CHAPTER 1: INTRODUCTION

1.1 General introduction

Polyhydroxyalkanoates (PHAs) are the biodegradable polyester composed of hydroxyl fatty acids which is produce naturally in numerous of pseudomonads (Brandl *et al.*, 1990; Steinbüchel and Valentin, 1995). PHAs served as energy storage materials in the cytoplasm granules of the microbial (Stubbe and Tian, 2003). Anderson and Dawes, (1990) reported that there are two groups of PHAs: short-chain-length polyhydroxyalkanoates (scl-PHA) and medium-chain-length polyhydroxyalkanoate (mcl-PHA).

The medium-chain-length polyhydroxyalkanaoate (mcl-PHA) could be produced by numerous pseudomonads using variety of substrates including carbohydrates, oil, alcohols, fatty acids hydrocarbons and others (Ashby and Foglia, 1998; Kim *et al.*, 2000). Indeed, *Pseudomonas putida* is a good mcl-PHA producer which is capable of accumulating PHA to about 40% of its cell dry weight (Marsudi *at al.*, 2007). The monomeric units of the mcl-PHAs are mainly β-hydroxyalkanoates with 4 to 16 carbon atoms (Brandl *et al.*, 1988).

P. putida PGA1 does not produce lipase, therefore it is incapable to metabolise oils directly as carbon source for PHA production (Tan *et al*., 1997). In order for *P. putida* PGA1 to synthesis the PHA using palm oil, the lipid compound has to be digested into simple fatty acids by saponification in order for the bacteria to uptake. However, the saponification process would increases the cost of PHA production and would not be practical for bulk production of PHA. Some pseudomonads had been reported to be able to utilize lipid for PHA bio-synthesis but the production is low (Solaiman *et al.*, 1999). For example, *Pseudomonas aeruiginosa* accumulated PHA about 15% of its cell dry weight and

Pseudomonas fluorescens accumulated PHA to only 1 to 2% of its cell dry weight (Huisman *et al.*, 1989).

The objective of this study was to construct an *Escherichia coli* strain which is capable of synthesizing PHA using palm oil as carbon source. For this objective, two recombinant *E. coli* strains were generated: recombinant *fab* B^{-} *E. coli* LS1298 (1) that harbours both *lip* gene obtained from *P. fluorescens* ATCC13525 and *phaC1* gene from *P. putida* PGA1 and (2) that harbours only *phaC1* gene from *P. putida* PGA1. The recombinant *E. coli* strains were then tested for PHA synthesis using several carbon sources including palm oil. This study was carried out in five major experiments which consisted of:

1. Amplification and cloning of the *lip* gene from *P. fluorescens* ATCC13525 and *phaC1* gene from *P. putida* PGA1.

2. Construction of recombinant *E. coli* which is capable of utilizing palm oil as carbon substrate for PHA synthesis.

3. Determination of the heterologous gene expression in mRNA level by reverse transcriptase polymerase chain reaction technique.

4. Determination the lipase activity in the lipase producing recombinant *E. coli* strains using spectrophotometric.

5. Determination of the monomers composition and estimation of PHA content produced by the constructed recombinant *E .coli* strains using gas chromatography analysis.

CHAPTER 2: LITERATURE REWIEW

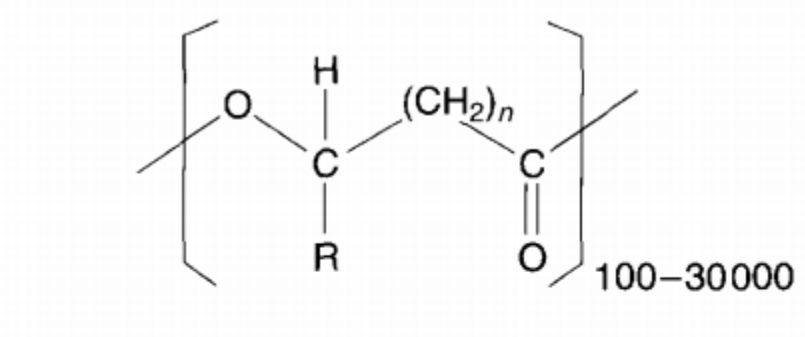
2.1 Polyhydroxyalkanoates (PHAs)

For the past decades, synthetic polymers (plastics) have been widely used due to its physical structure which can be chemically manipulated according to the desired shapes, strengths and elasticity (Reddy *et al.*, 2003). However, the synthetic plastics with high molecular size are one of the factors that render to its non-biodegradability (Fakirov and Bhattacharyya, 2007). The public anxiety of the harmful effects of incomplete degradation of those conventional synthetic plastics to the Earth led to the discovery of comparable materials that can be eliminated from the biosphere.

Hence, the potential commercial values of the biodegradable plastic materials demand an intensive study to determine their productions and applications. PHA is a group of polyester compounds that can be found in the cytoplasm of various bacteria as energy storage (Madison and Huisman, 1999). Generally, PHA producers convert the available carbon source such as lipids and carbohydrates into PHA granules in the cells (Anderson and Dawes, 1990). Although the needs of biodegradable materials to replace the non-biodegradable plastics in various applications have increased tremendously, PHA is not practical to use as the cost for PHA production is relatively high due to its low yield and low efficiency in its downstream process (Witholt & Kessler, 1999). Therefore, an effective approach to reduce the cost of PHA production is necessary to overcome this problem. In order to fulfill the high demands of PHA in the market, several important issues are required to be resolved (Byrom, 1987). These include the production cost, the quality of the PHA produced, the method used to produce PHA, the selection of the raw materials as well as the handling of the waste products (Reddy *et al.*, 2003).

2.2 Properties of PHAs

PHA is mainly made up of (R)-β-hydroxyl fatty acid monomers with different carbon chain length (Figure 2.1) whereby the R group can varies from C1 to C16 (Lee, 1996).



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 structure of PHA (Lee, 1996).

There are numerous types of homopolyester or copolyester (with diverse hydroxyalkanoic acids) generated by microorganism such as poly-3-hydroxybutyrate (P3HB), copolymers of hydroxybutyrate and hydroxyvalerate Poly-(3HB-co-3HV) and copolymers of hydroxybutyrate and hydroxyoctanoate Poly-(3HB-co-3HO) (Antonio *et al.,* 2000; Steinbüchel and Valentin, 1995).

Based on the number of carbon atoms in the monomer units, PHAs are categorized into two main groups (Anderson and Dawes, 1990). The first group of PHA is the short-chain-length PHA (scl-PHA) which consists of hydroxyl fatty acid monomers with repeat units of 3 to 5 carbon atoms such as the polyhydroxybutyrate (PHB) (Poirier *et al.*, 1995). Another group of PHA is the medium-chain-length PHA (mcl-PHA) comprising of hydroxyl fatty acid monomers with repeat-units of 6 to 14 carbon atoms such as (3)-β-polyhydroxyoctanoate (Rehm and Steinbüchel, 1999). Table 2.1 (Chen, 2001; Doi *et al.*, 1995; Galegoa *et al.*, 2000; Khanna and Srivastava, 2005; Ljungberg and Wesslen, 2002; Spyros and Marchessault, 1996; Steinbüchel, 1991; Sudesh *at al.*, 2000; Suyatma *et al.*, 2004; Wang *et al.*, 2009; Zhang and Sun, 2004) shows the material characteristics of melting temperature (T_m), glass-transition temperature (T_g), tensile strength (MPa) and the elongation at break of various PHAs.

Table 2.1: Properties of various PHAs

Polymers	T_m (°C)	T_g (°C)	Tensile strength (MPa)	Elongation at break (%)
poly-3-hydroxybutyrate (PHB)	177	4	43	5
Polypropylene	170	-	34	400
Poly-4-hydroxybutyrate P(4HB)	60	-50	104	1000
PHA	60 to 177	-50 to 4	17 to 104	2 to 1000
Poly-lactic acid (PLA)	175	60	49.6 to 61.6	5.2 to 2.4

2.3 Applications of PHAs

PHAs have a wide range of applications due to their novel biodegradable and biocompatible features (Zhao *et al.*, 2003). Besides, PHAs could be produced from renewable resources such as plant oils. Thus, PHAs have become a suitable eco-friendly material that can replace the synthetic plastics (Koning and Witholt, 1996). The bio-degradation of the synthetic plastics in nature is slower than that of natural polyesters and this advantage has apart the PHAs from conventional plastics. It depends on the environmental factors as well as the microorganisms that involved in their surroundings (Albertsson *et al.*, 1994; Cruz-Pinto *et al.*, 1994).

Till date, there are few commercially-available PHA products. For example, copolyesters produced from 3-hydroxybutyrate and 3-hydroxyvalerate (BIOPOL^{TM}) are distributed in the U.S. from the company Monsanto and Metabolix. Brandl *et al.* (1990) had reported that biopolymers are widely used in the packaging industry.

Besides, the biocompatibility and biodegradation characteristics of PHA are the ideal criteria for the usage of biomaterial in surgical applications (Ueda and Tabata, 2003). This is to avoid immune-rejection and inflammation in the patients as well as to eliminate the non-degradable foreign substance that remains in patients during surgery. Moreover, it is suitable if the implanted device can be absorbed and disappears during the recovery after the surgery (Nebe *et al.*, 2001). This can prevent the risk of a second operation to remove the device which may cause complications in patients. There are a numbers of medical materials commercially available for medical application and pharmaceutical industries such as, dental implants, bone fixation, implants for plastic surgery, orthopedic implants,

surgical staple and surgical joints for orthopedics applications, cardiovascular patches, articular cartilage repair devices, tendon repair devices and others.

The traditional method to repair damage skin caused by burns and skin diseases is to harvest the healthy skin from other parts of patient's body and transfer to the infected sites. However, this technique has its disadvantage as the patient may not have enough skin available and this may create more skin damages (Loss *et al.*, 2000). Patients with severe skin damage need a relatively safe, rapid and effective wound management in order to prevent further aggravation (Van der Veen *et al.*, 2010). Using artificial skin as a protection membrane to cover the damaged skin is one way to avoid dehydration and infection (Cooper *et al.*, 1991). It functions as a barrier to the wound from direct exposure to the outside environment which can help in infection and inflammation resistance as well as to prevent dehydration.

In tissue engineering, a medical materials scaffold is normally required to support the growth of the regeneration tissue (progenitor cells or stem cells) (Ishuang *at al.*, 1997). The physical properties of the scaffold for example the porosity and pore size, affect the growth factor delivery and cells attachment, migration, differentiation and proliferation (Ma and Lui, 2003). Indeed, polyhydroxyalknoates with the biodegradation, and biocompatible characteristic is the good preference to engineer this scaffold such as poly-lactic acid (PLA), poly-glycolic (PGA) and poly-ε-caprolactone (PCL) (Ueda and Tabata, 2003). Kinoshita *at al.* (1993) reported that the clinical application using poly-L-lactides (PLLA) with bone marrow transplant for the mandibular defects.

2.4 Natural PHA producing bacteria

PHAs are synthesized and accumulated intracellularly in many bacteria species. Under controlled conditions, PHA can be accumulated up to 90% of the cell dry weight in some bacteria (Anderson and Dawes, 1990; Madison and Huisman, 1999). Studies showed that pseudomonads, rhizobacteria and bacillus are the common PHA-producing bacteria (Trainer and Charles 2006; Wang and Bakken, 1998). In natural environments, PHA is accumulated in the cell when the bacteria are exposed to stress conditions such as lack of essential nutrients (nitrogen, phosphorus, oxygen or magnesium) and with an excess of carbon sources (Anderson and Dawes 1990). Under such conditions, the growth of bacteria is suppressed due to the absence of nutrients, but they are capable to deposit the excess water soluble carbon substrates into insoluble granules in the cytoplasm as energy reserves (Figure 2.2) (Reddy *et al.*, 2003). This becomes an alternative tactics to support the survival of bacteria when the growth conditions are not in favor (Zhao *et al.*, 2007).

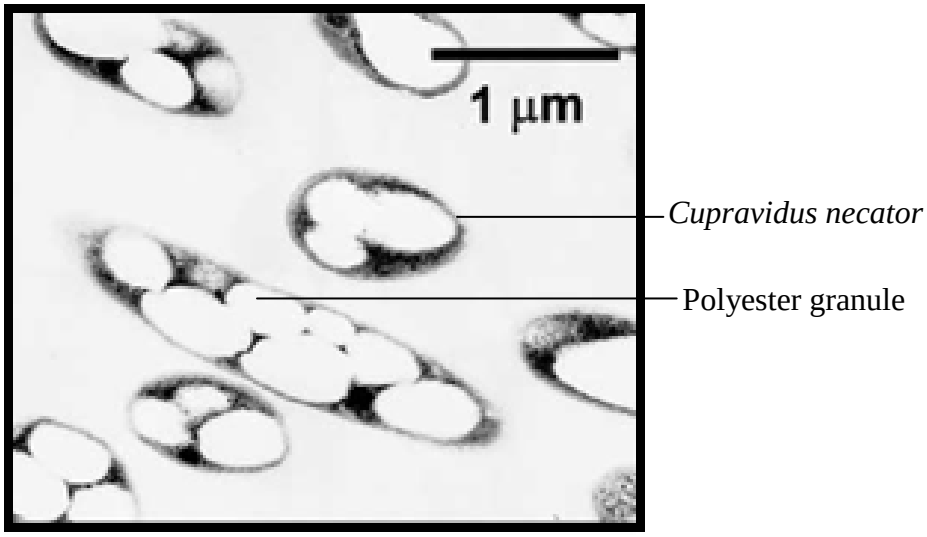


Figure 2.2: PHB granules accumulated in *Cupravidus necator* (formerly known as *Ralstonia eutropha*) strains under nutrient limitation (modified from Stubbe and Tian, 2003).

2.5 PHA synthases

PHA synthase is the key enzyme for PHA polymerization in microbial system. Generally, PHA synthases can be classified into four major classes (Table 2.2) based on the composition of the protein subunits and substrate specificity of the enzyme (Rehm, 2003). Generally, Class I PHA synthase consists of one protein subunit with molecular mass of 60 kDa to 73 kDa and polymerizes (R)-3-hydroxy fatty acids with 3 to 5 carbon atoms to scl-PHA. *Cupravidus necator* (formerly known as *Ralstonia eutropha*) is the representing bacteria species Class I PHA synthase (Jia *et al.*, 2001, Rehm *et al.*, 2002).

Table 2.2: Four classes of polyester synthase (modified from Rehm, 2003)

Class	Subunits	Species	Substrate
I	PhaC ~60-73 kDa	*Cupriavidus necator*	$3HA_{SCL}$-CoA (~C3-C5) $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA, $3MA_{SCL}$-CoA
II	PhaC ~60-65 kDa	*Pseudomonas aeruginosa*	$3HA_{MCL}$-CoA (~≥C5)
III	PhaC ~40 kDa PhaE ~40 kDa	*Allochromatium vinosum*	$3HA_{SCL}$-CoA ($3HA_{MCL}$-CoA [~C6-C8], 4HA-CoA, 5HA-CoA)
IV	PhaC ~40 kDa PhaR ~22 kDa	*Bacillus megaterium*	$3HA_{SCL}$-CoA

In the case of Class II PHA synthase (commonly found in *Pseudomonas* spp.), it has a substrate preference of (R)-3-hydroxy fatty acids with 6 to 14 carbon atoms for PHA synthesis (Qi *et al.*, 2000). Similarly to Class I PHA synthase, Class II PHA synthase also consists of only one protein subunit with molecular mass about 60 kDa to 65 kDa.

PHA synthase from *Allochromatium vinosum* has been classified into Class III PHA synthase and it consists of two protein subunits and each is about 40 kDa (Aneja *et al.*, 2009). The PHA synthase from this group also prefers (R)-3-hydroxy fatty acid with 3 to 5 carbon atoms as PHA substrates. The Class IV PHA synthase which can be found in genus *Bacillus* is made up of two protein subunits and has similar substrate specificity to those of Class I and Class III PHA synthases (Tajima *et al.*, 2003).

2.6 Biosynthesis pathway of PHA production

The biosynthesis of PHA involves several enzymatic reactions and the (R)-β-hydroxyacyl-CoA is an important precursor in PHA polymerization (Rehm and Steinbüchel, 1999). This intermediate can be produced from several metabolic pathways: the glycolysis pathway, the fatty acid β-oxidation pathway and fatty acid *de novo* biosynthesis pathway (Sudesh *et al.*, 2000).

2.6.1 PHA synthesis using carbohydrates

Chen *et al.* (2001) reported that there are bacterial strains which can produce polyester using carbohydrates such as *Cupravidus necator*. Such bacteria produce PHA from glycolysis pathway (Figure 2.3).

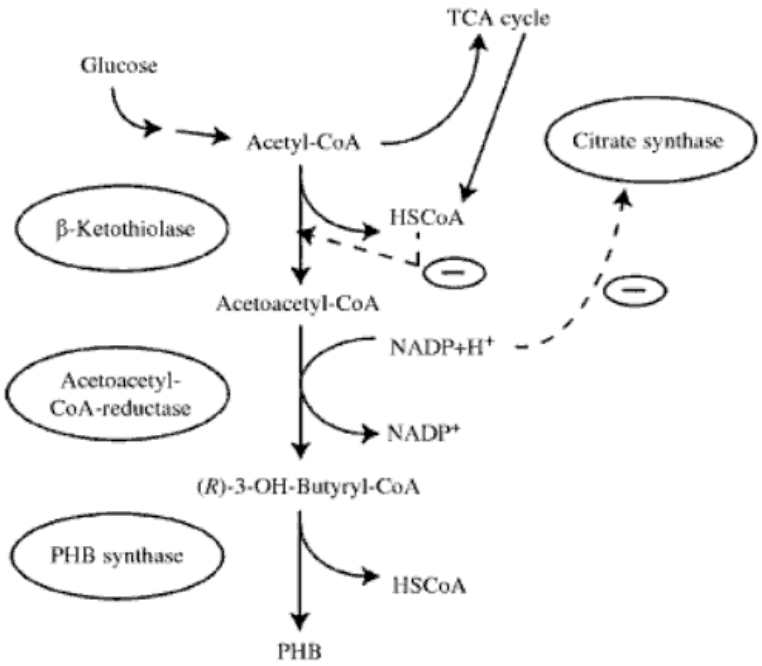


Figure 2.3: PHB synthesis pathway in *Cupravidus necator*. Modified from Kessler and Witholt, (2001).

In this pathway, two acetyl-CoA resulted from sugar degradation are condensed to form acetoacetyl-Co A by β-ketothiolase (encoded by *phaA*). The acetoacetyl-CoA is further reduced to (R)-3-hydrozybutyryl-CoA by NADPH-dependent reductase (encoded by *phaB*). Lastly, PHA synthase (encoded by *phaC*) polymerizes the (R)-3-hydrozybutyryl-CoA into poly(3-hydroxybutyrate).

2.6.2 PHA synthesis via fatty acid β-oxidation pathway

PHA synthesis in the bacteria via fatty acid β-oxidation pathway uses fatty acids as substrates (Lageveen *et al.*, 1988). The fatty acids being uptake by the bacteria are activated by acyl-CoA synthase to give fatty acyl-CoAs before entering the β-oxidation pathway (Figure 2.4). In the β-oxidation pathway, the acyl-CoAs undergo three enzymatic reactions. Firstly, acyl-CoA is oxidized by acyl-CoA-dehydrogenase to give trans-2-enoyl-CoA. The trans-2-enoyl-CoA then undergoes hydration reaction to form L-β-hydroxyacyl CoA by 2-enoyl-CoA hydratase. Subsequently, (L)-β-hydroxyacyl CoA is converted to (R)-β-hydroxyacyl-CoA by 3-hydroxyacyl-CoA epimerase. Lastly, the carboxyl group of the (R)-β-hydroxyacyl-CoA undergoes esterification by PHA synthase with hydroxyl group of another monomer to form PHA (Lu *et al.*, 2003).

Other intermediates in β-oxidation biosynthesis pathway also participate in the PHA biosynthesis indirectly. For example, the key enzyme such as enoyl-CoA hydratase also channels the other intermediates from fatty acid oxidation pathway for the PHA biosynthesis (Taguchi *at al.*, 1999). On the other hand, 3-ketoacyl-CoA intermediates from β-oxidation can also undergo reduction to form (R)-β-hydroxyacyl-CoA by 3-ketoacyl-ACP-reductase which can be polymerized to PHA (Eggink *et al.*, 1995).

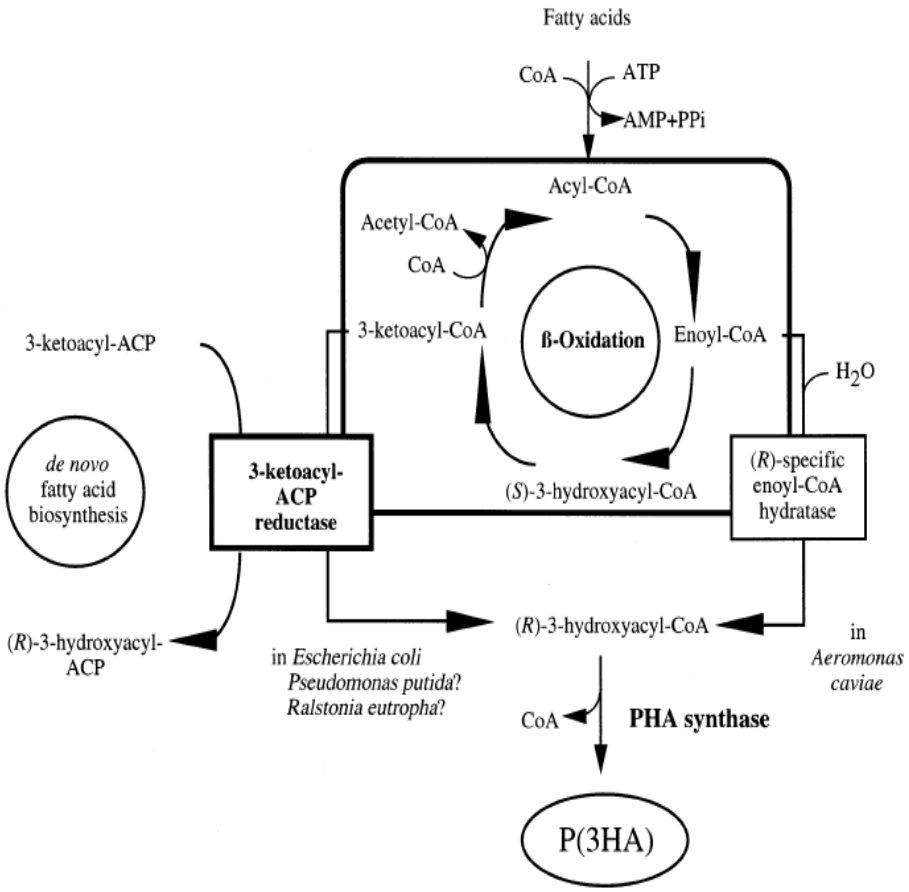


Figure 2.4: Carbon flux for poly(3-hydroxyalkanoate) biosynthesis from fatty acids. An intermediate in L-oxidation, 3-ketoacyl-CoA is converted into (R)-3-hydroxyacyl-CoA by 3-ketoacyl-ACP reductase in *E. coli*, not determined in *P. putida*, and *R. eutropha*, while enoyl-CoA is converted into (R)-3-hydroxyacyl-CoA by (R)-specific enoyl-CoA hydratase in *A. caviae* (Taguchi *et al.*, 1999).

2.6.3 PHA synthesis via *de novo* fatty acid synthesis pathway

Recent studies have shown that the PHA can be synthesised via *de novo* fatty acid synthesis pathway (Figure 2.5) when bacteria are fed with the non-related carbon substrates such as gluconate, acetate or ethanol (Fiedler *et al.*, 2000). An intermediate acetyl-CoA from carbohydrate degradation is carboxylated into malonyl-CoA by acetyl-CoA carboxylase. Subsequently, the malonyl-CoA is converted into malonyl-ACP by ACP- malonyl transferase.

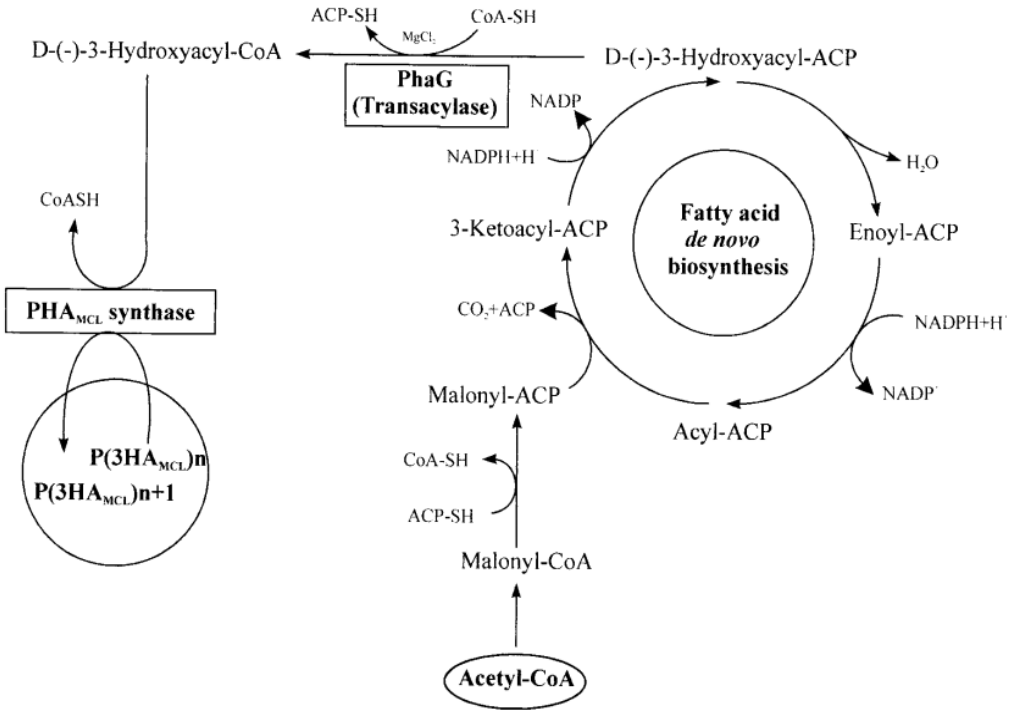


Figure 2.5: PhaG-mediated metabolic route of mcl-PHAsynthesis from acetyl-CoA. 3HA, 3-hydroxyalkanoate (Fiedler *et al.*, 2000).

In the first reaction of the *de novo* fatty acid synthesis pathway, acyl-ACP and malonyl-ACP are condensed by β-ketoacyl-ACP synthase and this result in the formation of 3-ketoacyl-ACP. Then, this 3-ketoacyl-ACP is further reduced to (R)-3-hydroxyacyl-ACP by β-ketoacyl-ACP reductase. In order to serve as substrate for PHA synthase, (R)-3-hydroxyacyl-ACP must be converted to the corresponding CoA derivative by 3-hydroxyacyl-ACP-CoA transacylase (encoded by *phaG*) which is the key enzyme in channeling the intermediates of *de novo* fatty acid biosynthesis pathway to PHA production from carbohydrates (Hoffmann *et al.*, 2000).

2.7 Approaches to increase PHA production

PHAs have attracted interest in the various industries as they have similar physical properties as other conventional synthetic thermoplastics. Furthermore, PHAs have great potential in medical applications because PHAs are found to be biocompatible to human and others mammals (Zinn *et al.*, 2001). However, the production cost of PHA is relatively high compared to the conventional thermoplastics.

Hence, ways to increase the PHA productivity have been proposed to ensure the efficiency in PHA production in order to meet the market demands. The most common strategy employed in enhancing the PHA yield is the improvement of bacterial strains used in PHA production together with optimization of the fermentation operations.

2.7.1 Strain improvement

Improvement for bacterial strains used in PHA production can be achieved by genetic modification techniques. The basic genetic engineering of bacteria can be accomplished by directly deleting, altering or adding desired nucleic acid of its original genome with the use of recombinant DNA technology (Brown, 2006). As a result, the characteristics of that particular living microorganism are manipulated at the molecular level to become a desired useful product.

The genetic metabolic engineering provides a wide range of strategies to perform PHA overexpression in natural PHA producer microorganism or in other expression host.). PHA depolymerases (encoded by *phaZ*) have been discovered as catabolic enzyme used by microorganism to hydrolyse PHA when carbon sources is required (Jendrossek and

Handrick, 2002). Therefore, PHA yield in microorganism could be improved via removing the PHA degradation mechanism. As reported by Cai *et al.* (2009) the PHA production in *Pseudomonas putida* KT2442 was improved when the PHA depolymerase gene was removed by chromosomal gene knockout technique.

Based on the knowledge of the PHA biosynthesis, various PHA synthase from different bacteria strains had been successfully cloned and heterologous expressed in recombinant strains (Kolibachuk *et al*. 1999; Qi *et al.*, 1997; Ren *et al.*, 2005; Steinbüchel *et al.*, 1998). Additionally, Lu *et al.* (2003) had been reported that the PHA production was improved when precursor supply of enoyl-CoA was enhanced in *E. coli* via manipulation of the fatty acid β-oxidation pathway.

2.7.2 PHA production in *Escherichia coli*

Various *E. coli* strains are widely used for heterologous gene expression, however it has been reported that wild-type *E. coli* are relatively poor in PHA synthesis (Steinbüchel *et al.*, 1998) because *E .coli* is problematic in providing major precursor for PHA polymerization from the fatty acid synthesis pathway (Park *et al.*, 2005).

Qi *et al.* (1997) had reported that, one of the solutions was to create a mutant *E. coli* strain which would be able to support the polyester synthesis by inhibition of key enzymes in the β-oxidation pathway such as *fad A* and *fad B* (Figure 2.6). This inhibition of the β-oxidation pathway led to the accumulation of the precursor (S)-3-hydroxyacyl-CoA which was then channelled for PHA synthase. As reported by Langenbach e*t al.* (1997), the *fad B*$^{-}$ *E. coli* LS1298 have the strongest PHA accumulation using various fatty acids.

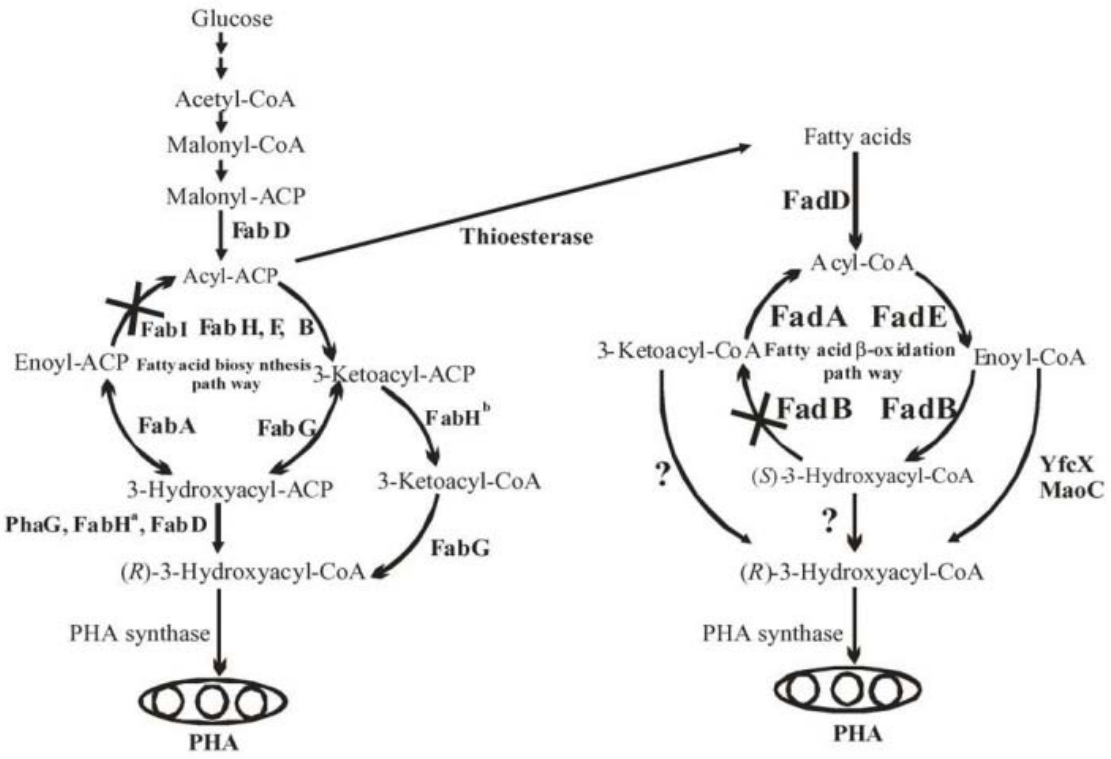


Figure 2.6: Metabolic engineering of the fatty acid biosynthesis pathway for production of PHA (Park *et al.*, 2005).

2.8 Involvement of lipase in PHA production

Lipases are the extracellular enzymes that hydrolyse the ester bonds of water insoluble lipid substrates and convert triglyceride to glycerides and fatty acids. There are numerous lipases found in bacteria, plant and animal (Saxena *et al.*, 1999). It has been reported that microbial lipases are more stable compare with the animal or plant lipases (Hasan *et al.*, 2006).

The selection of carbon substrates used in PHA production is significantly crucial which is influencing the production cost. Basically, oil is a potential substrate for PHA production because it is relatively cheap renewable carbon resources, readily available in large quantity and containing long fatty acid chain which can be incorporated into mcl-PHA.

Tan *et al.* (1997) had reported that high yield production of mcl-PHA in *P. putida* using saponified palm kernel oil (SPKO). However, the *P. putida* could not utilize the unsaponified palm kernel oil (PKO) for PHA synthesis due to its inability to produce lipase in the cell. Additional chemical process to breakdown the palm oil by saponification for PHA synthesis is not cost effective in bulk production. Therefore, further studies on utilizing lipase to hydrolyse oil in PHA production are essential.

2.8.1 Lipase secretion mechanisms in bacteria

Translocation of bacterial lipases through cytoplasmic membrane is largely dependent on the differences in their protein folding structures. As reported by Rosenau and Jaeger (2000), there are three mechanisms of lipase secretion in bacteria: the ABC exporter system, secreton-mediated secretion system and the auto-transporter system (Figure 2.7).

In ABC exporter system, the lipases that secreted by *P. fluorescens* contain C-terminal targeting signals which are recognized by the ABC exporter (Binet *et al.*, 1997). The lipases are then transported extracellularly via a pore structure to cross over the periplasm between the cytoplasmic membrane and the outer membrane of the bacteria.

The second type of lipase secretion in bacteria is by secreton, a complex secretion machinery made up of 14 or less different proteins (Pugsley *et al.*, 1993). The Xcp protein is located both in inner and outer membrane, creating a pore which allows the transportation of lipase (Bitter *et al.*, 1998). This secretion mechanism can be found in *P. aeruginosa* (Jaeger *et al.*, 1994). There are some cases that the secreted lipase is firmly bound to surface of the bacterial cells. This type of lipase consists of N-terminal that is responsible for the catalytic activity and C-terminal which encodes for auto-transporter protein used in lipase translocation (Henderson *at al.*, 1998).

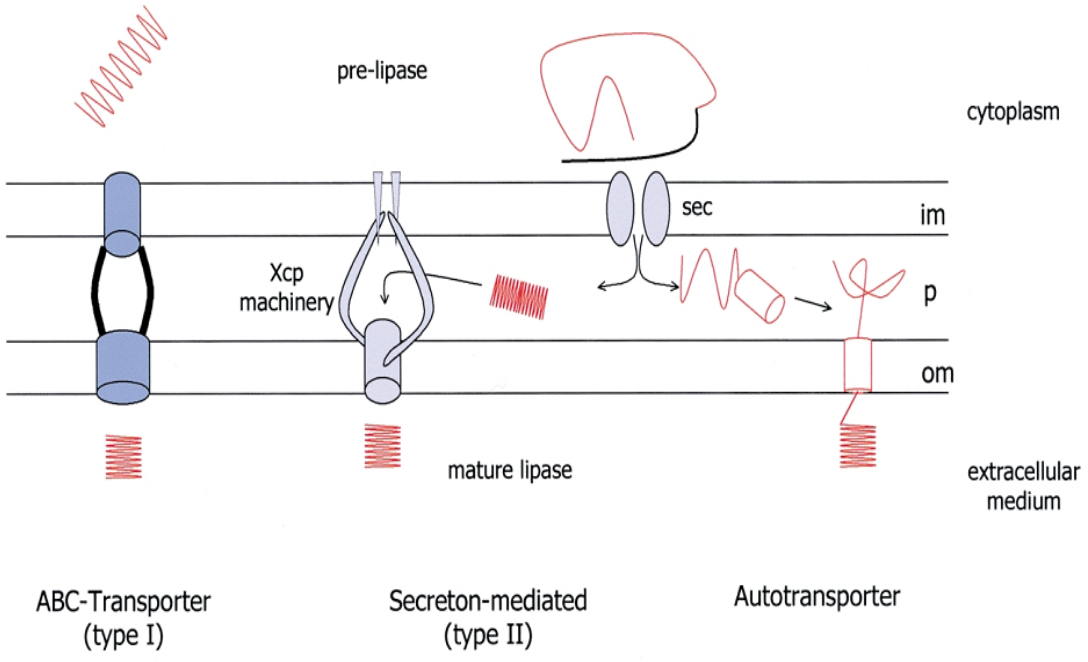


Figure 2.7: Secretion pathway used by lipolytic enzymes of gram-negative bacteria. (Rosenau and Jaeger, 2000).

2.8.2 Pseudomonads lipases

The lipases produce from *Pseudomonas* spp. are categorized into three groups based on their amino acid sequences homology which is Group I, II and III (Jaeger *et al.,* 1994). Lipases categorized in Group I (representative strain is Burkholderia *cepacia*, formerly known as *Pseudomonas cepacia*) and Group II (*P. aeruginosa*) have molecular mass of 28 to 33 kDa with approximately 300 to 320 amino acid residues. The *lip* genes coding the lipases in these two groups contain an export signal sequence and disulfide bridge (existence of cysteine residue) at the N-terminal. Lipases from these groups require a helper protein which plays a crucial role in assisting the folding of the lipase into a functioning conformation for translocation. This chaperone-like protein is encoded by the gene located at the downstream of *lip* gene.

Contrary to Groups I and II, lipase in Group III (*P. fluorescens*) consist of proteins of larger molecular mass (55 kDa). The *lip* gene coding for Group III lipase does not contain an export signal sequence and thus a helper protein is not required. In this case, lipase is secreted extracellularly via an ABC-exporter system (Duong *et al.,* 1994).

Higgins *et al*. (1992) had reported that the ABC exporter protein consists of four membrane-associated domains which involved in membrane transporting system. Two of these domains are hydrophobic and membrane-embedded which create a channel for the substrate to travel across the membrane. The remaining two domains, located at the cytoplasmic side of the membrane are associated with ATP for transport purposes.

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacteria strains and culture condition

All the bacterial strains used in this study (Table 3.1) were routinely cultured in Nutrient Broth medium (Merck, USA) at 30°C for 16 h in the orbital shaker incubator at 160 rpm.

Table 3.1: Bacteria strains used in this study

Bacterial strains	**Description (relevant genotype, phenotype)**	**Source**
One Shot® Top10 *E. coli* (OS)	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ* ΔM15Δ*lacX74 rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen , USA
- OS_ pT-*phaC1*	One Shot® Top10 *E. coli* containing pT-*phaC1*	In this study
- OS_ p2T-*lip*	One Shot® Top10 *E. coli* containing p2T-*lip*	In this study
fad B¯ *E. coli* LS1298 (LS)	F-tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1 λ-*fad B*::Kan	Concetta C. DiRusso, University of Nebraska, Lincoln, USA.
- LS_M3	*E. coli* LS1298 containing both pT-*phaC1* and p2T-*lip* vector	In this study
- LS_ pT-*phaC1*	*E. coli* LS1298 containing pT-*phaC1* vector	In this study
- LS_pT-*lacZ*	*E. coli* LS1298 containing pT-*lacZ* vector	In this study
Pseudomonas fluorescens	ATCC 13525	ATCC
Pseudomonas putida PGA1	Wild type	Eggink, G., Agrotechnological Research Institute, Wageningen, Netherlands.

The One Shot® Top10 *Escherichia coli* strains (Table 3.1) were cultivated in Luria-Bertani (LB) Broth (containing 1.0% (wt/vol) Tryptone, 0.5% (wt/vol) Yeast Extract, 1.0% (wt/vol) Sodium Chloride) (Merck, USA) at 37°C for 16 h in the orbital shaker incubator at 160 rpm.

3.2 Identification and amplification of *lip* gene and *phaC1* gene

To clone a functioning lipase (*lip* gene) and PHA synthase (*phaC1* gene) genes from *Pseudomonas* spp., the genes of interest were identified and amplified by Polymerase Chain Reaction (PCR) technique. Genomic DNA of *Pseudomonas fluorescens* (ATCC 13525) and *Pseudomonas putida* (PGA1) were used as PCR template. The combination of forward and reverse oligo primers (LF & LR and C1F & C1R to amplify *lip* gene and *phaC1* gene respectively) were designed using GeneFisher software according to its functional amino acid sequences retreated from GenBank database. The designed oligo primers were subjected to Primer-BLAST analysis to ensure the oligo primers are specific to the targeted sequences in the desired bacteria. All oligo primers (Table 3.2) were custom-synthesized by First BASE Laboratories Sdn. Bhd., Malaysia.

Table 3.2: Oligo primers used in this study

Primers	**Sequences**	**Targeted priming site**
C1F C1R	5'-ATG AGT AAC AAG AAC AAC GAT GAG C-3' 5'-GCC ACG GCG CTG TAA CTC A-3'	*phaC1* gene in *P. putida* (*PGA1*)
TrxF pBADR pBADF	5´-TTC CTC GAC GCT AAC CTG-3 5'-GAT TTA ATC TGT ATC AGG -3' 5'-ATG CCA TAG CAT TTT TAT CC -3'	Sequencing primers for pBAD TOPO vector
LF LR	5'-CAC CAT GGG TAT CTT TGA CTA TAA A-3' 5'-ACC GCC ACC GGT TTA TTA CG-3'	*lip* gene in *P. fluorescens* (ATCC13525)

In the PCR reaction, the bacteria lysate was used as DNA template. One mL of freshly-grown bacteria cells in 100 mL of Nutrient Broth (Merck, USA) was collected by centrifugation at 10,000 ×*g* for 3 min and the cell pellet was washed with sterile distilled water twice. Cells were then resuspended in 200 µL sterile distilled water and were boiled at 95°C for 10 min before being used in PCR reaction.

PCR gradient reaction was performed to determine the optimum annealing temperature for the oligo primers. The following thermal cycle condition was used: 94°C for 5 min, followed by 30 repeated cycles of 94°C for 30 s, gradient annealing temperature ranging from 50°C to 65°C for 30 s and extension at 72°C for 2 min and a final incubation at 72°C for 10 min (Mycycler ™, thermal cycler, Bio-Rad Laboratories Inc., CA). The PCR reaction mixture was prepared as shown in Table 3.3.

Table 3.3: Reaction mixture for PCR

Component	Volume (µl)	Final Concentration
5× green or colourless GoTaq® Flexi Buffer	20	1×
$MgCl_2$ solution (25 mM)	6	1.5 mM
PCR nucleotide mix (10 mM each)	2	0.2 mM each dNTP
Upstream primer (10 mM)	5	0.5 µM
Downstream primer (10 mM)	5	0.5 µM
GoTaq® DNA Polymerase (5 U/µL)	0.5	1.25 U
DNA template	1	-
Sterile distilled water	60.5	-
Total	100	

To amplify a blunt end PCR fragment, 100 µL of *Pfx* DNA polymerase reaction mixture (Invitrogen, USA) was prepared as showed in Table 3.4. The amplification thermal cycle was as follow: 94°C for 5 min followed by 25 cycles 94°C for 15 s, 52.9 °C for annealing temperature which has been determined earlier for 30 s and extension at 68°C for 2 min and a final incubation for extension was at 68°C for 8 min.

Table 3.4: Reaction mixture for blunt end PCR fragment

Component	Final Volume (µL)	Final Concentration
10× *Pfx* Amplification Buffer	10	1×
$MgSO_4$ solution (50 mM)	2	1.0 mM
10 mM dNTP mixture	3	0.3 mM each
Upstream primer (10 mM)	5	0.5 µM
Downstream primer (10 mM)	5	0.5 µM
Platinum *Pfx* DNA Polymerase	0.8	1 U
DNA template	1	
Sterile distilled water	73.2	
Total	100	

The PCR products were then analyzed by agarose gel electrophoresis which was carried out in TAE buffer (50 mM tris-acetate, pH 8; 1 mM EDTA) at 90V for 50 min. For each sample, 5 µL of PCR amplified product was mixed with 1 µL of 6× loading dye and was loaded into the well of 1.5% (wt/vol) agarose gel in 1× TAE buffer. The agarose gel was then stained with ethidium bromide and visualized under UV transilluminator (Syngen Bio Imaging, UK). The size of the amplified DNA product was determined by referring to the 1kb DNA ladder (Promega, USA).

3.3 Construction of recombinant plasmids

The amplified *phaC1* PCR fragment from *P. putida* and *lip* PCR fragment from *P. fluorescens* were purified by using MEGAquick-spin™ PCR & Agarose Gel DNA extraction kit (INTRON Biotechnology, INC., Korea). The purification steps were carried out as recommended by manufacturer.

For each sample, 5 volume of BNL Buffer was added to the PCR reaction product and was mixed using vortex. The mixture was then transferred to the spin column assembly provided in the kit and centrifuged at 13,000 rpm for 1 min. The filtrate was discarded and the spin column was placed into the same collection tube. Subsequently, 700 μL of washing buffer was added to each column and again centrifuged at 13,000 rpm for 1 min. After discarding the filtrate, the column was then spin-dried by centrifugation at 13,000 rpm for 1 min. The spin column was then placed into a sterile 1.5 mL microcentrifuge tube and 50 μL of Elution Buffer was directly added to the center of the column without touching the membrane with the pipette tip. The column was incubated at room temperature for 1 min and then centrifuged at 13,000 rpm for 1 min. The filtrate in the microcentrifuge tube contained purified DNA fragments and was stored at -20°C.

The plasmids used in this study were listed in Table 3.5. To construct recombinant plasmid pT-*phaC1* and p2T-*lip*, the purified PCR products of *phaC1* and *lip* genes were ligated into pBAD-TOPO vector and pBAD202/D-TOPO vector respectively (Promega, USA). The reaction mixture for ligation was prepared as shown in Table 3.6. The mixtures were incubated for 5 min at room temperature and then placed on ice. The remaining reaction mixture was stored in -20°C for future use.

Table 3.5: Plasmids used in this study

Plasmids	**Description (relevant genotype, phenotype)**	**Source**
pBAD-202 TOPO vector (p2T)	Expression vector: Km^R	Invitrogen, USA
pBAD-TOPO vector (pT)	Expression vector: Amp^R	Invitrogen, USA
pT-*phaC1*	pBAD-TOPO vector containing a 1.7 kb PCR product (*phaC1* gene*)* cloned from *P. putida*	In this study
p2T-*lip*	pBAD containing a 1.7 kb PCR product (*lip* gene) cloned from *P. fluorescens*	In this study
pUC19	Control vector	Invitrogen, USA
pBAD-TOPO/*lacZ*/V5-His (pT-*lacZ*)	Control vector containing *β-galactosidase* gene	Invitrogen, USA

Table 3.6: Reaction mixture for TOPO® cloning

Reagent	Standard Reaction	Positive Control	Background Control
pBAD202/D-TOPO Vector (10 ng/µL) or pBAD-TOPO Vector	1 µL	1 µL	1 µL
PCR product	0.5 to 5 µL	-	-
Control Insert DNA	-	1 µL	-
Salt Solution (1.2 M NaCl and 0.06 M $MgCl_2$)	1 µL	1 µL	1 µL
Deionized water to a final volume of	6 µL	6 µL	6 µL

3.4 Transformation

This transformation method references the protocol reported by Sambrook and Russel, (2001). Firstly, the recombinant plasmids were transformed into One Shot® Top10 *E. coli* strain and the *fad B*¯ *E. coli* LS1298 by heat shock method. *E. coli* One Shot® Top10 and LS1298 strains were cultured overnight in 100 mL Luria-Bertani (LB) Broth. Prior to transformation procedure, 1 mL of each culture was inoculated into 100 mL of fresh LB broth and incubated for 3 h at 37°C in an orbital shaker incubator until the OD_{600} of the culture was 0.5. The number of viable cells for cloning purpose should not exceed 10^8 cells /mL which give approximately 0.5 at OD_{600}.

The fresh-grown cells were then aseptically transferred into an ice-cold microcentrifuge tube and were kept on ice for 10 min. Cells were harvested by centrifugation at 1,500 × *g* for 2 min at 4°C. Cell pellet was washed twice with 1 mL of ice-cold 0.1 M of $CaCl_2$ and resuspended in 0.5 mL cold 0.1 M of $CaCl_2$ followed by incubation on ice for 30 min. For each transformation reaction, 100 µL of cell suspension was used. Finally, 4 µL of the recombinant plasmid (from ligation mixture) were added into 100 µL of competent cells and incubated on ice for another 30 min.

The cells were heat-shocked at 42°C for 30 s and placed on ice immediately after the heat-shock treatment. Then, 100 µL of pre-warmed SOC medium (containing 2% tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added into the cell suspension and incubated with shaking (160 rpm) at 37 °C for 1 h. After the incubation period, 50 µL of the transformed cells was spread on a pre-warmed selective agar plate (LB agar medium containing 50 µg/mL of kanamycin or 100 µg/mL ampicillin or both (Sigma, USA)) and incubated at 37°C. After 16 h of incubation,

white colonies were carefully picked and grown in 100 mL of fresh LB broth medium for subsequent analysis.

For preparation of negative control, 2 µL of plasmid pBAD-TOPO/*lacZ*/V5-His (10 ng/µl) was transformed into *fad* B^{-} *E. coli* LS1298 using the heat shock protocol as mentioned before. Blue colonies were then selected from the selective agar plates with 100µg/mL of ampicillin and 100 µg/ml of X-Gal. Meanwhile, pUC19 plasmid was used to determine the transformation efficiency of the *E. coli* One Shot® Top10. Here, 10 pg of pUC19 uncut plasmid which is provided in the TOPO cloning kit was used for transformation using the same procedure as mentioned before. Similarly, 50 µL of transformant cells were plated on LB agar containing 100 µg/mL of ampicillin and 100 µg/mL of X-Gal. The transformation efficiency should be about 1×10^{8} cfu/µg DNA in which ~ 250 colonies were obtained from average of four plates.

3.5 Transformants analysis

3.5.1 PCR technique

The presence and orientation of the insert in the recombinant transformant was confirmed by PCR technique. The PCR reaction mixture was prepared according to the description in Table 3.3. The primers that were used in this PCR procedure were the combination of TrxF primer or the pBADF primer and a primer that hybridizes within the insert (Table 3.2). The PCR products were then visualized and analyzed by 1.5% (wt/vol) of agarose gel electrophoresis as mentioned in section 3.2.

3.5.2 DNA sequencing analysis

The selected white colony was grown in 100 mL of fresh LB broth medium with appropriate concentration of antibiotics. The plasmid was then harvested by using DNA-spin^{TM} Plasmid DNA Purification Kit (INTRON, Korea) following the provided kit protocol. For each sample, 3 mL of overnight culture was harvested by centrifugation at 13,000 rpm for 30 s in a microcentrifuge tube. The cell pellet was resuspended in 250 µL of Resuspension Buffer using vortex (RNase A Solution that provided in the kit was added into Resuspension Buffer before used). Subsequently, 250 µL of Lysis Buffer was added and the solution was mixed by inverting the tube several times with closed lid. Then, 350 µL of Neutralization Buffer was added and the solution was mixed gently by inverting the tube several times with closed lid until cloudy and a flocculent precipitate was formed.

The mixture was then incubated at 4°C for 5 min to enhance the precipitation. After centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant was transferred into a spin

column provided in the kit. The column was centrifuged at 13,000 rpm for 60 s. The filtrate in the collection tube was discarded and the collection tube was re-used in subsequent step. Then, 500 µL of Washing Buffer A was added and the column was centrifuged at 13,000 rpm for 60 s. The filtrate in the collection tube was again discarded.

Next, 700 µL of Washing Buffer B was added and the column was centrifuged at 13,000 rpm for 60 s. The excess buffer solution that was trapped in the membrane of the spin column was removed by centrifugation at 13,000 rpm for 60 s. Lastly, the spin column was placed into a sterile 1.5 mL microcentrifuge tube and 50 µL of Elution Buffer was directly added to the center of the column without touching the membrane with the pipette tip. It was then incubated at room temperature for 1 min and then centrifuged at 13,000 rpm for 1 min. The filtrate in the microcentrifuge tube containing extracted plasmids was then stored at -20°C. The DNA sequencing was performed by First BASE Laboratories Sdn Bhd, Malaysia using ABI BigDye Terminator V3.1 kit in ABI377-96 upgrade and ABI 3100 Genetic Analyzer. The retrieved nucleotide sequences were analyzed with Molecular Evolutionary Genetics Analysis software version 4.0 (MEGA) (Tamura *et al.*, 2007) and BLAST search in the National Center for Biotechnology Information (NCBI) database.

3.6 Reverse transcriptase polymerase chain reaction (RT- PCR)

3.6.1 RNA extraction from bacterial cells

An overnight cultured bacterial cell (1mL) was harvested by centrifugation at 500 × *g* at 4°C for 5 min. Cell pellet was resuspended by vortex in 100 µL of the lysosyme solution which containing 10nM Tris-HCl (pH 8.0), 0.1 mM EDTA and 1mg of lysozyme. For each tube, 0.5 µL of 10% (wt/v) SDS solution was added to the mixture and was mixed by vortex. After 5 min of incubation at room temperature, 350 µL Lysis Buffer containing of 1% (v/v) of 2-mercaptoethanol were added and mixed well by vortex. The lysate were transferred to a homogenizer inserted in an RNase-free tube and centrifuged at 12,000 × *g* for 2 min at room temperature.

Then, 250 µL of absolute ethanol was added to each volume of bacterial cell homogenate and mixed thoroughly by vortex. The mixture was then transferred to a Spin Cartridge and centrifuged at 12,000 × *g* at room temperature for 15 s. The RNA sample that trapped in the Spin Cartridge were washed by 700 µL Wash Buffer I once and 500 µL Wash Buffer II for twice. The washing buffer was removed every time by centrifugation at 12,000 × *g* for 15 s. The Spin Cartridge was centrifuge again at 12,000 × *g* for 1 min at room temperature to dry the membrane with trapped RNA. The Spin Cartridge was then inserted to a RNase- free Recovery Tube. Then, 50 µL of RNase-free water was loaded to the center of Spin Cartridge and incubated for 1 min. The RNA was then harvested into the Recovery Tube by centrifugation at 12,000 × *g* for 2 min.

3.6.2 DNase treatment

RNA samples were DNase treated to remove residual DNA. Briefly, 1 µL extracted RNA samples (10 µg/µL) were mixed with 1 µL of 1 U/µL DNase I (Invitrogen, USA), 1 µL of 10× DNase I Buffer and 7 µL of DNase-free H_20. This mixtures were then incubated at room temperature for 15 min and followed by heat inactivation at 65°C for 15 min after 1µL of 25nM EDTA were added.

3.6.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

The total RNA was reverse transcripted to cDNA using SuperScript ™ III First-Strand System (Invitrogen, USA). Firstly, RNA-primer mixture were prepared in a total volume of 10 µL which contain 1 pg to 5 µg of total RNA, 5 µM of oligo $(dT)_{20}$, 0.2 nM dNTP mix and DEPC-treated H_2O. The mixture was incubated at 65°C for 5 min and then placed on ice for at least 1 min.

Then, the cDNA synthase mixture were prepared which containing 2 µL of 10× RT buffer, 4 µL of 25 mM $MgCl_2$, 2 µL of 0.1M DTT, 1 µL of RNaseOUT™ (40 U/µL), 1 µL of SuperScript™ III RT (200 U/µL) and 10 µL RNA-primer mixture. Followed by that, both cDNA synthase mixture and RNA-primer mixture were mixed and incubated at 50°C for 50 min and heat inactivated at 85°C for 5 min. After reaction mixture was chilled on ice, 1 µL of RNase H were added and incubated at 37°C for 20 min. The cDNA samples were used as the template for normal PCR immediately following section 3.2.

3.7 Determination of lipase activity in the recombinant strains

3.7.1 Trioleoylglycerol agar plate

Trioleoylglycerol agar was used to determine lipolytic reaction of bacteria (Kouker and Jaeger, 1987). Trioleoylglycerol agar contained 10 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride, 20 g of agar and 0.3% (vol/vol) of emulsified olive oil per 1 L of medium. The emulsified olive oil was prepared by adding 100 mL of olive oil into 400 mL of warm distilled water with 1mL of Tween 80. The mixture was then homogenized in high speed blender and sterilize in autoclave at 121°C for 15 min. Then 30 mL of emulsified oil was added to the lukewarm sterilized media and was mixed thoroughly. The recombinant transformants of the *E. coli* OS- p2T-*lip* and LS_M3 were cultured on trioleoytlglyrecol agar plate and incubated at 37°C for 48 h. Clear halo formation indicated positive lipolysis of recombinant strains.

3.7.2 Lipase activity assay

The constructed recombinant strains were cultivated in the LB broth containing 0.3% (vol/vol) of olive oil. The lipase activities of the recombinant strains were examined by spectrophotometric method using *p*-nitrophenyl laurate as a substrate. For each reaction sample, 25 µL of culture fluid were dissolved in 725 µL of 50 mM phosphate buffer pH 7.0 and 100 µL of 25 mM *p*-nitrophenyl laurate in absolute ethanol. After 10 min of incubation period at 37°C, 250 µL of Na_2CO_3 were added to the mixture and were centrifuged at 13000 rpm, 4°C for 10 min. Culture fluid were substituted with distilled H_2O to serve as blank for the spectrophotometer. The reaction mixture was examined using a

spectrophotometer (UV-1601, UV-visible spectrophotometer, Shimadzu, Japan) at 420 nm. This experiment was repeated three times for each sample.

3.8 PHA analysis in recombinant strains

3.8.1 Cultivation of recombinant strains in different carbon sources

The recombinant strains harboring functioning PHA synthase gene was tested for the PHA production by shake-flask technique. To prepare the inoculums for PHAs production, the pure recombinant isolate was grown in 90 mL nutrient broth at 37°C in orbital shaking incubator at 180 rpm with five different concentrations of inducer, L-arabinose (0.00002%, 0.0002%, 0.002%, 0.02% and 0.2% (wt/vol) respectively), together with 50 mg/mL of kanamycin or 100 µg/mL of ampicillin or both. After 12 h of incubation, 10 mL of carbon substrate such as emulsified palm kernel oil (PKO), sodium octanoic acid, saponified palm kernel oil (SPKO) and glucose were added respectively into the culture to achieve a final carbon substrate concentration of 0.2% (wt./vol). The culture was further incubated for another 36 h.

The palm kernel oil used in this study was kindly supplied by Southern Acids (M) Ltd., Klang, Selangor. The saponified palm kernel oil (SPKO) was prepared by mixing 8 g of palm kernel oil with ethanolic sodium hydroxide solution (11.2 g of sodium hydroxide dissolved in 400 mL of absolute ethanol). The mixture was refluxed for 1 h at 75°C. Next, the semi-solid sodium salt of fatty acid was collected by removing the ethanol in the solution using rotary evaporator. The SPKO was then further dried in the over at 60°C and stored in room temperature.

After 36 h of incubation period, the culture was harvested by using large capacity refrigerated centrifuge (Continent-R, Hanil Science Industrial, Korea) at 8,000 × *g* for 30 min at 10°C. The harvested cell pellet was then washed with 0.9 % saline to remove access supernatant. Finally, the harvested cells pellet was heat-dry in oven at 50°C to remove access water.

3.8.2 Microscopic analysis for PHA production in recombinant strains

This analysis references the method reported by Ostle and Holt, (1982). After 48 h of incubation period of the bacteria culture, the heat-fixed smears of bacterial cells were stained with 1% (vol/vol) Nile Blue A at 55°C for 10 min in a coplin staining jar and washed with tap water to remove excess stain with 8% (vol/vol) acetic acid for 1 min. The stained smear were then washed and dried, remoistened with water, and covered with cover slip. The cover slip is necessary as standard immersion oil will extract some of the fluorescent dye and obscure the field with a general yellow fluorescence. The cover slip thus protects the stained cells from immersion oil. The slides were then examined under fluorescent microscope using 450 to 490 nm filter. Nile Blue A have the affinity with hydrocarbon compound and PHA granules accumulated within the bacteria cell will show fluorescing bright orange.

3.8.3 Gas chromatography analysis of PHA production in recombinant strains

The PHA content in the bacterial cells was subjected to methanolysis treatment (Brandl *et al.*, 1988) and the resulting methyl esters of PHA monomers were analysed using gas chromatography. For each sample, 25 mg of the dry cells were resuspended in the mixture of 1 mL chloroform and 1 ml of methanol containing 15% (vol/vol) H_2SO_4 in a screw-capped test tube. The mixture was then incubated at 100°C for 2 h with occasional shaking. After heating, the reaction was cooled to room temperature and 0.5 mL of deionized water was added. The mixture was then thoroughly homogenized using vortex and halted to separate the organic phase from the aqueous phase. The organic phase solution that set at the bottom layer was collected by Pasteur pipettes and transferred into a screw-cap sample vial.

Then, 1 µL of collected organic phase were injected and analyzed by GC-2014 gas chromatograph (Shimadzu, Japan) equipped with BP20 capillary column (30.0 m by 0.25 mm; SGE Analytical Science) operating in split mode (split ration 1:20). The column was temperature programmed with initial temperature at 120ºC for 2 min and followed by the temperature increment at rate 20°C/min until reached temperature at 230ºC and hald for 10 min. Hydrogen was used as the carrier gas, the injection temperature was at 225ºC however the separated compound was detected at 230ºC by SFID1 column detector.

The concentration of methyl esters compound were analyzed and calculated based on the peak area by GC Solution software (version 2.3). Each peak at different retention time in the gas chromatogram represents one compound of the sample mixture. By referring to the concentration of standard solution, concentration of the PHA that accumulated in the recombinant strains can then be determined. The compound of the mixture was identified by comparing to the retention times of the samples with the standard. The standard included methyl 3-hydroxybutanoate (C4), methyl 3-hydroxyhexanoate (C6), methyl 3-hydroxyoctanoate (C8), methyl 3-hydroxynonanoate (C10), methyl 3-hydroxydecanoate (C12), methyl 3-hydroxydodecanoate (C14) and methyl 3-hydroxyhexadecanoate (C16) that were used in the experiment were purchased from Larodan Fine Chemicals (Sweden).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Verification of lipase activity in *Pseudomonas flourescens* ATCC 13525 and PHA synthase activity in *Pseudomonas putida* PGA1

There have been comprehensive studies on lipase activity in *P. fluorescens* (Tan *et al.*, 1992). The lipase production in *P. fluorescens* ATCC 13525 purchased from American Type Culture Collection was verified by using trioleoylglycerol agar plate assay (Figure 4.1) whereby halo was observed around the culture after 48 h incubation on the plate. On the other hand, the ability of PHA accumulation in *P. putida* PGA1 was confirmed using Nile Blue A fluorescence micrograph (Figure 4.2). It was reported that *P. putida* PGA1 could accumulate mcl-PHA up to 40% of the cell dry weight in previous studies (Tan *et al*,. 1997).

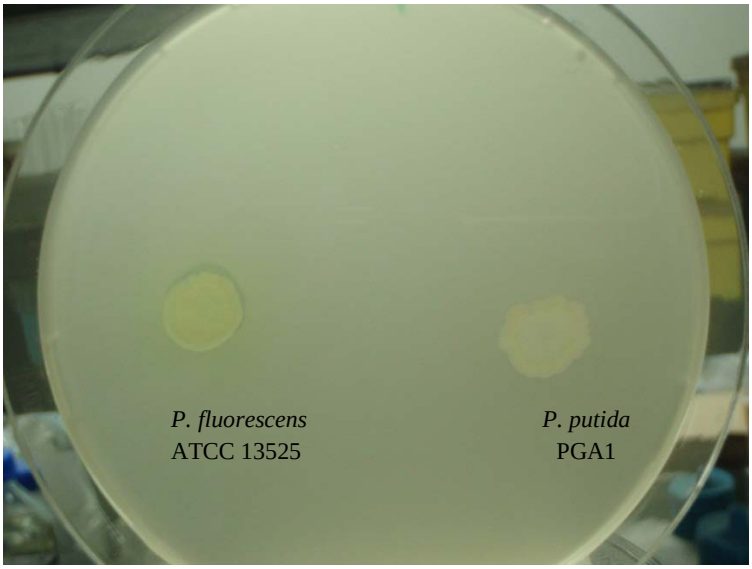


Figure 4.1: Lipase production by *P. fluorescens* ATCC13525 on trioleoylglycerol agar plate. Lipolytic activity as indicated by a clear zone around the culture in comparison , *P. putida* PGA1 did not produce lipase.

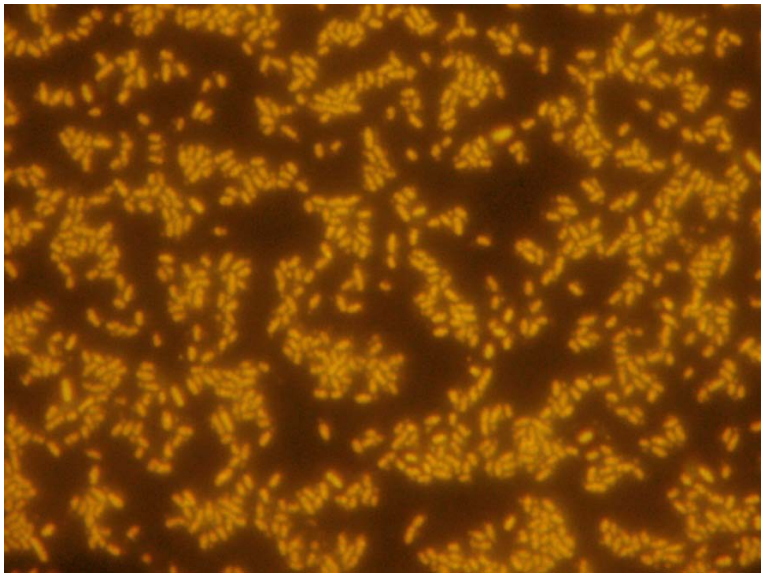


Figure 4.2: Nile Blue A fluorescence micrograph of PHA accumulation in *P. putida* PGA1.

Hence, the desired enzymatic reactions in *P. fluorescens* ATCC 13525 and *P. putida* PGA1 were confirmed and thus they were used as the donor of the *lip* gene and *phaC1* gene respectively in further analysis.

4.2 PCR amplification of *lip* gene and *phaC1* gene

In order to amplify *lip* gene from *Pseudomonas fluorescens* ATCC 13525 and *phaC1* gene from *Pseudomonas putida* PGA1, oligo primers were designed according to their coding regions in the respective strains. The DNA sequences of the *lip* gene in *P. fluorescens* PrtB (AF216702, region: 3814-5400) and *phaC1* gene in *P. putida* KT2440 (AE015451, region: 5699514-5701193) were retrieved from GenBank database and were used as the references in designing the oligo primers. The oligonucleotide primers PfF and PfR were designed to amplify the *lip* gene, whilst primers C1F and C1R were to amplify the *phaC1* gene.

The optimal annealing temperature of the designed oligo primers were determined by gradient PCR using annealing temperature ranging from 50.0°C to 65°C (Figure 4.3). The agarose gel picture revealed that *lip* gene fragment from *P. fluorescens* ATCC 13525 (~1586 bp) was amplified at annealing temperatures 50.0°C, 51.0°C, 52.9°C and 55.5°C (Figure 4.3, lane 1 to 4). On the other hand, *phaC1* gene fragments (~1680bp) from *P. putida* PGA1 were able to be amplified within the annealing temperature of 50°C to 65°C (Figure 4.3, lanes 9 to16). The size of amplified *lip* gene and *phaC1* gene were coincided with the predicted sizes based on the retrieved sequences from GenBank database. Hence, the suggested optimum annealing temperature for both *lip* gene and the *phaC1* gene was 52.9°C.

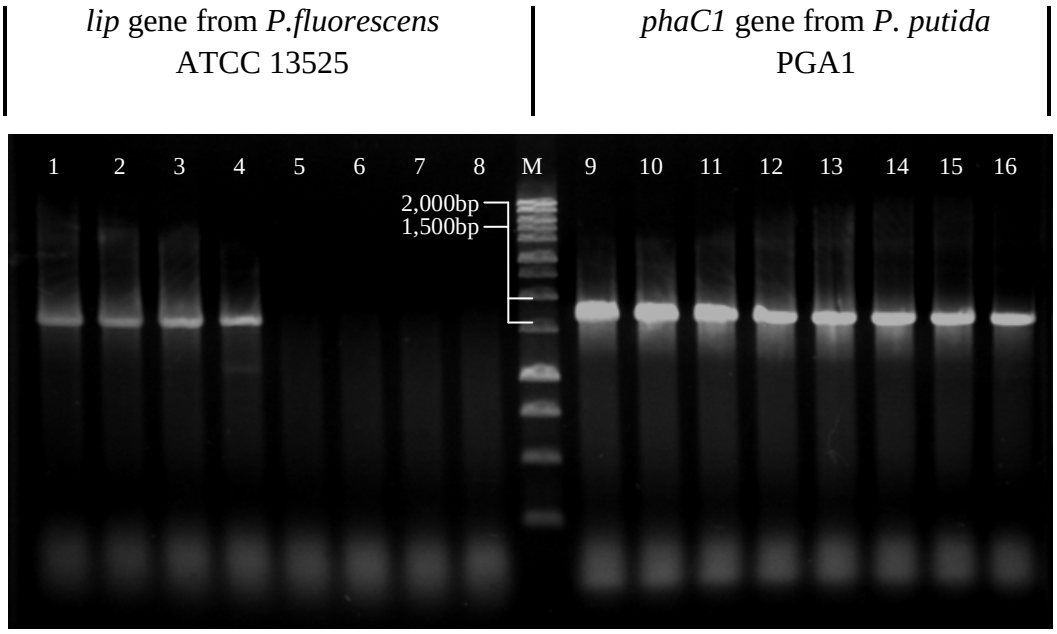


Figure 4.3: Agarose gel electrophoresis analysis of PCR-amplified *lip* gene from *P. fluorescens* ATCC 13525 and *phaC1* gene from *P. putida* PGA1. Lanes 1 and 9: 50.0°C; lanes 2 and 10: 51.0°C; lanes 3 and 11: 52.9°C; lanes 4 and 12: 55.5°C; lanes 5 and 13: 59.1°C; lanes 6 and 14: 62.0°C; lanes 7 and 15: 63.8°C; lanes 8 and 16: 65.0°C; M: 1kb DNA ladder (Promega).

4.3 Screening of *Escherichia coli* transformants harbouring the *lip* gene or the *phaC1* gene

Plasmid pT-*phaC1* was constructed by ligating *phaC1* DNA fragment into the pBAD-TOPO vector, which was then transformed into the One Shot® Top10 *E. coli* by heat shock treatment. The orientation of the inserted fragment in the transformants was determined by PCR using a combination of primers pBADF (targeted to vector located at distance about ~150 nt upstream from the cloning side) and C1R (hybridized to *phaC1* gene).

The vector with correctly-orientated insertion showed an amplified DNA fragment (~1830 bp which includes the 150 bp flanking end from the vector sequence before the inserted *phaC1* gene). The agarose gel electrophoresis revealed that 4 out of 8 selected transformants contained vector with the correct-orientated insertion (Figure 4.4, lanes 1, 5, 6 and 7). The transformant analysed at lane 7 was designated as OS_ pT-*phaC1* and used in subsequent experiments.

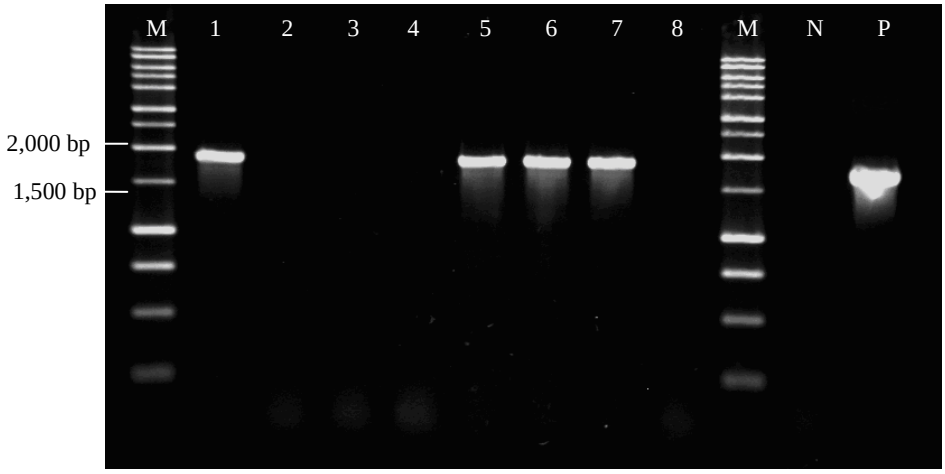


Figure 4.4: Agarose gel electrophoresis analysis of PCR-amplified *phaC1* gene from selected transformants. Lanes 1 to 8 represent 8 individual transformant; M: 1kb DNA ladder (Promega); N: negative control; P: *phaC1* gene fragment from *P. putida*.

Plasmid p2T-*lip* was constructed by ligating amplified *lip* DNA fragment from *Pseudomonas flourescent* into pBAD-202 TOPO vector and was transformed into the One Shot® Top10 *E. coli*. The p2T-*lip* positive clones harboring *lip* gene were selected by selective medium LB agar with 50 μg/mL of kanamycin. Similarly, the orientation of the insertion in transformants was also verified by PCR technique. A combination primers set TrxF (hybridized to vector located at distance about ~150 nt upstream from the cloning side) and LR were used for this PCR. Agarose gel electrophoresis showed that all 8 selected clones contained the correctly orientated insertion (Figure 4.5). The transformant analysed at lane 8 was designated as OS_p2T-*lip* and used in subsequent experiments.

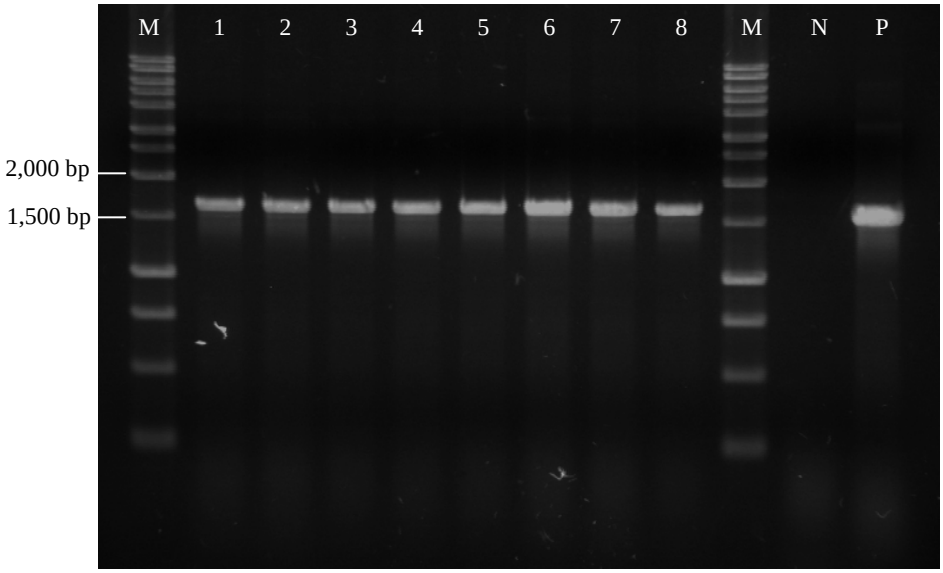


Figure 4.5: Agarose gel electrophoresis analysis of PCR-amplified DNA *lip* gene from selected transformants. Lanes 1 to 8 represent 8 individual transformant; M: 1kb DNA ladder (Promega); N: negative control; P: *lip* gene fragment from *P. fluorescens*.

4.4 Nucleotides sequences analysis

The vector p2T-*lip* from *E. coli* OS_p2T-*lip* was sequenced (Appendix A) and the analyzed nucleotide sequences of the *lip* gene by ClustalW revealed that a distinctive repeat of C-terminal signal sequence GGXGXD was found at the C-terminus (Appendix B). This repeated sequence was significantly homologous to the lipases from *P. fluorescens* retrieved from GenBank database (Appendix B). Ahn *et al.* (2001) reported that this glycine-rich consensus sequence is recognized by the ABC transporter, one of the lipase secretion mechanisms. Besides, the amino acid sequences that encode for substrate-binding domain of lipase enzyme (VVVSGHSLGGLA) were also identified and showed significant homology to others *P. fluorescens* strains by multiple-alignment using ClustalW (Tan *et al.*, 1992).

The purified plasmid from *E. coli* OS_ pT-*phaC1* was sequenced by using primers pBADF and pBADR (Appendix C). The multiple-alignment result showed that the amplified *phaC1* synthase from *P. putida* PGA1 strain have conserved catalytic residues (His, Cys and Asp) which is significantly homologous to other class II PHA synthase (Appendix D). In a previous study by Amara and Rehm (2003), these catalytic residues at C terminal region in *phaC1* synthase belong to the active site of *α/β*-hydrolase superfamily domain.

The Class II PHA synthase consists of two PHA synthase genes, *phaC1* and *phaC2* which are located at the PHA gene cluster and separated by the PHA depolymerase gene (*phaZ*) (De Eugenio *et al.*, 2007). In this study, *phaC1* from *P. putida* was amplified for cloning purpose. It has been proposed that *phaC1* and *phaC2* genes have same properties and they are capable to operate independently in transgenic host. Rehm and Steinbüchel (1999) had reported that both PHA synthase perform similar properties and exert a similar substrate specificity and that 3-hydroxydecanoyl-CoA is the main substrate.

4.5 Screening of *Escherichia coli* LS1298 transformants with the *lip* and/or *phaC1* genes

The *fab B*$^{-}$ *E. coli* LS1298 strain was used as the host for lipase as well as PHA synthase expression in this study. Expression of *phaC1* in *E. coli* LS1298 was performed by transformation of pT-*phaC1* vector which was extracted and purified from *E. coli* OS_ pT-*phaC1*. Transformants were selected on LB agar containing 50 μg/mL of kanamycin and 100 μg/mL of ampicillin. The existence of plasmid pT-*phaC1* in the transformants was verified by PCR technique using oligo primers set pBADF and C1R, whereby the positive transformant will give a DNA fragment of ~1830 bp. The agarose gel picture showed 8 selected transformants which contained vector pT-*phaC1* (Figure 4.6). Transformant analysed at lane 1 was designated as LS_pT-*phaC1* and used in subsequent experiments.

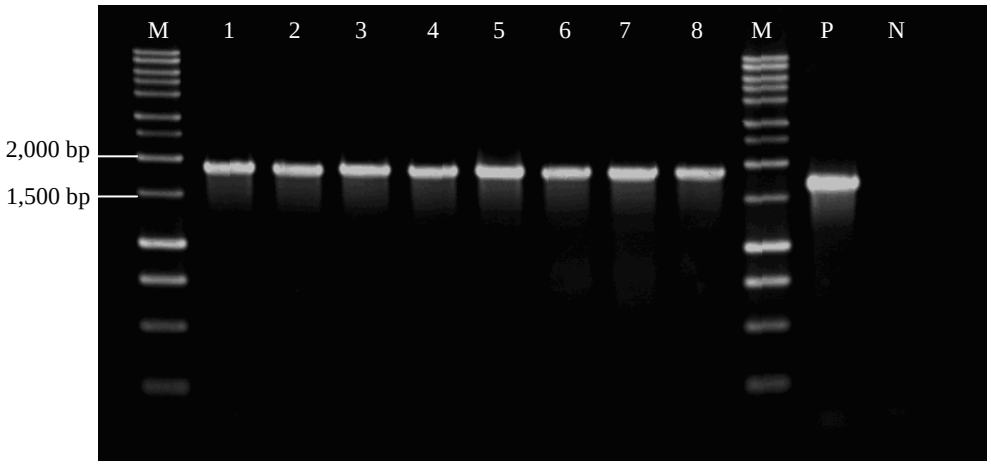


Figure 4.6: Agarose gel electrophoresis analysis of PCR-amplified *phaC1* gene fragment from selected *E. coli* LS1298 transformants with pT-*phaC1* vector. Lanes 1 to 8 represent 8 individual transformant; M: 1kb DNA ladder (Promega); N: negative control; P: *phaC1* gene fragment from *P. putida*.

Construction of *fab B*$^{-}$ *E. coli* that was capable of expressing both the *lip* and *phaC1* was accomplished by inserting equal volume of both vectors p2T-*lip* and pT-*phaC1* harvested from OS_ p2T-*lip* and OS_ pT-*phaC1* respectively into the same cell. PCR was performed

to screen the transformed *E. coli* LS1298 strain harboring both vectors p2T-*lip* and pT-*phaC1* by using a combination of oligo primers TrxF and LR (for confirmation of *lip*), as well as pBADF and C1R (for confirmation of *phaC1*). Agarose gel electrophoresis showed that 3 out of 8 picked transformants (Figure 4.7, lane 3, 4 and 8) were found to contain both vectors. Transformant analysed at lane 3 was designated as LS_M3 for subsequent experiments.

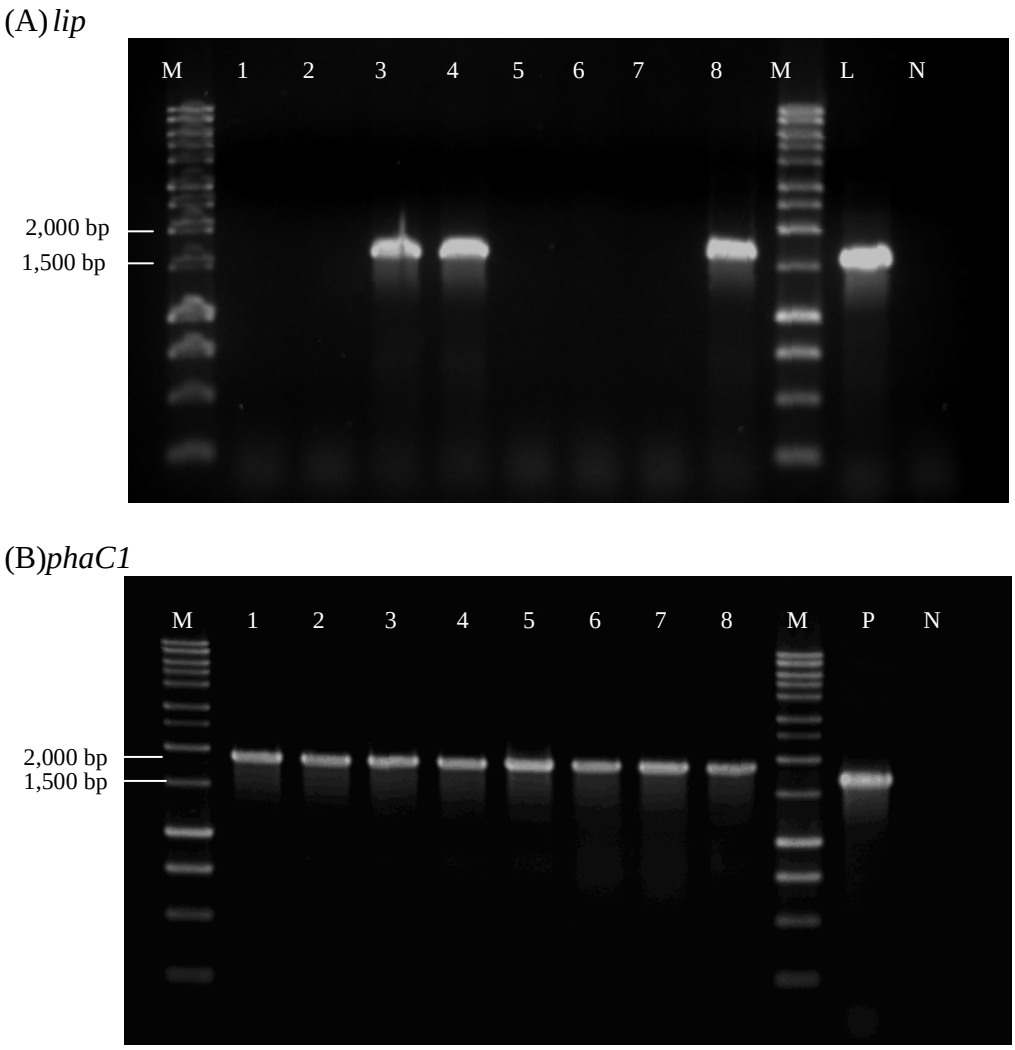


Figure 4.7: Agarose gel electrophoresis analysis of PCR-amplified *lip* gene (A) and *phaC1* gene (B) fragment from selected *E. coli* LS1298 transformants with both p2T-*lip* and pT-*phaC1* vectors. Lanes 1 to 8 represent 8 individual transformant; M: 1kb DNA ladder (Promega); L: *lip* gene fragment from *P. fluorescens*; N: negative control; P: *phaC1* gene fragment from *P. putida*.

4.6 Detection of mRNA for *lip* and *phaC1* genes in the transformants *fad B*$^{-}$ *Escherichia coli* LS1298

Reverse transcription polymerase chain reaction (RT-PCR) was used to detect the mRNA in the recombinant *E. coli*. It is a useful indicator of gene expression within the cells even in a low gene copy number. The expression of *lip* gene from *P. fluorescens* and *phaC1* gene from *P. putida* in the respective recombinant *E. coli* strains was studied at the mRNA level. The mRNA in the recombinant strains was first reverse-transcribed into cDNA. A frequent problem encountered in performing RT-PCR is DNA contamination. Therefore, the extracted total RNA was first treated with DNase. This precautionary measure was to prevent potential false positive results.

Figure 4.8 (lanes A1 and A2) illustrates that *lip* gene was detected in cDNA from *P. fluorescens* and *E. coli* LS_M3 (containing both *lip* and *phaC1* genes) using primers set LF & LR. However, in Figure 4.9 (lanes X1, X2 and X3) *phaC1* gene was detected in cDNA from *P. putida*, *E. coli* LS_M3, *E. coli* LS_pT-*phaC1* (containing only *phaC1* gene) using primers C1F & C1R.

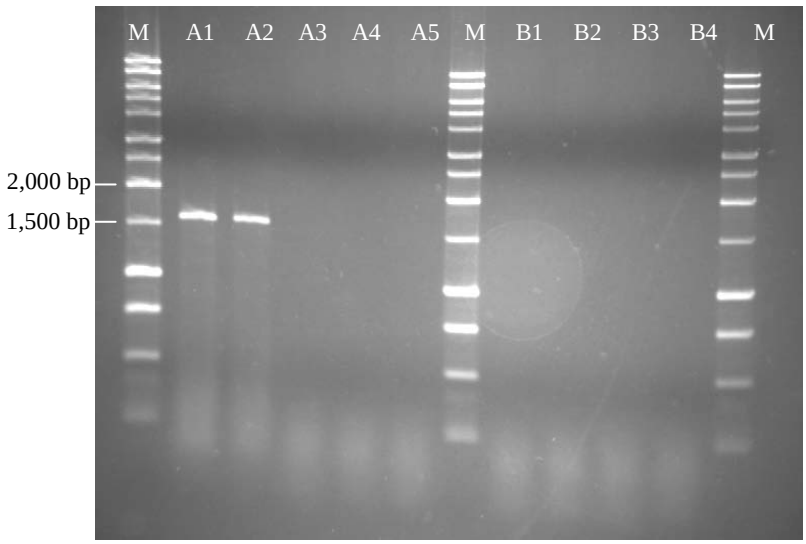


Figure 4.8: Agarose gel electrophoresis analysis of PCR-amplified targeted *lip* gene from cDNA (A) and mRNA (B) of various bacteria strains. M: 1kb DNA ladder (Promega). Lanes A1 and B1: *P. fluorescens*; A2 and B2: *E. coli* LS_M3; A3 and B3: *E. coli* LS_pT-*phaC1*; A4: LS_pT-*lacZ*; A5: PCR negative control.

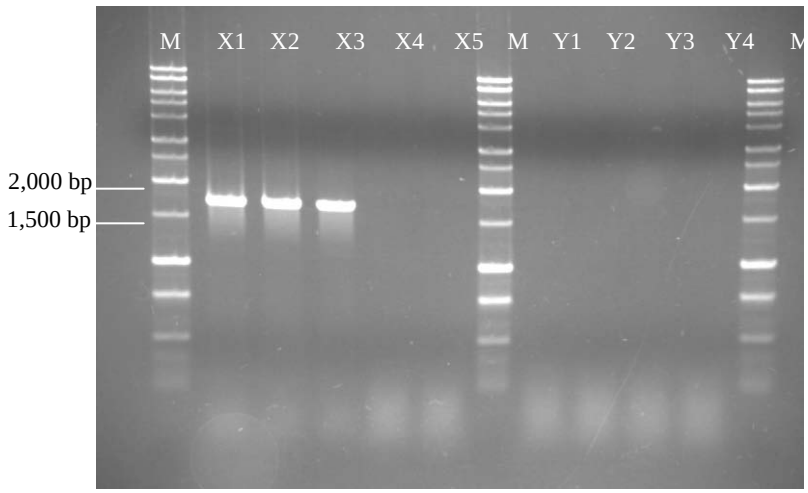


Figure 4.9: Agarose gel electrophoresis analysis of PCR-amplified targeted *phaC1* gene from cDNA (X) and mRNA (Y) of various bacteria strains. M: 1kb DNA ladder (Promega). Lanes X1 and Y1: *P. putida*; X2 and Y2: *E. coli* LS_M3; X3 and Y3: *E. coli* LS_ pT-*phaC1*; X4: LS_pT-*lacZ*; X5: PCR negative control.

4.7 Determination of lipase activity in recombinant strains

The lipolytic activity in the constructed recombinant strains was preliminarily examined on the trioleoylglycerol agar plate (method refer to section 3.7.1). Wild-type *P. fluorescens*, One Shot® Top10 *E. coli*, the *fad B*⁻ *E. coli* LS1298 recombinant strain *E. coli* OS_p2T-*lip* and LS_M3 were spot cultivated on the trioleoylglycerol agar plate containing 0.002% (vol/vol) of arabinose and 0.3% (vol/vol) of olive oil. Wild-type *P. fluorescens* and the recombinant strains *E. coli* OS_p2T-*lip* and LS_M3 showed distinctive halo area around the culture (Figure 4.10), indicating the lipolytic activity in those two strains. Both the One Shot® Top10 *E. coli* and the *fad B*⁻ *E. coli* LS1298 did not show any halo area.

Lipase production by the recombinant strains were then quantified by spectrophotometric assay method using *p*-nitrophenyl laurate as substrate (method refer to section 3.7.2). A lipase activity standard curve was first constructed (Figure 4.11) based on the absorbance reading of reaction mixture with known lipase concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mU/mL of purified lipase enzyme from *Pseudomonas* sp. (Sigma-Aldrich, Germany, Cat. No.: L9518)). The standard curve showed that the *p*-nitrophenol liberated in the lipase activity assay was directly proportional to the concentration of lipase used. However, the standard graph plateaued off at lipase concentrations above 0.40 mU/mL. Therefore, a best fitting line was drawn using the data from lipase concentrations of 0.05 mU/mL to 0.40 U/mL. The linear regression line showed the correlation value (R^2) was 0.864.

For lipolytic analysis in bacteria culture, 1 mL of culture from the LB broth containing 0.002% (vol/vol) of arabinose and 0.3% (vol/vol) of olive oil were collected and subjected for lipase activity determination by spectrophotomety. The value of estimated lipase in the bacteria culture was calculated based on the lipase standard linear regression line and was presented in Figure 4.12. *P. fluorescens* ATCC 13525 showed the highest lipase production in comparison to the recombinant strains OS_p2T-*lip* (harboring only *lip* gene) and LS_M3

(harbouring both *lip* and *phaC1 genes*), whereas no lipase was detected in One Shot® Top10 *E. coli* and *E. coli* strain LS_pT-*lacZ*. This coincided with the trioleoylglycerol agar plate assay whereby lipase was detected in *P. fluorescens*, *E. coli* stains LS_M3 and LS_ pT-*phaC1*.

The results also showed that the lipase activity in recombinant *E. coli* strains OS_p2T-*lip* and LS_M3 were 19% and 53% lower in comparison to wild-type *P. fluorescens*. The possible reasons that the lower the lipase expression efficiency in recombinant strains *E. coli* LS-M3 in comparison to LS_ pT-*phaC1* could be attributed to the to the presence of other expression vector in *E. coli* LS-M3 and yet to be determined.

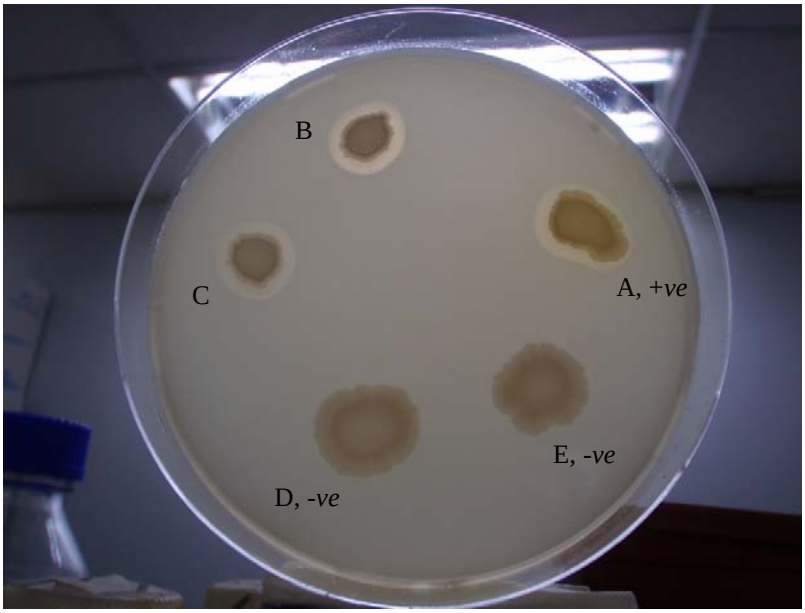


Figure 4.10: Lipolytic production of *P. fluorescens* ATCC13525 and recombinant *E. coli* strains on trioleoylglycerol agar plate. The bacteria strains A: *P. fluorescens* (positive control), B: recombinant strain *E. coli* OS_p2T-*lip*, C: recombinant strain *E. coli* LS_M3, D: One Shot® Top10 *E. coli* (negative control) and (E) *E. coli* strain LS_pT-*lacZ* (negative control) Lipolytic activity as indicated by a clear zone around the culture in comparison D and E (negative control).

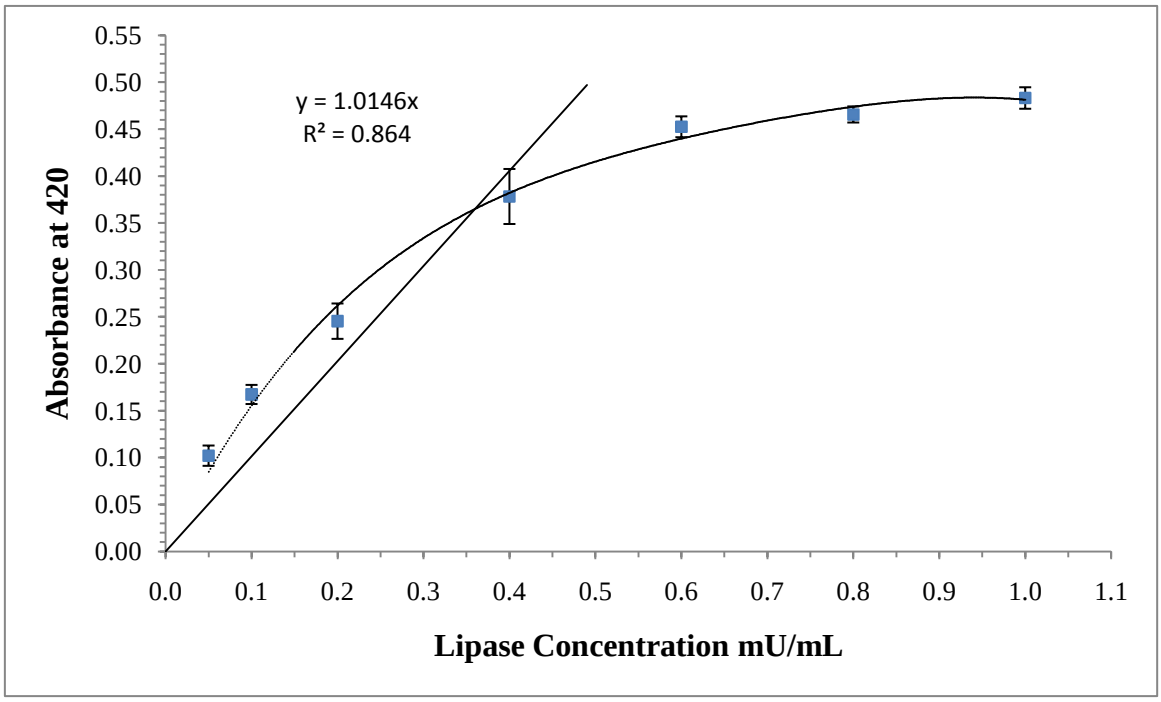


Figure 4.11: Lipase activity standard curve. Vertical bars denote standard deviations from independent experiments done in triplicate.

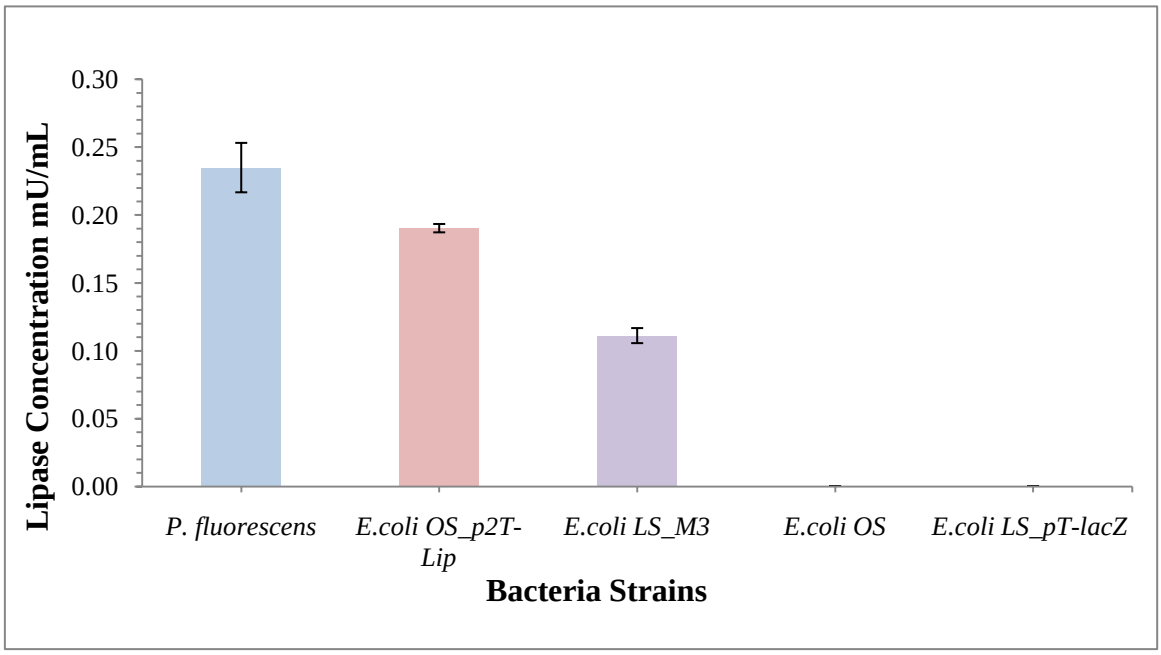


Figure 4.12: Lipase activity assay of different bacteria strains. Vertical bars denote the standard deviation from independent experiments done in triplicate.

4.8 PHA synthesis in recombinant strains

4.8.1 Detection of PHA accumulation by Nile Blue A staining

The ability of *fad* B^{-} *E. coli* LS1298 harboring *phaC1* gene for the PHA accumulation was initially verified by cultivating it in sodium octanoate. The expression of PHA synthase in recombinant strains was tested in various concentration of inducer (L-arabinose). Nile Blue A staining was used for the primary detection of PHA granules in the recombinant strains (Figure 4.13). The microscopic analysis showed increasing intensity in bright orange fluorescence when the recombinant *E. coli* strain LS_pT-*phaC1* was cultivated in increasing concentration of L-arabinose (Figure 4.13). The culture with 0.002% L-arabinose had the highest intensity out of the five tested concentrations (Figure 4.13c), hence 0.002% of L-arabinose was used in the following experiments.

The microscopic observation also revealed that recombinant *E. coli* strains LS_pT-*phaC1* cells with accumulated PHA have elongated shape compared to the *E. coli* cells without PHA accumulation (rod-shaped). This change could be caused by the accumulated PHA granules in the cells cytoplasm, but this is yet to be determined by transmission electron microscopy (TEM).

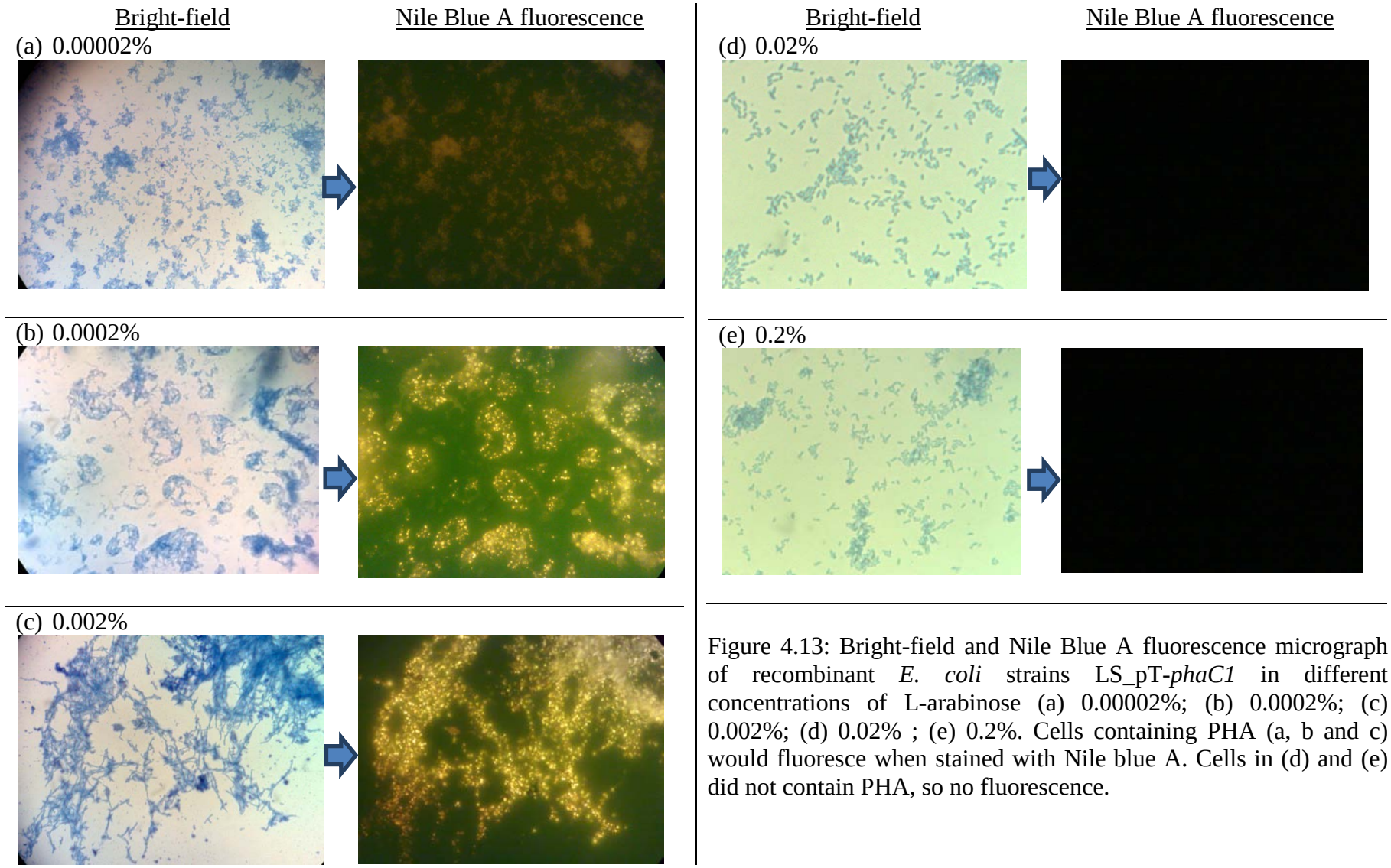


Figure 4.13: Bright-field and Nile Blue A fluorescence micrograph of recombinant *E. coli* strains LS_pT-*phaC1* in different concentrations of L-arabinose (a) 0.00002%; (b) 0.0002%; (c) 0.002%; (d) 0.02% ; (e) 0.2%. Cells containing PHA (a, b and c) would fluoresce when stained with Nile blue A. Cells in (d) and (e) did not contain PHA, so no fluorescence.

4.8.2 Detection of monomers composition and estimation of PHA content by gas chromatography (GC)

The PHA production in LS_M3 and LS_pT-*phaC1* using various carbon substrates was quantified by GC technique. According to the PHA standard gas chromatogram, the retention times for each methyl esters of 3-hydroxy fatty acids standards were determined: methyl 3-hydroxybutanoate (C4) at 4.01 min; methyl 3-hydroxyhexanoate (C6) at 5.13 min; methyl 3-hydroxyoctanoate (C8) at 6.49 min; methyl 3-hydroxynonanoate (C10) at 7.71 min; methyl 3-hydroxydecanoate (C12) at 9.02 min; methyl 3-hydroxydodecanoate (C14) at 10.75 min and methyl 3-hydroxyhexadecanoate (C16) at 13.42 min (Figure 4.14). However, the peak presented at retention time of 8.5 min in Figure 4.15, 4.16 and 4.17 was ignored due to this peak was detected in the negative control (*E. coli* strain LS_pT-*lacZ*) and the identity of this compound is yet to be determined.

Based on the GC results, both the recombinant *E. coli* strains containing the *phaC1* gene were able to accumulate PHA to 6.1% and 8.1% of cell dry weight respectively (Table 4.1). The GC results in this study was similar to the study by Ren *et al.* (2005) which demonstrated their recombinant *E. coli* JMU193 with *phaC1* from *P. putida* GPo1 was able to accumulate PHA up to 8% of total cell dry weight when in cultivated in LB supplemented with 15 mM octanoate. However, the production was found to be much lower than that in the wild-type *P. putida* (30%). In contrary, a similar study by Langenbach *et al.* (1997) had revealed that the recombinant strain *fab* B^{-} *E. coli* LS1298 harbouring *phaC1* gene from *P. aeruginosa* PAO1 capable to acuumulate21% of the cell dry weight using 0.5 (wt/vol) decanoate as carbon substrate.

The GC analysis revealed that PHA was detected in recombinant *E. coli* strain LS_pT-*phaC1* cultivated with sodium octanoate or saponified palm kernel oil (SPKO) as carbon

source but not with glucose or palm kernel oil (PKO) as carbon source. GC analysis demonstrated that PHA accumulation of 8.1% of cell dry weight in recombinant *E. coli* strain LS_pT-*phaC1* which cultivated in LB supplemented with sodium octanoate and containing only 3-hydroxyoctanoate monomer. PHA was also detected in *E. coli* strain LS_pT-*phaC1* when cultivated in LB broth with SPKO in which contributing approximately 8.1% of cell dry weight. The PHA monomers were composed of 51.8% 3-hydroxyoctanoate, 35.8% 3-hydroxynonanoate and 12.4% 3-hydroxydecanoate.

PKO is a triglycerides which mainly made up of saturated fatty acids like lauric acid (C12), myristic acid (C14), palmitic acid (C16) and oleic acid (C18:1). It had been reported that the fatty acids derivatives from the PKO are the suitable carbon substrate for the production of mcl-PHA (Tan *et al.*, 1997). Normally, PKO can be broken down to its fatty acid compounds by chemical (such as saponification) or enzymatic hydrolysis by lipase. Many of the pseudomonads are known to accumulate PHA from a variety of carbon substrates (Eggink *et al.*, 1995) but only few of them can utilize lipid directly for PHA synthesis, such as *Pseudomonas oleovorans* (Füchtenbusch *et al.*, 2000) and *P. aeruginosa* (Solaiman *et al.*, 1999). In this study, a recombinant *E. coli* strain LS_M3 which harboured both *lip* and *phaC1* genes have the capability to accumulate PHA by utilizing PKO as carbon substrate.

GC analysis showed that *E. coli* LS_M3 could produce PHA up to 7.8% of cell dry weight using PKO as carbon substrate, and the monomeric composition comprised 49.6% wt of 3-hydroxyoctanoate, 36.9% wt of 3-hydroxynonanoate and 13.5% wt of 3-hydroxydecanoate. The level of PHA production in *E. coli* LS_M3 was much lower than the values reported by a similar study (Solaiman *et al.*, 2001), whereby a genetically-modified PHA producer *P. putida* (pCN51lip-1) with lipolytic ability could accumulate PHA up to 53% of the cell dry weight.

As *Pseudomonas* members are naturally PHA producers, they have advantage over non-PHA-producing bacteria such as *E. coli* as some of the pathways involved in PHA production are readily available. The *phaC1* gene is the key enzyme to polymerize the monomers into long-chained polymers. However, other enzymes involved in formation of these PHA monomers might not be present in *E. coli*. Thus, this could be one of the contributing factors that cause the low production of PHA in recombinant *E. coli* strain in this study.

On the other hand, when *E. coli* LS_M3 was fed with sodium octanoate, about 6.6% of cell dry weight PHA was produced and the only constituent was 3-hydroxyoctanoate. On the other hand, PHA up to 8.0% of cell dry weight was observed in the *E. coli* LS_M3 that fed with SPKO (with PHA constituents 50.3% 3-hydroxyoctanoate, 36.8% 3-hydroxynonanoate and 12.9% 3-hydroxydecanoate). However, PHA was not detected when the recombinant strains was fed with glucose. No PHA was detected in both recombinant strains *E. coli* strains LS_pT-*lacZ* and LS_pT-*phaC1* which were used as negative controls.

PHA was detected in both recombinant strains that cultivated in sodium octanoate, PKO and SPKO even though biomass was much lower in comparison with cells grown in LB. In contrary, although glucose seems to have highest biomass in both recombinant strains, no PHA was detected. The possible reasons to this occurrence could be attributed to the substrate specificity of the PHA synthase. Indeed, the PHA synthase from *Pseudomonas* spp. was reported to utilize intermediate (R)-3-hydroxyacyl-CoA from the β-oxidation pathway as main substrate (Huijberts *et al.*, 1992). However, there are several pseudomonads capable to accumulate PHA from non-related carbon source such as gluconate (Timm *et al.*, 1990; Lee, 1996) and it is involves in the conversion of an intermediate from the fatty acid *de novo* synthesis pathway by transacylase phaG to the

desired substrates in order to be recognized by PHA synthase for polymerization (Rehm, *et al.*, 1998). Since the *E. coli* strain might not have the desired metabolic pathways to convert the non-related carbon source to a precursor which can be recognized by the PHA synthase with *Pseudomonas* origins, it is expected that the recombinant *E. coli* strains were incapable to produce PHA from glucose.

In addition to that, it has been reported that glucose has a negative effect in activating arabinose (*ara*) operon (Miyada *et al.*, 1984) in transgenic host. Basically, there are two activation events which mediate the activation of the *ara* operon (Schleif, 2000). One of the events is the binding of the inducer arabinose to the activator protein encoded by *araC* gene. Another is the CAP-cAMP catabolite repression system like in the *lac* operon; cyclic AMP activates a molecule (CAP), which then binds to the lac promoter and regulate the transcription. Both the AraC-arabinose complex and CAP-cAMP complex act as activator protein which activates transcription of the *ara* operon and allow transcription to begin. However, in the presence of glucose, it lowers the levels of 3', 5'-cyclic AMP and thus decreases its binding to CAP (Guzman *et al.*, 1995). As a result, the transcriptional process is drastically decreased. Hence, the addition of glucose in the recombinant *E. coli* culture might give an adverse effect on the PHA production.

Table 4.1: PHA content and monomer composition of recombinant *E. coli* LS_M3 and *E. coli* LS_pT-*phaC1* cultivated in various carbon sources

Strain	Carbon source	Cell dry weight (g/L)	PHA content % CDW (wt/wt)	Composition of PHA Monomer (% wt)		
				3HO	3HN	3HD
E. coli LS_M3	LB [ab] + Sodium octanoate[c]	0.755	6.6	100	-	-
	LB [ab] + PKO [c]	1.168	7.8	49.6	36.9	13.5
	LB [ab] + SPKO [c]	1.102	8.0	50.3	36.8	12.9
	LB [ab] + Glucose [c]	2.237	0	-	-	-
	LB [ab]	1.759	0	-	-	-
E. coli LS_pT-*phaC1*	LB [ab] + Sodium octanoate[c]	0.786	8.1	100	-	-
	LB [ab] + PKO [c]	1.184	0	-	-	-
	LB [ab] + SPKO[c]	1.100	8.1	51.8	35.8	12.4
	LB [ab] + Glucose[c]	2.313	0	-	-	-
	LB [ab]	1.833	0	-	-	-
E. coli LS_pT-*lacZ*	LB [b] + PKO [c]	1.203	-	-	-	-
	LB [b] + SPKO [c]	1.051	-	-	-	-
E. coli OS_pT-*lacZ*	LB [b] + PKO [c]	1.099	-	-	-	-
	LB [b] + SPKO [c]	1.164	-	-	-	-

LB: *Luria-Bertani broth*

[a] *Supplement :* 50 μg/mL of kanamycin, [b] *Supplement :* 100 μg/mL ampicillin, [c] 0.2% (wt/vol) of carbon source was added into the culture after 12 hours incubation in the *LB.*

3HO: 3-hydroxyoctanoate; 3HN: 3-hydroxynonanoate; 3HD: 3-hydroxydecanoate; 3HDD: 3-hydroxydodecanoat.

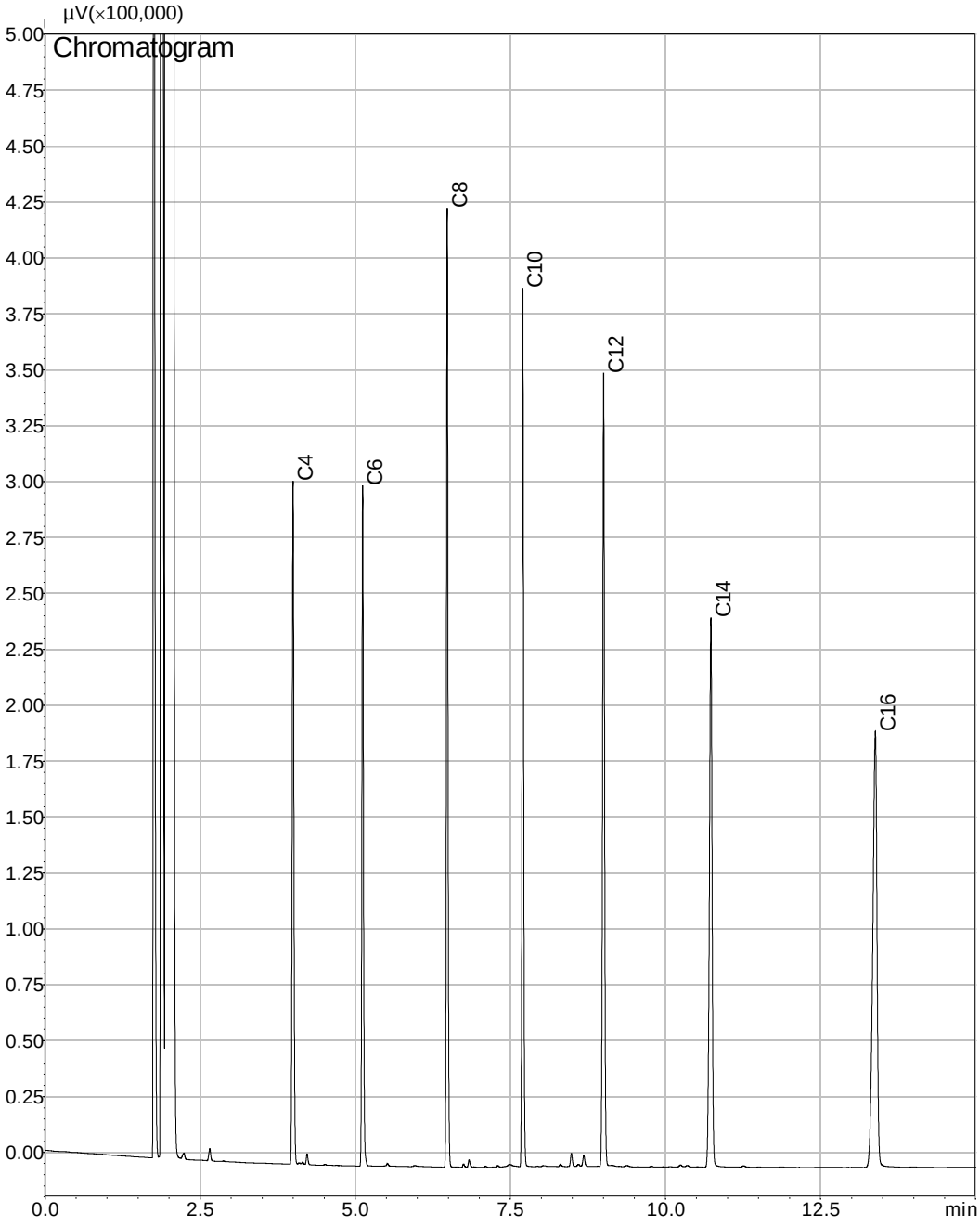
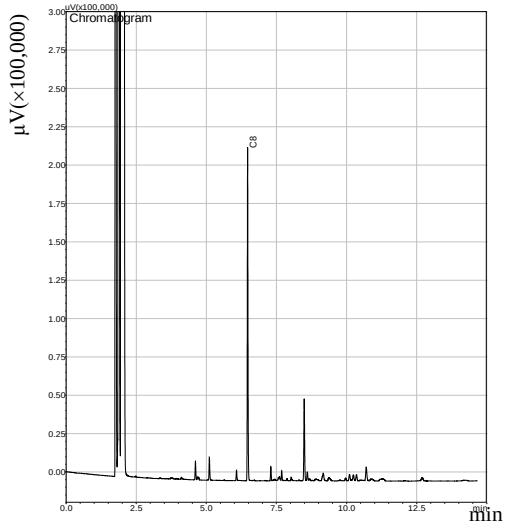
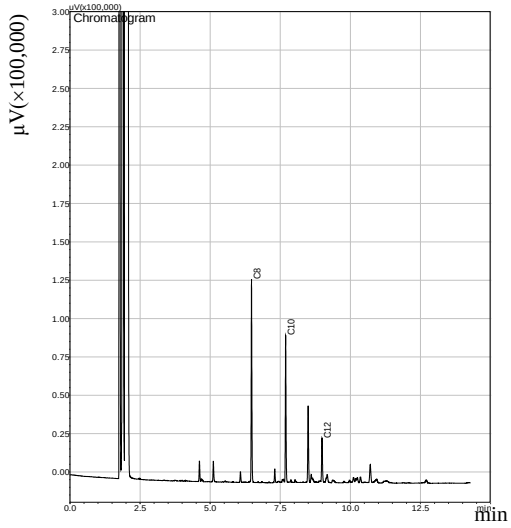


Figure 4.14: Chromatogram of methyl esters standards analysis by gas chromatography (GC). The text above the peak refers to the monomer of the PHA component, C4: methyl 3-hydroxybutanoate; C6: methyl 3-hydroxyhexanoate; C8: methyl 3-hydroxyoctanoate; C10: methyl 3-hydroxynonanoate; C12: methyl 3-hydroxydecanoate; C14: methyl 3-hydroxydodecanoate; C16: methyl 3-hydroxyhexadecanoate.

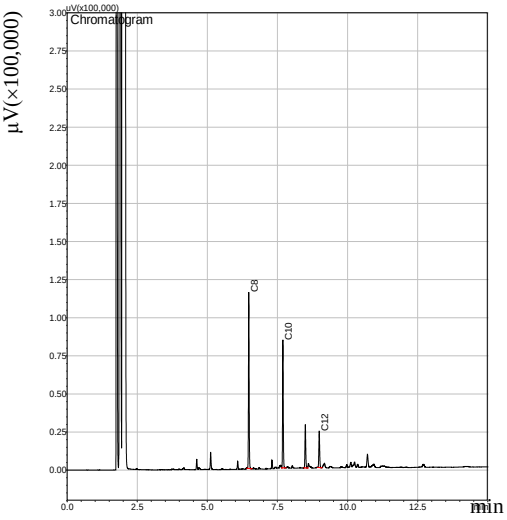
Sodium octanoate



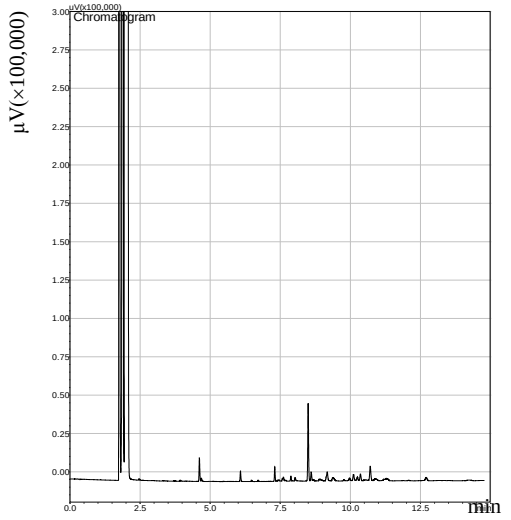
PKO



SPKO



LB



Glucose

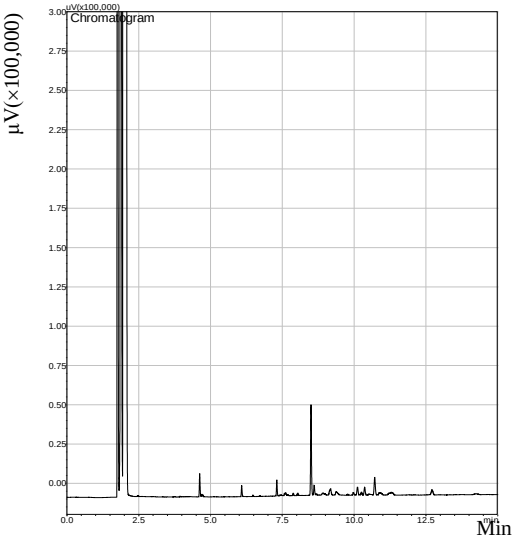


Figure 4.15: Chromatogram of methyl esters PHA by GC separation from the dry cell of recombinant *E. coli* LS_M3 by using different carbon sources. The text above the peak refers to the monomer of the PHA component that detected by GC, C8: methyl 3-hydroxyoctanoate; C10: methyl 3-hydroxynonanoate; C12: methyl 3-hydroxydecanoate.

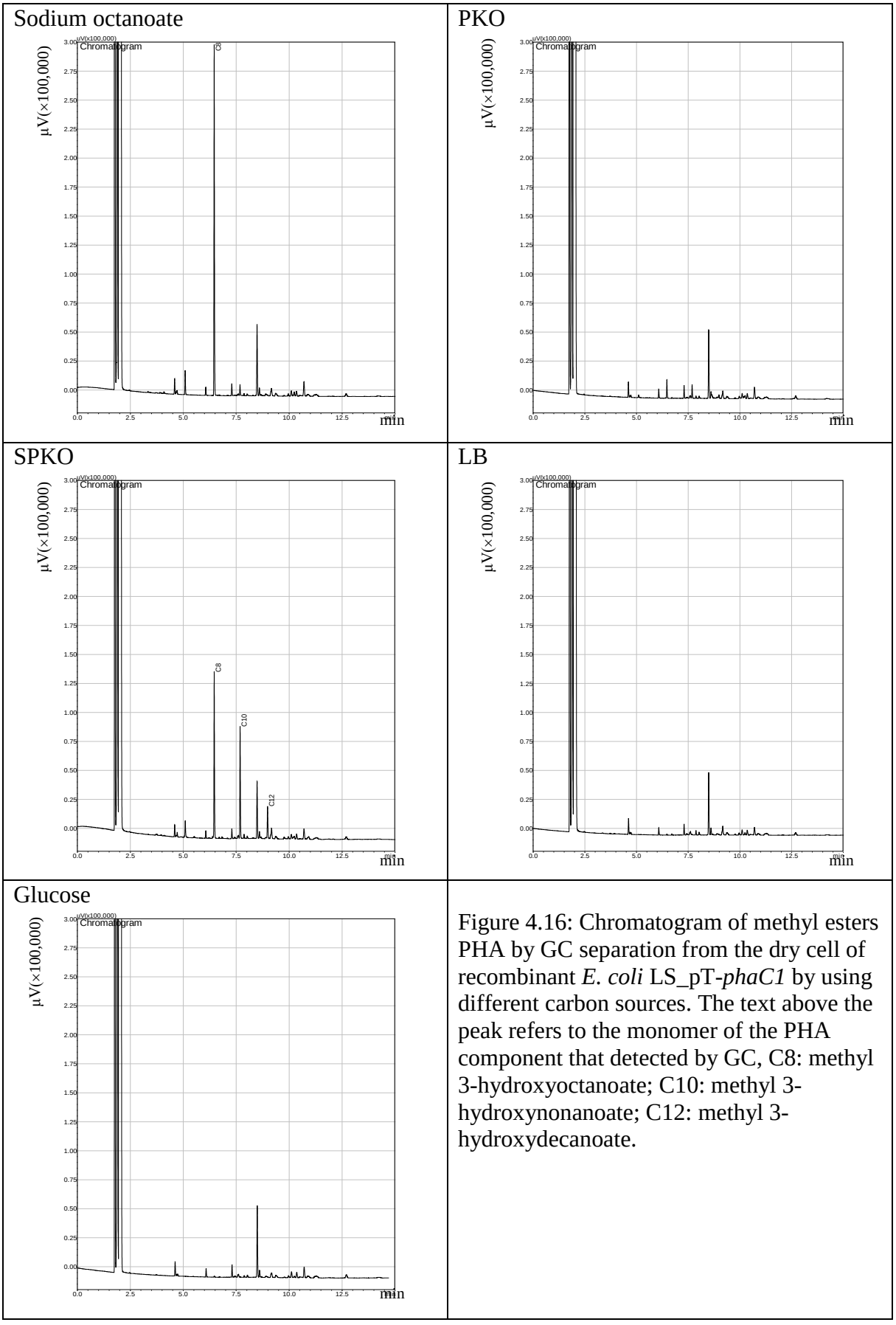
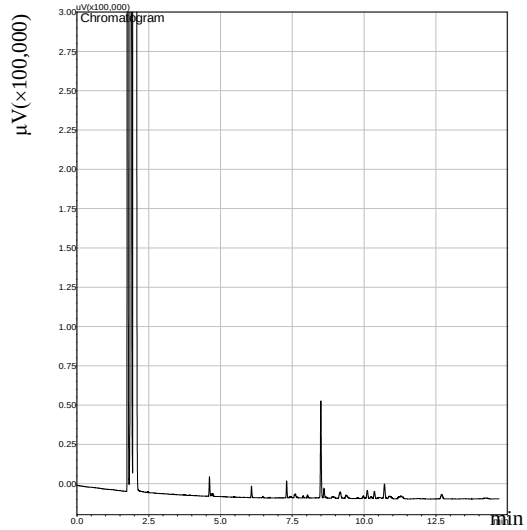


Figure 4.16: Chromatogram of methyl esters PHA by GC separation from the dry cell of recombinant *E. coli* LS_pT-*phaC1* by using different carbon sources. The text above the peak refers to the monomer of the PHA component that detected by GC, C8: methyl 3-hydroxyoctanoate; C10: methyl 3-hydroxynonanoate; C12: methyl 3-hydroxydecanoate.

PKO



SPKO

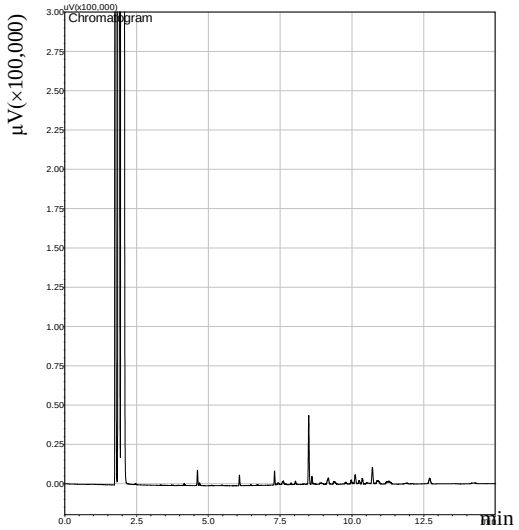


Figure 4.17: Chromatogram of methyl esters PHA by GC separation from the dry cell of recombinant *E. coli* LS_pT-*lacZ* by using different carbon sources. The text above the peak refers to the monomer of the PHA component that detected by GC, C8: methyl 3-hydroxyoctanoate; C10: methyl 3-hydroxynonanoate; C12: methyl 3-hydroxydecanoate.

4.9 Problems encountered in heterologous expression of *lip* and *phaC1* genes in recombinant *Escherichia coli*

Heterologous expression of PHA synthase from various bacteria strain in *E. coli* has successfully achieved for the past decades (Langenbach *et al.*, 1997; Park *et al.*, 2005; Qi *et al.*, 1997; Ren *et al.*, 2000). This approach was widely applied because the expression of heterologous protein in recombinant *E. coli* by genetic engineering has been reported as a well-developed approach in order to obtain high yields of the desired protein (Elvin *et al.*, 1990). One of the major criteria to be fulfilled by the bacteria host in order for the heterologous expression of PHA synthase in *E. coli* is containing a suitable PHA biosynthesis pathway.

Despite the development of sophisticated cloning techniques, there are difficulties encountered in expressing a foreign gene in recombinant *E. coli*. Based on the overall result that presented in this study, neither the PHA production nor the lipase activity in the constructed recombinant strains achieved to the levels similar to those expected levels in the wild-type bacteria and other reported recombinant strains. One of the factors that may contribute to the low yield of PHA and lipase in the recombinant strains is the stability of the antibiotic subjected during the bacterial cultivation.

In this study, the selectable markers (ampicillin or kanamycin resistance gene) on the expression vector were used to distinguish the recombinant strains harboring desired gene from the plasmid-free strain. Indeed, the population of the recombinant strain that contain the desired plasmid was dependent on the stability of the antibiotic. It has been reported that some of the marker antibiotics such as ampicillin was degraded over time by *E. coli* (Sambrook and Russel, 2001). The degradation of the antibiotic may cause the loss of the desired plasmid and eventually it led to the plasmid-free cells overgrew the recombinant cell population (Dong *et al.*, 1995).

Another contributing factor could be the choice of promoter used in the gene expression. In order for the transcription to take place, the promoter plays an important role for the polymerase to bind to. Larsen *et al.* (1984) had reported that the induction of a heterologous protein by a strong promoter can lead to uncontrollable overproduction which might induce stress response in the host cell and eventually termination of its growth. Moreover, it has been reported that protein accumulation to 30% of the total protein within the cells can cause damage to the rRNA and ribosome which was unfavorable for cell growth and viability (Dong *et al.*, 1995).

The promoter is normally located near the genes they regulate and it is the key element of an expression vector to initiate the gene expression in the beginning stage. Most of the frequently used promoters in the expression vectors are *lac* promote and *trp* promoter. The expression of the heterologous gene in the recombinant *E. coli* can be regulated either by using its own native promoter or by an external promoter within the expression vector. However, Deretic *et al.* (1989) had reported that the transcription of many pseudomonads genes is highly regulated by their own promoter and which might not be recognized by the *E. coli* RNA polymerase. This coincides to the finding reported by Ren *et al.* (2005) in which no PHA was produced in genetic engineered *E. coli* harboring the PHA synthase gene and the native promoter from *P. putida*.

An inducer that regulates foreign genes expression of the promoter in vector systems also influences the yield of the desired protein. Hypothetically, high concentration of the inducer regulating the heterologous gene expression by the promoter in the recombinant *E. coli* results in increasing the heterologous protein production.

However, looking at the Nile Blue A staining micrographs (Figure 4.13), no PHA was detected in recombinant strains tested with the highest concentration of L-arabinose (2%) while the recombinant strains induced with 0.002% of L-arabinose had high fluorescence intensity. This revealed that an appropriate concentration of the inducer is very crucial in gene expression. Excessive overproduction of heterologous protein by high concentration of inducer might cause toxicity which is detrimental to *E. coli* (Blum *et al.*, 1992). But, overall PHA yield in the recombinant *E. coli* strains in 0.002% concentration of inducer was still comparatively low. This could be due to the degradation of the antibiotic and led to the over-growth of the plasmid-free *E. coli* in the overall population which contributed to the total biomass.

There are relationship between the cell growth and its PHA content in which the cell density defines the amount of PHA accumulated. A low cell density with high PHA content or high concentration cell density with low PHA content will result in low final PHA concentration. However the best PHA productivity can be obtained in high concentration of cell density with high PHA content. Therefore, the cell growth and the PHA synthesis need to be balanced to reach a point in order to achieved maximum PHA accumulation in the bacteria.

CHAPTER 5: CONCLUSION

Some pseudomonads were found to be capable of synthesizing mcl-PHA by utilizing oil as sole carbon substrate but the yield was relatively low when compared with those produced from carbohydrates, alcohols and fatty acids. The aim of this study was to construct a recombinant *Escherichia coli* strain which capable to utilize palm kernel oil directly as carbon substrate for PHA synthesis.

In this study, *phaC1* gene from *Pseudomonas putida* and *lip* gene from *Pseudomonas fluorescens* ATCC13525 were amplified and inserted into pBAD-TOPO and pBAD202/D-TOPO vectors respectively. The two recombinant plasmids were cloned into fad B^{-} *E. coli* LS1298 and the resulting recombinant E. coli strains were obtained: LS_pT-phaC1 with *phaC1* gene only, and LS_M3 with both phaC1 gene and *lip* gene.

The lipase activity of the recombinant *E. coli* LS_M3 was quantified by using lipase activity assay. Results showed that 1.01 mU/mL of lipase was produced by recombinant LS_M3 and was approximately 53% lower than that in *P. fluorescens* ATCC13525, the *lip* gene donor strain.

The PHA production in both recombinant strains was tested by growing in the LB media containing different carbon substrates, followed by gas chromatography analysis to quantify the PHA in the cell. The PHA yield of both recombinant *E. coli* strains were much lower (6 to 8.1% of its cell dry weight) as compare to the PHA production in *P. putida* (40% of its cell dry weight), which as the *phaC1* gene donor strain. However, recombinant *E. coli* LS_M3 showed low amount of PHA production using palm kernel oil, whilst *P. putida* cannot synthesis PHA from oil.

CHAPTER 6: FUTURE WORK

In practice, the satisfactory yield of enzyme in recombinant bacteria is often difficult to obtain. The levels of PHA accumulation as well as the lipase activity of constructed recombinant *E. coli* stains in this preliminary study were not optimized and further improvement should be pursued in future studies. The PHA synthase plays a crucial role in PHA polymerization, however other functionally genes encoding proteins participating in the PHA metabolism are also important. These include the *phaJ* gene encoding (R)-specific enoyl-CoA hydratase (Fiedler *et al.*, 2002) and *phaP* gene encoding phasing enzyme involved in stabilize PHA granules (Steinbüchel *et al.*, 1995).

Thus, many other research activities can be performed in order to understand the PHA synthesis and its pathway in the recombinant strains in order to resolve the problem encounter during this study that a feasible high yield of PHA production can be achieved. Improvements such as heterologous expression of desired genes in a high performance cloning vector and optimization of inducer concentration for gene expression in the recombinant strain could be carried out.