DUAL SYNTHESIS OF POLY(3-HYDROXYBUTYRATE-*co*-4-HYDROXYBUTYRATE) COPOLYMER AND YELLOW PIGMENT BY *Cupriavidus* sp. USMAHM13 AND ITS CHARACTERIZATION

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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
°C/min	Degree Celsius per minute
ΔH_m	Heat of Fusion
g	Gravity
cm	Centimeter
C_L	Dissolved oxygen concentration
C^*_L	Dissolved oxygen concentrations in equilibrium with mean gaseous oxygen concentration
CFU/mL	Colony-forming unit per mililiter
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_w	Weight-average molecular weight

M_n	Number-average molecular weight
M_w /M_n	Polydispersity index
mg	Miligram
mg/mL	Miligram per mililiter
mL/mg	Mililiter per miligram
mL	Mililiter
mm	Milimeter
mM	Milimolar
mol%	Mole percentage
MPa	Mega Pascal
nm	Nanometer
psi	Pounds per square inch
QO_2X	Oxygen uptake rate of cells
R	Correlation coefficient
R^2	Determination coefficient
rcf	Rotation centrifugational force
rpm	Rotation per minute
t	Time
T_g	Glass transition temperature
T_m	Melting temperature
µg/mL	Microgram per mililiter
µL	Microliter
µm	Micrometer
v/v	Volume per volume

vvm	Volume per volume per minute
wt%	Weight percentage
wt% C	Weight percentage of carbon
w/v	Weight per volume
w/w	Weight per weight
3D	3-dimensional
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyryl-CoA
4HB	4-hydroxybutyrate
4HB-CoA	4-hydroxybutyryl-CoA
ACP	Acyl carrier protein
ANOVA	Analysis of variance
ATCC	American type culture collection
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
CH_3COONH_4	Ammonium acetate
CME	Caprylic methyl ester
CoA	Coenzyme A
$CoCl_2 \cdot 2H_2O$	Cobalt (II) chloride hexahydrate
$CoSO_4 \cdot 7H_2O$	Cobalt sulphate heptahydrate
$CuCl_2 \cdot 2H_2O$	Copper (II) chloride dihydrate

DO	Dissolved oxygen
DOT	Dissolved oxygen tension
DSC	Differential Scanning Calorimeter
fadA	β-ketoacyl-CoA thiolase
fadB	β-ketoacyl-ACP synthase I
fadE	acyl-CoA dehydrogenase
$FeSO_4 \cdot 7H_2O$	Iron (II) sulphate heptahydrate
FID	Flame Ionization Detector
GC	Gas Chromatography
GPC	Gel Permeation Chromatography
HA	Hydroxyalkanoate
IS	Internal standard
K_2HPO_4	Di-potassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
MBC	Minimal Bactericidal Concentration
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
MVA	Mevalonate
NA	Nutrient agar
NaCl	Sodium chloride
Na_2SO_4	Sodium sulphate

NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NB	Nutrient broth
NMR	Nuclear magnetic resonance
NR	Nutrient rich
OD	Optical density
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(4HB)	Poly(4-hydroxybutyrate)
PDH	Pyruvate dehydrogenase
PDI	Polydispersity index
PHA	Polyhydroxyalkanoate
PhaA	*β*-ketothiolase
PhaB	Acetoacetyl-CoA reductase
PhaC	PHA synthase
PhaG	(*R*)-3-hydroxyacyl-acyl carrier protein-CoA transferase
PMMA	Polymethylmethacrylate
PTFE	Polytetrafluoroethylene
RSM	Response Surface Methadology
scl	Short-chain-length
TCA	Tricarboxylic acid
UV-Vis	Ultraviolet-Visible
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate heptahydrate

DWI SINTESIS KOPOLIMER POLI(3-HIDROKSIBUTIRAT-*ko*-4-HIDROKSIBUTIRAT) DAN PIGMEN KUNING OLEH *Cupriavidus* sp. USMAHM13 DAN PENCIRIANNYA

ABSTRAK

Kopolimer poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) [P(3HB-*ko*-4HB)] telah menarik perhatian yang intensif dalam pelbagai aplikasi. *Cupriavidus* sp. USMAHM13 didapati mempunyai keupayaan menghasilkan kopolimer P(3HB-*ko*-4HB) seiring dengan penghasilan pigmen kuning. Kajian awal menunjukkan ekstrak pigmen mempamerkan aktiviti antimikrob terhadap pelbagai bakteria. Kajian ini dimulakan dengan menggunakan pelbagai strategi untuk menilai kesan kepelbagaian prekursor karbon terhadap biosintesis P(3HB-*ko*-4HB) dan pigmen kuning. Gabungan 1,4-butanadiol dengan 1,6-hexanadiol menghasilkan pertumbuhan bakteria sebanyak 1.21 g/L hingga 6.72 g/L, kandungan PHA sebanyak 28% (b/b) hingga 47% (b/b) dan kepekatan pigmen sebanyak 0.05 g/L hingga 0.18 g/L. Peningkatan kopolimer P(3HB-*ko*-4HB) dan pigmen kuning berjaya dicapai dengan menggunakan 0.42% (b/b) C 1,4-butanadiol dengan 0.14% (b/b) C 1,6-hexanadiol pada C/N=25 yang menunjukkan peningkatan pada kandungan PHA dan kepekatan pigmen sebanyak 52% (b/b) dan 0.25 g/L, masing-masing. Kajian lanjut terhadap penghasilan kopolimer P(3HB-*ko*-4HB) dan pigmen kuning juga dijalankan melalui pengoptimuman medium. Penambahan ammonium asetat didapati sumber nitrogen terbaik untuk meningkatkan penghasilan P(3HB-*ko*-4HB). Bagi mengoptimumkan penghasilan pigmen kuning, saringan suplemen tambahan yang terbaik dilakukan dan menghasilkan pigmen yang tinggi apabila menggunakan ekstrak yis pada kepekatan optimum 2 g/L. Metodologi permukaan respons (RSM) digunakan untuk menilai dan mengoptimum kesan

kepekatan berbeza 1,4-butanadiol, ammonium asetat dan ekstrak yis. Selepas pengoptimuman, kandungan PHA sebanyak 53% (b/b) yang mewakili peningkatan 15% dan kepekatan pigmen sebanyak 0.35 g/L yang mewakili peningkatan 21% telah diperoleh pada 0.56% (b/b) C 1,4-butanadiol, 1.14 g/L ammonium asetat dan 2 g/L ekstrak yis. Namun demikian, biojisim residu (5.87 g/L) menunjukkan sedikit pengurangan sebanyak 9.5%. Melalui kajian terhadap pelbagai kepekatan oksigen terlarut, kesan terhadap kadar pengadukan dari 100 rpm ke 500 rpm mendorong *Cupriavidus* sp. USMAHM13 menghasilkan P(3HB-*ko*-4HB) yang tinggi sebanyak 3.5 kali ganda dan pigmen kuning dengan peningkatan 31%. Selain itu, kadar pengadukan yang lebih tinggi telah mempamerkan sifat fizikal polimer yang baik dari segi berat molekul (M_w) dan pemanjangan sehingga putus yang lebih tinggi. Berat molekul dan pemanjangan sehingga putus yang tertinggi adalah 1513 kDa dan 818.8%, masing-masing diperolehi pada kadar pengadukan tertinggi iaitu 500 rpm. Dalam keseluruhan kajian, penggunaan pelbagai sumber karbon dicadangkan memberi kesan yang tinggi terhadap kedua-dua penghasilan seiring, terutamanya dengan kehadiran oksigen yang mungkin mempunyai kaitan dengan aliran elektron dalam laluan biosintesis P(3HB-*ko*-4HB) dan pigmen kuning yang berkemungkinan terlibat dalam *Cupriavidus*. sp USMAHM13. Ujian kepekatan perencatan minimum (MIC) membuktikan aktiviti antimikrob pigmen kuning yang bagus dengan julat 3200 μg/mL hingga 12800 μg/mL. Pembangunan perancah bersalut pigmen kuning diuji dengan teknik pengkolonian bakteria dimana pengurangan kolonisasi bakteria berlaku pada sela 6 jam pertama dan kemudiannya direncatkan sepenuhnya selepas 24 jam. Kajian ini telah berjaya membuktikan keupayaan *Cupriavidus* sp. USMAHM13 menghasilkan kopolimer P(3HB-*ko*-4HB) dengan ciri fizikal yang baik serta pigmen kuning yang bersifat antimikrob daripada pelbagai sumber karbon dalam keadaan optimum.

DUAL SYNTHESIS OF POLY(3-HYDROXYBUTYRATE-*CO*-4-HYDROXYBUTYRATE) COPOLYMER AND YELLOW PIGMENT BY *Cupriavidus* sp. USMAHM13 AND ITS CHARACTERIZATION

ABSTRACT

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer has attracted intensive attention in various applications. *Cupriavidus* sp. USMAHM13 was found to have the ability of producing P(3HB-*co*-4HB) copolymer and yellow pigmentation simultaneously. Preliminary study had shown that the pigment extract exhibited antimicrobial activity against various bacteria. This present study was first conducted by applying various strategies to evaluate the effect of various carbon precursors on the biosynthesis of P(3HB-*co*-4HB) and yellow pigment. Combination of 1,4-butanediol with 1,6-hexanediol yielded a bacterial growth of 1.21 g/L to 6.72 g/L, PHA content of 28 wt% to 47 wt% and pigment concentration of 0.05 g/L to 0.18 g/L. Enhancement of P(3HB-*co*-4HB) copolymer and yellow pigment production were successfully achieved using 0.42 wt% C of 1,4-butanediol with 0.14 wt% C of 1,6-hexanediol at C/N=25 which resulted in an increased PHA content and pigment concentration of 52 wt% and 0.25 g/L, respectively. Further study on P(3HB-*co*-4HB) copolymer and yellow pigment production was also carried out through medium optimization. Addition of ammonium acetate was found to be the best nitrogen source in enhancing the P(3HB-*co*-4HB) accumulation. Prior to optimization of the yellow pigment production, screening for the best additional supplement was performed; resulting a high yield of yellow pigment when using yeast extract with optimal concentration of 2 g/L. Response surface methodology (RSM) was employed to evaluate and optimize the effect of different concentration of 1,4-butanediol,

ammonium acetate and yeast extract. After optimization, significantly higher PHA content of 53 wt% which represents 15% increment with pigment concentration of 0.35 g/L that represent 21% increment was achieved at 0.56 wt% C of 1,4-butanediol, 1.14 g/L of ammonium acetate and 2 g/L of yeast extract. However, residual biomass (5.87 g/L) showed a slight reduction by 9.5%. By examining various concentration of dissolved oxygen, increased agitation speed from 100 rpm to 500 rpm had induced *Cupriavidus* sp. USMAHM13 to accumulate high levels of P(3HB-*co*-4HB) by 3.5-folds and yellow pigment with 31% increment. Additionally, effect of higher agitation speeds had revealed good physical properties of polymer in term of higher molecular weight (M_w) and tensile strength. The highest molecular weight and elongation-at-break were 1513 kDa and 818.8%, respectively obtained at the highest agitation speed of 500 rpm. In all these experimental conditions, the usage of various carbon sources could be suggested to give high effect on both production simultaneously, especially with the presence of oxygen which might have a correlation with the electron flow in a possible biosynthesis pathway of P(3HB-*co*-4HB) and yellow pigment of *Cupriavidus*. sp USMAHM13. The results for minimal inhibitory concentration (MIC) test evidenced a good antimicrobial activity of the yellow pigment, ranging from 3200 µg/mL to 12800 µg/mL. Development of yellow pigment-coated scaffold was tested with bacterial colonization technique whereby reduction of bacteria colonization happened at first 6 hours interval and was fully inhibited after 24 hours. This study had successfully revealed the capability of *Cupriavidus* sp. USMAHM13 to produce P(3HB-*co*-4HB) copolymer with good physical properties as well as an antimicrobial yellow pigment when grown under optimized conditions.

1.0 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a class of polyester found in many microorganisms. To date, there are various PHAs being synthesized in the form of homopolyesters, copolyesters, terpolyesters and polyesters blends, depending on the types of carbon sources fed and the living organisms. There is a diverse range of PHA-producing organisms including microorganisms and plants. However, bacteria have been reported as the most promising candidate in regard to large-scale PHA production (Bohmert-Tatarey *et al.,* 2002). The usage of plant as PHA-producing organisms is less favorable because it leads to a low production of PHA, which is less than 10% (w/w) of dry weight. Somehow, at high levels of PHA [10-40% (w/w) of dry weight] might potentially retard the plant's growth. In contrast, PHA produced by bacteria can be as high as 90% (w/w) of the dry cell mass in bacterial cells (Steinbüchel and Everslöh, 2003). The PHAs exist as insoluble materials in form of granules and are synthesized in the cytoplasm of bacterial cells under stress condition and act as energy reserves during starvation period (Sudesh *et al.,* 2000).

Currently, PHAs are receiving much attention in many research studies as scientists find these polymers are among the suitable replacement for synthetic plastics, owing to several of their interesting and beneficial characteristics (Chen *et al.,* 2006). Biocompatibility, biodegradability and renewability are some of the superior features that make PHAs favourable (Verlinden *et al.,* 2007). PHAs are considered as environmental friendly bioplastics because they can be degraded into water and carbon dioxide by the surrounding bacteria (Yong, 2009). These characteristics are expected to solve several environmental issues such as global warming and solid-waste management problem due to the influenced of conventional plastics (Lee *et al.,* 2008).

Apart from its importance in ecosystem functions, PHAs also have potential as biomedical and biotechnological materials (Koller *et al.,* 2011). They can be applied in the medical field as heart valves (Rai *et al.,* 2011), surgical sutures, bone plates, osteosynthetic materials (Steinbüchel and Füchtenbusch, 1998), medical temporary implants and chiral monomers (Witholt and Kessler, 1999). It also has been described as the bio-based polymer with therapeutic interests. Similar to polypropylene, PHAs also exhibit thermoplastic and elastomeric properties (Sato *et al.,* 2005).

PHAs are widely produced by microorganisms (Madison and Huisman, 1999). Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] is one of the copolymer with great use due to its beneficial mechanical properties and its biocompatibility (Williams and Martin, 2002). The mechanical properties of P(3HB-*co*-4HB) copolymer are found to exhibit a unique degree of crystallinity (Lu *et al.,* 2011). It can be highly crystalline to strong elastomeric rubber-like material due to the incorporation of 4HB monomer units (Saito *et al.,* 1996; Martin and Williams, 2003). The biocompatibility of P(3HB-*co*-4HB) copolymer is due to the fact of the existence of both 3HB and 4HB monomers which are known to be the common metabolites found in human (Sudesh *et al.,* 2000). The biocompatibility of copolymer, added with a controllable degradation rate may possess a good features such as the ability to bent and stretch; which make it as an excellent biomaterial in the field of medical sciences especially in soft tissue engineering (Chen *et al.,* 2009; Chanprateep *et al.,* 2010) and drug delivery system (Chee *et al.,* 2008).

However, in medical application, there is a common and frequent problem encountered with PHA and other biomaterial known as biomaterial infections (Gottenbos *et al.,* 2002). The biomaterial related infections are caused by the biofilm formation. Biofilm is known as an adherent polymeric matrix produced by a sessile

community of microbial cells. Infection towards medical implants has been a serious case in which binding of bacteria favors hydrophobic surfaces like polymer-based implants. Experiment had proved that under these biofilm, the microbial cells can be resistant to antibiotic and immune responses (Wu *et al.,* 2015). The formation of biofilm is widely found in natural surroundings in human sickness and also affect medical devices (Hall-Stoodley *et al.,* 2004). Therefore, it is an urgent state for clinicians to find a method to overcome this problem; or in some other way to find a treatment to the biofilm infections.

Thus, a new discovery of the naturally-associated, antibacterial properties polymer is one of promising characters to overcome the problem of biofilm formations. It can also suit various applications especially in medical application. In the present study, a novel bacterium, *Cupriavidus* sp. USMAHM13 was found as a new P(3HB-*co*-4HB)-accumulating strain which also exhibits yellow pigmentation (Ramachandran and Amirul, 2013a). Preliminary study had proved that the crude pigment extract exhibited antibacterial activity. As a correlation in producing a medically suitable polymer with strong antimicrobial properties, it is believes that by producing high production of P(3HB-*co*-4HB) with high yield of yellow pigment may result in giving out a good property of biomaterial. Previously, *Cupriavidus* sp. USMAHM13had noticeably stood up as a potential microorganisms that able to produce two types of byproducts simultaneously.

With the advantages that can be gained, the attempt of this study was to produce and characterize the P(3HB-*co*-4HB) copolymer and yellow pigment by *Cupriavidus* sp. USMAHM13. Various carbon sources with different combinations and concentrations were used in order to gain the best production of P(3HB-*co*-4HB) and yellow pigment. The improvement of both P(3HB-*co*-4HB) and yellow pigment production were

employed based on selected medium parameter and process parameter. Enhancement of the P(3HB-*co*-4HB) and pigment production were achieved through statistical analysis by employing response surface methodology (RSM). By using the optimized condition, batch fermentation via 3.6 L bioreactor were employed to further study its effect of dissolved oxygen level which can increase the yield of the copolymer and yellow pigment. Apart from that, characterization of the produced P(3HB-*co*-4HB) and yellow pigment were also conducted.

The objectives of this study are:

1) To screen the combination of various carbon sources for the production of P(3HB-*co*-4HB) and yellow pigment.
2) To improve the production of P(3HB-*co*-4HB) and yellow pigment based on medium and process parameter via response surface methodology (RSM) analysis.
3) To evaluate the mechanical and antimicrobial properties of P(3HB-*co*-4HB) copolymer scaffold.

2.0 LITERITURE REVIEW

2.1 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are natural, biodegradable polymers which represent a group of biopolyesters produced microbially by wide variety of microorganisms (Anderson and Dawes, 1990). At present, there are at least 75 different genera of bacteria found to be able to synthesize various PHAs (Reddy *et al.,* 2003; Madsen, 2015). PHAs are polyesters of hydroxyalkanoates (HAs) and exhibit many structural variations (Table 2.1). PHAs have properties ranging from thermoplastics to elastomers, biodegradable and biocompatible making them useful for the production of bulk and specialty plastics.

Depending on the number of carbon atoms in the PHAs chain, HAs are generally divided into two classes; short-chain-length HA (scl-HA) and medium-chain-length HA (mcl-HA). Scl-HA monomers consist of 3 to 5 carbon atoms whereas mcl-HA monomers are comprised of aliphatic and/or aromatic (*R*)-hydroxyalkanoates with 6 to 14 carbon atoms (Tsuge, 2002; Kim *et al.,* 2007b). These differences occur mostly due to substrate specificity of PHA synthase. Biosynthesis of all PHAs is possible with the present of PHA synthase. PHA synthase catalyzes the polymerization of monomer in various PHA biosynthetic pathways (Steinbüchel and Lütke-Eversloh, 2003). Different PHA synthases from different microorganisms can polymerize HAs from only certain range of carbon length. The PHA synthase of *Pseudomonas oleovorans* can synthesize HAs of 6 to 14 carbon atoms whereas that present in *Cupriavidus necator* can only accept HAs consisting of 3 to 5 carbon atoms (Khanna and Srivastava, 2005). Although most PHA-producing bacteria strain accumulates either scl-PHAs or mcl-PHAs, some

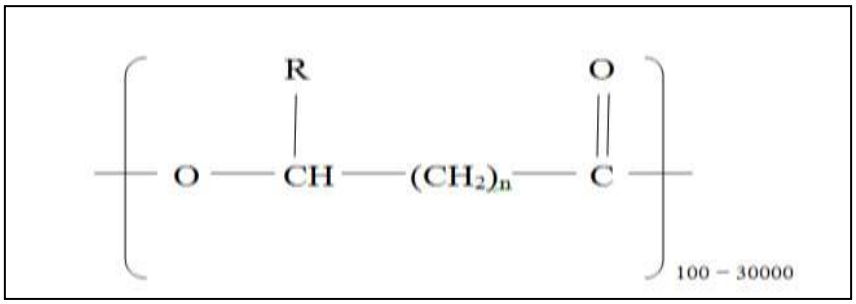
strains have been reported to produce microbial copolyesters consisting of scl-HAs and mcl-HAs (Steinbüchel and Hein, 2001).

PHAs are synthesized by a wide range of bacteria through the fermentation of alkanoic acids, alkenes, alkanes, sugars and lipids (Anderson and Dawes, 1990). Bacteria produce and deposit PHAs as carbon and energy storage in form of polymerized inclusion bodies (Anderson and Dawes, 1990). PHAs are stored intracellularly in order to enhance the survival of bacteria under unfavorable condition (Anderson and Dawes, 1990; Madison and Huisman, 1999; Sudesh *et al.,* 2000). A potential degrader, *Bacillus megaterium* was reported to increase in survival using PHA as energy (López et al., 1998). Meanwhile, stored PHBHHx copolymers in *Aeromonas hydrophila* 4AK4 show survival capability under various stress conditions such as osmotic shock, nutrient-limited environment, cold treatments, UV-irradiation, hydrogen peroxide and ethanol (Zhao *et al.,* 2007). Due to their low solubility and high molecular weight, PHAs constitute an ideal carbon-energy reserve material which enforces insignificant osmotic pressure to the cell (Sudesh *et al.,* 2000). Under anaerobic condition, PHAs can act as an electron sink that conduct the recycling of reducing equivalents (Campisano *et al.,* 2008). When employing toxic substances that could inhibit the growth of microorganisms, PHA accumulation was not only normally stored, yet it is as an alternative way to cope with the existence of stress by metabolizing the excess fatty acid (López-Cortés *et al.,* 2008).

As PHA is a biodegradable polymer, it degrades naturally in the environment by the enzymatic activities of microorganisms (Jendrossek *et al*., 1996). Although PHAs are hydrophobic, water-insoluble and partially crystalline polymers, they can be degraded by a diverse range of microorganisms. PHA can be degraded either intracellularly by intracellular depolymerases of the accumulating strain (Merrick and

Doudoroff 1964; Handrick *et al.,* 2004), or extracellularly by extracellular depolymerases (Volova *et al.,* 2010; Ansari *et al.,* 2013). There are four types of enzymes found in PHA-producing cell which are related to PHA biosynthesis, namely PHA polymerases, PHA depolymerases, phasins, and PhaR. Phasins function as granule stabilizer while PhaR act as phasins regulator (Pötter and Steinbüchel, 2005).

Figure 2.1: General chemical structure of PHAs (Reddy *et al.,* 2003).



		Types of monomer	Ref.
n = 1	R = methyl	3-hydroxybutyrate; 3HB	
	R = ethyl	3-hydroxyvalerate; 3HV	
	R = propyl	3-hydroxyhexanoate; 3HHx	
	R = pentyl	3-hydroxyoctanoate, 3HO	
	R = nonyl	3-hydroxydodecanoate, 3HDDA	
	R= nonanoate	3-hydroxynonanoate, 3HN	(Sun *et al.,* 2007; Jiang *et al.,* 2013)
n = 2	R = hydrogen	4-hydroxybutyrate; 4HB	
n = 3	R = hydrogen	5-hydroxyvalerate; 5HV	
	R = ethyl	5-hydroxyhexanoate, 5HHx	
n = 4	R = hexyl	6-hydroxydodecanoate, 6HDDA	

Table 2.1: Types of monomer in PHAs. n represents the number of monomer units. R represents the side chain.

2.2 Biosynthetic pathway of PHA

Naturally-occurring biosynthesis of PHA has different metabolic pathway in different microorganisms. Most biosynthetic pathway of PHA consists of two steps of enzymatic reaction which includes the supply of substrate monomer and polymerization of monomers (Taguchi *et al.,* 2001). Over the past many years, eight pathways of biosynthesis of PHA were identified (Chen, 2010). Most biosynthetic pathway proceeds in three known metabolic pathways, involving pathway I, pathway II and pathway III (Philip *et al.,* 2007) (Figure 2.1). Pathway I applied in *C. necator* is the one that is well-studied among biosynthetic pathway of PHA (Anderson and Dawes, 1990; Philip *et al.,* 2007).

Pathway I involves acetyl coenzyme A (acetyl-CoA), a most common molecule derived from tricarboxylic acid (TCA) cycle (Zinn *et al.,* 2001; Tsuge, 2002). Acetyl-CoA acts as a key component to provide 3-hydroxyalkanoyl-CoA with different length, depending on the various specificities of substrates for PHA synthase (Chen, 2010). In this pathway, two acetyl-CoA moieties are condensed via dimerization catalyzed by *β*-ketothiolase (PhaA) to produce acetoacetyl-CoA molecules (Philip *et al.,* 2007). After that, the product undergoes reduction to form (*R*)-3-hydroxybutyryl-CoA by NADPH-dependent acetoacetyl-CoA reductase (PhaB). This reaction takes place stereospecifically as only the (*R*)-configuration are taken as substrates (Zinn *et al.,* 2001). The resulting (*R*)-3-hydroxybutyryl-CoA are then undergoing polymerization by PHA synthase to form poly(3-hydroxybutyrate) [P(3HB)] polymer chain (Fukui *et al.,* 1998; Tsuge, 2002). In *C. necator*, the enzyme *β*-ketothiolase, acetoacetyl-CoA

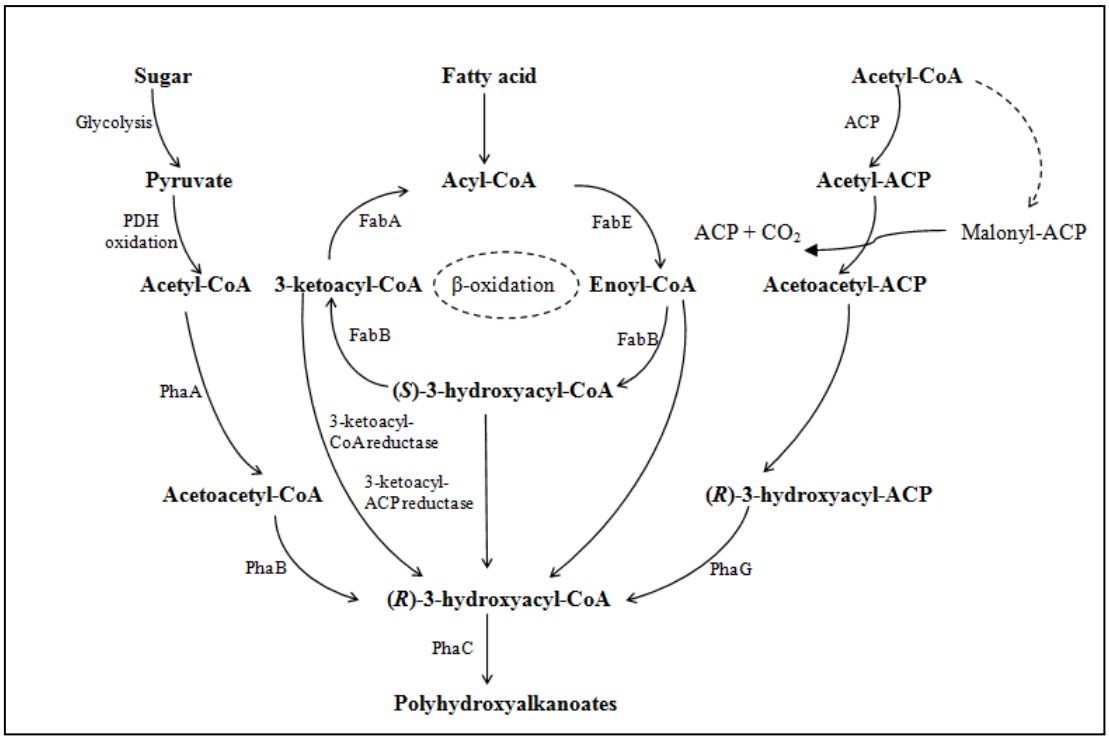


Figure 2.2: PHAs biosynthetic pathways involving sugar catabolism, fatty acid *β*-oxidation and intermediary pathway. PhaA, *β*-ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, (*R*)-3-hydroxyacyl-acyl carrier protein-CoA transferase; ACP, acyl carrier protein; PDH, pyruvate dehydrogenase; FabA, *β*-ketoacyl-CoA thiolase; FabB, *β*-ketoacyl-ACP synthase I; FabE, acyl-CoA dehydrogenase (Chen, 2009; Gumel *et al.,* 2013).

reductase and PHA synthase constitute the *phaCAB* operon (Rehm, 2003). All these enzymes reside in the cytosol of the cell (Anderson and Dawes, 1990). When the cell encounters nutrient-rich condition, the level of free acetyl-CoA moieties is high, causing the TCA cycle to be favorable. However, under growth with nutrient-limiting condition, CoA level is reduced and this favors the P(3HB) synthesis (Zinn *et al.,* 2001).

Pathway II involves fatty acid degradation (*β*-oxidation). In any pathways related to fatty acid metabolism, different HA monomers will be generated (Lageveen *et al.,* 1988). Fatty acid can be utilized by PHA-accumulating pseudomonads such as *Pseudomonas aeruginosa* (Steinbüchel and Lütke-Eversloh, 2003). These microbes are able to produce PHA_{mcl} from alkanoates, alkenes and alkanes. The monomer composition is related to the carbon sources used (Lageveen *et al.,* 1988). This enables fermentation to operate in one-phase and allows PHA synthesis that works independently from alkane oxidizing enzyme system (Steinbüchel and Lütke-Eversloh, 2003). Besides that, it is found that carbon chain length of the 3-hydroxyalkanoic acids converted into PHAs was related to the carbon chain length of the fatty acid used as carbon source (Gross *et al.,* 1989). In the case for non-substituted aliphatic and saturated fatty acids, the fatty acid is first changed into acyl-CoA thioesters and oxidized through trans-2-enoyl-CoA and (*S*)-3-hydroxyacyl-CoA to 3-ketoacyl-CoA. As a result, two formations of moieties are obtained, namely acetyl-CoA and acyl-CoA. Further cycles take place until the particular acyl-CoA is completely converted into acetyl-CoA (Steinbüchel and Lütke-Eversloh, 2003). Under a condition that promotes PHA accumulation, intermediates of *β*-oxidation and the incomplete acetyl-CoA degradation are both partly or entirely withdrawn and converted into PHAs. Another problem arised when the available PHA synthases could not accept the intermediates as substrates. Thus, the presence of three different enzymes namely epimerases, reductase

and hydratase had established other pathways which made PHA accumulation possible by supplying (*R*)-3HA-CoA (Tsuge, 2002; Steinbüchel and Lütke-Eversloh, 2003).

Pathway III is the fatty acid *de novo* biosynthetic pathway which involves the yield of monomers from simple, unrelated carbon sources such as glucose, fructose and sucrose. This pathway helps pathway II to generate monomers for PHA synthesis (Philip *et al.,* 2007). This pathway mainly generates mcl-(*R*)-3HA monomers from fatty acid *β*-oxidation intermediates in wide range of fluorescent pseudomonads (Tsuge, 2002). Acyl-ACP-CoA transacylase (*phaG*) enzyme plays an important role in fatty acid synthesis and PHA biosynthesis (Rehm *et al.,* 1998). It converts (*R*)-3-hydroxydecanocyl-acyl carrier protein (ACP) from fatty acid biosynthetic pathway into coenzyme A (CoA) by transferring hydroxyacyl moiety from ACP to CoA; forming a substrate for PHA synthase (Steinbüchel and Lütke-Eversloh, 2003; Philip *et al.,* 2007). In *Pseudomonas putida*, the supply of 3-hydroxyacyl has contributed to approximately 90% of the monomers from the accumulated PHA_{mcl} through this pathway (Huijbert *et al.,* 1994). In a case where both glucose and fatty acids undergo via Pathway II and III or other pathways, copolymer will be produced (Aldor and Keasling, 2003).

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

The incorporation of 4HB units into P(3HB) produce copolymer of P(3HB-*co*-4HB) which is a very promising material focusing on biodegradable materials. Copolymer of P(3HB-*co*-4HB) is a type of short-chain-length polymer and was identified in 1988 by Doi and colleagues. The discovery of this copolymer was described when 4-hydroxybutyric acid was fed to *C. necator* as carbon source (Doi, 1990). A similar observation reported by Saito *et al*. (1996) which demonstrated the synthesis of P(3HB-*co*-4HB) by *C.necator* when grown in media containing 4-

hydroxybutyric acid, γ-butyrolactone and alkanediols of even number. *C. necator* has been extensively studied and capable of producing copolymer P(3HB-*co*-4HB) with various compositions of 4HB monomer units ranging from 0-100 mol% (Kim *et al.,* 2005).

P(3HB-*co*-4HB) copolymer can be synthesized by providing various, specialized 4HB carbon precursors with even number of carbon such as γ-butyrolactone, 4-hydroxybutyric acid, 1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol (Saito *et al.,* 1996; Amirul *et al.,* 2008a). The usage of various carbon sources related with the types of PHA constituents produced. This is due to the broad substrate specificity of PHA synthase enzyme in different microorganisms. Microorganisms have the ability to produce PHA from wide range of carbon sources due to the involvement of various metabolic pathways. Besides that, specialized type of carbon source based on the number of carbon has somehow related with the type of PHA monomer produced. Besides that, biosynthesis of P(3HB-*co*-4HB) copolymer can be carried out using unrelated carbon sources such as glucose (Lee *et al.,* 2007) and the combination of succinic acid and ethanol (Valentin and Dennis, 1997) by recombinant bacteria. Other factors can also affect the P(3HB-*co*-4HB) copolymer production which are by controlling the culture conditions such as culture aeration and cell concentration in order to obtain various 4HB molar fractions (Vigneswari *et al.,* 2009b). Manipulation of other elemental compositions such as phosphate and nitrogen may also enhance the formation of microbial products. The relationship between carbon and nitrogen known as carbon to nitrogen (C/N) ratio is the most important relationship in which their proportions can give impact to product formation especially in PHA production. Previous study by Chanprateep *et al.* (2008) had demonstrated the effect of changing C/N ratio on P(3HB-*co*-4HB) production by *Ralstonia eutropha* strain A-04. Result

showed that the increased of 4HB molar fraction is proportional to the increased of carbon (4-hydroxybutyric acid) added into the culture medium. At C/N ratio range of 4 to 20, maximum production of 4HB molar fraction is obtained. This shows that an appropriate level of C/N ratio is important and can be maintained to promote better PHA accumulation (Patwardhan and Srivastava, 2008).

Among the diverse types of PHAs, P(3HB-*co*-4HB) is found to have beneficial mechanical properties. Depending on the monomer compositions, this copolymer exhibits various material properties from crystalline plastic to elastic rubber. As 4HB content increased, the melting temperatures (T_m), glass transition temperature (T_g), enthalpy of fusion (ΔH_m) and crystallinity degree of P(3HB-*co*-4HB) copolymer decreased at lower 4HB content range and increased at high 4HB content range. This is due to the increased integrated units acting as defect in the matrix crystal lattice (Cong *et al.,* 2008). This condition will lead to secondary crystallization and cause brittleness at ambient temperature (Madbouly *et al.,* 2007). Contrarily, only P(4HB) lattice was observed in P(3HB-*co*-4HB) copolymers with compositions of 78-100 mol% of 4HB fractions (Saito *et al*., 1996). P(3HB-*co*-4HB) has a narrow temperature range of processing due to slow thermal degradation at temperature above its melting point (An *et al.,* 1997; Li *et al.,* 2015). Saito *et al.* (1996) had reported the melting point of P(3HB-*co*-4HB) film decreased from 178°C to 130°C as 4HB fraction decreased. Meanwhile, the glass-transition temperature decreased from 4 to -48°C as 4HB fraction increased from 0 to 100 mol%. Saito *et al.* (1996) have demonstrated an increase in tensile strength from 17 MPa to 104 MPa of P(3HB-*co*-4HB) copolymer with increasing of 4HB monomer compositions from 64 mol% to 100 mol%. Under isothermal crystallization, the P(3HB) crystals were presumed to omit the 4HB units in the copolymer chain due to its different fiber repeat and conformation (Hsieh *et al.,*

2006). The rate of degradation also varies with different 4HB molar fractions (Doi *et al.,* 1992). However, it is much faster degraded than P(3HB) due to the presence of 4HB comonomer (Doi *et al.,* 1990a).

2.3.1 Biosynthetic pathway of P(3HB-*co*-4HB)

Several wild-type bacterial strains are potential producers of P(3HB-*co*-4HB) copolymer. Among the bacteria that can synthesis PHAs, the earlier biosynthesis of P(3HB-*co*-4HB) is produced using *C. necator. C. necator* have the capability to utilize variety of substrates such as γ-butyrolactone, 4-hydroxybutyric acid and alkanediols (1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol) to produce random P(3HB-*co*-4HB) copolymer (Saito *et al.,* 1996; Steinbüchel and Lütke-Eversloh, 2003). These types of carbon sources can promote 4HB constituents in P(3HB-*co*-4HB) biosynthesis (Lee *et al.,* 2004). Copolymers with different compositions are produced through mixing of different carbon sources with different compositions in a culture medium (Plackett, 2010).

In the biosynthesis of P(3HB-*co*-4HB) (Figure 2.2), carbon sources used are converted into both monomers of P(3HB-*co*-4HB) namely 3HB and 4HB. As γ-butyrolactone enters the biosynthetic pathway, it will be hydrolytically cleaved into 4-hydrobutyric acid. Simillar to γ-butyrolactone, 1,4-butanediol and 1,6-hexanediol will converted into 4-hydroxybutyric-CoA through two times oxidation reaction. First reaction forms γ-hydroxybutyraldehyde intermediate which is further oxidized into 4-hydroxybutyric acid. Part of it will undergo polymerization by PHA synthase. However, another portion of hydroacyl-CoA undergo condensation to form enoyl-CoA, which enter the 3HB synthesis pathway as *R*(-)-3-hydroxybutyryl-CoA (Braunegg *et al.,*

1998). Formation of 3-hydroxybutyryl-CoA is more likely to happen with the presence of succinate, pyruvate, acetyl-CoA and semialdehyde from 4-hydroxybutyrate (Valentin *et al.,* 1995).

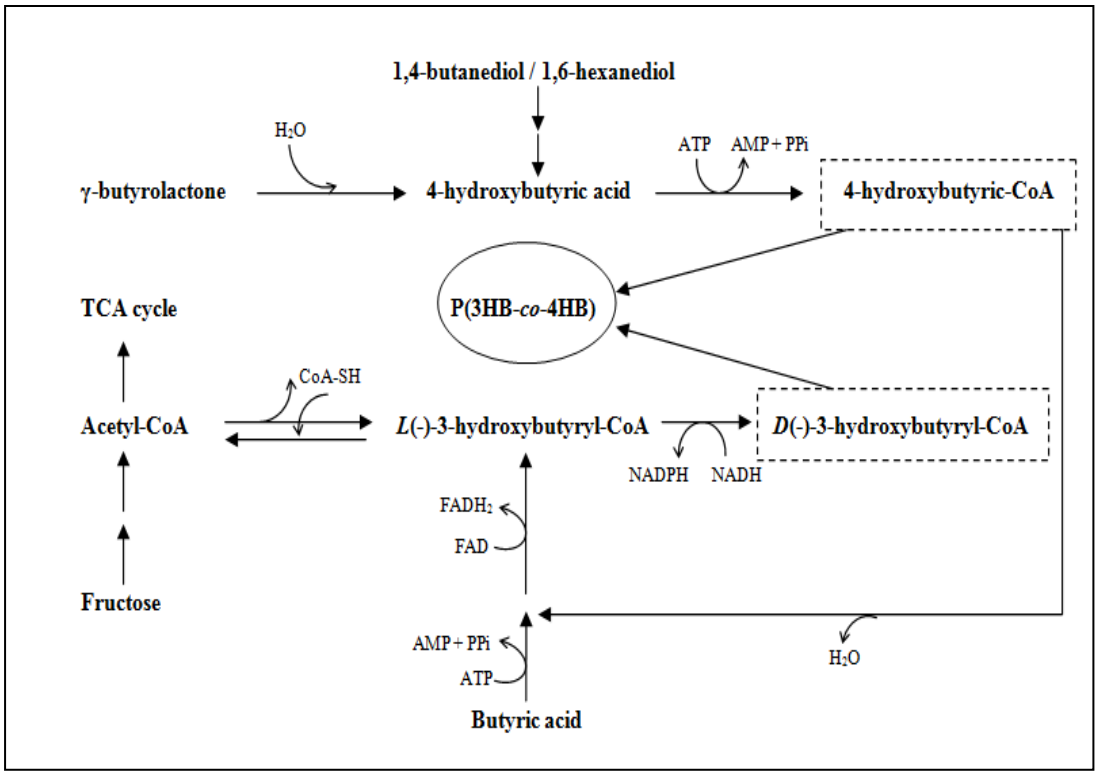


Figure 2.3: Biosynthetic pathway of P(3HB-*co*-4HB) copolymer by *Cupriavidus necator* (Doi, 1990).

In recombinant bacteria, biosynthesis of P(3HB-*co*-4HB) using unrelated carbon source (such as glucose) is differ from the usage of 4HB carbon precursors. It occurs from citric cycle via succinate or succinyl-CoA. The succinyl-CoA is first reduced to form succinate semialdehyde (SSA) and then forming 4HB monomer. CoA transferase then converts 4HB to 4HB-CoA which is a substrate for polymerization by PHA synthase (Steinbüchel and Lütke-Eversloh, 2003; Li *et al.,* 2010).

There are some cases that can interfere during the biosynthesis of P(3HB-*co*-4HB). It was reported that when fructose and γ-butyrolactone are fed together, *R*(-)-3-hydroxybutyryl-CoA will form, causing the 4HB synthesis within copolymer to decrease (Doi, 1990). To some extent, the presence of butyric acid in a medium is able to prevent the formation of 4-hydroxybutyryl-CoA into *R*-3-hydroxybutyrl-CoA. However, a complete inhibition of 3HB synthesis from 4HB acid by ammonium sulfate and citrate stated by Nakamura *et al.* (1992) is somewhat not been explained (Braunegg *et al.,* 1998). Different case with *Delftia acidovorans* DS-17, this bacterium is unable to produce 3HB monomers instead synthesizing 4HB monomers when grown on 1,4-butanediol or 4-hydroxybutyric acid. This phenomenon happened due to the inhibition of 4-hydroxybutyryl-CoA to convert to acetyl-CoA, resulting in the production of P(4HB) homopolymer.

2.4 Fermentation strategy of P(3HB-*co*-4HB) production

Industrial interest in biopolymers has stimulated many ideas to develop bioprocessing strategies. The development of biopolymer fermentation strategy need to provide good production of PHA in term of final yield of cell biomass, cell growth rate, final PHA content in cell dry weight, efficiency of substrate-to-product transformation, low production cost, efficient process, time taken to obtain high cell density and the ability to obtain biopolymers of desired properties (Chen, 2009a). High PHA productivity is an important aspect in which it can reduce the cost of equipment needed, while PHA content affects multiple aspects on process economics (Choi and Lee, 1999). Depending on the objectives of a research, different fermentation processes involve different mode of fermentation and the usage of optimization strategy.

Batch fermentation can be divided into one-stage cultivation, two-stage cultivation and three-stage cultivation. One-stage and two-stage cultivations are highly favourable for biosynthesis of P(3HB-*co*-4HB). One-stage cultivation involves a direct inoculation of bacterial culture from nutrient broth to PHA production medium, namely mineral salt medium (MSM). There is a limited supply of nitrogen available in the production medium. This condition can favour polymer synthesis concurrently with cell growth. Rahayu *et al.* (2008) had demonstrated a one-stage fermentation process to obtain P(3HB-*co*-4HB) copolymer using oleic acid and 1,4-butanediol (C/N=20) by *Cupriavidus* sp. USMAA2-4. Promising results were obtained with high cell dry weight of 5.78 g/l, 44 wt% of PHA content and 27 mol% of 4HB compositions. Another strain namely *Cupriavidus* sp. USMAA1020 was also reported to have pronounced synergistic influence of substrate mixtures towards one-stage production of P(3HB-*co*-4HB), getting a maximum PHA content of 74 wt% with 70 mol% of 4HB molar fractions (Huong *et al.,* 2013). On the other hand, two-stage cultivation involved two distinct

steps, whereby the first step is to promote cell growth with minimum amount of P(3HB) and the second step is to enhance PHA accumulation (Choi *et al.,* 1999). Most production of P(3HB-*co*-4HB) copolymer favours two-stage cultivation over one-stage cultivation (Choi *et al.,* 1999; Kimura *et al.,* 2008; Hsieh *et al.,* 2009; Vigneswari *et al.,* 2009a). An effective production of P(3HB-*co*-4HB) copolymer by *Wautersia eutropha* had obtained maximum 4HB composition of 94 mol% when cultivated under two-step fermentation using 20 g/L of 4-hydroxybutyric acid plus with 2 g/L of propionic acid (Kimura *et al.,* 2008). This showed that the implementation of two-stage cultivation can be a useful strategy for efficient production of 4HB-rich P(3HB-*co*-4HB) copolymer over one-stage fermentation.

The application of mode of fermentations such as continuous cultures, fed-batch and cyclic/repeated fed-batch are among the common fermentation strategies to have high concentration of biomass and high production of desired product (Ienczak *et al.,* 2011). Fed-batch mode is the main culture to achieve high cell productivity through feeding strategy. Since fed-batch fermentation is performed by managing the feed of limiting substrate, this mode of fermentation is considered as the most suitable method for PHA biosynthesis. By using *Ralstonia eutropha* ATCC 17699, Kim *et al.* (2005) reported the development of feeding strategy for high cell density of P(3HB-*co*-4HB) fed-batch fermentation, in which an appropriate amount of γ-butyrolactone can be controlled to avoid cell growth inhibition. This strategy was able to decrease the toxicity effect of organic acids, hence obtaining enough amounts of 4HB fractions from 0 to 50 mol%. Other successful fed-batch fermentation was demonstrated by Park and Kim (2011). They showed an improved cell growth and P(3HB-*co*-4HB) production through the co-feeding of soybean oil and γ-butyrolactone.

Optimization of fermentation conditions has proved to be able to increase high yield of product and productivity in many bioprocesses (Choi *et al.,* 1996; Kulpreecha *et al.,* 2009). In general, optimization involves finding a critical value for each factor that satisfies the desired response. This strategy had been widely used in bioprocesses, analytical applications and industry (Candioti *et al.,* 2014). One-variable-at-a-time technique was the traditional way for optimizing processes. Other than that, the application of statistical analysis such as response surface methodology (RSM) is one of the method for optimization. RSM is proved to be a useful tool to optimize culture conditions for PHA biosynthesis (Md. Iqbal and Amirul, 2014). Ramachandran and Amirul (2014) had implemented the usage of RSM to produce P(3HB-*co*-4HB) by *Cupriavidus* sp. USMAHM13. Result showed that optimum usage of 1,4-butanediol with slightly increased of ammonium acetate had positively affected the PHA content with high 4HB composition (50 mol%). A similar experiment was conducted by Md. Iqbal and Amirul (2014) whereby five variables were applied using RSM. The optimum variables demonstrated an increase of residual biomass and PHA concentration, from 2.9 g/L to 4.9 g/L and 4.2 g/L to 7.6 g/L, respectively.

2.5 Application and potential of P(3HB-*co*-4HB) copolymer

Delftia acidovorans is known as a potential P(3HB-*co*-4HB)-producing bacteria for medical applications. The PHAs produced by *Delftia acidovorans* had been tested and proved to be safe for its genotoxicity, cytotoxicity and implant test (Siew *et al.,* 2009). At present, attention on PHAs has increased towards economical production and commercialization. As PHAs come with new characteristics, they lead to various applications. PHAs application can cover areas such as in industrial, agriculture, medicine, polymer blend and nanocomposites (Philip *et al.,* 2007).

The biodegradablility and durability of PHAs like P(3HB-*co*-4HB) copolymer are one of the factors that make them unique and sustainable for the production of bioplastics. Furthermore, the product from the degradation of this copolymer namely 3-hydroxybutyrate and 4-hydroxybutyrate are known as the natural metabolites found in human bodies. Its low melting temperature makes it easier for product processing. In biomedical applications, biomaterials need to meet these characteristics: (1) biocompatility, (2) support cell growth and adhesion, (3) no prolonged inflammation, (4) no toxic effect and (5) the product from biomaterial degradation must be non-toxic or can be metabolized by the body (Philippe *et al.,* 2010).

Having novel features as biocompatible, thermostable and biodegradable polymers, P(3HB-*co*-4HB) have beneficial impact in wide scope of medical fields such as tissue engineering (Wu *et al.,* 2009), cardiovascular (Hoerstrup *et al.,* 2000), implants (Korkusuz *et al.,* 2001; Martin and Williams, 2003), wound dressing (Ishikawa, 1996), drug delivery system (Türesin *et al.,* 2000; Chee *et al.,* 2008) and cell encapsulation and nanoencapsulation (Grage *et al.,* 2009). P(3HB-*co*-4HB) is capable of releasing drugs and hormones in slow rate. Tang *et al.* (2008) had demonstrated a potential ability of P(3HB-*co*-97%4HB) through histology observation as it degrades in 4 weeks with the presence of macrophages along the interphase period, indicating the occurrence of a good progression of healing. Besides that, P(3HB-*co*-4HB) has also been used as implantable rods in osteomyelitis therapy (Chen and Wu, 2005). Korkusuz *et al.* (2001) evaluated the effectiveness of therapy of implant-related osteomyelitis by developing an antibiotic carrier made of P(3HB-*co*-4HB). Results showed that drug to polymer ratio of 1:1 (w/w) were competent when treating on bone infection by hemolytic bacterium *Staphylococcus aureus*. Besides that, evaluation of *in vitro* biocompatibility of P(3HB-*co*-4HB) copolymer had been tested in fibrolast cells by Siew *et al.* (2006). The authors

demonstrated that P(3HB-*co*-4HB) showed good biocompatibility on V79 and L929 fibroblast cells. Moreover, the P(3HB-*co*-4HB) tested with alkaline comet assay showed no genotoxic effects after 72 hours under treatment. This evidenced the potential usage for future medical applications.

PHA blend is an alternative method to add advantage to the polymer to suit specific applications. PHA blending is the blending of PHAs with other material in order to enhance its material properties. This method has been widely employed nowadays. For example, P(3HB-*co*-4HB) which is blended with collagen showed significant improvement in cell adhesion as compared to unblended P(3HB-*co*-4HB) copolymer scaffold (Vigneswari *et al.,* 2015). Likewise, P(3HB-*co*-4HB)/collagen/vitamin E blend scaffold showed increased cell proliferation through cellular response *in vitro* with negligible cytotoxicity (Rao e*t al.,* 2010).

Nanocomposites are one of a new generation of polymers. It is a hybrid of polymer matrix with fiber, particle or pallet obtained in nanometer size known as nanoparticles (Pandey *et al.,* 2005). Among variety of nanocomposites nowadays, layered structure or fillers with lamellar nanocomposites are economically favourable because of its higher degradation rate (Philip *et al.,* 2007; Wang *et al.,* 2012). Recently, nanocomposites had been prepared with various nanoparticles such as silica (Han *et al.,* 2011), nanoclay, halloysite, boron nitride and layered double hydroxides (Wang *et al.,* 2012). The usage of silica acts as nanofiller that contain abundant reactive groups on its surface. It had been widely employed to boost polymer performances and fabricate polymer/silica nanocomposites (Han *et al.,* 2011). The P(3HB-*co*-4HB)/silica nanosomposites prepared by Han *et al.* (2011) exhibited an improved thermal stability and mechanical properties. The enzymatic degradation also accelerated proportionally with increased content of silica. It happens due to effective enzyme degradation towards

the ester groups of the copolymer chains as silica has high hydrophilicity (Han *et al.,* 2003). Incorporation with nanoclay also gives pronounced improvement in mechanical, thermal and optical transparency by reinforcing 5 wt% of claytone into P(3HB-*co*-70%4HB). Besides that, this nanocomposite also showed antimicrobial properties and can be enhanced with the increased of clay concentration (Wang *et al.,* 2012).

The biocompatibility of P(3HB-*co*-4HB) has been tested on mouse fibroblast cell line (L929) inoculated on P(3HB-*co*-4HB) films with different 4HB compositions and evaluated using various level of drug loading and poly(DL-lactide-*co*-glycolide) (50:50). As a result, it was found that as 4HB composition increases, the cell viability also increases from 2.7 x 10^5 to 12.2 x 10^5 cells/mL. This is due to the changes in film surface morphology of the polymer from coralloid to smoother one, as it can enhance cell proliferation. This condition was observed using P(3HB-*co*-4HB) as comparable to poly(lactic-*co*-glycolic acid) (PLGA) (Chee *et al.,* 2008). The same experiment was conducted by Türesin *et al.* (2000) in studying the potential of different types of copolymer in drug loading application. It was found that P(3HB-*co*-4HB) counterpart was able to release 72% of the initial drug at rate of 5.4 mg of Sulperazone (tested antibiotic) per day. As compared to P(3HB-*co*-3HV) samples, this copolymer released a bit slower with 70% from their initial drug content in two weeks. This shows that P(3HB-*co*-4HB) has the potential to be used for future drug delivery technology.

2.6 Microbial pigment

Recent increasing concern in banning of synthetic colorants was due to its toxicity (Cañizares-Villanueva *et al.,* 1998), terratogenicity and carcinogenicity (Babitha, 2009) which had caused many health problems. There has been a high demand towards replacement of synthetic origin with natural pigments. To overcome

this problem, microbial and plant source of pigments are some of good alternatives. In spite of the usage of pigments from plants, microbial pigments have grown interest due to their safety to use, easy down-streaming process and their naturally-produced characteristic (Babitha, 2009; Malik *et al.,* 2012).

There are several common natural pigments produced from microbes such as flavonoids, tetrapyrroles and carotenoids (Cañizares-Villanueva *et al.,*1998). Flavonoids compound is a purple-coloured pigment and was currently getting a new research prospect in microbial pigment production (Du *et al.,* 2011). Flavonoids are able to meet many applications such as to treat hormone-related disorder and as antioxidant (Shaik *et al.,* 2006). It is also was found as a potential health-promoting substance and it is available as supplements to meet general public needs (Harborne and Williams, 2000). Tetrapyrrole, a macrocyclic compound is commonly used to synthesize various molecules in microorganism which include chlorophyll and phycobilliproteins (Schulze *et al.,* 2006). Meanwhile, carotenoid is a family of yellow to orange-red pigment and is diversely found in bacteria, fungi, algae and plants. Like other pigments, carotenoid serves a variety of functions such as colorants, antioxidants and precursors of vitamins (Cheng, 2007).

The purpose for microorganisms to produce pigments comes with the natural functions of the pigments. Some pigments are secreted in order to protect themselves from unfavourable conditions and some of them possess a fighting effect prior for survival. For example, red-pigmented bacterium *Serratia marcescens* produces prodigiosin which exhibit antimicrobial activity against other microbes (Ibrahim *et al.,* 2014; Lapenda *et al.,* 2015). Differ with a fungal pathogen called *Cryptococcus neoformans* and *Histoplasma capsulatum*, they produce an antimicrobial pigment which protect themselves against natural antimicrobial compounds synthesized by other

microbes (Duin *et al.,* 2002). A species of fungi called *Sporothrix schenckii* produce melanin to overcome the effect of ultraviolet radiation (Romero-Martinez *et al.,* 2000). Meanwhile, *Wangiella dermatitidis* synthesized black pigment named dihydroxynaphthalene melanin to survive under extreme heat and cold (Paolo *et al.,* 2006). In other cases involving a golden-coloured *Staphylococcus aureus*, it was reported that this bacterium frequently caused skin infection in human. The loss of pigmentation can cause a significant decrease in virulence activity of the bacteria (Liu *et al.,* 2005; Liu *et al.,* 2008). This phenomenon accounts the importance of pigment production for microbes.

Other than towards the protection against unfavourable conditions, microbial pigments are also being produced to acquire complex nutrients such as iron. This microbial activity has been described in many iron-reducing bacteria such as *Pseudomonas aeruginosa* (Cox, 1980), *Escherichia coli* (Fischer *et al.,* 1990), *Listeria monocytogenes* (Barchini and Cowart, 1996) and many more. Besides that, cyanobacteria produce chlorophyll as acquisition of energy via photosynthesis (Chew and Bryant, 2007).

2.6.1 Bacterial pigment

Pigmented bacteria are abundant and become one of the vital characteristic for identification of microorganisms (Joshi *et al.,* 2003). Colours give an important means for various microorganisms such as bacteria in the way they were categorized due to their distinctive colour of their colonies. Colour featured them as pathogenic microbes from the colour they viewed at different conditions. Some bacteria produce pigments as part of their normal metabolism. Some bacteria tend to change their colour under stress