SINGLE-STAGE BIOSYNTHESIS OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) USING *Cupriavidus* sp. USMAA1020 VIA MIXED-SUBSTRATE CULTIVATION STRATEGY

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Growth associated term
β	Non-growth associated term
e	Exponent
μ_{max}	Maximum specific growth rate
^{1}H-NMR	Proton-Nuclear magnetic resonance analysis
^{13}C-NMR	Carbon- Nuclear magnetic resonance analysis
ΔH_m	Enthalpy of fusion
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
3HHp	3-hydroxyheptanoate
3HHx	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
4HB	4-hydroxybutyrate
A_a	Area under amorphous hump
A_c	Area under crystalline peak
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
C	Carbon atom
C/N	Carbon-to-nitrogen ratio
CoA	Coenzyme A
C=O	Carbonyl
CAM	Chick chorioallantoic membrane

$CDCl_3$	Deuterated chloroform
$CaCl_2 \cdot 2H_2O$	Calcium chloride dehydrate
$CuCl_2 \cdot 2H_2O$	Copper (II) chloride dihydrate
CME	Caprylic methyl ester
$CoSO_4 \cdot 7H_2O$	Cobalt (II) sulphate heptahydrate
D	Randomness
D_i	Dispersity
DO	Dissolved oxygen
$FeSO_4 \cdot 7H_2O$	Iron (II) sulphate heptahydrate
g	Gravity
GC	Gas chromatography
GC-MS	Gas chromatography- mass spectroscopy
HA(s)	Hydroxyalkanoate(s)
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
ICI	Imperial Chemical Industries
kDa	Kilo Dalton
K_2HPO_4	Dipotassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
K_La	Volumetric mass transfer coefficient
LCL	Long chain length
m_s	Maintenance coefficient
mol%	Mol percentage
M_w	Weight-average molecular weight
M_n	Number-average molecular weight
MCL	Medium chain length

$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
$MnCl_2 \cdot 4H_2O$	Manganese (II) chloride tetrahydrate
MSM	Mineral salt medium
NA	Nutrient agar
NADH	Nicotinamide adenine dinucleotide
$(NH_4)_2SO_4$	Ammonium sulphate
N.D.	Not detected
NMR	Nuclear magnetic resonance
NR	Nutrient rich
OD	Optical density
PHA(s)	Polyhydroxyalkanoate(s)
PhaA	*β*-kethiolase
PhaB	Acetoacetyl-CoA reductase
PhaC	PHA synthase
PhaG	(*R*)-3-hydroxyacyl-ACP-CoA-transferase
PhaP	Phasin
PhaR	Regulator protein for phasing expression
PhaZ	PHA depolymerase
PLA	Polylactic acid
P(3HB)	P(3-hydroxybutyrate)
P(4HB)	Poly(4-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-4HV)	Poly(3-hydroxybutyrate-*co*-4-hydroxyvalerate)
P(3HB-*co*-3HV-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate)

P(3HB-*co*-3HV-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-4-hydroxybutyrate)
P(3HHx-*co*-3HO-*co*-3HD)	Poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate)
P(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD)	Poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate)
P	Product
P value	Probability value
PDI	Polydispersity index
PTFE	Polytetrafluoroethylene
rpm	Rotations per minute
s	Substrate
SCL	Short chain length
SSADH	Succinate semialdehyde dehydrogenase
SEM	Scanning electron microscope
sp.	Species
spp.	Several species
TEM	Transmission electron microscope
TCA	Tricarboxylic acid
TMS	Tetramethylsilane
T_c	Crystallization temperature
T_g	Glass transition temperature
T_m	Melting temperature
UV	Ultraviolet
vvm	Gas volume flow per unit of liquid volume per minute (volume per volume per minute)
v/v	Volume per volume
x	Biomass

x_c	Crystallinity
x_m	Biomass at t = 0
x_m	Maximum biomass
X_c	Degree of crystallinity
w/v	Weight per volume
wt%	Weight percentage
wt% C	Weight percentage of carbon
$Y_{x/s}$	Yield coefficient of biomass over substrate
$Y_{p/s}$	Yield coefficient of product over substrate
$Y_{p/x}$	Yield coefficient of product over biomass
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate heptahydrate

BIOSINTESIS SATU PERINGKAT POLI(3-HIDROKSIBUTIRAT-*ko*-4-HIDROKSIBUTIRAT) MENGGUNAKAN *Cupriavidus* sp. USMAA1020 MELALUI STRATEGI PENGKULTURAN SUBSTRAT CAMPURAN

ABSTRAK

Kopolimer poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) [P(3HB-*ko*-4HB)] merupakan polihidroksialkanoat (PHA) yang penting dalam bidang biofarmaseutikal dan perubatan. Produktiviti kopolimer dengan komposisi 4HB yang tinggi sering dicapai melalui pengkulturan dua-peringkat tetapi ini menyukarkan dengan meningkatkan masa dan usaha pengendalian, dan risiko kontaminasi dalam proses penghasilan berskala besar. Kajian ini bertujuan untuk membangunkan strategi fermentasi satu-peringkat yang berkesan menggunakan strategi pengkulturan substrat campuran sebagai pengganti fermentasi dua-peringkat. Strategi ini menunjukkan kesan sinergi substrat campuran yang ketara terhadap pengumpulan P(3HB-*ko*-4HB) secara satu-peringkat dengan peningkatan kandungan dan kepekatan PHA sebanyak 74% (b/b) dan 6.9 g/L masing-masing; dan komposisi monomer 4HB dari 7 mol% ke 70 mol%. Ianya adalah selaras dengan eksperimen menggunakan karbon tunggal yang menunjukkan penghasilan PHA tertinggi sebanyak 57% (b/b), 3.1 g/L dan 48 mol% 4HB. Manipulasi substrat campuran dan nisbah di dalam bioreaktor menunjukkan bahawa kebanyakan proses fermentasi telah menghasilkan kandungan PHA yang melebihi 50% (b/b), dengan satu siri kopolimer yang mengandungi komposisi monomer 4HB yang pelbagai dari 12 mol% ke 55 mol%. Penghasilan PHA yang tinggi sebanyak 73% (b/b) telah diperolehi, dan seterusnya menyumbangkan kepekatan dan hasil PHA yang tinggi sebanyak 8.6 g/L dan 2.7 g/g.

Ciri-ciri kopolimer tersebut telah dipengaruhi oleh jenis substrat dan strategi pencampuran. Bioproses satu-peringkat yang dijalankan menepati dengan baik model logistik dan Luedeking-Piret (dan yang diubahsuai), dibuktikan dengan nilai R^2 yang tinggi dari 0.9153 ke 0.9957. Penggunaan kedua-dua substrat dan penghasilan P(3HB-*ko*-4HB) adalah pada kadar yang tinggi pada fasa pertumbuhan eksponen bakteria, dicirikan oleh nilai pemalar pertumbuhan-berkait penggunaan substrat (γ) dan pengumpulan P(3HB-*ko*-4HB) (α) yang tinggi, masing-masing dari 0.0690 g/g ke 0.2863 g/g dan 0.0660 g/g ke 0.1886 g/g. Hal ini adalah selari dengan nilai pemalar pertumbuhan tak-berkait, δ dan β yang menunjukkan kadar penggunaan substrat dan penghasilan kopolimer yang rendah, masing-masing dari 3.9×10^{-5} g/g/h ke 0.0011 g/g/h dan 0.0092 g/g ke 0.0459 g/g pada fasa pertumbuhan pegun. Kopolimer P(3HB-*ko*-30 mol% 4HB) yang mempunyai berat molekul, 927 kDa dan pemanjangan-sehingga-putus tinggi dan 1637% telah dibiosintesiskan daripada 0.56% (b/b C) 1,4-butanadiol dan 1,6-hexanadiol dengan nisbah 3:1. Peningkatan kadar goncangan dari 150 rpm ke 250 rpm telah menunjukkan peningkatan kadar penghasilan P(3HB-*ko*-4HB) yang efisien masing-masing dari 28 % (b/b) ke 63% (b/b) dan 3.1 g/L ke 6.5 g/L, yang meminimumkan penggunaan substrat terhadap pertumbuhan bakteria dari 8.0 g/L ke 3.8 g/L. Kepelbagaian kadar pengudaraan dari 0.5 ke 1.0 vvm mengekalkan residu biojisim di antara 4.0 g/L dan 4.8 g/L tetapi diiringi dengan peningkatan kepekatan PHA dari 4.8 g/L ke 7.2 g/L. Pengkulturan substrat campuran telah terbukti sebagai strategi yang boleh meningkatkan produktiviti kopolimer, komposisi monomer 4HB dan sifat kopolimer dalam fermentasi secara satu-peringkat.

SINGLE-STAGE BIOSYNTHESIS OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) USING *Cupriavidus* sp. USMAA1020 VIA MIXED-SUBSTRATE CULTIVATION STRATEGY

ABSTRACT

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer is polyhydroxyalkanoate (PHA) with biopharmaceutical and medical significance. Copolymer productivity with high 4HB monomer composition is usually achieved via two-stage cultivation but this reduces the ease of handling by increasing the time, effort and risk of contamination in large-scale production. This study aims to by-pass the two-stage fermentation by developing an efficient single-stage fermentation strategy using mixed-substrate cultivation. This strategy showed significant synergistic effect towards the single-stage production of P(3HB-*co*-4HB) with an enhanced PHA content and concentration up to 74 wt% and 6.9 g/L respectively; and 4HB monomer composition from 7 mol% to 70 mol%. This is in relative to experimentation using sole carbon sources which recorded the highest PHA accumulation of 57 wt%, 3.1 g/L and 48 mol% 4HB. Manipulation of the substrate mixture and its mixing ratios in bioreactor revealed that most of the fermentations resulted in PHA content of more than 50 wt%, with a series of copolymers of 4HB monomer compositions ranged from 12 mol% to 55 mol%. A remarkably high PHA accumulation of 73 wt% was obtained, contributing to high PHA concentration and yield of 8.6 g/L and 2.7 g/g respectively. The copolymer characteristics were predominantly affected by the nature of substrates and the mixing strategies. The single-stage fermentation fit well to the logistic and Luedeking-Piret (and modified) models, evidenced by high adjusted R^2 values of 0.9153 to 0.9957. Both substrates

utilization and P(3HB-*co*-4HB) accumulation were at higher rate during bacterial exponential growth phase, characterized by higher values of growth-associated substrate utilization (γ) and P(3HB-*co*-4HB) accumulation (α) constants of 0.0690 g/g to 0.2863 g/g and 0.0660 g/g to 0.1886 g/g respectively. This is in relative to non-growth associated constants, δ and β which demonstrated lower substrates utilization and copolymer accumulation rate during stationary phase respectively at 3.9×10^{-5} g/g/h to 0.0011 g/g/h and 0.0092 g/g to 0.0459 g/g. A P(3HB-*co*-30 mol% 4HB) copolymer with high molecular weight and elongation at break values of 927 kDa and 1637% was biosynthesized from 0.56 wt% C 1,4-butanediol and 1,6-hexanediol at ratio of 3:1. An increase of agitation from 150 rpm to 250 rpm has resulted in increase efficiency of P(3HB-*co*-4HB) accumulation from 28 wt% to 63 wt% and 3.1 g/L to 6.5 g/L respectively which concurrently minimized the utilization of substrates towards bacterial growth from 8.0 g/L to 3.8 g/L. Variation of aeration from 0.5 vvm to 1.0 vvm kept the residual biomass constant in between 4.0 g/L and 4.8 g/L but accompanied with an increase of PHA concentration from 4.8 g/L to 7.2 g/L. The mixed-substrate cultivation was proven to be promising strategy to enhance copolymer yield, 4HB accumulation and characteristics in single-stage fermentation.

CHAPTER 1: INTRODUCTION

1.1 Research background

Polyhydroxyalkanoates (PHAs) are group of biopolymers made up of diverse range of monomers. To date, over 150 monomer constituents have been identified as building blocks of PHAs (Gao et al., 2011; Urtuvia et al., 2014; Jia et al., 2016). Of all PHAs, poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer is known to be suitable absorbable biomaterial in medical and pharmaceutical applications. It can be specifically applied for wound healing, orthopedics, therapeutics, drug delivery, tissue engineering and etc (Koller et al., 2007; Chee et al., 2008; Wu et al., 2009; Brigham and Sinskey, 2012; Luef et al., 2015; Phong et al., 2016).

There are few reasons directing the usage of the copolymer as high value medical product. Unlike other PHAs, this copolymer was found not only degraded by depolymerases, but also capable of hydrolyzation by lipases and esterases from both prokaryotes and eukaryotes (Doi et al., 1992; Saito et al., 1996; Li et al., 2010; Hsieh et al., 2009; Vigneswari et al., 2016). This is due to the absence of alkyl side chain as pendant group on 4HB monomers being attached to the copolymer chain (Mukai et al., 1993; Saito et al., 1996). The introduction of 4HB monomer results in copolymers with higher *in vivo* degradation rate than other PHAs (Loo and Sudesh, 2007; Li et al., 2010). Besides, the degradation of P(3HB-co-4HB) releases 3-hydroxybutyric acid and 4-hydroxybutyric acid which are natural metabolites presence in human bloodstream (Bessman and Fishbein, 1964; Lee, 1996a; Martin and Williams, 2003). This enhances the biocompatibility as the copolymer has the ability to tolerate inflammatory responses with tissue, without imposing detrimental

side effect on human body. In term of the material properties, the copolymer exhibits wide variety of mechanical strengths from highly crystalline plastic to elastomeric rubber-like material, upon combining both 3HB and 4HB monomer constituents at varying molar fractions (Doi et al., 1990; Saito et al., 1996; Loo and Sudesh, 2007; Cesário et al., 2014). This improves the potential of biomaterial applications since the resulting copolymers can be tailored to meet specific needs. P(3HB-*co*-4HB) copolymer is considered to be one of the most valuable biopolymer among the vast number of PHAs being synthesized by numerous microorganisms (Amirul et al., 2008; Park and Kim, 2011).

1.2 Problem statement

P(3HB-*co*-4HB) is produced via fermentation process rather than chemical synthesis (Martin and Williams, 2003). To date, most of the fermentations targeting on this copolymer uses two- or three-stage cultivation in order to obtain high PHA productivity and copolymers with high 4HB monomer composition (Doi et al., 1988; Kunioka et al., 1989a; Doi et al., 1990; Nakamura et al., 1992; Saito et al., 1996; Choi et al., 1999; Mitomo et al., 2001; Kim et al., 2005; Lee et al., 2007; Amirul et al., 2008; Hsieh et al., 2009; Vigneswari et al., 2009 Rao et al., 2010a). This includes the production of poly(4-hydroxybutyrate) [P(4HB)] homopolymer as well (Saito and Doi, 1994; Saito et al., 1996; Choi et al., 1999). Obtaining copolymers with higher 4HB monomer composition is desirable as it is generally concluded that this would enhance the copolymer's application as biomaterial in medical and bio-pharmaceutical (Martin and Williams, 2003). However, multi-stages fermentation strategy is not feasible when it comes to industrial scale production. This bioprocess requires a minimum of two fermentation vessels which involves the use of nutrient-rich medium for bacterial growth phase, followed by nitrogen-free medium for PHA

accumulation phase in separate vessels. This reduces the ease of handling, prone to the risk of contamination during inocula transfer and indirectly increases the operational cost and effort (Rahayu et al., 2008). Production of P(3HB-*co*-4HB) via single-stage cultivation is less frequently employed and usually returned with either low copolymer productivity and/or low 4HB monomer composition. For example, Park and Kim (2011) were able to produce high biomass of 10 g/L to 20 g/L but resulted in accumulation of low 4HB monomer composition of 6 mol% to 10 mol%. Kang et al. (1995) were able to produce P(3HB-*co*-4HB) with high 4HB monomer composition up to 83 mol% but with low PHA concentration of 0.4 g/L. In spite of this, single-stage cultivation still offers an advantage in industrial-wise for the production of P(3HB-*co*-4HB).

To date, the production of this copolymer has not been extensively explored in bioreactor scale through single-stage fermentation. This is also true even for two-stage fermentation process which normally resulted in production of homopolymer P(4HB) or copolymer P(3HB-*co*-4HB) with high 4HB monomer composition. Most of the two-stage fermentations were carried out in shake-flask fermentation (Renner et al., 1996; Saito et al., 1996; Choi et al., 1999; Mitomo et al., 2001; Lee et al., 2004; Kim et al., 2005; Chai et al., 2009; Rao et al., 2010b; Vigneswari et al., 2010). Several recent studies have reported the copolymer production through single-stage fermentation in bioreactor but these did not return with copolymer of higher productivity and higher 4HB monomer composition (Park and Kim, 2011; Cavalheiro et al., 2012; Cesário et al., 2014). The copolymers produced have 4HB monomer compositions of less than 15 mol%.

Overall, there are some limitations on the upstream biosynthesis of the copolymer. This will eventually reduce the feasibility of the copolymers to be

applied in healthcare application. In light of this, investigation and search for an efficient approach that will optimally produce the copolymer is significantly important.

1.3 Objectives

In the present study, the P(3HB-*co*-4HB) copolymer was biosynthesized by *Cupriavidus* sp. USMAA1020 isolated from Malaysian environment. The novelty of the work comes from the idea of relating the mixed-substrate cultivation strategy on the single-stage production of the P(3HB-*co*-4HB) copolymer. To the best of my knowledge, there are limited publications which discussed the effect of the strategy on P(3HB-*co*-4HB) production in details. It is hypothesized that substrate mixture strategy would overcome the limitation in single-stage cultivation, leading to the development of efficient bioprocess strategy for the copolymer production.

The objectives of this study are:

1. To evaluate the applicability of mixed-substrate cultivation in improving single-stage production of P(3HB-*co*-4HB) through shake-flask scale fermentation.

2. To produce P(3HB-*co*-4HB) copolymers from single-stage mixed-substrate cultivation in controlled bioreactor system.

3. To study the production kinetics and characteristics of P(3HB-*co*-4HB) biosynthesized by *Cupriavidus* sp. USMAA1020 in single-stage mixed-substrate cultivation system.

4. To investigate the parameters affecting the upscaling process of P(3HB-*co*-4HB) from lab to pilot scale fermentation.

CHAPTER 2: LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHAs): The next generation bioplastics

2.1.1 PHAs in general

Polyhydroxyalkanoates (PHAs) are a group of natural biopolymer consisting of a repeated chain of hydroxyalkanoates (HAs) monomer. These biopolymers are synthesized by a wide variety of microorganisms under stress conditions whereby the surrounding environments are limited of nutrients such as nitrogen, magnesium, phosphorus or oxygen but sufficient with carbon sources (Anderson and Dawes, 1990; Liu et al., 2016).

PHAs are identified as one of bacterial storage components analogous to starch and glycogen (Dawes and Senior, 1973; Olukoshi and Packter, 1994; Sudesh et al., 2000). PHAs are lipoidic and belong to one of the four principal classes of the storage polymers namely lipids, carbohydrates, polyphosphates and nitrogen reserve compounds found in microorganisms (Dawes, 1985). Under the restricted microbial growth conditions, the carbon sources are converted to PHAs, which serves as intracellular carbon and energy storage compounds. PHAs exhibit high degree of polymerization by the action of PHA synthase, with the resulting molecular weight up to several million Daltons (Anderson and Dawes, 1990; Valappil et al., 2007).

PHAs exist in the form of discrete inclusion bodies, typically 0.2 μm to 0.5 μm in diameter; and localized in a mobile amorphous state within the cytoplasm of the cells (Dawes and Senior, 1973; Lee, 1996a; Sudesh et al., 2000). These granules are highly refractive and can be clearly visualized under phase contrast and electron microscopic observation (Dawes and Senior, 1973; Lee, 1996a; Sudesh et al., 2000). PHAs are water-insoluble. This makes PHA an excellent energy reserve material

since it does not contribute to any significant increase in osmotic pressure within the bacterial cytoplasm, even in a high quantity of PHAs (Loo and Sudesh, 2007). As the physiological fitness of the cells is not affected, this would prevent the leakage of valuable compounds out of the cells which in turn securing the stored nutrients at low maintenance cost (Peters and Rehm, 2005; Rehm, 2006; Verlinden, 2007).

PHAs are synthesized by various bacterial species from eubacteria and archeabacteria with a total of more than 90 generas (Zinn et al., 2001; Kessler et al., 2001; Rehm, 2006). This includes numerous Gram-positive and Gram-negative bacteria in which the former is prominently represented by *Bacillus* sp., *Streptomyces* sp., *Rhodococcus* sp., *Clostridium* sp., *Staphylococcus* sp., *Corynebacterium* sp., and *Nocardia* sp.; whereas the latter is notably denoted to *Cupriavidus* sp., *Pseudomonas* sp., *Methylobacterium* sp., *Azotobacter* sp., *Burkholderia* sp. and recombinant *Escherichia coli* (Lee, 1996a; Valappil et al., 2007; Liu et al., 2016).

Among all these, the Gram-negative *Cupriavidus necator* (formerly *Ralstonia eutropha*, *Alcaligenes eutrophus* or *Waustersia eutropha*) is the representative of PHA producers and extensively studied in PHA research (Jendrossek, 2009; Tanadchangsaeng and Yu, 2012). This is because the bacterium, of strictly respiratory and facultative chemolithoautotrophic is able to utilize a diverse range of carbon sources from simple, defined to complex and inexpensive for growth and PHA biosynthesis (Kim et al., 1994; Sugimoto et al., 1999; Valappil et al., 2007; Ashby et al., 2011). Besides, the bacterium can accumulate high quantities of PHA up to 90 wt% of the biomass under appropriate nutrient depleted conditions (Jendrossek and Handrick, 2002; Khanna and Srivastava, 2005; Chen, 2009; Tanadchangsaeng and Yu, 2012).

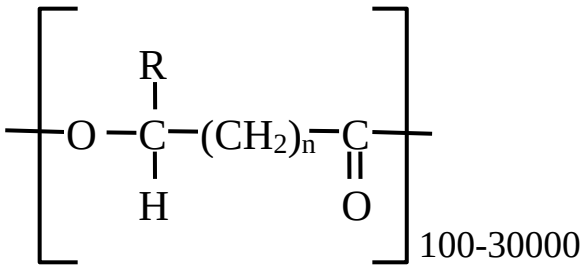
At present, PHA producing microorganisms are found in either natural or engineered ecosystems such as in soil, marine, wastewater treatment plant, salt marsh and activated sludge process (Liu et al., 2016). The production of PHAs is not feasible via chemical synthesis but through *in vivo* accumulation by microorganism itself as processing factory (Valappil et al., 2007; Wang et al., 2014). Therefore, industrial scale production of successfully commercialized PHAs has usually been performed using enzyme and bioprocess engineering approach (Liu et al., 2016).

Among the various biodegradable polymers, PHAs are excellent substitutes for petroleum-based synthetic plastics because they exhibit plastic-like feature in term of the physical and thermal properties. They are water resistance, oxygen impermeable and piezoelectric thermoplastics and/or elastomers (Valappil et al., 2007; Keshavarz and Roy, 2010). Unlike conventional plastics, PHAs are known and recognized to be the only waterproof thermoplastics that are completely biodegradable in terrestrial and aquatic ecosystems by microorganisms (Philip and Roy, 2007). These polymers are degraded into harmless by-products, releasing carbon dioxide and water under aerobic conditions; methane and water under anaerobic conditions (Jendrossek and Handrick, 2002; Lim et al., 2005; Sridewi et al., 2006; Chanprateep, 2010). Without releasing any toxic waste, this resolves the issue of toxicity caused by the accumulation of non-degradable plastic waste.

In addition, PHAs are completely being biosynthesized form renewable resources. This resolves the issue of over dependence on synthetic plastics which uses fossil fuels that are facing depletion as raw materials (Zinn et al., 2001; Steinbüchel, 2005; Chanprateep, 2010). PHAs have also caught attention due to their biocompatibility. They are immunologically inert which is promising in fabricating high-value medically related products.

PHAs are group of biopolymers made up of a diverse range of monomers. Depending on the substrate specificity of PHA synthase of the particular strain, various homopolymers, copolymers, terpolymers, blocks and blends PHAs could be synthesized. To date, there are more than 150 monomer constituents found to be produced by more than 300 microorganisms. The structural diversity of the monomers and compositions has resulted in PHAs with a wide range of physical and thermal characteristics. This has opened up to various opportunities for exploitation as there are many possibilities yet to be discovered in various applications. Figure 2.1 shows the general chemical structure of various PHAs.

Owing to the renewable, eco-friendly and the diversification of the material properties, PHAs are therefore can be applied into various sectors from disposable goods packaging, agriculture, aquacultural, to medical and biopharmaceutical industries (Chen and Wu, 2005; Tanadchangsaeng and Yu, 2012). PHAs are envisioned to be one of the next generation materials which will return lots of benefits to mankind and the world.



n = 1	R = hydrogen	Poly(3-hydroxypropionate)
	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hydroxyvalerate)
	R = propyl	Poly(3-hydroxyhexanoate)
	R = pentyl	Poly(3-hydroxyoctanoate)
	R = nonyl	Poly(3-hydroxydodecanoate)
n = 2	R = hydrogen	Poly(4-hydroxybutyrate)
	R = methyl	Poly(4-hydroxyvalerate)
n = 3	R = hydrogen	Poly(5-hydroxyvalerate)
	R = methyl	Poly(5-hydroxyhexanoate)
n = 4	R = hexyl	Poly(5-hydroxydodecanoate)

Figure 2.1: General structure of poly(3-hydroxyalkanoates) in which R refers to the length of side chain and n refers to size of alkyl group. PHAs are made up of carbon, hydrogen and oxygen atoms (Lee, 1996b)

2.1.2 History and development of PHAs

PHAs have gone through for almost a century since they have been discovered (Keshavarz and Roy, 2010). Although the commercialization of PHAs have still returned in limited success, tremendous efforts have been carried out which expands the fundamentals of the microbial physiology in PHA biosyntheses, leading to the established manipulation of the production through multi-level omics (Chen, 2009). According to Sudesh et al. (2000), the science and technology development of PHAs is significantly marked by three stages: discovery of P(3HB), identification of various hydroxyalkanoate monomers other than 3HB and manipulation of PHA biosynthesis genes. Along this timeline, the development is also closely related to the establishment and the improvement of PHA quantification method.

The presence of lucent granules in bacterial cytoplasm was first reported by Beijerinck in 1888 (Amara and Moawad, 2011; Tanadchangsaeng, 2014). However, the composition of PHA was only characterized at the mid of 1920s, by a French microbiologist, Maurice Lemoigne who identified the inclusion bodies found in *Bacillus megaterium* as poly(3-hydroxybutyrate) [P(3HB)] (Anderson and Dawes, 1990). By 1950s, accumulated studies from genus *Bacillus* suggested that P(3HB) functions as intracellular carbon and energy reserve. Dawes and Senior (1973) later on reviewed the physiological roles and concluded that the function of P(3HB) is analogous to starch and glycogen which are well known bacterial storage materials.

Lemoigne was also the first who reported the analytical method for P(3HB). This has been performed via long processes including extraction, purification, saponification and distillation. These processes resulted in volatile and non-volatile fractions which both contains the polymer respectively in different forms, *trans*-2-

butenoic acid (dehydrated 3-hydroxybutyric acid) and 3-hydroxybutyric acid. The compounds were weighed and represented as accurate amount of P(3HB) found in the bacterial cells.

Lemoigne's method was accurate but requires large biomass quantity and the whole process was time-consuming. To overcome this, various improvements have been carried out such as using spectrophotomeric method by Williamson and Wilkinson (1958), Slepecky and Law (1960) and Law and Slepecky (1961); glass fibre disk assay method by Ward and Dawes (1973) which permits faster and handling of smaller samples. Karr et al. (1983) and Hesselmann (1999) developed the P(3HB) quantification using ion exchange high-performance liquid chromatography (HPLC) and UV measurement.

The 3-hydroxybutyrate (3HB) was the only known hydroxyalkanoate (HA) to be the building block of this polymer storage until 1970s. Wallen and Rohwedder (1974) discovered the presence of HAs other than 3HB in the chloroform extracts of activated sewage sludge. Analysis using nuclear magnetic resonance (NMR) revealed the presence of longer hydroxy fatty acids meanwhile the subsequent analysis by gas chromatography-mass spectroscopy (GC-MS) confirmed the presence of 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx). Findlay and White (1983) detected that at least 11 HAs with 3HB and 3HV being major constituents in the polymer extracted from marine sendiments whereas 3HB, 3-hydroxyheptanoate (3HHp) and 3-hydroxyoctanoate (3HO) were found in the polymer extracted from the monocultures of *Bacillus megaterium*. Odham et al. (1986) reported the presence of 3HB, 3HHx, and 3HO from activated sewage sludge. de smet et al. (1983) and Lageveen et al. (1988) significantly reported the accumulation of 3HO and 3HHx without 3HB from *Pseudomonas oleovorans*, when

grown on n-octane. The PHA quantification method has also come to its major advancement to this end. The method which consists of acidic based extraction, hydrolysis and methylation developed by Braunegg et al. (1978) provides accurate and more reproducible analytical measurement. The quantification method using gas chromatography (GC) was applied and adapted widely by many authors.

The identification of other HA units marks a significant impact in PHA research, development and commercialization. This is due to the fact that homopolymer P(3HB) is brittle that limited the application as a substitute for synthetic plastic. Therefore, incorporation of foreign HA units into the P(3HB) polymer chain could enhance the overall material properties which extends the material applications.

In early 1960s, PHA received its commercial attention by W.R. Grace & Co. (U.S.A) which patented the fermentation process of P(3HB) and its fabrication into absorbable prosthetic devices. However, the industrial scale exploitation of P(3HB) are remained inactive. Following the petroleum crisis in 1970s, the demand of searching alternatives for plastic industry has boosted. The Imperial Chemical Industries, ICI from United Kingdom (currently known as Zeneca Bio Products) has initiated the production of 70 wt% P(3HB) from *Acaligenes latus*. Besides, P(3HB-*co*-3HV) copolymer was also produced and marketed under the trademark BIOPOL® by the same company. Monsanto, a splitting of ICI has later acquired the BIOPOL® and produces various range of P(3HB-*co*-3HV) copolymers in April 1996. After two years, Metabolix Inc. obtained license from Monsanto and launched a company named Tepha Inc. which developed the medical devices using PHAs (Braunegg et al., 1998; Philip and Roy, 2007). Biotechnologische Forchungsgesellschaft is another company in Austria that has also developed the

pilot scale production of P(3HB) in 1990 (Hänggi, 1990; Hrabak, 1992; Kessler et al., 2001). The process which used sucrose as sole carbon source would able to accumulate the homopolymer up to 80% of cell dry weight. A biomass density of 60 g/L was achieved but the production process stopped in 1993 (Kessler et al., 2001).

The revolution of molecular biology approach in life science research has encouraged the understanding of PHA biosynthesis both at genetic and enzymatic level. By 1980s, genes that are responsible for PHA biosynthesis were successfully cloned from *Ralstonia eutropha* into *Escherichia coli*. *Ralstonia eutropha* was extensively studied as model for PHA biosynthesis and this revealed three important enzymes responsible in producing P(3HB) from acetyl-CoA. Up to year 2003, there was about 60 PHA synthase structural genes were cloned and sequenced from various eubacteria (Steinbüchel and Lutke-Eversloh, 2003; Kadouri et al., 2005).

Generally, the development of PHAs can be categorized into upstream and downstream processing in which the former involves the biosynthesis of PHAs and the latter involves the characterization and application of PHAs as presented at Figure 2.2. The upstream processing of PHAs includes the isolation of microorganisms, screening and optimization, genetic modification and biosynthesis at various fermentation scales; meanwhile the downstream processing of PHAs covers the recovery and purification of the biopolymers, degradation, quantification, characterization as well as the application of PHAs.

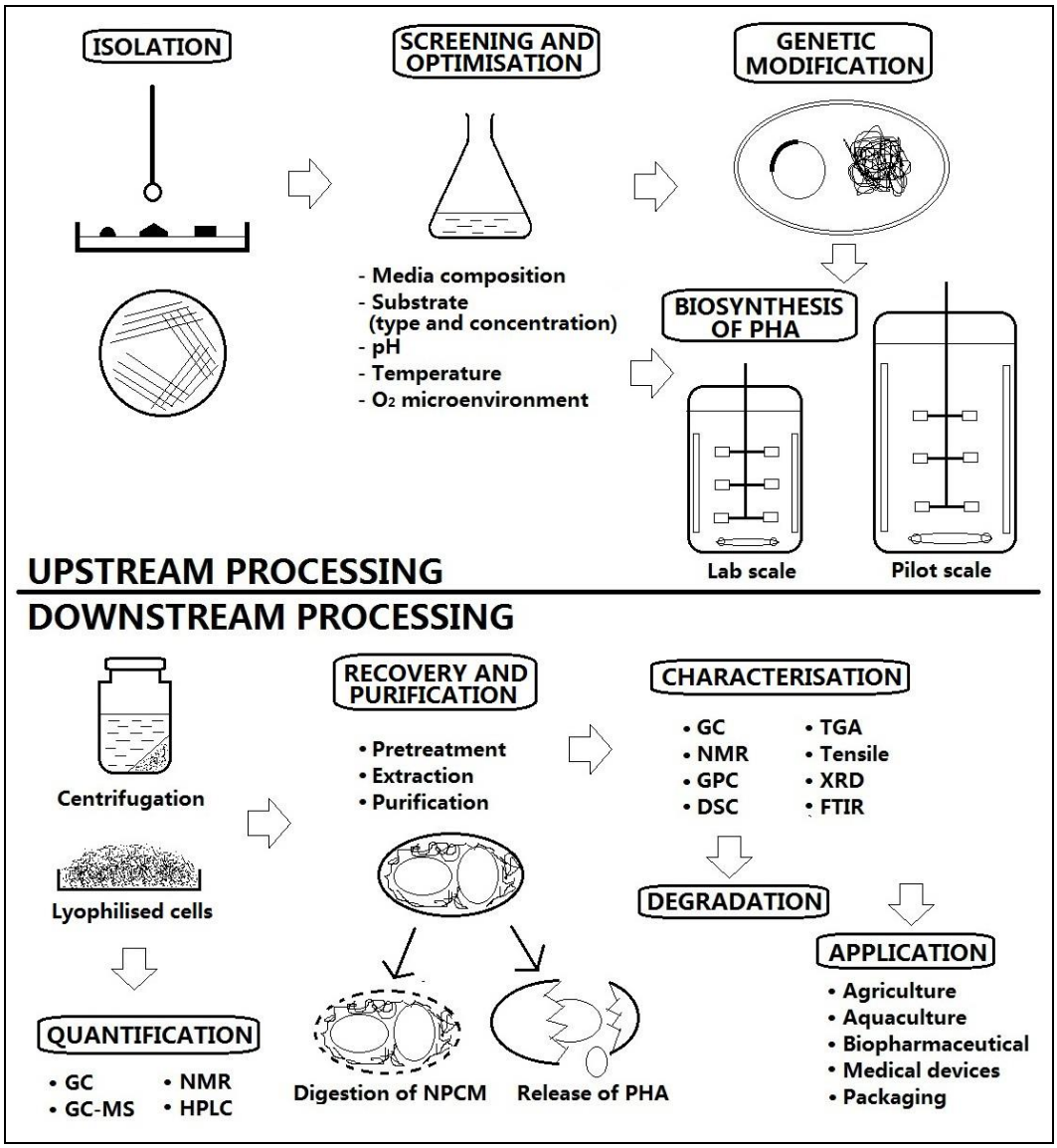


Figure 2.2: The major pipelines in upstream and downstream processing of PHAs (Amirul et al., 2015)

2.1.3 Classification and properties of PHAs

PHAs can be categorized based on its monomeric units. PHAs with three carbons (C3) to five carbons (C5) monomers are classified as short-chain-length (SCL-). PHAs with the range from C6 to C14 are classified as medium-chain-length (MCL).

SCL-PHAs are stiff, brittle and crystalline materials that contains methyl or ethyl group as small side chains (Verlinden et al., 2007). *Cupriavidus necator* is one of the bacteria that produces SCL-PHAs (Matsusaki et al., 1998). Homopolymer P(3HB) is a typical example of SCL-PHA. It possesses high tensile strength that is comparable to commodity plastic such as polypropylene, but with a low elongation at break (Anderson and Dawes, 1990; Khanna and Srivastava, 2005). P(3HB) is rigid and brittle with low elasticity. This is due to its perfect stereoregularity which acquires detrimental aging process at ambient temperatures (de Koning and Lemstra, 1993). It is highly crystalline which consists of large spherulites and possesses post-crystallization behaviours. As a result, formation of the irregular pores on the surface limits the flexibility of amorphous polymer chain (Shishatskaya and Volova, 2004; Wen et al., 2012; Hong et al., 2011). The homopolymer exhibits high melting temperature at 170°C to 180°C. As a whole, this homopolymer is difficult to process and therefore unfavourable to be applied for industrial use.

Even though P(3HB) is the most studied and well-characterized PHA, it should be noted that its characteristics do not represent the overall material properties of SCL-PHAs. By incorporating other hydroxyalkanoate units into the P(3HB) macromolecular chain, this will result in enhanced material properties following the disturbed and reduced crystal lattice arrangement of P(3HB) (Doi, 1995). This is

mainly accomplished by the addition of carbon precursors as co-substrate during the upstream biosynthesis process, which subsequently copolymerizes 3-hydroxybutyrate and other monomers. The PHAs with improved material properties such as P(3HB-*co*-3HV), P(3HB-*co*-4HB), P(3HB-*co*-4HV), P(3HB-*co*-3HV-*co*-4HB) are some of the known examples of SCL-PHAs.

The discovery of the first MCL-PHA was dated on 1983, in which a polyester containing mainly of 3-hydroxyoctanoic acid from an axenic culture of *Pseudomonas oleovorans* (de smet et al., 1983; Koller et al., 2013). It was later found out that most of the bacteria from genus *Pseudomonas* (belong to rRNA-homology-group I) are capable to synthesize MCL-PHA, with only a few exceptions (Steinbüchel and Lutke-Eversloh, 2003). Typical MCL-PHAs includes P(3HHx-*co*-3HO-*co*-3HD) and P(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD). These polymers are generally elastomeric and semi crystalline, which possesses low tensile strength and high elongation to break. In term of thermal properties, MCL-PHAs exhibits lower glass transition temperature, lower degree of crystallinity and broader melting range as compared to SCL-PHAs. Apart from that, these polymers also possess lower molar mass due to a low degree of polymerization (Koller et al., 2013). Table 2.1 shows general difference in between SCL-PHAs and MCL-PHAs.

Since SCL-PHAs are stiff and brittle whereas MCL-PHAs are more elastomeric and flexible, incorporation of both SCL- and MCL- monomers will result in a SCL- MCL- copolymer which possesses the properties in between two states. The resulting polymer has superior properties as compared to either SCL- or MCL-PHAs. According to Chen (2009), SCL-MCL-PHAs are ideal in advancing the on-going development in various applications since they exhibit flexible mechanical properties. An attempt in producing this combination of copolymers started since

1988 by Brandl et al. (1989) who studied the ability of *Rhodospirillum rubrum*, a phototrophic bacterium to produce various kinds of PHAs. Among all the SCL-MCL-PHAs produced, P(3HB-*co*-3HHx) is one of the successful polymer in this category been produced up to an industrial scale (Chen, 2009). P(3HB-*co*-3HV-*co*-3HHx) terpolymer is another SCL-MCL-PHA which has returned novel and attractive properties to be employed in various application (Bhubalan et al., 2008). Pocter & Gamble has also trademarked SCL-MCL-PHA copolymers as Nodax^{TM} which comprising of C4 and C6-C12 monomers (Bugnicour et al., 2014).

Table 2.1: General differences of SCL-PHAs and MCL-PHAs (Koller et al., 2013; Amirul et al., 2015)

SCL-PHAs	**Properties**	**MCL-PHAs**
Around 0 °C	**Glass transition temperature (T_g)**	Around -40 °C
Up to 70%	**Degree of crystallinity (X_c)**	Not more than 40 %
Higher, up to 180 °C	**Melting point (T_m)**	Broad range of melting temperature
Higher	**Molar mass**	Lower
Higher	**Polydispersity (PDI)**	Lower

2.1.4 Biosynthesis of PHAs

Since the first discovered PHA, P(3HB) was found to play significant role as carbon and energy source during imbalanced growth conditions, this has led to a remarkable interest in the metabolic pathways and regulatory metabolism of the PHA biosynthesis. The biosynthesis and utilization of PHAs are mediated by important enzymes such as PHA synthases (PhaC) and PHA depolymerases (PhaZ). PHA synthase is among the key enzyme that exclusively involved in PHA accumulation since it polymerizes the *β*-hydroxyalkanoyl-CoAs into poly-*β*-hydroxyalkanoate (Kadouri et al., 2005). PHA synthases are localized onto the surface of PHA granules together with phasins (PhaP)/regulator (PhaR) system which also involves in promoting PHA accumulation (York et al., 2002).

Bacterial PHA synthases can be classified into Class I, Class II, Class III and Class IV PHA synthases in according to the subunit composition (either only PhaC, or PhaC with PhaE) and the substrate specificity (resulting in either SCL-PHAs or MCL-PHAs). Class I PHA synthases are known as SCL-PHA synthases, composed one type of subunit and use C3-C5 monomers as substrates; the MCL-PHA synthases are Class II synthases which also composed of one type of subunit but using C6-C14 as substrates; meanwhile Class III PHA synthases uses C3-C5 substrates and composed of two different subunits. The three classes of the PHA synthases are generally represented by *Ralstonia eutropha* (*Cupriavidus spp*.), *Pseudomonas aeruginosa* (*Pseudomonas spp*.) and *Allochromatium vinosum* respectively (Stainbuchel and Lutke-Eversloh, 2003; Kadouri et al., 2005; Philip and Roy, 2007; Hyakutake et al., 2014; Ushimaru et al., 2014). Tsuge et al. (2015) highlighted the recent investigation of newest classification of PHA systhases. The class IV synthases composed of PhaC and PhaR subunit and are prevalent among

microorganism from *Bacillus* genus such as *Bacillus megatarium* and *Bacillus cereus*.

The biosynthesis of P(3HB) is the best known PHA biosynthetic pathway. This SCL-PHA involves a three-step enzymatic reaction. In this pathway, *β*-kethiolase (PhaA) catalyzes the formation of acetoacetyl-CoA from two acetyl-CoAs via condensation reaction. This is followed by the action of acetoacetyl-CoA reductase (PhaB) which reduces acetoacetyl-CoA to 3-hydroxybutyryl-CoA by NADH. Finally, PHA synthase (PhaC) polymerizes the 3-hydroxybutyryl-CoA to form P(3HB) via esterification reaction and release coenzyme-A. P(3HB) biosynthesis is regulated by intracellular concentration of acetyl-CoA and 3-kethiolase. During balanced growth conditions, acetyl-CoAs are channeled towards tricarboxylic (TCA cycle). This releases a high amount of free coenzyme A which inhibits the activity of 3-kethiolase. When the growth is limited, the level of NADH and acetyl-CoA increases. Thus, the concentration of free coenzyme A reduced. This promote the activity of 3-kethiolase which stimulates P(3HB) biosynthesis (Zinn et al., 2001; Loo and Sudesh, 2007; Verlinden et al., 2007). Figure 2.3 shows the metabolic pathway of P(3HB) biosynthesis.

The biosynthesis of SCL-PHAs other than P(3HB) occurs by feeding the structurally related precursor substrates to the microorganism. The production of these PHAs depends on the metabolism level required to convert the substrate precursors into corresponding hydroxyacly-CoA thioesters, as well as the broad substrate range of the PHA synthases (Steinbüchel and Valentin, 1995; Steinbüchel and Fuchtenbusch, 1998). For example, P(3HB-*co*-3HV) copolymer can be produced in most P(3HB) accumulating strain by feeding substrates such as pentanol, propionic acid, and valeric acid with odd number of carbon atoms. These substrates

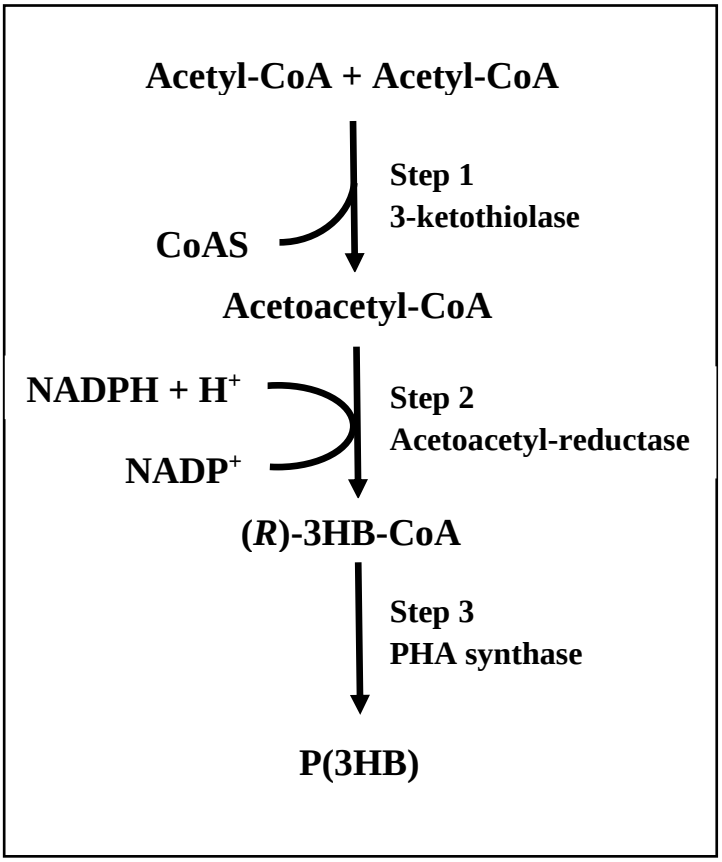


Figure 2.3: Metabolic pathway of P(3HB) synthesis (Anderson and Dawes, 1990)