

STUDY THE REACTION MECHANISM OF THE PRODUCTION OF NOVEL
BIOPOLYMER {POLY (3-HYDROXYBUTYRATE) [PHB]} FROM THE PALM
OIL SEED

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Thesis submitted in fulfillment of the requirements
For the award of the degree of
Bachelor in Chemical Engineering

Faculty of Chemical Engineering and Natural Resources
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JUNE 2012

ABSTRACT

Poly(3-hydroxybutyrate) (PHB), is a biodegradable plastic that recognized as a potential alternative to substitute conventional petrochemical-based plastic. This biodegradable plastic is derived from bacterial fermentation using various carbon sources. Since the cost of carbon substrates has been identified as the major constrain in PHB production, inexpensive and renewable carbon substrate were currently investigated as alternative to conventional sugar-based substrate. Palm oil, massive-readily edible plant oil, may be an alternative substrate to conventional carbon source for PHB production. PHB is synthesized by *Alcaligenes eutrophus* in a production medium containing palm oil. Highest cell-dry weight was obtained by *A. eutrophus* when 20 g/L of palm oil and 0.6 g/L of urea were used. Cell-dry weight increased when the addition of starter inoculum volume increased up to 10 mL. Highest cell-dry weight was obtained at 48 to 60 hour of incubation. In a cell growth kinetic study, the total cell-dry weight obtained in presence of 20 g/L palm oil was greater than 12g/L and 16 g/L. Bacteria fermentation in the presence of 0.6 g/L urea shows greater total cell-dry weight compared to fermentation system without any addition of urea. This study proved that palm oil is a feasible and excellence carbon substrate for PHB biosynthesis by *A. eutrophus*. In this study, the evaluation of optimum condition for PHB biosynthesis by bacterial fermentation was accomplished.

ABSTRAK

Poly(3-hydroxybutyrate) (PHB), plastik boleh biodegradasi yang diiktiraf sebagai alternatif yang berpotensi untuk menggantikan plastik konvensional berasaskan petrokimia. Plastik boleh biodegradasi ini dihasilkan dari fermentasi bakteria menggunakan pelbagai sumber karbon. Sejak kos sumber karbon telah dikenal pasti sebagai kekangan utama dalam pengeluaran PHB, sumber karbon yang murah dan boleh diperbaharui kini disiasat sebagai alternative kepada sumber konvensional berasaskan gula. Minyak sawit, minyak tumbuhan secara besar-besaran dan sedia ada yang boleh dimakan, boleh menjadi sumber alternatif kepada sumber konvensional untuk pengeluaran PHB. PHB yang disintesis oleh *Alcaligenes eutrophus* dalam medium pengeluaran yang mengandungi minyak sawit. Berat biojisim kering yang tertinggi telah diperolehi oleh *A. eutrophus* apabila 20 g/L minyak sawit dan 0.6 g/L urea telah digunakan. Berat biojisim kering meningkat apabila penambahan jumlah inokulum permulaan meningkat sehingga 10 mL. Berat biojisim kering yang tertinggi telah diperolehi pada tempoh inkubasi selama 48 hingga 60 jam. Dalam satu kajian kinetik pertumbuhan sel, jumlah berat biojisim kering yang diperolehi dengan kehadiran 20 g/L minyak sawit adalah lebih besar berbanding daripada 12 g/L dan 16 g/L. Fermentasi bakteria dengan kehadiran urea sebanyak 0.6 g/L menunjukkan jumlah berat biojisim kering yang lebih tinggi berbanding dengan sistem fermentasi tanpa apa-apa tambahan urea. Kajian ini membuktikan bahawa kelapa sawit merupakan sumber karbon yang boleh dilaksanakan dan baik untuk biosintesis PHB oleh *A. eutrophus*. Dalam kajian ini, penilaian keadaan optimum untuk biosintesis PHB dari fermentasi bakteria telah dicapai.

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LIST OF ABBREVIATION

DA	-	Dalton Molar Mass
EU	-	European Union
GC-FID	-	Gas Chromatography-Flame Ionized Detector
^1H NMR	-	Proton Nuclear Magnetic Resonance
OD	-	Optical Density
PHA	-	Polyhydroxyalkanoate
PLA	-	Poly lactides
PHV	-	Polyhydroxyvalerate
PHH	-	Polyhydroxyhexanoate

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Over the years, conventional plastics have brought economic, environmental, and social advantages. In 2009, around 230 million tonnes of plastic were produced; around 25 per cent of these plastics were used in the EU (Mudgal et al., 2010). About 50 per cent of plastic is used for single-use disposable applications, such as packaging, agricultural films and disposable consumer items (Hopewell et al., 2009). It is estimated that plastics save 600 to 1300 million tonnes of CO₂ through the replacement of less efficient materials, fuel savings in transport, contribution to insulation, prevention of food losses and use in wind power rotors and solar panels (PlasticsEurope, 2010). However, their reputation and popularity has also meant a rise in plastic waste, which are proven harmful for environment. Nowadays, abundance of plastic waste has brought environmental issues, economic crumble, and social health crisis. Furthermore, the main raw material to produce conventional plastic, which is petroleum, is in the brink of depletion. The petroleum sources are only as much as necessary to sustain global fuel demands. Further consumption of petroleum in order to produce plastic will increase the rate of petroleum depletion, thus making it very expensive in society market. Plastics consume approximately 8 per cent of world oil production. 4 per cent as raw material for plastics and 3 to 4 per cent as energy for manufacture (Hopewell et al., 2009). Although the figure is not exceeding 10 per cent, the plastic demand shows increasing trend for the next upcoming years especially in several critical industries such as medial, pharmaceutical, and automotive. Years after, the consumption would likely continue to be increased to sustain global demand of plastics.

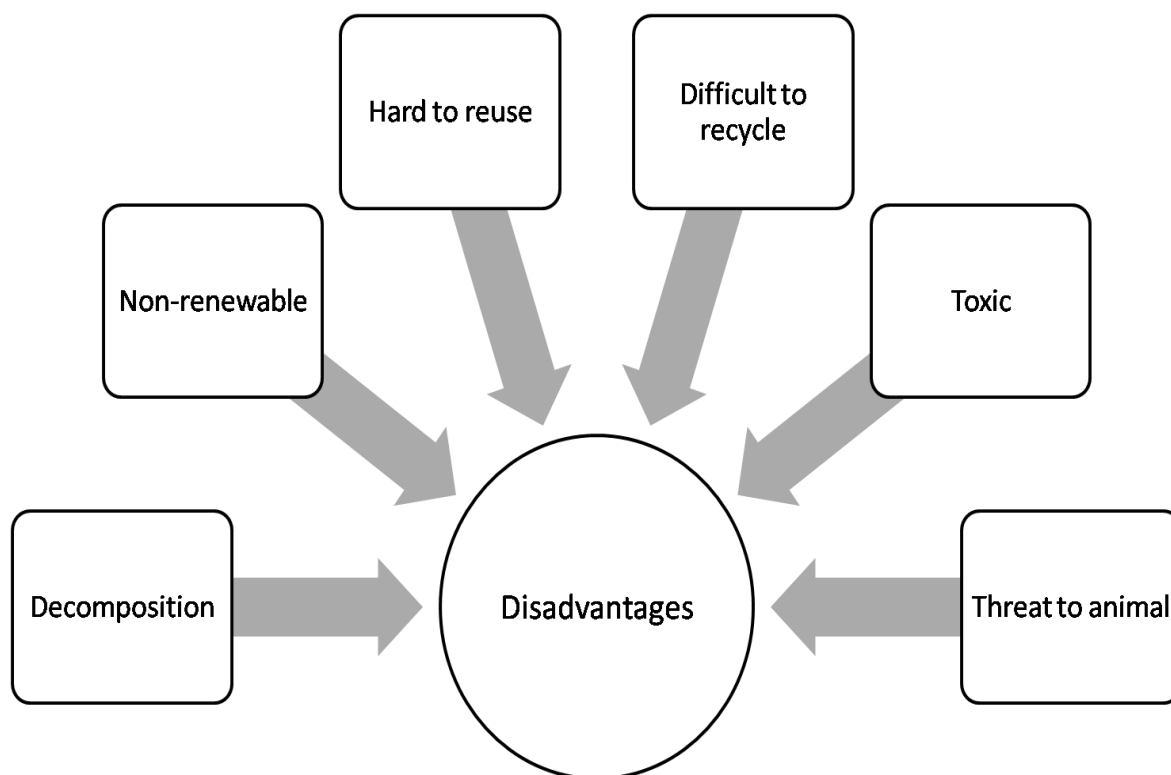


Figure 1.1: Disadvantages of conventional plastic

Plastics-based products are certainly ubiquitous, from cell-phones and computers, bicycle helmet and car interiors, to plastic bags and bottles. These plastic, while convenient, do have disadvantages when used on as wide a scale. While most of these disadvantages are environmental in nature, the consequences could have widespread economic consequences in the long term. Figure 1.1 illustrates the disadvantages of conventional plastic. The major set-back of conventional plastics is the sheer amount of time they acquire in order to decompose. The average conventional plastic requires almost 500 years decomposing. Plastics are hard to reuse and difficult to recycle. There are several types of plastic that cannot be recycled due to its properties. The time cycle to reuse plastic also shorter compared to other material such as glass. If often reuse, conventional plastic is more likely to become less and less sturdy over time. Abundance Plastic waste in ocean and seas is also known as “plastic soup”, which is a threat to every aquatic life.

To date, there are several effort and attempts to find the solution for problematic issues concerning conventional plastics. Large attraction is concentrated on the recycling of the conventional plastic. However, this method is not very effective in economical aspect for the long time. The recycle of plastic also brought some major issues, as it is hard to separating and sorting the wide variety of plastics and there are also alterations in the plastics material, such as additives, which limiting its further application range (Flechter, 1993). Focus and effort should be directed to alter the life time and biodegradability of conventional plastic, while maintaining the nature of conventional plastic. By doing this, plastic abundance can decompose and degrade easily in a short period of time. Substitution of non-biodegradable by degradable plastics is a major interest of both decision-makers and the plastic industry (Song et al., 1999). Producing and commercializing eco-friendly products such as bioplastics is one such reality that can help us overcome the pollution problem cause by conventional plastics (Reddy et al., 2003).

Polymeric structure of conventional plastics is a factor that contributes to disability of degradation. Therefore, by modifying the polymeric structure of conventional plastics will presents promising result of degradability. There are three types of biodegradable plastic, which are photodegradable, semi-degradable and completely biodegradable (Reddy et al., 2003). Photodegradable plastic degrades when extensive ultraviolet radiation disintegrates the modified polymeric structure which contain light-sensitive group directly attached to the backbone of the polymer as additive (Kalia et al., 2000). Disintegrate action rendering the polymeric structures open for further bacterial degradation. This reaction occurs within several weeks to months.

However, landfill cannot provide sufficient sunlight, thus their remains un-degrade. Starch-linked plastic is an example of semi degradable plastic. Starch is attached and cross-linked to the polymeric chain. Upon disposal, the starch link will be degraded by starch-utilized bacteria in the soil, leaving the polymer fragments that will further degraded by other bacteria. So far, it is reported that bacteria did degrade the starch but the polymer fragments are remain non-degradable. (Johnstone., 1990). The last type of biodegradable plastic, which is completely biodegradable, is rather new and

promising. This biopolymer is produced from substrate-utilizing bacteria and degraded by several others widespread bacteria.

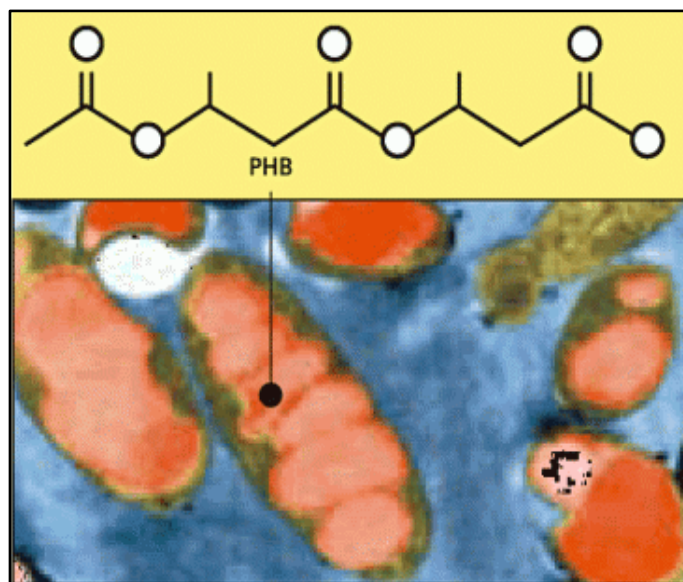


Figure 1.2: Polymeric Structure of PHB

Source: <http://www.foodylife.com/food-package/867/bioplastic-poly-3-hydroxybutyrate-phb-and-its-importance/>

This study is focusing on the third type of biodegradable, which is the complete biodegradable. There are several well-known biopolymer categorized in this types including polyhydroxyalkanoate (PHA), polylactides (PLA), aliphatic polyester, polysaccharides, copolymers and/or blend of all above (Reddy et al., 2003). PHA is 100% biodegradable, thermoplastic, elastomer, insoluble in water, non toxic and biocompatible polymers. These polyesters have characteristics similar to those of the polyethylene and polypropylene, and can therefore be used instead of conventional plastics (Lopez et al., 2010). Poly(3-hydroxybutyrate) (PHB) is the best characterized PHA. PHB in *Bacillus megaterium* was discovered and firstly described by French microbiologist, Lemoigne, in 1925. Since then, several of bacteria strain among archaeobacteria (Doi.,1990), gram positive (Findlay., 1983), and gram negative bacteria (Forsyth et al., 1958), and photo synthetic bacteria (Hassan et al., 1998) including cyanobacteria (Jau et al., 2005) have been identified to accumulate PHB.

These bacteria strain able to accumulated PHB both aerobically and anaerobically. In 1973, PHB is recognized as a bacterial storage material that has similar function as starch and glucose. PHB homopolymer is accumulated by *b.megaterium* when the ration of glucose to nitrogen is high (Macrae and Wilkinson., 1958) and the following intercellular degradation of PHB occurred in the absence of carbon and energy sources. The first commercial production of PHB started in 1960's but the first industrial production came true in 1970's. The first produced commercial product was packed under name of "BIOPOL™".

To date, there are significant different of market price between expensive PHA bioplastic and affordable conventional plastic. This has been the major obstacle for PHB to become replacement of conventional plastic in market (Bidy, 2002). The price of PHA depends on the substrate cost which is usually pure such as glucose and propionate for P(HB-HV) production, PHA yield, as well as on the downstream process used for polymer extraction (Lee, 1996). In order to make the PHA production economically viable, many goals have to be addressed simultaneously. Economic biotechnological plastic production depends on highly productive microorganisms and on low-cost substrates. The nature of the substrate not only determines the PHA content but also its composition, which subsequently affects the final polymer properties. Furthermore, raw materials may account for 40 to 50% of the total operating costs (Meesters, 1998).

1.2 Problem Statement

The environmental concern regarding to the pollution caused by conventional plastic have drive several individual and organization to invest in biodegradable material. Existing landfill facilities cannot sustain abundance of conventional plastic waste for a lot longer. It is time that society to aware about these environmental issues related to conventional plastic waste. In order to achieve this, biodegradable plastic should able to replace conventional plastic in market so that consumer can get benefit from it. However, the commercial price of PHB bioplastic is far enough to be able to compete with conventional plastic. The PHB bioplastic price is rather expensive for household application compared to conventional plastic. After all, major source of plastics waste is originally generated from household. Comparatively, the mount of conventional plastic produced every years is tremendous and exceeding all bioplastic production amount. If the production amount of bioplastic can exceed the conventional plastic, it is possible to reduce conventional plastic usage, thus reducing the amount of conventional waste generated. A satisfying and feasible process to produce PHB is not yet been achieved. The main obstacle is to find the suitable carbon substrate to produce PHB. The choice of carbon substrate is an important factor in optimization of PHB production. The nature of carbon substrate determines the yield PHB content as well as the properties of the monomer. Consequently, the final properties of polymer are also affected by the nature of carbon substrate. Amongst of all final PHB production cost based on the variety carbon substrate, 80% of them is affected the by carbon substrate that were used in the production process. Therefore, the possibility to further reduce the production cost is achievable if a cheap carbon substrate can be identified and utilized. Various carbon substrates especially forest seed oil such as soybean oil, palm oil, olive oil, sunflower oil and coconut oil, have been evaluated and found to be excellent carbon sources for PHA production. These, carbon substrate are categorized as food-grade oil. It is both wasteful and unethical to convert food sources into bioplastics. However, this issue is no longer presents any obstacle since palm oil production amount is in excess. In addition, there are no sufficient data that can be used as the precursor to the large scale production. All parameters that related to the PHB biosynthesis can be optimized, thus providing good information to produce large scale of PHB.

1.3 Objective of Study

- i) To produce PHB with palm oil through single stage fermentation.
- ii) To identify the best value of parameters that yields the highest content of cell dry weight and PHB.
- iii) To analyze the trend of the bacterial growth and PHB biosynthesis.

1.4 Scope of Study

In order to achieve the objectives stated above, the following scopes of studies have been drawn:

- i) To study different parameters (Inoculum density, urea concentration, oil concentration and incubation period) that give optimum condition to produce PHB.
- ii) To analyze the bacterial growth trend over time in order to understand the mechanism of PHB biosynthesis.

1.5 Rationale and Significance of Study

Forest seed oils have been found to result in high-yield PHA production. Palm oil is renewable and readily available resource in oil palm-rich countries such as Malaysia. Malaysia, being the world's second largest producer of palm oil, is able to ensure continuous supply of palm oil products for sustainable PHA production. The biosynthesis and characterization of various types of PHA using palm oil products have been described in detail in this review. Besides, by-products and waste stream from palm oil industry have also demonstrated promising results as carbon sources for PHA biosynthesis. Various palm oil products and by-products have yielded positive results when used as carbon source for PHA biosynthesis. The studies provide preliminary results on the efficiency of palm oil bioconversion into PHA and future implementation of these substrates for larger and continuous PHA production systems.

CHAPTER 2

LITERATURE REVIEW

2.1 Polyhydroxyalkanoate

Polyhydroxyalkanoates (PHA) are biodegradable polymer that belongs to polyester group. PHAs are synthesized from microbial fermentation using a variety of carbon substrates. This polyester consists of a variety of hydroxyalkanoates that can be synthesized by at least 75 genera of gram-positive and gram-negative bacteria (Reddy et al., 2003). PHAs polymers are also known as storage polyester that accumulated as intracellular granules produced by bacteria in certain condition. These intracellular granules act as carbon and energy reserves under limited growth condition such as nutrient stress. Such growth condition allows the accumulation of PHA intercellular to extents as high as 90% of cell dry weight (Madison and Huisman, 1999). To date, there are more than 100 monomer units that have been identified as components of reserve PHA (Reddy et al., 2003). This fact creates numerous possibilities of producing various types of biodegradable polymers with wide range of properties. The molecular weight of PHAs is varies with their producer. The molecular weights of PHAs are normally ranging from 50,000 to 1,000,000 Da (Madison and Huisman, 1999). Several PHA produced by bacteria have a sufficient molecular mass to form polymer characteristics that identical to most petrochemical-based polymer such as polypropylene or polyethylene. PHAs can be categorized based on the number of carbon atoms of their monomer unit. There are two typical groups of PHAs which are known as short chain length and medium chain length. The chain length refers to carbon atoms that construct the whole monomer.

Short chain PHA (PHA_{SCL}) is composed with 3 to 5 carbon atoms. On the other hand, medium chain PHA (PHA_{MCL}) is composed with 6 to 14 or more carbon atoms (Yee et al., 2010). The first type of PHAs discovered in 1925 was a PHA_{SCL} known as Poly(3-hydroxybutyrate) PHB. PHB is the well-characterized PHA. PHB have been identified in the cytoplasmic membrane and cytoplasm of *Escherichia coli*. Since then, the fermentative product of PHB has been known as the simplest and the most common occurring type of PHA. The PHB normally consists of 1000 to 30000 hydroxy fatty acid monomer. However, there are several other polymer of PHA's class including Polyhydroxyvalerate (PHV), Polyhydroxyhexanoate (PHH), Polyhydroxyoctanoate (PHO) and copolymer.

PHAs also presence in the form of copolymer and the most common copolymer is poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Lee et al., 1999). PHBV can be formed by co-feeding of substrates and may result in the formation of polymer consisting 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers. The combination of 3HV into PHB results in a PHBV that is less stiff but more brittle than PHB. Nowadays, PHAs that are commercially produced up to 500 tons per annum are from the PHA_{SCL} group (Luengo et al., 2003). The PHA_{MCL} group polymer is yet to make a significant breakthrough in commercial status. In fact, PHA_{MCL} is not a viable choice to be produce in bulk quantities. One of the major contributors of this problem is high production cost even for testing purposes.

PHA is thermoplastic, elastomeric and non-toxic polymers that are almost identical to petrochemical-based plastics. Moreover, PHA have a high degree of polymerization and relatively high crystalline whereby this characteristic is quite similar to those conventional plastic such as polyethylene (Reddy et al., 2003). In addition, PHA is biocompatible, optically active and isotactic (stereochemical regularity in repeating units), piezoelectric and insoluble in water. These features make them extremely useful in medical application (Yee et al, 2010). Furthermore, the unique property that distinguishes PHA from all petrochemical-based plastics is their total biodegradability in nature. It is believe that PHA degraded upon exposure to soil, fertilizer, or marine deposit.

There are several factors that affect and contribute biodegradation phenomenon such as exposed area condition and microorganism activity surrounding exposed area. The exposed area condition such as pH, moisture, humidity and temperature also play important roles in biodegradation activities (Boopathy, 2000). Molecular weight, polymer composition and polymer crystallinity are assumed to be significant factors specifically in PHA biodegradation (Lee, 1996). Biodegradability of PHA also depends on nature of monomer unit of PHA. For instance, copolymers that consisting PHB monomer units have been found to be degrade more rapidly than PHB and PHBV copolymer (Reddy et al., 2003).

Biodegradation of PHAs can occur under either aerobic or anaerobic condition. Products of aerobic degradation are carbon dioxide and water. In contrast, anaerobic degradation omits carbon dioxide and methane. Study has shown that PHA able to compostable in extensive range of temperature. The maximum compostable temperature recorded at 60°C with moisture level at 55%. Other studies have shown that 85% of PHAs were degraded in 7 weeks (Flechter, 1993). In addition, PHA has been recorded to degrade in marine environments at temperature below 6°C and within 254 days. The well-known biodegradability of PHAs has become attraction for numerous researchers to study and enhance PHAs production. Therefore, PHAs has been identified as a potential substitute to daily commercial plastic that derived from petrochemical.

The novel features of PHA offer an extensive range of application in many industries. Initially, the PHA was used as films for packaging purposes of some packaging industries. These packaging films are usually used in bags, containers, and paper coating (Reddy et al., 2003). Afterward, These PHA films are upgraded to sheets by combining PHA with other polymer such as polyvinyl alcohol. PHA also serves similar application as conventional commodity plastic in human daily life such as disposable item and cosmetic packaging. PHA also useful as stereoregular compound which mainly utilized as biodegradable carries for medicines, hormones, insecticides, and herbicides. Furthermore, due to PHA biocompatibility and piezoelectric properties, PHA is frequently used as osteosynthetic material in the stimulation of bone growth. PHA also used as synthetic bones, surgical structure, replacement of blood vessels, and suture filaments (Wang and Bakken, 1998). In addition, there are also some recent

applications of PHA wastewater treatment. This fact shows the increasing diversity of PHA usage which alternately reflecting the figure of increasing demand for PHA biodegradable polymer.

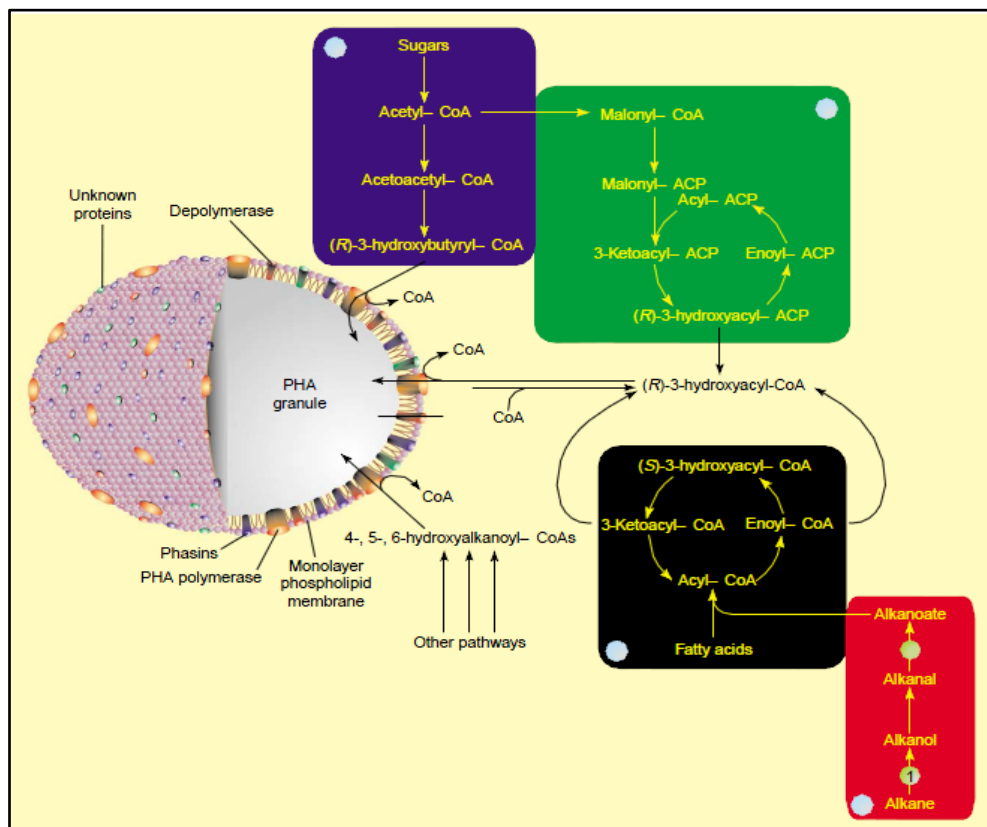


Figure 2.1: PHB metabolic pathways

Source: J. M. Luengo et al, 2003

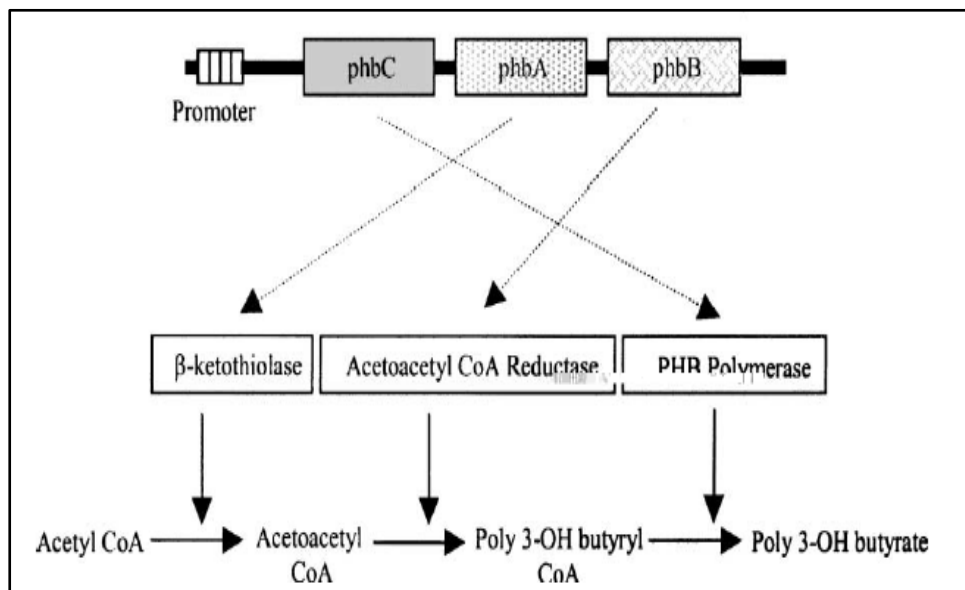


Figure 2.2: Biosynthetic pathway of PHB

Source: C.S.K Reddy et al., 2003

Extensive knowledge and information on PHB metabolic activity, biochemistry and physiology since 1987 has become a precursor to advance engineered PHA production. The early available information has been enriched with further molecular genetic studies. Numerous data involving PHA synthesis and degradation has been characterized from many different microorganisms. The picture is now clear that there is diversity in nature of PHA formation, each suited with ecological need of various PHA producing microorganisms. Genetic studies have exposed the regulation of PHA formation with respect of growth conditions. In order to study more sophisticated design of recombinant organism for advance PHA production, the basic foundation of knowledge of PHB biosynthesis must be understand.

The basic biosynthesis of PHB consists of three stages. These three stages are basically three enzymatic reaction catalyzed by three different enzyme. The first stage of the enzymatic reaction involves condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β -ketoacetylCoAthiolase (en-coded by *phbA*).

The second stage of the enzymatic reaction involves the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phbB*). Finally, the (*R*)-3-hydroxybutyryl-CoA monomer are polymerized into PHB by P(3HB) polymerase, encoded by *phbC* (Luengo et al., 2003). Figure 2.1 and Figure 2.2 illustrates the biosyntheses pathway of PHB. This basic foundation of PHB biosynthesis has been enhanced to a greater potential for various PHA monomer. Advance PHA production technology has been developed by utilizing recombinant bacteria, genetically engineered plant, and anaerobic digestion of biological waste. Several of these efforts have been dedicated to reduce the production cost of PHA and making them feasible to commercialize. The principle enzyme of PHB and oligomers derived from polymer depolymerase. The microorganism secrete enzyme that break down the polymer into hydroxyacids. There are few prominent microorganisms which PHB depolymerase. Among them are *Alcaligenesfaecalis*, *Rhodospirillumrubrum*, *Bacillus megaterium*, *Alcaligenesbeijerinckii*, and *Pseudomonas lemoignei* (Reddy et al., 2003). Figure 2.3 shows the PHA granular accumulated in various PHA producers.

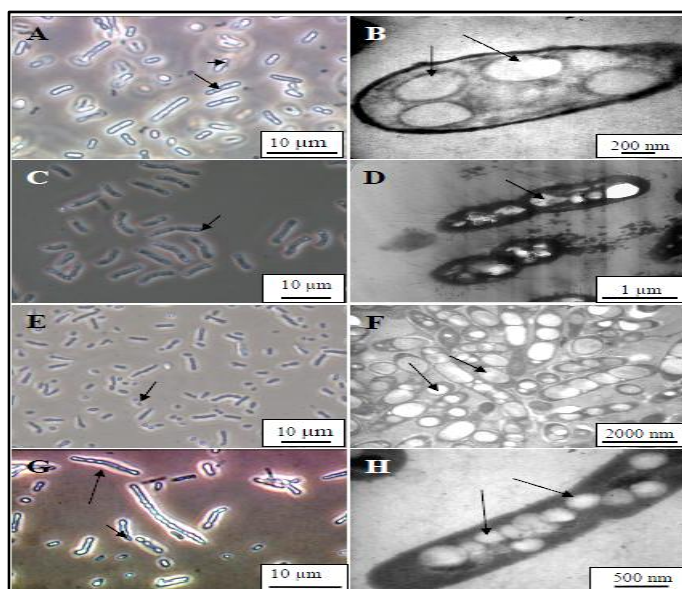


Figure 2.3: Observation of PHA granular, under phase-contrast and transmission electron

Source: Yee et al., 2010

2.2 Natural Producer , Recombinant Bacteria and Transgenic Plant

PHAs are natural product that are synthesized and catabolised by various types of microorganisms. These microorganisms are either natural isolates or recombinant bacteria. More than 250 various natural PHA-producing bacteria are recorded. However, only a few of these bacteria have been employed to synthesis PHA. To date, more than 90 genera of microorganism are employed to accumulate and catabolise PHAs (Luengo et al., 2003). These microbes are aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes.

There are several well-known natural PHA-producing bacteria such as *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Staphylococcus*, *necator* and *Micrococcus*. These bacteria are capable to utilizing different carbon sources including plant oil and waste to produce PHA. Table 2.1 shows production of PHA from various PHA producers. Most extensive studies and common application are often related to *C. necator*. However, Natural PHA-producing bacteria have some limitation.

Natural PHA-producing bacteria have a long generation time and relatively low optimal growth temperature. Moreover, these cells are usually difficult to lyse and contain pathway for PHA degradation (Reddy et al., 2003). Consequently, the process to recovers PHA turns out to be difficult. It is desirable to eliminate such limitations in order to enhance the production rates of PHA. It is crucial to develop strains that capable to achieve high final cell density in a short period of time and produce high PHA yield from simple, cheap and renewable substrate. Recombinant bacteria strains have been developed through results of extensive studies on genetic engineering.

The main objective for development of recombinant bacteria is to produce PHA from cheap renewable resources efficiently. *Escherichia Coli* are unable to synthesis or degrade PHA. However, *E. coli* grows rapidly, even at high temperature and it does lyse easily. Owing these advantages, *E. coli* are often become a prominent choice to be genetically modified. Fast growing cell will enables it to accumulate more amount polymer in short period of time. Moreover, the PHA granules recovery cost will be much cheaper due to easy cell lysis of *E. coli*. Genetically developed recombinant *E.*