EXPRESSION OF *phaC1* AND *phaC2* FROM *Pseudomonas* sp. USM4-55 IN *Escherichia coli* EXPRESSION SYSTEM

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by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

September 2007

PENGEKSPRESAN *phaC1* DAN *phaC2* DARIPADA *Pseudomonas* sp. USM4-55 DI DALAM SISTEM PENGEKSPRESAN *Escherichia coli*

oleh

BALQIS BT AB. GHANI

Tesis yang diserahkan untuk memenuhi keperluan bagi Ijazah Sarjana Sains

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TABLE OF CONTENTS

CHAPTER 3: MATERIALS AND METHODS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

A	Absorbance
bp	Basepair
BSA	Bovine serum albumin
CoA	Coenzyme A
CTAB	Cetyl trimethyl ammonium bromide
Da	Dalton
DCPK	Dicyclopropylketone
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	Dithiotreitol
DCW	Dry cell weight
EDTA	Ethylenediamine tetraacetic acid
GTE	Glucose Tris-EDTA
His	Histidine
IPTG	Isopropyl-β-D-thiogalactoside
kb	Kilobase pairs
kDa	KiloDalton
lacO	Lac operator
LA	Luria Bertani agar
LB	Luria Bertani
LCL	Long chain length
LDPE	Low density polyethylene
M	Molar

MCL	Medium chain length
MCS	Multiple cloning sites
mM	Milimolar
µg/mL	Microgram per mililitre
µL	Microlitre
µM	Micrometer
N	Normal
NA	Nutrien agar
NADH	Nicotinamide adenine dinucleotide (reduced form)
ng/mL	Nanogram per mililitre
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometer
OD	Optical density
ORF	Open reading frame
P(3HB)	Poly(3-hydroxybutyrate)
P(4HB)	Poly(4-hydroxybutyrate)
P(3HB-*co*-67 mol % HP)	Poly(3-hydroxybutyrate-*co*-hydroxypentanoate) containing 67 mol % of HP
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-6 mol % 3HA)	Poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoate) containing 6 mol % of 3HA
P(3HHx-*co*-3HO)	Poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate)
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

PHA	Polyhydroxyalkanoate
PhaC	Polyhydroxyalkanoate synthase
PHAs	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
psi	Pound per square inch
PT5	T5 promoter
PVP-360	Polyvinylpyrolidone average molecular weight is 360 000
(R)-3HB-CoA	(R)-3-hydroxybutyrate-Coenzyme A
RBS	Ribosome binding site
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SCL	Short chain length
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide Gel Electrophoresis
TAE	Tris-Acetic Acid-EDTA
TCA	Tricarboxylic acid
TE	Tris-EDTA
TEMED	N,N,N,N -tetramethyl-ethylenediamine
U/mg	Unit per milligram
U/mL	Unit per mililitre
V	Volt
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PENGEKSPRESAN *phaC1* DAN *phaC2* DARIPADA *Pseudomonas* sp. USM4-55 DI DALAM SISTEM PENGEKSPRESAN *Escherichia coli*

ABSTRAK

Polihidrosialkanoat (PHA) disintesiskan secara intrasel dalam pelbagai mikroorganisma sebagai bahan simpanan untuk sumber karbon dan tenaga. PHA sintase (PhaC) merupakan enzim utama dalam pempolimeran PHA. *Pseudomonas* sp. USM4-55 adalah pencilan tempatan yang berupaya menghasilkan campuran polimer berantai pendek dan sederhana panjang. Gen-gen yang mengkodkan PhaC (*phaC1* and *phaC2*) daripada *Pseudomonas* sp. USM4-55 diamplifikasi (setiap satunya bersaiz 1.7 kb) daripada DNA genomik, diklon ke dalam pGEM®-T untuk tujuan penjujukan DNA dan seterusnya diekspres. Bagi kajian pengekspresan protein, pQE-30 telah digunakan sebagai vektor pengekspresan yang dikawal oleh promoter PT5. Klon-klon ini dinamakan sebagai pQE-C1 dan pQE-C2, kemudian klon-klon tersebut diaruh dengan 0.01 mM isopropil-β-D-thiogalaktosida (IPTG) apabila OD_{600} mencapai 0.6. Protein-protein rekombinan ini telah diekspres di dalam *E. coli* M15 yang masing-masingnya bersaiz ~60 kDa dan ~61 kDa selepas diaruh dengan 0.01 mM IPTG. Kedua-dua protein telah berjaya ditulenkan menggunakan turus Ni-NTA dengan bantuan tag 6x Histidin. Akhirnya, protein lakuran ini ditunjukkan mempunyai aktiviti PhaC dengan menggunakan 3.1 mM (R)-3-hidroksibutirat CoA sebagai substrat di dalam analisis pengasaian enzim. Aktiviti spesifik bagi PhaC1-His dan PhaC2-His adalah masing-masingnya 0.002 U/mg dan 0.003 U/mg.

EXPRESSION OF *phaC1* AND *phaC2* FROM *Pseudomonas* sp. USM4-55 IN *Escherichia coli* EXPRESSION SYSTEM

ABSTRACT

Polyhydroxyalkanoates (PHAs) are synthesized intracelullarly by a variety of microorganisms as reserve materials for carbon and energy source. PHA synthase (PhaC) is the key enzyme in the polymerization of PHAs. *Pseudomonas* sp. USM4-55 is a local isolate which is able to produce a blend of short chain length and medium chain length of polymer. Genes coding for PhaC (*phaC1* and *phaC2*) from *Pseudomonas* sp. USM4-55 were amplified (each with a size of 1.7 kb) from the genomic DNA, cloned into pGEM®-T for sequencing and then expressed. For expression studies, pQE-30 was used as the expression vector under the control of PT5 promoter. These clones were named pQE-C1 and pQE-C2, and were induced with 0.01 mM of isopropyl-β-D-thiogalactoside (IPTG) at OD_{600} of 0.6. These recombinant proteins were expressed in *E. coli* M15 with sizes of ~60 kDa and ~61 kDa, respectively upon induction with 0.01 mM of IPTG. Both proteins were successfully purified by using Ni-NTA column with the help of 6x Histidine tag. Finally, these fusion proteins were shown to have PhaC activity by using 3.1 mM of (R)-3-hydroxybutyrate CoA as a substrate in the enzyme assay analysis. The specific enzyme activities for PhaC1-His and PhaC2-His are 0.002 U/mg and 0.003 U/mg protein, respectively.

CHAPTER 1: INTRODUCTION

1.1 General introduction

Several studies have reported the production of polyhydroxyalkanoic acids (PHAs) in many different microorganisms found throughout the world (Jendrossek *et al.*, 1996). Most of these microorganisms are able to accumulate poly(3-hydroxybutyric acid), poly(3HB) and other PHAs as storage materials in the cells (Qi *et al.*, 2000). They can also degrade these materials in the cytoplasm with the help of PHA depolymerase enzymes encoded by the gene *phaZ* (Rehm and Steinbuchel, 1999). These polyesters have a great potential in industrial and medical applications because of their interesting properties such as plasticity, biodegradability, and biocompatibility (Steinbuchel, 1996). PHA synthases are the enzymes responsible for PHA biosynthesis by catalysing the polymerisation of 3-hydroxyacyl-CoA substrates to PHA with the concomitant release of CoAs (Steinbuchel, 1991).

In an environmentally conscious society, this type of polymer is believed to be a potential candidate to substitute synthetic plastics which are available in the market today. PHAs are bio-friendly to the environment rather than synthetic plastics. The synthetic plastics will release dangerous gases and particles to the environment whenever they are decomposed. However, the production of PHAs from fermentation technology is considered costly even using varieties of renewable sources compared to synthetic plastics. Thus, many studies around the world have been done comprehensively in producing PHAs at a lower cost. As a result, manipulation of DNA is one way to produce PHAs in a short time

and in large amount in recombinant organisms in natural hosts, *E. coli* or transgenic plants.

In this study, *phaC1* and *phaC2* were amplified and subcloned from *Pseudomonas* sp. USM4-55. This bacterium was isolated from local soil, and the PHA synthase genes had been cloned and characterized (Baharuddin, 2001). There are three genes clustered together; *phaC1, phaZ* and *phaC2*. The PHA synthase is a very interesting enzyme and the structure is still unknown. Our research is now focused on getting a high yield of soluble and functional PHA synthase, suitable for protein crystallization screening which will enable us to determine its structure.

1.2 Research objectives

This research was carried out with four objectives which consist of:

1) Isolation of PHA synthase (*phaC*) from *Pseudomonas* sp. USM4-55.
2) Expression of *phaC1* and *phaC2* in *E. coli* expression system.
3) Purification of recombinant PHA synthases (PhaC).
4) Determination of the recombinant PhaC enzymes activities.

The overview of this study was sketched out as shown in Figure 1.1.

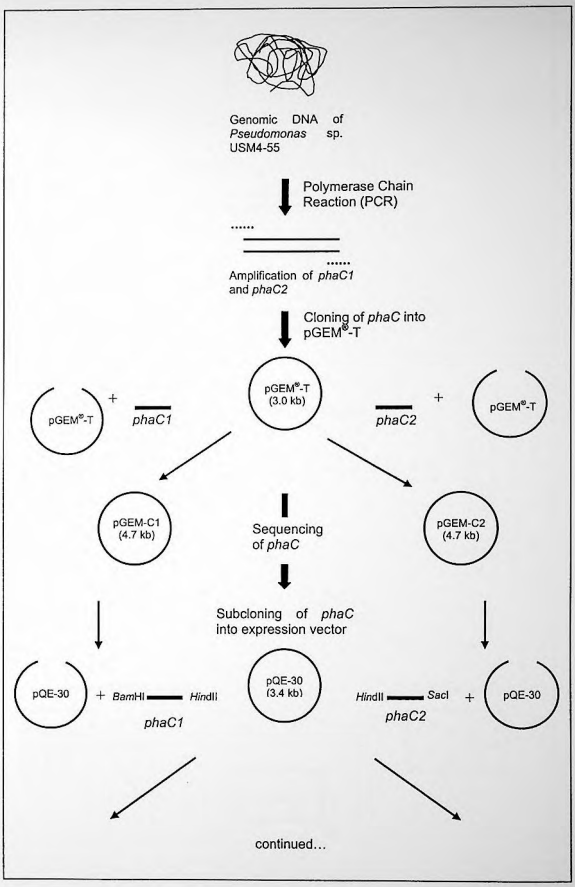


Figure 1.1 Overview of the study carried out.

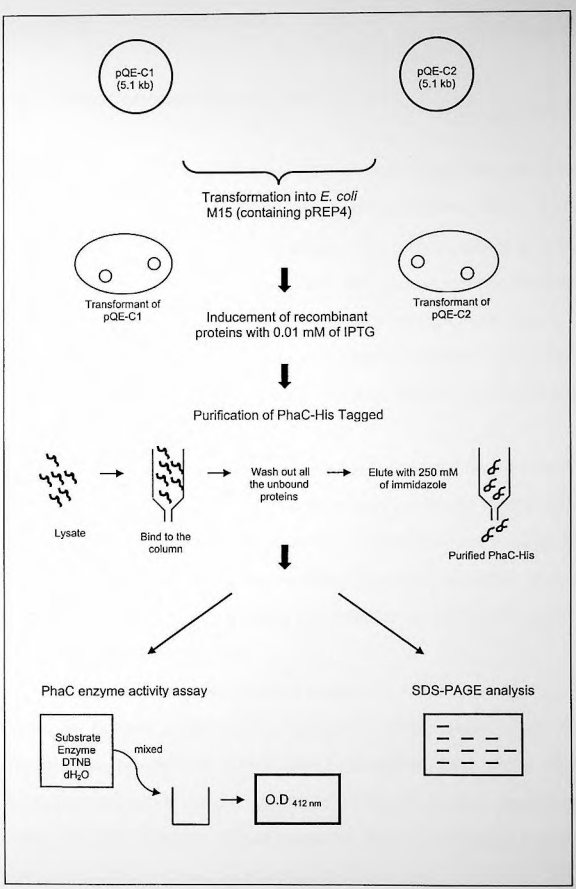


Figure 1.1 ...ended.

CHAPTER 2: LITERATURE REVIEW

2.1 POLYHYDROXYALKANOATES (PHA)

2.1.1 Background

Polyhydroxyalkanoate (PHA) is a group of microbial polyesters which are produced by various microorganisms in the world (Steinbuchel, 1991). These polymers are deposited in the cell cytoplasm of the microorganisms as water insoluble inclusions (polymer granules) and are used as reserve materials. Microorganisms usually accumulate the polymers when there is an excess of carbon source and depletion of one or more essential nutrients in the growth medium such as nitrogen (Schlegel *et al.*, 1961), potassium, sulfur (Wilkinson and Munro, 1967) or oxygen (Senior *et al.*, 1972).

In 1985, Tal and Okon had demonstrated that *Azospirillium brasilense* cells with high polyhydroxybutyrate [P(3HB)] content are able to survive better than those cells with a lower P(3HB) content. P(3HB) can also serve as an endogenous carbon and energy source during sporulation in *Bacillus* species (Slepecky and Law, 1961) and during cyst formation in *Azotobacter* species (Stevenson and Socolofsky, 1966).

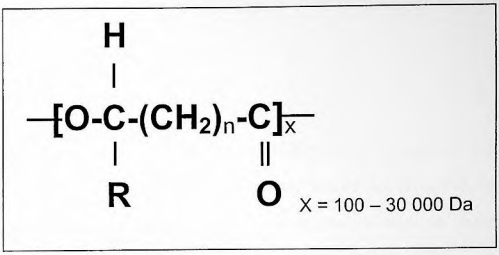
PHA granules were first observed by Meyer in *Azotobacter chroococum* during his research at the beginning of the 20th century (Meyer, 1903). He reported that the granules were soluble in chloroform. Later on, in 1925 Lemoigne successfully isolated granules of P(3HB) in *Bacillus megaterium* using chloroform extraction method (Lemoigne, 1926). He identified that the composition of the granules was poly-β-hydroxybutyric acid.

PHA becomes so fascinating because of its biodegradable and biocompatible characteristics. These polymers can be degraded in the environment mainly by bacteria and fungi to carbon dioxide and water (Braunegg *et al.*, 1998). Hence, PHAs can also be metabolized intracellularly by depolymerase enzymes to use as carbon and energy sources. The polymers are also highly valued in medical, agricultural and industrial applications due to its biocompatibility. To date, PHA still cannot completely replace the synthetic plastics currently available because the production of biodegradable plastics is costly. Thus, various studies are extensively done in order to reduce the cost of producing biodegradable plastics.

2.1.2 Classification of PHAs

Generally PHA can be divided into three groups which are short chain length PHA (scl-PHA), medium chain length PHA (mcl-PHA) and long chain length PHA (lcl-PHA) (Lee, 1996). Classification of PHAs is based on the number of carbon atoms in the polymer. Short chain length PHA (scl-PHA) consists of 3 to 5 carbon atoms and medium chain length PHA (mcl-PHA) consists of 6 to 14 carbon atoms. However, there is another unrecognized group called long chain length PHA (lcl-PHA) consisting of more than 14 carbon atoms. Basically these groups are different because of the substrate specificity of PHA synthases to incorporate 3HAs of a certain range of carbon length (Anderson and Dawes, 1990). For example, *Alcaligene eutrophus* is able to produce scl-PHA, while *Pseudomonas oleovorans* can produce mcl-PHA and lcl-PHA is very rarely produced by bacterium but it still exists. The chemical structure of PHA is shown in Plate 2.1 (Sudesh, 2000). The plate shows that

PHA has an R absolute configuration [D(-) in traditional nomenclature] in the chiral center of 3-hyroxybutyric acid. The molecular weight of this polymer ranges from 2×10^5 to 3×10^6 Da and it depends on the microorganism and the growth conditions applied (Byrom, 1994).



n = 1	R = hydrogen	Poly(3-hydroxypropionate)
	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hyrdroxyvalerate)
	R = propyl	Poly(3-hyrdroxyhexanoate)
	R = pentyl	Poly(3-hyrdroxyoctanoate)
	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	Ploy(5-hydroxyhexanoate)
n = 4	R = hexyl	Poly(6-hydroxydodecanoate)

Figure 2.1 General structure of polyhyrdoxyalkanoate and some of its monomers (Sudesh, 2000).

2.1.3 Biosynthesis of PHA

There is a wide range of bacterial strains which can produce PHA from numerous carbon sources such as alkanoic acids, alcohol and alkanes. There are three types of metabolic pathways in PHA production. The pathways will be discussed with appropriate examples. A metabolic pathway of PHA biosynthesis is illustrated in Figure 2.2.

2.1.3.1 Scl-PHA biosynthesis

Biosynthesis of scl-PHA [P(3HB)] can be represented by the pathways in *Wautersia eutropha* (formerly known as *Ralstonia eutropha*), *Zooglea ramigera* or *Azotobacter beijerinckii*. The pathway and regulation of P(3HB) synthesis have been studied extensively and is well established (Pathway I).

The pathway of scl biosynthesis in *W. eutropha* (Anderson and Dawes, 1990) starts with the conversion of an appropriate carbon substrate (for example sugars, alcohols, organic acids or carbon dioxide) to acetyl-coenzyme A (acetyl-CoA). Two molecules of acetyl-CoA are condensed by the action of β-ketothiolase (acetyl-CoA acetyltransferase) which is encoded by *phaA* gene. This reaction will release a free coenzyme A (CoASh) and form acetoacetyl-CoA. The intermediate is then reduced to (*R*)-isomer of 3-hyrdoxybutyryl-CoA by NADPH- dependent reductase encoded by *phaB*. Finally, polymerization of (*R*)-3-hydroxybutyryl-CoA by the action of *phaC* will produce P(3HB) or scl-PHA.

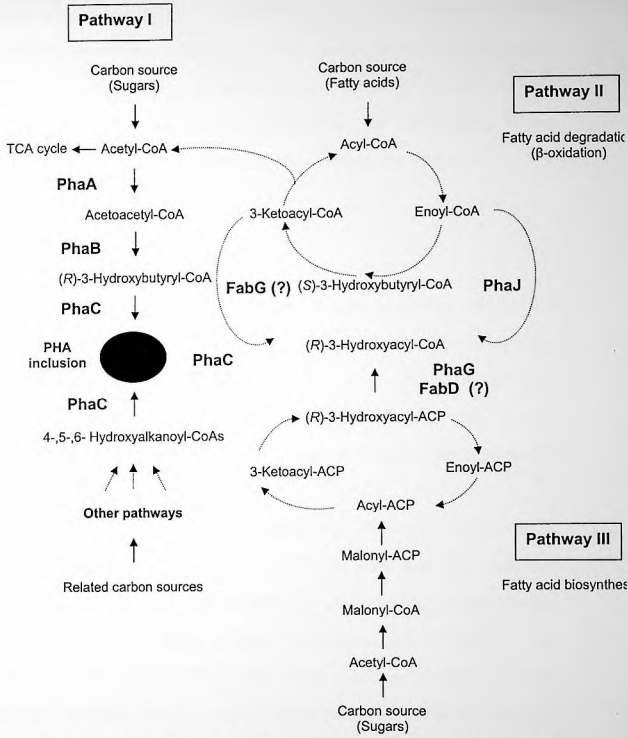


Figure 2.2 Metabolic pathways that supply hydroxyalkanoate monomers for PHA biosynthesis (Sudesh, 2000).

PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (*R*)-enoyl-CoA hydratase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-CoA-reductase.

β-ketothiolase is a key regulatory enzyme (Oeding and Schegel, 1973; Senior and Dawes, 1973) in the production of scl-PHA. The enzyme is inhibited when the concentration of free Coenzyme A is high. Acetyl-CoA will enter the tricarboxylic acid (TCA) cycle to generate energy and to form amino acids when there is excess oxygen. In other words, when the concentration of free coenzyme A is high under balanced growth conditions, the synthesis of scl-PHA is inhibited.

2.1.3.2 Mcl-PHA biosynthesis

Biosynthesis of mcl-PHA [P(3HA)] from various alkanes, alkanols and alkanoates are represented by the event in fluorescent pseudomonad which belong to the rRNA homology group I (Lageveen *et al.*, 1988). There are three types of pathways involved in producing mcl-PHA such as fatty acids β-oxidation (fatty acids degradation), *de novo* fatty acids (fatty acids biosynthesis) and chain elongation.

Fatty acids β-oxidation is represented by *Pseudomonas oleovorans,* which is able to utilize a wide variety of n-alkanoic acids, n-alkanals, n-alkanols and n-alkanes when equipped with the OCT plasmid harboring the essential genes for the initial oxidation of alkanes to produce mcl-PHA (Beilen *et al.*, 1994; Schwartz and Mc Choy, 1973). This bacterium produces mcl-PHA through β-oxidation pathway, but it cannot produce PHA when grown on fructose, glucose and glycerol. In the β-oxidation cycle, 2-trans-enoylCoA, S-3-hydroxyacyl-CoA and 3-ketoacyl-CoA are used as precursors to form the PHA polymerase substrate (*R*)-3-OH-acyl-CoA. There are two different polymerases present in the bacterium such as PhaC1 and PhaC2.

PHA biosynthesis involving the fatty acids biosynthesis pathway (Pathway III) can be represented by *Pseudomonas aeruginosa*, *Pseudomonas aureofaciens*, *Pseudomonas citronellolis*, *Pseudomonas mendocina* and *Pseudomonas putida*. *phaG* produces 3-hydroxyacyl-CoA-ACP transferase, which is capable of channelling the intermediates of the *de novo* fatty acids biosynthesis to PHA biosynthesis. (*R*)-3-hydroxyacyl-ACP will be converted to (*R*)-3-hydroxyacyl-CoA form and channeled to PHA biosynthesis cycle.

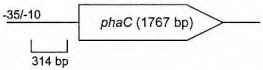
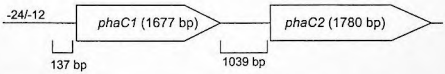
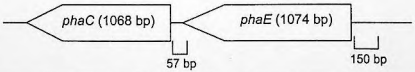
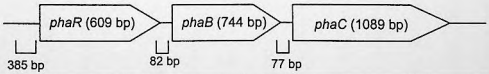
In the chain elongation reaction, acyl-CoA is extended with acetyl-CoA by forming ketoacyl-CoA, then ketoacyl-CoA is converted to (*R*)-3-OH-acyl-CoA by ketoacyl-CoA reductase to be a PHA synthase substrate.

2.1.4 Types of PHA synthases (PhaC)

PHA synthase (PhaC) is the key enzyme in producing PHA by microorganisms. Rehm (2003) reported that there are almost 59 PhaC nucleotide sequences from 45 microorganisms which have been identified. PHA synthases are classified into four different groups based on their subunit compositions, substrate specificities and primary structures (Table 2.1). Cysteine residue is conserved in all PHA synthases and is potentially an active site in PHA polymerization (Griebel *et al.*, 1968). Organization of the genes in four different groups of PHA synthases is shown in Table 2.2.

Type I PHA synthase is represented by *Ralstonia eutropha* which produces scl-PHA containing three to five carbon atoms. PhaC from this type consists of one subunit with the molecular weight ranging from 60 to 73 kDa (Qi and Rehm, 2001). This enzyme preferentially utilizes CoA thioesters from various (*R*)-3-hydroxyfatty acids comprising three to five carbon atoms.

Table 2.2 Organization of PHA synthases genes (Rehm, 2003).

Type of PHA synthase	Molecular organization of PHA synthase
Type I (*Wautersia eutropha*)	
Type II (*Pseudomonas oleovorans*)	
Type III (*Chromatium vinosum*)	
Type IV (*Bacillus megaterium*)	

The second type of PHA synthase produces mcl-PHA containing 6 to 14 carbon atoms. It is represented by two PHA synthases (PhaC1 and PhaC2) from Pseudomonad groups and consists of one subunit of PhaC of about 60 to 73 kDa. These enzymes favor CoA thioesters of various (*R*)-3-hydroxyfatty acids comprising 6 to 14 carbon atoms (Amara and Rehm, 2003; Slater *et al.*, 1992; Slater *et al.*, 1988; Schubert *et al.*, 1988; Peoples and Sinskey, 1989).

Type III PHA synthase consists of two different subunits type called C-subunit (~40 kDa) and E-subunit (~40 kDa). C-subunit has 21 to 28 % amino acid identity with type I and II PHA synthases, whereas E-subunit does not show any similarity to other types of PHA synthases. However, both of the subunits are important for the function of PHA synthase. This type of PHA synthase is represented by *Chromatium vinosum* and utilizes CoA thioesters of (*R*)-3-hydroxyfatty acids comprising three to five carbon atoms (Liebergesell *et al.*, 1992, Yuan *et al.*, 2001).

Type IV PHA synthase is represented by *Bacillus megaterium* which also comprises two subunits, namely PhaC subunit which is approximately 40 kDa and PhaR which is about 20 kDa.

2.1.5 Physical properties of PHAs

Physical properties of PHAs are highly dependent on the composition of monomer units and molecular weight. P(3HB) or scl-PHA is the most well studied biopolymer because the accessibility of the polymer is wider than mcl-PHA. P(3HB) is stiffer, brittle and also highly crystalline compared to mcl-PHA (De Koning *et al.*,1992). The physical property of P(3HB) is almost similar to conventional plastics (polypropylene) but it has a high melting temperature of

about 177°C (Doi *et al.*, 1990). PHA copolymer such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] is less stiffer and tougher compared to P(3HB). It also has higher elongation to be broken and the melting point reduces from 160°C to 100°C totally depends on the composition of polymer (Holmes, 1985).

Unlike scl-PHA, mcl-PHA has much lower crystallinity and higher elasticity with low melting point, low tensile strength and high elongation to be broken (Preusting *et al.*1990), thus it has been suggested to be used for a wide range of applications. Presently, there are many bacteria that are able to produce a blend of scl and mcl polymer which has better material properties for general application. Physical properties such as crystallinity, melting point, stiffness and toughness can be improved by increasing the molecular weight or by incorporation of hydroxyl-acids units to form PHA copolymers (Shilpi and Ashok, 2005). Table 2.3 shows comparison of various polymer properties.

2.1.6 Applications of PHAs

PHAs have been so attractive to commercial applications due to their biocompatibility, biodegradability and thermo plasticity features. These features make them very competitive towards petro-chemical plastics. Mcl-PHA are rubbery and flexible materials with a lower crystallinity level than scl-PHA. Therefore mcl-PHA has a wide range of applications compared to scl-PHA (Gross *et al.*, 1989; Preusting *et al.*, 1990). PHA can be blended, either modified

Table 2.3 Comparison of polymer properties

Polymer*	Melting point (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)	Glass transition temperature (°C)
P(3HB)	179	3.5	40	5	4
P(3HB-*co*-3HV)					
3 mol% 3HV	170	2.9	38	nd	nd
9 mol% 3HV	162	1.9	37	nd	nd
14 mol% 3HV	150	1.5	35	nd	nd
20 mol% 3HV	145	1.2	32	nd	nd
25 mol% 3HV	137	0.7	30	nd	nd
P(3HB-*co*-4HB)					
3 mol% 4HB	166	nd	28	45	nd
10 mol% 4HB	159	nd	24	242	nd
16 mol% 4HB	nd	nd	26	444	nd
64 mol% 4HB	50	30	17	591	nd
90 mol% 4HB	50	100	65	1080	nd

Table 2.3 ...continued on next page

Polymer*	Melting point (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)	Glass transition temperature (°C)
P(4HB)	53	149	104	1000	nd
P(3HHx-*co*-3HO)	61	nd	10	300	nd
P(3HB-*co*-6 mol% 3HA)	133	0.2	17	680	-8
P(3HB-*co*-67 mol% HP)	44	nd	nd	nd	-19
P(3HB-*co*-3HHx)	52	nd	20	850	-4
Convensional plastics					
Polyethylene-terephalate	262	2.2	56	7300	3400
Polypropylene	170	1.7	34.5	400	45
Polystyrene	110	3.1	50	nd	21
LDPE	130	0.2	10	620	-30

* P(3HB) is poly(3-hydroxybutyrate), P(3HB-*co*-3HV) is poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-4HB) is poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), P(4HB) is poly(4-hydroxybutyrate), P(3HHx-*co*-3HO) is poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate), P(3HB-*co*-6 mol% 3HA) is poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoate) containing 6 mol% of 3HA, P(3HB-*co*-67 mol% HP) is poly(3-hydroxybutyrate-*co*-hydroxypentanoate) containing 67 mol% of HP, P(3HB-*co*-3HHx) is poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) and LDPE is low density polyethylene. (Byrom, 1994; Doi *et al.*1990; Kunioka *et al.*1989; Saito and Doi, 1994).

nd is not determined.

at the surface or composted with other polymers in order to adjust their mechanical properties depending on the requirements for different applications (Guo-Qiang and Qiong, 2005).

Since PHAs are very unique, they are used in numerous applications such as industrial, medical, pharmaceuticals, agricultural and food industries. In industrial applications, PHAs are used in packaging films mainly in bags, containers and paper coatings. Disposable items include razors, utensils, diapers, feminine hygiene products, cosmetic containers and cups. PHAs are also useful as biodegradable carriers for long term dosage of herbicides, fungicides, insecticides or fertilizers (Reddy *et al.*, 2003). In addition, they are also being considered as sources for the synthesis of enantiomerically pure chemicals and as raw materials for the production of paint (Muller and Seebach, 1993).

In medical applications, PHAs are used as osteosynthetic materials in the stimulation of bone growth due to their piezoelectric properties, in bone plates, surgical sutures and blood vessel replacements (Reddy *et al.*, 2003). PHAs also can be applied as matrix in retardant materials for the slow release drugs and hormones in medicine. Scl-PHA also shows a high biocompatibility to various cell lines, such as osteoblastic, epithelial cell and ovine chondrocytes (Rivard *et al.*, 1996; Nebe *et al.*, 2001). Akhtar (1990), reported that the scl-PHA can be used to induce prolonged acute inflammatory responses and chronic inflammatory responses. Other potential applications of PHAs in medical are shown in Table 2.4.

Table 2.4 Potential applications of PHA in medicine (Zinn *et. al.*, 2001).

Type of application	Products
Wound management	Sutures, skin substitutes, nerve cuffs, surgical meshes, staples, swabs
Vascular system	Heart valves, cardiovascular fabrics, pericardial patches, vascular grafts
Orthopedics	Scaffolds for cartilage engineering, spinal cages, bone graft substitutes, meniscus regeneration, internal fixation devices (e.g., screws)
Drug delivery	Micro and nanospheres for anticancer therapy
Urology	Urological stints
Dental	Barrier material for guided tissue regeneration in periodontitis
Computer assisted tomography and ultrasound imaging	Contrast agent

2.1.7 Degradation of PHAs

One of the most important characteristic of PHAs is its biodegradability. PHAs easily degrade into water and carbon dioxide either intracellularly or extracellularly. Basically, degradation of PHAs occurs in two steps. Firstly, PHAs are degraded depending on the type of depolymerases which hydrolyzes polymers into monomers (Jendrossek *et al.*, 1993), monomers and dimers (Schirmer *et al.*, 1993) or a blend of oligomers (Nakayama *et al.*, 1985). Secondly, the oligomers are cleaved by oligomer hydrolase to form monomers (Delafield *et al.*, 1965, Shirakura *et al.*, 1983). In aerobic condition, the monomers will be taken up and metabolized by the cells to yield carbon dioxide and water. Methane, carbon dioxide and water are released into the environment where the monomers are metabolized in anaerobic condition.

Moreover, intracellular degradation is an active degradation by the bacterium which produces the polymer itself. Usually, this bacterium will synthesize PHA depolymerase to hydrolyze the polymers in the cell. Intracellular PHAs are also called native PHA granules which exist in the amorphous state and the molecules are mobile. These granules have particular surface layer which consists of proteins and phospholipids that is sensitive to physical or chemical stress (Amov *et al.*, 1991).

Table 2.5 lists microorganisms which are able to degrade PHA in various environments. These microorganisms will excrete extracellular depolymerases to hydrolyze the PHA into dimers and/or monomers in the environment. The end products then will be absorbed and utilized as nutrients. They degrade PHA widely in various ecosystems such as soil (Mergaert *et al.*, 1993), compost,

Table 2.5 PHA degrading microorganisms were isolated from various environments (Shilpi and Ashok, 2005).

Source from which isolated	Microorganism	Reference
Soil	*Aspergillus fumigatus*	Mergaert *et al.* 1993
	Acidovorax faecalis	Mergaert *et al.* 1993
	Comamonas sp.	Jendrossek *et al.* 1993
	Pseudomonas lemoignei	Delafield *et al.* 1965
	Variovorax paradox	Mergaert *et al.* 1993
Activated sludge	*Acidovorax faecalis*	Tanio *et al.* 1985
	Pseudomonas fluorescens	Schirmer *et al.* 1993
Sea water	*Comamonas testeroni*	Kasuya *et al.* 1994
Lake water	*Pseudomonas stutzeri*	Mukai *et al.* 1994
Anaerobic sludge	*Ilyobacter delafieldii*	Jansen and Harfoot, 1990

aerobic and anaerobic sewage sludge, fresh and marine water (Kunioko *et al.*, 1989), estuarine sediment and air. It has been found that the rate of biodegradation of PHA in the environment depends on temperature, moisture level, pH and nutrient supply (Boopathy, 2000). Besides that, the physical properties of PHA materials such as composition, crystallinity, additives and surface area are also important (Lee, 1996).

2.2 *Pseudomonas* sp. USM4-55

Pseudomonas sp. USM4-55 was isolated by Anthony from an oil palm plantation in Tasik Chini, Pahang. This bacterium is able to utilize free fatty acids and sugars to produce a blend of scl-and mcl-PHAs (Few, 2001). When grown on oleic acid as the carbon source, *Pseudomonas* sp. USM4-55 is able to produce poly(3-hydroxybutyrate) homopolymer and mcl-PHAs that consists of 3-hydroxyhexanoate (C_6), 3-hydroxyoctanoate (C_8), 3-hydroxydecanoate (C_{10}), 3-hydroxydodecanoate (C_{12}), 3-hydroxy-cis-5-dodecanoate ($C_{12:1}$) and 3-hydroxytetradecanoate (C_{14}). The molecular weight of P(3HB) and mcl-PHAs are approximately 1×10^6 Da and 0.4×10^4 Da, respectively.

Further studies were done to clone and characterize the molecular organization of PHA synthases genes in *Pseudomonas* sp. USM4-55. The studies showed that there were two types of PHA synthases (*phbC* and *phaC*) involved in the biosynthesis of PHA. It was found that the open reading frames (ORF) of *phaC1* and *phaC2* were clustered with PHA depolymerase (*phaZ*) (Baharuddin, 2001). The molecular organization of the three clustered genes is shown in Figure 2.3. *phaC1* is 1680 bp in length, while *phaC2* is 1683 bp long.

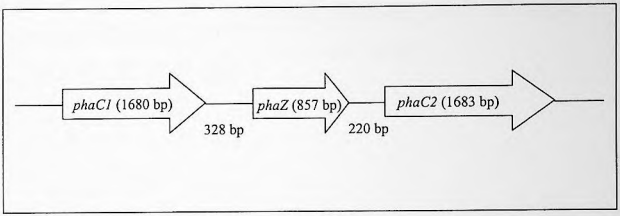


Figure 2.3 Molecular organization of the three clustered genes; *phaC1* and *phaC2*; genes encoding PHA synthase and phaZ, gene encoding PHA depolymerase in *Pseudomonas* sp. USM4-55 (Baharuddin, 2001).

The third PHA synthase (*phbC*) has also been successfully cloned and characterized. It is suggested that the genetic organization of *phb* locus showed a putative promoter region, followed by *phbB* (NADPH-dependent acetoacetyl-Coenzyme A reductase), *phbA* (β-ketothiolase) and *phabC* (polyhydroxybutyrate synthase) (Neo, 2006). Figure 2.4 shows the molecular organization of the *phbC* operon. PhbC was confirmed to be functionally active when heterologous expression in *E.coli* JM109 was employed. pGEM"ABex (containing $phbA_{Re}$ and $phbB_{Re}$) harboring *phbC* was able to accumulate P(3HB) homopolymer up to 40 % of dry cell weight (DCW).

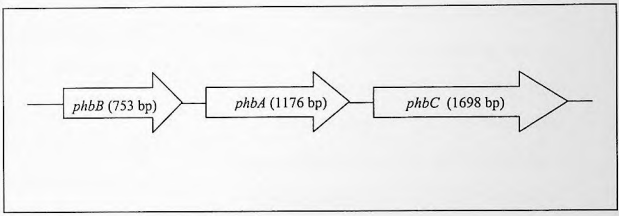


Figure 2.4 Molecular organization of *phbC* operon which consists of *phbB* gene encoding NADPH-dependent acetoacetyl-Coenzyme A reductase, *phbA* gene encoding β-ketothiolase and *phbC* encoding PHB synthase (Neo, 2006).