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

SHANTINI KANNUSAMY

UNIVERSITI SAINS MALAYSIA

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by

SHANTINI KANNUSAMY

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

OCTOBER 2015
ACKNOWLEDGEMENTS

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<thead>
<tr>
<th>Symbols and Abbreviations</th>
<th>Full name</th>
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<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>ΔH_m</td>
<td>Heat of fusion</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μM</td>
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<td>μmol</td>
<td>Micromole</td>
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<td>3HB</td>
<td>3-hydroxybutyrate</td>
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<td>3-hydroxybutyryl Coenzyme A</td>
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<td>3-hydroxyvalerate</td>
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<td>ANOVA</td>
<td>Analysis Of Variance</td>
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<td>C</td>
<td>Carbon atom</td>
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<td>CaCl₂</td>
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<td>Chloroform</td>
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<tr>
<td>Cₗ</td>
<td>Dissolved oxygen concentration</td>
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<table>
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<td>C*&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Dissolved oxygen concentrations in equilibrium with mean gaseous oxygen concentration</td>
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<td>CME</td>
<td>Caprylic methyl ester</td>
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<td>CPKO</td>
<td>Crude palm kernel oil</td>
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<tr>
<td>CPO</td>
<td>Crude palm oil</td>
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<td>Flame ionization detector</td>
</tr>
<tr>
<td>g</td>
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</table>
g
Gram

GC
Gas chromatography

g/L
Gram per liter

GPC
Gel permeation chromatography

h
Hour

H
Hydrogen atom

HCl
Hydrochloric acid

H₂O
Water

HPLC
High-performance liquid chromatography

H₂SO₄
Sulphuric acid

ICI
Imperial Chemical Industries

IS
Internal standard

J/g
Joule per gram

Kb
Kilo base pairs

kDa
Kilodalton

kg
Kilogram

KH₂PO₄
Potassium dihydrogen phosphate

Kₒₐ
Volumetric oxygen transfer coefficient

KOH
Potassium hydroxide

L
Liter

LB
Luria Bertani

M
Molar
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<td>mcl</td>
<td>Medium chain length</td>
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<td>min</td>
<td>Minute</td>
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<td>mg</td>
<td>Milligram</td>
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<td>Magnesium sulphate heptahydrate</td>
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<tr>
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<td>mol percent</td>
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<tr>
<td>MPa</td>
<td>Megapascal</td>
</tr>
<tr>
<td>MSM</td>
<td>Mineral salt medium</td>
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<td>Mₔ</td>
<td>Weight-average molecular weight</td>
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<tr>
<td>Mₔ/M₀</td>
<td>Polydispersity index</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium hydrogen phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Ammonium chloride</td>
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</table>
NiCl$_3$·6H$_2$O  
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  
$\beta$-ketothiolase; gene encoding $\beta$-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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>scl</td>
<td>Short chain length</td>
</tr>
<tr>
<td>sd</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>sp.</td>
<td>Species</td>
</tr>
<tr>
<td>$T_c$</td>
<td>Crystallization temperature</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TES</td>
<td>Trace elements</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>wt%</td>
<td>Dry weight percent</td>
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<tr>
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<td>Weight per volume</td>
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LIST OF PUBLICATIONS

PEER REVIEWED JOURNALS


CHAPTERS IN BOOK


BOOK


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)

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)


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 gencangan 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
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 gongcangan 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 penculik tempatan ini didapat 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 keseluruhan, 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 keseluruhan, strategi fermentasi yang diperolehi melalui kajian ini daripada kelalang gongcangan 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™. 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 $2 \times 10^5$ to $3 \times 10^6$ 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 (Pachen e 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 C₆ (3-hydroxyhexanoate) to C₁₂ (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 \( \text{R(−)-3-hydroxybutyryl-CoA} \). 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
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-ketoacetyl-CoA, 3-ketoheptanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketoctanoyl-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-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.
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™ 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

![Chemical structure of P(3HB-co-3HV)](image)

Figure 2.3: Poly(3-hydroxybutyrate) 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)

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

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-hydroxybutyryl-CoA 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-hydroxybutyrlyl-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 β-ketothiolase named bktB. This enzyme has a higher tendency for propionyl-CoA as compared to the β-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