CHAPTER 1

INTRODUCTION

1.1 Rationale

Conventional plastic materials (i.e. petroleum-derived) are widely used in everyday life primarily because they are strong, inexpensive, and chemically inert (Liu *et al.*, 2000). However, the persistence of plastics in the environment is increasingly considered to be a source of ecological problems. After being used, they are disposed into the garbage and sanitary embankments already saturated with plastic residues, causing an accumulation of garbage, hindering the circulation of liquids and gases and retarding the stabilization of the organic matters. The environmental impact caused by the disposal of plastics and the progress of medicine have motivated the development of biodegradable and biocompatible materials.

Polyhydroxyalkanoates (PHA), a highly potential candidate of alternative biodegradable plastics, are biological polyesters synthesized by a wide range of bacteria and accumulated as intracellular carbon and energy storage material in enormous quantities under various nutritional and environmental conditions. Since PHA can be produced by biosynthesized from renewable carbon sources such as sugars and vegetable oils, PHA has been recognized as an eco-friendly plastic material compared with non-biodegradable and petroleum-based ones. Nowadays, PHA continues to attract industrial interests as a renewable, biodegradable and biocompatible thermoplastic that can be used for marine, agricultural, and medical applications. Various studies have shown that PHA can be biologically synthesized from a diverse range of carbon substrates (e.g. glucose, fatty acid, oil, etc) and consisted of over 100 different monomeric components. The study (Reddy *et al.*, 2003), also displayed that the produced PHA has a similar properties as compared to the petroleum based plastics.

Although the studies of PHA production have been carried out in various carbon sources, not much is known about the production of commercially important PHA from glycerol, mainly a by-product (so-called waste) in the biodiesel production industry. Moreover, the critical factor limiting the use of PHA plastics is their high cost of production. Broader use of biodegradable plastics in packaging and disposable products as a solution to environmental problems depends heavily on further reduction in their costs and on the discovery of novel biodegradable plastics with improved properties. Using activated sludge as a source of bacteria to convert organic contents and nutrients into PHA would produce biodegradable plastics(Yu *et al.*, 1999).

Therefore, the main purpose of this research was to convert glycerol to PHA bioplastics using mixed culture under various cultivation procedures. Additionally, microbial community dynamics during PHA production processes using the sludge as a seed bacterial in order to find dominant bacteria that could endure to restrictive conditions would be analyzed. Chemical composition and structure, and chemical/physical properties of the produced PHA would be examined.

1.2 Literature Review

1.2.1 Bioplastic-producing bacteria

Natural PHA-producing bacteria contain pathways for PHA degradation have a long generation time and require relatively low optimal growth temperature. Metabolic engineering was being intensely explored to introduce new metabolic pathways to broaden the utilizable substrate range, to enhance PHA synthesis and to produce novel PHA. Recombinant *E. coli* strains harboring the *Alcaligenes eutrophus* PHA biosynthesis genes in a stable high-copy-number plasmid have been developed and used for high PHA productivity (Zhang *et al.*, 2001). The production of various PHA using natural isolates and recombinant bacteria with different substrates were presented in Table 1.1.

Table 1.1 Bacterial strains capable of producing PHAs from different carbon sources

Bacterial strain	**Carbon source**	**Biopolymer** [1]	**Reference**
Aeromonas hydrophila	Lauric acid, oleic acid	mcl-PHA	Han *et al.* (2004)
Alcaligenes latus	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB	Wong *et al.* (2005)
Bacillus cereus	Glucose, ε-caprolactone, sugarbeet molasses	PHB, terpolymer	Labuzek *et al.* (2001)
Bacillus sp.	Nutrient broth, glucose, alkanoates, ε-caprolactone, soy molasses	PHB, PHBV, copolymers	Shamala *et al.* (2003); Tajima *et al.* (2004); Yilmaz *et al.* (2005)
Burkholderiasacchari sp. nov.	Fructose, lactose, maltose, raffinose, rhamnose, sorbitol, sucrose, trehalose, xylitol	PHB, PHBV	Bramer *et al.* (2001)
Burkholderia cepacia	Palm oil, crude palm oil, oleic acid, xylose, levulinic acid, sugarbeet molasses	PHB, PHBV	Alias *et al.* (2005); Celik *et al.* (2005)
Caulobacter crescentus	Caulobacter medium, glucose	PHB	Rehm *et al.* (2001)
Escherichia coli mutants	Glucose, glycerol, palm oil, ethanol, sucrose, molasses	(UHMW) PHB	Kahar *et al.* (2005); Nikel *et al.* (2006); Sujatha *et al.* (2006)

[1] mcl-PHA: medium-chain-length polyhydroxyalkanoates, PHB: poly(3-hydroxybutyrate), PHBV: poly(3-hydroxybutyrate-co-valerate), UHMW: ultra-high molecular weight

Table 1.1 Bacterial strains capable of producing PHAs from different carbon sources (CONT')

Bacterial strain	**Carbon source**	**Biopolymer** [1]	**Reference**
Halomonas boliviensis	Starch hydolysate, maltose	PHB	Quillaguaman*et al.* (2006)
Pseudomonas aeruginosa	Glucose, waste free fatty acids, waste free frying oil	mcl-PHA	Hoffmann *et al.* (2005)
Pseudomonas oleovorans	Octanoic acid	mcl-PHA	Foster *et al.* (2005)
Pseudomonas putida	Glucose, octanoic acid	mcl-PHA	Tobin *et al.* (2005)
Pseudomonas putida, P. fluorescens, P. jessenii	Glucose, aromatic monomers	aromatic polymers	Ward *et al.* (2005)
Pseudomonas stutzeri	Glucose, soybean oil, alcohols, alkanoates	mcl-PHA	Xu *et al.* (2005)
Rhodopseudomonas palustris	Acetate, malate, fumarate, succinate, propionate, malonate, gluconate, butyrate, glycerol, citrate	PHB, PHBV	Mukhopadhyay *et al.* (2005)

[1] mcl-PHA: medium-chain-length polyhydroxyalkanoates, PHB: poly(3-hydroxybutyrate), PHBV: poly(3-hydroxybutyrate-co-valerate), UHMW: ultra-high molecular weight

Table 1.1 illustrates both pure culture sand mixed cultures that are able to biosynthesize PHA. Less study on the PHA production by the mixed culture, compared with that by the pure culture, was investigated. Especially, the PHA contents were lower in the production of PHA by mixed culture (activated sludge), compared with the production of PHA by pure culture. For instant in batch culture, the maximum consumed sugar and sugar utilization efficiency were attained after 100 h whereas ammonia was completely assimilated after 80 h to obtain the highest cell dry weight being 10.18 and 8.73 g L^{-1} after 100 and 80 h incubation by *Ralstonia eutropha* ATCC 17697 and *Alcaligen eslatus* ATCC 29712, respectively (El-Sayed *et al.*, 2009). Then, *Bacillus megaterium* ATCC 6748 could produce the highest PHB production was observed after 45 h of growth when 4% molasses and 4% corn steep liquor were used, whereas the highest biomass (7.2 g L^{-1}) was obtained at 4% molasses and 6% corn steep liquor (Chaijamrus *et al.*, 2008).

Many papers describe the conversion of glycerol to PHA using pure cultures such as *Burkholderia* sp. USM which was capable of converting triglycerides into up to 60 wt% of PHB could be obtained when glycerol was fed (Chee *et al.*, 2010). Previous works by Ashby *et al.* (2005) had revealed the possibilities of producing PHA from glycerol. A mixed culture fermentation consisting of wild type *P. oleovorans* NRRL B-14682 and *Pseudomonas corrugate* 388 was used. Both bacteria with similar growth requirements produced different PHA from the same substrate. *P. oleovorans* NRRL B-14682 and *P. corrugata*388 were able to convert glycerol to 20 wt% of PHB and 40 wt% of mcl-PHA blends, respectively. Recently, Moralejo-Gárate *et al.* (2011) reported the potential of using microbial community engineering for production of the PHA from glycerol was explored, subsequently fed batch PHA production step under growth-limiting conditions, the enriched mixed community produced PHA up to a dry weight content of 67 wt.%.

On the other hand, mixed culture biotechnology is a promising alternative to pure culture biotechnology for the production of the PHA. Mixed culture biotechnology employed open undefined mixed culture and ecological selection principles to produce a product such as bioplastics, ideally from a waste stream. It therefore combined the methodology of environmental biotechnology with the goals of industrial biotechnology (Katja *et al.*, 2010).

The idea of PHA production using mixed cultures arose from the recognition of PHA's role as a metabolic intermediate in microbial processes for wastewater treatment. Biological wastewater treatment usually occurred under dynamic conditions (Leeuwen *et* *al.*, 1997). Activated sludge, a well-known mixed culture, was able to store PHA as carbon and energy storage material under unsteady conditions arising from an intermittent feeding regime and variation in the presence of an electron acceptor. Microorganisms which were able to quickly store available substrate and consume the storage to achieve a more balanced growth had a strong competitive advantage over organisms without the capacity of substrate storage (Leeuwen *et al.*, 1997). Moreover, an interesting alternative to pure culture was the use of mixed culture such as activated sludge from wastewater treatment plants (Chua *et al.*, 2003; Dionisi *et al.*, 2004; Reis *et al.*, 2003). The use of mixed culture instead of pure culture brought the advantages that sterile conditions were not needed and continuous cultures could be obtained without the risk of culture contamination.

Open-mixed culture biotechnology based on natural inocula with a high microbial diversity allows for the operation of bioprocesses under non-sterile conditions with no risk of strain degeneration (Kleerebezem *et al.*, 2007). As opposed to pure culture processes, mixed culture furthermore allow for the use of continuous processing, and were capable of dealing with mixtures of substrates of variable composition. All these aspects significantly reduce the costs of mixed culture processes compared to pure culture-based industrial fermentations. The impact of the operational conditions on glucose fermentation by mixed cultures has been studied extensively (Hawkes *et al.*, 2007; Temudo *et al.*, 2007; Zoetemeyer *et al.*, 1982).

In mixed culture fermentations, control of the carbon and nitrogen concentrations in the broth and appropriate staggering of inoculations by the component cultures are key factors determining the efficiency of the process. To do this, fed-batch operation had been preferred in many laboratory-scale fermentations, and final PHA concentrations up to 12 g L^{-1} have been reported (Tohyama *et al.*, 2002). It had also been shown that this may be increased more than three-fold by more complex control of the ammonia feed rate (Tohyama *et al.*, 2002).

1.2.2 Pathway involving polyhydroxyalkanoate productions

The biosynthetic routes to PHA monomers compete with and/or rely on important pathways such as the tricarboxylic acid (TCA) cycle, fatty acid degradation (β-oxidation) and fatty acid biosynthesis for precursors, and involve central metabolites such as acetyl-CoA and cofactors such as PhaA, PhaB, PhaC, etc. (Figure 1.1).

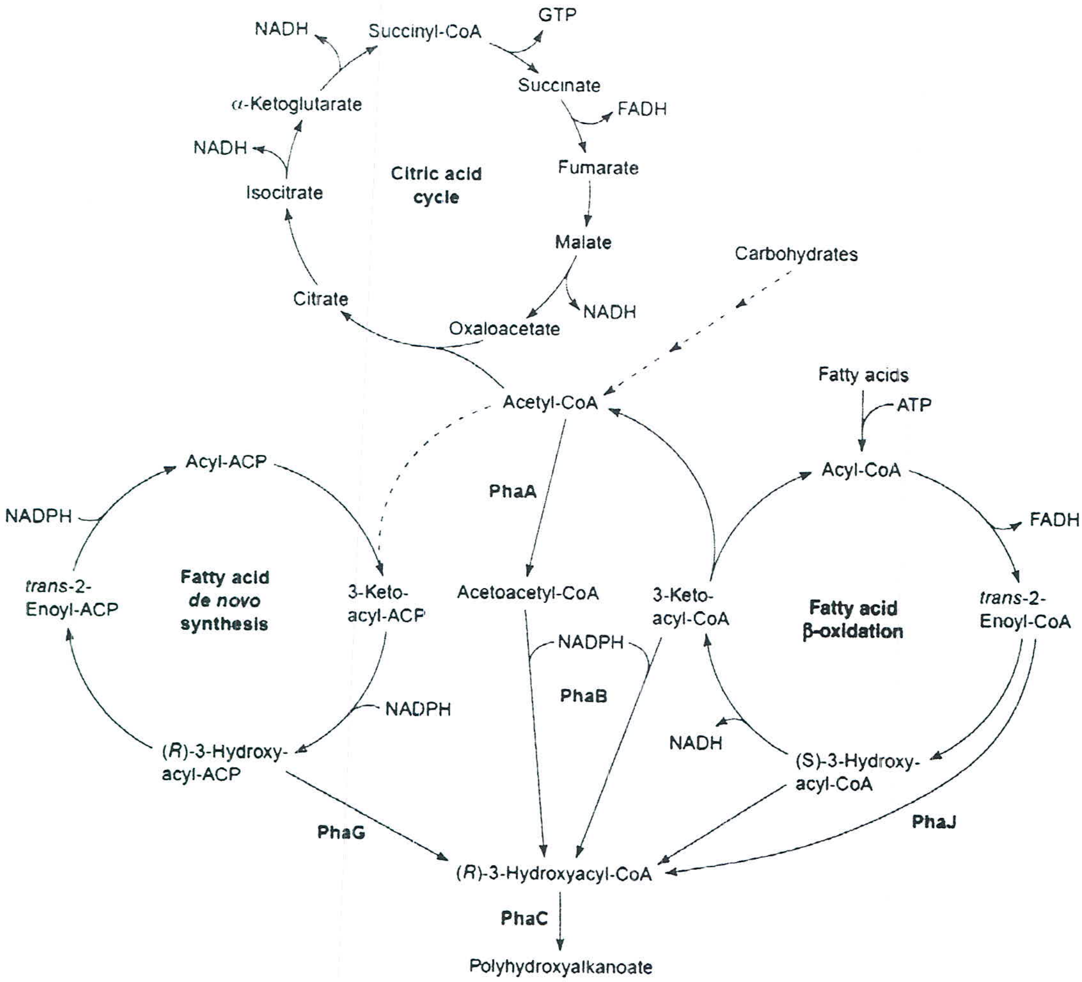


Figure 1.1 PHA biosynthesis in the context of microbial metabolism, the major enzymes involved in PHA biosynthesis are in red. Abbreviations: PhaA, 3-ketothiolase; PhaB, (R)-3-ketoacyl-CoA reductase (for PHB biosynthesis, this enzyme is acetoacetyl-CoA reductase); PhaC, PHA synthase or polymerase; PhaG, (R)-3-hydroxyacyl ACP: CoA transacylase; PhaJ, (R)-specific enoyl-CoA hydratase. Dotted lines represent reactions where intermediate metabolic steps are not included. PhaC is specific for enantiomeric monomers in the (R) configuration (Aldor *et al.*, 2003)

Conclusion: three main pathways are involved in the synthesis of PHA precursors as follows:

- Chain elongation: by which acyl-CoA is extended with acetyl-CoA through citric acid cycle (TCA cycle)
- Fatty acid β-oxidation: fatty acid degradation by β-oxidation is the main metabolic route of fatty acids, such as (R)-specific enoyl-CoA hydratase (PhaJ), which

catalyze the (R)-specific hydration of β-oxidation intermediate, 2-trans-enoyl-CoA, to (R)-3-hydroxyacyl-CoA, plays a critical role in supplying monomer units from β-oxidation to PHA synthesis (Fiedler *et al.*, 2002)

- Fatty acid *de novo* biosynthesis: *de novo* fatty acid biosynthesis pathway, which produces (R)-3-hydroxyacyl-CoA precursors from non-related carbon sources such as glucose and gluconate

Meanwhile, several different metabolic links between fatty acid metabolism and PHA biosynthesis have been revealed (Figure 1.1). Knowledge of these metabolic links and the circumstance that fatty acid *de novo* synthesis and β-oxidation occur in almost any organism were utilized to establish PHA biosynthesis in various non-PHA producing organisms (Steinbuchel *et al.*, 2003). For example, in the bacterium *Alcaligenes eutrophus*, polyhydroxybutyrate (PHB), a thoroughly characterized PHA is synthesized from acetyl-CoA by a sequential action of three enzymes, as shown in Figure 1.2.

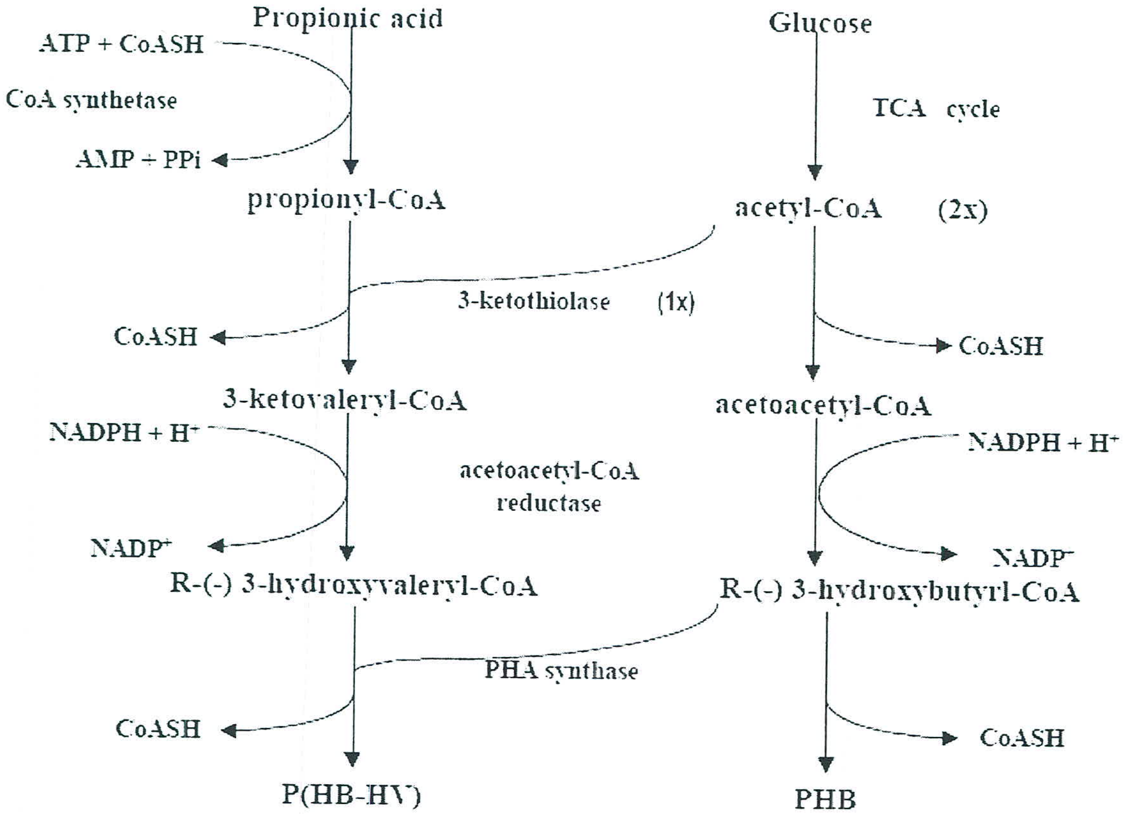


Figure 1.2 The biosynthetic pathway of PHB and P(HB-HV) in *Alcaligenes eutrophus* (Hustede *et al.*, 1993)

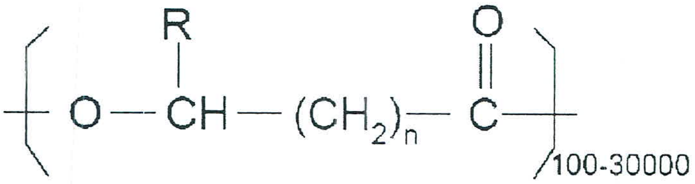
The first enzyme, in the pathway 3-ketothiolase promotes the condensation of two acetyl CoA moieties in a reversible manner to form acetoacetyl-CoA. This is followed by the action of acetoacetyl-CoA reductase which reduces acetoacetyl-CoA to R(-)-3-hydroxybutynl-CoA. The PHA synthase then polymerizes the R(-)-3-hydroxybutyrl-CoA to form PHB (Lee *et al.*, 1996; Poirier *et al.*, 1995). *A. eutrophus* can accumulate PHB as inclusion up to 80% of the dry weight which the bacterial are cultivated in media containing excess carbon such as glucose, but limited in one essential nutrient, such as nitrogen or phosphate. Thus, addition of 4-hydroxybutyrate or 5-hydroxyvalerate in the growth media leads to synthesis of the corresponding CoA thioesters and incorporation into PHA by the PHA synthase (Figure 1.2). One few bacteria are known to synthesize PHA short chain length (scl) containing monomers other than 3HB when grown on simple sugar (Haywood *et al.*, 1991). Some *pseudomonads* species have been reported to accumulate PHA copolymers containing medium chain length (mcl) monomer for instance *Pseudomonas oleovorans*and *Pseudomonas putida*, when grown on alkanoic acid (Steinbuchel *et al.*, 1991). The composition of PHA produced is usually related to the substrate used for growth, which is mostly 2nd carbon shorter than the substrate used (Poirier *et al.*, 1995). The analysis of biosynthetic pathways of these species have been discussed extensively (Fuller *et al.*, 1992; Huijberts *et al.*, 1992; Lageveen *et al.*, 1988).

Recently, there has been a very significant increase in the production of glycerol as a by-product in the processes of biodiesel production. This trend has caused a sharp fall in the cost of glycerol, which is now considered a waste product that must be disposed of (Ashby *et al.*, 2005). As a result, glycerol has become a very attractive substrate for bacterial fermentations. One potential use of glycerol is in industrial fermentation where it can be employed as a carbon substrate for microorganisms (Shum-Tim *et al.*, 1999) such as the use of glycerol for microbial PHA synthesis has been studied in natural PHA producers, such as *Methylobacterium rhodesianum* (Bormann *et al.*, 1999) and several *Pseudomonas* strains (Madison *et al.*, 1999), and also in recombinant *E. coli* carrying the PHB synthesis genes from *Streptomyces aureofaciens* (Almeida *et al.*, 2007; Mahishi *et al.*, 2003). Then, Almeida *et al.*(2007) studied the effect of PhaP on bacterial growth and PHB accumulation from glycerol in bioreactor cultures of recombinant *E. coli* carrying PhaBAC and PhaP of *Azotobacter* sp. strain FA8. Additionally, Bormann and Roth (1999) cultivated *Methylobacterium rhodesianum* and *Ralstonia eutropha*to produce PHB using media which contained glycerol and casein hydrolysates as C/N-substrates. In addition to, Brioudea *et al.* (2007) reported polyesters were prepared from the polycondensation between 10% wt glycerol and adipic acid using dibutyltindilaurate as catalyst.

1.2.3 Classification of polyhydroxyalkanoate

1.2.3.1 Number of carbon atom in monomer units

PHA can be classified into 2 categories based on the side chain length (R-group) in the PHA monomer (Figure 1.3).



$$\left[-O-\overset{\displaystyle R}{\overset{|}{C}}H-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\right]_{100\text{-}30000}$$

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

Figure 1.3 The general structure of polyhydroxyalkanoates (Ojumu *et al.*, 2004)

1. **Short chain length polyhydroxyalkanoate (scl-PHA)**contains4 to 5 carbon monomers; polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) contain 4 carbon monomers (Allmendinger *et al.*, 2003) and 5 carbon monomers [3-(R)-hydroxyvalerate], respectively. *Ralstonia eutropha, Allochromatium vinosum* and *Bacillus megaterium* are representative scl-PHA producers. Class I (PhaC), class III (PhaC, PhaE), and class IV (PhaC, PhaR) PHA synthases were active toward short chain length 3-hydroxyalkanoate-CoA (scl-3HA-CoA) containing three to five carbons for the production of scl-PHA.

2. **Medium chain length polyhydroxyalkanoate (mcl-PHA**) contains six to fourteen carbon monomers. *Pseudomonas putida, P. oleovorans,* and *P. aeruginosa* are representative mcl-PHA producers. Class II PHA synthase PhaC was active toward medium chain length 3-hydroxyalkanoate-CoA (mcl-3HA-CoA) containing six to fourteen carbons for the production of mcl-PHA.

The PHA shares physical and material properties which recommended them for applications in various areas. They was thermoplastics and/or elastomerics, insoluble in water, enantiomeric pure, non-toxic, biocompatible, and exhibit a high degree of polymerization and molecular weights of up to several million (Steinbuchel *et al.*, 2003). The typical weight-average molecular mass of PHA was in the order of 400 kDa and the melting point (T_m) range was 163 – 174°C. However, the most important feature was the biodegradability of PHA.

The PHA also serves as a model to develop various kinds of biodegradable polymers, either by chemical or combination of biotechnological and synthetic processes. There are several types of PHA that co-exist with aliphatic compounds under the family of polyesters. Figure 1.4 illustrated general terms of the two main groups of polyesters, aromatic polyesters had excellent thermal and mechanical properties however were resistant to microbial attack. Aliphatic polyesters on the other hand were biodegradable but their properties were less durable than aromatic polyesters. The development of both aliphatic and aromatic polyesters had seen enormous improvements in their mechanical properties. This development takes into consideration the need for materials which had a lesser impact on non-renewable resources and the environment by the end of their useful life.

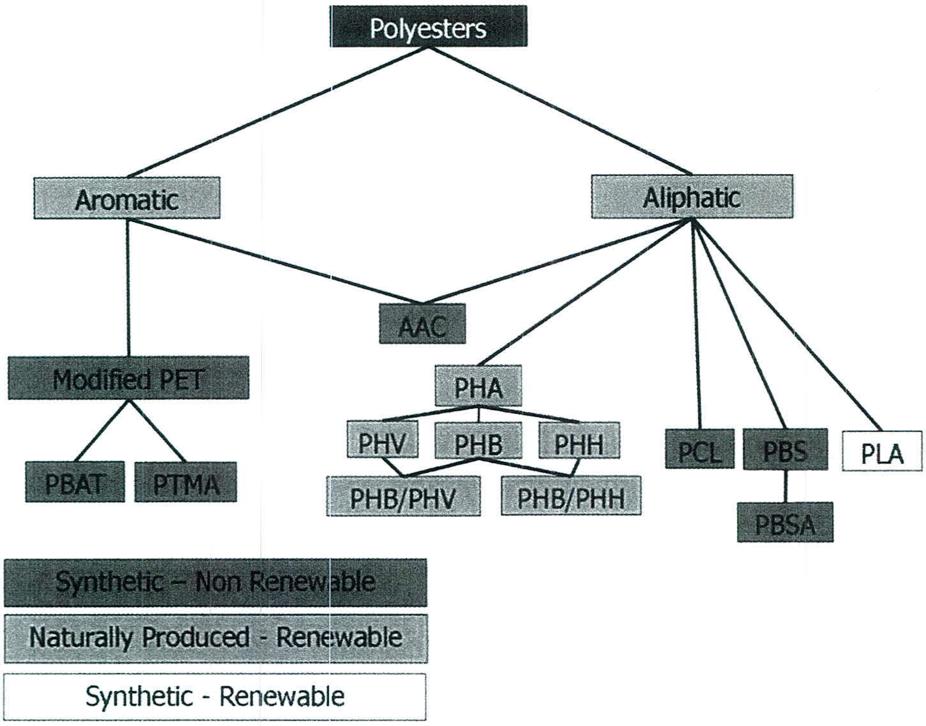


Figure 1.4 A general overview isa list of these polyesters and their grouped relationships. PHA – polyhydroxyalkanoates; PHB – polyhydroxybutyrate; PHH - polyhydroxyhexanoate PHV – polyhydroxyvalerate; PLA - polylactic acid; PCL – polycaprolactone; PBS - polybutylene succinate; PBSA - polybutylene succinate adipate; AAC - aliphatic-aromatic copolyesters; PET - polyethylene terephthalate; PBAT - polybutyleneadipate/terephthalate and PTMA- polymethyleneadipate/terephthalate (Madison *et al.*, 1999)

1.2.3.2 Rearrangement of monomers in polymer chains

1. Homopolymeris composed of only one monomer in polymer chains, such as polyhydroxybutyrate (PHB), a polyhydroxyvalerate (PHV), etc.
2. Copolymer features the least of 2 monomers incorporated into polymer chains, e.g. poly-hydroxybutyrate-co-hydroxylvalerate (PHBV), etc.

Because homopolymer was a highly crystallized polymer, not easily molded, and therefore, not extensively commercialized. On the other hand, copolymer had a lower melting point and much better flexibility and had been commonly used in many applications (Yu *et al.*, 2005).

1.2.4 Applications of polyhydroxyalkanoate

The PHA are polyesters produced by microorganisms under unbalanced growth conditions. They were generally biodegradable and thermoprocessable, making them attractive as biomaterials for applications in both conventional medical devices and tissue engineering (Chen *et al.*, 2005). The applications of bacterial PHA had concentrated on 3 principal areas: medical and pharmaceutical, agricultural, and commodity packaging (Holmes, 1985). According to Lafferty *et al.* (1988), the most advanced development of bacterial PHA was in the medical field, especially pharmaceutical applications although they had a considerable potential as consumer goods products.

1.2.4.1 Medical and pharmaceutical applications

One type of PHA is polyhydroxybutyrate (PHB), which was strongly flexible, and it can be degraded in vivo at least in part by a surface erosion process. While the chemical structure of PHB was similar to that of current absorbable polyesters used in implantable medical products. The strength of PHB fibers prepared by melt extrusion compared well with that of traditional suturing materials; however, PHB was typically more flexible. PHB should find use in a wide variety of medical fields such as cardiovascular, wound healing, orthopedic, drug delivery, and tissue engineering (Lafferty *et al.*, 1988; Lee, 1996).

Therefore, it is plausible that it is biocompatible with animal tissues and can be implanted into animal tissues without any toxic effects. Some possible applications of bacterial PHA in the medical and pharmaceutical applications include: biodegradable carriers for long-term dosage of drugs inside the body, surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements, and stimulation of bone growth and healing by piezoelectric properties. The advantages of using biodegradable plastics during implantation are that it is biodegradable, i.e. the need for surgical removal is not necessary.

1.2.4.2 Agricultural applications

The PHA is biodegradable in soil. Therefore, the use of PHA in agriculture was very promising. They could be used as biodegradable carrier for long-term dosage of insecticides, herbicides, or fertilizers, seedling containers and plastic covers protecting saplings, biodegradable matrix for drug release in veterinary medicine, and tubing for crop irrigation. Here again, it was not necessary to remove biodegradable items at the end of the harvesting season.

1.2.4.3 Biodegradable commodity packaging

According to Lafferty *et al.* (1988), the PHB homopolymer and PHB-PHV copolymer have some properties, i.e. tensile strength and flexibility, similar to polyethylene and polystyrene. Also, small additions of PHA improved the property of some conventional polymers, e. g., addition of a small amount of PHA reduces the melt viscosity of acrylonitrile. Tsuchikura (1994) reported that "BIOPOL" with high PHV content was more suitable for extrusion blow moulding and extrusion processes, e.g., made into films, sheets, and fibres, while "BIOPOL" with low PHV content was more suitable for general injection moulding processes. One particular property of PHB films that made it possible to be used for food packaging was the relatively low oxygen diffusivity. Plastics produced from PHA had been reported to be biodegraded both in aerobic and anaerobic environments (Page, 1995). In summary, possible applications of PHA were used for commodity goods include packaging films, bags and containers, disposal items such as razors, utensils, diapers, and feminine products.

1.2.5 Physicochemical compositions

The PHA were more than 100 different types of known basic building blocks for PHA polymers reported (Simon *et al.*, 1983; Steinbüchel *et al.*, 1995). Among PHA, medium-chain-length PHA (mcl-PHA) consisting of monomers of 3-hydroxyhexanoate (HHx), 3-hydroxyoctanoate (HO), 3-hydroxydecanoate (HD), 3-hydroxydodecanoate (HDD), or even higher-chain-length monomers show amorphous and elastic properties compared with short chain-length PHA (scl-PHA), which has a high degree of crystallinity and rigidness (Ashby *et al.*, 2000; Noda *et al.*, 2004; Ouyang *et al.*, 2007).

A difference in polymer composition determined with chemical analysis is often required to determine their monomeric compositions. Recently, several modern analytical techniques such as gas chromatography (GC), IR spectroscopy and NMR spectroscopy have been used to determine PHA structure in bacterial dry cells. Hence, the NMR spectroscopy and Fourier transforms infrared spectroscopy (FTIR) analysis are widely used for this purpose.

1.2.5.1 Nuclear magnetic resonance (NMR)

The NMR technique was utilized to identify the polymers synthesized by the strains. The utility of this technique for the identification of the specific biopolymer had been demonstrated previously (Patel *et al.*, 2009). Barnard and Sanders (1989) reported that high resolution NMR spectroscopy of live cells had been used to show that the PHB was predominantly in a mobile state within the storage granules of *Ralstonia eutropha*, *Methylobacterium extorquens*, and *Methylobacterium* AM1. By comparison of chemical and NMR analysis of PHB indicated that about 70% of the polymer in *R. eutrophus* gave sharp observable resonances. Then, Valentin *et al.*(1999) claimed the simultaneous accumulation of two homopolyesters by *Burkholderia* sp. was confirmed by NMR spectroscopic analysis. In addition, the ^{1}H NMR signals was used in order to reveal the PHA produced by *Comamonas* sp. EB172 was a PHBV and confirmed the speculation (Zakaria *et al.*, 2008). Therefore, the NMR spectroscopic analysis was fundamental method that could be determined the PHA-producing bacteria structural analysis.

1.2.5.2 Fourier-transformed infrared spectroscopy (FTIR)

The FTIR has been demonstrated to be a powerful tool for studying microorganisms and their cell components in intact form (Helm *et al.*, 1991; Naumann *et al.*, 1995). It was reported that PHB was observable in FTIR spectra in intact bacteria (Helm *et al.*, 1995; Nicols *et al.*, 1984). The FTIR spectroscopy was a particularly suitable technique for investigating the specific intermolecular interaction. On this basis could evaluate the structure formed by H-bonds and its effected on the PHB crystallization. The advantage of FTIR spectroscopy was used to verify the presence of intermolecular hydrogen bonds (inter-H-bonds) between poly-(3-hydroxybutyrate co-3-hydroxyvalerate) (PHBV) (Fei *et al.*, 2004). By monitoring the spectral changes during PHBV crystallization and blends dissociation, the absorptive ratio of C=O bonds in crystalline and amorphous regions in PHBV and that of H-bonded and free C=O in PHBV/BPA blends were experimentally determined as 1.40 and 1.68, respectively (Fei *et al.*, 2004).

Moreover, FTIR spectroscopy was easy to use and was able to provide information about the molecular structure of samples under study in “real-time”. It is recently being increasingly for investigation of the crystallization of PHA (Kansiz *et al.*, 2007). Therefore, in our study we extended the observation to find the PHB can be detected rapidly by the FTIR technique in intact cells.

1.2.6 Increasing polyhydroxyalkanoate production

Biodegradable polymers costs more to produce than do petro-chemical polymers (Yamane, 1993), such as polyethylene and polystyrene. Thus, improvement strategies for productivity of PHB (Kim *et al.*, 1994; Suzuki *et al.*, 1986) and an optimal production strategy for PHB (Choi *et al.*, 1997) had been developed.

Fermentation strategy was also developed for the efficient production of PHA by high cell density culture. The best strategy PHA-production should satisfy several demands, such as a fast growing population, being able to utilize cheap carbon and having a high production rate (Lopez-Cortes *et al.*, 2008). Although high PHA content could be achieved by using pure culture fermentation process, the cost of PHA production was still too high for PHA to become a competitive commodity plastic material. As to reduce the expensiveness of PHA, a novel PHA production strategy, which was to utilize the mixed bacterial culture in activated sludge for PHA production had been proposed in the last decade (Chua *et al.*, 2003). Cost of raw materials was a major factor in economics of production media for PHA fermentation. So, reducing PHA production costs had been many ways for instant increasing products *via* limiting nitrogen source and/or selection of suitable carbon source. Accumulation of PHA occurred when the cells becoming limited for an essential nutrient but had excess of carbon source (Page, 1989). During the initial balanced growth phase, cell mass was produced. Nutrient limitation when imposed in second phase, leaded to PHA accumulation in cells (Wang *et al.*, 2007).

In the first stage, it was essential to optimize the operational conditions for the enrichment of PHA-accumulating microorganisms, so that PHA production capability of mixed cultures could be improved. In the second stage, carbon substrate such as glucose was fed to the acclimatized sludge and limiting nitrogen source in containing medium for induce of PHA production.

1.2.7 Life cycle assessment (LCA) of bioplastics

As a consequence of the development of new markets, energy consumption and waste production were increasing at a fast rate. Emerging countries needed more and more fossil resources whilst the developed countries were late and hesitant in introducing energy saving programs and controlling the release of greenhouse gases. The amount of goods produced and packed was also growing, making waste disposal an emergency for several nations. These problems represent a powerful driving force which had stimulated much interest in a potential solution, bio-based and biodegradable polymers. At an industrial level, several biopolymers had been developed and biodegradable products were already present in the market (e.g. compostable bags for the at source collection of organic waste, packaging, mulch films for agriculture, compostable cutlery and tableware etc) (Satkofsky, 2002). Since the biodegradable "green" products displace consumer products made with traditional polymers, the question soon arose whether the claimed environmental superiority of the biopolymer and bio-products was actually founded on fact.

Biodegradability and compostability was verified thanks to a testing strategy that was well defined by the harmonized European standard EN 13432 (Degli, 2003). The content of renewable carbon could be quantified by using the radiometric test described in the ASTM D6866-05 standard. The overall environmental impact of products and services can be determined by applying LCA which was based on the ISO 14040 and ISO 14044 series of standards was currently advocated as an important means for performing comparison between products and services.

LCA has emerged as a powerful method that takes into account the products' energy and resources consumed, as well as the generation of emissions and wastes, of the products entire life span from cradle to grave (Hauschild, 2005; Landis *et al.*, 2007). LCA could be applied in a wide range of scientific research and industrial areas and could bring to attention unexpected environmental outcomes. This kind of application was important to ensure that, while solving a particular environmental concern; negative impacts were not passed from one stage to another or from one environmental compartment to another.

Some bioplastics can even be produced through both sugar platforms and thermo-chemical platforms (Figure 1.5). It was then only a strategy choice depending on the feedstock availability and cost of material since the biotechnology route could be more efficient (Figure 1.5), or not.

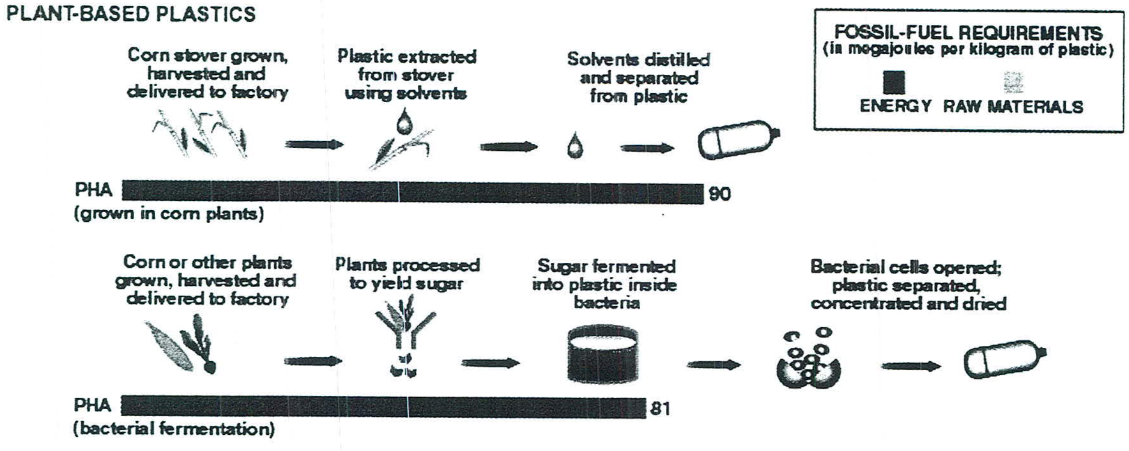


Figure 1.5 Processing routes for PHA production (Comstock *et al.*, 2004)

Many reports and research papers was available regarding the sugar platform and the environmental potential of those processes. The bioengineering platform based polymers were driven by the need of specific new functionalities of polymers like biodegradability or compostability and most of the research focuses on biodegradable/compostable biopolymers (European Bioplastic, formerly IBAW, 2005). The most available manufacturing processes for production of bioplastics were based on the sugar platform whereas the existing thermochemical platforms stop at the production of biofuel. However, it was possible to go further downstream and to make polymers like polyolefins out of biomass, but those processes remain relatively unexplored in terms of environmental potential.

The main representatives of polyhydroxyalkanoates are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). For this family of polymers, no full LCA were available, but only studied and estimated comparing the energy requirements and CO_2, or greenhouse gas emissions, among them the papers by Gerngross and Slater (2000); Gerngross (1999) and Kurdikar *et al.* (2001). In Table 1.2, their data were compared to LCA data for petrochemical polymers, according to Boustead (commissioned by APME).

Table 1.2 Energy requirements for plastics production (Boustead, 1999; Gerngross *et al.*, 2000)

	Cradle-to-factory gate fossil energy requirements, in GJ/tonnes plastic		
	Process energy	Feedstock energy	Total
PHA grown in corn plants	90	0	90
PHA by bacterial fermentation	81	0	81
HDPE	31	49	80
PET (bottle grade)	38	39	77
PS (general purpose)	39	48	87

*Data for PHA from Gerngross and Slater (2000); Data for petrochemical polymers from Boustead (1999)

Table 1.2 shows that the total cradle-to-factory gate fossil energy requirements of PHA can compete with polyethylene (HDPE) depending on the type of the PHA production process. Compared to polyethylene terephthalate (PET), the minimum total energy input for PHA production (fermentation) was somewhat higher while it was lower compared to polystyrene (PS). In contrast, the process energy requirements of PHA were two to three times higher than for petrochemical polymers (Table 1.2). Limiting the discussion to these process energy data Gerngross and Slater (2000) drew the conclusion that polyhydroxyalkanoates did not offer any opportunities for emission reduction (Gerngross, 1999). This finding was valid for certain system boundaries, e.g. for the system 'cradle-to-factory gate', the output of which were plastics pellets. The conclusion was also correct if all plastic waste has been deposited in landfills. In contrast, the finding was not correct if other types of waste management processes were assumed within the 'cradle-to-grave' concept. Total fossil energy requirements (Table 1.2) were practically identical for PE and PHA manufactured by bacterial fermentation. Hence, if combusted in a waste incinerator (without energy recovery), both plastics result in comparable CO_2 emissions throughout the life cycle.

1.3 Research Objectives

In this study, a microbial consortium obtained from sludge was used as a seed starter for converting commercial glycerol to PHA bioplastics. The research objectives are as follows:

1. Analyze microbial communities that are capable of converting the commercial glycerol to polyhydroxyalkanoate (PHA) bioplastics.
2. Examine PHA production in 10-L reactor systems.
3. Characterize the microbial communities present in fermentation sludge and isolate microorganisms responsible for bioplastic production by culture dependent methods and molecular biological techniques, such as denaturing gradient gel electrophoresis (DGGE) and sequencing of 16S rRNA genes.
4. Characterize and determine the chemical composition and structure, and chemical/physical properties of the produced PHA purification.