BIOSYNTHESIS AND CHARACTERIZATION OF NOVEL POLYHYDROXYALKANOATE BY *Cupriavidus necator* PHB^{-}4 TRANSFORMANT HARBORING PHA SYNTHASE GENE OF *Chromobacterium* SP. USM2

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

PHA	Polyhydroxyalkanoate
3HB	3-hydroxybutyrate
3HD	3-hydroxydecanoate
3HDD	3-hydroxydodecanoate
3HDde	3-hydroxydodecenoate
3HHx	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
3HTde	3-hydroxytetradecenoate
3HV	3-hydroxyvalerate
3H2MB	3-hydroxy-2-methylbutyrate
3H2MV	3-hydroxy-2-methylvalerate
3H4MV	3-hydroxy-4-methylvalerate
P(3HB)	Poly(3-hydroxybutyrate)
P(3HHp)	Poly(3-hydroxyheptanoate)
P(3HV)	Poly(3-hydroxyvalerate)
P(4HB)	Poly(4-hydroxybutyrate)
PHO	Poly(3-hydroxyoctanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3H4MV)	Poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-3HV-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate)

P(3HB-*co*-3H4MV-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate -*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV-*co*-3H4MV-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxy-4-methylvalerate -*co*-3-hydroxyhexanoate)
PLA	Poly(lactic acid)
HA	Hydroxyalkanoate
C. necator PHB$^-$4 (*phaC*$_{Cs}$)	Transformant of *C. necator* PHB$^-$4 harboring the PHA synthase gene of *Chromobacterium* sp. USM2
C. necator PHB$^-$4 (*phaC*$_{Ac}$)	Transformant of *C. necator* PHB$^-$4 harboring the PHA synthase gene of *Aeromonas caviae*
PhaA	3-ketothiolase
PhaB	NADPH-dependent acetoacetyl-CoA reductase
PhaC	PHA synthase
PhaG	(*R*)-3-hydroxyacyl-ACP-CoA transferase
PhaJ	(*R*)-enoyl-CoA hydratase
PhaZ1 to PhaZ7	Extracellular PHA depolymerases
FabG	3-ketoacyl-ACP reductase
FabB	Epimerase
phaC	PHA synthase gene
phaA, phaB, phaG, phaJ	Monomer supplying encoded genes
C6:0	Caproic
C8:0	Caprylic
C10:0	Capric
C12:0	Lauric
C14:0	Myristic
C16:0	Palmitic
C18:0	Stearic

C20:0	Arachidic
C16:1	Palmitoleic
C18:1	Oleic
C18:2	Linoleic
C18:3	Linolenic
CPO	Crude palm oil
CPKO	Crude palm kernel oil
PO	Palm olein
PAO	Palm acid oil
PKAO	Palm kernel acid oil
4MV	Isocaproic acid
SCL	Short-chain-length
MCL	Medium-chain-length
CME	Caprylate methyl ester
DCW	dry cell weight
DEAE	Diethylaminoethyl cellulose
DMF	Dimethylformamide
DSC	Differential scanning calorimetric
FDA	Food and Drug Administration
FT-IR	Fourier transform infrared
GC	Gas chromatography
GPC	Gel permeation chromatography
IUPAC	International Union and Applied Chemistry
Km	Kanamycin
MM	Mineral salts medium

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
ND	Not detected
NR	Nutrient rich
O.D.	Optical density
PTFE	Polytetrafluorethylene
SEM	Scanning electron microscopic
TCA	Tricarboxylic acid
TDW	Total dry weight
TES	Trace elements solution
TGA	Thermogravimetric
TMS	Tetramethylsilane
VFA	Volatile fatty acids
KRS-5 (TiBr/T11)	KRS5 Thallium Bromo-Iodide
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon nuclear magnetic resonance
ΔH_m	Enthalpy of fusion
M_n	Number-average molecular weights
M_w	Weight-average molecular weights
M_w/M_n	Polydispersity index
T_d	Temperatures at 5 % weight loss
T_g	Glass transition temperature
T_m	Melting temperature
v/v	Volume per volume
w/v	Weight per volume

w/w	Weight per weight
A_{3HB}	Area below 3HB monomer peak
A_{3HHx}	Area below 3HHx monomer peak
A_{3HV}	Area below 3HV monomer peak
A_{3H4MV}	Area below 3H4MV monomer peak
A_{CME}	Area below CME peak
K	GC constant
k_{3HB}	3HB monomer constant
k_{3HHx}	3HHx monomer constant
k_{3HV}	3HV monomer constant
k_{3H4MV}	3H4MV monomer constant
$CaCl_2$	Calcium chloride
$CDCl_3$	Deuterated chloroform
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate
$CrCl_3 \cdot 6H_2O$	Chromium chloride hexahydrate
$CuSO_4 \cdot 5H_2O$	Copper sulfate pentahydrate
$FeCl_3$	Iron (III) Chloride
HCl	Hydrochloride
KH_2PO_4	Potassium dihydrogen phosphate
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
Na_2HPO_4	Sodium phosphate
Na_2SO_4	Sodium sulphate anhydrous
NH_4Cl	Ammonium chloride
$NiCl_2 \cdot 6H_2O$	Nickel chloride hexahydrate
%	Percentage

ºC	Degree Celsius
°C/min	Degree Celsius per minute
cm	Centimeter
cm^{-1}	Per centimeter
Da	Dalton
g	Gram
g/L	Gram per liter
h	Hour
Jg^{-1}	Joule per gram
kPa	Kilopascal
kV	Kilovolt
mg	Milligram
mg/L	Milligram per liter
mg/mL	Milligram per milliliter
MHz	Megahertz
min	Minute
mL	Milliliter
mL/L	Milliliter per liter
mL/min	Milliliter per minute
mm	Millimeter
mm/min	Millimeter per minute
mol %	Mole percent
MPa	Mega pascal
N	Normality
nm	Nanometer

ppm	Parts-per-million
Psi	Pounds per square inch
rpm	Revolutions per minute
V	Volt
wt %	Weight percent
µL	Microliter
µL/min	Microliter per minute
µm	Micrometer
x *g*	Times gravity

Biosintesis dan pencirian polihidroksialkanoat novel yang dihasilkan oleh transforman *Cupriavidus necator* PHB⁻4 yang menampung gen sintase PHA daripada *Chromobacterium* sp. USM2

ABSTRAK

Penyatuan monomer baru, 3-hydroxy-4-methylvalerat (3H4MV) pada rangkaian monomer polihidroksialkanoat (PHA) didapati berjaya memperbaiki ciri polimer yang dihasilkan. Dalam kajian ini, transforman *Cupriavidus necator* PHB⁻4 yang menampung gen sintase PHA daripada *Chromobacterium* sp. USM2 [*C. necator* PHB⁻4 (*phaC*$_{Cs}$)] dinilai potensinya untuk mensintesis PHA novel iaitu PHA rantaian-pendek dan rantaian-sederhana-panjang (SCL-MCL) yang mana ia mengandungi 3H4MV. Fruktosa atau minyak isirong kelapa sawit mentah (CPKO) telah digunakan sebagai sumber karbon utama bersama dengan asid isokaproik (4MV) sebagai prekursor untuk menghasilkan ciri-ciri PHA SCL-MCL yang cemerlang. Kepekatan dan masa pembekalan prekursor 4MV telah dikenalpasti sebagai parameter penting yang mempengaruhi kandungan PHA dan komposisi monomer bagi polimer yang dihasilkan. PHA novel yang terdiri daripada 78 mol % 3HB, 1 mol % 3HV, 3 mol % 3H4MV dan 18 mol % 3HHx telah diperoleh apabila 5 g/L CPKO dan 1 g/L 4MV dibekalkan ketika inokulasi awal. PHA ini mempunyai ciri-ciri yang sangat menarik – sifat kenyal seperti getah, walaupun setelah setahun penuaan. Ia boleh menarik kembali setelah dibebaskan daripada perengangan. Sifat ini belum dilaporkan dalam mana-mana laporan PHA. Transforman lain iaitu *Cupriavidus necator* PHB⁻4 yang menampung gen sintase PHA daripada *Aeromonas caviae* [*C. necator* PHB⁻4 (*phaC*$_{Ac}$)] juga telah digunakan untuk perbandingan kemampuannya dalam menghasilkan PHA yang mengandungi komposisi monomer 3H4MV yang berbeza. PHA SCL-MCL novel yang dihasilkan oleh *C. necator*

PHB$^-$4 (*phaC*$_{Cs}$) telah dianalisa dengan lebih lanjut untuk lebih memahami ciri-cirinya yang cemerlang. Pencirian struktur telah dikaji dengan menggunakan spektroskopi ^{1}H NMR dan ^{13}C NMR sementara FT-IR dilakukan untuk mengesahkan kewujudan kumpulan berfungsi PHA. Selain itu, sifat terma telah ditentukan oleh TGA dan DSC untuk membandingkan PHA SCL-MCL dengan kopolimer P(3HB-*co*-3HHx). Keputusan menunjukkan bahawa pengabungan monomer 3H4MV dan peningkatan komposisi monomer 3HHx dalam PHA telah menurunkan T_g, T_m dan ΔH_m. Berat molekul polimer adalah di antara 7 - 10 x 10^5. Ujian mekanikal juga telah dilakukan. Kekuatan tegangan menunjukkan nilai yang didapati adalah di antara 15 - 27 MPa, modulus Young berada dalam lingkungan 84 - 623 MPa dan pemanjangan pada saat pemutusan adalah sekitar 84 - 478 %. Aplikasi serat halus PHA telah berjaya difabrikasikan dengan menggunakan mesin putaran elektro berdasarkan parameter yang terkawal. Di samping itu, keputusan degradasi filem PHA SCL-MCL yang dilakukan pada permukaan tanah telah menunjukkan bahawa ia terdegradasi dengan lebih cepat berbanding dengan filem P(3HB) dan filem P(3HB-*co*-3HHx).

Biosynthesis and characterization of novel polyhydroxyalkanoate by *Cupriavidus necator* PHB⁻4 transformant harboring PHA synthase gene of *Chromobacterium* sp. USM2

ABSTRACT

The incorporation of a newly identified 3-hydroxy-4-methylvalerate (3H4MV) monomer into the backbone of polyhydroxyalkanoate (PHA) is thought to improve the properties of the resulting polymer. In this study, a transformant of *C. necator* PHB⁻4 harboring the PHA synthase gene of *Chromobacterium* sp. USM2 [*C. necator* PHB⁻4 (*phaC*$_{Cs}$)] was evaluated for its ability to synthesize novel short-chain and medium-chain-length (SCL-MCL) PHA containing 3H4MV. Fructose or crude palm kernel oil (CPKO) was used as main carbon source together with isocaproic acid (4MV) as precursor to produce the excellent SCL-MCL PHA. 4MV concentration and its feeding time had been identified as important parameters affecting PHA content and monomer composition of the resulting polymer. A novel PHA that consists of 78 mol % 3HB, 1 mol % 3HV, 3 mol % 3H4MV and 18 mol % 3HHx was obtained when 5 g/L CPKO and 1 g/L 4MV were fed during initial inoculation. This PHA has a very interesting characteristic – rubber-like character even after one year of aging. It can retract after being released from the stretching. This property has not been found in any of the previously reported PHA. Another transformant of *C. necator* PHB⁻4 harboring the PHA synthase gene of *Aeromonas caviae* [*C. necator* PHB⁻4 (*phaC*$_{Ac}$)] was used as well for comparison of its ability to produce PHA that contained different monomer compositions of 3H4MV. The novel SCL-MCL PHA that was produced by *C. necator* PHB⁻4 (*phaC*$_{Cs}$) was characterized to further understand its excellent properties. Structural characterizations were carried out by ^{1}H NMR and ^{13}C NMR spectroscopes while FT-IR was done to confirm the presence of the functional groups of PHA. Besides, thermal properties

determined by TGA and DSC to compare these SCL-MCL PHA with P(3HB-*co*-3HHx) copolymer showed that the incorporation of 3H4MV monomer and enhanced 3HHx monomer composition in the PHA have lowered the T_g, T_m and ΔH_m. The molecular weights of the polymers were in the range of 7 - 10 x 10^5. Mechanical tests were also carried out. The tensile strength was in the range of 15 - 27 MPa, Young's modulus in the range of 84 - 623 MPa and elongation at break in the range of 84 - 478 %. Application of fine PHA fiber was successfully fabricated using an electrospinning machine with controlled parameters. Besides, degradation of the SCL-MCL PHA film done on the surface of soil showed that it degraded faster compared to P(3HB) and P(3HB-*co*-3HHx) films.

1.0 INTRODUCTION

Many new technologies have been developed to make human life more comfortable and convenient. A good example is synthetic plastics, which are derived from petrochemicals. Plastics have become one of the most commonly used materials in our daily life. While these materials have benefited mankind, they have also brought numerous environmental pollution problems. Furthermore, the eventual depletion of petroleum, which is the principal source of conventional plastics, has urged scientists to identify a renewable source to replace it.

Recently, "green" products have become very popular in the action of saving our mother earth. Biodegradable plastics have driven the interest of researchers to replace the synthetic plastics. One of the well known classes of biodegradable plastics (also known as bioplastics) is polyhydroxyalkanoate (PHA). A number of discoveries have been directed towards the development of PHA due to its potential biodegradable and biocompatible properties. Poly(3-hydroxybutyrate) [P(3HB)], as the most widespread or extensively studied PHA, was first discovered in *Bacillus megaterium* but it has limited applications due to its very high crystallinity and brittle properties (Sudesh *et al.*, 2000). Substantial efforts have been directed towards the development of this environmentally friendly biopolymer. However, the existing PHA has poor characteristics compared to the conventional plastics.

Several studies have shown that copolymers and terpolymers with short-chain-length (SCL) and medium-chain-length (MCL) monomers have improved properties compared to SCL or MCL homopolymers (Doi *et al.*, 1995, Chanprateep & Kulpreecha, 2006, Zhao & Chen, 2007). The incorporation of novel MCL monomers, such as 3-hydroxy-4-methylvalerate (3H4MV), into the homopolymer of

PHA has shown significant improvement in the properties of PHA (Tanadchangsaeng *et al.*, 2009, Lau *et al.*, 2010, Tanadchangsaeng *et al.*, 2010). The addition of this monomer into the homopolymer has caused it to become more ductile. It was also found that the ductility can be maintained for quite a long period without significant deterioration that is caused by secondary crystallization. This monomer provides a significant additional advantage to the homopolymer of PHA and can be used for practical application.

Cupriavidus necator H16 is a famous model bacterium for PHA production because of its ability to produce high dry cell weight (DCW) and PHA content. Its PHA-negative mutant strain, PHB$^-$4 has been used in many studies to produce transformant strains by inserting PHA synthase gene from other bacterial strains. Recently, a local bacterium, *Chromobacterium* sp. USM2 was isolated. The PHA synthase gene of this bacterium has been cloned and transformed into *C. necator* PHB$^-$4 (Bhubalan *et al.*, 2010a). This transformant strain was found able to produce PHA containing SCL and MCL monomers when fed with only crude palm kernel oil (CPKO). It is used in this study to produce various types of PHA consisting of 3H4MV monomer. The objectives of this study are as follows:

1. To biosynthesize novel compositions of PHA containing 3H4MV monomer and to study the properties of this PHA.
2. To compare the capability of *Chromobacterium* sp. and *Aeromonas caviae* synthase genes to incorporate 3H4MV monomer into PHA.
3. To study the parameters in fabrication of electrospun PHA films containing 3H4MV monomer.
4. To investigate the degradation of PHA containing 3H4MV in the environment.

2.0 LITERATURE REVIEW

2.1 PHA

PHA is intracellular granules where energy and carbon are reserved when there is imbalance in nutrient supplies (Anderson & Dawes, 1990). The conditions that favor the accumulation of PHA granules in cells are the lack of nitrogen, phosphorous, magnesium and oxygen but excess carbon source is present (Ryu *et al.*, 1997, Lee *et al.*, 2000, Kek *et al.*, 2008). PHA can enhance the survival rate of the microorganism when it is under environmental stress condition (Kadouri *et al.*, 2005, Ayub *et al.*, 2007). This natural, renewable and fully biodegradable bioplastic is environmentally friendly and biocompatible. However, the production cost of PHA is currently far more expensive when compared to that of conventional plastics. Therefore, it is used mostly in medical applications in which conventional plastics cannot perform (Verlinden *et al.*, 2007). In order to promote this bioplastic, research has been focusing on the use of inexpensive substrates, new extraction methods, genetically enhanced strains and mixed cultures to make it commercially attractive. In addition, efforts to improve its physical properties are also being carried out.

2.2 PHA synthesis in living organism

PHA is found in many living organisms including prokaryotes and eukaryotes (Reusch *et al.*, 1992, Chen, 2010b, Poirier & Brumbley, 2010). However, the type of PHA found in eukaryotes are comprised of very low molecular weight P(3HB) that consists of about 100 - 150 3HB monomers. This type of P(3HB) found in eukaryotes are complexed with membrane proteins. On the other hand, the PHA synthesized by prokaryotes is usually of very high molecular weights and have

plastic-like properties. Bacteria are normally used for large scale PHA production (Chen, 2010a). The first PHA discovered is P(3HB) (Lemoigne, 1926). It is the most widespread PHA but has limited applications due to its highly crystalline property and brittleness (Sudesh *et al.*, 2000).

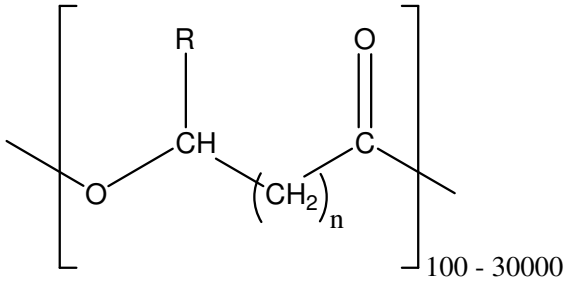
Plants can be used to produce PHA as well, but the PHA yield is low. When the PHA yield is high, there will be negative effect on the growth and development of the plants (Bohmert *et al.*, 2002). On the other hand, bacteria can accumulate very high content of PHA up to 90 % of the DCW (Steinbüchel & Lütke-Eversloh, 2003). In a particular study, a very high yield of PHB up to 93 % (w/w) of total dry weight (TDW) was achieved due to microbial autolysis at the end of fermentation (Xu *et al.*, 2010).

PHA is accumulated as intracellular granules and it is insoluble in water. This property ensures that the general physiological fitness of the organism is not affected (Peters & Rehm, 2005). PHA granule surface is thought to be coated with a layer of phospholipids and granule associated proteins such as phasins. The phasins will affect the number and size of the PHA granules (Pötter *et al.*, 2002, Pötter & Steinbüchel, 2005).

2.3 Chemical structure of PHA

In addition to P(3HB), there are many other types of PHA, which are consisted of 3-hydroxy fatty acids. In the metabolism of bacterial cells, excess carbon sources are converted into hydroxyacyl-CoA thioesters. Ester bond will be formed between the carboxyl group of one monomer and the hydroxyl group of neighboring monomer. This polymerization reaction is catalysed by PHA synthase (Verlinden *et*

al., 2007). Figure 1 shows the general structure of PHA (Steinbüchel, 1991, Steinbüchel & Valentin, 1995).



n= 1	R= hydrogen	Poly(3-hydroxypropionate)
	R= methyl	Poly(3-hydroxybutyrate)
	R= ethyl	Poly(3-hydroxyvalerate)
	R= propyl	Poly(3-hydroxyhexanoate)
	R= pentyl	Poly(3-hydroxyoctanoate)
	R= nonyl	Poly(3-hydroxydodecanoate)
n= 2	R= hydrogen	Poly(4-hydroxybutyrate)
	R= methyl	Poly(4-hydroxyvalerate)
n= 3	R= hydrogen	Poly(5-hydroxyvalerate)
	R= methyl	Poly(5-hydroxyhexanoate)
n= 4	R= hexyl	Poly(6-hydroxydodecanoate)

Figure 2.1 General structures of PHA and some representative members

Based on the number of carbon atoms in monomer units, PHA is divided into two groups: SCL PHA which contains 3 - 5 carbon atoms and MCL PHA which contains 6 - 14 atoms of carbon.

When mixed carbon substrates are used, such as starch and valerate, copolymers of PHA, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] are formed (Sheu *et al.*, 2009). When a mixture of substrates is used, consequential polymer will be random copolymer. However, when the substrates are fed alternated overtime, PHA block copolymer is possible to be attained (Pederson *et al.*, 2006).

2.4 Bacterial strains that produce PHA

PHA is produced by different bacterial strains. One of the most studied strains is *C. necator* (formerly known as *Wautersia eutropha, Ralstonia eutropha* or *Alcaligenus eutrophus*). It was used in industrial production by Imperial Chemical Industries (ICI plc) to produce P(3HB-*co*-3HV) under the trade name of Biopol™. Recently, the Biopol™ patents were acquired by Metabolix Inc. (USA) (Verlinden *et al*., 2007). Until now, *C. necator* is still been used widely for bacterial fermentation as it is a more cost-effective strain. Even if the production is switched to other bacterial strains or agricultural crops, its PHA synthase gene is most likely expressed in the PHA-producers used (Verlinden *et al*., 2007).

Other important strains are *Bacillus* spp*., Alcaligenes* spp*., Pseudomonas* spp*., Aeromonas hydrophila, Rhodopseudomonas palustris, Escherichia coli, Burkholderia sacchari* and *Halomonas boliviensis* (Verlinden *et al*., 2007).

Spore-forming *Bacillus* strains are able to produce novel terpolymer (Łabużek & Radecka, 2001). However, the environmental conditions favorable for PHA

production are also encouraging spore production. Hence, these two metabolic processes may interfere with one another resulting in reduced PHA production. Taking this into consideration, nonspore-forming mutant types of *Bacillus* were evaluated for the PHA production potential.

On the other hand, genetic engineering is an useful tool to design strains that are improved in PHA production and the properties of the polymer produced (Verlinden *et al*., 2007).

2.5 Carbon sources

When there is a lack of growth nutrients except carbon source in bacterial cells, the surplus carbon is metabolized via a few metabolic pathways to produce PHA. The type of the carbon sources supplied greatly affects the biosynthesis of PHA. The carbon sources can be categorized into two groups, namely, structurally related carbon source and structurally unrelated carbon source (Taguchi *et al.*, 2004). The former group includes the carbon sources which result in hydroxyalkanoate (HA) monomers that have similar chemical structures to them. The latter group consists of carbon sources that generate monomers which have completely unrelated chemical structure to them.

The most common carbon sources used in PHA production are mainly sugars such as glucose (Łabużek & Radecka, 2001, Valappil *et al.*, 2007), gluconate (Hoffmann & Rehm, 2004, Valappil *et al*., 2007), sucrose (Zhang *et al.*, 1994, Valappil *et al.*, 2007) and fructose (Tsuge *et al.*, 2005, Valappil *et a*l., 2007). However, the high cost of these sugars makes PHA production cost incomparable to that of the conventional plastics. Regardless of the type of carbon source used and monomers produced, the concern on the production cost is always affecting the wide

use of PHA. Many efforts have been done to reduce the production cost in order to compete with the price of conventional petrochemical plastics.

In order to reduce the production cost of PHA, the use of reasonably priced carbon sources such as plant oils or fatty acids, wastes from agricultural or food industries and even carbon dioxide have been studied (Tsuge, 2002). In one of the examples on efforts to reduce the production cost, volatile fatty acids (VFAs) (Kourmentza *et al.*, 2009) were used in mixed culture. It led to better final yields of PHA/VFA and more rapid accumulation rates.

Wastewater (Pijuan *et al.*, 2009, Rodgers & Wu, 2010) was found to be effective as a sustainable recovered source for the production of PHA. At the same time, sludge can be reduced. As such, the production costs of PHA production and sludge disposal can be decreased. Besides that, the feasibility of using wheat-based biorefinery for the production of P(3HB) was studied (Xu *et al.*, 2010). In that research, a very high TDW yield up to 93 % (w/w) was obtained due to microbial autolysis at the end of fermentation. This bacterial autolysis latent has shortened the downstream process in fermentation for the purification of P(3HB). In another example, the use of combination of dairy waste, rice bran and sea water as sources in the production of P(3HB) was carried out (RamKumar Pandian *et al.*, 2010). It can serve as an effective end-of-pipe technology in disposing dairy industrial waste.

In addition to the aforementioned examples, milk and ice-cream processing wastewater (Chakravarty *et al.*, 2010), fermented sugar cane molasses (Bengtsson *et al.*, 2010), waste glycerol (Cavalheiro *et al.*, 2009) and methanol (Mokhtari-Hosseini *et al.*, 2009) were also found to be economically competitive carbon sources in the PHA production.

Besides waste products, renewable plant oils are another examples of inexpensive carbon sources that can be used to reduce the PHA production cost while reducing the pollutant wastes that are harmful to our environment. Soybean oil (Tsuge *et al.*, 2004), palm oil products (Loo *et al.*, 2005, Bhubalan *et al.*, 2008, Kek *et al*., 2008), spent palm oil (Rao *et al.*, 2010) were excellent carbon sources for efficient production of PHA. Studies have shown that plant oils such as soybean oil and palm oil are good and inexpensive carbon sources that give high yield of PHA and cell biomass (Kahar *et al.*, 2004, Loo *et al*., 2005, Kek *et al.*, 2008). In addition, plant oils that consist of triglycerides with long chain fatty acids have higher carbon content per unit weight, which lead to higher PHA production when compared to glucose (Akiyama *et al.*, 2003).

2.5.1 Palm oil products

Palm oil and the by-products derived from it have initiated the interest of researchers in Malaysia to use these as raw materials because they can be found abundantly throughout the country. They are renewable and can be counted as steady source of raw materials for some industries such as bio-diesel (Sumathi *et al.*, 2008) and also can be used as good carbon feedstock in PHA production (Loo *et al*., 2005, Kek *et al*., 2008).

Palm oil is extracted from the fruit of oil palm tree *Elaeis guineensis*. It can be found in various forms for examples, crude palm oil (CPO), palm olein (PO) and CPKO. CPO is extracted from the mesocarp of the palm fruits while CPKO is obtained from the endosperm (kernel) of the palm fruits. Both CPO and CPKO are extracted from palm fruits but they are different in terms of fatty acid compositions. The unwanted free fatty acids present in CPO and CPKO are removed through a

chemical refining process, saponification to produce palm acid oil (PAO) and palm kernel acid oil (PKAO) respectively (Kek *et al*., 2008).

Table 2.1 shows the summary of fatty acid compositions of palm oil products which is obtained from Unitata Ltd (Kek *et al*., 2008).

Table 2.1 Fatty acid compositions of palm oil products (Source: Unitata Ltd.) (Kek *et al*., 2008)

Fatty acid	CPO	PAO	CPKO	PKAO
Saturated				
Caproic (6:0)	-	-	-	-
Caprylic (8:0)	-	-	3.9	2.0
Capric (10:0)	-	-	3.5	2.5
Lauric (12:0)	0.1	0.8	48.5	44.1
Myristic (14:0)	0.9	1.1	16.2	17.8
Palmitic (16:0)	43.8	44.7	7.5	10.8
Stearic (18:0)	4.0	3.7	2.6	3.1
Arachidic (20:0)	-	-	-	-
Unsaturated				
Palmitoleic (16:1)	-	-	-	-
Oleic (18:1)	42.1	40.3	15.7	17.3
Linoleic (18:2)	8.9	9.4	2.1	2.3
Linolenic (18:3)	0.2	-	-	-

CPO: crude palm oil
PAO: palm acid oil
CPKO: crude palm kernel oil
PKAO: palm kernel acid oil

2.5.2 Isocaproic acid (4MV)

The International Union and Applied Chemistry (IUPAC) name of isocaproic acid is 4-methylpentanoic acid. It is also known as 4-methylvaleric acid and isohexanoic acid. It has the following chemical structure:

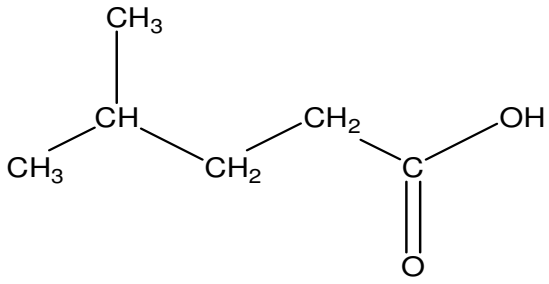


Figure 2.2 Chemical structure of 4MV

This acid is a new structurally related carbon source that can be used as a precursor in the PHA production to produce the new 3H4MV monomer (Tanadchangsaeng *et al.*, 2009, Lau *et al.*, 2010). 4MV has a similar structure with leucine and therefore it may be metabolized through the leucine pathway to produce the new monomer. Besides leucine, it also has similar structure with valeric acid, a precursor that is used to synthesize 3HV monomer.

2.6 Existing important PHA

2.6.1 Homopolymer PHA

2.6.1(a) P(3HB)

P(3HB) was the first discovered PHA (Lemoigne, 1926) and it is the most common biological polyester that can be naturally produced by various type of bacteria. *C. necator*, a well-known PHA producer can accumulate P(3HB) as insoluble inclusions from various type of carbon sources, for examples sugars and plant oils (Tsuge, 2002). P(3HB) is very stiff and brittle (Sudesh *et al.*, 2000). It alone does not have appropriate material properties for practical application.

2.6.1(b) Other reported non-P(3HB) homopolymer

Besides P(3HB), there are some other reported non-P(3HB) homopolymers as stated below:

i. Poly(4-hydroxybutyrate) [P(4HB)] (Steinbüchel *et al.*, 1994)

ii. Poly(3-hydroxyvalerate) [P(3HV)] (Steinbüchel & Schmack, 1995, Shen *et al.*, 2009)

iii. Poly(3-hydroxyheptanoate) [P(3HHp)] (Wang *et al.*, 2009) and

iv. Poly[(*R*)-3-hydroxyundecanoate] and poly[(*R*)-3-hydroxydecanoate] homopolymer (Chen, 2010b).

2.6.2 Copolymer

As mentioned above, P(3HB) homopolymer has poor properties. In order to improve its physical properties, many researches have been carried out to incorporate different HA units into P(3HB) homopolymer to form copolymer. There are various types of bacteria, which are capable of producing copolymers. The type of the produced copolymers depends on the biosynthethic pathway found in the bacteria and carbon sources fed. The copolymers of PHA possess useful and flexible mechanical properties, thus making them the preferred materials for application development (Noda *et al.*, 2010).

2.6.2(a) SCL copolymer

SCL copolymer consists of 2 monomers made up of 3 - 5 carbon atoms. Examples of SCL copolymers are listed as below:

i. Poly(3-hydroxypropionate-*co*-3-hydroxybutyrate) (Shimamura *et al.*, 1994, Valentin *et al.*, 2000)

ii. Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] (Saito *et al.*, 1996, Lee *et al.*, 2004, Kim *et al.*, 2005, Rao *et al.*, 2010) and

iii. P(3HB-*co*-3HV) (Alderete *et al.*, 1993, Lee *et al.*, 2008, Sheu *et al*., 2009, Sankhla *et al.*, 2010).

2.6.2(b) SCL-MCL copolymer

Besides SCL copolymers, there are copolymers which are made up of 1 SCL and 1 MCL monomers as well. Examples:

i. Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] (Doi *et al.*, 1995, Asrar *et al.*, 2002, Tsuge *et al*., 2004, Loo *et al*., 2005). *A. hydrophila* transformant was used to produce this copolymer consisting of high 3HHx content up to 94.5 mol % (Jian *et al.*, 2010).

ii. Poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate) [P(3HB-*co*-3H4MV)] (Tanadchangsaeng *et al*., 2009, Lau *et al*., 2010). It is a flexible and ductile material with moderate toughness. It was shown that this newly identified 3H4MV unit is a promising monomer in improving the 3HB-based polymers' material properties and stability.

2.6.2(c) MCL copolymer

Many *Pseudomonas* spp. are able to accumulate MCL PHA copolymers containing C_6 - C_{12} monomers, for example poly(3-hydroxydecanoate-3-hydroxydodecanoate) (Chen, 2010b).

2.6.3 Terpolymer

PHA that consists of 3 monomers is called terpolymer PHA. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HV-*co*-3HHx)] produced by *A. hydrophila* 4AK4 transformant harboring genes *phaAB* is a

terpolyester with higher thermal stability and elongation at break compared to the homopolymer P(3HB) and its copolymers P(3HB-*co*-3HV) or P(3HB-*co*-3HHx). In addition, this terpolyester had lower melting temperatures (T_m) and enthalpy of fusions (ΔH_m) than P(3HB) (Zhao & Chen, 2007, Zhang *et al.*, 2009). In another study, palm kernel oil was used as main carbon source together with sodium propionate or sodium valerate as 3HV-precursors for the synthesis of novel compositions of P(3HB-*co*-3HV-*co*-3HHx) terpolymers which had interesting elastomeric behaviors (Bhubalan *et al*., 2008).

Another terpolymer poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-4HB-*co*-3HHx)] was found to have better thermal stability due to the introduction of 4HB and 3HHx monomers into P(3HB) (Xie & Chen, 2008). It has lower crystallinity and better flexibility compared to P(3HB) homopolymer and their two monomers containing copolymers. Besides, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3HV-*co*-4HB)] showed superior properties over those of 3HB and its copolymer (Madden *et al.*, 2000, Chanprateep & Kulpreecha, 2006).

2.6.4 PHA with more than 3 monomers

In some of the researches, PHA with more than 3 monomers were discovered as well, for examples a SCL-MCL polymer containing five types of monomers, namely 3HB, 3HV, 3-hydroxy-2-methylbutyrate (3H2MB), 3-hydroxy-2-methylvalerate (3H2MV) and 3HHx (Bengtsson *et al*., 2010). In addition, there are even PHA that contained up to 10 different types of 3-hydroxyalkanoic acid units, including saturated 3-hydroxyacids from C_4 to C_{14}, and unsaturated monomers as 3-hydroxydodecenoate (3HDde) and 3-hydroxytetradecenoate (3HTde) (Simon-Colin

et al., 2008). These are mainly semi-crystalline polymers. In another similar research, PHA that is composed of C_6 - C_{14} 3-hydroxyacids monomers, with a predominance of 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD) were found (Simon-Colin *et al.*, 2009).

2.6.5 Industrial production of PHA

Among these various types of polymers, PHA that have been produced in industrial scale are mainly P(3HB), P(3HB-*co*-3HV), P(3HB-*co*-4HB) and P(3HB-*co*-3HHx) (Chen, 2010b).

2.7 Metabolic pathway

2.7.1 Naturally occurring metabolic pathway

The three best-studied PHA synthesis metabolic pathways are summarized in Figure 2.2 (Tsuge, 2002). Pathway I is the most common pathway occurring in many bacteria. In this pathway, (*R*)-3HB monomers are generated from acetyl-CoAs. The best representative bacterium is *C. necator.* In this bacterium, two acetyl-CoA are condensed to become acetoacetyl-CoA by 3-ketothiolase or also called PhaA. After that, acetoacetyl-CoA is reduced by NADPH-dependent acetoacetyl-CoA reductase (PhaB) to become (*R*)-3HB-CoA and further polymerized by PHA synthase (PhaC) to yield P(3HB).

Pathway II and pathway III which involve fatty acid β-oxidation and fatty acid biosynthesis are the main pathways that are involved in MCL monomers production. In these pathways, intermediates are converted to 3HA-CoA monomers for PHA synthase with the help of specific enzymes. From *trans*-2-enoyl-CoA and (*R*)-3-hydroxyacyl-ACP, (*R*)-3-hydroxyacyl-CoA is produced in the presence of (*R*)-

specific enoyl-CoA hydratase (PhaJ) and (*R*)-3-hydroxyacyl-ACP-CoA transferase (PhaG). From fatty acid β-oxidation and fatty acid biosynthesis, 3-ketoacyl-ACP reductase (FabG) is found to accept 3-ketoacyl-CoA and 3-ketoacyl-ACP as substrates and produces MCL-(*R*)-3HA-CoA, which is further converted to PHA by PhaC.

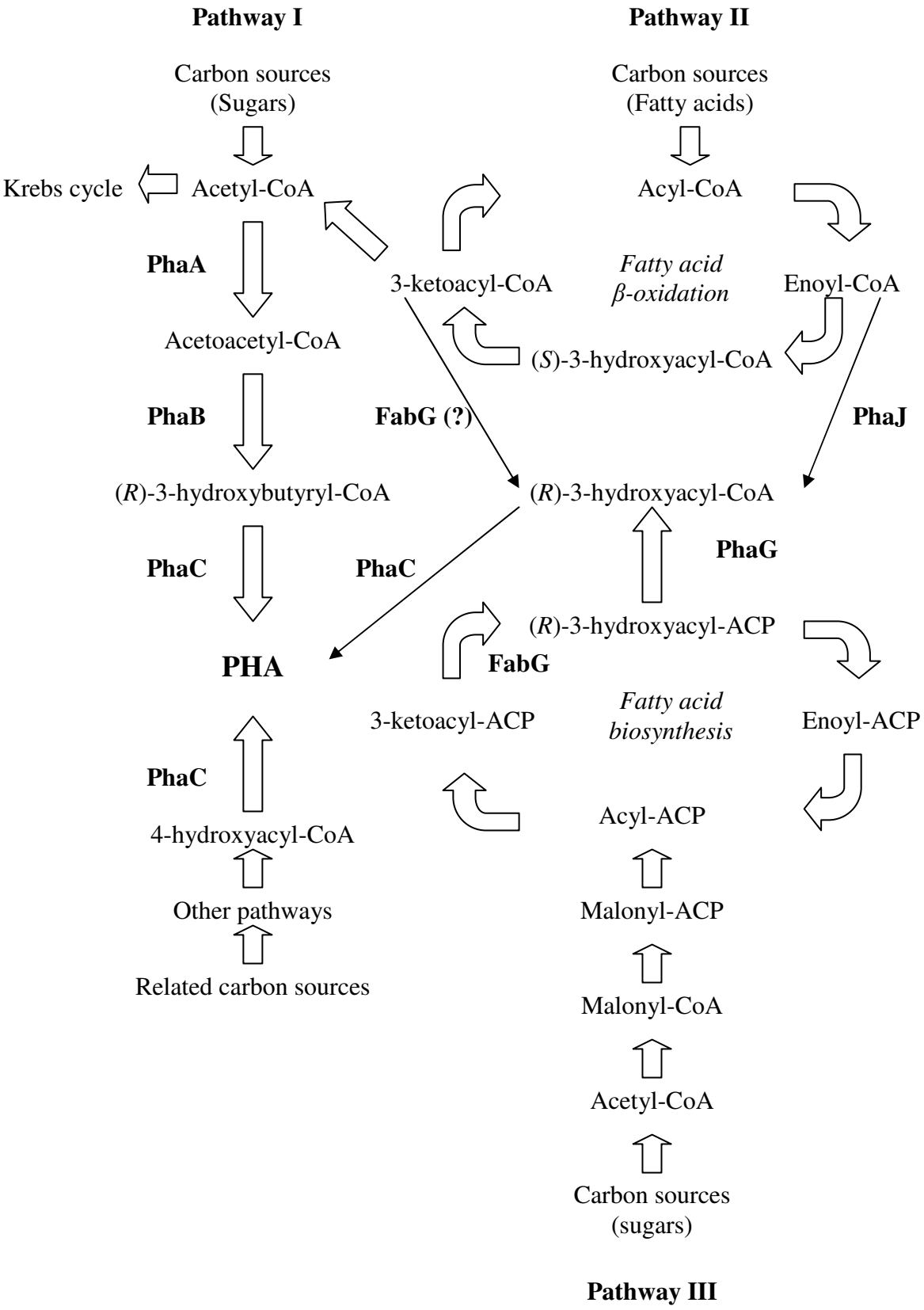


Figure 2.3 Metabolic pathways that supplying monomers for PHA synthesis (Tsuge, 2002). PhaA, 3-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (*R*)-specific enoly-CoA hydratase; FabG, 3-ketoacyl-ACP reductase

2.7.2 Engineered pathways

To produce PHA with desirable monomer compositions, genetic engineering technology has been used. The desirable genes have been cloned from PHA-producing-bateria, for examples, PHA synthase gene (*phaC*) and monomer supplying encoded genes (*phaA, phaB, phaG, phaJ*). These genes can encode the products which have inherent substrate specificity. Hence, the bacteria transformants can produce controlled monomer compositions of PHA. Besides that, the supplied carbon sources also affect the monomer composition of PHA produced (Tsuge, 2002). In consequences, some of the bacteria transformants are able to produce PHA with desirable monomer compositions and some may have enhanced the DCW and PHA content compared to the wild type bacteria (Fukui & Doi, 1997, Tsuge *et al*., 2004, Bhubalan *et al*., 2010a, Jian *et al*., 2010).

2.8 Physical Properties

P(3HB) homopolymer is highly crystalline, stiff and brittle. These properties have limited its application (Sudesh *et al*., 2000). In the efforts of enhancing its useful properties, the incorporation of second monomer into the backbone of P(3HB) is effective. Copolymers for example P(3HB-*co*-3HV), P(3HB-*co*-4HB), P(3HB-*co*-3HHx) or MCL-PHA are less stiff and brittle but retaining some of the other mechanical properties of P(3HB). By varying the compositions and molecular structure, the physical and thermal properties of the polymers can be regulated (Sudesh *et al*., 2000). These polyesters have a wide variety of compositions which display different properties, from hard crystalline plastics to elastic rubbers. On the other hand, terpolymer has higher thermal stability and elongation to break compared to P(3HB) and its copolymers (Zhao & Chen, 2007). It also changed the crystallinity

and flexibility of the polymers (Chanprateep & Kulpreecha, 2006, Xie & Chen, 2008).

Some PHA show multiple melting peak behavior and melting-recrystallization-remelting (Gunaratne & Shanks, 2005). It is important to know the thermal degradation point of the biopolymer during the processing. P(3HB) starts to degrade at 246.3 ºC while P(3HB-*co*-3HV) at 260.4 ºC. This shows that the presence of second monomer in the backbone of P(3HB) increases the thermal stability of the polymer (Carrasco *et al.*, 2006). Comparison of some P(3HB) homopolymer, copolymers and terpolymers' properties are shown in Table 2.2.

Table 2.2 Properties of some P(3HB) homopolymer, copolymers and terpolymers

Polymer	T_m [a] (ºC)	T_g [b] (ºC)	Tensile strength (MPa)	Elongation to break (%)	Reference
P(3HB)	177	4	43	5	(Tsuge, 2002)
P(3HB-*co*-20 mol % 3HV)	145	-1	20	50	(Tsuge, 2002)
P(3HB-*co*-16 mol % 4HB)	150	-7	26	444	(Tsuge, 2002)
P(3HB-*co*-10 mol % 3HHx)	127	-1	21	400	(Tsuge, 2002)
P(3HB-*co*-22 mol % 3H4MV)	126	-2	11	380	(Tanadchangsaeng *et al*., 2009)
P(3HB-*co*-3 mol % 3HV-*co*-93 mol % 4HB)	55	-52	14	430	(Chanprateep & Kulpreecha, 2006)
P(3HB-*co*-7 mol % 4HB-*co*-20 mol % 3HHx)	-	-8	1	364	(Xie & Chen, 2008)
P(3HB-*co*-2 mol % 3HV-*co*-7 mol % 3HHx)	144	-3	22	312	(Bhubalan *et al*., 2008)

[a] Melting temperature
[b] Glass-transition temperature

2.9 Application

Most of the PHA applications are to replace petrochemical synthetic plastics. Currently, in packaging and coating industries, the plastics can be replaced partly or completely by PHA (Verlinden *et al*., 2007). Some of the other applications are shown in Figure 2.4 (Chen, 2010b).

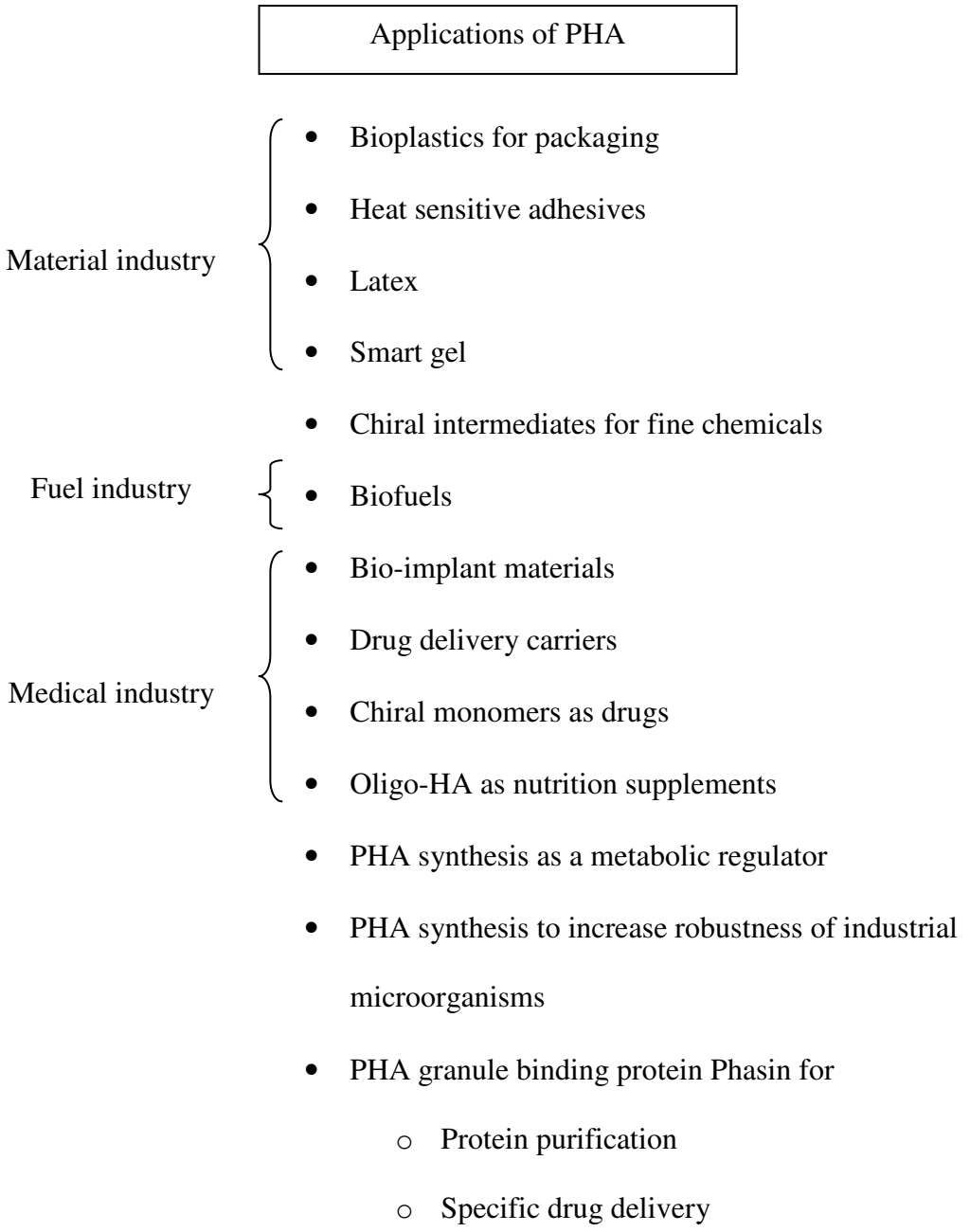


Figure 2.4 Industrial value chain of PHA (Chen, 2010b)

2.10 Fabrication of electrospun PHA film

Electrospinning is one of the effective techniques for polymer nanofibers fabrication. Such nanofibers appear to possess several amazing characteristics, for examples, very large surface area-to-volume ratio, flexibility in surface functionalities and enhanced performance in mechanical properties (for examples tensile strength and stiffness) (Huang *et al.*, 2003).

In electrospinning process, the polymer is dissolved in proper amount of solvent to form polymer solution (Huang *et al*., 2003). The solution fluid is then introduced into the capillary tube. There are three basic components in the process: a high voltage supplier, a capillary tube with a small diameter needle and a metal collecting plate. A high voltage is applied to generate an electrically charged jet of polymer solution out of the needle. The solution jet evaporates before reaching the collecting plate and forms interconnected web of small fibers on it. There are two electrodes, one is placed into the solution while the other is attached to the collector. Electric field is supplied to the end of the capillary tube which has the solution fluid held by its surface tension. This stimulates a charge on the surface of the liquid. A force that is directly opposite to the surface tension is formed when there is mutual charge repulsion and contraction of the surface charges to the counter electrode. Repulsive electrostatic force then forms due to the further increase of the electric field and this eventually overcomes the surface tension, resulting in the ejection of charged jet of the fluid from the needle tip. The elongation process that allows the jet to become very long and thin occurs as the discharged polymer solution jet undergoes instability. At the same time, the solvent evaporates and thus a charged polymer fiber is collected on the metal collecting plate.

There are a few parameters which influence the nanofibers formation in the electrospinning (Huang *et al*., 2003). They are (a) properties of the polymer solution such as elasticity, viscosity, conductivity and surface tension, (b) major variables such as hydrostatic pressure in the capillary tube, electric potential at the capillary tip, distance between the tip and the collecting plate, and (c) ambient parameters such as solution temperature, humidity and air velocity in the chamber.

PHA nanofibers are useful in medical devices such as sutures, bone plates, surgical mesh, cardiovascular patches (Chen & Wu, 2005) and scaffolds (Tang *et al.*, 2008). In the field of tissue engineering, P(3HB), P(4HB), P(3HB-*co*-4HB), P(3HB-*co*-3HV) and P(3HO-*co*-3HHx) have been evaluated as biomaterial. In mammalian body, the hydroxyl acids that are released when PHA is broken down *in vivo* are found to be considerably less acidic and less inflammatory than the other currently used polymers such as poly(lactic acid) (PLA) (Taylor *et al.*, 1994). Electrospun biomaterials, which have very high surface area-to-volume ratio and high porosity, can help better cell attachment and perfusion. Besides, the morphology and architecture of electrospun structure are similar to some extracellular matrix (Li *et al.*, 2002). Due to the biodegradability, flexible mechanical properties, and excellent biocompatibility, electrospun PHA is found to be a promising biomaterials for scaffolds (Tang *et al*., 2008).

2.11 Biodegradability

Biodegradation of PHA is divided to intracellular PHA degradation and extracellular PHA degradation. Intracellular degradation or mobilization refers to the degradation activities occurring in PHA accumulating bacterium on its own endogeneous storage reservoir (Jendrossek & Handrick, 2002). Intracellular PHA

depolymerases are the enzymes that catalyze this process. On the other hand, extracellular degradation is the consumption of an exogeneous polymer by a not-necessarily accumulating microorganism such as bacteria or fungi that secretes extracellular PHA depolymerases which are carboxyesterases (Jendrossek & Handrick, 2002, Choi *et al.*, 2004). This extracellular PHA is released by the PHA accumulating cells after death and cell lysis.

Extracellular PHA-degrading microorganisms can be aerobic or anaerobic organisms that are found in different types of ecosystems such as soil, compost, sewage sludge, fresh and marine water (including deep sea), estuarine sediment, and air (Jendrossek & Handrick, 2002). These bacteria vary in the type of PHA that they can degrade. They are specific to either SCL-PHA or MCL-PHA. However, some bacteria are able to consume a large variety of polyesters including SCL-PHA and MCL-PHA (Schirmer *et al.*, 1995). These types of bacteria are able to synthesize more than one PHA depolymerase in respect to the substrate specificity for SCL-PHA and MCL-PHA. For example, *Streptomyces exfoliates* K10 can degrade both P(3HB) and poly(3-hydroxyoctanoate) (PHO) with SCL-PHA depolymerase and at least one additional depolymerase with specificity for MCL-PHA (Klingbeil *et al.*, 1996). A well-known and unique PHA-degrading bacterium, *Paucimonas* (*Pseudomonas*) *lemoignei* can synthesize at least 7 extracellular PHA depolymerases (PhaZ1 to PhaZ7) (Jendrossek & Handrick, 2002). Besides bacteria, many PHA-degrading fungi have been recognized (Gonda *et al.*, 2000, Sanyal *et al.*, 2006).

Many extacellular PHA depolymerases have common characteristics: (a) high stability at wide range of pH, temperature and ionic strength, (b) a relatively small M_r (< 70 kDa) consisting of only one polypeptide, (c) alkaline pH optimum (7.5 to 9.8), (d) most of the SCL-PHA depolymerases are inhibited by reducing agents and by

serine hydrolase inhibitors, (e) many depolymerases do not bind to anion exchangers such as DEAE (at neutral pH) but have strong affinity to hydrophobic materials (Jendrossek & Handrick, 2002).

The degradation of PHA starts by enzymatic surface erosion of the polyester to water-soluble monomers and/or oligomers (Jendrossek & Handrick, 2002). The properties of PHA such as (a) accessibility of the polymer surface, (b) monomeric composition, (c) crystallinity and (d) stereoregularity have a strong effect on its biodegradability. The enzymes activities may differ as they depend on the conditions of the environment. The degradation rate of PHA films is faster for those films that are buried in the sediment compared to those that are placed on the sediment surface. This is because buried films are continually in contact with the sediment surrounding it. So, degradation occurs on all surfaces as there are more exposed surface area for microbial attack (Sridewi *et al.*, 2006). On the other hand, polymer composition does have an effect on the degradation rate. For example P(3HB-*co*-5 mol % 3HHx) are degraded faster than P(3HB-*co*-5 mol % 3HV) and P(3HB). Copolymer degrades faster than homopolymer because of the differences in surface morphology and crystallinity. Higher crystallinity reduces the degradation rate of the polymer. The incorporation of second monomers, for examples, 3HV and 3HHx increases the amorphous proportion and hence increases the degradation rate of the PHA films. The surface morphology also affects the degradation rate. Films that have more porous will degrade faster. Besides that, molecular weight (M_w) is another factor which determines the biodegradable rate of the PHA. Low M_w is more preferred for biodegradability (Tokiwa & Calabia, 2004). Melting temperature (T_m) has great effect on biodegradability of polymer as well. In general, the higher the T_m, the lower the biodegradability (Tokiwa & Calabia, 2004).

Besides polymer's properties, conditions of the environment do have significant effects on PHA degradation. The factors of the environment are: microbial population, humidity, pH, nutrient supplies and temperature of the given environment (Tokiwa & Calabia, 2004).

2.12 Renewable nature and life cycle

The production of PHA is bioresource-based and renewable (Braunegg *et al.*, 2004). Agricultural feeds such as fatty acids and sugars can be used as carbon sources in the biosynthesis of PHA (Kadouri *et al.*, 2005) which reduce the production cost of the process. The biosynthesis and biodegradation of PHA are compatible to the carbon cycle as shown in Figure 2.3 (Gross & Kalra, 2002, Verlinden *et al*., 2007). As compared to other non-degradable conventional plastics, PHA starts to receive great attention because their production is renewable-based.