BIOSYNTHESIS AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) COPOLYMER FROM *Cupriavidus* sp. USMAHM13

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols and Abbreviations	Full name
%	Percentage
β	Beta
γ	Gamma
°C	Degree Celsius
ΔH_m	Heat of fusion
g	Gravity
C_L	Dissolved oxygen concentration
C^*_L	Dissolved oxygen concentrations in equilibrium with mean gaseous oxygen concentration
Da	Dalton
g	Gram
g/l	Gram per liter
J/g	Joule per gram
kDa	KiloDalton
kg	Kilogram
K_La	Volumetric oxygen transfer coefficient
l	Liter
M	Molar
M_n	Number-average molecular weight
M_w	Average molecular weight
M_w/M_n	Polydispersity index
mg	Milligram
mg/ml	Milligram per milliliter
ml	Milliliter

mm	Millimeter
mmol/l	Millimole per liter
mM	Millimolar
Mol%	Mole percentage
MPa	Mega Pascal
nm	Nanometer
ppm	Parts per million
psi	Pounds per square inch
QO_2X	Oxygen uptake rate of the cells
R	Correlation coefficient
R^2	Determination coefficient
rcf	Rotation centrifugational force
rpm	Rotation per minute
T_g	Glass transition temperature
T_m	Melting temperature
T_c	Crystallization temperature
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microliter
µm	Micrometer
v/v	Volume per volume
wt%	Weight percent
w/v	Weight per volume
w/w	Weight per weight
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyryl-CoA
4HB	4-hydroxybutyrate

4HB-CoA	4-hydroxybutyryl-CoA
ACP	Acyl carrier protein
ANOVA	Analysis of variance
ASTM	American society for testing and materials
ATCC	American type culture collection
BLAST	Basic local alignment search tool
C/N	Carbon-to-nitrogen ratio
$CaCl_2 \cdot 2H_2O$	Calcium (II) chloride dihydrate
CCD	Central composite design
$CDCl_3$	Deuterated chloroform
CDW	Cell dry weight
CME	Caprylic methyl ester
CoA	CoenzymeA
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
$CoSO_4 \cdot 7H_2O$	Cobalt sulphate heptahydrate
CPKO	Crude palm kernel oil
CPO	Crude palm oil
$CuCl_2 \cdot 2H_2O$	Copper (II) chloride dihydrate
DO	Dissolved oxygen
DSC	Differential scanning calorimeter
DSMZ	Deutsche sammlung von mikroorganismen und zellkulturen
EMBL	European molecular biology laboratory
FabG	3-ketoacyl-CoA reductase
$FeSO_4 \cdot 7H_2O$	Iron (II) sulphate heptahydrate
FID	Flame ionization detector
GC	Gas chromatography

GP	Glycerine pitch
GPC	Gel permeation chromatography
HA	Hydroxyalkanoate
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
HMDS	Hexamethyldisilazane
ICI	Imperial chemical industries
IS	Internal standard
KH_2PO_4	Potassium dihydrogen phosphate
MCL	Medium-chain-length
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
MIC	Minimal inhibitory concentration
$MnCl_2 \cdot 4H_2O$	Manganese (II) chloride tetrahydrate
MSM	Mineral salts medium
NA	Nutrient agar
NaCl	Sodium chloride
Na_2SO_4	Sodium sulphate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
NH_4Cl	Ammonium chloride
$(NH_4)_2SO_4$	Ammonium sulphate
NMR	Nuclear magnetic resonance
NR	Nutrient rich
OD	Optical density

OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
PDI	Polydispersity index
PHAs	Polyhydroxyalkanoates
PhaA	*β*-ketothiolase
PhaB	NADPH-dependent acetoacetyl-CoA dehydrogenase
PhaC	PHA synthase
PhaG	3-hydroxyacyl-ACP-CoA transferase
PhaJ	Enoyl-CoA hydratase
PCR	Polymerase chain reaction
PO	Palm olein
RDP	Ribosomal database project
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
SCL	Short-chain-length
SEM	Scanning electron microscope
TCA	Tricarboxylic acid
TEM	Transmission electron microscope
TMS	Tetramethylsilane
UV-Vis	Ultraviolet-Visible
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate heptahydrate

BIOSINTESIS DAN PENCIRIAN KOPOLIMER POLI(3-HIDROKSIBUTIRAT-*ko*-4-HIDROKSIBUTIRAT) DARIPADA *Cupriavidus* sp. USMAHM13

ABSTRAK

Penghasilan poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) [P(3HB-*ko*-4HB)] dengan menggunakan kombinasi gliserin buangan dan karbon pelopor daripada bakteria adalah masih sangat terhad. Oleh sebab itu, kajian ini dijalankan untuk (i) memencilkan dan mengenalpasti bakterium yang berupaya menghasilkan kopolimer P(3HB-*ko*-4HB) dengan kandungan PHA dan komposisi monomer 4HB yang tinggi, (ii) meneroka keupayaan bakterium tersebut dalam menghasilkan kopolimer P(3HB-*ko*-4HB) dengan menggunakan pelbagai karbon yang boleh diperbaharui dan murah, (iii) mengoptimumkan penghasilan kopolimer P(3HB-*ko*-4HB) dengan menggunakan tar gliserin melalui fermentasi kelalang goncangan dengan menggunakan metodologi permukaan respon (RSM) dan (iv) menilai sifat-sifat bahan kopolimer P(3HB-*ko*-4HB) dengan pelbagai komposisi monomer 4HB. Dalam kajian ini, suatu bakteria novel berpigmen kuning yang mempamerkan keupayaan untuk menghasilkan kopolimer P(3HB-*ko*-4HB) telah dipencilkan dengan jayanya dari Perak, Malaysia dan telah dilabelkan sebagai *Cupriavidus* sp. USMAHM13. Berdasarkan pada analisis fenotip dan genotip, ia boleh dicadangkan bahawa *Cupriavidus* sp. USMAHM13 mewakili suatu spesis novel dalam genus *Cupriavidus*. Penyaringan awal substrat karbon untuk penghasilan kopolimer P(3HB-*ko*-4HB) oleh *Cupriavidus* sp. USMAHM13 telah mendedahkan bahawa komposisi 4HB monomer yang lebih tinggi (43 mol%) dengan berat kering sel dan kandungan PHA sebanyak 6.0 g/l dan 49% (b/b), masing-masing dicapai melalui kombinasi tar

gliserin (5 g/l) dan 1,4-butanadiol (5 g/l) secara pengkulturan satu peringkat. Kajian ini juga mendedahkan bahawa gliserin mentah yang diasingkan daripada tar gliserin paling menyumbang kepada sintesis kopolimer P(3HB-*ko*-4HB) dibandingkan dengan komponen lain yang diasingkan. Peningkatan pengumpulan monomer 4HB juga dicapai melalui penambahan ammonium asetat sebagai sumber nitrogen yang bertindak sebagai perangsang 4HB. Pengoptimuman medium dengan menggunakan RSM melalui fermentasi kelalang goncangan telah menjurus kepada pengumpulan tertinggi monomer 4HB (51 mol%) dengan berat kering sel dan kandungan PHA sebanyak 10.1 g/l dan 53% (b/b), masing-masing dengan menggunakan kombinasi tar gliserin (10 g/l), 1,4-butanadiol (8.14 g/l) dan ammonium asetat (2.39 g/l). Biosintesis kopolimer P(3HB-*ko*-4HB) dengan komposisi monomer 4HB berjulat daripada 3 mol% kepada 40 mol% juga dicapai melalui fermentasi berkelompok dalam bioreaktor dengan memanipulasi kepekatan ammonium asetat. Kopolimer-kopolimer yang dihasilkan mempamerkan berat molekul, sifat haba dan mekanikal yang berjulat luas bergantung kepada komposisi monomer dan jenis substrat karbon.

BIOSYNTHESIS AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) COPOLYMER FROM *Cupriavidus* sp. USMAHM13

ABSTRACT

Production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] using combination of waste glycerine and carbon precursor by bacteria is still very limited. Therefore, this research was conducted to (i) isolate and identify a bacterium that able to produce high PHA content and 4HB monomer composition, (ii) explore the ability of the bacterium to produce P(3HB-*co*-4HB) using various inexpensive and renewable carbon sources, (iii) optimize the P(3HB-*co*-4HB) copolymer production using glycerine pitch through shake-flask fermentation using response surface methodology (RSM) and (iv) evaluate the material characteristics of the P(3HB-*co*-4HB) copolymers with various 4HB monomer compositions. In this study, a novel yellow-pigmented bacterium which exhibited ability of producing P(3HB-*co*-4HB) copolymer was successfully isolated from Perak, Malaysia and designated as *Cupriavidus* sp. USMAHM13. Based on the phenotypic and genotypic analyses, it could be suggested that *Cupriavidus* sp. USMAHM13 represents a novel species within the genus *Cupriavidus*. Preliminary screening of carbon sources for biosynthesis of P(3HB-*co*-4HB) copolymer by *Cupriavidus* sp. USMAHM13 revealed that high 4HB monomer composition (43 mol%) with cell dry weight and PHA content of 6.0 g/l and 49 wt%, respectively was achieved through combination of glycerine pitch (5 g/l) and 1,4-butanediol (5 g/l) via one-stage cultivation. This study also revealed that recovered crude glycerine from glycerine pitch contributed the most for the synthesis of P(3HB-*co*-4HB) copolymer compared to the other

recovered components. Enhancement of 4HB monomer accumulation was also attained through the addition of ammonium acetate as nitrogen source which acted as 4HB stimulator. Medium optimization using RSM through shake-flask fermentation had led to the highest accumulation of 4HB monomer (51 mol%) with cell dry weight and PHA content of 10.1 g/l and 53 wt%, respectively using combination of glycerine pitch (10 g/l), 1,4-butanediol (8.14 g/l) and ammonium acetate (2.39 g/l). Biosynthesis of P(3HB-*co*-4HB) copolymer with 4HB monomer compositions ranged from 3 mol% to 40 mol% was also achieved through batch fermentation in bioreactor by manipulating the concentration of ammonium acetate. The P(3HB-*co*-4HB) copolymers produced exhibited a wide range of molecular mass, thermal and mechanical properties depending on the monomer compositions and type of carbon sources.

1.0 INTRODUCTION

The current prominence on sustainability, eco-efficiency and green chemistry has generated tremendous search for materials that are renewable and environmentally friendly. Biopolymers are one of the renewable materials from microorganisms which can provide a source of sustainable alternative to petroleum derived plastics. A variety of biodegradable polymers such as polyhydroxyalkanoates (PHAs), poly(ε-caprolactone) (PCL), polylactide (PLA), poly(*p*-dioxanone) (PPDO) and poly(butylene succinate) (PBS) are being studied for different applications ranging from industrial to medical applications (Akaraonye *et al*., 2010).

Polyhydroxyalkanoates (PHAs) are one of the versatile classes of biodegradable polymers which constitute a group of microbial biopolyesters with important ecosystem functions and high biotechnological potentials (Akaraonye *et al*., 2010; Koller *et al*., 2011). It is well established that PHAs are synthesized by bacteria and some archaea as an intracellular carbon and energy storage material through various pathways when experience metabolic stress in the environments of fluctuating availability and limitation of nutrient (Koller *et al*., 2011; Anderson and Dawes, 1990). PHAs have evoked great interest among researchers due to their inherent biocompatibility and biodegradability which is not surprising as monomer of PHAs, 3-hydroxybutyric acid is a normal constituent of human blood that has been considered in industries such as food supplement, pharmaceutical and other fine chemicals (Ren *et al*., 2010).

Even though both industries and governments have increased their efforts in the commercialization of biodegradable polymers, high production costs (4-6 USD/kg), limited microbial strains and difficulty in recovering the polymer have hampered the widespread applications of these high-quality polymers (Akaraonye *et*

al., 2010). Development of superior PHA-producing strains and fermentation strategies as well as the current progress in downstream process technology will make the prices of PHA products to be competitive with their synthetic counterparts. The isolation and development of PHA-producing microorganism that has the ability to utilize inexpensive and renewable carbon substrates has to be pursued intensively since half of the production cost accounts on the substrate cost (Kim, 2000; Ren *et al*., 2010; Sudesh *et al*., 2011).

Among the diverse types of PHAs that have been revealed, copolyester poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] has been explored as biopolymer porous substrates in tissue engineering applications due to their biocompatibility and desirable mechanical properties (Williams and Martin, 2002). In fact, existence of 4-hydroxybutyric acid as normal constituent in the extracts of brain tissue of rat, pigeon and man has classified it as one of the most valuable biopolymer among the vast number of different PHAs synthesized by microorganisms (Sudesh *et al*., 2000; Williams and Martin, 2002).

Exploring the utilization of waste materials is a good example of reducing the substrate cost by eliminating the necessity for supplementing with the more expensive carbon source. Recycling of wastes generated from industrial plants for PHA production is not only crucial in improving the economics of microbial PHA production but also for waste management (Solaiman *et al*., 2006; Akaraonye *et al*., 2010). Currently, the rising demand for the biodiesel worldwide has led to the excess discharge of by-product glycerine which is considered as an unrefined raw product. This waste glycerine is the principal by-product generated during the transesterification of vegetable oils and animal fats in the presence of catalyst. Unrefined glycerine has become a potential environmental pollutant because

majority of the cosmetics, pharmaceuticals and food industries prefer purified glycerine as a raw material. Nevertheless, glycerine purification process is an expensive process and recently, it has become economically unfeasible due to low prices of glycerine (Leoneti *et al*., 2012; da Silva *et al*., 2009).

It is of great importance for scientists to explore alternative potential uses of unrefined glycerine in order to reduce its excess accumulation and to control the economics of biodiesel production (Solaiman *et al*., 2006). Numerous papers have published direct utilization of unrefined glycerine in various applications such as feedstock in the production of different chemical products, hydrogen synthesis, additives for automotive fuels and ethanol or methanol production. Other interesting uses that have been considered are such as animal feed, co-digestion, co-gasification and waste treatment. Bioconversion into high value products through microbial fermentation is one of the most promising applications for the use of unrefined glycerine (Yang *et al*., 2012).

The excess production of waste glycerine is also creating problems for oleochemicals industries due to the collapse in crude glycerine prices which have fallen from about $0.25 per pound to $0.05 per pound. The producers need to pay to remove the crude glycerine from their plants and incinerate it. One of the US government agencies, Department of Energy has adopted the promotion of new glycerine platform chemistry and product families as one their most important goals to meet the need for obtaining new chemicals (Yang *et al*., 2012; Dharmadi *et al*., 2006).

Oleochemicals industry in Malaysia has been diversifying significantly due to the plentiful supply of kernel and palm oils as raw materials as well as the high demand for downstream products such as glycerine, fatty alcohols and fatty acids.

However, environmental awareness is growing rapidly in Malaysia because oleochemicals industry is one of the palm-oil based industries that possess risk to the environment. Approximately, 494 kg of glycerine pitch is generated daily in Malaysia and it is treated and disposed at prescribed premises. The cost for landfill is ~163 US$ whereas for incineration is ~260 US$ - 1172 US$ per tonne (Hazimah *et al*., 2003; Hidawati and Sakinah, 2011).

1.1 Problem statements

About two million species of microbes has been estimated in Malaysia as a major resource for innovative biotechnological products processes. However, microbial diversity remains an unexploited resource as only about 17% of the total number of estimated numbers of bacteria and fungi have been reported by the year 2000 (Vikineswary, 1998; Krishnapillay *et al*., 2003). At present, only two bacteria that capable of producing P(3HB-*co*-4HB) copolymer with various 4HB monomer compositions have been isolated from Malaysian environment. The bacteria are *Cupriavidus* sp. USMAA1020 and *Cupriavidus* sp. USMAA2-4 that isolated from Lake Kulim, Kedah and Sg. Pinang, Penang, respectively (Amirul *et al*., 2008; Chai *et al*., 2009). Therefore, the search for new P(3HB-*co*-4HB)-producing bacterial strains from Malaysian environment still remains of interest as Malaysia is one of the world's twelve mega diversity areas with exceptionally rich biological resources.

Disposal of combustible wastes like glycerine pitch has been a major problem to the community. Burning the waste can literally mean converting it into acrolein, a highly volatile compound and well-known for its toxicity and very hazardous to life (Hazimah *et al*., 2003). Biological conversion of glycerine pitch as potential carbon substrate into microbial polyester would give positive impact on both economic and environmental aspect. Production of P(3HB-*co*-4HB) copolyester using combination

of waste glycerine with addition of carbon precursor are still limited. It is imperative to study the variable factors affecting the P(3HB-*co*-4HB) copolyester accumulation using glycerine pitch and to systematically monitor the compositions of waste glycerine as such information could help to define acceptable range for feedstock variability of this waste carbon.

1.2 Objectives

In the present study, bioprospection of P(3HB-*co*-4HB)-accumulating bacteria was performed from Malaysian environment. The potential bacterium was selected based on its ability to convert carbon precursor into high 4HB monomer composition. The isolated bacterium was identified and characterized based on physiological and molecular analysis. Production of P(3HB-*co*-4HB) copolymer by the isolated bacterium using glycerine pitch was focused throughout the study.

The objectives of this study were;

1. To isolate and identify a bacterial strain with ability to produce high PHA content and 4HB monomer composition

2. To explore the ability of the newly isolated strain to produce P(3HB-*co*-4HB) using various inexpensive and renewable carbon sources

3. To optimize the P(3HB-*co*-4HB) copolymer production using glycerine pitch through shake-flask fermentation using response surface methodology (RSM)

4. To evaluate the material characteristics of the P(3HB-*co*-4HB) copolymers with various 4HB monomer compositions

2.0 LITERATURE REVIEW

2.1 Polyhydroxyalkanoates: The prospect green biopolymer

Polyhydroxyalkanoates (PHAs) are one of the greatly fascinating families of microbial polyesters of 3, 4, 5 and 6 hydroxyacids that have promising potentials in various industrial and medical applications due to their wide range of characteristics (Akaraonye *et al*., 2010; Philip *et al*., 2007). These polymers with imperative ecosystem roles and high biotechnological potentials are synthesized naturally by a diverse range of bacterial species from at least 75 different genera (Koller *et al*., 2011; Reddy *et al*., 2003). The polymers are usually accumulated as insoluble inclusions in the cytoplasm of bacterial cells during the depletion of essential nutrients such as nitrogen, magnesium or phosphorus in the presence of abundant carbon sources (Tian *et al*., 2009).

Most of the bacteria accumulate these polymers as storage materials in the form of mobile, amorphous, liquid granules for their survival under stress or hostile conditions (Luengo *et al*., 2003). PHAs also serve as a sink for reducing equivalents for some microorganisms. The insolubility of PHAs inside the bacterial cytoplasm causes insignificant increase in the osmotic pressure, thus preventing the leakage of these valuable compounds out of the cells while securing the stored nutrient at a low maintenance cost (Rehm, 2006; Verlinden *et al*., 2007).

PHAs exhibit thermoplastic and elastomeric properties after they are extracted from the cells. These biopolymers are the only waterproof thermoplastic materials available that are fully biodegraded in the terrestrial and aquatic ecosystems by microorganisms through both aerobic and anaerobic conditions (Philip *et al*., 2007; Lee, 1996). PHAs can be used in various ways similar to many

non-biodegradable synthetic plastics by varying their toughness and flexibility, depending on their formulations (Verlinden *et al*., 2007).

In addition, PHAs have caught the attention of many researchers due to their inherent biocompatibility. The medically attractive characteristics of these biopolymers have become the focus of many investigations. PHAs are more favourable for the development of tissue-engineered scaffold because they have been proven biocompatible in tissue engineering. They also exhibit medically important characteristics that are not found in the present synthetic absorbable polymer such as polyglycolic acid (PGA). Hence, these polymers serve as excellent substitute for synthetic plastics due to their ease of processability, tailor-made physical characteristics, biodegradability and biocompatibility (Sudesh *et al*., 2000; Valappil *et al*., 2006).

The incorporated 3-hydroxyalkanoic monomers units of the PHAs are all in the *R*(–) configuration due to the stereospecificity of the polymerizing enzyme, PHA synthase. Therefore, PHAs containing *R*(–) 3HA monomers units represent a family of the optically active microbial polyesters. Small portion of *S* monomers are detected only in unusual cases. Biosynthesis of PHAs by bacteria will warrant the incorporation of *R*(–) HA monomers which is indispensable for the biodegradability and biocompatibility of these polymers (Sudesh *et al*., 2000; Akaraonye *et al.*, 2010; Zinn and Hany, 2005). Hydrolysis of PHAs will produce *R*-hydroxyalkanoic acids that can be used as chiral starting materials in fine chemicals, pharmaceutical and medical industries (Philip *et al*., 2007).

The material properties of the polymers such as melting temperature, glass transition temperature and crystallinity are greatly influenced by the length of the side chain and the functional group of polymers. Most of the PHAs exhibit thermal

and mechanical properties that are comparable to petroleum-based plastics, such as polypropylene. The variable properties of the polymer will determine the final application of these polymers in various industrial and medical fields (Akaraonye *et al.*, 2010; Brigham and Sinskey, 2012).

2.1.1 History of PHAs

In 1963, Chowdhury reported that the PHA granules which occur as refractile bodies in the bacterial cells were first observed under the microscope by Beijerinck in 1888. However, only in 1927, the composition of PHAs was firstly reported by the French scientist, Maurice Lemoigne. Inclusion bodies found in *Bacillus megaterium* that primarily consists of poly(3-hydroxybutyrate) [P(3HB)] were characterized by Lemoigne who worked at the Lille branch of the Pasteur Institute - France. Lemoigne was the first to report that the bacterial granules are not ether soluble as in lipids and act as reserve material components (Braunegg *et al*., 1998; Amara, 2008).

In 1974, Wallen and Rohwedder reported the presence of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) as major monomers with C6 and probably C7 as minor components from the activated sewage sludge that extracted using chloroform. This heteropolymer showed distinguishable properties with P(3HB) as it exhibited lower melting temperature and was soluble in hot ethanol. This was the first report on the presence of other 3-hydroxyacids than 3HB (Anderson and Dawes, 1990).

De Smet *et al*. (1983) reported significant progress whereby *Pseudomonas oleovorans* was found to accumulate 3-hydroxyoctanoate (3HO) and an unidentified fatty acid when grown on n-octane (50%, v/v). Subsequently, a more detailed investigation by Lageveen *et al*. (1988) disclosed that the unidentified fatty acid

accumulated by *Pseudomonas oleovorans* was (*R*)-3-hydroxyhexanoate (3HHx). P(3HB) is not synthesized by *Pseudomonas oleovorans* from either n-alkanes or glucose.

At least 11 short-chain 3-HAs with 3HB and 3HV being the major components were detected using gas chromatography analysis in the polymer extracted from marine sediments. Presence of 95% 3HB, 3% 3-hydroxyheptanoate (3HHp), 2% 3HO and trace amounts of three other 3-HAs were detected in the purified polymer extracted from *Bacillus megaterium* (Findlay and White, 1983). This was followed by the discovery of PHAs containing C4, C6 and C8 monomers from sewage sludge (Odham *et al*., 1986).

The production of P(3HB) was fully developed on the industrial scale only in the early 1960s. Several patents were obtained by Baptist and Werber at W.R. Grace & Co. (U.S.A) for their pioneering works related to P(3HB) production by fermentation and fabrication of absorbable prosthetic devices. Tremendous increase in the search for alternative plastics was boosted by the oil crisis in 1970. This opportunity was taken by the Imperial Chemical Industries (ICI) from United Kingdom to formulate conditions that able to produce 70 wt% of P(3HB) homopolymer using *Alcaligenes latus.* A novel P(3HB-*co*-3HV) copolymer under trademark BIOPOL® was also produced by ICI. In April 1996, a range of P(3HB-*co*-3HV) marketed under the trademark BIOPOL® was produced using *Cupriavidus necator* by Monsanto which purchased the BIOPOL® business from Zeneca Bio (branch of ICI). In 1998, Metabolix Inc. obtained the licence from Monsanto and launched a new spin off company named Tepha following the alliance between Metabolix Inc. and Children's Hospital, Boston. Tepha, Inc. is a medical device company headquartered in Lexington, Masachussetts, US which develops innovative

medical devices based on PHA polymers such as P(4HB) homopolymer through advancement of biotechnology and material sciences to be used in procedures for surgical repair and regenerative medicine (Philip *et al*., 2007; Braunegg *et al*., 1998).

2.1.2 Types of PHAs and their physical properties

Although PHAs are considered consumer-oriented and environmentally friendly biopolymer due to their biodegradability and biocompatibility, commercialization of these biopolymers is stringently dependent on the material properties that satisfy the requirement of the targeted market application. At present, more than 200 different monomer constituents are found either as homopolyester or in combination as copolyester (Gomez *et al*., 2012). Wide substrate range of the PHA synthase has resulted in the versatility of the monomer compositions which is a clear advantage because the monomer variation provides PHAs an extended spectrum of associated properties. PHAs are classified into three classes according to their monomer compositions; Short-chain-length (SCL)-PHAs, medium-chain-length (MCL)-PHAs and short-chain-length-medium-chain-length (SCL-MCL)-PHAs (Steinbüchel and Lütke-Eversloh, 2003; Chen, 2009).

2.1.2.1 Short-chain-length (SCL)-PHAs

SCL-PHAs are polymers of 3-HA monomers with a chain length of three to five carbon atoms. They are stiff materials that have methyl and ethyl groups as small side chains. These polymers exhibit high tensile strength and crystallinity but with low elongation to break depending on their monomer compositions (Doi *et al*., 1995). Homopolymer P(3HB) is the most well-known microbial polyester produced by wide ranges of microorganisms and has comparable material properties with polypropylene (Anderson and Dawes, 1990). Nevertheless, P(3HB) is a rigid and

brittle polymer with low elasticity, thus making it unfavourable for industrial use due to its limited applications. The brittleness of P(3HB) is due to its perfect stereoregularity which requires it to undergo a detrimental aging process at ambient temperatures (de Koning and Lemstra, 1993). It is also difficult to process this homopolymer because it exhibits high melting temperature of 170°C (Sudesh *et al*., 2000).

Manipulating the side chains and compositions of P(3HB) polymers through incorporation of other monomers can generate different types of polymers with favourable material properties as the polymers will confer less stiffness and tougher properties. Introducing the methyl and ethyl groups as side chains into the polyster backbone can improve the ductility of P(3HB) by disturbing or reducing the crystal lattice of P(3HB) (Doi *et al*., 1995). Among the short-chain-length polymers that have been studied with such material properties are copolymers poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] and terpolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3HV-*co*-4HB)] (Park *et al*., 2012).

P(3HB-*co*-3HV) copolymer is one of the most well-characterized polyester that has attracted industrial attention (Bhubalan *et al*., 2008). Incorporation of 3HV monomers into the 3HB monomer chains will increase the Young's modulus, elasticity, tensile strength and toughness of the P(3HB-*co*-3HV) copolymer (Madden *et al*., 2000; Ojumu *et al*., 2004). Decrease in the melting temperature with incorporation of 3HV monomer has allowed better thermal processing of P(3HB-*co*-3HV) copolymer and even better biodegradation process because decrease in the

melting temperature is coupled without any changes in the degradation temperature (Bluhm *et al*., 1986).

P(3HB-*co*-4HB) copolymer is a very promising but insufficiently studied PHA. This copolymer exhibits good biocompatibility, resorbability, elastomeric properties and are biodegraded *in vivo* and in the environment at high rates (Vigneswari *et al*., 2012; Zhila *et al*., 2011). Fabrication of P(3HB-*co*-3HV-*co*-4HB) terpolymer is initiated for producing a hybrid polymer possessing the superior and desirable physical and mechanical properties of both P(3HB-*co*-3HV) and P(3HB-*co*-4HB) copolymers. According to Aziz *et al*. (2012), enhancement of the mechanical and physical properties of the terpolymer P(3HB-*co*-3HV-*co*-4HB) can be achieved by incorporating different proportions of both 3HV and 4HB monomer units into the terpolymer chain. Terpolymers with superior material properties are desirable in the medical and pharmaceutical fields. Ramachandran *et al.* (2011) has reported production of terpolymer P(63%3HB-*co*-4%3HV-*co*-33%4HB) with high Young's modulus (101 MPa) and elongation to break (937%) which is suitable for medical applications such as sutures, cardiovascular stents and vascular grafts.

2.1.2.2 Medium-chain-length (MCL)-PHAs

MCL-PHAs consist of monomers with 6 to 14 carbon atoms. The first discovery of MCL-PHAs is a polyester containing 3-hydroxyoctanoic acids (3HO) synthesized by *Pseudomonas oleovorans* (Steinbüchel and Lütke-Eversloh, 2003). Typical 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). These PHAs are usually represented by the

most versatile PHA accumulators, *Pseudomonads* which belong to the rRNA-homology-group I. This group of bacteria derives the 3-hydroxyacyl-CoA from the intermediates of fatty acid *β*-oxidation pathway for the polymerization reaction by MCL-PHA synthase when they are grown on aliphatic alkanes or fatty acids (Sudesh *et al*., 2000).

The monomer composition of MCL-PHAs produced is usually related to the substrate used with most units have 2 carbon atoms lesser than the provided carbon source (Ojumu *et al*., 2004; Braunegg *et al*., 1998). Enoyl-CoA hydratase (PhaJ) and 3-ketoacyl-CoA reductase (FabG) are the specific enzymes that involved in the conversion of intermediates of fatty acid *β*-oxidation into the suitable monomers used in the PHA polymerization by MCL-PHA synthase. Copolyester consisting of (*R*)-3HO as main monomer and (*R*)-3HHx as minor monomer is accumulated by *Pseudomonas putida* cultivated on octanoic acid as carbon source (Steinbüchel and Lütke-Eversloh, 2003).

MCL-PHAs can also be synthesized by most of the *Pseudomonas* sp. using structurally unrelated carbon sources such as gluconate, fructose, acetate, glycerine and lactate. The precursors for these polymers are provided by *de novo* fatty acid synthesis and converted from acyl carrier protein (ACP) form to CoA through catalytic reaction by 3-hydroxyacyl-CoA-ACP transferase (PhaG) (Yu, 2007). Monomers for MCL-PHAs biosynthesis are also generated by malonyl-CoA-ACP transacylase (FabD) which is an over-expressed transacylating enzyme. *P. aeruginosa, P. aureofaciens, P. citronellolis, P. mendocina and P. putida* are among the *Pseudomonads* that have been revealed to synthesize MCL-PHAs through this pathway (Sudesh *et al*., 2000). Production of MCL-PHAs comprising of 7 different monomers; 3HD (major constituent), 3HHx, 3HO, saturated and mono-unsaturated

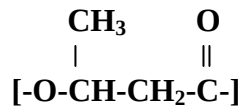
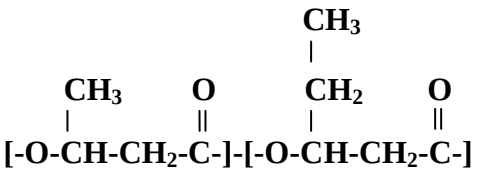
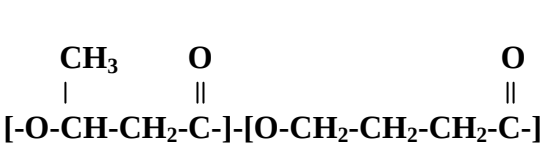
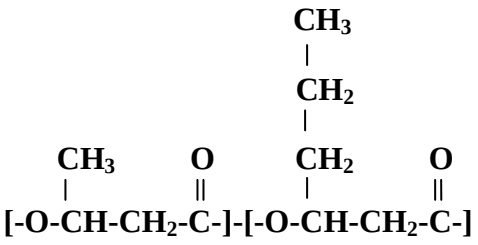
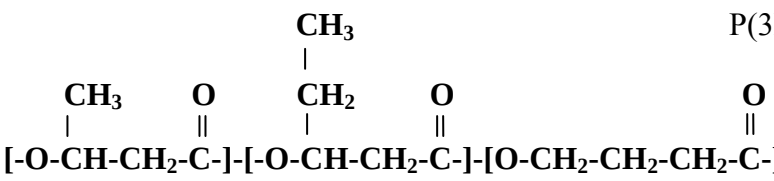
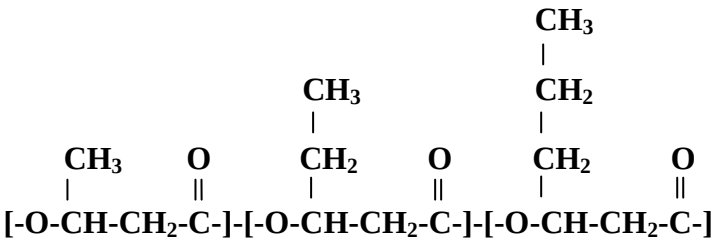
monomers of 12 and 14 carbon atoms by *P. putida* grown on glucose has been reported by Huijberts and Eggink (1996).

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

According to Chen (2009), copolyesters of SCL and MCL monomers are the ideal biomaterials for the advancement of various applications because they exhibit useful and flexible mechanical properties. MCL-PHAs are more elastomer in nature compared to SCL-PHAs which are often stiff and brittle. Incorporating both monomers will result in SCL-MCL PHA copolymers exhibiting properties between the two states which will depend on the different proportions of SCL and MCL monomers. This copolymer has superior properties compared to the SCL and MCL homopolymer. Therefore, it is desirable to elucidate new and low cost ways to synthesize SCL-MCL-PHAs with a small molar fraction of MCL monomers from renewable resources (Nomura *et al*., 2004).

P(3HB-*co*-3HHx) copolymer is one of the successful SCL-MCL-PHAs that is produced on an industrial scale (Chen, 2009). High yield production of P(3HB-*co*-3HHx) copolymer has been obtained successfully using renewable soybean oil by *Cupriavidus necator* and its recombinant (Kahar *et al*., 2004). Bhubalan *et al*. (2008) has proven the good choice of palm kernel oil as the primary carbon source together with the addition of sodium propionate and sodium valerate as 3HV carbon precursors for the production of P(3HB-*co*-3HV-*co*-3HHx) terpolymers having novel compositions with attractive properties. SCL-MCL-PHA copolymers comprising C4 and C6-C12 have been trademarked as Nodax^{TM} by US-based Procter & Gamble (Noda *et al*., 2010). Table 2.1 shows the chemical structure of common PHAs.

Table 2.1: The chemical structure of common PHAs (Braunegg *et al*., 1998; Brigham and Sinskey, 2012)

Chemical structure	Polymer
	P(3HB)
	P(3HB-*co*-3HV)
	P(3HB-*co*-4HB)
	P(3HB-*co*-3HHx)
	P(3HB-*co*-3HV-*co*-4HB)
	P(3HB-*co*-3HV-*co*-3HHx)

2.1.3 Biosynthesis of PHAs

2.1.3.1 Biosynthesis of SCL-PHAs

In 1969, Richie and Dawes revealed the involvement of an acyl carrier protein (ACP) and CoA esters as the intermediates in the PHA synthesis (Dawes, 1988). Three main ezymes involved in the biosynthesis route from acetyl-CoA are; 3-ketothiolase, acetoacetyl-CoA reductase and PHA synthase. The controlling enzyme in the PHA biosynthesis is 3-ketothiolase with CoA as key effector metabolite. Two molecules of acetyl-CoA are coupled in a condensation reaction by 3-ketothiolase (PhaA) to generate acetoacetyl-CoA through the release of the CoA (Anderson and Dawes, 1990). This reversible reaction catalyzed by 3-ketothiolase that inhibited by the presence of excess free CoA is discovered by Senior and Dawes (1973). Subsequently, the acetoacetyl-CoA is stereoselectively reduced to *R*(–)-3-hydroxybutyryl-CoA by acetoacyl-CoA reductase (PhaB). Two acetoacetyl-CoA reductases (NADH and NADPH) possessing different substrate and coenzyme specificities have been found in *Cupriavidus necator*. Since PHA synthase of *Cupriavidus necator* is specific for *R*(–)-substrates, only the NADPH reductase involved in the PHA synthesis from acetyl-CoA (Kessler and Witholt, 2001). According to Dawes (1988), the acetoacetyl-CoA reductase, a typical thiol enzyme is five times more active with NADPH than NADH.

PHA synthase (PhaC) polymerizes the monomers with the release of CoA. This enzyme which is bound with the membrane of the PHA granules, determines the type of PHAs synthesized by bacteria. PHA synthase is distinguished into three types based on the substrate specificities and primary structures. The active site of the PHA synthase that takes part in the polymerization process is a strictly conserved

cysteine residue (Sudesh *et al*., 2000). PHA synthase of *Cupriavidus necator* is active with C4 and C5 substrate and also specific for *R*(–) enantiomers. This indicates active preferences of the PHA synthase of *Cupriavidus necator* towards SCL monomers. Since the position of the oxidized carbon in the PHA monomers is usually not a vital factor, this enzyme can incorporate 4-HA and 5-HA besides the common 3-HA (Anderson and Dawes, 1990; Sudesh *et al*., 2000). Polymerization involves the reaction of soluble components of the cytoplasm at a surface to form a hydrophobic product which apparently accumulates in a very hydrophobic environment within the granules through a two-stage process reaction. This reaction involves the generation of an acyl-enzyme intermediate through a functional thiol group on the enzyme (Dawes, 1988).

2.1.3.2 Biosynthesis of MCL-PHAs

Biosynthesis of MCL-PHAs consisting of (*R*)-3-hydroxy fatty acids is performed through conversion of fatty acid metabolism intermediates to the (*R*)-3-hydroxyacyl-CoA. Conversion of fatty acid *β*-oxidation intermediates into suitable monomers for the polymerization process by the PHA synthase requires the involvement of specific enzymes, enoyl-CoA hydratase (PhaJ) and 3-ketoacyl-CoA reductase (FabG) (Rehm, 2006; Sudesh *et al*., 2000). The (*R*)-specific hydration of 2-enoyl-CoA is catalyzed by the (*R*)-specific enoyl-CoA hydratase (PhaJ) to supply the (*R*)-3-hydroxyacyl-CoA monomer which is the substrate for the polyester synthase (PhaC) (Fukui *et al*., 1998).

Biosynthesis of *de novo* fatty acid intermediates which exclude the fatty acid *β*-oxidation pathway are the alternative route for MCL-PHA synthesis in the bacteria. This pathway is the main route employed by the bacteria cultivated on unrelated

carbon sources such as carbohydrate, acetate or ethanol to synthesize 3-hydroxyacyl-CoA (Suriyamongkol *et al*., 2007). In this pathway (FabD pathway), the conversion of the (*R*)-3-hydroxyacyl moiety of the respective ACP (acyl carrier protein) thioester to its corresponding CoA thioester is catalyzed by the malonyl-CoA-ACP transacylase (FabD). 3-hydroxyacyl-ACP-CoA transferase (PhaG) also involves in the conversion of (*R*)-3-hydroxyacyl-ACP to (*R*)-3-hydroxyacyl-CoA which is a substrate for PHA synthase (Yu, 2007; Sudesh *et al*., 2000). Figure 2.1 illustrates the biosynthetic pathways involve in synthesizing various types of PHAs.

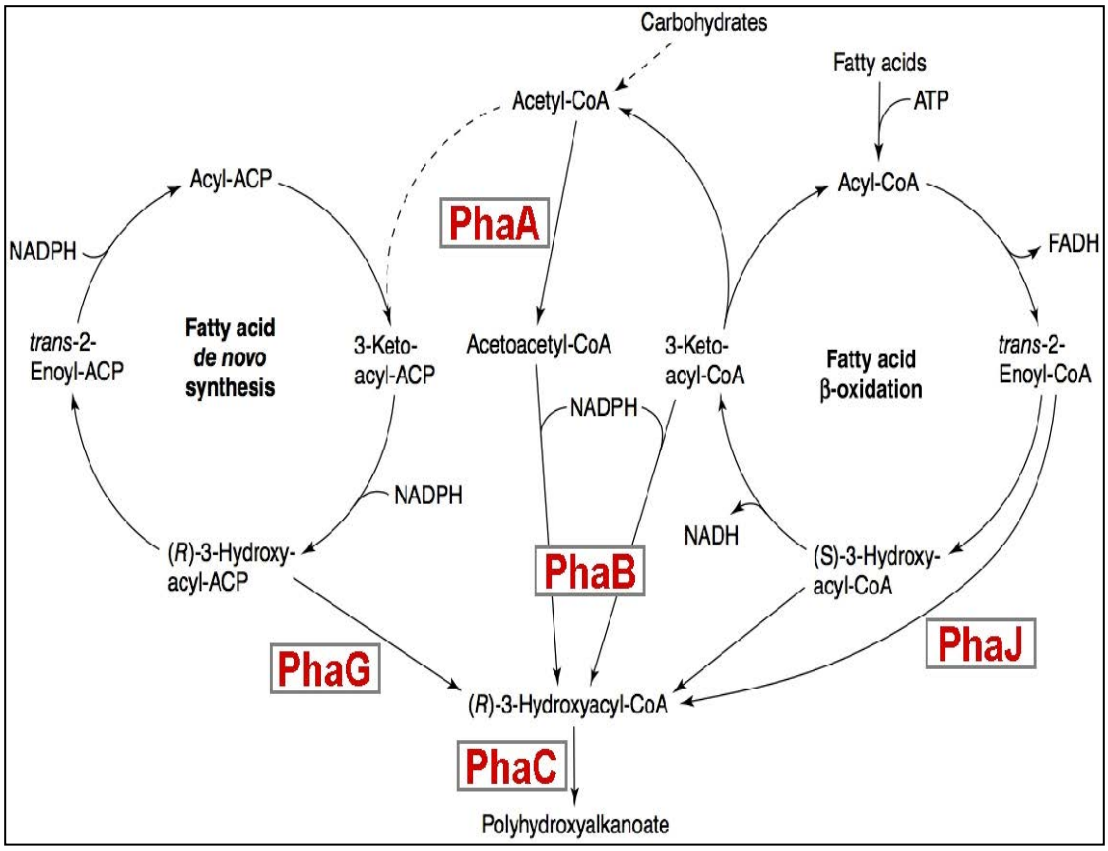


Figure 2.1: Biosynthetic pathways of short-chain-length (SCL)-PHA, medium-chain-length (MCL)-PHA and short-medium-chain-length (SCL-MCL)-PHA from carbohydrates. PhaA, *β*-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (*R*)-specific enoyl-CoA hydratase. Dotted lines represent reactions where intermediate metabolic steps are not included (Aldor and Keasling, 2003; Sudesh *et al*., 2000).

2.2 Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)]

2.2.1 Biosynthesis of P(3HB-*co*-4HB) copolymer by various microorganisms

A very promising and interesting candidates for biomaterial is the P(3HB-*co*-4HB) copolymer due to the existence of 4-hydroxybutyrate monomer that reduces the crystallinity of polymer but enhances the polymer's flexibility characteristic. (Chanprateep *et al*., 2010; Zhila *et al*., 2011). Production of PHAs consisting of 4HB monomer by various microorganisms has been investigated since early 1990s. Wild-type strains capable of biosynthesizing P(3HB-*co*-4HB) copolymer from different carbon sources are *Cupriavidus necator* (Doi, 1990; Nakamura and Doi, 1992; Valentin *et al*., 1995; Lee *et al*., 2000; Kim *et al*., 2005; Chanprateep *et al*., 2008; Chanprateep *et al.*, 2010; Rao *et al*., 2010; Saito *et al*., 1996; Volova *et al*., 2011), *Alcaligenes latus* (Hiramitsu *et al*., 1993; Saito *et al*., 1996; Kang *et al*., 1995), *Comamonas testosteronii* (Renner *et al*., 1996), *Delftia acidovorans* (Kimura *et al*., 1992; Saito *et al*., 1996; Sudesh *et al*., 1999; Lee *et al*., 2004; Mothes and Ackermann, 2005; Hsieh *et al*., 2009; Ch'ng *et al*., 2012), *Hydrogenophaga pseudoflava* (Choi *et al*., 1999) and *Chromobacterium* sp. (Zhila *et al*., 2011).

Saito *et al*. (1996) reported similar observation as made by Kunioka Masao in 1988 who demonstrated production of random copolymer of P(3HB-*co*-4HB) by *Cupriavidus necator* using γ-butyrolactone, 4-hydroxybutyric acid and alkanediols of even number (1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol). Molar fraction of 4HB ranging from 9 mol% to 34 mol% was produced using various carbon sources. Decrease in the 4HB molar fraction was observed when fructose or butyric acid was added into the nitrogen-deficient medium containing 4-hydroxybutyric acid or γ-butyrolactone. Similar synthesis of P(3HB-*co*-

4HB) copolymer using various carbons was also carried out using *Delftia acidovorans* DS-17 that isolated from activated sludge. This bacterium did not accumulate 3HB monomer when grown on 1,4-butanediol or 4-hydroxybutyric acid which proposes the restriction of 4-hydroxybutyryl-CoA metabolism to acetyl-CoA. Inability of this bacterium to metabolize 4-hydroxybutyryl-CoA into (*R*)-3-hydroxybutyryl-CoA had resulted in the synthesis of P(4HB) homopolymer with PHA content ranged from 21 wt% to 28 wt%.

According to Kim *et al*. (2005), bacterial growth was inhibited by high concentration of fructose (> 20 g/l) and γ-butyrolactone (> 6 g/l) in the biosynthesis of P(3HB-*co*-4HB) copolymer by *Cupriavidus necator*, suggesting that a controlled feeding rate of fructose and γ-butyrolactone should be employed as one of the strategies in the fed-batch fermentation. Acetate as well as propionate were also used as stimulator at concentration of 2 g/l to increase the 4HB monomer incorporation from 38 mol% to 54 mol%.

High proportions of 4HB unit (60 mol%-100 mol%) was also produced by *Cupriavidus necator* using 4-hydroxybutyric acid supplemented with additives such as ammonium sulphate and potassium dihydrogen citrate, however the polyester content was found to decrease (Saito *et al*., 1996). Regulation of 4HB molar fraction through supplementation of propionate was also reported by Lee *et al*. (2000), suggesting that increment of 4HB monomer composition from 12 mol% to 52 mol% through addition of propionate in small amount together with γ-butyrolactone was due to the inhibition of ketolysis reaction which catalyzes the lysis of 4HB-CoA to two units of acetyl-CoA.

Chanprateep *et al*. (2008) demonstrated efficient accumulation of 4HB monomer by newly isolated *Cupriavidus necator* strain A-04 through shake-flask

fed-batch cultivation that was influenced by the carbon precursors such as γ-hydroxybutyric acid and 1,4-butanediol. It was suggested that monomer compositions of 4HB could be regulated from 0 mol% to 70 mol% by manipulating the concentrations of two carbon substrates and the carbon-to-nitrogen ratio. Specific production rate of 3HB monomer was the highest at C/N 200 whereas maximum specific production rate of 4HB monomer was attained when C/N ratio used is between 4 and 20.

The feasibility production of P(3HB-*co*-4HB) copolymer by *Cupriavidus necator* using the spent palm oil left after frying activities and 1,4-butanediol was demonstrated by Rao *et al*. (2010) who reported high PHA yield (0.75–0.8 g/g of spent palm oil) with constant accumulation of 4HB monomer (15 mol%) that led to a conclusion that 4HB monomer accumulation was not influenced by the cultivation period and the existence of polar solids in the spent palm oil.

Cavalheiro *et al*. (2012) presented the first report on the production of P(3HB-*co*-4HB) copolymer from waste glycerine using high-cell density fed-batch cultures of *Cupriavidus necator* DSM 545. Incorporation of 4HB monomers was initiated by adding γ-butyrolactone. P(3HB-*co*-4HB) copolymers with 11 mol% to 22 mol% of 4HB monomer were attained by manipulating the dissolved oxygen concentration and cultivation time. Monomer of 4HB was increased by 2-fold using propionic acid as a stimulator but it had resulted in the formation of P(3HB-*co*-3HV-*co*-4HB) terpolymer because propionic is a precursor for formation of HV monomer.

Delftia acidovorans possesses the most efficient metabolic pathway for the biosynthesis of P(3HB-*co*-4HB) copolymer and generally, PHAs extracted from this bacterium are safe as tested based on cytotoxicity, genotoxicity and implant tests. Even though *Delftia acidovorans* is a potential strain for the production of P(3HB-

co-4HB) copolymer for medical applications, but it has inferior ability of controlling the monomer compositions in a wide range (Ch'ng *et al*., 2012; Siew *et al*., 2009). According to the study carried out by Hsieh *et al*. (2009), P(3HB-*co*-94%4HB) copolymer was achieved with cell concentration and PHA content of 2.5 g/l and 13 wt%, respectively using *Delftia acidovorans* cultivated under optimal conditions as follows; 1,4-butanediol (10 g/l), pH (7), incubation time (72 hours) and temperature (26ºC). *Delftia acidovorans* was also reported to produce P(3HB-*co*-4HB) copolymers with extremely high 4HB compositions (93-99 mol%) with PHA content of 12 wt% to 18 wt% using 1,4-butanediol (5 g/l) as sole carbon source through two-stage cultivation process (Kimura *et al*., 1992).

According to Lee *et al*. (2004), besides adjusting the concentration of carbon, increase in 4HB monomer composition accumulation by *Delftia acidovorans* could also be achieved by adjusting pH and aeration. It was reported that influence of pH was greater on the generation/incorporation of 3HB monomers rather than on 4HB monomers directly, leading to the suggestion that pH affected the intracellular concentration of acetyl-CoA. The authors also reported that molar fraction of 4HB could be significantly enhanced without adversely affecting the PHA content by reducing the aeration as the conditions prevented the incorporation of 3HB monomer.

Effect of Mg^{2+} concentrations on the compositions of P(3HB-*co*-4HB) copolymer produced by *Delftia acidovorans* using glucose and 1,4-butanediol had been reported by Lee *et al*. (2007). The authors demonstrated that 3HB monomer decreased with the increase of Mg^{2+} which was due to the decrease in the uptake of glucose by the bacteria as the stability of membrane was disrupted by the ionic interactions with the phosphonyl group. Concentration of Mg^{2+} influences the

transport of glucose across the membrane which eventually affects the generation of acetyl-CoA required to synthesize the 3HB monomer. P(3HB-*co*-4HB) copolymer content was also very low in the absence of Mg^{2+}, suggesting that enzymes involve in the conversion of glucose and 1,4-butanediol to 3HB and 4HB monomer require Mg^{2+} as a cofactor which will bind to the substrate to orient them properly for the reaction.

Choi *et al*. (1999) reported that 4HB contents up to 66 mol% could be produced by *Hydrogenophaga pseudoflava* cultivated using combination of glucose and γ-butyrolactone through one-step cultivation process. Higher 4HB monomer compositions (89-95 mol%) was achieved through two-step cultivation processes. Random P(3HB-*co*-4HB) copolymers having broad range of 4HB monomer compositions from 0 mol% to 83 mol% were produced by *Alcaligenes latus* using combination of 3-hydroxybutyric and 4-hydroxybutyric acids (Kang *et al*., 1995).

Bacterial strain, *Comamonas testosteronii* had been investigated also for its potential of producing P(3HB-*co*-4HB) copolymer using various carbon sources and precursors which yielded molar fraction of 4HB monomer above 90 mol%. The remarkably high 4HB monomer composition accumulated by this strain was attributed to the very low degradation of 4HB-CoA into 3HB-CoA via acetyl-CoA. Although high concentration of 1,4-butanediol presents in the medium, the bacterium only utilized the stored PHA as main carbon and energy source when the acetate as carbon source became limited. The inability to metabolize 4-hydroxybutyric acid as carbon and energy source seems to be the reason for the accumulation of high 4HB monomer by *Comamonas testosteronii* (Renner *et al*., 1996).

In recent years, wild-type strains of *Cupriavidus* sp. USMAA1020 (Amirul *et al*., 2008; Amirul *et al*., 2009; Vigneswari *et al*., 2009; Vigneswari *et al*., 2010) and

Cupriavidus sp. USMAA2-4 (Chai *et al*., 2009; Rahayu *et al*., 2008) and recombinant strains of *Cupriavidus necator*, *Escherichia coli*, *Aeromonas hydrophila* and *Pseudomonas putida* (Zhang *et al*., 2009; Valentin and Dennis, 1997; Li *et al*., 2010) are described as competent P(3HB-*co*-4HB) producers.

Cupriavidus sp. USMAA1020 and *Cupriavidus* sp. USMAA2-4 were isolated from Malaysian environment. Both strains have the ability to produce P(3HB-*co*-4HB) copolymer with wide ranges of 4HB monomer compositions through one-stage and two-stage cultivation processes using various carbon precursors (Amirul *et al*., 2008; Chai *et al*., 2009). High 4HB monomer composition of 99 mol% with PHA content of 28 wt% was attained using *Cupriavidus* sp. USMAA2-4 grown in medium containing 1,6-hexanediol through two-stage cultivation stage (Chai *et al*., 2009). P(3HB-*co*-27%4HB) of 44 wt% content was produced by *Cupriavidus* sp. USMAA2-4 cultivated for 60 hours through one-stage cultivation using combination of oleic acid (0.5 wt% C) and 1,4-butanediol (0.5 wt% C). Different yields of P(3HB-*co*-4HB) contents ranging from 47 wt% to 58 wt% were obtained by employing new strategy of adding oleic acid and 1,4-butanediol with different concentrations together and separately. Higher PHA content of 58 wt% was obtained by adding 1,4-butanediol into medium containing oleic acid after 48 hours of cultivation (Rahayu *et al*., 2008).

Cupriavidus sp. USMAA1020 was able to produce 4HB molar fractions ranged from 6 to 14 mol% with high PHA content of 47 wt% to 60 wt% using γ-butyrolactone when C/N ratio was increased from 10 to 60 through one-stage cultivation. Higher 4HB molar fraction of 60 mol% was achieved using γ-butyrolactone (20 g/l) through two-stage cultivation (Amirul *et al*., 2008). Effect of culture conditions such as phosphate ratio, cell concentration and aeration on the