solvent. The recovered P(3HB-*co*-3HHx) were then purified and used to develop controlled-release fertilizer. Finally, the efficacy of the CRF was evaluated on oil palm seedlings.

1.1 Objectives of the study

The objectives of the study were:

1. To produce P(3HB-*co*-3HHx) from palm olein through fed-batch fermentation using Recombinant *C. necator* Re2058/pCB113.
2. To develop a new biological recovery process of PHA using mealworms and characterize the purified polymer.
3. To formulate PHA-based controlled release fertilizer and evaluate its efficiency on oil palm seedlings.

CHAPTER 2

LITERATURE REVIEW

2.1 Fertilizers and environmental problems

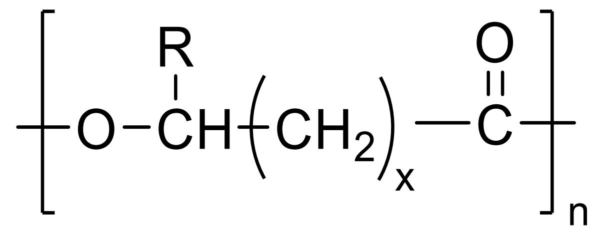
The global usage of NPK (nitrogen, phosphorous and potassium) fertilizer is forecasted to increase at the rate of 3.5 Metric tons in 2016/17 to fulfil the agriculture demand (Heffer and Prud'homme, 2012). Commonly, NPK fertilizers are used as plant nutrition. These NPK fertilizers are water soluble and applied in the form of uncoated pellets. Therefore, most of these nutrients are leached out and mixed with the ground water. It was reported by U.S Environmental Protection Agency (USEPA) that the level of nitrate in drinking water exceeding 10 mg/L is not safe for humans (Broschat, 1995; Entry and Sojka, 2007). Therefore, many studies have been carried out in order to develop controlled released fertilizer (CRFs) using non-biodegradable and biodegradable polymers. However, biodegradable polymers have gained much attention for the development of controlled release fertilizer as compared to the non-biodegradable polymers.

2.2 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are biopolymers of various hydroxyalkanoates (HAs) synthesized as intracellular carbon and energy storage compounds by various bacteria (Lee and Choi, 1999). PHA can be accumulated by Gram positive and Gram negative bacteria as well as Archaea (Reddy et al., 2003; Rehm, 2003). PHA accumulation has been shown to enhance bacteria survival during unfavourable conditions and serves as energy source for the formation of spores in microbes such as *Bacillus* species. PHA granules can be viewed under phase contrast

light microscope due to their high refractivity (Sudesh et al., 2000; Prabu and Murugesan, 2010) .

A French scientist by the name Lemoigne (1926) first discovered P(3HB) in *Bacillus megaterium*. Later, it was found that the accumulation of P(3HB) is affected by growth conditions. Apart from *B. megaterium*, P(3HB) can be produced by a wide range of microorganisms such as *Pseudomonas* sp. (Doudoroff and Stanier, 1959) and *Sphaerotilus* sp. (Rouf and Stokes, 1962). These microorganisms are capable of accumulating large amounts of P(3HB) granules. Besides P(3HB), approximately 150 different monomers have been identified and polymerized by various microorganisms which gave rise to polymers with wide range of physical and mechanical properties (Steinbüchel and Lütke-Eversloh, 2003). For example, the incorporation of 4-hydroxybutyrate (4HB) and 5-hydroxyvalerate (5HV) into polymer by *C. necator* was observed when chlorinated valeric acid was used as carbon source (Kunioka et al., 1988; Kunioka et al., 1989a). Polymer consisting of 3HAs copolymers other than 3HB was produced by *Pseudomonas oleovorans* using alkane (C6- C12) (Lageveen et al., 1988), alcohols (C6-C10) (Haywood et al., 1989) and alkanoates (C8-C10) (Haywood et al., 1990) as sole carbon source. A *Pseudomonas* strain was found to be incorporating longer chain monomer into polymer such as 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), and 3-hydroxydodecanoate (3HDD) (Abe et al., 1994; Kato et al., 1996). The general PHA structures are shown in Figure 2.1.



Number of repeating units, x	Alkyl group, R	Polymer type
1	Hydrogen	Poly(3-hydroxypropionate)
	Methyl	Poly(3-hydroxybutyrate)
	Ethyl	Poly(3-hydroxyvalerate)
	Propyl	Poly(3-hydroxyhexanoate)
	Pentyl	Poly(3-hydroxyoctanoate)
	Nonyl	Poly(3-hydroxydodecanoate)
2	Hydrogen	Poly(4-hydroxybutyrate)
3	Hydrogen	Poly(5-hydroxyvalerate)

Figure 2.1: The general PHA structures

PHA produced with variable monomeric composition can be used for various applications due to their physical and chemical properties (Jendrossek and Handrick, 2002; Steinbüchel and Lütke-Eversloh, 2003). There are two main types of PHA, depending on the number of carbon atoms in the monomer units. Short chain-length PHAs (scl-PHAs) are those containing monomers with 3-5 carbon atoms. Medium chain-length PHAs (mcl-PHAs) are those containing monomers with 6-14 carbon atoms. Mixed chain-length PHAs are copolymers containing both scl- and mcl-PHA monomers (Madison and Huisman, 1999).

2.3 Biosynthesis of PHA

PHA synthases are the major enzymes involved in the biosynthesis of PHA. They polymerize the substrates *(R)*-3-hydroxyacyl-CoA, 4-hydroxybutyryl-CoA, 5-hydroxyvaleryl-CoA and 6-hydroxyhexanoyl-CoA by releasing the Coenzyme A (CoA) (Łabużek and Radecka, 2001; Chuah et al., 2013b; Tsuge et al., 2015). More than 59 distinctive PHA synthases have been cloned and also sequenced from 46 distinctive bacteria. PHA synthases are divided into 4 major class (Table 2.1) based on substrate specificity, subunit and primary structure (Rehm, 2003; Pötter and Steinbüchel, 2006). Class I and class II PHA synthases consists of only one type of subunit (PhaC) with an average molecular weight between 60 to 73 kDa which are represented by *Cupriavidus necator* and *Pseudomonas aeruginosa* respectively.

Class I PHA synthases favour the synthesis of homopolymer or copolymer of scl-PHA from the scl-HA-CoA monomers. However, a PHA synthase from *A. caviae* of the same class was found to incorporate 3HHx monomer.

Table 2.1: Classes of PHA synthase (Rehm, 2007)

Class	Gene /Subunit	Representative species	Substrate
I	PhaC	*Cupriavidus necator*	$3HA_{scl}$-CoA (C3-C5)
	~60-73 kDa	*Sinorhizobium meliloti*	$4HA_{scl}$-CoA
		Burkholderia sp.	$5HA_{scl}$-CoA
II	PhaC1; PhaC2	*Pseudomonas aeruginosa*	$3HA_{mcl}$-CoA (C5)
	~60-65 kDa	*Pseudomonas putida*	
III	PhaC (~40 kDa) ;	*Allochromatiumvinosum*	$3HA_{scl}$-CoA
	PhaE (~40 kDa)	*Thiocapsa pfennigii*	$3HA_{mcl}$-CoA (C6-C8)
		Synechocystis sp.PCC6803	$4HA_{scl}$-CoA, $5HA_{scl}$-CoA
IV	PhaC (~40 kDa)	*Bacillus megaterium*	$3HA_{scl}$-CoA
	;PhaR (~22 kDa)	*Bacillus* sp.INT005	

Class II PHA synthases highly prefer to react with mcl-HA-CoA monomer comprising of 6 to 14 carbon atoms. PHA synthases under the classes III and IV possess two different subunits. Class III PHA synthases from *Allochromatium vinosum* consist of PhaC and PhaE (PHA synthase expression protein) and the molecular weight of both sub units are approximately 40 kDa. Class IV PHA synthases in *Bacillus megaterium* are similar to class III PHA synthases but PhaR was found in place of PhaE. The substrate specificity is also similar to class III PHA synthases (Rehm and Steinbüchel, 1999; Rehm, 2003; Pötter and Steinbüchel, 2006; Jendrossek, 2009). The biosynthesis route for P(3HB) in *C. necator* H16 is one of the simplest and well-studied biosynthetic pathways (Figure 2.2). Three enzyme reactions are involved in this pathway. The pathway starts with the enzyme *β*-ketothiolase (PhaA) which produces acetoacetyl-CoA by condensing two molecules of acetyl-CoA. Then, the enzyme NADPH-dependent acetoacetyl-CoA reductase (PhaB) catalyses the conversion of acetoacetyl-CoA to *(R)*-3-hydroxybutyryl-CoA (3HB-CoA). Finally, the PHA synthase polymerizes the *(R)*-3-hydroxybutyryl-CoA to P(3HB). It is well known that the activity of PHA synthase in *C. necator* towards the polymerization of scl-HA monomers. Nevertheless, the incorporation of 4- and 5-HA monomers besides the more common 3-HA clarified that the position of the oxidized carbon in the monomer is apparently not a crucial factor (Sudesh et al., 2000; Philip et al., 2007).

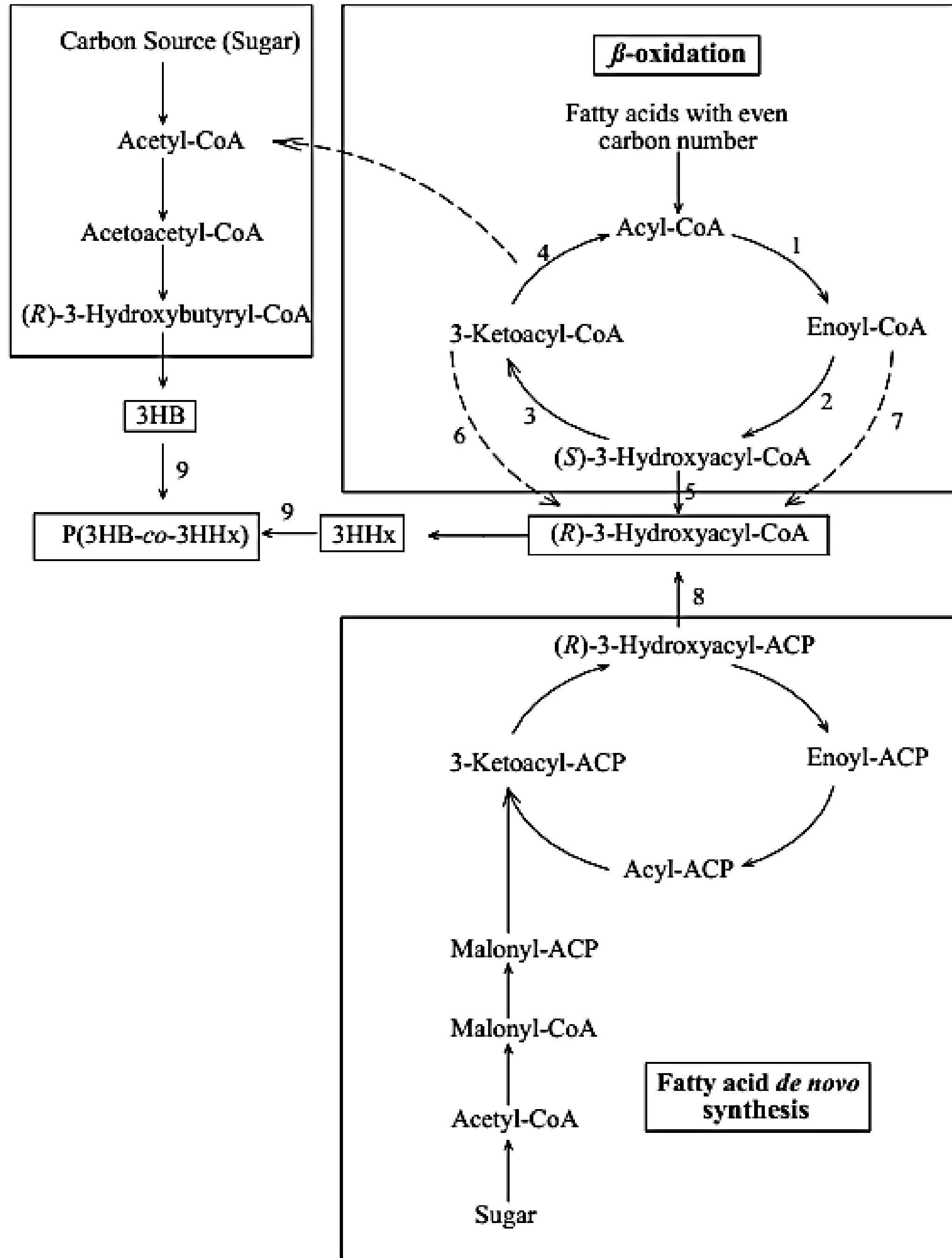


Figure 2.2: Proposed metabolic pathway for the biosynthesis of P(3HB-*co*-3HHx) from the fatty acids consisting of even carbon number. Enzymes: (1) acyl-CoA dehydrogenase; (2) enoyl-CoA hydratase; (3) 3-hydroxyacyl-CoA dehydrogenase; (4) 3-ketoacyl CoA thiolase; (5) epimerase; (6) 3-ketoacyl-CoA reductase; (7) (*R*)-specific enoyl-CoA hydratase; (8) (*R*)-3-hydoxyacyl-ACP-CoA transferase; (9) PHA synthase (Tsuge, 2002; Steinbüchel and Lütke-Eversloh, 2003)

PHA synthases play a major role in determining the characteristics of PHAs such as productivity, monomer composition, molecular weight and polydispersity. Therefore many studies have been carried out in order to understand the mechanism of polymerization through the crystal structure of PHA synthase. Unfortunately, the crystallization of PHA synthase was not successful for more than 30 years (Jia et al., 2000; Bernd et al., 2001). Recently, Kim et al. (2016a) reported the crystal structure and function of C-terminal domain and N-terminal domain (Kim et al., 2016b) of *Cupriavidus necator* PHA synthase. The discovered crystal structure of PHA synthase will be useful to tailor-made PHA for specific application (Chen, 2016).

2.4 Poly(3-hydroxybutyrate) [P(3HB)]

P(3HB) homopolymer is one of the common types of PHA that is extensively studied. It is highly crystalline and demonstrates good mechanical strength but poor ductility. Doi et al. (1995b) reported that the tensile strength and elongation at break for P(3HB) is 43 MPa and 5% respectively. High-resolution ^{13}C nuclear magnetic resonance (NMR) spectroscopy has revealed that the P(3HB) granules are in amorphous state when they are inside the bacterial cell. The results obtained from DSC also indicates that the presence of water molecules that are apparently hydrogen bonded to the PHA chains in the native granules (Barnard and Sanders, 1988; Barnard and Sanders, 1989; Jeremy, 1993; Porter and Yu, 2011). The P(3HB) granules become crystalline after they are isolated from the bacterial cell (Barnard and Sanders, 1989; Amor et al., 1991).The molecular weight of P(3HB) are in a range of 200 to 300 kDa depending on bacteria and growth conditions (Hinrichsen, 1995). The melting temperature (T_m) of P(3HB) is around 180 °C. The reduced

crystallinity and melting temperature and increased elongation to break is achieved by copolymerizing with co-monomer such as 3HV into P(3HB) (Tsuge, 2002).

2.5 Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)]

The P(3HB-*co*-3HHx) is synthesized naturally when the *Aeromonas caviae* is grown with fatty acids with even number of carbons. The enzyme PHA synthase and (*R*)-specific enoyl-CoA hydratase (PhaJ) is responsible for the biosynthesis of P(3HB-*co*-3HHx). The monomers are supplied for the P(3HB-*co*-3HHx) synthesis via fatty acid *de novo* and *β*-oxidation pathway (Figure 2.2). The intermediate product of enoyl-CoA is converted into (*R*)-3-hydroxyacyl-CoAs in *β*-oxidation pathway by the enzyme PhaJ. Firstly, the fatty acids will be converted to acyl-CoA thioesters by the enzyme thiokinase and oxidised to form acetyl-CoA and acyl-CoA. The intermediate product consists of two carbon atoms lesser than the number of carbon atoms that have entered into the first cycle. For fatty acids with even carbon number, the cycle will continue until all the acyl-CoA is completely converted into acetyl-CoA. The final products of acetyl-CoA and propionyl-CoA are formed from the fatty acids with odd carbon number in *β*-oxidation pathway (Steinbüchel and Lütke-Eversloh, 2003).

P(3HB-*co*-3HHx) copolymers are among the most attractive PHA for commercial exploitations because of their resemblance to some commodity plastics such as low density polyethylene (LDPE). The physicochemical properties of P(3HB-*co*-3HHx) depends on the 3HHx molar fraction. Therefore, it is necessary to control the composition of 3HHx. High P(3HB-*co*-3HHx) production was obtained using the recombinant strains of *C. necator* grown with plant oil and sugar (Fukui and Doi, 1998; Kichise et al., 2002). *C. necator* was genetically modified in order to

utilize fructose or soybean oil and produce P(3HB-*co*-3HHx) at high yield besides controlling the composition of 3HHx in the copolymer (Kahar et al., 2004). Various methods have been developed to enable the biosynthesis of P(3HB-*co*-3HHx) such as taking advantage of improved PHA synthase specificity and co-monomer alteration by using carbon sources. The PHA synthase (PhaC) enzyme specificity towards 3HHx from *Chromobacterium* sp. strain USM2 was enhanced through saturation point mutagenesis. This increased the 3HHx incorporation 4-fold and P(3HB-*co*-3HHx) production 1.6-fold compared to wild-type synthase (Chuah et al., 2013a). By using sodium salts of alkanoic acids and olive oil as the carbon source, *A. caviae* produced random copolymer of 3HB and 3HHx (Doi et al., 1995b). *Aeromonas hydrophila* and *Pseudomonas putida* GPp104 (pMON25806) are able to produce P(3HB-*co*-3HHx) with 3HHx unit fractions ranging from 2.5 to 9.5 mol% (Doi et al., 1995b; Fukui and Doi, 1998; Asrar et al., 2002). Sugars and plant oils could be used as carbon sources for PHA accumulation in various types of bacteria. It has been demonstrated that plant oils typically serve as a more robust carbon source compared to sugars (Brigham et al., 2010). When the production of various plant oils are compared, palm oil has the highest yield per area (Basiron, 2007). Since Malaysia is the leading producer and exporter of palm oil it provides the option of producing PHA using commercially produced palm oil (Wong et al., 2012).

2.6 *Cupriavidus necator*

Cupriavidus necator is a Gram negative beta-proteobacterium formerly known as *Ralstonia eutropha, Alcaligenes eutrophus* or *Wautersia eutropha.* It is considered as the highest PHA producing organism. *C. necator* accumulates up to 80 wt% from the dry cell weight under nitrogen limitation conditions (Fukui et al.,

1998). It is known that *C. necator* is not a spore forming bacteria and able to grow and accumulate PHA. However, it can only accumulate scl-PHA as an intracellular compound. The bacterium is capable of incorporating 3HHx (mcl monomer) into 3HB (scl monomer) after successful genetic modification (Fukui et al., 1998; Tsuge et al., 2004)

2.6.1 Recombinant *Cupriavidus necator* Re2058/pCB113

C. necator H16 has been shown to produce large quantities of scl-PHA as an intracellular storage compound. Since it is capable to produce high amounts of scl-PHA, Budde and co-workers created a recombinant strain from *C. necator* H16 wild type (ATCC 17699) capable of producing copolymers that consist of scl-mcl PHA. Genetically modified recombinant *C. necator* Re2058/pCB113 could accumulate P(3HB-*co*-3HHx) using plant oil as the sole carbon source under nitrogen limiting condition (Budde et al., 2011) (Figure 2.3).

The PHA synthase from *R. aetherivorans* I24 (*phaC*2) was used to replace the native PHA synthase from *C. necator* wild type. The synthase from *Rhodococcus* species was found to incorporate mcl monomers into PHA (Williams et al., 1994; Hori et al., 2009) and the PHA synthase from *R. aetherivorans* I24 was able to produce copolymers of P(3HB-*co*-3HHx) when introduced into *C. necator*. The enoyl-CoA hydratase (*phaJ*) from *P. aeruginosa* together with PHA synthase from *R. aetherivorans* I24 (*phaC*2) were expressed in the plasmid pCB113. The *phaJ* was inserted in order to increase the supply of 3HHx into the polymer. The *proC* gene (pyrolline-5-carboxylate reductase) was removed from the native genome and expressed via plasmid to ensure the plasmid stability without using kanamycin.

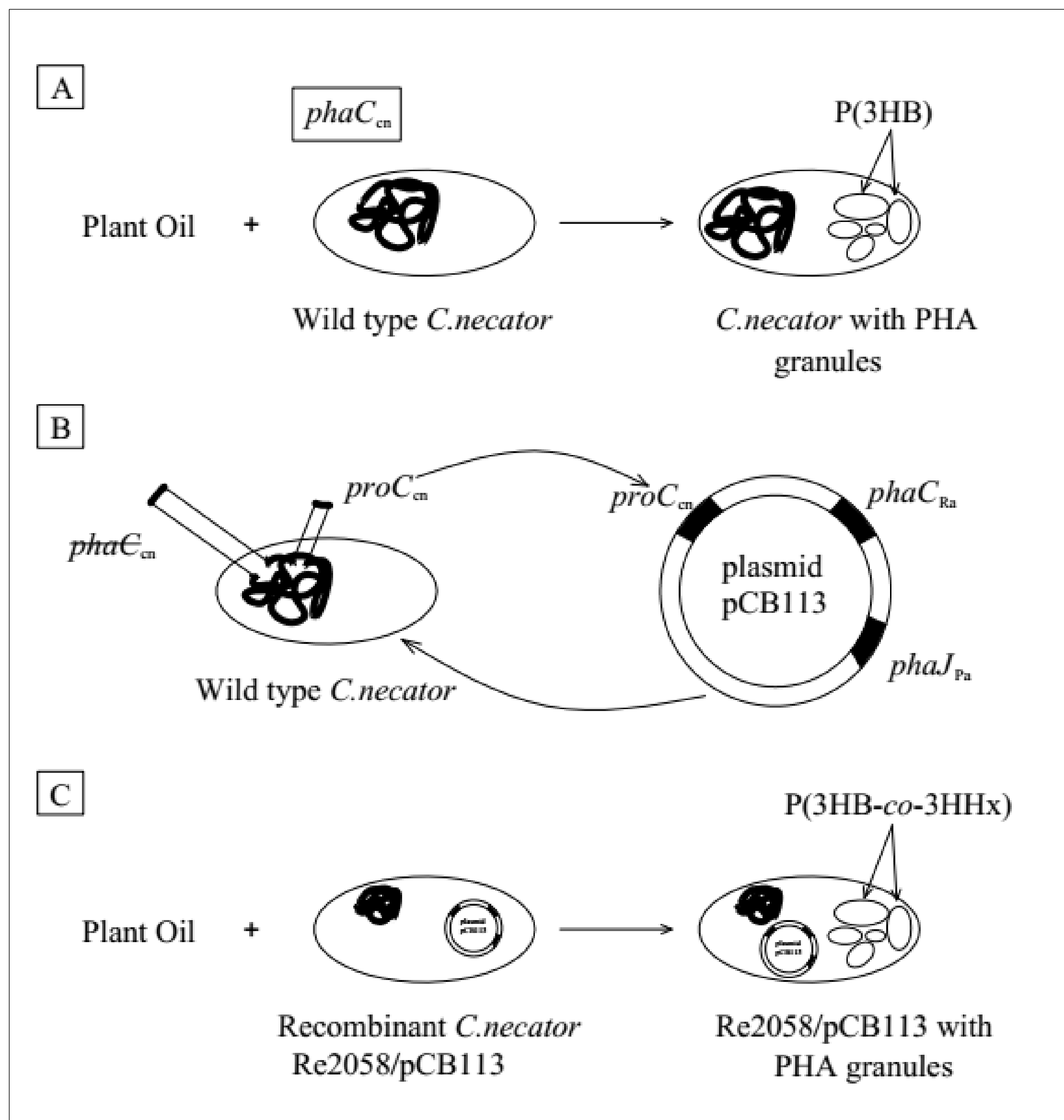


Figure 2.3: P(3HB) synthesis by wild type *C. necator* using plant oil (A), Construction of recombinant *C. necator* Re2058/pCB113 (B), P(3HB-*co*-3HHx) synthesis by recombinant *C. necator* Re2058/pCB113 using plant oil (C). $phaC_{Cn}$, PHA synthase gene of *C. necator*; $phaC_{Ra}$, PHA synthase gene of *R. aetherivorans*; $phaJ_{Pa}$, enoyl- CoA hydratase gene of *P. aeruginosa*; *proC*, pyrolline-5-carboxylate reductase.

The stable recombinant *C. necator* Re2058/pCB113 was constructed and shown to produce high amounts of P(3HB-*co*-3HHx) when grown on plant oil. Steinbüchel and Lütke-Eversloh (2003) proposed a metabolic pathway for the biosynthesis of mcl-PHA. The proposed metabolic pathway was established by linking the fatty acid β-oxidation and fatty acid *de novo* biosynthesis pathways. The conversion of fatty acid to acetyl-CoA takes place through four different steps in β-oxidation. Before entering into β-oxidation, fatty acid was converted to acyl-CoA thioesters and it is oxidised by the acyl-CoA dehydrogenase to form enoyl-CoA. (*S*)-3-hydroxyacyl-CoA is formed by the addition of water with the help of enoyl-CoA hydratase. This intermediate product is withdrawn from β-oxidation and incorporated as a 3HHx monomer into the polymer chain. The type of monomer is based on the length of fatty acid from the carbon source. The 3HHx monomer is accumulated in *Aeromonas hydrophila* by converting enoyl-CoA with the help of (*R*)-specific enoyl-CoA hydratase (*phaJ*) (Fukui et al., 1998). The intermediate product of (*S*)-3-hydroxyacyl-CoA has to be converted into (*R*)-3-hydroxyacyl-CoA in order to form the mcl-PHA. The cycle continues using non-converted intermediate to form 3-ketoacyl-CoA followed by acetyl-CoA and finally produce acyl-CoA by shortening two carbons. The acyl-CoA will be used for the formation of 3HB monomer and the *β*-oxidation cycle will be continued.

2.7 Thermal Properties of PHA

The P(3HB) homopolymer is highly crytalline, has a melting temperature (T_m) of 177 °C (Doi et al., 1995b). The glass transition temperature (T_g) was found to be 4 °C with a cooling rate of −100 °C (Scandola et al., 1988). The addition of small molecules such as plasticizer changes the thermal properties of P(3HB). The suitable

and effective plasticizer for P(3HB) is acetyl tributyl citrate (ATBC) because it reduces the melting and glass transition temperature to 163 °C and −10 °C, respectively (Wang et al., 2008). The melting temperature of P(3HB-*co*-3HV) random copolymer slightly lower than P(3HB) but the melting temperature depends on the composition. The melting temperature is significantly reduced when the 3HV composition is increased up to 40 mol% (Kunioka et al., 1989b). The thermal properties are also greatly influenced by the incorporation of 3HHx into the copolymer. Various research groups have studied the crystallization kinetics and melting behaviour of the P(3HB-*co*-3HHx) (Xu et al., 2002; Padermshoke et al., 2004a; Sato et al., 2004; Chen et al., 2005; Hu et al., 2007; Mori et al., 2008). It was found that the 3HHx monomers do not co-crystallize together with 3HB in the crystalline domain due to the bulkiness of the propyl (3C) side group in the 3HHx monomer. The increase in 3HHx unit composition from 0 to 25 mol% decreases the melting temperature from 178 °C to 52 °C and a decrease in the degree of crystallinity was observed (Doi et al., 1995b). Asrar et al. (2002) found a similar trend when the 3HHx composition was increased from 2.5 to 35 mol%. Commonly, isothermally crystallized semi crystalline polymers were found to have multiple melting temperatures (Kong and Hay, 2003; Mai et al., 2003; Song et al., 2006). The melting peak of solvent casted P(3HB) film has an extra shoulder peak on the heating curve at the heating rate of 2 °C/min (Owen et al., 1992). The copolymer P(3HB-*co*-17 mol% 3HV) has two melting temperatures at 142 °C and 153 °C under the same condition. The multiple melting behaviour of P(3HB) and P(3HB-*co*-3HV) was studied systematically using wide-angle x-ray diffraction (WAXRD), hot stage optical microscope, conventional differential scanning calorimeter (DSC), step scan DSC and it was found that the secondary crystallization was due to the specific

thermal history of the sample (Gunaratne and Shanks, 2005a; Gunaratne and Shanks, 2005b; Gunaratne and Shanks, 2006). The multiple melting temperatures was found to be due to the slower rate of crystallization (Gunaratne et al., 2004). The melting behaviour of the P(3HB-*co*-3HHx) has broader temperature range (50-130 °C) compared to P(3HB-*co*-3HV) (Xu et al., 2002). Two melting endotherms were observed with P(3HB-*co*-15 mol% 3HHx) and isothermal crystallization temperature (at ~60-80 °C) due to the thermal transition between crystalline and amorphous region (Padermshoke et al., 2004b). Three endothermic peaks were observed with P(3HB-*co*-12 mol% 3HHx) during subsequent heating scan (Hu et al., 2007).

2.8 Melt viscoelastic properties of PHA

The melt rheological property of PHA is not well studied compared to crystalline, thermal and mechanical properties. The capillary and oscillatory rheometer were used to study the melt viscosity of P(3HB) (El-Hadi et al., 2002). It is agreed that the complex viscosity was related to steady shear viscosity. The melt rheology properties of P(3HB) was studied using rotational rheometer equipped with parallel plate geometry (Choi et al., 1995; Park et al., 2001). It was found that the shear viscosity of P(3HB) decreased with slow shear rate but shear thinning was also observed (Choi et al., 1995). The rheological analysis showed the thermal degradation of P(3HB) and suggested that the analysis begins with lower shear frequency at 1 rad/s. The viscosity values were found to be higher when the P(3HB) was mixed with 20% polyethylene oxide (PEO) (Choi et al., 1995). Arakawa et al. (2008) measured the steady shear viscosity of P(3HB-*co*-12 mol% 3HV) using capillary rheometer and observed the shear thinning behaviour of the polymer. The same copolymer, P(3HB-*co*-12 mol% 3HV) was used to measure the oscillatory

shear viscosity. It was reported that the zero viscosity $\eta 0$ and relaxation time λ at 160 °C was 3200 Ps/s and 0.35s, respectively. The P(3HB-*co*-3HHx) was also used to study the viscoelastic properties and kinetics of thermal degradation during the rheological measurements. Daly et al. (2005) studied the dynamic and transient rheological analysis using P(3HB-*co*-3HHx). The viscosity of the copolymer decreased with time at constant temperature and it decreased significantly when the temperature increased to more than 165 °C, indicating the occurrence of polymer degradation and reduction of molecular weight. The apparent activation energy calculated for thermal degradation process was 189 kJ/mol. The method was developed to prove the relationship between thermal degradation and molecular weight of the PHA (Harrison and Melik, 2006).

2.9 Single cell protein (SCP)

Food with adequate protein content is necessary to meet the growing demand. So it is necessary to find an inexpensive protein food source to feed the growing populations. In 1996, fungi, bacteria or yeast was used as a source of SCP. As an alternative source of protein, these microorganisms are produced in large scale and used as protein source for animal feed and human food (Kuhad et al., 1997; Ravindra, 2000). The advantages of producing microorganisms, are i) wide range of substrate and simple methods can be used, ii) substrate conversion efficiency is higher, iii) the growth rate is faster and productivity is high and iv) the growth of microorganisms is not dependant on climatic conditions (Roth, 1982). The growth rate and nutritional composition of microorganisms can be altered by genetic modification. The protein content of the common food is lower than that in microorganisms. Besides that, the microorganism consists of lipids, vitamins, amino

acids, carbohydrates and minerals. Large scale production of microorganisms from the agricultural wastes makes the production of protein source become economically viable. Moreover, it requires small space and less water compared to the production of conventional livestock. The amount of waste generated is also lesser compared to other food production processes (Kihlberg, 1972; Kuhad et al., 1997).

The focus towards the bacteria for the production of microbial protein is due to the faster growth rate. Bacteria grow in a wide range of organic substrates such as carbohydrates, liquid hydrocarbon and gases. However, the microorganisms should be cultivated under the controlled conditions such as pH, temperature, carbon, nitrogen and aeration (Kuhad et al., 1997). Example of micro-organisms used as a source of microbial protein are *Methylophilus methylotrophus* and *C. necator* H16 (Calloway and Kumar, 1969; Stringer, 1982). The benefit of SCP from any sources depends on its nutritional value. The nutritional composition is more or less the same for most microorganisms. It contains about 50% of protein, 15% of nucleic acids, 20% are the cell membrane and other substances. The elemental compositions of cell substances of all bacteria are very similar. The composition may differ depending on the storage compound, capsular substances and cell wall (Schlegel and Lafferty, 1971). The nucleic acid is one of the main factors affecting the usage of bacteria as SCP when compared to algae and yeast. The SCP from bacteria requires further processing to reduce the amount of nucleic acid. High amount of nucleic acids causes health problems to human and animals. The nucleic acid is not considered as a toxic substance in food but it shows some physiological effects and imbalance in other essential nutrients when it is consumed in excess (Kihlberg, 1972; Nasseri et al., 2011). Even though, it is consumed in excess by the animals such as pigs and rats,

they produce uricase enzyme to convert uric acid to allantoin and soluble metabolites that can be excreted by kidney. It is still possible to use the bacteria-derived SCP as animal feed without any further processing (Kihlberg, 1972; Giesecke and Tiemeyer, 1982; Stringer, 1982). One of the possible disadvantage is that the size of bacteria and their low density culture makes the downstream process expensive and not economically viable (Nasseri et al., 2011). Moreover, when some bacteria are consumed as food they create health problems. It is important to create awareness that not all bacteria is harmful to humans. Bacterial SCP can be considered as a suitable alternative food for farmed animals. Unlike wild animals that consume food from the natural environment, animals fed with food formulated based on nutritional and economical concern (Sapkota et al., 2007).

2.9.1 *Cupriavidus necator* H16 as a source of SCP

Cupriavidus necator H16 is a gram-negative, soil bacterium widely used for the production of PHA. However, it has been shown that this wild type bacterium can be used as SCP based on the nutritional composition (Waslien and Calloway, 1969). The *C. necator* H16 cells consist of high protein content and were studied as an alternative source of SCP for animal feed during the 1970s. Apart from protein source, these bacterial cells contain some important amino acids similar to casein such as valine, methionine, threonine and lysine. An analysis of the nutritive value of the bacterial cells showed that the concentrations of certain important amino acids such as lysine, methionine, threonine and valine were high. The amino acid composition of *C. necator* H16 and *Pseudomonas saccharophila* were compared and it was found that both bacteria consisted of similar concentration of amino acids except for lysine, methionine and threonine. *C. necator* contained more lysine and

methionine but less threonine than *Pseudomonas*. Interestingly, the lysine content in the bacterial cells are higher than cereal grains. Most of the proteins derived from bacteria are digestible by animals (Calloway and Kumar, 1969).

2.10 Use of PHA in animal feed

PHA is known as a carbon and energy storage compound in micro-organisms. Recently, PHA is gaining attention as animal feed in order to increase the metabolic energy (Forni et al., 1999a; Forni et al., 1999b; Peoples et al., 2001; Boon et al., 2013). However, PHA is not completely digested by the animals due to the lack of certain enzymes and the microorganisms present in the gastrointestinal tract were not able to digest the polymeric chains. Moreover, the retention time of the PHA in the gastrointestinal tract is short. The degradation of PHA such as P(3HB-*co*-3HV) is hindered by high crystallinity. In order to improve the digestibility, the size of polymer chain is reduced by pre-treatment using sodium hydroxide before feeding to the animals. It was found that the PHA digestibility is improved after the pre-treatment using sodium hydroxide (Forni et al., 1999a). Some changes was observed in gut micro-flora due to the formation of short-chain fatty acids (SCFAs) such as 3-hydroxybutyric acids as the by-product of P(3HB) degradation when bacterial cells with PHA was used as animal feed (Ushida and Kuriyama, 2004; Boon et al., 2013). It was found that the growth of intestinal cells was promoted by SCFAs and it activated the ion transportation as well as increased the blood flow in large intestine mucosa (Ushida and Kuriyama, 2004). SCFAs could be used as bio-control agents in production of animals. However, the physiological and physiochemical characteristics of the environment affect the bacteriostatic activity of the short chain fatty acids (Defoirdt et al., 2009). It is possible for PHA to be degraded by the large

intestinal microflora in the animal gut and provide physiological benefits by releasing short-medium chain fatty acids instead of toxic effects (Ushida and Kuriyama, 2004).

2.11 Recovery of PHA from bacterial cells and purification methods

PHAs are accumulated in the form of water insoluble granules in the bacterial cell cytoplasm. It is necessary to break or lyse the bacterial cell wall in order to obtain the PHA granules. In addition, the PHA granules are closely associated with several types of proteins which are located on the surface of the granules (Sudesh et al., 2004; Bresan et al., 2016). Most of the PHA extraction processes are developed using organic solvents such as chloroform and dichloromethane (Choi and Lee, 1999b). Recently, a method was developed for disruption of cells containing PHA by using linear alkyl benzene sulfonic acid LAS-99 as alternative for the SDS (Yang et al., 2011). The newly developed method requires only 20% of total amount surfactant used for SDS based recovery methods. The purity and yield obtained was 88% and 86% respectively when the PHA recovered from the cell under acidic pH. However, this type of recovery method has some disadvantages such as consumption of large quantity of clean water. Furthermore, the waste water generated from this process contains large amount of SDS which cannot be reused and it may lead to increased cost in waste water treatment. Also, recovery method was developed by using enzyme based digestion of cell constituents to recover the PHA. Detergents combined with chelating agents improved the efficiency of PHA recovery efficiency as compared to solvent-extracted PHA. Alternative methods such as live bacterial cells were also studied for the degradation and utilization of non-PHA cells materials and release the PHA from the cells, to substitute the usage of costly purified enzymes