CHAPTER I
INTRODUCTION

Nowadays plastic materials are an integral part of our life due to their excellent properties such as ease of molding, light weight, chemical resistance and their durability. Thus, the use of plastics has increased rapidly over the past few decades. Based on the report by Global Industry Analysts, global plastic consumption will reach 297.5 million tons by 2015. However, the accumulation of the plastic wastes has become a critical environmental problem as most conventional plastics are produced from petroleum oil which take several centuries to be degraded. To solve this problem, biodegradable plastic is considered a promising alternative (1-3).

One such compound is polyhydroxyalkanoates (PHAs). PHAs is a family of microbial polyester with potential use as bio-plastic for many applications such as food packaging, drug delivery, medical implants, agriculture/horticulture, automotive and household appliances. PHAs can be produced from a range of renewable carbon substrates. PHAs are produced by some types of bacteria as intracellular granules for carbon and energy storage under unbalance growth conditions in the presence of excess carbon source (4). Unlike the petroleum-based plastics, PHAs are biodegradable, thus reducing waste accumulation and greenhouse gas emission. Moreover, environmental impact analysis shows that life cycle of bio-based plastics are superior to petroleum-based plastic (5).

Depending on the monomer structure, PHAs can be classified into three groups which are Short-Chain-Length PHAs (SCL-PHAs) containing 3 to 5 carbon atoms in the monomer, Medium-Chain-Length PHAs (MCL-PHAs) containing 6 to 14 carbon atoms in the monomer and SCL-MCL-PHA copolymer containing both SCL-and MCL-monomers (6). The difference in monomer compositions affects the properties of PHAs. SCL-PHAs such as poly(3-hydroxybutyrate) or P(3HB) have high crystallinity. MCL-PHAs, on the other hand, have high flexibility. SCL-MCL-PHA copolymers combine the advantages of SCL-PHAs and MCL-PHAs. The properties of

SCL-MCL-PHA copolymers range from high crystallinity to elasticity, depending on the molar fraction of the various monomer constituents. For example, a copolymer composed of a high fraction of 3HB and a low fraction of 3-hydroxyhexanoate (3HHx) possesses properties similar to low-density polyethylene (7).

In nature, only a few bacteria are known to produce SCL-MCL-PHA such as *Pseudomonas* sp. 61-3, which produces a random copolymer consisting of C4 to C12 monomer units from gluconate (8), *Aeromonas caviae* and *Aeromonas hydrophila*, which produce P(3HB-*co*-3HHx) from alkanoic acids (9, 10). Unfortunately, *Aeromonas* spp. have been reported as pathogens (11). Therefore, they are not practical to use for large scale production. Moreover, it is difficult to adjust the content and composition of copolymers produced by these bacteria, as their metabolic pathways are poorly characterized and no tools for genetic modification are available. A more practical approach to the production of copolymers with specific properties for industrial application is genetic engineering of model microorganisms (*e.g.*, *Escherichia coli*) that do not naturally produce PHAs.

There are three well-known pathways for PHAs biosynthesis in bacteria (Fig 1.1) (12). Pathway I is found in many bacteria and has been extensively studied in *Ralstonia eutropha*, which produces SCL-PHA. This species produces SCL-PHA through the action of β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC) enzymes. PhaA and PhaB generate the SCL-monomer: (*R*)-3-hydroxybutyryl-CoA [(*R*)-3HB-CoA] by sequentially catalyzing acetyl-CoA to acetoacetyl-CoA and to (*R*)-3HB-CoA respectively. PhaC then polymerizes (*R*)-3HB-CoA monomers to the P(3HB) polymer. Pathways II and III are found mostly in the *Pseudomonas* bacteria, and are mainly involved in the production of MCL-PHAs. They are linked to the fatty acid β-oxidation and fatty acid biosynthesis pathway *via* (*R*)-specific enoyl-CoA hydratase (PhaJ) and (*R*)-3-hydroxyacyl-ACP-CoA transferase (PhaG) enzymes, respectively.

The production of SCL-MCL-PHA copolymers in both wild type and recombinant strains has been previously investigated (13-16). However, the use of PHAs as commercialized material is still limited. This is because of their high production cost. For example the market price of P(3HB) from Biomer (Germany) is €12/kg while the market price of polypropylene is only €0.74/kg (1). According to

Choi and Sang (1997), the price of PHAs mainly depends on substrate cost which accounts for 40-48% of the total production cost (17). Therefore, one approach to reduce the production cost is by using economical substrates such as waste materials as alternative carbon source. Many waste materials have been studied for use as alternative substrates for PHA production (1). Recently, glycerol, a by-product from the growing biodiesel industry, is becoming a promising inexpensive carbon source (18). Thus it is interesting to develop the bio-production system for SCL-MCL-PHA from crude glycerol to reduce the production cost of PHAs.

In this study, the conversion of glycerol to SCL-MCL-PHA copolymers was investigated. Based on the understanding of the PHA biosynthetic pathway in native strains (12), a hybrid pathway (Fig 1.2) was constructed in *E. coli* by genetic engineering approach. The pathway contains two monomers supplying routes including β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) for providing SCL-monomer: (*R*)-3HB-CoA and enoyl-CoA hydratase (PhaJ) for providing MCL-monomer. PhaC polymerizes the sythesized monomers to copolymers. Additionally, to study the effects of different *pha* genes combination on the composition of copolymers, three wild type PHA-producing bacteria, *Ralstonia eutropha* (*R. eutropha*), *Aeromonas hydrophila* (*A. hydrophila*) and *Pseudomonas putida* (*P. putida*), were selected as candidates due to their ability to produce different types of PHAs. $PhaA_{Re}$ and $PhaB_{Re}$ of *R. eutropha* with three *phaC* genes which are *phaC* of *R. eutropha* ($phaC_{Re}$), *A. hydrophila* ATCC 7966 ($phaC_{Ah}$) and *P. putida* KT2440 ($phaC2_{Pp}$) and three *phaJ* genes which are $phaJ_{Ah}$, $phaJ1_{Pp}$ and $phaJ4_{Pp}$ were heterologously co-expressed in recombinant *E. coli*. Two groups of recombinant plasmids were created. The first group harbors *phaA, phaB* and *phaC* genes (pETDuet-*ABC*s). The second group harbors *phaJ* genes (pCDFDuet-*J*s). The ability of recombinant *E. coli* to synthesize PHAs from glycerol was investigated by culturing the cell in minimum medium containing glycerol as the sole carbon source. The physical and thermal properties of the produced polymers were also characterized. This study provides the basis for cost-effective production of PHA copolymers with properties suitable for industrial application to improve the competitiveness of the bio-plastic industry.

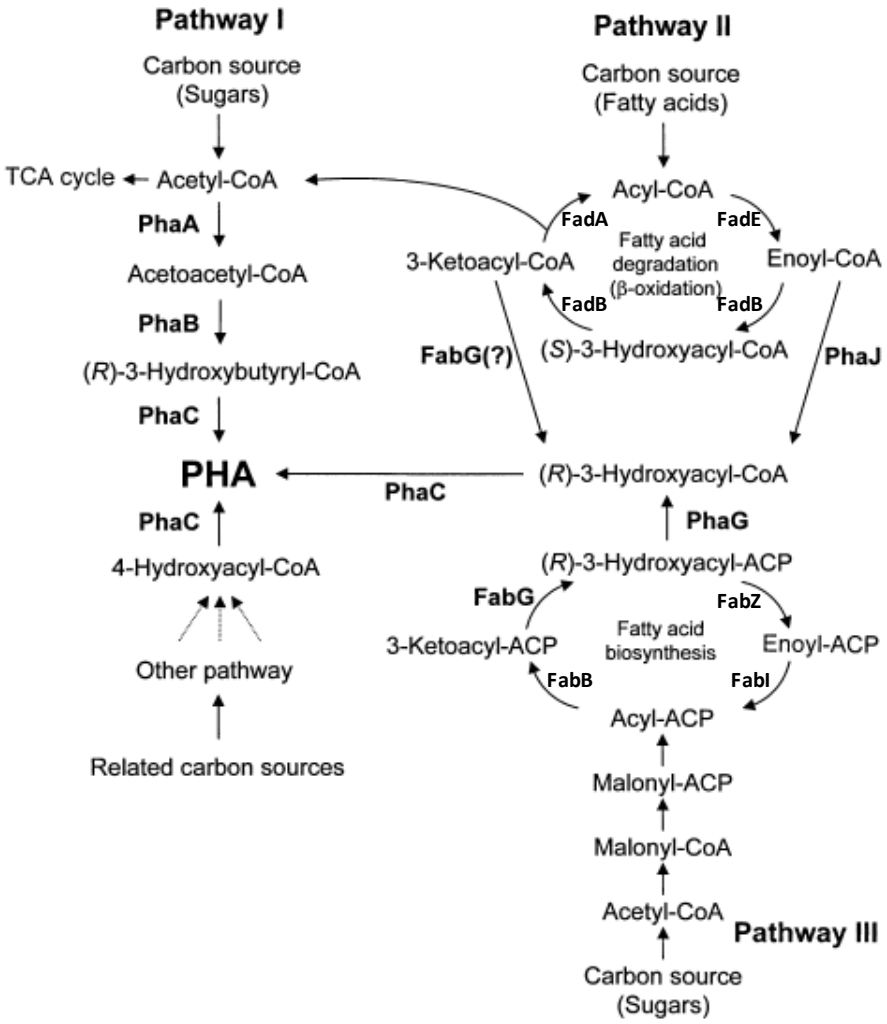


Figure 1.1 Three metabolic pathways that provide various hydroxyalkanoate (HA) monomers for PHAs biosynthesis [modified from (12) with permission from Elsevier].

There are three well-known PHA biosynthetic pathways. Pathway I provides SCL-monomer from acetyl-CoA. Pathway II and pathway III provide SCL- and MCL-monomers from the intermediates of fatty acid β-oxidation and fatty acid biosynthesis pathway, respectively. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaJ, (*R*)-specific enoyl-CoA hydratase; PhaG, 3-hydoxyacyl-ACP-CoA transferase; FadA, 3-ketoacyl-CoA thiolase; FadB, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; FadE, acyl-CoA dehydrogenase; FabB, 3-ketoacyl-ACP synthase I; FabG, 3-ketoacyl-ACP reductase; FabI, enoyl-ACP reductase; FabZ, 3-hydroxyacyl-ACP dehydratase.

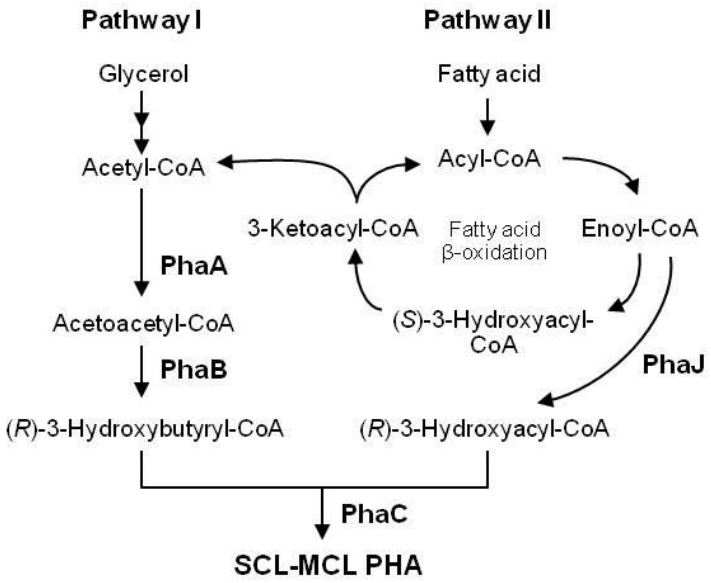


Figure 1.2 The hybrid pathways for biosynthesis of SCL-MCL-PHA copolymers in recombinant *E. coli* [reproduced from (19) with permission from JSBA].

The hybrid pathway was designed by combining two monomer-supplying pathways with one polymerization pathway. Glycerol was used as a carbon source for generating (*R*)-3-Hydroxybutyryl-CoA (SCL-monomer) by the activities of β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB), whereas fatty acid was used as a carbon source for generating (*R*)-3-Hydroxyacyl-CoA (MCL-monomer) by the activity of (*R*)-specific enoyl-CoA hydratase (PhaJ). The final step is polymerization by PHA synthase (PhaC) to biosynthesize SCL-MCL-PHA.

CHAPTER II
OBJECTIVES

1. To establish the pathway for biosynthesis of SCL-PHA and SCL-MCL-PHA copolymers in recombinant *E. coli*.

2. To investigate the effect of the substrate specificity of three different PHA synthase enzymes and three different (*R*)-specific enoyl-CoA hydratase enzymes for PHA biosynthesis.

3. To investigate the types and content of PHAs produced by recombinant *E. coli* growing under pure or crude glycerol as carbon substrate and to investigate the effect of fatty acids using as co-substrate.

4. To investigate the physical and thermal properties of PHAs produced by recombinant *E. coli*.

CHAPTER III
LITERATURE REVIEW

3.1 History of polyhydroxyalkanoates (PHAs) research

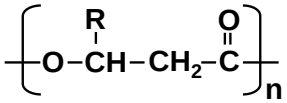
In 1926, poly(3-hydroxybutyrate) [P(3HB)] was firstly discovered and extracted from *Bacillus megaterium* by Lemoigne (20). But further research on P(3HB) failed to spread because of the limited methods for detection and characterization. The accumulated P(3HB) granule was later proven at the end of 1950s that it was an intracellular storage granule for carbon and energy (21, 22). Twenty years later, PHAs containing 3-hydroxyalkanoic acid (3HA) other than 3-hydroxybutyric acid (3HB) were identified (23, 24). This finding encouraged the research on PHAs. Numerous other 3HA constituents such as 3-hydroxyvaleric acid (3HV), 3-hydroxyhexanoic acid (3HHx), 3-hydroxyheptanoic acid (3HHp) and 3-hydroxyoctanoic acid (3HO) were discovered by the end of 1980s (25, 26). At the same time, the genes encoding for the enzymes involved in PHA biosynthesis from *Ralstonia eutropha* were cloned and the key enzyme for polymerization was named as PHA synthase (27, 28). To date, about 59 PHA synthase enzymes have been identified (29).

3.2 Chemical structure and properties of PHAs

PHAs are a family of linear polyester composed of several units of hydroxyalkanoate. The general chemical structure of PHAs is shown in Figure 3.1, where R is alkyl side chain which varies from C0 to C11 and n is number of monomer units in each polymer chain which varies from 100 to 30,000. Moreover, the monomers which contain hydroxyl groups at positions 4, 5 or 6 and the side chain group containing unsaturations or substitutions are also found. About 150 different monomer constituents of PHAs have been reported (29). Generally, PHAs can be

classified into three major groups, depending on their monomer structures. Short-Chain-Length PHAs (SCL-PHAs) compose of monomers containing 3 to 5 carbon atoms, for example, poly(3-hydroxybutyrate) P(3HB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)]. Medium-Chain-Length PHAs (MCL-PHAs) compose of monomers containing 6 to 14 carbon atoms, for example, poly(3-hydroxyoctanoate) P(3HO) and poly(3-hydroxyoctanoate-*co*-3-hydroxydecanoate) [P(3HO-*co*-3HD)]. Short-Chain-Length-*co*-Medium-Chain-Length PHAs (SCL-MCL-PHAs) contain both SCL-monomers and MCL-monomers for example, Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)].

The differences in monomer compositions affect the properties of PHAs. SCL-PHAs have high crystallinity whereas MCL-PHAs have higher flexibility (30). Therefore, the properties of SCL-MCL-PHAs can range from high crystallinity to elasticity, depending on the molar fraction of various monomer constituents. These SCL-MCL-PHAs possess superior material properties compared to those of SCL-PHAs and MCL-PHAs (16, 31). Moreover, it was found that copolymer: P(HB-*co*-HHx) has better biocompatibility than P(3HB) homopolymer (32). Due to these highly diverse PHA-monomer constituents, PHAs can be used in many applications comparable to petroleum based plastic. Table 3.1 compares the properties of PHAs to petroleum-based plastics (4, 12, 33).



R group	Type of PHA	PHA name
$-CH_3$	SCL-PHA	Poly(3-hydroxybutyrate)
$-C_3H_7$	MCL-PHA	Poly(3-hydroxyhexanoate)
$-CH_3$, $-C3H_7$	SCL-MCL-PHA	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)

Figure 3.1 General structure of polyhydroxyalkanoates (PHAs) and their examples.

PHAs are divided into three groups which are SCL-PHAs, MCL-PHAs and SCL-MCL-PHAs based on their monomer structure. The difference in monomer structure depends on the alkyl side chain group (R group). In general, R group varies from C0 to C11 and n is number of monomer units which vary from 100 to 30,000.

Table 3.1 Properties of some PHAs and petroleum based plastic [data from (4, 12, 33)].

Properties	**P(3HB)**	**P(3HB-*co*-3HHx) (10 mol% 3HHx)**	**P(3HO-*co*-3HHx) (11 mol% 3HHx)**	**PP**	**LDPE**
T_m (°C)	177	127	61	176	130
T_g (°C)	4	-1	-36	-10	-36
Crystallinity (%)	60	34	30	50-70	20-50
Extension to break (%)	5	400	300	400	620
Tensile strength (Mpa)	43	21	-	38	10

PP, polypropylene; LDPE, low density polyethylene; T_m, melting temperature; T_g, glass transition temperature

3.3 PHAs biosynthesis

PHAs can be produced by many types of microorganism from various renewable substrates such as sugar, starch, and plant oil. In most cases, bacteria produce PHAs under unbalance growth conditions such as limitation of nitrogen, phosphorus, or oxygen in the presence of excess carbon. PHAs are accumulated inside the cells as intracellular granules for carbon and energy storage (Fig 3.2) (34, 35). It is an ideal storage granule due to its high molecular weight and low solubility which does not affect the bacteria osmotic pressure (36). Also, bacteria containing PHA granules are able to survive during starvation in the absence of exogeneous carbon source by utilizing the accumulated PHAs (37).

(A) (B)

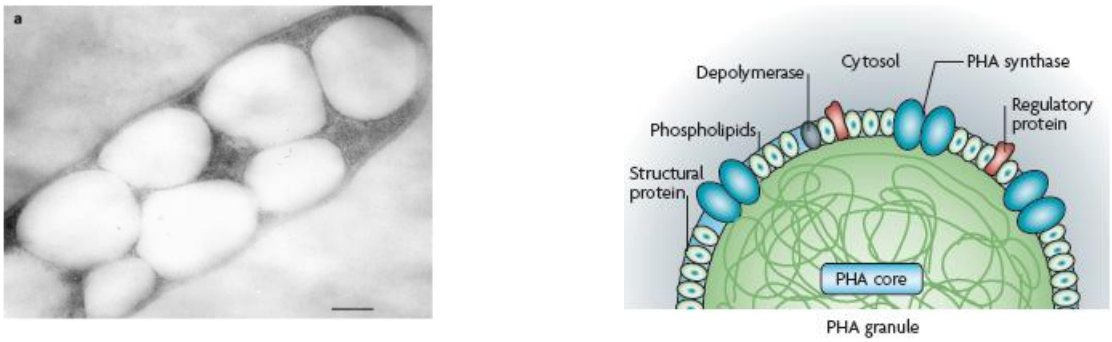


Figure 3.2 PHA granule characteristic. (A) PHA granules accumulated inside *Ralstonia eutropha*. Bars, 0.2 µm. (B) Cartoon drawing represents the compositions of PHA granule [(A) is reproduced from (35) with permission from Society for General Microbiology and (B) is reproduced from (34) with permission from Nature Publishing Group].

PHA is deposited as spherical intracellular granules with an amorphous hydrophobic core surrounded by proteins involving in PHA metabolism. In PHA-producing bacteria, PHAs are produced when the carbon source is excess while the other nutrients are limited. When the carbon starvation occurs, the reserved PHA granules are mobilized by intracellular PHA depolymerase.

3.3.1 PHA biosynthetic pathways

The constituent of PHAs depends on the carbon sources, PHA synthases, and monomer supplying metabolic routes (38, 39). There are three well-known PHA biosynthesis pathways (Fig 1.1) (12).

Pathway I produces SCL-PHAs. Acetyl-CoA is a central molecule for P(3HB) biosynthesis. Under normal growth condition, acetyl-CoA is oxidized through tricarboxylic acid cycle (TCA) to CO_2. However, under nutrient limitation, the accumulation of acetyl-CoA and NADH, starts P(3HB) biosynthesis (40). Three main catalysis enzymes: β-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC), are responsible for P(3HB) biosynthesis. Two molecules of acetyl-CoA are condensated to acetoacetyl-CoA by the function of PhaA. Then acetoacetyl-CoA is subsequently reduced to (*R*)-3-hydroxybutyryl-CoA by PhaB. (*R*)-3-hydroxybutyryl-CoA molecules are then be polymerized by PhaC to produce P(3HB).

Pathway II and III produce MCL-PHAs. Pathway II involves fatty acid β-oxidation cycle. When bacteria are grown on fatty acids, fatty acids are catabolized by β-oxidation, providing precursors for MCL-PHAs biosynthesis. β-oxidation of saturated fatty acids has four basic steps. First, fatty acyl-CoA is dehydrogenated by acyl-CoA dehydrogenase (FadE) yielding *trans*-2-enoyl-CoA. In the second step, water is added to the double bond of *trans*-2-enoyl-CoA, yielding (*S*)-3-hydroxyacyl-CoA by the activity of enoyl-CoA hydratase (FadB). The third step, (*S*)-3-hydroxyacyl-CoA is dehydrogenated to form 3-ketoacyl-CoA by the activity of 3-hydroxyacyl-CoA dehydrogenase (FadB). The fourth step, 3-ketoacyl-CoA thiolase (FadA) catalyzes (*S*)-3-hydroxyacyl-CoA to produce acyl-CoA shortened by two carbon atoms and acetyl-CoA.

Two intermediates including *trans*-2-enoyl-CoA and 3-ketoacyl-CoA seem to be the precursors of PHAs. To channel these two intermediates to PHA biosynthesis, it is necessary to convert them to (*R*)-3-hydroxyacyl-CoAs which are the monomer substrates of PHA synthase. Two enzymes, (*R*)-specific enoyl-CoA hydratase (PhaJ) and 3-ketoacyl-ACP reductase (FabG), are responsible for converting *trans*-2-enoyl-CoA and 3-ketoacyl-CoA to (*R*)-3-hydroxyacyl-CoAs, respectively.

Thus, monomer molecules varying in the carbon atom number are produced by shortening two carbon of acetyl-CoA out of each cycle of β-oxidation (41).

Pathway III involves the fatty acid biosynthesis pathway (*de novo* fatty acid synthesis). There are four steps in fatty acid elongation cycle. Two carbon atoms elongation is started with condensation of acyl-ACP with malonyl-ACP by the activity of 3-ketoacyl-ACP synthase I (FabB) or II (FabF) resulting in 3-ketoacyl-ACP. The second step, 3-ketoacyl-ACP is reduced by 3-ketoacyl-ACP reductase (FabG) yielding (*R*)-3-hydroxyacyl-ACP. The third step, (*R*)-3-hydroxyacyl-ACP is dehydrated by 3-hydroxyacyl-ACP dehydratase (FabZ) yielding enoyl-ACP. The fourth step, enoyl-ACP is reduced by enoyl-ACP reductase (FabI) resulting in acyl-ACP with two added carbon atoms (42). An intermediate: (*R*)-3-hydroxyacyl-ACP seems to be a precursor for PHAs. (*R*)-3-hydroxyacyl-ACP is converted to (*R*)-3hydroxyacyl-CoA which is a monomer substrate of PHA synthase by the activity of 3-hydoxyacyl-ACP-CoA transferase (PhaG) (43). Unlike pathway II, paythway III is considered as a non-substrate related pathway (such as from glucose and gluconate).

3.3.2 PHA-producing bacteria

There are several bacterial strains that can produce PHAs. The overview of bacterial strains used for PHAs production is shown in Table 3.2 (30). Based on the type of PHAs, three native PHA-producing bacterial strains have been studied intensively. These include *Ralstonia eutropha* producing SCL-PHAs, *Aeromonas* strains producing SCL-MCL-PHAs, and *Pseudomonas* strains producing MCL-PHAs. In addition, several recombinant *E. coli* strains have been constructed for PHAs production (28, 44-46).

Table 3.2 Overview of bacterial strains used for PHAs production [reproduced from (30) with permission from John Wiley and Sons].

Bacterial strain (s)	Carbon source (s)	Polymer (s) produced	Reference
Aeromonas hydrophila	Lauric acid, oleic acid	mcl-PHAs	(Lee *et al.* 2000; Han *et al.* 2004)
Alcaligenes latus	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB	(Wong *et al.* 2004, 2005)
Bacillus cereus	Glucose, ε-caprolactone, sugarbeet molasses	PHB, terpolymer	(Labuzek and Radecka 2001; Yilmaz and Beyatli 2005; Valappil *et al.* 2007)
Bacillus spp.	Nutrient broth, glucose, alkanoates, ε-caprolactone, soy molasses	PHB, PHBV, copolymers	(Katircioglu *et al.* 2003; Shamala *et al.* 2003; Tajima *et al.* 2003; Yilmaz *et al.* 2005; Full *et al.* 2006)
Burkholderia sacchari sp. nov.	Adonitol, arabinose, arabitol, cellobiose, fructose, fucose, lactose, maltose, melibiose, raffinose, rhamnose, sorbitol, sucrose, trehalose, xylitol	PHB, PHBV	(Brämer *et al.* 2001)
Burkholderia cepacia	Palm olein, palm stearin, crude palm oil, palm kernel oil, oleic acid, xylose, levulinic acid, sugarbeet molasses	PHB, PHBV	(Keenan *et al.* 2004; Nakas *et al.* 2004; Alias and Tan 2005; Çelik *et al.* 2005)
Caulobacter crescentus	Caulobacter medium, glucose	PHB	(Qi and Rehm 2001)
Escherichia coli mutants	Glucose, glycerol, palm oil, ethanol, sucrose, molasses	(UHMW)PHB	(Mahishi *et al.* 2003; Kahar *et al.* 2005; Park *et al.* 2005a; Nikel *et al.* 2006; Sujatha and Shenbagarathai 2006)
Halomonas boliviensis	Starch hydolysate, maltose, maltotetraose and maltohexaose	PHB	(Quillaguaman *et al.* 2005, 2006)
Legionella pneumophila	Nutrient broth	PHB	(James *et al.* 1999)
Methylocystis sp.	Methane	PHB	(Wendlandt *et al.* 2005)
Microlunatus phosphovorus	Glucose, acetate	PHB	(Akar *et al.* 2006)
Pseudomonas aeruginosa	Glucose, technical oleic acid, waste free fatty acids, waste free frying oil	mcl-PHAs	(Hoffmann and Rehm 2004; Fernández *et al.* 2005)
Pseudomonas oleovorans	Octanoic acid	mcl-PHAs	(Durner *et al.* 2000; Foster *et al.* 2005)
Pseudomonas putida	Glucose, octanoic acid, undecenoic acid	mcl-PHAs	(Tobin and O'Connor 2005; Hartmann *et al.* 2006)
Pseudomonas putida, P. fluorescens, P. jessenii	Glucose, aromatic monomers	aromatic polymers	(Tobin and O'Connor 2005; Ward and O'Connor 2005; Ward *et al.* 2005)
Pseudomonas stutzeri	Glucose, soybean oil, alcohols, alkanoates	mcl-PHAs	(Xu *et al.* 2005)
Rhizobium meliloti, R. viciae, Bradyrhizobium japonicum	Glucose, sucrose, galactose, mannitol, trehalose, xylose, raffinose, maltose, dextrose, lactose, pyruvate, sugar beet molasses, whey	PHB	(Mercan and Beyatli 2005)
Rhodopseudomonas palustris	Acetate, malate, fumarate, succinate, propionate, malonate, gluconate, butyrate, glycerol, citrate	PHB, PHBV	(Mukhopadhyay *et al.* 2005)
Spirulina platensis (cyanobacterium)	Carbon dioxide	PHB	(Jau *et al.* 2005)
Staphylococcus epidermidis	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB	(Wong *et al.* 2004, 2005)
Cupriavidus necator	Glucose, sucrose, fructose, valerate, octanoate, lactic acid, soybean oil	PHB, copolymers	(Kim *et al.* 1995; Kichise *et al.* 1999; Taguchi *et al.* 2003; Kahar *et al.* 2004; Khanna and Srivastava 2005a; Volova and Kalacheva 2005; Volova *et al.* 2005)
Cupriavidus necator H16	Hydrogen, carbon dioxide	PHB	(Pohlmann *et al.* 2006)

3.3.2.1 *Ralstonia eutropha*

Ralstonia eutropha is a Gram-negative, facultative chemolithoautotrophic bacterium and is a model strain for the study of PHA metabolism. It is able to synthesize and accumulate P(3HB) in the cytoplasm as carbon and energy storage from simple carbon sources such as glucose, fructose and acetic acid (47, 48). In 2006, Pohlmann et al., sequenced the whole genome of *R. eutropha* H16 (49). Therefore, many key components of PHA metabolism in *R. eutropha* have been revealed (Fig 3.3). For P(3HB) biosynthesis, three key enzymes, β-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC), are responsible for a three-step reaction starting with the condensation of two acetyl-CoA to acetoacetyl-CoA then reduces to (*R*)-3-hydroxybutyryl-CoA and polymerizes to P(3HB) (Pathway I in Fig 1.1). These enzymes are encoded by *phaA*, *phaB*, and *phaC* respectively. These genes are organized in one operon as *phaCAB* (Fig 3.4A) (50).

It was reported that *R. eutropha* accumulated large amount of P(3HB) at 76% of cell dry weight when cultured in glucose with fed-batch fermentation (51). In fact, *R. eutropha* can produce PHAs consisting of various SCL-3-hydroxyalkanoic acid (SCL-3HA), for example, P(3HB-*co*-3HP) (52) and P(3HB-*co*-3HV) by co-feeding with propionic acid (53). Moreover, Green et al., 2000 reported that *R. eutropha* can produce PHAs consisting not only SCL-3HA but also MCL-3HA (at a minor fraction) when sodium acrylate was added to the culture medium to inhibit the 3-ketoacyl-CoA thiolase activity in the final step of β-oxidation (54). The result suggested that PHA synthase of *R. eutropha* ($PhaC_{Re}$) has broad range substrate specificity and can polymerize both SCL-3HA and MCL-3HA monomers.

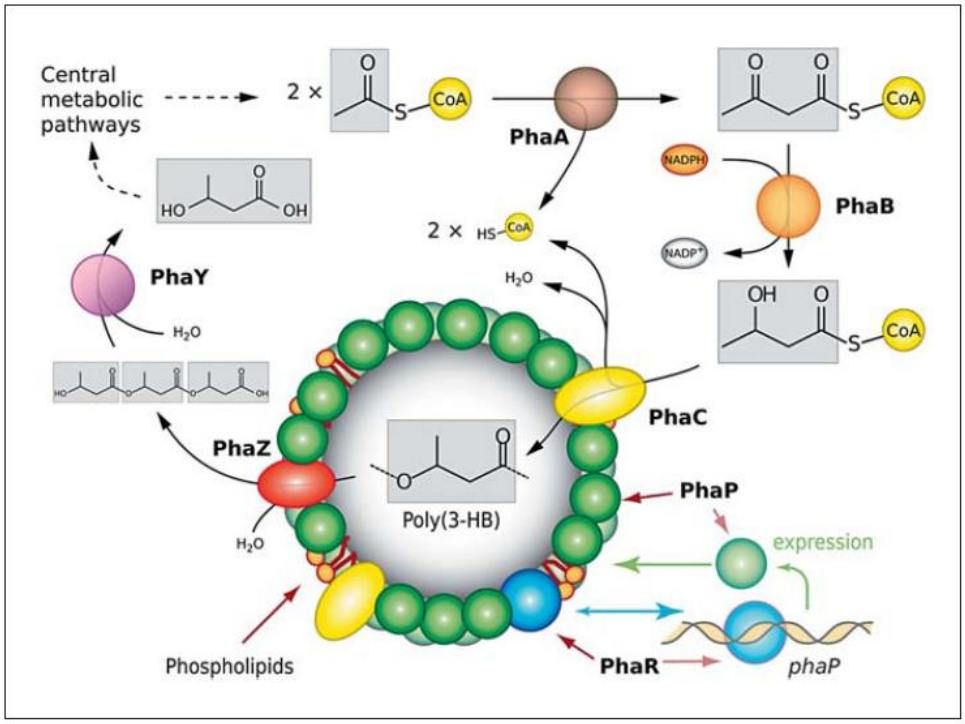


Figure 3.3 The elements involved in P(3HB) metabolism in *R. eutropha* H16 [reproduced from (48) with permission from S. Karger AG].

The enzymes and proteins involved directly in P(3HB) metabolism are represented in bold text. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaZ, P(3HB) depolymerase; PhaY, 3HB-oligomer hydrolase; PhaP, Phasin; PhaR, Transcriptional regulator of Phasin expression.

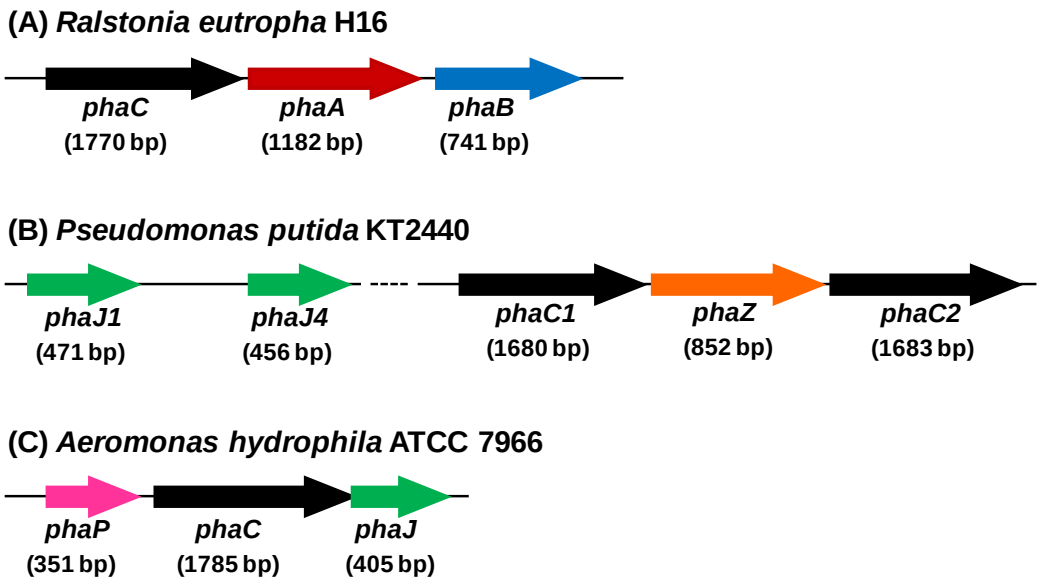


Figure 3.4 The organization of *pha* genes in three native PHA-producing bacteria. (A) a complete *phaCAB* operon in *Ralstonia eutropha*; (B) two *phaC*s, *phaC1* and *phaC2*, are separated by *phaZ* in *Pseudomonas putida* KT2440 downstream of *phaJ1* and *phaJ4*; (C) *phaPCJ* operon in *Aeromonas hydrophila* ATCC 7966. *phaA*, β-ketothiolase; *phaB*, NADPH-dependent acetoacetyl-CoA reductase; *phaC*, PHA synthase; *phaJ*, (*R*)-specific enoyl-CoA hydratase; *phaP*, phasing (PHA granule associated protein)*; phaZ*, PHA depolymerase.

3.3.2.2 *Pseudomonas* spp.

Some *Pseudomonas* spp. can synthesize and accumulate PHAs of various MCL-3HAs under stressed condition when grown on related-carbon sources such as plant oil, fatty acids or alkanoic acids (55-57) as well as non-related carbon sources such as glucose or gluconate (43, 58, 59). The biosynthesis routes of PHAs in *Pseudomonas* spp. are linked with fatty acid β-oxidation and *de novo* fatty acid biosynthesis pathway (pathway II and III in Fig 1.1) (60). Among many strains of *Pseudomonas*, an attractive strain is *Pseudomonas putida* KT2440. This strain is a metabolically versatile saprophytic soil bacterium which can utilize various carbon sources such as glycerol, gluconate, and fatty acids (61, 62). In 2002, the whole genome of *Pseudomonas putida* KT2440 was sequenced and the putative genes encoding enzymes involved in the PHA biosynthesis have been identified (63). There are two *phaC* genes (*phaC1* and *phaC2*) separated by *phaZ* (Fig 3.4B) which are similar to that from *Pseudomonas* strains such as *P. oleovorans* (64), *P. aeruginos*a (65), and *Pseudomonas* sp. strain 61-3 (66). PhaC1 of *P. putida* KT2440 was cloned and expressed in *E. coli* revealing its substrate specificity toward MCL-monomers: 3-hydroxyoctanoyl-CoA (C8) and 3-hydroxydecanoyl-CoA (C10) (67). Moreover, three homologs of *phaJ* (*phaJ1*, *phaJ3*, and *phaJ4*) of *P. aeruginosa* are found based on genome database search (62, 68). PhaJ1 and PhaJ4 were heterologously expressed in *E. coli* revealing that they exhibit high substrate specificity toward MCL-enoyl-CoA. By monitoring the gene expression level and characterization of hydratase activity in *P. putida* cytosolic protein, it was suggested that PhaJ4 is one of the main contributor involved in PHA biosynthesis *via* fatty acid β-oxidation (62, 69).

3.3.2.3 *Aeromonas* spp.

Aeromonas spp. are Gram-negative, facultative anaerobic bacteria which are found in aquatic environment (70). They are reported to be the cause of some diseases such as bacterial gastroenteritis, prostatitis, and hemolytic-uremic syndromethese in invertebrates and vertebrates such as fish, frogs, bird, domestic animal, and human (11). Nonetheless, *Aeromonas strains* are intensively studied for their ability to synthesize PHA consisting of both SCL-3HA and MCL-3HA units (9, 71, 72). In fact, there are only a few bacterial strains that can

produce SCL-MCL-PHAs; for example, some *Pseudomonas* strains (73, 74), *Rhodocyclus gelatinous* (75), and *Rhodococcus rubber* (76). *Aeromonas* spp. produce P(3HB-*co*-3HHx) from alkanoic acid or from plant oil. The P(3HB-*co*-3HHx) biosynthesis pathway of *Aeromonas caviae* was proposed by Fukui et al. (Fig 3.5) (13, 77). The key enzymes for P(3HB-*co*-3HHx) biosynthesis in *A. caviae* are PHA synthase (PhaC) and (*R*)-specific enoyl-COA hydratase (PhaJ). The precursors for P(3HB-*co*-3HHx) synthesis are supplied through fatty acid β-oxidation via the activity of (*R*)-specific enoyl-CoA hydratase (PhaJ) (pathway II in Fig 1.1). In 2006, the genome of *Aeromonas hydrophila* ATCC 7966 was sequenced and revealed its PHA biosynthesis genes system (70). The genes are organized in the genome as *phaPCJ* operon (Fig 3.4C) which is similar to that of *A. caviae* (77).

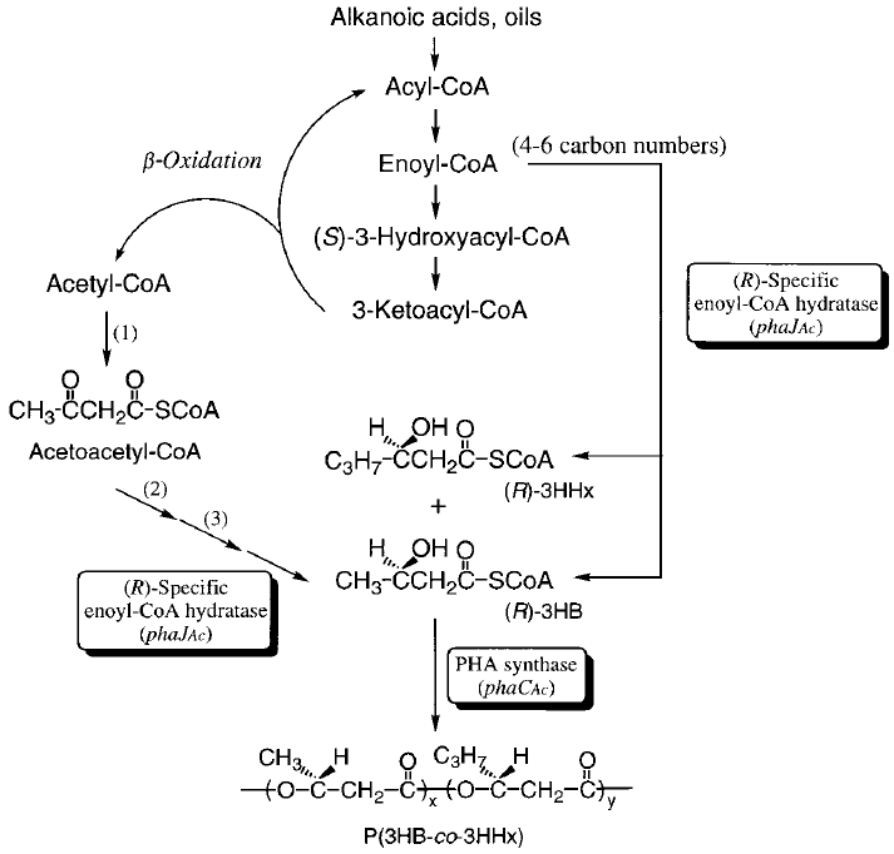


Figure 3.5 Proposed pathway of P(3HB-*co*-3HHx) biosynthesis in *A. caviae* from alkanoic acids or oils. (1), β-ketothiolase; (2), NADH-acetoacetyl-CoA dehydrogenase; (3), crotonase [(*S*)-specific enoyl-CoA hydratase] [reproduced from (77) with permission from American Society for Microbiology].

3.3.2.4 Recombinant *Escherichia coli*

Escherichia coli is a useful host strain for production of several products. There are many advantages of using *E. coli* as a producer over wild-type PHA-producing bacteria. They are: fast growth to high cell density, no special cultivated conditions required, the ability to utilize several carbon substrates, relatively easy extraction and purification of PHAs produced from *E. coli*, lack of intracellular PHA depolymerase and is safe for large scale production (78, 79). Moreover, biosynthesis of PHAs in host organisms that naturally do not produce PHAs allows modulation and regulation of the biosynthetic pathway (80). Several metabolic engineering strategies, either individually or in combination, have been used to construct recombinant *E. coli* for production of a variety of PHAs with desired compositions and properties. These include host genome manipulation, recombinant gene expression, protein engineering, external substrate manipulation and addition of inhibitor (Fig 3.6) (81).

3.3.2.4.1 SCL-PHA production

Among many type of SCL-PHAs, P(3HB) is the best characterized. Several studies have established the P(3HB) biosynthesis pathway in recombinant *E. coli* and has developed strategies for high production of P(3HB) (79). As mentioned above, there are two key enzymes, β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB), which are involved in supplying 3-hydroxybutyryl-CoA (3HB-CoA) for PHA synthase (PhaC) (pathway I). The *phaCAB* operons of various P(3HB)-producing bacteria, for example *Alcaligenes eutrophus*, *Methylobacterium extorquens*, *Rhizobium meliloti*, and *Streptomyces aureofaciens*, have been identified and heterologously expressed in *E. coli* which lead to P(3HB) accumulation (28, 44-46). Moreover, the other factors that affect the biosynthesis of P(3HB) in *E. coli* have also been manipulated. Acetyl-CoA, serving as a precursor to P(3HB) monomer, was modulated by inactivation of the *pta* gene encoding a phosphotransacetylase of *E. coli* host genome (82). NADPH, required as a reducing power for acetoacetyl-CoA reductase (PhaB), was increased by knocking out the *pgi* gene encoding phosphoglucose isomersase (83) or co-transformation of *tkt* gene encoding transketolase with *phaCAB* (84).

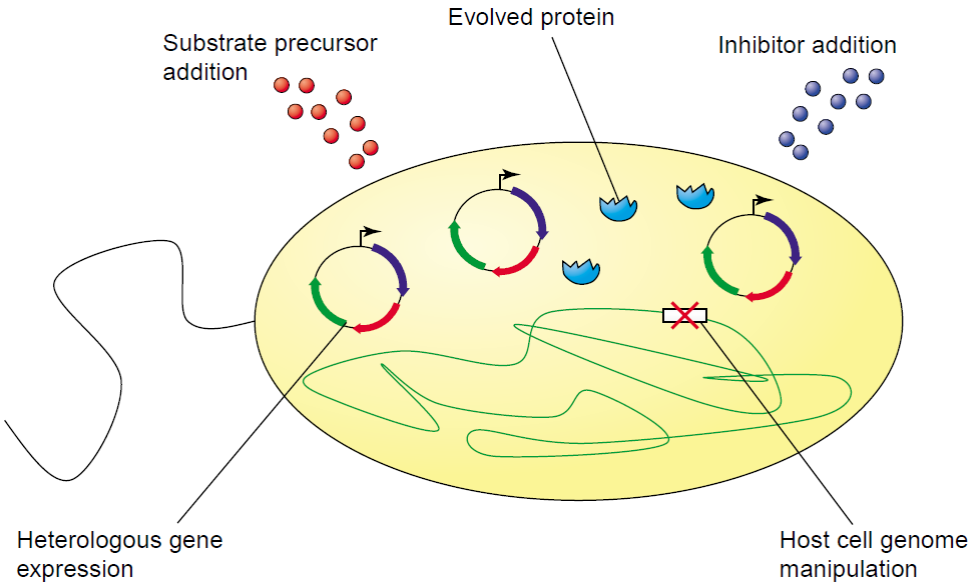


Figure 3.6 Schematic of various strategies for metabolic engineering [reproduced from (81) with permission from Elsevier].

Many approaches can be used for metabolic engineering. For example, substrate precursors can be added into the culture media. Protein evolution and mutagenesis are used to enhance the enzyme activity. Adding some inhibitors into the culture media or host cell genome manipulation can be used to inhibit some competitive pathways. Heterologous gene expression is used to introduce target enzymes into the recombinant host.

3.3.2.4.2 MCL-PHA production

MCL-PHA production is linked with fatty acid metabolism (Pathway II and III). Some intermediates of fatty acid β-oxidation and *de novo* fatty acid biosynthesis including enoyl-CoA, 3-ketoacyl-CoA, (*S*)-3-hydroxyacyl-CoA, and 3-hydroxyacl-ACP are seen to be major precursors of PHA biosynthesis. The enzymes involved are (*R*)-specific enoyl-CoA hydratase (PhaJ) (68, 77, 85, 86), 3-ketoacyl-ACP reductase (FabG) (87, 88), and 3-hydoxyacyl-ACP-CoA transferase (PhaG) (43, 89) were identified and cloned from native PHA-producing bacteria. Table 3.3 shows the list of these enzymes and other enzymes which have been successfully expressed in recombinant *E. coli* for PHA biosynthesis from fatty acid metabolism (41).

Metabolic engineering of fatty acid metabolism pathway for supporting PHA biosynthesis in recombinant *E. coli* is considered in two ways (individually or in combination). The former is inhibition of the enzymes in fatty acid metabolism and the later is amplification of enzymes that convert the intermediates from fatty acid metabolism pathway to monomers of PHAs. For enzyme inhibition, *fadA* and/or *fadB* mutant *E. coli* strains are popular (87, 90-93). The first successful biosynthesis of MCL-PHA in recombinant *E. coli* from fatty acids was established by expressing *phaC1* gene of *P. aeruginosa* in *fadB* mutant *E. coli* (93). In *fadB* mutant *E. coli* strain, *E. coli* MaoC which is homologous to *P. aeruginosa* (*R*)-specific enoyl-CoA hydratase (PhaJ) was found to be important for supplying (*R*)-3HA-CoA from the intermediate of β-oxidation pathway to PHA biosynthesis pathway. However, it was found that only PhaC expression in *E. coli* with a functional β-oxidation pathway did not lead to the production of PHA suggesting that the activity of *E. coli* MaoC toward enoyl-CoA is weaker than FadB in β-oxidation pathway (94).
The second way is amplification of enzymes that convert the intermediates from fatty acid metabolism pathway to monomers of PHAs. Various enoyl-CoA hydratase (PhaJ) with different substrate specificities from various natural PHA-producing bacteria have been cloned and expressed in *E. coli* (68, 77, 86, 95). Here, however, high-level expression of PhaJ is needed to compete with the β-oxidation pathway enzyme for channeling its intermediates to PHA biosynthesis pathway.

Table 3.3 List of enzymes which have been successfully expressed in recombinant *E. coli* for PHA biosynthesis from fatty acid metabolism [reproduced from (41) with permission from Elsevier].

Key enzymes for PHA biosynthesis via fatty acid metabolism in recombinant *E. coli*

Enzymes	Characteristic	Representative sources	Substrates[a]	References
PHA synthase				
PhaC1, PhaC2	Polymerization of HA-CoAs	Pseudomonads	C6–C12	[5]
PhaC		Aeromonads	C4, C6	[36]
Enoyl-CoA hydratase				
PhaJ	Hydration of enoyl-CoA	Aeromonads	C4, C6	[21,23]
ePhaJ[b]		*A. caviae*	C4–C12	[25]
PhaJ1, PhaJ2, PhaJ3, PhaJ4		*P. aeruginosa*	C6–C12	[24,26]
MaoC		*E. coli*	C6–C10	[37]
YfcX[c]		*E. coli*	C4–C12	[38]
PaaG[c], PaaF[c], YdbU[c]		*E. coli*	C6–C12	[39]
Ketoacyl-CoA reductase				
FabG	Reduction of 3-ketoacyl-CoA	*E. coli*	C6–C12	[18–20]
RhlG		*P. aeruginosa*	C6–C10	[18]
3-Hydroxydecanoyl-ACP:CoA transacylase				
PhaG	Conversion of 3-hydroxyacyl-ACP to 3-hydroxyacyl-CoA	*P. putida*	C6–C12	[35]
FabH, FabD		*E. coli*	C4	[40]
eFabH[d]		*E. coli*	C4–C10	[41]

[a] Substrate specificity was assumed based on the monomer composition of PHA copolymers.
[b] Engineered *A. caviae* PhaJ.
[c] Multifunctional enzyme carrying out hydration of enoyl-CoA and dehydration of 3-HA-CoA.
[d] Engineered *E. coli* FabH.

3.3.2.4.3 SCL-MCL-PHA production

Only a few bacteria with the ability to synthesize SCL-MCL-PHAs have been discovered; for example, *Aeromonas caviae* and *Aeromonas hydrophila* which produce P(3HB-*co*-3HHx) from alkanoic acids (9, 10) and *Pseudomonas* sp. 61-3 which produces random copolymer consisting of C4-C12 monomer units from gluconate (8). Metabolic pathways for production of SCL-MCL-PHAs have successfully been established in recombinant *E. coli*. Several combination patterns of *pha* genes have been created. For example, PHA synthase gene ($phaC_{Re}$) of *R. eutropha* was co-expressed with two monomer supplying enzymes encoded by β-ketothiolase ($phaA_{Re}$) and acetoacetyl-CoA reductase ($phaB_{Re}$) genes in *E. coli* mutant in fatty acid β-oxidation (*E. coli fad*B mutant LS1298). The recombinant strain produced SCL-MCL-PHAs consisting of 3HB and 3HO when using octanoate or decanoate as carbon source (14). The pathway for biosynthesis of copolymers from glucose was established in *E. coli* JM109 by co-expression of various mutant 3-ketoacyl-acyl carrier protein synthase III genes (*fabH*) with *phaC1* of *Pseudomonas* sp. 61-3 resulting in production of copolymers consisting of C4 to C10 monomers with variation of %molar fraction (15). Moreover, the hybrid pathway which combines two monomer supplying routes including PhaA and PhaB from

Bacillus sp. 256 and PhaJ from *P. aeruginosa* was constructed in *E. coli* and they were co-expressed with PhaC of *P. aeruginosa*. The recombinant strain can produce copolymers from glucose and fatty acids (96).

3.4 Biodegradation of PHAs

In the environment, PHAs can be degraded by both heat and the activity of some microorganisms such as bacteria (*Bacillus* sp., *Pseudomonas* sp., and *Streptomyces* sp.) and fungi (*Aspergillus ustus* and *Penicillium* sp.) (37, 97, 98). Microorganisms attack to the surface of PHA and secrete the enzyme called extracellular PHA depolymerase to solubilize PHA to its oligomers. The oligomers are then absorbed into the cell and hydrolyzed by some hydrolase to its monomers. The monomers are then used as nutrient (99). The rate of PHAs biodegradation depends on the characteristic of PHAs such as monomer compositions, crystallinity, and molecular weight as well as on the environmental conditions such as temperature, pH, and moisture. For example, P(3HB) takes a few months to be degraded in anaerobic compost but takes a year to be degraded in seawater (100-102). Besides the degradation of PHAs in the environment, PHAs used in biomedical material like surgical sutures, implant patches, and drug delivering can be degraded in the living organism by some enzymes in blood and tissue such as lipase and esterase (103, 104). The products from the PHAs degradation have no toxicity effects on the living organisms (105, 106).

3.5 Applications of PHAs

PHAs have a wide range of applications due to their properties, ranging from thermoplastic to elastomers (Fig 3.7) (107). Detailed information of three major applications including industrial, medical, and agricultural are:

3.5.1 Industrial applications

Earlier PHAs were developed for everyday-use applications. For example, P(3HB) was used to produce combs, pens, and bullets by Biomer company (Germany). Due to the gas barrier property of P(3HB-*co*-3HV), it is suitable for use in food packaging, plastic beverage bottles, coated paper and film. P(3HB-*co*-3HHx) has been used to make the flexible products such as flexible packaging, binder, and nonwovens (99).

3.5.2 Medical applications

Due to the biodegradability and biocompatibility property of PHA, the recent development has been focused on their medical applications; for example, implant materials (bone marrow scaffolds, cardiovascular patches, and surgical sutures) and drug delivery (tablets, drug controlled-release matrices) (108).

3.5.3 Agricultural applications

PHAs are useful in agriculture applications since they can be degraded in both aerobic and anaerobic soil conditions (101). PHAs have been used for coating of seeds, fertilizer, insecticide, and herbicides, for the slow release in soil. In addition they are used as mulch film and plastic containers as there is no need for removal after harvesting process (30).

Figure 3.7 Applications of PHAs [reproduced from (107) with permission from Springer].

PHAs can be used to produce many kinds of plastic products such as for food packaging and fiber. Chiral-(*R*)-form of PHA monomers can be used as starting materials in industrial fermentation. PHAs are used as bio-implants and drug encapsulators. PHAs are catalyzed to hydroxyalkanoate methyl esters which are used as biofuels. (*R*)-3HB hydrolyzed from P(3HB) has therapeutic potential on Alzheimer's disease.

3.6 Glycerol

3.6.1 Glycerol from biodiesel production

Glycerol is becoming an interesting carbon source in biotechnology development since it is a by-product from biodiesel production. Recently, biodiesel which is an alternative fuel has seen an increased in production. Approximately 10% (w/w) crude glycerol was produced as a by-product from transesterification of vegetable oils and animal fat in biodiesel production (Fig 3.8). It is estimated that the world biodiesel market would come to 37 billion gallons by 2016, which means that approximately 4 billion gallons of crude glycerol will be produced. The compositions of crude glycerol are varied with biodiesel production process (types of catalyst, the nature of the starting materials, the alcohol/oil ratio, the temperature of reaction, water content, and free fatty acid content) (109). In general it is composed of glycerol as a major component, mono-/di-/tri glycerides, soap and a substantial content of methanol and salts (110). The flow chart of alkaline catalyzed biodiesel production is depicted (Fig 3.9). Although purified glycerol is used in many applications such as in food, cosmetic, and pharmaceutical industries (111), the surplus glycerol still exists, which leads to remarkable reduction in its price in the world market from 650-700 €/ton in 1996 to 150-200 €/ton in 2005 (112).

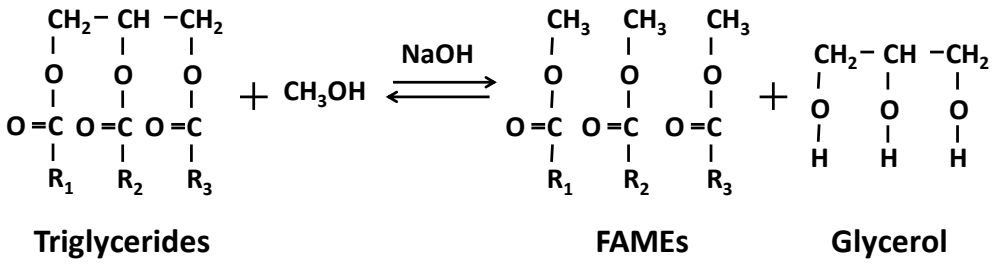


Figure 3.8 Biodiesel reaction.

Transesterification of triglycerides with methanol using NaOH as a catalyst producing biodiesel or fatty acid methyl esters (FAMEs) and glycerol.

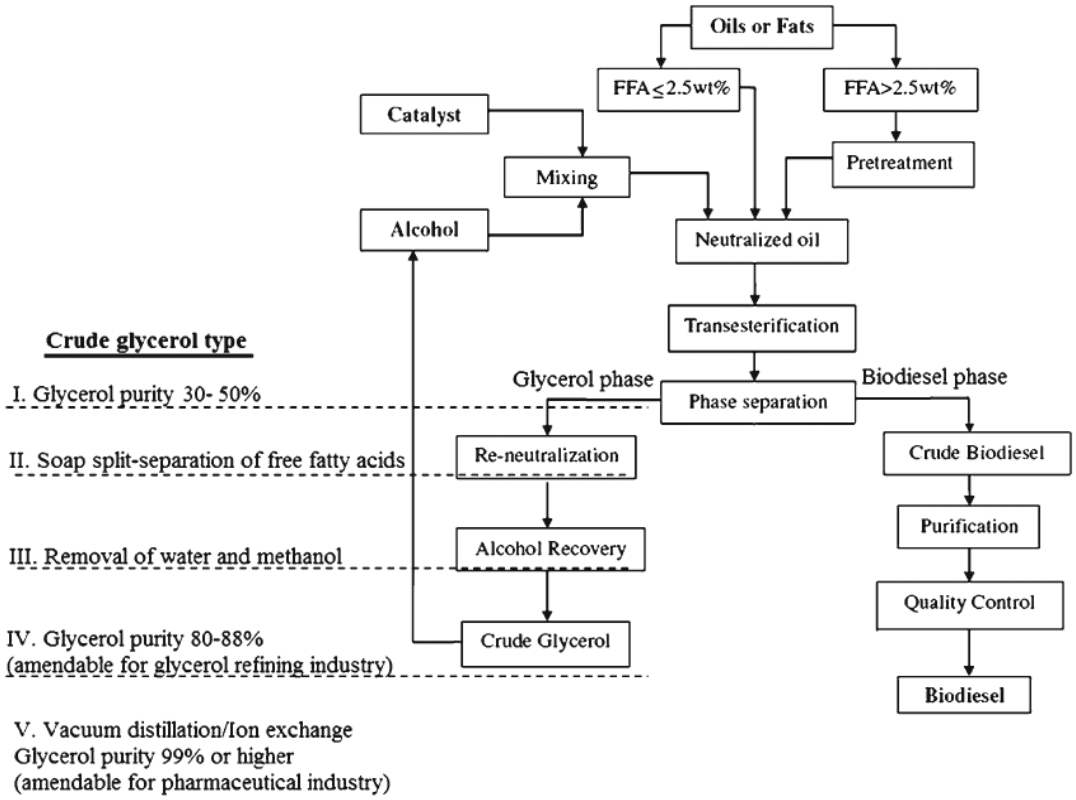


Figure 3.9 Flow chart of alkaline catalyzed biodiesel production [reproduced from (109) with permission from Springer].

Compositions of crude glycerol depend on starting materials (oil/fat, alcohol, and catalyst), the transesterification method (molar ratio of alcohol-to-oil and temperature), and the process for separation.

3.6.2 Glycerol catabolism in *E. coli*

Glycerol is a polyol compound that has three hydroxyl groups. *E. coli* catabolizes glycerol through the functions of glycerol transporter, ATP-dependent glycerol kinase, and glycerol-3-phosphate dehydrogenase (Fig 3.10) (113).

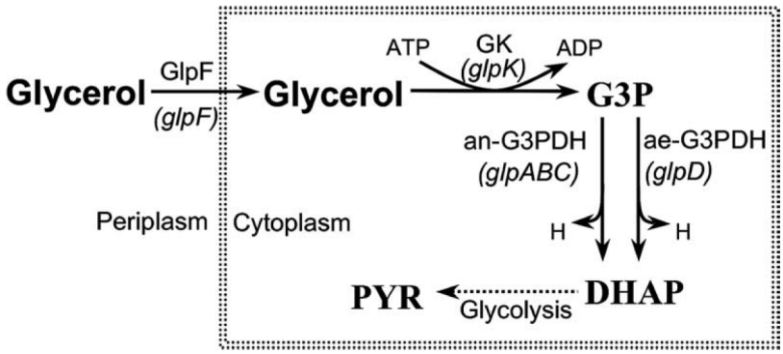


Figure 3.10 Glycerol catabolism in *E. coli* [reproduced from (113) with permission from American Society for Microbiology].

Glycerol catabolism in *E. coli* involves a glycerol transporter (encoded by *glpF*), an ATP-dependent glycerol kinase (GK encoded by *glpK*), glycerol-3-phosphate dehydrogenase (G3PDH encoded by *glpD*).

3.6.3 Conversion of glycerol to PHAs

Many studies investigated the alternative uses of crude glycerol, for example, combustion for heat (114), use in animal feeding (115), and conversion by thermochemical process to higher-value products (114, 116). Moreover, glycerol can be used as a carbon feed stock for microorganism for production of value-added products such as hydrogen, ethanol, 1,3-propanediol, and polyhydroxyalkanoates (PHAs) (117, 118). Figure 3.11 shows the overview of the pathways and products converted from glycerol. Production of PHAs from glycerol has been reported using either wild-type or recombinant bacterial strains (Table 3.4)

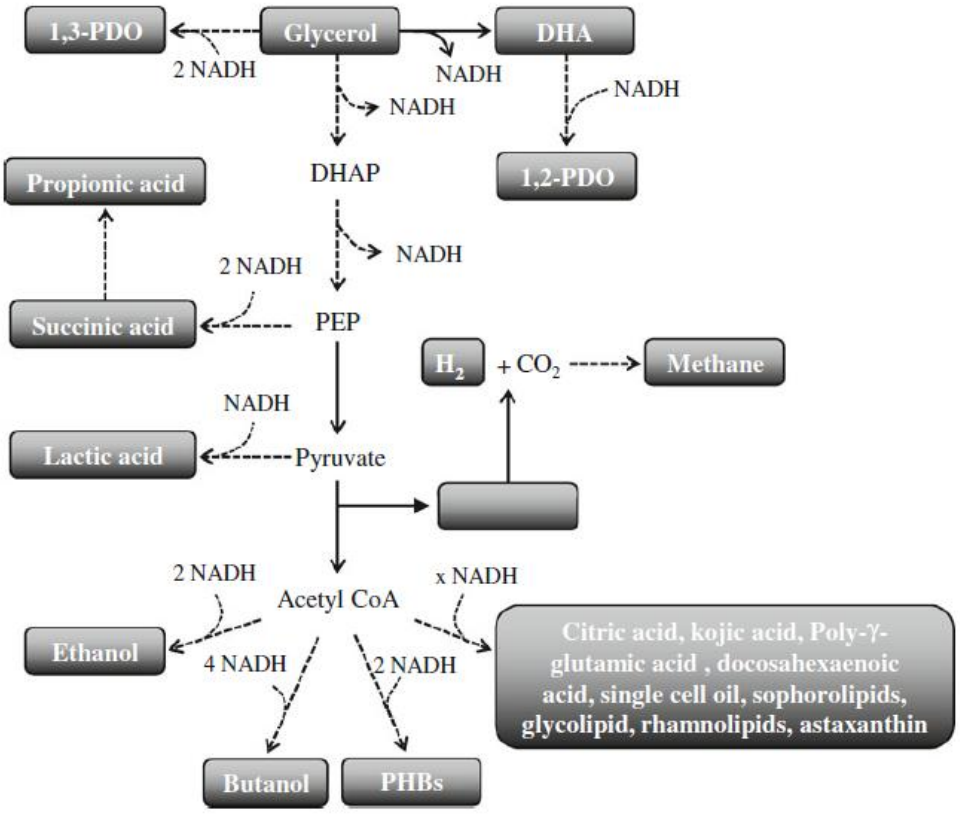


Figure 3.11 Overview of the pathways and products converted from glycerol [reproduced from (117) with permission from Springer]. Solid lines represent single step reaction and dotted lines represent multiple step reactions. PDO, propanediol; DHA dihydroxyacetone; PHB, poly(3-hydroxybutyrate); H, reducing equivalents (NADH, NADPH, FADH2, H2).

Table 3.4 PHAs production from glycerol.

Strain	Type of PHA	Operation mode	Culture medium (carbon source)	Cultivation time (h)	% PHA content	PHA (g/L)	Reference
P. putida GO16	MCL-PHA	Fed-batch	Crude glycerol+ Sodium terephthalate	48	-	5.7	Kenny ST et al., (2012) (119)
Mixed microbial communities	P(3HB)	Fed-batch	Crude glycerol	-	67	-	Moralejo-Garate H et al., (2011) (120)
Recombinant *E. coli* (deletion: *ptsG*, *sdhA*, and *pta* genes, heterologous expression: *phaC1* of *P. aeruginosa*	MCL-PHA	Flask (50 mL)	Pure glycerol+ decanoate	-	4.5	0.2	Kang Z et al., (2011) (121)
		Fed-batch (3 L)	Pure glycerol + decanoate	> 90	5.6	0.5	
Halomonas sp. KM-1	P(3HB)	Flask (20 mL)	Pure glycerol	60	40.5-44.8	1.9-2.3	Kawata Y and Aiba S (2010) (122)
			Crude glycerol		39	1.6	

Table 3.4 PHAs production from glycerol (Cont.).

Strain	Type of PHA	Operation mode	Culture medium (carbon source)	Cultivation time (h)	% PHA content	PHA (g/L)	Reference
Bacillus sonorensis	P(3HB)	Flask (100 mL)	Crude glycerol	96	71.8	-	Shrivastav A et al., (2010) (123)
Halomonas hydrothermalis					75	-	
Burkholderia cepacia ATCC 17759 (pathogen)	P(3HB)	Flask (100 mL)	Crude glycerol	96	81.9	4.8	Zhu C et al., (2010) (124)
		Fed-batch (200 L)		120	31.4	7.4	
Recombinant *E. coli*	P(3HP)	Fed-batch (2 L)	Pure glycerol	92	12	1.4	Andreesen B et al., (2010) (125)
			Crude glycerol		5	0.3	
Zobellella denitrifican	P(3HB)	Flask (50 mL)	Pure glycerol	48	53.6	1.7	Ibrahim MH and Steinbuchel A, 2010 (126)
				96	80.1	2.8	
			Pure glycerol + NaCl	48	79.7	2.9	
				96	87	4.2	

Table 3.4 PHAs production from glycerol (Cont.).

Strain	Type of PHA	Operation mode	Culture medium (carbon source)	Cultivation time (h)	% PHA content	PHA (g/L)	Reference
Burkholderia sp. USM (JMC 15050)	P(3HB)	-	Pure glycerol + palm oil derivertivies + fatty acid	72	22-27	-	Chee JY et at., 2010 (3)
Cupriavidus necator	P(3HB)	Fed-batch	Pure glycerol	34	62	51.2	Cavalheiro et al., 2009 (2)
			Crude glycerol		50	38.1	
Zobellella denitrificans MW1	P(3HB)	Fed-batch	Pure glycerol	50	67	54.3	Ibrahim MH and Steinbuchel A, 2009 (127)
Recombinant *E. coli*	P(3HB)	Batch 5.6 L-reactor	Pure glycerol	48	42	3.5	Pablo I et al., 2008 (128)
		Fed-batch 5.6 L-reactor		60	51	10.8	

Table 3.4 PHAs production from glycerol (Cont.).

Strain	Type of PHA	Operation mode	Culture medium (carbon source)	Cultivation time (h)	% PHA content	PHA (g/L)	Reference
Recombinant *E. coli* (harboring *phaBAC* of *Azotobacter* sp strain FA8)	P(3HB)	flask	Pure glycerol	48	9.8	0.6	Almeida A et al., 2007 (129)
		5.6 L-bioreactor			46.1	3.4	
Recombinant *E. coli* (harboring *phaBACP* of *Azotobacter* sp strain FA8)		Flask			38.2	3.1	
		5.6 L-bioreactor			51.9	7.9	
Paracoccus denitrificans and *Cupriavidus necator*	P(3HB)	Fed-batch	Pure glycerol	-	70	-	Mothes G et al., 2007 (130)
			Crude glycerol	-	48	-	
High osmophilic microorganism	P(3HB-*co*-3HV)	Batch 42-L bioreactor	Crude glycerol + peptone	170	76	16.2	Koller et al., 2005 (131)
Pseudomonas NRRLB-14682	P(3HB)	Flask (500 mL)	Crude glycerol	72	13-27	-	Ashby RD et al., 2004 (132)

Table 3.4 PHAs production from glycerol (Cont.).

Strain	Type of PHA	Operation mode	Culture medium (carbon source)	Cultivation time (h)	% PHA content	PHA (g/L)	Reference
Recombinant *E. coli*	P(3HB)	Flask (50 mL)	Pure glycerol + peptone	48	60	-	Mahishi LH et al., 2003 (133)
Recombinant *Salmonella enteric*	P(3HB-*co*-3HV)	Flask	Pure glycerol	-	27	-	Aldor IS et al., 2002 (134)
Methylobacterium rhodesianum	P(3HB)	Flask	Pure glycerol + casein peptone	92	39	-	Borman EJ and Roth M, 1999 (135)

CHAPTER IV
MATERIALS

4.1 Bacterial strains

4.1.1 PHA-producing strains

Ralstonia eutropha (TISTR 1095), a PHA-producing bacteria, was obtained from the Thailand Institute of Scientific and Technological Research, Thailand. Two other strains of PHA-producing bacteria which are *Aeromonas hydrophila* (ATCC 7966), and *Pseudomonas putida* (ATCC 47054), were obtained from the American Type Culture Collection, USA.

4.1.2 *Escherichia coli* strains

Escherichia coli DH5α [*sup*E 44, Δ*lac* U169, (Φ80 *lac*Z ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*1 *rel*A1], was used as a host for plasmid propagation.

Escherichia coli BL21(DE3) [F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 *ind*1 *sam*7 *nin*5])], was used as a host for protein expression.

4.2 Plasmid vectors

Plasmid vectors used in this study are shown in Figures 4.1-4.3. The plasmid pTZ57R/T vector (Fermentas, Lithuania) is used for all cloning steps (Fig 4.1). Two vectors, pETDuet-1 and pCDFDuet-1 (Novagen, Germany) were employed for PhaA, PhaB, PhaC and PhaJ expression (Fig 4.2 and 4.3) and were kindly provided by Dr. Sarin Chimnaronk.

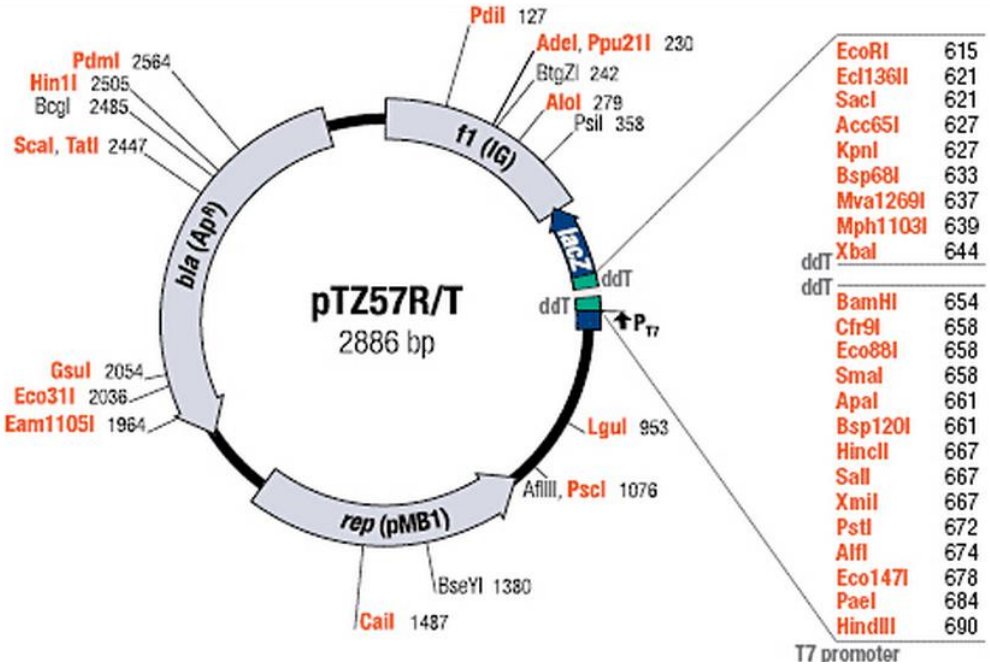


Figure 4.1 A physical map of pTZ57R/T vector (taken from Fermentas website: http://www.fermentas.com/en/products/all/molecular-cloning/kits/k121-instaclone-pcr-cloning?print).

The figure illustrates a TA cloning vector, pTZ57R/T, containing *lacZ* gene, multiple cloning sites, origin of replication in *E. coli* and ampicillin resistant gene.

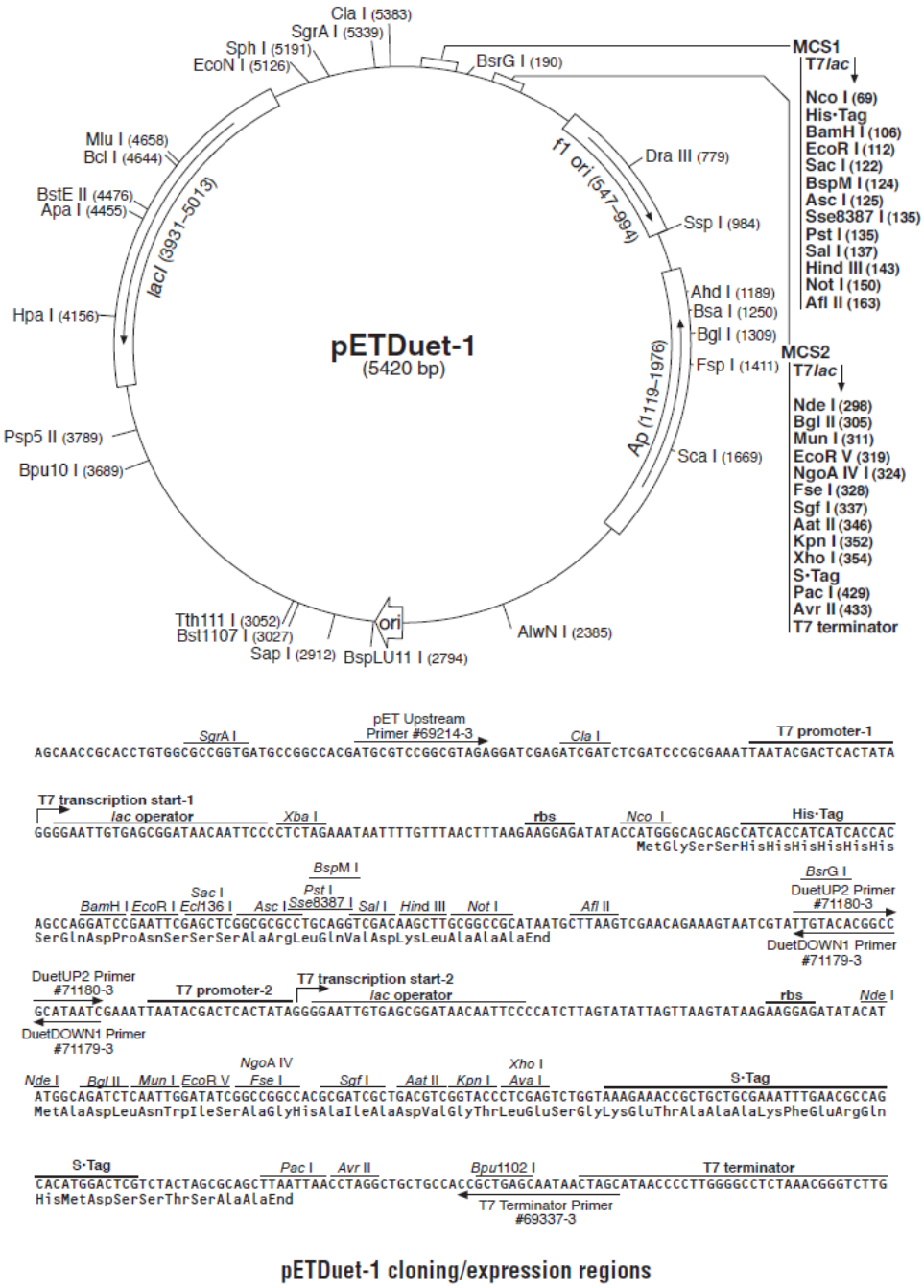
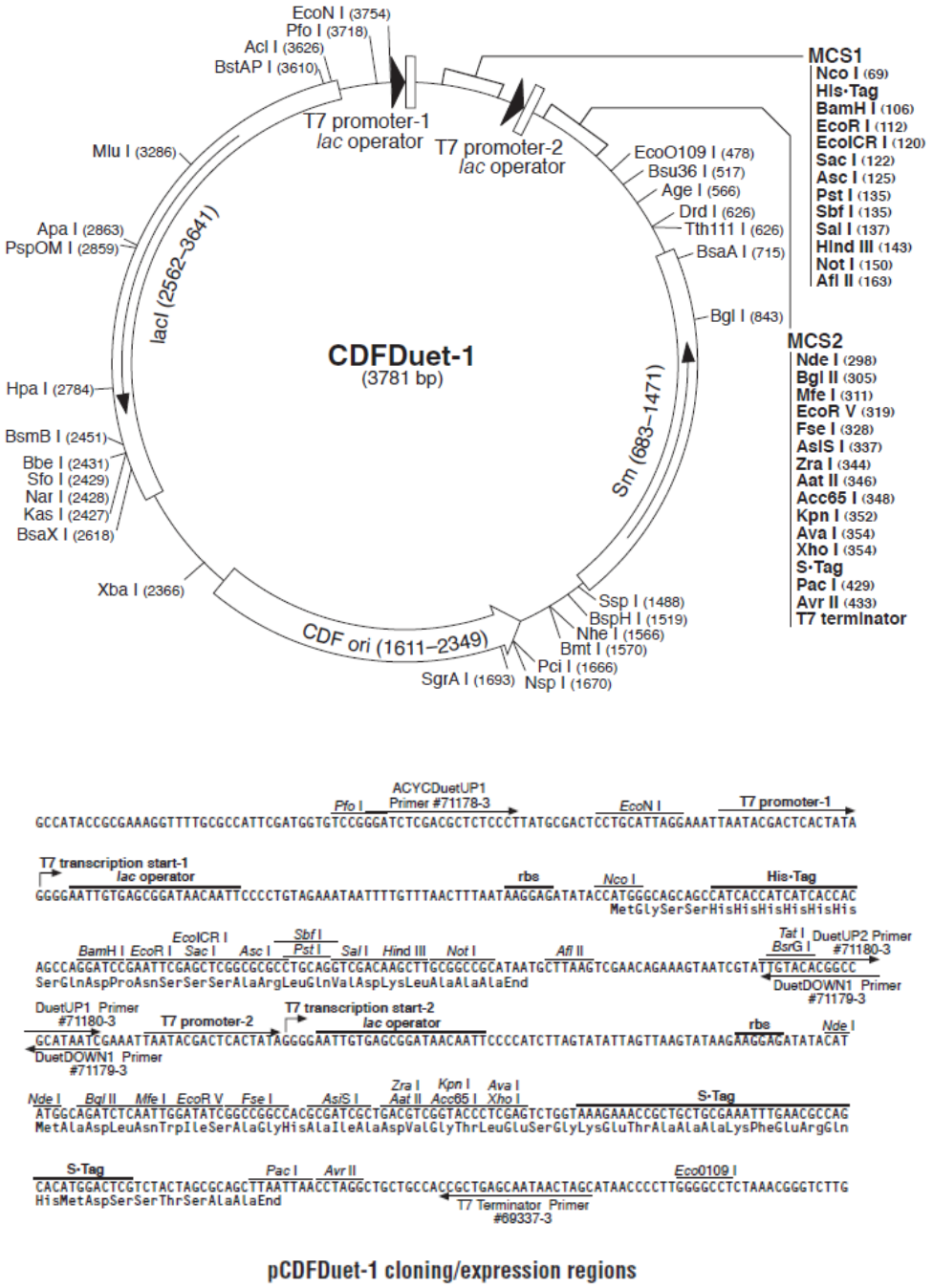


Figure 4.2 A physical map of pETDuet-1 vector (taken from Novagen's user protocol TB340 Rev. E 0305).

The figure illustrates a pETDuet-1 plasmid containing 2 multiple cloning sites driving by separated T7 promoters, His_6-tag, T7 transcription terminator, ampicillin resistant gene and ColE1 (pBR322) origin of replication.



pCDFDuet-1 cloning/expression regions

Figure 4.3 A physical map of pCDFDuet-1 vector (taken from Novagen's user protocol TB340 Rev. E 0305).

The figure illustrates a pCDFDuet-1 plasmid containing 2 multiple cloning sites driving by separated T7 promoters, His_6-tag, T7 transcription terminator, streptomycin resistant gene and CloDF13 origin of replication.

4.3 Synthetic oligonucleotides

All nucleotide primers were synthesized by BioDesign Co., Ltd., Thailand.

4.4 Culture media

4.4.1 Culture media for native PHA-producing bacteria

Ralstonia eutropha and *Aeromonas hydrophila* were cultivated in nutrient broth medium (BD Difco, USA) at 25^{o}C and 30^{o}C, respectively, with continuous shaking at 200 rpm. *Pseudomonas putida* was cultivated in LB medium at 37^{o}C with continuous shaking at 200 rpm.

4.4.2 Culture media for *E. coli* harboring pTZ57R/T vector (for cloning)

E. coli DH5α, was grown in Luria Bertani (LB) medium [1% (w/v) tryptone (Difco, USA), 1% (w/v) NaCl (Carlo Erba, France), and 0.5% (w/v) yeast extract (Difco, USA). For LB plate agar, 1.5% (w/v) bacteriology agar was added. The *E. coli* transformants were grown in LB containing 100 µg/mL of ampicillin. For blue/white colonies screening, 25 µl of a stock solution of 20 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in dimethylformamide and 50 µl of a stock solution of 100 mg/mL isopropylthio-β-D-galactoside (IPTG) (Thermo Scientific, USA) were spreaded on the plate agar.

4.4.3 Culture media for *E. coli* harboring pETDuet-1 and/or pCDFDuet-1 vector (for expression)

E. coli BL21(DE3), was cultivated in Luria Bertani (LB) medium containing 100 µg/mL ampicillin and/or 50 µg/mL streptomycin. IPTG was added into the cultured media at the final concentration at 1 mM to induce the heterologous protein expression.

4.4.4 Culture media for P(3HB) biosynthesis

Recombinant *E. coli* harboring *phaA*, *phaB* and *phaC* were cultivated in M9 medium [0.3% (w/v) KH_2PO_4 (Carlo Erba, France), 0.05% (w/v) NaCl (Carlo Erba, France), 0.1% (w/v) NH_4Cl (Carlo Erba, France), 0.68% (w/v) Na_2HPO_4 (Carlo Erba, France), 1 mM $MgSO_4$ (Carlo Erba, France), 1 mM $CaCl_2$ (Carlo Erba, France)] with or without 0.1% (w/v) yeast extract (Difco, USA). Pure glycerol or crude glycerol or glucose was used as sole carbon source with 100 µg/mL ampicillin. IPTG was added into the cultured media at the final concentration at 1 mM to induce the heterologous protein expression.

4.4.5 Culture media for SCL-MCL-PHAs biosynthesis

Recombinant *E. coli* harboring *phaA*, *phaB*, *phaC* and *phaJ* were cultivated in M9 medium containing pure glycerol or crude glycerol and sodium dodecanoate (Sigma, USA) whose concentration ratio was varied as indicated in the method section, 50 µg/mL ampicillin and 50 µg/mL streptomycin. IPTG was added into the cultured media at the final concentration at 1 mM to induce the heterologous proteins expression. All antibiotics were purchased from Sigma, USA.

4.5 Chemicals

Crude glycerol was kindly provided by Trang Palm Oil Co. Ltd., Trang, Thailand. Other analytical grade chemicals and solvents were purchased from Carlo Erba, (France), Fluka, (Switzerland), Sigma, (USA), Merck, (Germany) or Thermo Scientific, (USA).

4.6 Enzymes

Restriction enzymes and other modification enzymes were purchased from Fermentas (Lithuania).

4.7 Antibodies

Monoclonal Anti-polyHistidine, clone HIS-1, produced in mouse and Anti-Mouse IgG (Whole molecules)-Alkaline Phosphatase, produced in goat were purchased from Sigma (USA).

4.8 Commercial kits

GeneJET™ Plasmid Miniprep kit (Fermentas, Lithuania), QIAquick PCR Purification kit (QIAGEN, German).