CHAPTER II
LITERATURE REVIEWS

This chapter describes information and previous works involved with definition of biopolymer especially in a group of polyhydroxyalkanoates, biosynthesis of PHAs, PHAs accumulating bacteria, isolation and screening of PHAs bacteria, raw material for biopolymer production, fermentation and parameters affecting on the process, statistical methodology for process optimization, advantage properties of PHAs and finally, applications of PHAs.

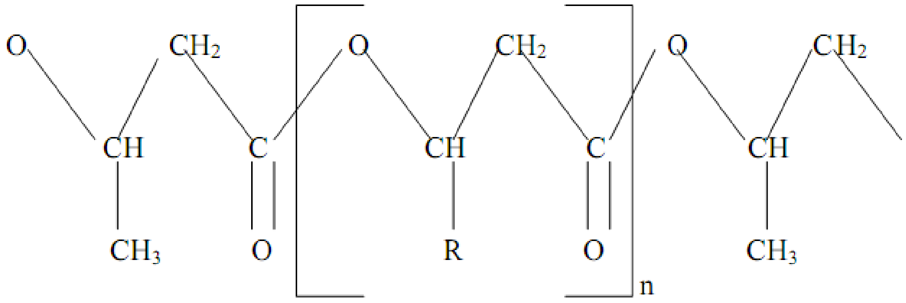
2.1 Biopolymer

Polymer is defined as molecules consist of a long, repeating chain of smaller units called as monomer. A biopolymer is a kind of organic polymer. Many biopolymers found in natural know as carbohydrate in form of starch which made up sugar monomers or protein and peptide biopolymers that consist of amino acid constituents.

American Society for Testing and Materials (ASTM D6400-99) has given the definition of biopolymer or biodegradable polymer is a polymer extracted from plant, animal, bacterial or fungi and including to synthesized monomer by bioprocesses. Similarly, in term of biodegradable plastic is a degradable plastic in which degradation results from the action of naturally occurring microorganisms such as bacteria, yeast, fungi or algae. The International Standard Organization (ISO 472, 1988) has also given the definition of biodegradable plastics that are plastic designed to undergo significant change in its chemical structure under specific environment conditions resulting in a loss of some properties that may vary as measure by standard test methods appropriate to the plastics and application in period of time that determines its classification. The change in chemical structure results in the action of naturally occurring microorganisms (Leejarkpai, 2006).

2.2 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates are biodegradable thermoplastics produced by many bacteria as intracellular carbon and energy reserves under balanced growth condition such as nutrient limitation (Wang, Inoue, 2001; Wang, Yu, 2000). Various microorganisms are produced in different properties of biopolymer. It depends on types of microorganisms and carbon sources used. Typically, most of PHAs known as polymers 3-hydroxy acids. The general formula as shown in Figure 2.1. The biopolymer in form of polyhydroxybutyrate (PHB) is mostly found in the microorganisms and is a main member of PHAs. However, the majority of the published research on PHAs rather than PHB has concentrated on two bacterial strains as *Alcaligenes eutrophus* and *Pseudomonas oleovorans* (Anderson, Dawes, 1990). The PHAs structure compost of a monomer of hydroxyalkanoic acid (HA) and is also used to classify a group of polymer following to the chain length. Their structure contains of (R)-3HA monomer units that are all in the R configuration due to the sterospecificity of polymerizing enzyme PHAs synthase.



n varies from 600 to 35000

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)

Figure 2. 1 PHAs structure, where R is the n-alkyl group of different chain length with or without the side group and length chain depends on amount of monomer unit (n) (Lee, 1996)

Typically, PHAs inclusions locates in the cell cytoplasm in a white form, with 0.2±0.5 µm in diameter and may be visualized quite clearly with a phase contrast light microscope due to their high refractivity (Dawes, Senior, 1973). There are 2 techniques mostly used to characterize the PHAs. Scanning electron microscope (SEM) is scanned the morphology and structure of microorganisms and transmission electron microscope (TEM) is used to monitor the PHAs accumulated in their cells. In Figure 2.2 shows the inclusion PHAs-containing bacteria observed by SEM and TEM.

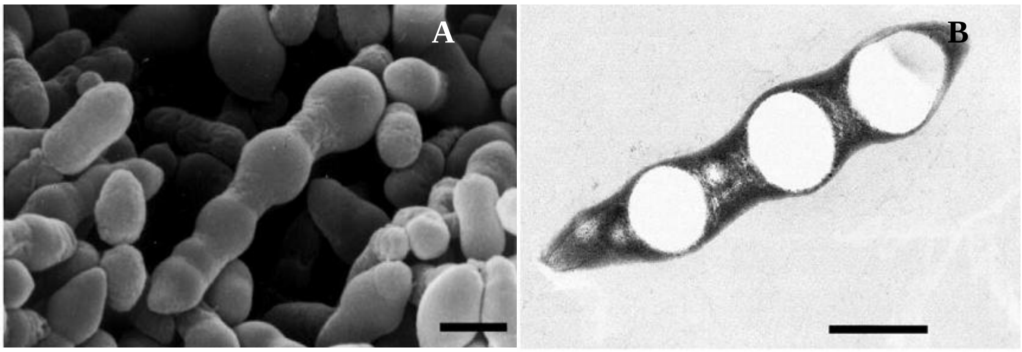


Figure 2.2 *Pseudomonas putida* U ΔfadBA β-oxidation mutant under two different electron microscopes (A) scanning electron microphotograph (SEM) and (B) transmission electron microphotograph (TEM) (Luengo et al., 2003)

Native PHAs inclusions can be stained by Sudan black B dye indicating that they are of a lipid nature (Burdon, 1946; Williamson, Wilkinson, 1958; Kallio, Harrington, 1960). However, they are not rigorous conditions to take up in this dye. Therefore, oxazine dye of Nile blue A perhaps is more specify to stain for PHAs. It exhibits a strong orange fluoresces at an excitation wavelength of 460 nm (Ostle, Holt, 1982). Recently, Nile Red is a fluorescent oxazone form that can be used to detect PHAs directly in growing bacterial colony (Spiekermann et al., 1999).

Protein is associated with PHAs inclusions. The surface of *in vivo* PHAs inclusions is boundary region where the protein involved in the biosynthesis by PHAs synthase and degradation and/or mobilization by intracellular PHAs depolymerase of PHAs are located (Merrick, Doudoroff, 1964; Griebel et al., 1968; Griebel, Merrick, 1971). Other proteins (phasins) thought be involved in the formation and stabilization of PHAs inclusions have also been identified on the surface (Steinbüchel et al, 1995).

The inclusions consist of a hydrophobic core of amorphous PHAs (Barnard, Sanders, 1989) that surrounded by a phospholipids monolayer membrane consisting of various catabolic and non-catabolic proteins which the catabolic proteins include the PHAs synthase, intracellular PHAs polymerase and non-catabolic proteins include a group of proteins designated as phasins (*PhaP*) (Steinbüchel et al, 1995). Figure 3 shows a model for possible structure of *in vivo* PHAs inclusions.

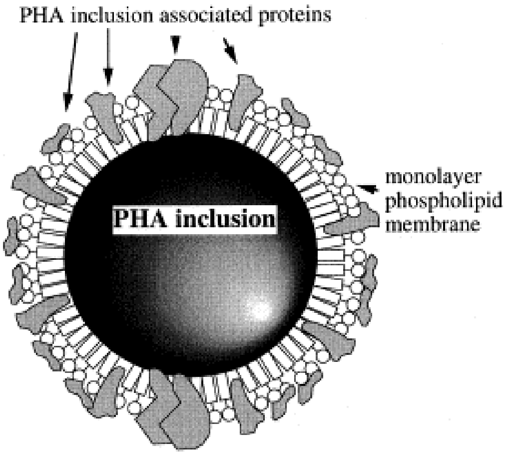


Figure 2.3 The structure model of *in vivo* PHAs inclusions and its association with specific proteins (not drawn according to actual scale) (Sudesh et al., 2000)

2.3 Biosynthesis of PHAs

In nature, different bacteria can produce and accumulate PHAs when they are grown with excess carbon source under nutrient-limiting conditions such as nitrogen, phosphorus, oxygen and high ratio of carbon-to-nitrogen. Many bacteria including gram-negative and gram-positive are able to biosynthesis and accumulation of PHAs in their cytoplasm as carbon and energy sources in form of granules (Keshavarz, Roy, 2010). In previous works state that, the intracellular accumulation of PHAs improves the survival of general bacteria under environmental stress conditions limited in water and soil (Kadouri et al., 2005; Zhao et al., 2007).

The roles of PHAs in bacterial environmental consistency are varied features. For example, the PHAs-producing bacteria of *Bacillus cereus* and *Clostridium botulinum* are accumulated maximally just prior to the formation of spores and degraded during the sporulation process (Valappil et al., 2007). Since, the PHAs may serve as a carbon and energy source or sporulation (Kominek, Halvorson, 1965; Nakata, 1965; Emeruwa, Hawirko 1973). Other works also mentioned that PHAs is utilized as a carbon and energy source during encystment in *Azotobacter vinelandii* (Lin, Sadoff, 1968; Sequra et al., 2003) although the mutations in the *phaB* and *phaC* genes in *A. vinelandii* had no effect on encystment or cyst viability under laboratory conditions. However, the metabolism of PHAs under natural conditions does has such effects cannot be ruled out (Sequra et al., 2003).

The metabolic pathway to synthesis PHAs depends on carbon source or substrate that was employed by bacterial. In addition, structure of gene to synthesize PHAs in bacteria was also varied. In Figure 2.4, the metabolic pathway, *PhaC* (PHAs synthase) has been identified as the key enzyme to synthesize PHAs inclusion. In addition, the genetic organization of representative polyester synthase genes encoding the various classes of enzymes found in several kinds of PHAs-producing microbes are shown in Figure 2.5

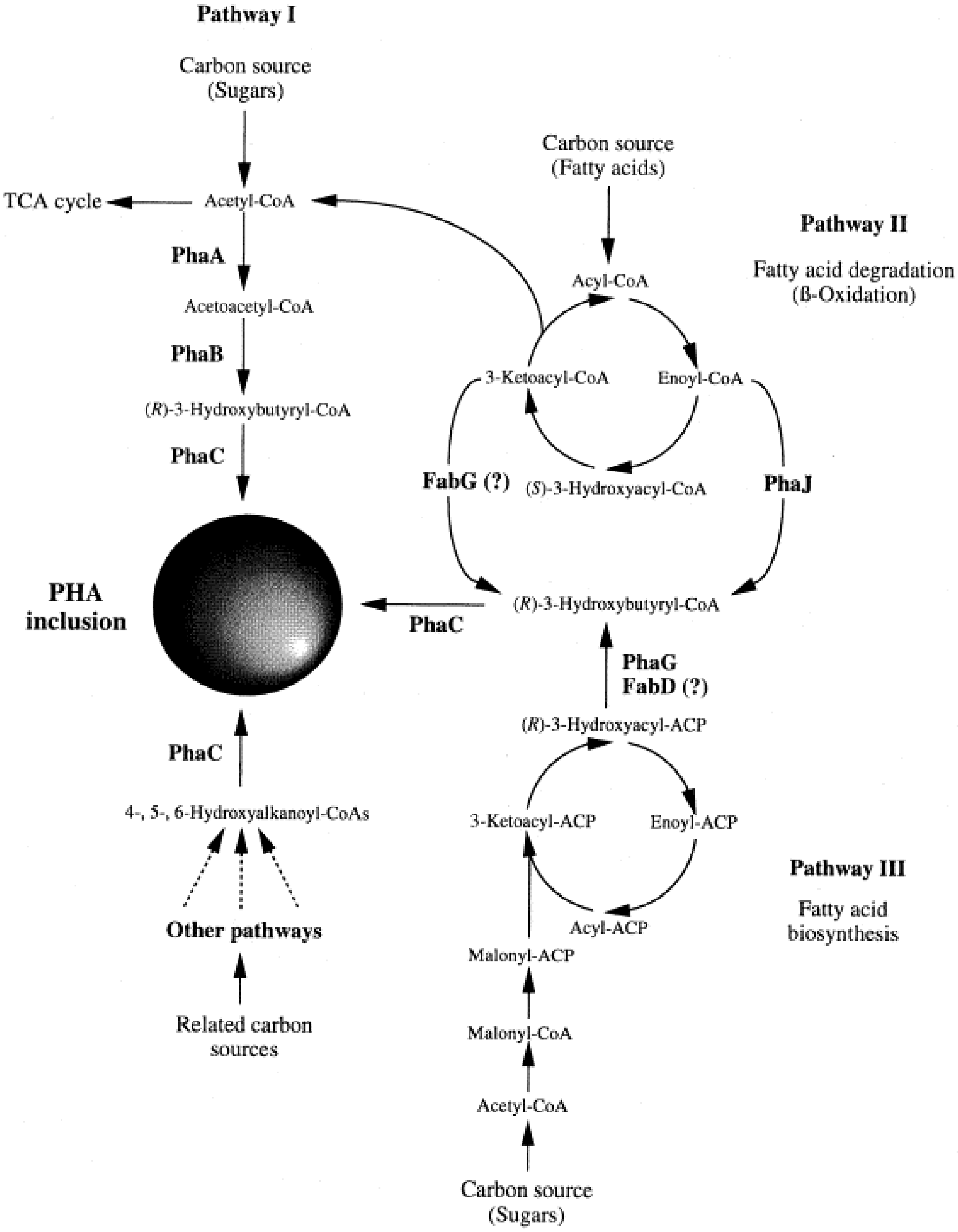


Figure 2.4 Metabolic pathways that supply hydroxyalkanoate monomers for PHA biosynthesis. *PhaA*, β-ketothiolase; *PhaB*, NADPH-dependent acetoacetyl-CoA reductase; *PhaC*, PHAs synthase; *PhaG*, 3-hydroxyacyl-ACP-CoA transferase; *PhaJ*, (*R*)-enoyl-CoA hydratase; *FabD*, malonyl-CoA-ACP transacylase; *FabG*, 3-ketoacyl-CoA reductase (Sudesh et al., 2000)

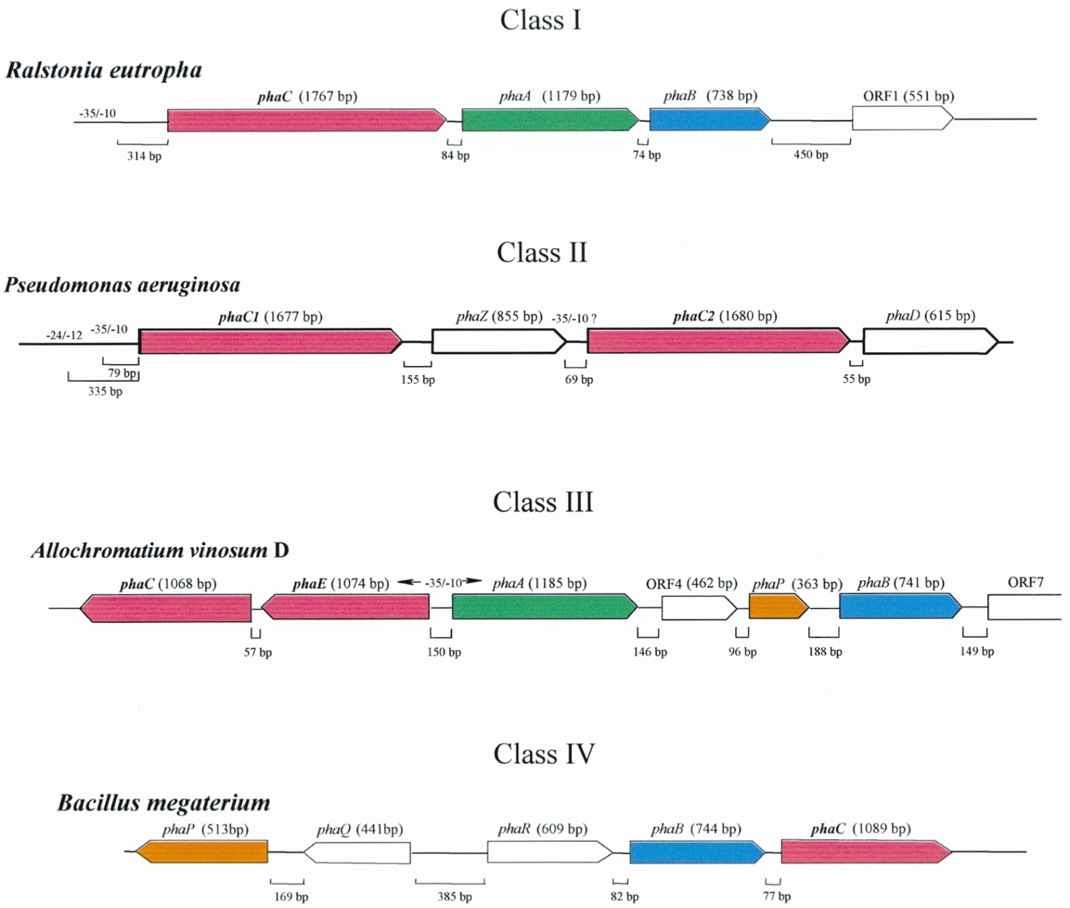


Figure 2.5 Genetic-organization of representative polyester synthase genes encoding the various classes of enzymes. *PhaC/C1/C2*, gene encoding PHAs synthase; *phaE*, gene encoding subunit of PHAs synthase; *pha*A, gene encoding β-ketothiolase; *phaB*, gene encoding acetoacetyl-CoA reductase; *phaR*, putative regulator for phasin protein; *phaZ*, gene encoding PHAs depolymerase; *phaD*, regulator involved in MCL-PHAs synthesis. (modified from Meyer, 1903).

2.4 PHAs accumulating bacteria

Typically, soil is a high diversity of microhabitats in which environmental condition can change rapidly (Postma et al., 1989). Therefore, bacteria in soil need to get acclimatization under varying including biotic and abiotic stress conditions (van Elsas, van Overbeek, 1993). After first discovery, the accumulation of PHAs was found in gram-positive bacteria. In present, there are more than 150 bacteria species mostly are gram-negative that can produce and accumulate PHAs in their cells especially, *Azotobacter* sp., *Alcaligenes* sp., *Azospirillun* sp., *Rhodobacter* sp., *Pseudomonas* sp. and etc.

PHAs is regulated by microorganisms grown in an liquid solution containing of sustainable resource such as glucose, sucrose, starch, fatty acid and even nutrients in wastewater at 30-37°C under the atmosphere pressure (Castro-Sowinsku et al, 2010). The most important reason for studying the feasibility of raw materials for PHAs production is consideration of the suitable bacterial strains. One of gram-negative bacteria, that be most interested by many researchers, is *Alcaligenes latus* because of its capability for utilizing many carbon sources such as glucose, fructose, sucrose and glycerol. Previously, sucrose-rich maple sap was used as a carbon source by *A. latus* (ATCC 29714) for the production of polyhydroxybutyrate (PHB). It was obtained at about 4.4±0.5 gL^{-1} biomass and 77.6±1.5% (dry cell weight) PHB content while when pure sucrose was used as a carbon source biomass and PHB content were reached at 2.9±0.3 gL^{-1} and 74.1+2.0% (dry cell weight) (Yezza et al., 2007). The results obtained really proved that capability of *A. latus* strain to produce PHB in lower cost of substrate. In other study, a gram-positive bacterium of *Bacillus megaterium* was employed to produce PHAs and sugarcane molasses was used as a carbon source. It was obtained at 44.6% (mg cell dry matter) that was almost the same value of 45.6% (mg cell dry matter) PHAs when glucose was used as a substrate (Gouda et al., 2001). Where else, Nikel et al. (2005) investigated to use a recombinant *Escherichia coli* containing of *Azotobacter* plasmid to produce PHB, the components of PHB production medium, optimal concentrations for maximal biomass and PHB production and the agro-industrial by-products such as corn steep liquor and milk whey as main carbon and nitrogen sources were also investigated. The systematic and statistical was applied to the screening and optimization using a Plackett-Burman screening design and response surface methodology. The results showed that three parameters of milk whey, corn steep liquor, and ionic strength were selected with significant at $p<0.05$ and represented the positive effect for PHB production when analyzed by Plackett-Burman screening design. Meanwhile, response surface methodology by central composite design (CCD) was carried out to optimize level of milk whey, corn steep liquor and ionic strength in the fermentation broth. The model for PHB production was significant at $p<0.05$ for regression and the coefficient (R^2) was 0.957, indicating the 4.3% of the variations in PHB concentration are not explained by the model. The validation of the model showed the predicted response and the actual response at 5.89 gL^{-1} and $6.12 \underline{+} 1.16$ gL^{-1} that implied the results of the validation experiments can be proven the applicability of the model.

2.5 Isolation of PHAs–producing bacteria

The important characteristic of PHAs and typical polymeric properties because of this polymer was produced from different renewable carbon sources. The varieties of different monomers can be incorporated into PHAs. Bacterial producers of PHAs have been isolated from various sources lead to the capable to utilize the carbon source. For example, waste stream of several treatments that often provide a mixture of substrates. Several foundation of natural isolates of *Actinobacillus, Azotobacter, Agrobacterium, Rhodobacter* and *Sphaerotilius* have focused on converting organic waste to bacterial PHAs (Madison, Huisman, 1999).

The isolation palm oil-utilising bacteria from oil mill effluent (POME) was attempted to select for palm oil-utilise bacteria for growth and synthesis of PHAs. As designed method, selective medium to isolate bacteria which incubated at 30ºC for 4 week with mineral and 1% (w/v) palm olein as sole carbon source and 2.5% (v/v) POME was added as a source of bacteria. Seven isolates were obtained from selective medium, two isolates could utilize palm olein for growth and production of poly(3-hydroxybutyrate) homopolyester. The higher producer was found in isolated FLP1. It is gram-negative and identified (BIOLOG) with 80% similarity to *Burkholderia cepacia* (Alias, Tan, 2005).

PHAs-producing bacteria belonging to *Bacillus cereus* group were isolated from ammunition-polluted soil. They are capable to produce P(3HB-co-3HV) copolymer from glucose as a carbon source. The molecular weight of PHAs in *B. cereus* YB-4 decreases drastically during 72 hr cultivation period (Mizuno et al., 2010). It provided new insights into PHAs biosynthesis in terms of copolymer composition and molecular weight changes by the members of *B. cereus* group. Although, a number of PHAs isolated from gram-positive bacteria were found in lower than gram-negative. However, lipopolysaccharide (LPS) endotoxin containing in outer membrane of gram-negative usually induces a strong immunogenic reaction in humans. In contrast, although gram-positive bacteria have thick outer membranes but they do not contain the LPS. Therefore, they have more potentially better source of PHAs to use in biomedical applications (Chen, Wu, 2005).

In case of bacterial strains isolated from marine sediment and screened for studying their ability to accumulate PHAs. Previous work has reported that four isolates were identified and closely to *Vibrio* spp. One isolate can accumulate PHAs as high as 41% of cell dry weight and all isolates showed the generation time less than 12 min without optimizing the growth condition. The fast-growing character of *Vibrio* spp. and easy to cultivate can be readily scaled up by fermentation. This characteristic may be an exploitable trait of bacterial strain for PHAs production in the industrial applications (Chien et al., 2007).

2.6 Raw material for PHAs production

The increasing of industrial interest exists in biotechnological production process of PHAs based on renewable resources such as carbohydrates, lipids, alcohols and organic acid. PHAs accumulation is preferred by adequate availability of suitable carbon source and a limiting supply with macronutrients such as nitrogen, phosphate, dissolved oxygen and micronutrients such as magnesium, sulphate, iron potassium, manganese copper, sodium cobalt, tin and calcium (Kim, Lenz, 2001; Helm et al., 2008). The economy of PHAs production is determined to a great extent up to 50% of the entire production costs by the cost of raw materials. The utilization of waste materials upgraded to the role of starting materials for PHAs biosynthesis constitutes a viable strategy for cost-efficient biopolymer production and may be helps industry to overcome disposal problems. The selection of appropriate waste stream as a feedstock mainly depends on the global region where a production plant will be constructed to save transportation costs, facilities for the production. Available wastes streams in different global regions following surplus whey from the dairy industry is available in large quantities in Europe and North America, whereas numerous amounts of non-wood lignocellulosic materials from rice, corn and sugarcane plants are found in many countries worldwide (Chen, 2010).

2.6.1 Nitrogen

Cost factor in typically phosphate-limited production processes for PHAs is cost of complex medium with small amounts of a complex nitrogen source. The major advantage of using complex nitrogen sources is a possible shortening of lag phase (during adaptation) in the early state of fermentation process. The availability of complete amino acids and peptides in complex nitrogen are easily to convert by the cells to synthesize proteinaceous material. Thus, a higher concentration of active biomass is able to accumulate PHAs in a short time.

2.6.2 Waste lipids

Different origins of waste lipids can be applied as substrates for bioprocess of PHAs production. For example, the production of PHAs from tallow, the cheapest fats available by *Pseudomonas resinovorans*. The result was found low amount of PHAs produced approximately 15% DCW because the process was not profitable (Cromwick et al., 1996). Later, the same substrate was used by *Ralstonia eutropha*. It was reported that the strain was successfully concerted to PHAs with relatively high yield (Taniguchi et al., 2003).

2.6.3 Whey and surplus

Whey and surplus materials from dairy industry are not only cheap raw materials but also cause a disposal problem for dairy industry. Because it contains high values of biochemical oxygen demand (BOD) and chemical oxygen demand (COD). The major carbohydrate in whey is lactose that can be served as a carbon source for growth and production of biopolymers such as PHAs and PLA (Kim et al., 1995).

2.6.4 Lignocellulosic wastes

Lignocellulosic material is mainly consisted of lignin, cellulose and hemicellulose. Selection of appropriate production strain for PHAs biosynthesis from lignocelluloses-derived substrates mostly depends on the conversion rates of hexose and pentose by the organisms. If the sugar are used in different rate and not parallel, the production process to be developed will be rather complicated. Then, sugar are not accepted as a substrate by bacterial strains or that are utilized considerably more slowly than other can pile up in the fermentation broth and may cause inhibitory effects that are likely to negatively influence growth and production kinetics and yields. The bacterial strains that use pentose beside hexose is rather limited, thus a multistep hydrolysis might be needed. After that, the different sugars will be separated (Chen, 2010). Previously, Young et al. (1994) studied the production of PHB from xylose by *Pseudomonas cepacia* ATCC 17759 and compared the production among other carbon sources of glucose and lactose. It was found that excellent specific growth rate were achieved at 0.3 Lh^{-1} on glucose and lactose while in case of xylose obtained at 0.1 Lh^{-1}, but rather low specific PHB production rates (less than 0.02 g g^{-1} h^{-1} and product yield less than 0.15 g g^{-1})

2.6.5 Starch

The highlight of starch as a carbon source is lower price than glucose. The processes for PHAs production based on starch require the conversion of starch to easily substrates such as glucose by enzymatic or chemical hydrolysis (Chen et al., 2006; Huang et al., 2006). After hydrolysis, major product of maltose was obtained after cultivation of three different strains of *A. latus* (DSM 1122, 1123 and 1124) in a 10 L fermentor. The results was compared with glucose found that specific rates for growth and product formation were lower using starch hydrolysate but the yields for biomass and PHAs were comparable (Braunegg et al., 1999).

2.6.6 Sugar

A pilot scale by the company PHB Industry in Brazil was started from sugarcane. The company products are saccharose and ethanol. The waste stream from the sugar and bioethanol productions are bagasse and fusel alcohols used for operate the PHAs production and making it economically competitive. The major by-product as bagasse can be hydrolysed and utilized by suitable microbial strains for PHAs synthesis. The successfully for using *Burkholderia sacchari* and *B. cepacia* hydrolysed bagasse as a carbon source for PHAs biosynthesis in a laboratory scale. The excellent results using *B. sacchari* IPT 101 on hydrolysed bagasse was obtained 62% PHAs per dry cell mass and reached at 0.39 g g^{-1} yield. These results were considerably better than yield obtained with pure sugars, but lower cell densities were achieved on hydrolysated bagasse (Silva et al., 2007). Molasses is a common waste material from the sugar industry. It contains of sucrose as the major carbohydrate beside other sugars and additional growth promoters such as vitamins and biotin. Utilization of molasses with the addition of the complex substrate corn-steep liquor by *Azotobacter beijerinckii* was produced about 3.7 gL^{-1} PHB after 24 hr of cultivation. In addition, the study encompassed detailed investigations of the beneficial effect of minor compounds such as metals contained in untreated molasses on microbial growth and production of PHAs (Purushothaman et al., 2001).

Sugarcane is an ancient crop with a complex genetic history. In 20th century, world-wide sugar industrial production from sugarcane (Hogarth, 1987). Scientific classification name of sugarcane is *Saccharum officinarum* L. It is a high biomass tropical crop and contains approximately 12-17% total sugar which is consisted of 90% is sucrose (Wheals et al., 1999). While, extracted sugarcane juice contains of approximately 14% (w/v) sucrose, 2% (w/v) glucose and 1% (w/v) fructose (Uppal et al., 2006). However, sugar concentration found in sugarcane juice depends on the variety, maturity and the time of harvest. Normally, it contains of sufficient organic nutrients and minerals for fermented sugars (Moreira, 2000). Thus, it could be considered as a substrate for production of biopolymer.

In Table 2.1 shows characteristics and composition of the sugarcane juice. Water is the main part (73-76%) of the juice while the remaining is the solid phase that is divided by into a soluble solid (10-16%) and a part of fiber (11-16%). However, if consider in a soluble solid. It is mainly composted of total sugar (up to 92%). For others, contain in forms of the soluble solid such as salts, organic acid, non sugars (protein, starch, gums, wax and fats) and others.

Table 2.1 Characteristics and composition of sugarcane juice

Characteristic	Percentage (%)
Water	73 – 76
Solid	24 – 27
soluble solid	10 – 16
Fiber	11 – 16
Sugarcane juice properties	Percentage of soluble solid (%)
Total sugar	75 – 92
sucrose	70 – 88
glucose	2 – 4
fructose	2 – 4
Salts	3.0 – 4.5
inorganic acid salt	1.5 – 4.5
organic acid salt	1.0 – 3.0
Organic acid	1.5 – 5.5
carboxylic acids	1.1 – 3.0
amino acid	0.5 – 2.5
other organic non sugars	
protein	0.5 – 0.6
starch	0.001 – 0.050
gums	0.30 – 0.60
wax and fats	0.05 – 0.15
Others	3.0 – 5.0

(modified from James, 1985)

Table 2.2 Summary of PHAs production by various carbon sources and bacteria

Microorganism	Carbon source	Time (hr)	Culture Condition	PHAs gL^{-1}	PHAs content[a] (%)	PHAs $g L^{-1} h^{-1}$	Reference
Ralstonia eutropha	Glucose	74	Fed-batch	232	82.0	3.14	Ryu et al., 1997
Ralstonia eutropha	Tropica hydrolysate	59	Fed-batch	60.77	58.0	1.03	Kim, Chang, 1995
Alcaligenes latus	Sucrose	20	Fed-batch	98.7	88.0	4.94	Wang, Lee, 1997
Alcaligenes latus DSM 1124	Malt waste	69	Fed-batch	22.632	70.1	0.328	Yu et al., 1999
Azotobacter chroococcum	Starch	58	Batch	0.8642	73.9	0.0149	Kim, 2000
Azotobacter vinelandii UWD	Molasses	36	Fed-batch	21.96	66.0	0.610	Page, Cornish, 1993

[a] PHAs content (%) dry cell weight

Table 2.2 Summary of PHAs production by various carbon sources and bacteria (Cont.)

Microorganism	Carbon source	Time (hr)	Culture Condition	PHAs gL^{-1}	PHAs content[a] (%)	PHAs $g\ L^{-1}\ h^{-1}$	Reference
Pseudomonas putida	Oleic acid	34	Fed-batch	60.7	36.05	1.79	Lee et al., 1999
Brevundimonas vesicularis	Acid-hydrolysated sawdust	120	Batch (flask)	162	64.0	1.35	Silva et al., 2007
Sphingopyxis macrogoltabida			Batch (flask)	231	72.0	1.925	
Bacillus megaterium CCM 2037	Whey	50	Batch (flask)	1.5	51.0	0.03	Obruca et al., 2011
Methylobacterium organophilum	Methanol	70	Fed-batch	130	52.0	1.86	Kim et al., 1996

[a] PHAs content (%) dry cell weight

2.7 Fermentation process

Bacteria are used for PHAs production can be classified into two groups based on the culture conditions required. First group is the bacteria require the limitation the essential nutrient for synthesis PHAs from excess carbon source. The bacteria in this group are *Alcaligenes eutrophus, Protomonas extorquens* and *P. oleovorans*. The second group includes *Alcaligenes latus,* a mutant strain of *Azotobacter vinelandii* and recombinant *Escherichia coli,* do not require nutrient limitation for PHAs synthesis and accumulate PHAs during growth. These characteristics have been to consider production process of PHAs (Khanna, Srivastrava, 2005). Typically, batch fermentations are kinetically limited with the exhaustion of essential nutrient (Zinn et al., 2004). The fed-batch or continuous fermentation process are obtained high productivity of PHAs. For fed-batch fermentation of bacteria belonging to non-nutrient limited, a nutrient feeding strategy is important to obtain a high yield of PHAs. Cell growth and PHAs accumulation require to be balanced to avoid incomplete accumulation of PHAs or premature termination of fermentation at low cell concentration (Khanna, Srivastrava, 2005). Continuous fermentation has been optimized for PHAs production and shown the PHAs content decreased with a higher specific growth (Durner et al., 2000). A clear advantage of chemostat cultivations is the tight control of the cell physiological functions by selected nutrient limitations (Egli, Zinn, 2003).

2.8 Parameters affecting on fermentation process

2.8.1 Carbon source

This is importance factor in production process especially the effect for microbial producing-biopolymer utilized and used as a carbon source. Previous study, the production of PHAs by *Bacillus megaterium* using different carbon sources such as fructose, glucose, lactose, xylose, sucrose, maltose, Na-gluconate and sugarcane molasse. Best production of PHAs found when glucose was used and 45.6% mg dry cell weight was obtained while 7.40% mg dry cell weight PHAs was obtained in case of xylose (Gouda et al., 2001).

2.8.2 Microorganism

It should be stated that different microbial strains have a capability to use carbon source for producing PHAs in various contents. For example, four *Vibrio* spp. as strains of M11, M14, M20 and M31 isolated from marine mangrove were found that they can utilize a variety of different carbons for synthesis of PHAs (in form PHB). Moreover, it also was found that they grew well on glycerol. Strains M11, M14, M20 and M31 can utilize a variety of different carbon sources for the synthesis of PHB. All strains tested grew well on glycerol and produced fairly large amount of PHB (content 24–43%). Strain M11 accumulated PHB in concentrations as high as 42.8% of cell dry weight when grown in glycerol as a carbon source while strain M31 was accumulated as 24.0% of cell dry weight (Chien et al., 2007).

2.8.3 Nitrogen source

The production of PHAs by *Bacillus megaterium* in organic nitrogen and inorganic nitrogen source, the greater PHAs production 40.10% mg dry cell weight was found in inorganic nitrogen source as ammonium chloride while organic nitrogen source as corn steep liquor, malt extract was obtained poor PHAs yield (Gouda et al., 2001). The best nitrogen source for *Vibrio* spp. strain MK4 was found in ammonium sulphate as 4.01 gL^{-1} PHB while 0.36 gL^{-1} PHB was obtained in protease peptone as a nitrogen source (Arun et al., 2009).

2.8.4 Acid/alkaline

Acid and alkaline condition mostly affect for microbial growth. The optimal condition of pH is neutral (pH 7.0). The increasing or decreasing of acid and base may reduce the production of PHAs (Arun et al., 2009).

2.8.5 Cultivation time

PHAs is a granule that synthesize for the energy source so long time of cultivation may be utilize or degrade by bacterial cell while short cultivation time may be harvested the cell but not yet accumulation of PHAs. Thus, the stage or cultivation time is depends on microbial growth profile.

2.8.6 Others

Other factors such as nutrient limitation, mineral or element, production process, temperature, aeration rate and/or agitation rate are affected on the PHAs production.

2.9 Statistical methodology for optimization condition

Optimization is the best alternative of the possibility to achieve the best case among the possible alternatives (Evans, 1982). The formal description of any optimization problem has three parts as following:

a) Set of variable, the optimization method can be controlled and used specify alternatives.
b) Set of requirements, the optimization method must achieve or satisfy.
c) Measurement of performance, to compare one alternative to another (Norback, 1980).

Response surface methodology (RSM) is a system or process can be described by the relationship between input and output as shown in Figure 2.6. It is a tool to understand the quantitative relationship between multiple input variable and output response or extendable to multiple responses and often with an emphasis in response optimization (Myers, Montgometry, 1995). The RSM consists of a collection of mathematical and statistical procedures including design of experiments (DOE), model selection and fitting, and optimization on the fitted model. Typical ways to apply RSM, one is sequential mode and the other a direct approach and a simpler model such as first order model can be used to move efficiently toward the vicinity of the optimum. Once the general region of the optimum is identified, a second- or higher-order response surface model is constructed by experimentation on the design points of a DOE of higher levels with experiment region set equal to those bounded by the process variables' upper and lower limits (Erdogdu, 2009).

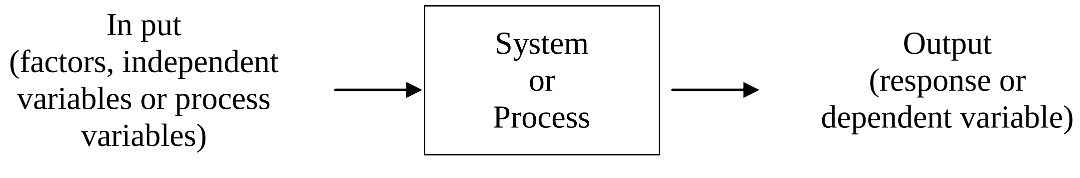


Figure 2.6 Schematic diagram for a system (Erdogdu, 2009)

The RSM is successful application by design of experiments such as two-level factorial design, central composite design (CCD) and Box-Behnken. The Analysis of variance (ANOVA) was examined the adequacy of the regression models. The procedure to implement/apply RSM comprises of

1) Performing screening experiment and experimental design
2) Manufacturing functional cream according to the experimental design
3) Building the RSM
4) Performing optimization
5) Verifying the optimal manufacturing conditions

In Figure 2.7 shows flow chart of applying RSM in step as

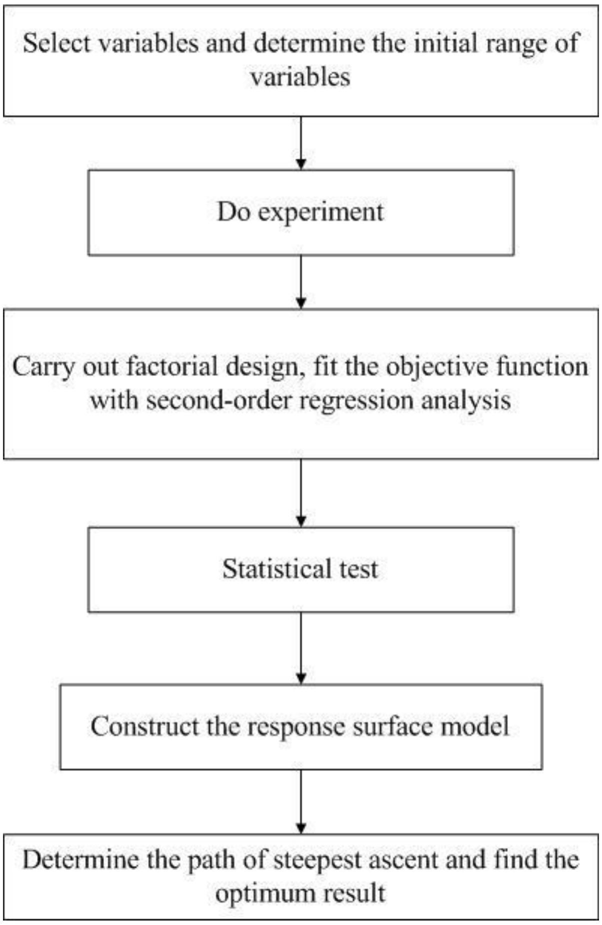
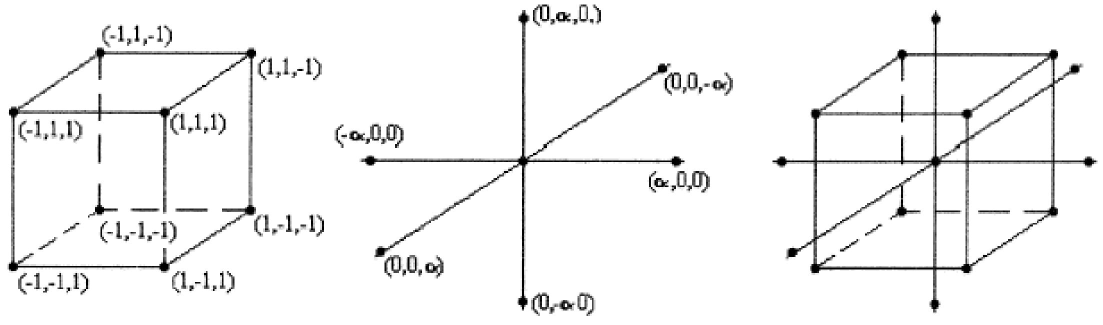


Figure 2.7 Flow chart of steps for response surface methodology (RSM) (Olabi et al., 2007)

Firstly, screening designs are used for observing the experimental region when little data is known about the target. The main effect information for each factor is derived, but cannot explain the interactions (Altekar et al., 2006).

Plackett and Burman design has been used for screening process variables that makes the greatest impact on the process. The results of the experimental design should not be used to draw conclusions about the interaction of the parameters in the system. Although, in a comparison made of several such designs for their potential use as statistical tools for processing experimental data (Williams, 1963). It was found to be the best for choosing the important variables. The Plackett and Burman design being a fractional factorial design allows efficient analysis of (N−1) variables in N experiments. However, it can only be applied efficiently for the process where the interactions between the variables are small when compared with their impact on the process being studied (Stowe, Mayer, 1966).

The central composite design (CCD) is a interest design of the second-order designs. It involves the use of two-level factorial points (2^k) axial points (2k) and multiple center points (n_c) for a total of $2^k + 2k + n_c$ design points with k is the number of input factors which show in Figure 2.8.



1) Factorial Design

2) Axial Points

3) Complete Design

Figure 2.8 Central composite experimental design for three variables (Williams et al., 1999)

All axial points have a distance of α from the center point and all coded of variables are zeros. The CCD can be made rotatable by setting the axial point values as $\alpha = \sqrt{k}$, the design is often referred to as a spherical design (Erdogdu, 2009). The central composite design is given as much information as a multilevel factorial, requires much fewer experiments than a full factorial and has been shown to be sufficient to describe the majority of steady-state process responses (Zhang et al., 2007). The complete quadratic model for k factors is given by the equation as follow.

$$y = \beta_0 + \sum_{i} \beta_i x_i + \sum_{i} \beta_{ii} x_{ii}^2 + \sum_{i<j} \sum_{i} \beta_{ij} x_i x_j$$

Where y was the predicted response, β_0 was a constant, β_i was linear regression coefficient, β_{ii} was the quadratic coefficient, β_{ij} was the interaction coefficient and x_i, x_j were independent variables.

In Figure 2.9 shows the general flowchart used to apply a central composite design in RSM to optimize the production process.

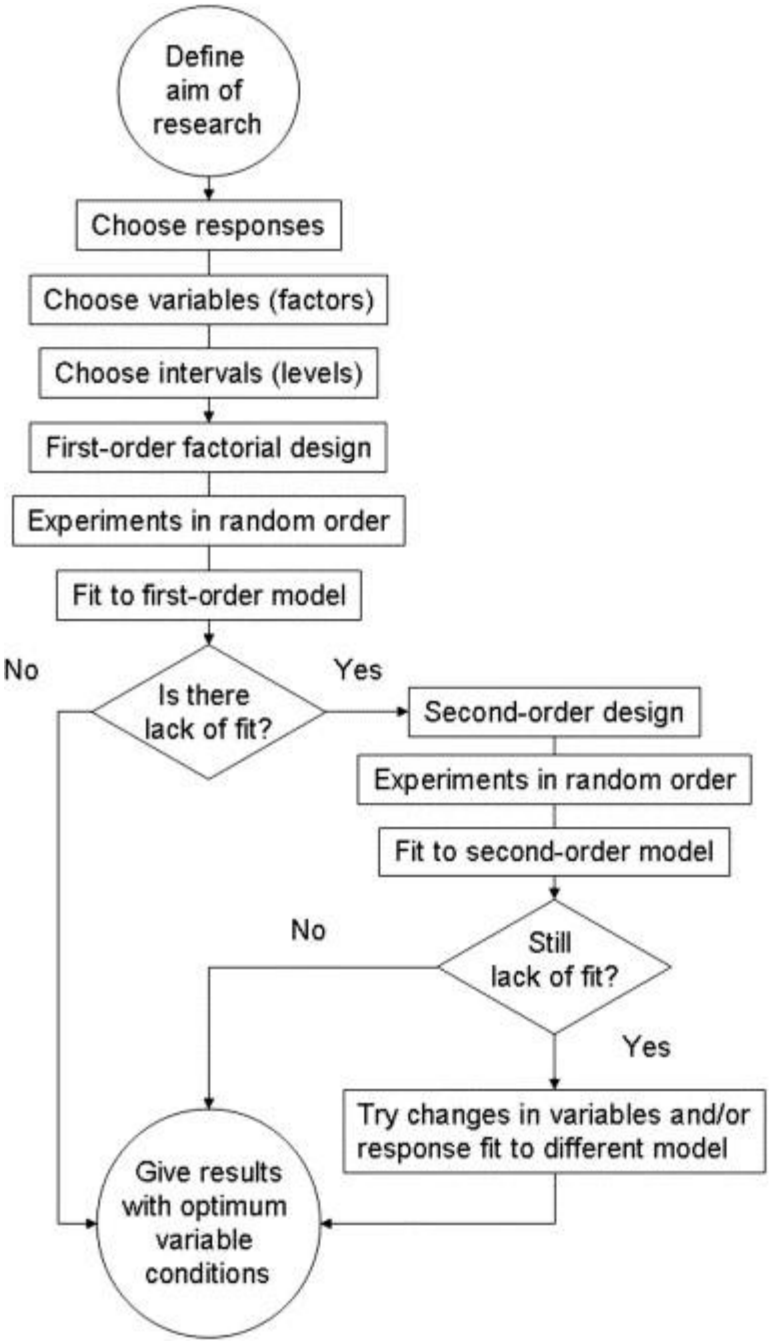


Figure 2.9 Flowchart of the central composite design (Vicente et al., 2007)

2.10 Advantage properties of PHAs

PHAs are completely degraded to water and carbon dioxide as the final products of the oxidation reaction. The advantage properties of PHAs can be concluded as follows. In addition, petroleum derived plastic in form of polypropylene (PP) and biopolymer of PHB has been compared in Table 2.4.

2.10.1 Biodegradability

PHAs have attracted much attention as environmentally compatible materials owing to their unique property of biodegradability. Unlike any other type of biodegradable plastics is biodegrade under not only aerobic but also anaerobic conditions (Federle et al., 2002). It can be degraded in natural environments such as soil, sludge, freshwater and seawater that many microorganisms utilize the degraded products as a carbon source. However, their biodegradability of PHAs do not assist to an increase of the landfill crisis. Hence, it has drawn more attention as a solution to problems concerning the global environment (Chen, 2010).

2.10.2 Bio-based nature

PHAs are produced from several renewable resources. Thus, they are independent of mineral oils or fossil fuels as feedstocks. The renewable resources for the production process such as materials are produced in agriculture and industrial by-product or waste stream such as surplus whey from dairy industry not only rich source of lactose, but also with minerals and protein residues that have good impacts on the microbial cultivation (Purushothman et al., 2001), starch or hydrolyzed starch as a carbon source that its lower price than glucose (Choi, Lee, 1999) and many bacterial strain can utilize starch as a carbon source such as newly isolated *Bacillus cereus* CFR06 was tested for PHAs production from various carbon sources include starch (Halami, 2008).

2.10.3 Carbon dioxide release

Carbon dioxide is released as the final mineralization product of biopolymers originates from the renewable carbon source for their biosynthesis. Furthermore, the photosynthetic fixation of the released carbon dioxide by plants generates renewable carbon sources again. The carbon flux in the synthesis and degradation of biopolymers is balanced. Therefore, PHAs do not contribute to global warming (Koller et al., 2010).

2.10.4 Biocompatibility

Special application especially for medical purposes of PHAs because of their biocompatibility. The ideal of PHAs are underlined by natural occurrence of (R)-3-hydroxybutyric acid (3HB), their low molecular weight oligomers and polymers in human body (Agus et al., 2006; Steinbüchel, Hen, 2001; Steinbüchel, Lütke-Eversloh, 2003; Zinn et al., 2001).

2.10.5 Hydrophobicity

PHAs have a hydrophobic properties restrict their applications as cell colonizing materials. The PHAs surfaces are quite inert and have no physiological activity that play an important role in the interaction between a biomaterial surface and cells (Tesema et al., 2005; Grøndahl et al., 2006).

Table 2.3 Properties of polypropylene (PP) comparisons of polyhydroxybutyrate (PHB)

Properties	Polypropylene (PP)	PHB
Melting point Tm [ºC]	171-186	171-182
Glass Transition Temperature Tg [ºC]	-15	5-10
Crystallinity [%]	65-70	65-80
Density [g cm^{-3}]	0.905-0.94	1.23-1.25
Molecular weight Mw (x10^{-5})	2.2-7	1-8
Molecular weight distribution	5-12	2.2-3
Flexural modulus [GPa]	1.7	3.5-4
Tensile strength [MPa]	39	40
Extension to break [%]	400	6-8
UV resistance	poor	good
Solvent resistance	good	poor
Oxygen permeability [cm^{3}m^{-2}atm^{-1}d^{-1}]	1700	45
Biodegradability	-	good

(modified according to Jogdand, 1999)

2.11 Applications of PHAs

The advantage features of PHAs such as non-toxic, biocompatible, biodegradable thermoplastic from renewable resource, high degree of polymerization, highly crystalline and insoluble in water that make them highly competitive with petrochemical-derived plastic as polypropylene (Reddy et al., 2003). Its has a wide range of applications. Initially, PHAs was used in packing films mainly in bags, containers and paper coatings including to diapers, disposable items, cups and cosmetic containers (Oeding, Schlegel, 1973; Senior, Dawes, 1973). Currently, applications of PHAs-based polymers or composites include packaging industry, medicine, pharmacy, agricultural, food industrial or the paint industrial (Anderson, Dawes, 1990). The details are explained and also shown in Figure 2.10 at the end of the section.

2.11.1 Packaging materials

The application of PHAs was initially used to make everyday articles such as shampoo, food and beverage bottles and also was developed as packaging films mainly for uses as shopping bags, paper coatings. Especially, PHB fibers with a high tensile strength were prepared by stretching the fibers after isothermal crystallization near the glass-transition temperature (Tanaka et al, 2007).

2.11.2 Biomedical implant materials

PHAs and their composites have been used to develop devices including sutures, rivers, tracks, staples, screws, bone plates and bone plating systems, slings, adhesion barriers (Dai et al., 2009). Tendon repair devices, bone marrow scaffolds, spin fusion cages, skin substitutes, bone graft substitutes, wound dressings and hemostats (Chen, Wu, 2005). The changing compositions of PHAs also allow suitable mechanical properties, biocompatibility and degradation times within desirable time frames under specific physiological conditions (Abe et al., 1995; Chen, Wu, 2005). Several PHAs following as PHB, Poly(hydroxybutyrate-co-valerate) (PHBV), poly(4-hydroxybutyrate) (P4HB), poly(hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx) and polyhydroxyoctanoate (PHO) are suitable in adequate quantities for application studies (Hrabak 1992; Byrom 1992; Chen et al. 2001).

Figure 2.10 Applications of polyhydroxyalkanoates (PHAs) in various fields (Chen, 2009)