OPTIMIZED PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYVALERATE) FROM *Cupriavidus* sp. USMAA2-4 AND ITS CHARACTERIZATION

SHANTINI KANNUSAMY

UNIVERSITI SAINS MALAYSIA

2015

OPTIMIZED PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYVALERATE) FROM *Cupriavidus* sp. USMAA2-4 AND ITS CHARACTERIZATION

by

SHANTINI KANNUSAMY

Thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy

OCTOBER 2015

ACKNOWLEDGEMENTS

One of the joys of completion is to look over the journey and remembering all the friends and family who have helped and supported me throughout this long but fulfilling road. I would like to take this opportunity to express my profound gratitude and deepest regards to my guide Prof. Dr. Amirul Al-Ashraf Abdullah for his exemplary guidance, monitoring, patience and constant encouragement throughout my study. I would also like to acknowledge with much appreciation the crucial role of my co-supervisor Assoc. Prof. Dr. Ahmad Ramli Mohd Yahya for guiding my research for the past several years and helping me to develop my background in the field of biochemical engineering and fermentation technology. His beneficial comments, remarks and advice throughout the research project are greatly appreciated. I appreciate the feedback offered by him. Dr. Kesaven Bhubalan, my field supervisor has offered much advice and insight throughout my work especially in the field of molecular biology. His attitude towards research has inspired me in many ways. I have been extremely lucky to have supervisors who cared so much about my work and who responded to my questions and queries promptly. I believe that the help, blessing, and guidance given by them will be useful in the journey of my life that I am about to embark.

I would like to express deepest appreciation to all of my lab members especially Kak Solehah, Kak Syahirah, Hezreen, Muzaiyanah, Kak Hemalatha, Nik Farhana Nadiah, Wawa, Kak Syifa, Rennukka, Kak Faezah, Kak Vigneswari, Kak Syikin and Hema. A special thanks to my juniors Iszaty, Azura, Azuraini, Sheeda, Kai Hee, Syafirah, Shakkilla, Adilah, Syafiq and Thina. They have been a good companion on an exhausting and during disappointing experiments. In many ways I

have learnt so much from them. I am thankful for being blessed with a friendly and cheerful group. I also would like to take this opportunity to express a deep sense of gratitude to Mr.Sekaran, En. Zahari and to other lab assistances who have helped me directly and indirectly. I am thankful to electron microscope staff members for their valuable information provided in their respective fields. Special thanks to USM for supporting me financially and for sourcing me a quality workstation.

Many friends have helped me stay sane through these difficult years. Their support and care helped me overcome setbacks and stay focused on my graduate study. I greatly value their friendship and I deeply appreciate their belief in me. I owe a very important debt to my close friend, Anitha. Without her support and encouragement, this dissertation would not have been materialized. Thanks dear for being there to listen and console me. A special thanks to Mr. Jaya Perkhass for his unconditional love and for sharing my wish to reach the goal of completing this wonderful task.

Of course no acknowledgments would be complete without giving thanks to our parents. I would like to thank my parents for their unconditional support, both financially and emotionally throughout my degree. In particular, the patience and understanding shown by my mum, dad and sister, Shalini during the honors' year are greatly appreciated. They have instilled many admirable qualities in me and given me a good foundation with which to meet life. They have taught me about hard work, self-respect, persistence and importance of being independent.

Above all, I thank God for his grace, wisdom, favors and protections. I may never have done this without the faith I have in you, the Almighty. Thank you Lord! There are many others whom I may have inadvertently left out and I sincerely thank all of them for their help too.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols and Abbreviations	Full name
%	Percentage
ß	Beta
°C	Degree Celcius
ΔH_m	Heat of fusion
µg	Microgram
µM	Micromolar
µmol	Micromole
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyryl Coenzyme A
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
^{13}C	Carbon-13
Abs	Absorbance
ANOVA	Analysis Of Variance
ASTM	American Society for Testing and Materials
C	Carbon atom
$CaCl_2$	Calcium chloride
CDW	Cell dry weight
$CHCl_3$	Chloroform
C_L	Dissolved oxygen concentration

C^*_L	Dissolved oxygen concentrations in equilibrium with mean gaseous oxygen concentration
CME	Caprylic methyl ester
CO_2	Carbon dioxide
CoA	Coenzyme A
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
CPKO	Crude palm kernel oil
CPO	Crude palm oil
$CrCl_3 \cdot 6H_2O$	Chromium chloride hexahydrate
$CuSO_4 \cdot 5H_2O$	Copper sulphate pentahydrate
$C_{12:0}$	Lauric acid
$C_{16:0}$	Palmitic acid
$C_{14:0}$	Myristic acid
$C_{18:1}$	Oleic acid
Da	Dalton
dH_2O	Distilled water
ddH_2O	Sterile distilled water
DO	Dissolved oxygen
DSC	Differential scanning calorimetry
$FeCl_3$	Iron (III) chloride
FID	Flame ionization detector
g	Gravity

g	Gram
GC	Gas chromatography
g/L	Gram per liter
GPC	Gel permeation chromatography
h	Hour
H	Hydrogen atom
HCl	Hydrochloric acid
H_2O	Water
HPLC	High-performance liquid chromatography
H_2SO_4	Sulphuric acid
ICI	Imperial Chemical Industries
IS	Internal standard
J/g	Joule per gram
Kb	Kilo base pairs
kDa	Kilodalton
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
K_La	Volumetric oxygen transfer coefficient
KOH	Potassium hydroxide
L	Liter
LB	Luria Bertani
M	Molar

mcl	Medium chain length
min	Minute
mg	Milligram
Mg	Magnesium atom
mg/L	Milligram per liter
$MgSO_4$	Magnesium sulphate
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
mL	Milliliter
mm	Millimetre
mM	Millimolar
M_n	Number-average molecular weight
mol%	mol percent
MPa	Megapascal
MSM	Mineral salt medium
M_w	Weight-average molecular weight
M_w / M_n	Polydispersity index
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
ng	Nanogram
NH_4Cl	Ammonium chloride

$NiCl_3 \cdot 6H_2O$	Nickel chloride hexahydrate
nm	Nanometre
NR	Nutrient rich
OD	Optical density
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PDI	Polydispersity index
PHA	Polyhydroxyalkanoate
PhaA; *phaA*	*β*-ketothiolase; gene encoding *β*-ketothiolase
PhaB; *phaB*	NADPH-dependent acetoacetyl-CoA dehydrogenase; gene encoding NADPH-dependent acetoacetyl-CoA dehydrogenase
PhaC; *phaC*	PHA synthase; gene encoding PHA synthase
PhaP; *phaP*	Phasin; gene encoding Phasin
PhaR; *phaR*	Regulator protein of the phasin expression; gene encoding regulator protein
PhaZ; *phaZ*	PHA depolymerase; gene encoding PHA depolymerase
PKO	Palm kernel oil
PO	Palm olein
psi	Pounds per square inch
RCDW	Residual cell dry weight
rcf	Rotation centrifugational force

rpm	Rotation per minute
scl	Short chain length
sd	Standard deviation
SEM	Scanning electron microscope
sp.	Species
T_c	Crystallization temperature
T_g	Glass transition temperature
T_m	Melting temperature
TCA	Tricarboxylic acid
TEM	Transmission electron microscope
TES	Trace elements
UV	Ultraviolet
V	Volt
v/v	Volume per volume
wt%	Dry weight percent
w/v	Weight per volume
w/w	Weight per weight

LIST OF PUBLICATIONS

PEER REVIEWED JOURNALS

1. K. Shantini., Ahmad R. M. Yahya., A. A. Amirul., Empirical modeling development for integrated process optimization of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production, *Journal Of Applied Polymer Science*, 125, 2155-2162. 2012

2. Shantini, K., Bhubalan, K., Yahya, A.R.M., Amirul, A.A., Productivity increment of biodegradable and biorenewable copolymer containing 3-hydroxyvalerate monomer initiated by alcohols as precursor substrate, *Journal Of Chemical Technology and Biotechnology*, 88 (7), 1364-1370. 2013

3. K. Shantini., Ahmad R. M. Yahya., A. A. Amirul., Influence of feeding and controlled dissolved oxygen level on the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer by *Cupriavidus* sp.USMAA2-4 and its characterization, *Applied Biochemistry and Biotechnology*, 175(5), 1315-1334. 2015

4. Huong Kai Hee., Shantini Kannusamy., Sumidtha Lim Yeong Hui., A.A. Amirul., Biosynthetic enhancement of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) through single stage cultivation by manipulating the substrate mixtures, *Journal of Biotechnology*, 42(9), 1291-1297. 2015

CHAPTERS IN BOOK

1. Ramachandran, H., K.Shantini., Huong, K.H., R. Mathava., Amirul, A.A., Blends of Polyhydroxyalkanoates (PHAs). In: Roy, I. and Visakh, P.M (eds). *Natural polymers series: Polyhydroxyalkanoates (PHAs) based blends, composites and nanocomposites*. Royal Society of Chemistry, Cambridge, UK, pg 66-97. 2015

2. K.Shantini., Kai-Hee Huong., Hema Ramachandran., A.A. Amirul., Microbial production of polyhydroxyalkanoates for agricultural and aquacultural applications. *Microbiology Monographs series*: *Beneficial microorganisms in agriculture and aquaculture*. Volume: 29,
   ISBN: 978-3-319-23182-2. 2016

BOOK

1. Amirul Al-Ashraf., Kai-Hee Huong., Hema Ramachandran., Shantini Kannusamy., Microbial-based polyhydroxyalkanoates: Upstream and downstream processing, *Smithers Rapra*. 2015

CONFERENCE PRESENTATION AND OTHERS

1. Ishak Muhammad Syafiq, K.Shantini, Al-Ashraf Abdullah Amirul, Kesaven Bhubalan. Production of P(3HB-*co*-4HB) copolymer with high 4HB monomer via additional synthase gene dosage using *Cupriavidus* sp. USMAA1020 transformant strain. International conference on Biological, Chemical and environmental sciences 2014, Phuket Thailand (Oral)

2. K.Shantini, K. Bhubalan, A.R.M. Yahya, A.A. Amirul. Production, optimization and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer from *Cupriavidus* sp. USMAA2-4. 9th Postgraduate Colloquium, School of Biological Sciences/IPS 2013. Universiti Sains Malaysia (Oral)

3. Shantini Kannusamy, Teh Yea Lu, Ahmad Ramli Mohd Yahya, Amirul Al-Ashraf Abdullah. The significance of feeding carbon source for the production of various monomer compositions. 31st Symposium of the Malaysian Society for Microbiology 2012, Kota Kinabalu Sabah (Poster)

4. Shantini Kannusamy, Teh Yea Lu, Ahmad Ramli Mohd Yahya, Amirul Al-Ashraf Abdullah. Effect of altering carbon to nitrogen ratio (C/N) on the production of a 3-hydroxyvalerate monomer. 7th International Symposium in Science and Technology 2012, Universiti Sains Malaysia (Poster)

5. Kai Hee Huong, K. Shantini, A. A. Amirul. Application of experimental design as a strategy for improving fermentation performance of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production by *Cupriavidus* sp. USMAA1020. Malaysian International Conference on Trends in Bioprocess Engineering 2012, Langkawi, Kedah Malaysia (Oral)

6. Shantini K, Yea L. T, Ahmad R. M. Y, Amirul. A. A. Regulation and characterization of the molar fraction of 3-hydroxyvalerate monomer by altering the carbon to nitrogen ratio (C/N). Malaysian International Conference on Trends in Bioprocess Engineering 2012, Langkawi, Kedah Malaysia (Oral)

7. Amirul A.A, Rahayu A, Nursolehah A.A, Vigneswari S, Shantini K, Majid M.I.A. Biosynthesis of various polyhydroxyalkanoate polymers as biodegradable and biocompatible material. FEMS 4th Congress of European Microbiologists 2011, Geneva Switzerland (Oral)

8. K. Shantini, Ahmad R. M. Yahya, A. A. Amirul. Comparison of poly(3 hydroxybutyrate-*co*-3-hydroxyvalerate) production by two different strains of *Cupriavidus* sp. using 1-pentanol and oleic acid. 2nd Symposium USM Fellowship 2011, Vistana Hotel, Penang Malaysia (Oral)

9. K. Shantini, Ahmad R. M. Yahya, A. A. Amirul. Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer by *Cupriavidus* sp. USMAA2-4 via one stage cultivation process. The 7th IMT-GT UNINET and The 3rd Joint International PSU-UNS Conference 2010, Prince Of Songkla University, Hat Yai Thailand (Poster)

10. Shantini K, Bhubalan K, Yahya A.R.M, Amirul A.A. Optimization of copolymer P(3HB-*co*-3HV) production by *Cupriavidus* sp. USMAA2-4 using Response Surface Methodology (RSM). 3rd Postgraduate Colloquium, School of Biological Sciences/IPS 2010, Universiti Sains Malaysia (Oral)

11. Shantini K, Bhubalan K, Yahya A.R.M, Amirul A.A. Optimization of copolymer P(3HB-*co*-3HV) production by *Cupriavidus* sp. USMAA2-4 using Response Surface Methodology (RSM). 4th Postgraduate Colloquium, School of Biological Sciences/IPS 2010, Universiti Sains Malaysia (Oral)

12. K. Shantini, Ahmad R. M. Yahya, A. A. Amirul. Production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer by *Cupriavidus* sp. USMAA2-4 via one stage cultivation process. The 2nd International Conference on Bio-based Polymers 2009, Universiti Sains Malaysia (Poster)

AWARDS

1. Best publication award (PPSKH) in conjunction with Malam Persada Kencana (2013), USM, Penang

2. Shantini Kannusamy, Teh Yea Lu, Ahmad Ramli Mohd Yahya, Amirul Al-Ashraf Abdullah. Effect of altering carbon to nitrogen ratio (C/N) on the production of a 3-hydroxyvalerate monomer. 7th International Symposium in Science and Technology 2012, Universiti Sains Malaysia (Best poster presenter)

3. K. Shantini, Ahmad R. M. Yahya, A. A. Amirul. Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer by *Cupriavidus* sp. USMAA2-4 via one stage cultivation process. The 7th IMT-GT UNINET and The 3rd Joint International PSU-UNS Conference 2010, Prince Of Songkla University, Hat Yai Thailand (Best poster presenter)

PENGHASILAN OPTIMUM POLI(3-HIDROKSIBUTIRAT-*ko*-3-HIDROKSIVALERAT) DARIPADA *Cupriavidus* sp. USMAA2-4 DAN PENCIRIANNYA

ABSTRAK

Polihidroksialkanoat (PHA) adalah termoplastik bersifat bioserasi dan biodegradasi yang mempunyai kebolehan sebagai pengganti sumber-sumber yang tidak boleh diperbaharui. PHA dihasilkan oleh mikroorganisma secara intraselular sebagai tenaga dan sumber karbon terpelihara. Homopolimer P(3HB) adalah rapuh manakala kopolimer poli(3-hidroksibutirat-*ko*-3-hidroksivalerat) [P(3HB-*ko*-3HV)] bersifat getah berdasarkan perubahan dalam komposisi monomer 3HV. Kajian ini meneroka potensi bakteria pencilan tempatan, *Cupriavidus* sp. USMAA2-4, bakteria Gram-negatif untuk menggunakan kombinasi asid oleik dan 1-pentanol untuk penghasilan kopolimer P(3HB-*ko*-3HV). Didapati bakteria pencilan tempatan ini berpotensi untuk menghasilkan pelbagai jenis kopolimer dan kombinasi sumber karbon ini tidak pernah dilaporkan. Didapati bakteria *Cupriavidus* sp. USMAA2-4 berupaya menghasilkan 58% (b/b) kandungan P(3HB-*ko*-3HV) dengan monomer 3-hidroksivalerat (3HV) sebanyak 8 mol% apabila campuran 0.50% (b/b C) asid oleik dan 0.06% (b/b C) 1-pentanol digunakan. Kepekatan kopolimer P(3HB-*ko*-3HV) sebanyak 4.5 g/L dan pertumbuhan bakteria sebanyak 3.4 g/L telah dihasilkan. Peningkatan kopolimer P(3HB-*ko*-3HV) dan pertumbuhan bakteria dalam kelalang goncangan diperolehi melalui pengoptimuman parameter kultur dengan menggunakan metodologi permukaan respon (RSM). Selepas pengoptimuman, penggunaan kepekatan 1-pentanol berkurang sebanyak 33%. Kandungan PHA meningkat 10.3% kepada 64% (b/b) dan kepekatan PHA meningkat 42% kepada 6.4

g/L. Monomer 3HV meningkat ke 10 mol% dengan pertumbuhan sebanyak 3.5 g/L. Peningkatan juga diperolehi melalui pengoptimuman bioreaktor secara fermentasi sesekelompok dengan menggunakan RSM dengan pertumbuhan signifikan sebanyak 9.8 g/L yang mewakili peningkatan 180% dan kepekatan PHA sebanyak 8.7 g/L yang mewakili peningkatan 36%. Produktiviti yang diperolehi dengan bioreaktor meningkat dua kali ganda kepada 0.16 g/L/h berbanding dengan fermentasi kelalang goncangan iaitu 0.088 g/L/h. Produktiviti PHA telah ditingkatkan dengan menggunakan kaedah kekerapan suapan yang berbeza dalam kultur suapan sesekelompok. Penghasilan kopolimer oleh bakteria pencilan tempatan ini didapati berkait dengan pertumbuhan; oleh itu kaedah suapan sesekelompok digunakan untuk meningkatkan pertumbuhan dan penghasilan kopolimer. Produktiviti tertinggi (0.48 g/L/h) yang mewakili peningkatan sebanyak 200% telah diperolehi dengan memberi sumber karbon dan sumber nitrogen sebanyak tiga kali dan juga dengan mengambil kira kadar penyerapan oksigen dan kadar pemindahan oksigen. Kepekatan P(3HB-*ko*-3HV) tertinggi iaitu 25.7 g/L dan kandungan PHA sebanyak 66% (b/b) yang lebih ketara telah diperolehi. Monomer 3HV sebanyak 24 mol% dan pertumbuhan sebanyak 13.3 g/L telah diperolehi. Kopolimer P(3HB-*ko*-3HV) yang dihasilkan telah dinilai ciri-cirinya. Secara keseluruhannya, didapati bahawa kopolimer yang dihasilkan melalui suapan tiga kali lebih fleksibel (Young's Modulus 187 MPa) dan mempunyai pemanjangan sehingga putus (193 %) yang lebih tinggi berbanding kopolimer yang lain. Berat molekul kopolimer di antara 233 hingga 849 kDa telah dihasilkan. Apabila kekerapan suapan sesekelompok meningkat didapati granul PHA juga meningkat dan ini berkait dengan index polidispersiti yang diperolehi. Secara keseluruhannya, strategi fermentasi yang diperolehi melalui kajian ini daripada kelalang goncangan sehingga bioreaktor boleh digunakan sebagai platform untuk

penghasilan kopolimer P(3HB-*ko*-3HV) oleh sektor industri memandangkan produktiviti yang tertinggi sebanyak 0.48 g/L/h telah diperolehi.

OPTIMIZED PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYVALERATE) FROM *Cupriavidus* sp. USMAA2-4 AND ITS CHARACTERIZATION

ABSTRACT

Polyhydroxyalkanoate (PHA) is a biocompatible and biodegradable thermoplastic that has the potential to replace non-renewable resources. It is produced by microorganisms as intracellular energy and reserve material. P(3HB) homopolymer is stiff while copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] was found flexible depending on the 3HV monomer composition. This study explores the potential of a local isolate, *Cupriavidus* sp. USMAA2-4, a Gram-negative bacterium to utilize the combination of oleic acid and 1-pentanol for the production of copolymer P(3HB-*co*-3HV). This local strain was found to have the potential for the production of various copolymers and the combination of this carbon source has never been reported. It was found that *Cupriavidus* sp. USMAA2-4 could produce 58 wt% of P(3HB-*co*-3HV) with 8 mol% 3-hydroxyvalerate (3HV) monomer by using 0.50 wt% C oleic acid and 0.06 wt% C 1-pentanol. The P(3HB-*co*-3HV) concentration obtained was 4.5 g/L with a growth of 3.4 g/L. Enhancement of P(3HB-*co*-3HV) production and growth were obtained through optimization of culture parameters by using the response surface methodology (RSM) in shake flasks. After optimization, the usage of 1-pentanol reduces 33%. The PHA content increased by 10.3% to 64 wt% and the PHA concentration increased 42% to 6.4 g/L. The 3HV monomer composition increased to 10 mol% with the growth of 3.5 g/L. Enhancement was also obtained through bioreactor optimization in batch fermentation using RSM whereby a significantly

higher growth of 9.8 g/L which represented a 180% increment and PHA concentration of 8.7 g/L which represented 36% increment. The productivity of the copolymer produced in the bioreactor increased by two fold to 0.16 g/L/h as compared to shake flask which was at 0.088 g/L/h. The productivity of PHA was improved by varying the frequency of feeding through the fed-batch culture. The production of copolymer by this local strain was found to be growth related; therefore method of feeding was used to increase the growth and copolymer production. The highest productivity (0.48 g/L/h) that represented 200% increment was obtained by feeding the carbon source and nitrogen source three times and also by considering the oxygen uptake rate (OUR) and oxygen transfer rate (OTR). A significantly higher P(3HB-*co*-3HV) concentration of 25.7 g/L and PHA content of 66 wt% were obtained. The 3HV monomer composition obtained was 24 mol% with the growth of 13.3 g/L. The P(3HB-*co*-3HV) copolymer produced were characterized. In general, the copolymer produced through three times feeding regimen exhibited greater flexibility (Young's Modulus 187 MPa) and elongation at break (193 %) as compared to the other copolymers. The weight average molecular weight of the copolymer ranged between 233 to 849 kDa. As the frequency of feeding increases, the number of granules also increased which correlated with the polydispersity value obtained. In general, the fermentation strategy obtained in this study from shake flask to bioreactor can be used as a platform for the production of P(3HB-*co*-3HV) copolymer by the industries as it has resulted in the highest productivity of 0.48 g/L/h.

CHAPTER 1: INTRODUCTION

Plastic, an important component of our life has gained its place as one of the most efficient, versatile, convenient and cheap material that can be used for various purposes (Luengo *et al*., 2003). The ever-growing accumulation of petrochemical based non-biodegradable plastic waste in the environment has always been an important issue of global concern. Besides its detrimental effects, constant production of these products also results in exhaustion of finite petroleum resources. A practical solution to this problem would be the use of biodegradable polymers derived from bio-based resources. However, complete substitution of established common plastics with biodegradable materials is still a challenge. This is mainly due to the production cost and material properties. Nevertheless, constant research regarding these issues has yielded many breakthroughs and brought forward some potential substitutes with compatible material properties and lowered production cost. Such an example is polyhydroxyalkanoate (PHA). PHA is such a potential candidate to replace non-renewable resources but the high production cost has become a limiting factor in furthering the usage of PHA.

PHA is an intracellular microbial reserve polymer of 3-hydroxy acids, which are produced by numerous bacteria during the depletion of nutrient such as nitrogen, oxygen, or phosphorus in the presence of excess carbon (Anderson and Dawes, 1990; Steinbuchel and Lutke-Eversloh, 2003). These polymers were found as granules in the cytoplasm of bacteria. Common producers of PHA are *Pseudomonas* species, *Rhodospirillum rubrum, Bacillus* species, *Aeromonas* species and *Cupriavidus necator* (previously known as *Alcaligenes eutrophus, Ralstonia eutropha* and *Wautersia eutropha*) (Anderson and Dawes, 1990). The intriguing biological

polymer has received considerable attention over the last decade because of the fact that PHA exhibit properties of biodegradable and elastic polymers (Sudesh *et al*., 2000).

Poly(3-hydroxybutyrate) [P(3HB)] is the most commonly occurring PHA in nature (Tsuge, 2002). The PHA synthase (PhaC) is the key enzyme involved in the incorporation of different monomers into the PHA polymer chain (Steinbuchel and Lutke- Eversloh, 2003). The properties of this microbial polymer can be regulated by introducing secondary monomer (Sudesh *et al*., 2000). The possibility to accumulate these diverse types of PHA is dependent entirely on the types of carbon precursors in which the bacteria was grown on, as well as the actively functioning metabolic pathways present in the cell (Loo and Sudesh, 2007; Amirul *et al*., 2008). Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV) is the first type of biopolymer that has been commercialized under the trademark BIOPOL^{TM}. The copolymer P(3HB-*co*-3HV) has been the center of attention in the bio-industrial fields, as it possesses superior mechanical properties compared to P(3HB) (Kim *et al*., 2009). The properties of the copolymer produced can be tailored by controlling the 3HV monomer composition.

PHA can be accumulated in various forms and types depending on the feedstock provided and the bacterial strain. The renewability of PHA reduces the dependence on fossil fuel (Sudesh *et al*., 2000). Very few microbes were found responsible for the production of SCL PHA copolymer that meets the commercial interest. Until today, *Cupriavidus necator* has been the species of choice to study the production of copolymer P(3HB-*co*-3HV) (Ramsay *et al*., 1990). Therefore the identification of a strain for the commercial production of copolymers is of special interest (Lee, 1996). Amirul *et al*. (2008) reported that *Cupriavidus* sp. USMAA2-4

is a local bacterium isolated from Sungai Pinang, Penang and was found responsible for the production of various copolymers.

The choice of carbon source is crucial in the production of PHA since half of the production costs depend on the substrate. There were many efforts taken to lower the production cost of PHA such as developing more efficient fermentation processes. According to Loo and Sudesh (2007), various carbon substrates such as 4-ketovaleric acid, propionic acid, valeric acid, heptanoic acid, nonanoic acid and amino acids such as valine, isoleucine, threonine and methionine can be used to synthesize the copolymer P(3HB-*co*-3HV). P(3HB-*co*-3HV) copolymers are commonly produced by adding precursor substrates such as propionic acid and valeric acid or their salts. These short-chain odd numbered carbon precursors are known to exert certain levels of toxicity to cells at high concentration (Volova and Kalacheva, 2005). Oleic acid, a cheap and renewable carbon source is one of the main fatty acids of palm oil (O'Brien 1998). Therefore, alcohol and oleic acid was added in order to achieve a higher cell biomass and copolymer content.

Since *Cupriavidus* sp. USMAA2-4 is a newly isolated strain from local environment, therefore it is necessary to optimize the production of the copolymer P(3HB-*co*-3HV) produced by this strain by using oleic acid and 1-pentanol. Besides that, optimization is necessary to obtain a standard formulation for the production of this copolymer and also to achieve a high PHA content. The optimization of the culture condition would allow widespread use of this copolymer and optimization methodology has been normally employed as the improvement strategies. It has been applied in many fields, such as in biological sciences, environmental sciences, pharmaceutical sciences and in food or industrial processes (Hanrahan and Lu, 2006). RSM is a statistically designed experimental protocol used for various

applications (Shih *et al*., 2008). It is a collection of statistical techniques for building models, designing experiments, evaluating the effects of factors, and searching for optimum conditions of factors for desirable responses (Mannan *et al*., 2007). Among these, the central composite design (CCD) is the most popular design as it is very efficient and flexible. It provides much information on experimental variable effects and overall experimental error.

Development of an efficient production technology for copolymer production would be of paramount importance. Industrial fermentation strategies involve producing high cell density product with high volumetric productivity (Cerrone *et al*., 2014). However, lower productivity of PHA when it comes to larger scale makes the overall production even more uneconomical. To overcome this, it is vital to design and develop proper fermentation strategies in order to improve the fermentation performance. Khanna and Srivastava (2005) reported that future studies should be carried out to increase the productivity by using alcohol for industrial PHA production. It is well established that high PHA productivity can be achieved by producing culture with high cell density, high PHA concentration or by reducing the cultivation time. In addition, the maximization of the carbon yield for PHAs relative to the carbon uptake for cell growth is of primary concern. Fed-batch cultivation is known as one of the strategy used to improve productivity. In fed-batch cultivations, one or more nutrients are added into the bioreactor while the existing components in the bioreactor are maintained. It has been well documented in the literature that fed-batch fermentation is a more suitable strategy in improving the P(3HB-*co*-3HV) production (Pena *et al*., 2014). Furthermore, there were also limited literature available on the production of P(3HB-*co*-3HV) copolymer with high productivity using locally isolated bacterium.

In this study, the ability of a locally isolated strain, *Cupriavidus* sp. USMAA2-4 to produce P(3HB-*co*-3HV) copolymer using oleic acid as the main carbon source and alcohol based 3HV precursor, 1-pentanol was investigated. Fermentation optimization process was carried out in shake flask as well as in bioreactor. Fed-batch strategy was carried out to improve the productivity of the copolymer and the material characteristics of the copolymer produced were determined. An optimized method for the production of copolymer P(3HB-*co*-3HV) in shake flask and bioreactor and improvement of copolymer productivity was focused throughout the study.

1.1 Objectives

The objectives of this study are:

1. To determine the potential of *Cupriavidus* sp. USMAA2-4 in utilizing oleic acid and 1-pentanol and to evaluate the production of P(3HB-*co*-3HV) copolymer production by using various parameters in single-stage cultivation.

2. To optimize the production of copolymer P(3HB-*co*-3HV) by using 1-pentanol and oleic acid through shake flask fermentation and bioreactor using response surface methodology (RSM).

3. To increase the productivity of copolymer P(3HB-*co*-3HV) by using fed-batch strategy in bioreactor.

4. To characterize the thermal and mechanical properties of the copolymer with various 3HV monomer compositions.

CHAPTER 2: LITERATURE REVIEW

2.1 Biobased polymers

Plastics are utilized in every manufacturing industry because it can be chemically manipulated to have a wide range of strengths and shapes. Plastics are recyclable, elastic, durable and chemically inert which contributes to various applications in many fields (Loo and Sudesh, 2007). However, plastics being xenobiotic are recalcitrant to microbial degradation. This gives a big impact on the environment. Synthetic plastics also produce toxins during the process of degradation (Suriyamongkol *et al*., 2007). Due to such circumstances, research has been carried out to produce a more environmental-friendly material that is natural, renewable and biocompatible (Verlinden *et al*., 2007). Replacement of non-biodegradable by biodegradable plastics is of major interest to the plastic industry (Reddy *et al*., 2003)

Biodegradable plastics are non-toxic and have the ability to degrade in a shorter period of time and this can reduce the environmental problem caused by petrochemical plastic (Gordon *et al.*, 2000; Flieger *et al*., 2003; Maeda *et al.*, 2005). One major discovery is the potential of synthesizing plastics using microorganisms. Biomaterials are natural products catabolised and synthesized by different organisms. Bioplastics are known as special type of biomaterials that can be assimilated by many species of microorganisms and do not cause toxic effects to the host (Chen, 2010).

The similar properties between the eco-friendly bioplastic and the synthetic plastics produced from fossil fuels make it as a good substitute for the conventional synthetic plastics used these days. The most widely produced bioplastics are polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and polylactides.

2.2 Polyhydroxyalkanoates (PHAs)

PHAs are simple macromolecules produced naturally by many species of microorganisms. It is an example of non-petroleum based biodegradable plastic that does not cause toxic effect to the host (Chien *et al*., 2007; Suriyamongkol *et al*., 2007). The building block of PHAs is 3-hydroxyalkanoic acid monomer unit (Figure 2.1). This bacterial storage material, which has monomers of 6 to 14 carbon atoms is a potential renewable and biodegradable plastics (Prieto *et al.*, 1999) and more than 75 genera are known to have the ability to synthesize and to accumulate PHAs intracellularly (Koller *et al*., 2011). PHAs are synthesized and accumulated inside numerous bacteria to a level as high as 80% of the cell dry weight when cultivated in media containing excess carbon but limitation of nitrogen or phosphorus (Ojumu and Solomon, 2004). This storage polymer acts as carbon and energy storage materials or as a sink for reducing power (Madison and Huisman, 1999; Doi, 1990). Due to the insolubility of PHAs inside the bacterial cytoplasm, insignificant increase in osmotic pressure was observed. This prevents the leakage of the polymer compound out of the cell (Verlinden *et al*., 2007). Bacteria that accumulate PHA showed enhanced tolerance towards environmental conditions such as heat, osmotic shock and UV irradiation (Kadouri *et al*., 2005).

PHAs are synthesized under stress conditions, especially when the nutrient supplies are imbalanced, for instance, depletion of nitrogen, phosphorus, sulphate, oxygen or other essential elements, but in the presence of excessive carbon sources (Reddy *et al.*, 2003). Besides, limitation of iron, magnesium, potassium and sodium was also found to stimulate the accumulation of PHA. This is to prevent starvation if an essential element becomes unavailable. In PHA production, it is important to maintain a suitable carbon to nitrogen (C/N) ratio because it is known to affect total

cell biomass and PHA production. This is due to the nitrogen content of the medium which directly affect the accumulation of acetyl-CoA that involve in PHA formation and bacterial growth (Lee *et al*., 2008).

These polyesters are deposited within water insoluble granule and are accumulated in the form of amorphous (Ren *et al*., 2000; Steinbuchel, 2005; Conte *et al.*, 2006). Once PHAs have been extracted from the cells, it exhibits the crystalline polymer behavior such as thermoplastic and elastomeric properties (Steinbuchel, 2005).

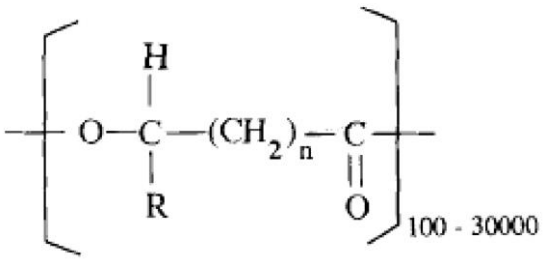


Figure 2.1: Chemical structure of PHA

R refer to side chain and n refers to the number of repeating units

The monomeric units of PHAs are enantiomerically pure and in the (*R*)-configuration. The monomers are all in the *R* configuration due to the stereospecificity of PHA synthase, the polymerizing enzyme. The *S* configuration monomers were detected only in rare cases (Haywood *et al*., 1991). The alkyl side chain of PHAs can be saturated, unsaturated, straight, branched chain containing aliphatic, aromatic, halogenated, epoxidized or nitrile side chain. Different type of functional group and the length of the side chain directly influence the properties of the polymer such as crystallinity and melting point (Eggink *et al*., 1995).

In prokaryotes, PHAs can be found in the cell cytoplasm as small insoluble inclusions which usually have 0.2 μm to 0.5 μm diameter in size (Sudesh *et al.*, 2000; Dennis *et al*., 2008). Observation under the electron and phase contrast microscope shows the granules to be highly refractile inclusions (Lee, 1996). The molecular weight of the polymer ranges from 2 x 10^5 to 3 x 10^6 daltons and the polydispersity index around 2.0 (Lee, 1996; Madison and Huisman, 1999; Agus *et al*., 2006).

Different species have different number and size of granule. The number of granules of *Cupriavidus necator* varies between the range of 8 to 12 granules whereas *Pseudomonas oleovorans* is estimated to have about one or two large granules (Zinn *et al.*, 2001). Observation of PHAs in various microorganisms can be identified by staining with Sudan black or Nile blue (Lee, 1996). The surface of the granule of these polyesters consists of a phospholipid layer separating two crystalline protein layers. The hydrophobic polyester core of the granule is largely amorphous *in vivo* (de Koning and Lemstra, 1992). In order to prevent crystallization of the polymer, water acts as plasticizer (Horowitz and Sanders, 1994).

Proteins responsible for PHA production can be divided into four classes: (I) PHA polymerases, (II) PHA depolymerases, (III) phasins (PhaPs) which have a granule stabilizing function, and (IV) PhaR that functions as regulator of phasins. All these granule associated proteins are found within the bacteria that produce PHAs (Zinn *et al*., 2001; Potter and Steinbuchel, 2005).

It was reported by Braunegg *et al*. (1998) that PHAs extracted from the bacterial cells show material properties that is similar to polypropylene. Its thermoplastic and elastomeric properties have been useful in many applications (O'Leary *et al.*, 2005; Nikodinovic *et al.*, 2008).

2.3 Discovery of PHAs

It was reported by Chowdhury in 1963 that lucent granules of PHAs in bacterial cells were first observed in 1888 by Beijerinck. Then, in 1926 French scientist Lemoigne discovered the composition of first PHA. He reported that *Bacillus megaterium* accumulate intracellular homopolymer that consisted of 3-hydroxybutyric acids. This homopolymer were linked through ester bonds between the carboxylic group and the 3-hydroxyl group of the next monomer (Zinn *et al.*, 2001; Suriyamongkol *et al.*, 2007).

Macrae and Wilkinson in 1958 reported the function of P(3HB) (Suriyamongkol *et al.*, 2007). Then, Wallen and Rohwedder in 1974 discovered PHAs containing 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) in chloroform extract of activated sewage sludge. They also reported that this heteropolymer has lower melting point compared to P(3HB) and was soluble in hot ethanol (Anderson and Dawes, 1990).

In 1983, a significant development was reported by De Smet when *Pseudomonas oleovorans* grown on 50% (vol/vol) n-octane was found to accumulate granules consist of 3-hydroxyoctanoic acid (3HO) and an unidentified fatty acids (De Smet *et al*., 1983).

This was followed by a more detailed investigation by Lageveen *et al*. (1988) who disclosed that the unidentified fatty acid was (*R*)-3-hydroxyhexanoate (3HHx). At least 11 short-chain 3-hydroxyacids (3-HAs) were detected in polymers extracted from marine sediment using the capillary gas chromatographic (GC) analysis.

About 95% 3HB, 3% 3-hydroxyheptanoate (3HHp), 2% 3HO and trace amounts of other 3-HAs were detected by them from the purified polymer of *B. megaterium* (Findlay and White, 1983). In 1986, discovery of PHAs containing C4,

C6 and C8 monomers from sewage sludge was reported by Odham *et al*. (1986). Imperial Chemical Industries (ICI) started investigating the production of P(3HB) by bacterial fermentation in 1976. The properties of P(3HB) was also recognized and this kept ICI's interest in P(3HB) after the oil crisis (Howells, 1982; Senior, 1984). However, due to the brittleness of the polymer, the usage of the polymer for various applications was limited. The discovery of P(3HV) in 1974 stimulate the ICI to patent various processes. Besides, the company also claimed the processes for production of various other monomers by using a variety of substrates (Philip *et al*., 2007).

In April 1996, Monsanto acquired the BIOPOL® from Zeneca Bio Products, England. Monsanto produces a range of P(3HB-*co*-3HV) under the trade name of BIOPOL® (Braunegg *et al*., 1998). In 1998, Metabolix Inc received the license from Monsanto. In the same year, a new spin off company was developed under the name of Tepha whereby medical devices were developed (Philip *et al*., 2007).

2.4 Types of PHAs

PHAs can be divided into three categories based on the number of carbon atoms present and their different proportion of monomer units. The enzyme responsible for PHAs biosynthesis is polyhydroxyalkanoate synthase (Zinn *et al*., 2001; Pantazaki *et al*., 2003; Chen *et al*., 2006; Ayub *et al*., 2007). The first category is the short chain length-PHAs (SCL)-PHAs, polymerizing C4-C5 carbon length monomers and the second category is medium chain length-PHAs (MCL)-PHAs whose carbon length ranges from C6-C12. The third category is the copolymer of short chain length and medium chain length whose monomer contains C3-C14

carbon atoms (SCL-MCL)-PHAs (O'Leary *et al.*, 2005). The composition of PHA depends on the substrate used (Huisman *et al*., 1989).

2.4.1 Short chain length-PHAs (SCL)-PHAs

The short chain length-PHAs (SCL)-PHAs constitutes 3–5 carbon atoms in the monomer. The (SCL)-PHAs are thermoplastics that have high melting temperature, stiffer in nature (Sujatha *et al.*, 2007), have high degree of crystallinity and rigidness (Chen *et al.*, 2006; Liu and Chen, 2007). An example of (SCL)-PHAs is P(3HB) which is the most common type of PHA (Ayub *et al.*, 2007). P(3HB) can be found accumulated in granular form in many bacteria (Akiyama *et al*.,1992). P(3HB) is also known to be optically active (Lenz and Marchessault, 2005). The granules isolated from *B. megaterium* that contain (SCL)-PHAs was found to be enwrapped by a monolayer of lipid and proteins on the outer side (Sudesh *et al*., 2000).

The mechanical properties of P(3HB) is comparable to polypropylene, however it has poor elongation at break compared to polypropylene (Iwata *et al*., 2003). This homopolymer also exhibited high melting temperature of 170ºC which is slightly lower than its degrading temperature (Sudesh *et al*., 2000). This makes the polymer difficult to be processed and this limits its applications. It is also brittle and highly hydrophobic (Pachene *et al.*, 2007). *Cupriavidus necator* formerly known as *Ralstonia eutropha*, is one of the bacteria that synthesize PHB (Lee *et al.*, 1999; Chien *et al.*, 2007). Besides, P(3HB) was also found to be a biocompatible polymer in human (Zinn *et al*., 2001). Copolymers can be formed by feeding precursors and this may result in the formation of a copolymer containing P(3HB) with 3-

hydroxyvalerate (3HV) monomer or 4-hydroxybutyrate (4HB) monomer (Huisman *et al*., 1992).

Another example of (SCL)-PHAs is poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] which is less stiff and brittle compared to P(3HB). It is produced by the incorporation of 3-hydroxyvalerate (3HV) monomer into P(3HB). Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB) is also a type of (SCL)-PHAs which is a strong and malleable thermoplastic with the tensile strength comparable to that of polyethylene. This copolymer is usually produced by using carbon substrates such as 1,6-hexanediol, 1,4-butanediol, 4-chlorobutyric acid or gamma-butyrolactone (Doi *et al*., 1989). It has elongation at break of 1000% resulting in a very elastic polymer. P(3HB-*co*-4HB) is mainly used in medical and pharmaceutical fields due to its biocompatibility and elastomeric properties (Martin and Williams, 2003).

2.4.2 Medium chain length-PHAs (MCL)-PHAs

The medium chain length (MCL)-PHAs consists of monomer ranging from C_6 (3-hydroxyhexanoate) to C_{12} (3-hydroxydodecanoate) (Hazenberg and Witholt, 1997). According to Steinbuchel and Lutke-Eversloh (2003) the first discovery of (MCL)-PHAs was 3-hydroxyoctanoic acid (3HO) produced by *Pseudomonas oleovorans*. Other example of MCL-PHAs are poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate) [P(3HHx-*co*-3HO-*co*-3HD)] and poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate) [P(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD) (Chen, 2010). Granules that has (MCL)-PHAs has a phospholipid layer separating two crystalline protein

layers and the granules were found to be larger than granules that contains (SCL)-PHAs (Stuart *et al*., 1995).

These PHAs are usually synthesized by *pseudomonads* belonging to the rRNA-homology-group I (Sudesh *et al.*, 2000). These (MCL)-PHAs can be produced from a number of organic compounds such as alkanes, aliphatic alcohols, carboxylic acids, and aromatic compounds using *Pseudomonas* strains (Lee *et al.*, 1999).

The (MCL)-PHAs are elastomers that has poor tensile strength and has high extension at break (Sujatha *et al.*, 2007). This (MCL)-PHAs are also a sticky materials which can be modified to make rubbers (Suriyamongkol *et al.*, 2007). The (MCL)-PHAs have lower level of crystallinity and higher elasticity as compared to P(3HB) or P(3HB-*co*-3HV) (Preusting *et al*., 1990).

2.4.3 Short chain length-medium chain length-PHAs (SCL-MCL)-PHAs

(SCL-MCL)-PHAs are known to exhibit properties in between (SCL)-PHAs and (MCL)-PHAs. This copolymer also has superior properties compared to SCL and MCL homopolymer (Nomura *et al*., 2004). It has a wide usage and is ideal for commercial uses (Sujatha *et al.*, 2007; Bhubalan *et al*., 2008). The properties of (SCL-MCL)-PHAs copolymer depend on the structure, monomer composition, distribution of monomer units, the average molecular weight and molecular weight distribution (Liu and Chen, 2007). Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] is an example of (SCL-MCL)-PHAs. It is usually synthesized by few microorganisms such as *Aeromonas caviae* and *Aeromonas hydrophilla*.

Incorporation of 3HHx was found to significantly reduce the melting point temperature thus improve the thermal processability (Doi *et al*., 1995). P(3HB-*co*-

3HHx) copolymers have properties almost similar to low density polyethylene (LDPE). PHAs with longer side chains are more ductile and can be molded easily.

2.5 Biosynthesis of PHA

Biosynthesis of PHA proceeds via three steps. First, the carbon source supplied to the bacteria will be transported by passive diffusion or with the help of specific transporters. Then the carbon source has to be converted into thioester of hydroxyalkanoic acid through metabolic pathways. Lastly, the CoA thioester produced will be used as a substrate for PHA synthase (Potter and Steinbuchel, 2006). There are many different metabolic pathways for the biosynthesis of PHA in bacteria. For all known bacterial PHA, there are three major different pathways identified.

2.5.1 Biosynthesis of SCL-PHAs

This pathway is found in *Cupriavidus necator* which starts from acetyl-CoA. Acetyl- CoA is converted to P(3HB) via three enzymatic steps. These enzymes are β-ketothiolase (phaA), acetoacetyl-CoA reductase (phaB) and PHA synthase (phaC). The biosynthetic β-ketothiolase catalyses the formation of a carbon–carbon bond by condensation of two acetyl-CoA moieties with the release of one CoA in a reversible manner (Anderson and Dawes, 1990; Poirer *et al*., 1995). An NADPH-dependent stereospecific acetoacetyl-CoA reductase catalyses the reduction of acetoacetyl-CoA to *R*(−)-3-hydroxybutyryl-CoA. This is linked to the growing chain of P(3HB) by an ester bond which is catalysed by PHA synthase with a CoA being liberated (Lee, 1996; Lee *et al.*, 1999; Verlinden *et al*., 2007). This reaction takes place only on the

surface of the granule (Zinn *et al.*, 2001). The polymerizing enzyme accepts only (*R*)-isomers as substrates (Tsuge *et al*., 2005).

Intracellular concentration of acetyl-CoA and free CoA is responsible for PHB synthesis. It was reported that PHB synthesis is stimulated by high intracellular concentration of NADH and high ratio of NADH/NAD. However, citrate synthase activity is inhibited by NADH. Citrate synthase which is responsible in TCA cycle is an important control point in PHB synthesis because it has the ability to control the availability of CoA that regulates the activity of β-ketothiolase. Once citrate synthase is inhibited, acetyl-CoA is converted to acetoacetyl-CoA by β-ketothiolase and the polymer production is favored. However, when the nutrient limitation is restored, the CoA concentration becomes high and these inhibit the β-ketothiolase thus channeling the acetyl-CoA to TCA cycle. This condition prevents the PHB synthesis (Anderson and Dawes, 1990)

It was found that *R. eutropha* has two β-ketothiolase (enzyme A and enzyme B) which are responsible to act in the biosynthetic pathway of P(3HB) synthesis. Enzyme A converts acetoacetyl-CoA and 3-ketopentanoyl-CoA whereas enzyme B cleaves acetoacetyl-CoA, 3-ketopentanoyl-CoA, 3-ketohexanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketooctanoyl-CoA and 3-ketodecanoyl-CoA. Initially, it was assumed that enzyme A is responsible in P(3HB) biosynthetic pathway whereas enzyme B is responsible for fatty acid degradation (Haywood *et al*., 1988). Slater *et al*. (1988) reported that enzyme B is the primary source for the formation of 3HV monomer.

There are two types of acetoacetyl-CoA found in *R. eutropha* which is NADPH-dependent acetoacetyl-CoA and NADH-dependent acetocetyl-CoA. NADH-dependent acetoacetyl-CoA is active with both *R*(-)- and *S*(+)-3-

hydroxyacyl-CoA and NADPH-dependent acetoacetyl-CoA is active only with *R*(-)-3-hydroxyacyl-CoA (Trotsenko and Belova, 2000). Only NADPH-dependent acetoacetyl-CoA was found involved in P(3HB) biosynthesis (Haywood *et al*., 1988).

It was reported by Jung *et al*. (2000) that the rate of biosynthesis of P(3HB) is controlled by β-ketothiolase and acetoacetyl-CoA reductase whereas the P(3HB) content is controlled by PHA synthase. The PHA synthase also plays an important role in controlling the polydispersity and molecular weight of the polymer (Sim *et al*., 1997). The three genes for PHA biosynthesis in *R. eutropha* are clustered and they form a single operon in the order of synthase-thiolase-reductase (*phaCAB*) (Peoples and Sinskey, 1989; Schubert *et al*., 1991).

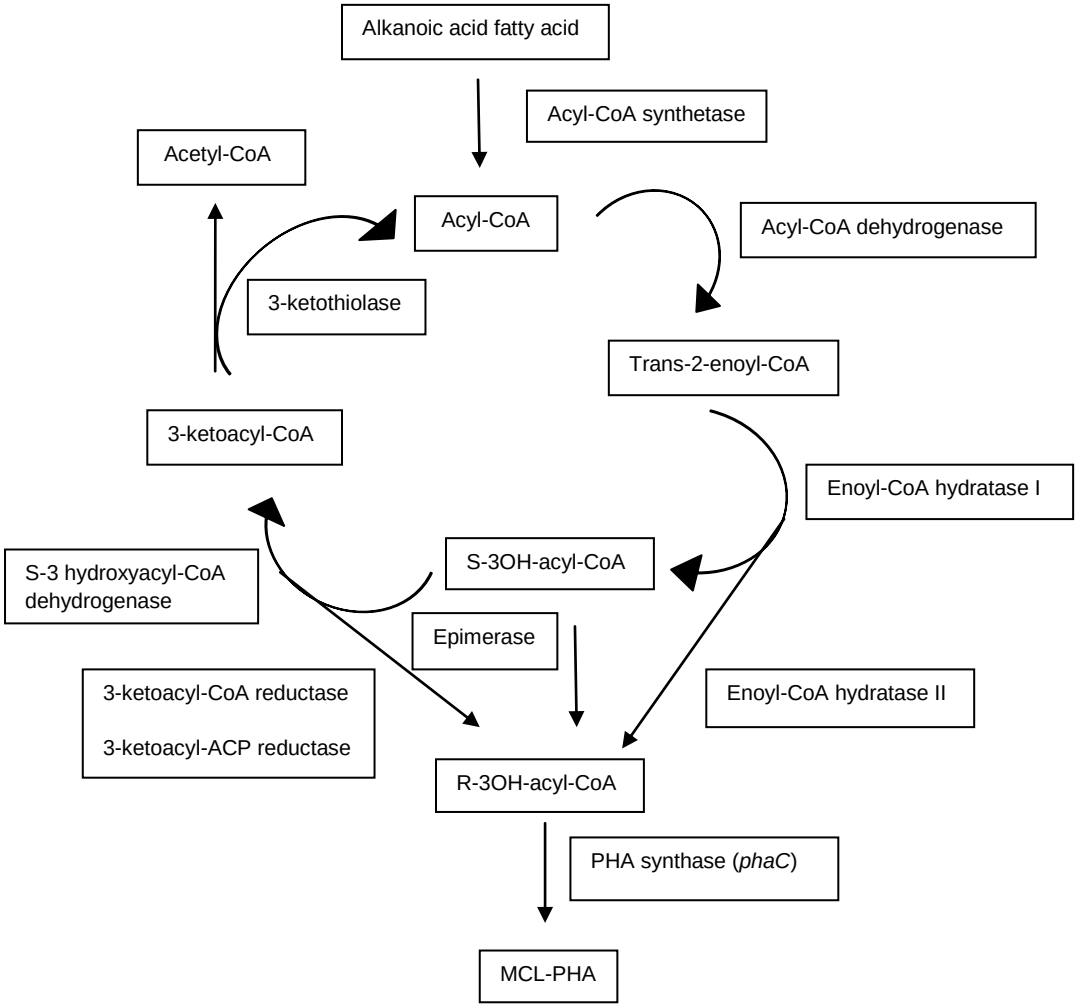
2.5.2 Biosynthesis of MCL-PHAs

The second type of PHA biosynthetic pathway is through fatty acid β-oxidation. This type of pathway can be found in *pseudomonas* and other *pseudomonas* belonging to the ribosomal RNA homology group I. They synthesize MCL-PHAs from various MCL-alkanes, alkanoates or alkanols (Lageveen *et al*., 1988). In this step, enoyl-CoA, 3-ketoacyl-CoA and (*S*)-3-hydroxyacyl-CoA are the intermediates of fatty acid β-oxidation pathway (Suriyamongkol *et al*.,2007). (*S*)-3-hydroxyacyl-CoA is required for the synthesis of the (*R*)-3-hydroxyacyl-CoA monomer (Madison and Huisman, 1999). In these bacteria, the carbon source added to the media directly influences the PHA produced.

The first step in this pathway involves the degradation of the fatty acids by removal of C_2 unit as acetyl-CoA to acyl-CoA before it is directed to β-oxidation pathway. Once the CoA has been generated, it is converted in a series of steps to 3-hydroxyacyl-CoA intermediates. The PHA synthase accepts only (*R*)-3-hydroxyacyl-CoA, however β-oxidation pathway only generates the (*S*)-3-hydroxyacyl-CoA (Schulz, 1991; Gerhart, 1993). Enzyme 3-hydroxyacyl-CoA epimerase was found responsible for the conversion of S isomer to R isomer.

The third type is found in *Pseudomonas aeruginosa* which use the fatty acid *de novo* biosynthesis pathway (Fab pathway). The bacteria that utilize this pathway can synthesize MCL-PHAs by using unrelated carbon source such as glucose (Anderson and Dawes 1990). During this pathway, (*R*)-3-hydroxyacyl-ACP-CoA transferase (PhaG) is responsible for the conversion of (*R*)-3-hydroxyacyl-ACP to (*R*)-3-hydroxyacyl-CoA (Suriyamongkol *et al*., 2007). Figure 2.2 illustrates the various biosynthetic pathways involved in synthesizing PHAs.

A


Alkanoic acid fatty acid
Acyl-CoA synthetase
Acetyl-CoA
Acyl-CoA
Acyl-CoA dehydrogenase
3-ketothiolase
Trans-2-enoyl-CoA
3-ketoacyl-CoA
Enoyl-CoA hydratase I
S-3 hydroxyacyl-CoA dehydrogenase
S-3OH-acyl-CoA
Epimerase
3-ketoacyl-CoA reductase
3-ketoacyl-ACP reductase
Enoyl-CoA hydratase II
R-3OH-acyl-CoA
PHA synthase (phaC)
MCL-PHA

B

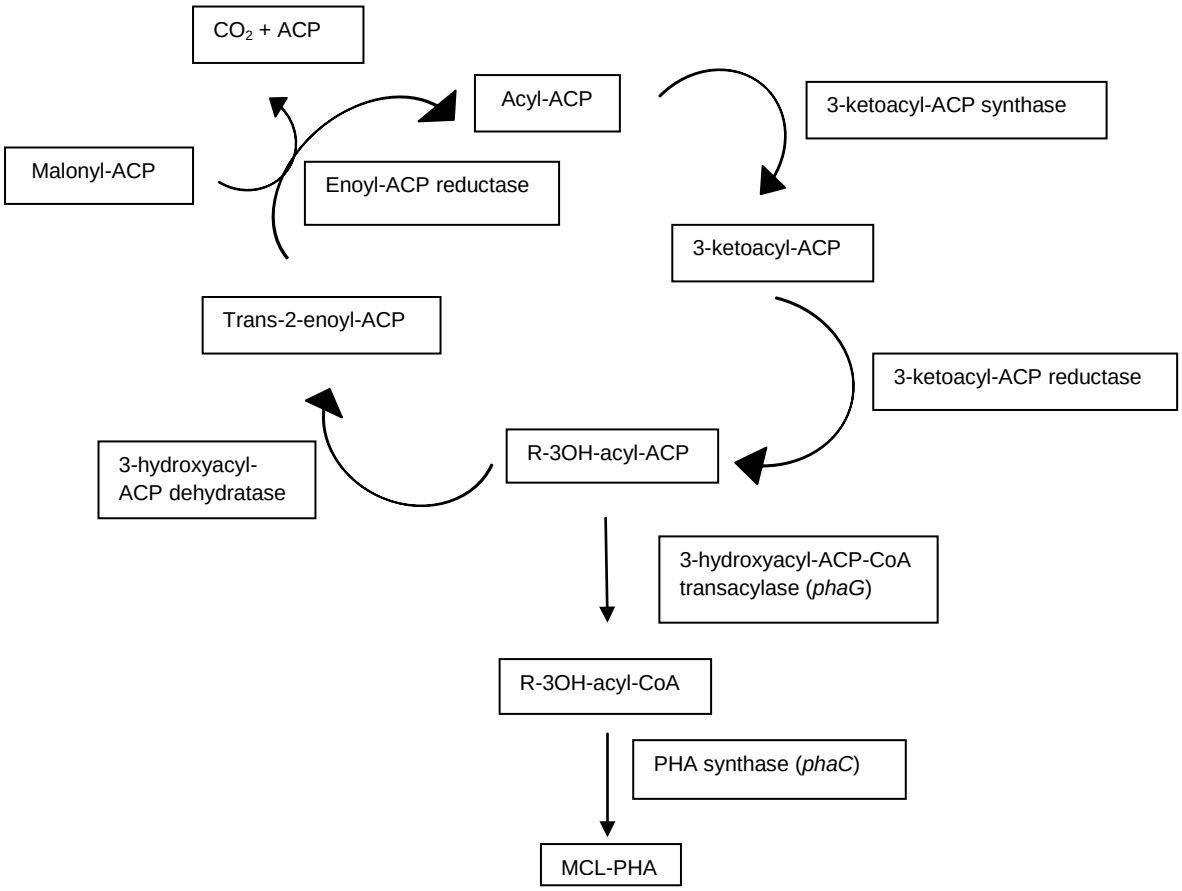


Figure 2.2: Pathway for MCL-PHAs synthesis. (A) Synthesis of MCL-PHAs using fatty acid β-oxidation pathway. (B) Synthesis of MCL-PHAs using fatty acid *de novo* biosynthesis pathway (Poirier, 2002).

2.6 Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)]

The improved properties of P(3HB) was obtained by incorporating 3HV monomer to P(3HB) to produce P(3HB-*co*-3HV) (Lee, 1996). Production of P(3HB-*co*-3HV) was carried out in large scale under the name of Biopol^{TM} and it has been the PHA marketing strategies by Zeneca and Monsanto (Poirier, 2002). The chemical structure of P(3HB-*co*-3HV) is shown in Figure 2.3

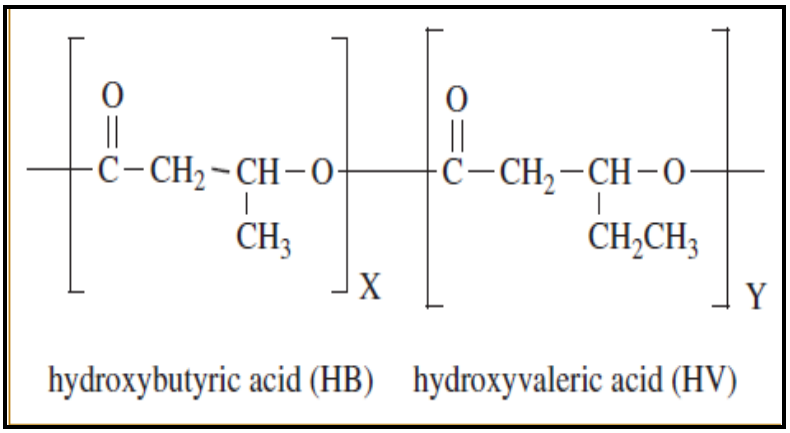


Figure 2.3: Poly(3-hyroxybutyrate) and copolymers with hydroxyvaleric acid. For a homopolymer of HB, Y = 0 (Pachene *et al*.,2007)

P(3HB-*co*-3HV) copolymer was found to be less crystalline, more flexible, and more readily processible. The properties of the copolymer are determined by varying the mole percentage of 3HV monomer in the polymer. As the fraction of 3HV monomer increases, increase in impact strength and decrease in Young's modulus was observed. This contributes to a tougher and more flexible copolymer (Pachene *et al*.,2007).

This copolymer also has a lower melting point as compared to P(3HB) without changing its degradation temperature. This allows the thermal processing of the copolymer for various applications (Lee, 1996). Table 2.1 shows thermal and mechanical properties of the copolymer produced with varying 3HV monomer

fraction to that of the synthetic polymers. [P(3HB-*co*-3HV) copolymer containing more than 20 mol% of 3HV units can be used to make films and fibers with different elasticity by controlling the processing conditions (Philip *et al*., 2007).

Table 2.1: Thermal and mechanical properties of various composition of P(3HB-*co*-3HV) copolymer (Doi, 1990; Loo and Sudesh, 2007)

	Melting temperature (°C)	Glass transition temperature (°C)	Young's Modulus (GPa)	Tensile strength (MPa)
Polypropylene	176	-10	1.7	38
Poly (ethylene terepthalate)	267	69	2.9	70
Nylon-6,6	265	50	2.8	83
Low density polyethylene	130	-30	0.2	10
P(3HB)	179	10	3.5	40
P(3HB-*co*-3 mol% 3HV)	170	8	2.9	38
P(3HB-*co*-9 mol% 3HV)	162	6	1.9	37
P(3HB-*co*-14 mol% 3HV)	150	4	1.5	35
P(3HB-*co*-20 mol% 3HV)	145	-1	1.2	32
P(3HB-*co*-25 mol% 3HV)	137	-6	0.7	30

It was reported that the copolymer P(3HB-*co*-3HV) exhibits isodimorphic characteristics. Below 30 mol% of 3HV monomer, the 3HV units crystallize in the PHB lattice whereas above 30 mol% of 3HV monomer, the 3HB units crystallize in the P(3HV) lattice (Bluhm *et al*., 1986; Anderson and Dawes, 1990). The spherulite growth rate of P(3HB) far exceeds the copolymer fraction and so at lower 3HV molar fraction, only the P(3HB) crystal growth was observed (Madden and Anderson, 1998). Two characteristics that accounts for isodimorphism are that the two different monomers have approximately the same shape and it occupy the same volume and the chain conformation of both the homopolymers compatible with either crystal lattice. The copolymers are termed isodimorphic when one crystalline

phase containing both monomer types are detected at all compositions. This phenomenon of isodimorphism accounts for many interesting properties displayed by these materials (Anderson and Dawes, 1990).

P(3HB-*co*-3HV) are synthesized using odd number carbon substrate. A random copolymer containing 3HB and 3HV were produced when valeric acid or propionic acid were added to the culture containing glucose (Steinbuchel and Schlegel, 1991). The usage of propionic acid was found to generate 3HB monomer together with 3HV monomer. Besides, the usage of propionic acid was also found to be highly toxic to the cell when used at a high concentration of propionic acid (Yu *et al*., 2002). Limited publication is available on the use of alcohols as precursor for 3HV by non-methylotrophic PHA producing strains such as *C. necator*. Berezina (2012) reported the usage of 1-pentanol as 3HV precursor by *C. necator* strain DSM 545. However, it was found that 1-pentanol exhibited negative influence on the biomass production and copolymer content.

Two parallel pathways are involved in the biosynthesis of the copolymer. One involves the synthesis of 3-hydroxybutyrate (3HB) and another one is the synthesis of 3HV monomers. The ß-ketothiolase enzyme (PhaA) encoded by *phaA* gene catalyzes the condensation of either two acetyl-CoA molecules or of one acetyl-CoA and one propionyl-CoA to form acetoacetyl-CoA or 3-ketovaleryl-CoA.

Then acetoacetyl-CoA or 3-ketovaleryl-CoA is reduced to 3-hydroxybutyryl-CoA (3HB-CoA) or 3-hydroxyvaleryl-CoA (3HV-CoA) by NADPH-dependent acetoacetyl-CoA reductase (PhaB) encoded by *phaB* gene. PHA synthase (PhaC) encoded by *phaC* gene involves in the polymerization of 3HB-CoA and 3HV-CoA randomly into a P(3HB-*co*-3HV) growing chain (Liu *et al*., 2009).

Condensation of acetyl-CoA with propionyl-CoA in *R. eutropha* is mediated by specific ß-ketothiolase named bktB. This enzyme has a higher tendency for propionyl-CoA as compared to the ß-ketothiolase (Slater *et al*., 1988).

2.7 Gene regulation in PHA biosynthesis

One of the proteins associated with the phospholipid granule is PHA synthase which is responsible for the stereoselective conversion of (*R*)-3-hydroxyacyl CoA thioester into PHAs with the concomitant release of coenzyme A (Rehm, 2003; Grage *et al*., 2009). It exists either in soluble form or are found bound to PHA granules (Gerngross *et al*., 1993).

The soluble PHA synthase was found to be less active as compared to the synthase bound to PHA granules (Martin and Gerngross, 1996). PhaC of *R. eutropha* exits as soluble protein in cytoplasm and becomes insoluble by granule binding after the PHA accumulation has been initiated (Gerngross *et al*., 1993). PHA synthase being the third key enzyme was found responsible for dispersion, molecular weight, monomer composition and in determining the final yield of PHA (Steinbuchel and Hein, 2001).

There are four classes of PHA synthase reported so far. PHA synthase that belongs to class I utilize (*R*)-3-hydroxy fatty acids with 3-5 carbon atoms and produces short chain length monomers. This type of PHA synthase can be found in *C. necator* (Ren *et al*., 2000; Rehm, 2003).

Class II PHA synthase can be found in *P. aeruginosa* that catalyze the medium chain length PHA. It utilizes (*R*)-3-hydroxy fatty acids with 6-14 carbon atoms. Both class I and class II PHA synthase consists of only one type of PhaC subunit that has molecular weight between 61 and 73 kDa (Qi *et al*., 1997; Qi and