BIOSYNTHESIS OF POLYHYDROXYALKANOATE (PHA) USING WASTE FISH OIL BY Cupriavidus necator H16

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by

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PUVANESWARY A/P KAESAVAN

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

±	Plus-minus
%	Percentage
A	Alpha
A_{3HB}	Area of 3HB monomer peak
A_{3HV}	Area of 3HV monomer peak
A_{4HB1}	Area of 4HB, γ-butyrolactone monomer peak
A_{4HB2}	Area of 4HB, 1, 4-butanediol monomer peak
A_{CME}	Area of internal standard (CME) peak
B	Weight of freeze-dried cells used for methanolysis in the unit of mg
B	Beta
Γ	Gamma
°C	Degree Celsius
µg	Microgram
µL	Microliter
Mm	Micrometer
µM	Micromolar
(*R*)-	*Rectus*-isomer
(*S*)-	*Sinister*-isomer
^{1}H	Proton
^{13}C	Carbon-13
3C	3 carbon atoms
3HB	3-hydroxybutyrate

3HB-CoA	3-hydroxybutyryl-CoA
3HHx	3-hydroxyhexanoate
3HHx-CoA	3-hydroxyhexanoate-CoA
3HO	3-hydroxyoctanoate
3HP	3-hydroxypropionate
3HV	3-hydroxyvalerate
4C	4 carbon atoms
4HB	4-hydroxybutyrate
4HV	4-hydroxyvalerate
5C	5 carbon atoms
5HHx	5-hydroxyhexanoate
5HV	5-hydroxyvalerate
6C	6 carbon atoms
7C	7 carbon atoms
8C	8 carbon atoms
A_{3HB}	Area of 3HB monomer
A_{3HHv}	Area of 3HV monomer
A_{CME}	Area of CME
ANOVA	Analysis of variance
BSA	Bovine serum albumin
C	Carbon atom
C5	Valeric acid
C11	11 carbon atoms
C19	19 carbon atoms
C14:0	Myristic acid

C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
C20:0	Arachidic acid
-CH	Methine group
$-CH_2$	Methylene group
$-CH_3$	Methyl group
$CaCl_2 \cdot 2H_2O$	Calcium chloride dehydrate
$CDCl_3$	Deuterated chloroform
CDW	Cell dry weight
CME	Caprylic methyl ester
CoA	Coenzyme-A
CPKO	Crude palm kernel oil
CoA	-Coenzyme-A
CoASH	Coenzyme-A with sulfhydryl functional group
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
$CrCl_3 \cdot 6H_2O$	Chromium chloride hexahydrate
$CuSO_4 \cdot 5H_2O$	Copper (II) sulphate pentahydrate
dH_2O	Distilled water
D	Polydispersity index
Da	Dalton
DMSO	Dimethyl sulfoxide
DTNB	5,5’-dithio-bis(2-nitrobenzoic acid)

DSC	Differential scanning calorimetry
$FeCl_3$	Iron (II) chloride
G	Gram
g/L	Gram per liter
GA	Gum Arabic
GC	Gas chromatography
GPC	Gel permeation chromatography
H	Hour
HA-CoA	Hydroxyacyl-CoA
HCl	Hydrochloric acid
ICI	Imperial Chemical Industries
K	Gas chromatography factor
k_{3HB}	3-hydroxybutyrate factor
k_{3HV}	3-hydroxyvalrate factor
kDa	Kilodalton
Kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
kPa	Kilopascal
L	Litre
LDPE	Low-density polyethylene
M	Molar
M_n	Number-average molecular weight
M_w	Weight-average molecular weight
M_w/M_n	Polydispersity index
Mcl	Medium-chain-length

mcl-PHA	Medium-chain-length PHA
Mg	Milligram
$MgCl_2$	Magnesium chloride
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
MHz	Megahertz
Min	Minute
mL	Mililiter
mM	Millimolar
MM	Mineral salts medium
mol%	Mole percent
MPa	Megapascal
N	Normality
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
$NiCl_2 \cdot 6H_2O$	Nickel chloride hexahydrate
Nm	Nanometer
NMR	Nuclear magnetic resonance
NR	Nutrient rich
OD	Optical density
OD_{595}	Optical density at wavelength 595 nm

OD_{412}	Optical density at wavelength 412 nm
OD_{600}	Optical density at wavelength 600nm
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)
PE	Polyethylene
PHA	Polyhydroxyalkanoate
PhaA	*β*-ketothiolase
PhaB	NADPH-dependent acetoacetyl-CoA dehydrogenase
PhaC	PHA synthase
PLA	Polylactic acid
PP	Polypropylene
Ppm	Parts per million
PTFE	Polytetrafluoroethylene
PUFAs	Polyunsaturated fatty acids
PVA	Polyvinyl alcohol
Rpm	Revolutions per minute
Scl	Short-chain-length
scl-PHA	Short-chain-length PHA
Sec	Second
Sp.	Species
TCA	Tricarboxylic acid
TCAA	Trichloroacetic acid
TGA	Thermogravimetric analysis
TMS	Tetramethylsilane

Tukey’s HSD	Tukey’s Honestly Significant Difference
T_d	Decomposition temperature
T_g	Glass-transition temperature
T_m	Melting temperature
U	Unit
v/v	Volume per volume
w/v	Weight per volume
WFO	Waste fish oil
wt%	Weight percent

POLIHIDROKSIALKANOAT (PHA) BIOSINTESIS MENGGUNAKAN SISA MINYAK IKAN OLEH Cupriavidus necator H16

ABSTRAK

Potensi penggunaan sisa minyak ikan sebagai sumber karbon untuk penghasilan polihidroksialkanoat (PHA) oleh *Cupriavidus necator* H16 telah disiasat. Pengumpalan sisa minyak ikan kelihatan terbentuk dan terapung di permukaan media. Fenomena ini mengehadkan bakteria tersebut mencapai dan mencernakan sumber karbon yang terkandung dalam sisa minyak ikan. Dalam satu percubaan yang dijalankan menggunakan gum Arab sebagai pengemulsi, didapati bahawa masalah pengumpalan dapat ditangani. Sisa minyak ikan telah didapati untuk menjadi satu sumber karbon yang berkesan untuk penghasilan PHA dengan kehadiran gum Arab sebanyak 2.5 g/L. Berat kering sel sebanyak 4.85 g/L dan kandungan PHA sehingga 73 wt% telah dicapai menggunakan 15 g/L sisa minyak ikan sebagai sumber karbon. Apabila media kultur disertakan dengan natrium 4-hidroksibutirat,1,4-butandiol, γ-butyrolaktone atau natrium valerat bersama-sama sisa minyak ikan, kopolimer poli(3-hidroksibutirat-*co*-4-hidroksibutirat, [P(3HB-*co*-4HB)] dan poli(3-hidroksibutirat-*co*-3-hidroksivalerat), [P(3HB-*co*-3HV)] Berjaya dihasilkan oleh *C. necator* H16. Sebanyak 63 mol% monomer 3HV telah digabungkan ke dalam kopolimer P(3HB-*co*-3HV) yang disintesis oleh *C. necator* H16. Antara prekursor 4HB yang dikaji, didapati bahawa γ-butyrolactone merupakan prekursor terbaik untuk penghasilan kopolimer P(3HB-*co*-4HB). Kopolimer P(3HB-*co*-4HB) dengan 36 mol% 4HB telah dihasilkan

Kehadiran serta komposisi dan kerawakan 3HB, 3HV dan 4HB, dalam polimer yang dihasilkan telah disahkan menggunakan analisis ^{1}H NMR and ^{13}C NMR. Sifat terma dan fizikal kopolimer juga dicirikan menggunakan kromatografi

penelapan gel (GPC), kalorimetri pengimbasan pembezaan (DSC), dan analisis termogravimetri (TGA). P(3HB) dan kopolimer P(3HB-*co*-3HV) dengan berat molekul dalam julat dari 1.5×10^6 hingga 2.0×10^6. Suhu lebur yang diperolehi berada dalam julat 67 sehingga 169 °C untuk P(3HB), P(3HB-*co*-3HV) dan P(3HB-*co*-4HB). Kajian ini jelas menunjukkan kemungkinan menggunakan sisa minyak ikan sebagai satu sumber karbon yang berpotensi untuk pertumbuhan sel dan pengeluaran PHA.

BIOSYNTHESIS OF POLYHYDROXYALKANOATE (PHA) FROM WASTE FISH OIL BY Cupriavidus necator H16

ABSTRACT

The potential use of waste fish oil (WFO) as carbon source for production of polyhydroxyalkanoates (PHAs) using *Cupriavidus necator* H16 was investigated. Small aggregates of the WFO were observed to be floating on the surface of culture medium, which limited the accessibility of the carbon source to the bacterium. An attempt of using Gum Arabic (GA) as an emulsifier was carried out to address the limited availability of WFO. WFO was found to be an effective carbon source for PHA production in the presence of GA at 2.5 g/L. Cell dry weight of 4.85 g/L and P(3HB) content up to 73 wt% were achieved by using 15 g/L of waste fish oil as the carbon source. Characterization of the PHA synthase enzyme showed 161 U/g activity for soluble fraction. Copolymers poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), [P(3HB-*co*-4HB)] and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), [P(3HB-*co*-3HV)] could be produced by *C. necator* H16 with respective addition of precursors such as sodium 4-hydroxybutyrate, 1,4-butanediol, γ-butyrolactone, or sodium valerate to the culture medium supplemented with WFO. Up to 63 mol% of 3HV monomer was incorporated into P(3HB-*co*-3HV) copolymer synthesized by *C. necator* H16. Among the 4HB precursors tested, γ-butyrolactone was found to be the best precursor for P(3HB-*co*-4HB) copolymer production. P(3HB-*co*-4HB) copolymer with 36 mol% of 4HB monomer was produced. The presence of the 3HB, 3HV and 4HB monomers, as well as their composition and randomness in the polymer produced were confirmed using ^{1}H NMR and ^{13}C NMR analyses. The physical and thermal properties of the copolymers were further characterized using

gel permeation chromatography (GPC), differential scanning calorimetry (DSC), and thermogravimetric analysis (TGA). P(3HB) and P(3HB-*co*-3HV) copolymers with a molecular weight range of 1.5×10^6 to 2.0×10^6 were produced. The melting temperature obtained were in the range of 67 to 169 °C for P(3HB), P(3HB-*co*-3HV) and P(3HB-*co*-4HB) copolymers. This study has demonstrated the possibility of using WFO as a potential carbon source for cell growth and PHA production.

1.0 Introduction

Plastics have permeated every facade of human life viz. packaging, consumer durables, construction, transportation to name a few and almost all industries. They have successfully replaced glass and paper in packaging. One of the reasons for great popularity of plastics is due to the tremendous range of properties exhibited by them such as their versatile qualities of strength, durability, lightness and resistance to degradation (Sudesh et al., 2000a; Sudesh et al., 2000b; Khanna and Srivastava, 2005; Sudesh and Iwata, 2008). Hence, the demand for plastics has been increasing in modern living. Concurrently, the production of plastics has increased substantially over the last 6 decades from around 1.7 million tonnes in 1950 to about 260 million tonnes today (Thompson et al., 2009). Durability of these plastics causes major concerns after they enter waste streams, as the consequences, rapid accumulation of non-biodegradable materials in our environment occurs and are also ingested by many aquatic animals (Thompson et al., 2004).

The cost for solid waste disposal is increasing and the hazards from waste incineration such as hydrogen cyanide emission from acrylonitrile-based plastics, makes synthetic waste management an arduous task (Atlas and Bartha, 1993). Thus, there is a pressing need to look for biodegradable as well as biocompatible polymers. One major source that has gained immense attention is a class of natural polyesters, known as polyhydroxyalkanoate (PHA). It is one of the most elaborately studied bio-based polymers. PHA is a group of storage polymers synthesized by many types of microorganism and functions as energy reserve. It is deposited in the cell cytoplasm as intracellular inclusion bodies in presence of excess carbon source and limited supply of nutrients such as nitrogen, phosphorous, sulfur, magnesium or oxygen

(Steinbüchel and Schlegel, 1991). Thus far, inclined on the microorganism and substrate used as feedstock, approximately 150 different structural analogs of PHA polymer have been identified. P(3HB), is the most common bacterial PHA found in nature with high crystallinity and also one of the most studied PHA. Various carbon sources have been used for P(3HB) assimilation since its discovery by Lemoigne in 1926 (Lemoigne, 1926; Chen, 2010). However, the applications of P(3HB) are normally limited due to its less desirable physical property such as brittleness (Madison and Huisman, 1999). Yet P(3HB) has several useful properties such as moisture resistance, water insolubility, optical purity and good oxygen permeability (Chakraborty et al., 2009).

Cupriavidus necator (formally known as *Wautersia eutropha*, *Ralstonia eutropha*, *Alcaligenes eutrophus* or *Hydrogenomonas eutropha*) is used as a model strain to study the biosynthesis of PHA and it is well-known to utilize sugars, fatty acids, and plant oils and convert them into PHA (Budde et al., 2011b). The cost of carbon substrate contributes to about > 50 % of total production cost of most biopolymer (Lee and Choi, 1998b; Titz et al., 2012). Therefore, the use of inexpensive and renewable agricultural as well as other industrial wastes could be highly advantageous to the economics of PHA production in large scale.

Reports of PHA production using oils or fats derived from animal source are limited. The major animal fats used for the PHA production are tallow, lard and butter oil. These sources have been evaluated for PHA production only using the *Pseudomonas* strains in their native form or recombinant versions. The major producers among *Pseudomonas* sp. were inefficient in utilizing the triacylglycerols (TAGs). So, researchers found it necessary to incorporate lipase artificially to make them efficient users of lipid substrates (Ashby and Foglia, 1998; Solaiman et al.,

2006). By far, crude Alaskan Pollock oil have been evaluated for PHA production as carbon source only using *Pseudomonas corrugata,* and this is the only report of fish oil being tried for PHA production (Ashby and Solaiman, 2008b).

The problem statement of this study

This study was conducted to evaluate the suitability of waste fish oil for the synthesis of PHA by *C. necator* H16 because it may be a potential feedstock to substitute food grade oil for large scale production of bioplastics in a sustainable way.

1.1 Objectives of this study:

1) To evaluate the efficacy of waste fish oil as the main carbon source to support the growth and PHA production by *C. necator* H16.
2) To produce P(3HB-*co*-3HV) and P(3HB-*co*-4HB) copolymer from waste fish oil with supplementation of precursors.
3) To characterize the physical properties of PHA produced from waste fish oil

2.0 Literature review

2.1 Storage of carbon and other compounds

Organisms throughout nature have developed various mechanisms to store vital nutrients such as carbon, phosphorous, and nitrogen. The storage most frequently implies the assimilation of polymers. These polymers could be depolymerized whenever the monomers are needed for synthesis of other metabolites or energy generation. In various instances the polymers form insoluble inclusions, which are of assistance since they are inert to reactions involving soluble substrates and also because the polymers do not contribute to the osmotic potential of the cell in which they are stored (Stubbe et al., 2005). Carbon storage molecules have more widespread and have greater industrial importance. Carbon storage is ubiquitous throughout the eukaryotic and prokaryotic organisms. Glycogens as well as triacylglycerols (TAGs) are stored by mammals as carbon and energy reserve. Plants on the other hand store starch and TAGs to provide nourishment for growing embryos in the form of endosperm. Prokaryotes store carbon in the form of glycogen, TAGs and PHA (Stubbe and Tian, 2003).

2.2 Bio-based and biodegradable polymers

"Bio-based polymers" are defined as organic macromolecules derived from renewable resources that have been polymerized by chemical and/or biological methods. Depending on the mode of synthesis, bio-based polymers are classified into three distinct following groups:

(i) biosynthetic polymers, e.g. PHA

(ii) bio-chemosynthetic polymers e.g. PLA, PBS and

(iii) modified natural polymers e.g. Starch and cellulose

Among these three groups of biopolymers, PHA has gained the major interest due to its excellent properties over the rest. Complete biodegradability nature of these polymers and also utilization of renewable resources for its synthesis makes it beneficial for numerous applications. Biodegradable polymers can be degraded or decomposed in natural environment. It is important to note that not all polymers synthesized by living organism are biodegradable. For instance, crystalline PLA, cellulose ester derivative and, polythioesters are not biodegradable. Utilization of biodegradable polymers i.e.: PHA for numerous purposes is believed to be capable of minimizing problems associated with waste disposal caused by conventional plastic materials (Sudesh and Iwata, 2008).

2.3 Polyhydroxyalkanoate (PHA)

PHA are polyoxoesters synthesized by a wide range of bacteria for carbon and energy storage. PHA was first discovered by Lemoigne in 1926 once he found that the polymer isolated from *Bacillus megaterium* was a polyester of 3-hydroxybutyric acid (Lemoigne, 1926). PHA is deposited in cells as intracellular inclusion bodies in presence of excess carbon source and limited supply of nutrient such as nitrogen, phosphorous, sulfur, magnesium or oxygen (Anderson and Dawes, 1990). Ultrastructure observation of thin sections of PHA containing cells by transmission electron microscope appears as electron dense bodies, discrete spherical particles with clear boundaries. PHA granules could be stained specifically with Sudan Black B or light fluorescent dyes such as Nile blue and Nile red. PHA granules could be observed as refractive inclusions under phase contrast light microscope. The average size of the PHA granules is 0.2 to 0.5 μm in diameter. Thus far, inclined on the microorganism

and substrate used as feedstock, a total of 150 different structural analogs of PHA polymer have been identified (Steinbüchel, 2005; Chen, 2010).

The properties of PHA bear close resemblance to some of the properties of commodity plastics such as polypropylene or low-density polyethylene. Most PHA is highly thermoplastic which allows for processing of stiff packaging materials or highly elastic elastomers (Anderson and Dawes, 1990; Sudesh and Iwata, 2008). PHA is biodegradable and biocompatible polyesters with oxygen impermeability and flexible mechanical properties (Brandl et al., 1990; Gross and Kalra, 2002; Chakraborty et al., 2012)

2.3.1 Types of PHA

The most common building blocks of PHA are (*R*)-3-hydroxyalkanoic acid monomer units. There are also some 4-hydroxyalkanoic acid monomer units. The most common naturally occurring microbial PHA is P(3HB). PHA is generally characterized as short-chain-length (scl-PHA, C3-C5) with carbon numbers in the range of 3 to 5 and medium-chain-length (mcl-PHA, C6-C14) with carbon numbers in the range of 6 to 14. A fusion polymer of scl-mcl-PHA can contain monomers with carbon numbers in the range of 3 to 14. The general structure of PHA is summarized in Table 2.1. The monomer units of microbial polyesters are all in the *R*(□) configuration due to the stereospecificity of PHA synthase which is essential for the biodegradability as well as biocompatibility of the material (Zinn and Hany, 2005).

The most commonly sudied scl-PHA includes homopolymer and copolymer consisting of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 4-hydroxybutyrate (4HB) monomers. Among them, P(3HB), P(3HB-*co*-3HV) and

P(3HB-*co*-4HB) are the well studied homopolymer and copolymer respectively. As for mcl-PHA, they comprise of various copolymers synthesized by a number of *Pseudomonas* strains from fatty acids and oily substrates (Hazenberg and Witholt, 1997). The scl-mcl-PHA is known for its combination of unique property of both scl- and mcl-PHA that can range from hard crystalline to increased elasticity. Terpolymers such as P(3HB-*co*-3HV-*co*-3HHx) and P(3HB-*co*-3HV-*co*-3HHp) are examples of scl-mcl-PHA. There are also other rare monomers such as 4-hydroxyvalerate (5C), 4-hydroxyhexanoate (6C), 5-hydroxyhexanoate (6C), 4-hydroxypentanoate (7C) and 4-hydroxyoctanoate (8C) that are able to be incoporated by some microorganism in lab scale experiments done by Valentin and co-workers.

Number of repeating units, n	Alkyl group, R	Polymer variety
1	Hydrogen	Poly(3-hydroxypropionate)
	Methyl	Poly(3-hydroxybutyrate)
	Ethyl	Poly(3-hydroxyvalerate)
	Propyl	Poly(3-hydroxyhexanoate)
	Pentyl	Poly(3-hydroxyoctanoate)
	Nonyl	Poly(3-hydroxydodecanoate)
2	Hydrogen	Poly(4-hydroxybutyrate)
3	Hydrogen	Poly(5-hydroxyvalerate)

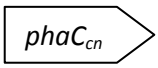
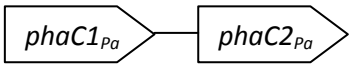
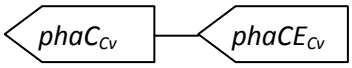
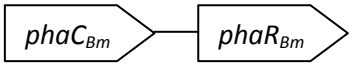
Figure 2.1 General structure of PHA (Lee, 1996)

*R refers to side group while n refers to the number of repeating units

2.4 Metabolic pathways and genes involved in PHA biosynthesis

PHA biosynthesis involves the uptake and subsequent conversion of carbon substrates into precursor molecules that are then polymerized by the enzyme PHA synthase. PHA synthase is the vital enzyme responsible for PHA biosynthesis, which catalyzes the polymerization of (*R*)-3-hydroxyacyl-CoA substrate to PHA. To date there are more than 59 different PHA synthases that have been sequenced and cloned from 46 different bacteria (Rehm, 2003; Rehm, 2007). Four major classes of PHA synthases have been distinguished by referring to their primary structures, substrate specificities and subunit composition (Table 2.1).

Table 2.1 Classes of PHA synthases and the representative species.

Class	**Gene structure**	**Subunits**	**Substrates**	**Representative species**
I		~60−73 kDa	3HA$_{scl}$-CoA	*C. necator* H16
II		~60−653 kDa	3HA$_{mcl}$-CoA	*Pseudomonas aeruginosa*
III		PhaC ~ 40 kDa PhaE~40 kDa	3HA$_{scl}$-CoA 3HA$_{mcl}$-CoA	*Allochromatium vinosum*
IV		PhaC~40 kDa PhaR~22 kDa	3HA$_{scl}$-CoA	*Bacillus megaterium*

2.5 Poly(3-hydroxybutyrate) P(3HB)

Among all the known PHA, P(3HB) is the first and also the most extensively studied PHA. P(3HB) is a homopolymer made up of (*R*)-3HB repeating units. P(3HB) biosynthetic pathway is the simplest which involves three enzymes and their respective encoding genes. Firstly, two acetyl-CoA molecules each consisting two carbon atoms (C2) are condensed into acetoacetyl-CoA with four carbon atoms (C4) catalyzed by β-ketothiolase (PhaA). Next, the acetoacetyl-CoA is then reduced to (*R*)-3-hydroxybutyryl-CoA by an NADPH dependent acetoacetyl-CoA dehydrogenase (PhaB). To complete the process, the synthesized (*R*)-3-hydroxybutyrl-CoA has to be polymerized into P(3HB) through catalytic reaction of the PHA synthase (PhaC) (Anderson and Dawes, 1990; Madison and Huisman, 1999).

β-ketothiolase is the enzyme which first acts as the regulatory enzyme in the pathway (Oeding and Schlegel, 1973). The acetyl-CoA produced is channeled into the tricarboxylic acid (TCA) cycle by citrate synthase and coenzyme A (CoASH) is released. The increasing amount of free CoASH will inhibit the β-ketothiolase and thus arresting the P(3HB) biosynthesis. Quite the opposite occurs during imbalanced growth condition such as limitation of nitrogen and the amino acid synthesis pathway would be blocked. The reduced concentration of free CoASH will no longer inhibit the β-ketothiolase, the overproduced acetyl-CoA will be chanelled into the P(3HB) biosynthesis pathway (Anderson and Dawes, 1990; Tsuge, 2002; Steinbüchel and Lütke-Eversloh, 2003; Verlinden et al., 2007).

The molecular weight (M_w) of P(3HB) synthesized is normally in the range of 200,000 to 3000,000 Da depending on the producer and growth conditions (Hinrichsen, 1995). P(3HB) is highly crystalline with its melting temperature (T_m)

around 180°C and glass transition temperature (T_g) at 4 °C (Tsuge, 2002). Though some of the thermal as well as mechanical properties of P(3HB) is comparable with polypropylene, it is a relatively stiff and brittle polymer (Sudesh et al., 2000a). However, the mechanical properties could be further upgraded by incorporation of other monomers to create copolymers such as P(3HB-*co*-3HV), P(3HB-*co*-3HHx) and also P(3HB-*co*-4HB).

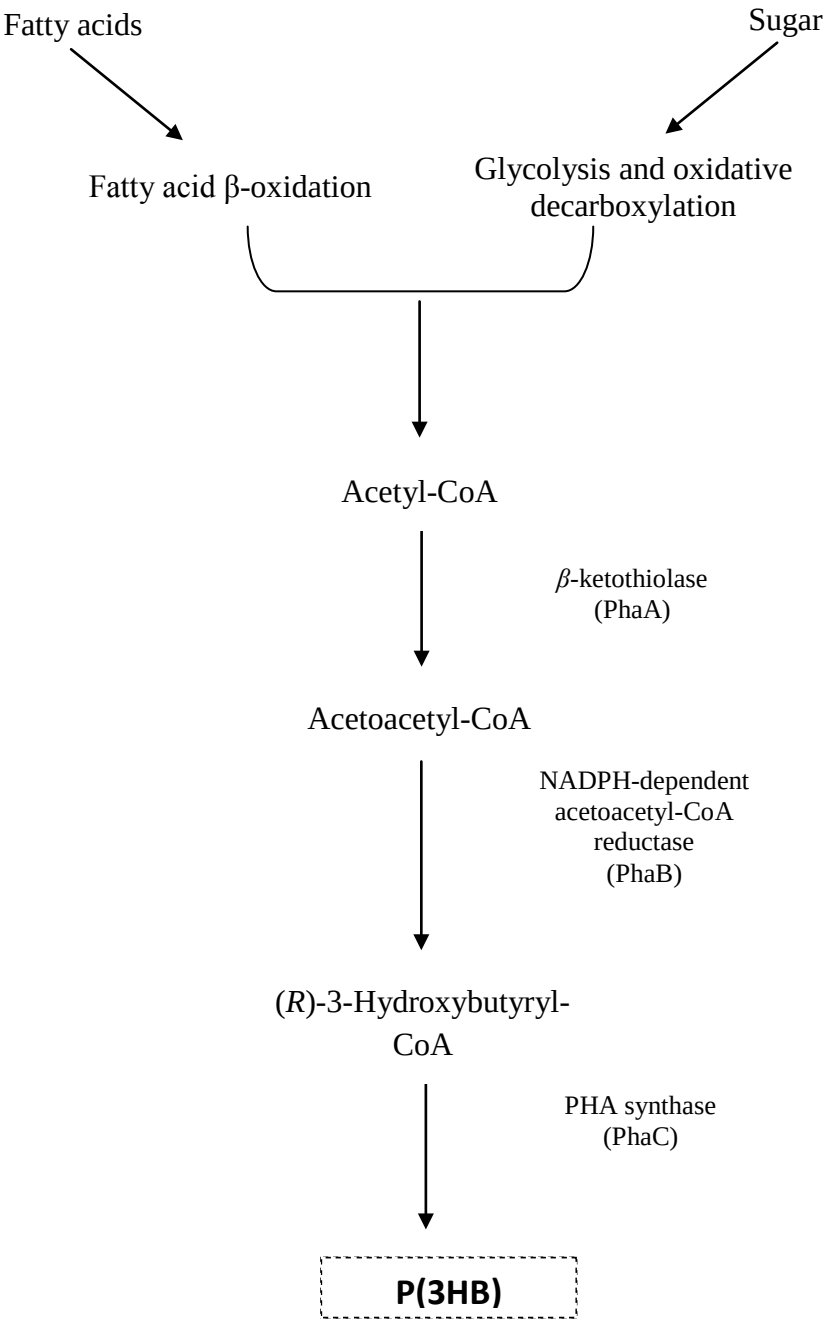


Figure 2.2 The established P(3HB) synthesis pathway (Anderson and Dawes, 1990; Madison and Huisman, 1999).

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

P(3HB-*co*-3HV) copolymer is known to exhibit superior properties compared to the properties of P(3HB) (Chen et al., 2006). The incorporation of 3HV monomer units assists in the lowering of melting temperature, crystallinity and also reducing the stiffness (Du et al., 2001). P(3HB-*co*-3HV) copolymer demonstrate isomorphic co-crystallization whereby the 3HB and 3HV monomer units may crystallize in a crystalline lattice. In addition, the formation of crystal structures is dependent upon the polymeric units containing various compositions of 3HB as well as 3HV monomer units (Bluhm et al., 1986; Na et al., 2001; Feng et al., 2002). As an isomorphic copolymer, P(3HB-*co*-3HV) with increasing 3HV composition have a tendency to show pseudoeutectic melting behavior allowing a structural transition of P(3HB) lattice to P(3HV) lattice (Bluhm et al., 1986; Mitomo et al., 1993; Mitomo et al., 1995). Below the pseudoeutectic point, 3HV monomer units cocrystallize in P(3HB) lattice on one side and on the other the reverse happens. The melting temperature (T_m) decreases from 170 to a minimum value about 70 °C and increase back to 102 - 112°C at a slower rate at the pseudoeutectic composition as the composition of 3HV increases from 0 to 100 mol% (Mitomo et al., 1995; Ha and Cho, 2002)

Production of P(3HB-*co*-3HV) copolymers is initiated by feeding of precursors such as propionic acid or valeric acid to the microorganisms such as in *C. necator* H16 (Doi et al., 1990). The incorporation of 3HV monomer into P(3HB) homopolymer chain results in P(3HB-*co*-3HV) copolymer. Generation of 3HV is dependent on the availability of 3-hydroxyvaleryl-CoA. The metabolic pathways for P(3HB-*co*-3HV) biosynthesis from mixture of glucose and propionic acid is shown in Figure 2.4**.** Firstly, propionic acid is converted to propionyl-CoA. At the same time,

acetyl-CoA is generated from TCA cycle. The converted propionyl-CoA and acetyl-CoA undergoes condensation by β-ketothiolase resulting in the formation of acetoacetyl-CoA and 3-ketovaleryl-CoA respectively. Next, the acetoacetyl-CoA and 3-ketovaleryl-CoAare reduced to (*R*)-3-hdroxybutyrl-CoA and (*R*)-3-hdroxyvaleryl-CoA respectively by NADPH-dependent acetoacetyl-CoA dehydrogenase which are then polymerized into P(3HB-co-3HV) by PHA synthase (PhaC) (Suriyamongkol et al., 2007).

Figure 2.3 General structure of P(3HB-*co*-3HV)
*x and y refers to the number of repeating units

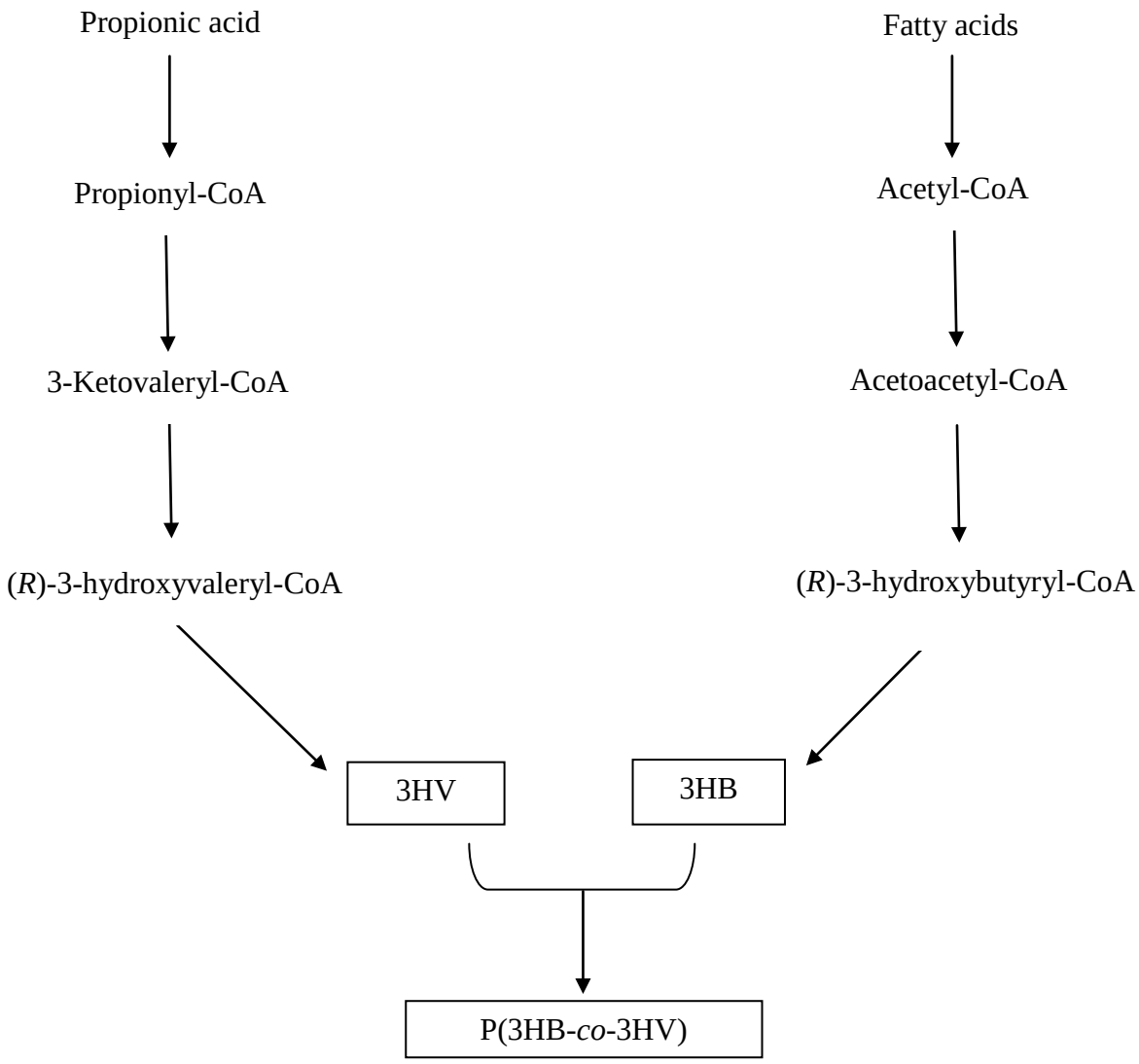


Figure 2.4 Biosynthesis pathway of P(3HB-*co*-3HV) copolymer in *C. necator* H16 with glucose and propionic acid as the 3HV precursor (Doi et al., 1990; Suriyamongkol et al., 2007)

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

P(3HB-*co*-4HB) copolymer is potentially one of the most useful PHA (Sudesh et al., 2000b) The carbon sources generally used to initiate 4HB monomer fraction is 4-hydroxybutyric acid, 1, 4-butanediol and γ-butyrolactone (Steinbüchel and Lütke-Eversloh, 2003). P(3HB-*co*-4HB) has been produced by a few microorganisms such as *Ralstonia eutropha, Alcaligenes latus, Comamonas acidovorans, Comamonas testosterone, Hydrogenophaga pseudoflava, Cupriavidus* sp and also several other recombinant strains (Park and Kim, 2011). Among these microorganism, *C. necator* H16 has been reported to produce P(3HB-*co*-4HB) with various 4HB monomer fraction ranging from 0 to 100 mol% and it has been extensively studied (Park and Kim, 2011). P(3HB-*co*-4HB) with 4HB monomer fraction of 0 to 6 mol% was produced by using *C. necator* H16 supplemented with soybean oil and γ-butyrolactone as carbon sources. (Park and Kim, 2011). Presently, microbial fermentation is the only successful method to produce P(3HB-*co*-4HB) copolymer which contains high molecular weight 4HB monomers (Saito et al., 1996; Hein et al., 1997; Sudesh et al., 1999).

P(3HB-*co*-4HB) copolymer containing high 4HB monomer fraction exhibits elastomeric property. This copolymer also possess lower T_m compared to P(3HB) homopolymer as the T_m ranges from 178 to 50 °C with increasing 4HB fraction. Previous studied reviewed that P(3HB-*co*-4HB) copolymers receives public attention to a great extent due to its suitability in biomedical and pharmaceutical application (Madden et al., 2000).

Figure 2.5 General structure of P(3HB-*co*-4HB)

*x and y refers to the number of repeating units

2.8 PHA production

PHA production by natural and also transformant microorganism has been well documented since the discovery of PHA last century. Microbial fermentation is the most established system of PHA production though plant-based production system has also been documented. Large-scale PHA production processes for commercialization purposes are currently derived from bacterial fermentations (Lee, 1996; Lee and Choi, 1998b; Choi and Lee, 1999c; Lee and Choi, 1999). The fed-batch cultivation mode is normally used in large-scale or industrial scale production (Chen et al., 2001). The fed-batch mode is known to produce high cell densities which consequently reduce the overall production cost (Lee and Choi, 1998b; Kellerhals et al., 1999b; Kellerhals et al., 1999a). Even though there are more than 300 different microorganisms producing PHA, only a few natural producers have been identified suitable for the production of PHA with high productivity. Besides bacteria, PHA production systems in other transgenic organisms have also been researched. Strains such as *C. necator* H16, *A. latus*, recombinant *E. coli* and some methylotrophs are

known for efficient production of PHA, with *C. necator* H16 as the most extensively studied (Lee, 1996; Choi and Lee, 1999c).

To initiate PHA production, generally one type of nutrient (generally nitrogen or phosphate) is limited while excess carbon source is provided in the PHA accumulation medium. The depletion of the chosen nutrient acts as the trigger for the metabolic shift to allow PHA biosynthesis. This phenomenon could be explained through the Entner-Doudoroff pathway in *C. necator* whereby the carbohydrate is catabolized to pyruvate which is then converted through dehydrogenation to acetyl-CoA. The acetyl-CoA then enters the TCA cycle and is utilized for reproductive growth (Braunegg et al., 1998). On the contrary, with the restriction of certain element, termination of protein synthesis results in high concentration of NADPH and NADH which inhibit citrate synthase and isocitrate dehydrogenase (Braunegg et al., 1998). The production of P(3HB) is regulated by citrate synthase by controlling the carbon flux into TCA cycle (Henderson and Jones, 1997). The inhibition of citrate synthase slows down the TCA cycle and channels acetyl-CoA towards P(3HB) biosynthesis (Dawes and Senior, 1973). The monomeric composition of PHA produced is dependent on the availability of carbon sources, metabolic pathways present in a microorganism and the substrate specificity of its PHA synthases.

One stage cultivation involves growth and PHA accumulation simultaneously while in two stage cultivation; cell growth phase is carried out in a separate nutrient enriched medium and then transferred into a nutrient limited medium for PHA accumulation phase. The cultivation period ranges from 24 to 96 h. During the cultivation period, the cells will go through a series of growth phases, such as lag

phase, exponential phase, PHA production, stationary phase and finally death phase (Chen et al., 2001; Kahar et al., 2004).

2.9 Carbon sources

Carbon source is one of the key factors which could reduce the cost of PHA production if a cheap and feasible carbon source could be identified. PHA is biosynthesized in bacterial cells from various renewable carbon sources. Previous study had estimated that contribution of substrate cost were approximately 28 – 50% compared to the total production (Lee and Choi, 1998b; Braunegg et al., 2004). For the large-scale production of PHA, sugars such as glucose, fructose and sucrose are the most common carbon sources utilized. Glucose was in fact used jointly with propionic acid during the early efforts towards commercializing P(3HB-*co*-3HV) copolymer by ICI under trade name BIOPOL ® (Byrom, 1992; Luzier, 1992). Nevertheless, the usage of these carbon sources accounted for much higher production cost (Choi and Lee, 1999c). As the carbon source is the ultimate contributor to the total substrate cost, efforts are still being devoted to exploit inexpensive fermentable raw materials as carbon sources that could be utilized efficiently for conversion to PHA.

Previous studied have revealed that by using plant oils as carbon source for PHA, the overall production cost and energy consumption could be reduced (Akiyama et al., 2003). Fatty acids are energetically advantageous carbon substrates due to their complete β-oxidation which generates more chemical energy in the form of ATP molecules compared to the complete oxidation of glucose molecule. Simply, a molecule of fatty acids (i.e.: C18:2) is able to generate nine acetyl-CoA molecules which is 4-fold of that generated from sugars, without releasing any carbon dioxide

from β-oxidation. Thus, more acetyl-CoA molecules are available for synthesis of more number of PHA polymers. This is mainly due to higher number of carbon atoms per gram of oil compared to sugar (Akiyama et al., 2003; Kahar et al., 2004).

Agriculture and its allied industries produce many feedstock as well as co-products that are attractive raw materials for PHA production. Fatty acids derived from agricultural triacylglycerols such as vegetable oils, animal fats and co-products derived thereof such as recycled grease have attracted the attention of researchers because they may perform as a better carbon source for microbial PHA production and they are also relatively cheaper, renewable and produce higher yields of polymer (Solaiman et al., 2006). Bioconversion of whey from dairy industry (Ahn et al., 2001; Povolo and Casella, 2003; Koller et al., 2007), molasses from sugar industry (Purushothaman et al., 2001; Albuquerque et al., 2010), starchy waste water (Yu, 2001), waste glycerol (Cavalheiro et al., 2009), waste cooking oil (Song et al., 2008), waste potato starch (Haas et al., 2008), hydrolyzed Alaskan Pollock oil from fishing industry (Ashby and Solaiman, 2008b), other industrial byproducts (Castilho et al., 2009) and slaughterhouse waste (Titz et al., 2012) have been investigated. Besides these, organic acids such as lactic acid, propionic acid and acetic acid derived as by-products of anaerobic fermentation processes had also been studied for PHA production (Tsuge et al., 1999; Tsuge et al., 2001). Utilizing the surplus waste materials for PHA production not only enhances the feedstock conversion into useful products but may also offer an alternative method to solve the disposal issues (Koller et al., 2010). The type of PHA polymer produced by some well-known bacterial strains with various carbon sources are summarized in Table 2.2.

Table 2.2 PHA production by several microorganisms from various carbon sources

Microorganisms	Type of PHA	Carbon source	References
A. caviae	P(3HB-*co*-3HHx) P(3HB-*co*-3HV)	Olive oil, sodium salts of fatty acids (C12−C18) Fatty acids (C11−C17)	(Doi et al., 1995a)
A. hydrophila transformant	P(3HB-*co*-3HV-*co*-3HHx)	Dodecanoic acid and propionic acid	(Zhao and Chen, 2007)
A. latus	P(3HB)	Sucrose	(Wang and Lee, 1997)
	P(3HB-*co*-3HP)	3-hydroxypropionic acid	(Shimamura et al., 1994)
C. necator	P(3HB)	Glucose,	(Doi, 1990)
		Soybean oil	(Kahar et al., 2004)
	P(3HB-co-3HV)	Glucose and propionic acid	(Du et al., 2001)
	P(3HB-*co*-4HB)	Fructose and γ-butyrolactone	(Kim et al., 2005)
	P(3HB-*co*-3HV-*co*-4HB)	Fructose + butyric acid + valeric acid	(Chanprateep and Kulpreecha, 2006)

C. necator PHB^{-}4 USMAA 1020	P(3HB-*co*-4HB)	γ-butyrolactone,4-hydrobutyric acid, 1, 4-butanediol	(Amirul et al., 2008)
C. necator PHB^{-}4 Transformants (phaC_{Cs})	P(3HB-*co*-3HHx)	Hexanoate, octanoate, soybean oil palm oil products	(Kichise et al., 1999) (Kahar et al., 2004) (Loo et al., 2005)
	P(3HB-*co*-3HV-*co*-3HHp)	fatty acids (C5−C13) heptanoate, nonaoate	(Fukui and Doi, 1997) (Kichise et al., 1999)
Chromobacterium sp. USM2	P(3HB-*co*-3HV)	Glucose, fructose + fatty acids	(Kolibachuk et al., 1999)
		Fructose/CPKO + sodium valerate	(Bhubalan et al., 2010)
	P(3HB)	CPKO	(Bhubalan et al., 2010)
E. coli transformants	P(3HB)	Glucose	(Choi and Lee, 1999b)
	P(3HB-*co*-3HV)	Glucose + propionic acid	(Choi and Lee, 1999a)
	P(3HB-*co*-3HV-*co*-3HHx)	Dodecanoic acid + fatty acids (5C – 9C)	(Park et al., 2001)
P. oleovorans	P(3HB) and mcl-PHA	Glucose, octanoic acid, oleic acid	(Ashby et al., 2002)
P. putida	mcl-PHA	Saponified palm kernel oil	(Annuar et al., 2007)
P. aeruginosa 47T2	mcl-PHA	Oily substrates	(Haba et al., 2007)

2.10 Precursors used for copolymer production

2.10.1 3-hydroxyvalerate precursors

Sodium valerate and sodium propionate are two common candidates for incorporation of 3HV monomers (Loo and Sudesh, 2007c). Apart from these two, sodium heptanoate was also reported to be capable of initiating 3HV monomer synthesis (Fukui et al., 1997; Park et al., 2001; Taguchi et al., 2003; Li et al., 2011). The additions of precursor are preferably done during accumulation stages in supplementation of essential carbon sources such as glucose, lactose, and sucrose or plant oils (Du et al., 2001). Previous reports by Doi and co-workers showed that, sodium valerate feeding produced much better 3HV monomer incorporation compared to sodium propionate. The reason behind this phenomenon was predicted to be caused by the conversion of sodium propionate into acetyl-CoA hence generating higher 3HB monomer synthesis. Conversely, the β-oxidation pathway of sodium valerate readily generates hydroxyvaleryl-CoA which is then polymerized into 3HV monomer and directly incorporated into the polymer chain without carbon chain breakdown (Doi et al., 1988). Additionally, propionic acid is of higher toxicity and is assumed to cause cell death with addition of higher concentrations (Yu et al., 2002).

2.10.2 4-Hydroxybutyrate precursors

Sodium 4-hydroxybutyrate, 1, 4-butanediol and γ-butyrolactone are commonly used 4HB precursors (Hein et al., 1997; Lee et al., 2004). The choice of a precursor depends on the type of microorganisms employed. Some bacteria possessing PHA_{SCL} synthase are capable to incorporate 4-hydroxybutyrate monomer (Steinbüchel and Lütke-Eversloh, 2003). The biosynthesis of copolymer starts as 4-hydroxybutyric acid

is metabolized and converted into 4HB-CoA either by transferase or thiokinase. γ-butyrolactone is a sutiable precursor for the synthesis of P(3HB-*co*-4HB) copolymer. This is because; lactone is hydrolytically cleaved to 4-hydroxybutyric acid. This reaction may be either catalyzed either by lactonases or esterases.

2.11 Surfactants

Surfactants are surface active agents with wide ranging properties such as lowering the surface and interfacial tensions of liquids. The presence of surfactants could functionally reduce the interfacial tension between immiscible fluids and enable them to be miscible through the creation of additional surfaces. In other words, surfactants are able to lead to an increase in the concentration of hydrophobic compounds in the water phase (Volkering et al., 1997). Surfactants have been shown to interact with microbes in many ways, it could either inhibit or stimulate growth of the cells depending on the molecule and the species of bacteria (Volkering et al., 1997). Sodium dodecyl sulfate (SDS), tween 80 (polyoxyethylene (20) sorbiton monooleate), and Triton X-100 [4-(1, 1, 3, 3-tetramethylbutyl)phenyl-polyethyleneglycol] are commonly found surfactants in biotechnology laboratories. Gum Arabic (GA) is a natural glycoprotein synthesized by the acacia tree (Qi et al., 1991; Goodrum et al., 2000). GA is used as thickener, emulsifier and stabilizer in the food processing industry. It is important to ensure that the surfactants used for PHA biosynthesis will not function as an alternate carbon source. Recent study conducted by Budde and coworkers showed that GA could function successfully as emulsifier for growth of *C. necator* on plant oil (Budde et al., 2011a).