THERMAL AND BIODEGRADATION PROPERTIES OF BIOPOLYMER PRODUCED FROM PAPER MILL WASTEWATER

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THERMAL AND BIODEGRADATION PROPERTIES OF BIOPOLYMER PRODUCED FROM PAPER MILL WASTEWATER

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A project report submitted in partial fulfilment of the requirements for the award of Bachelor of Engineering (Hons.) Petrochemical Engineering

Faculty of Engineering and Science Universiti Tunku Abdul Rahman

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DECLARATION

I hereby declare that this project report is based on my original work except for citations and quotations which have been duly acknowledged. I also declare that it has not been previously and concurrently submitted for any other degree or award at UTAR or other institutions.

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APPROVAL FOR SUBMISSION

I certify that this project report entitled **"THERMAL AND BIODEGRADATION PROPERTIES OF BIOPOLYMER PRODUCED FROM PAPER MILL WASTEWATER"** was prepared by HENG SU CIN has met the required standard for submission in partial fulfilment of the requirements for the award of Bachelor of Engineering (Hons.) Petrochemical Engineering at Universiti Tunku Abdul Rahman.

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ABSTRACT

Polyhydroxyalkanoate (PHA) is known as a type of biopolyester, which stored within the cells as energy storage materials by various microorganisms. The characterization of polyhydroxyalkanoate produced from papermill wastewater was investigated in this study. The papermill wastewater was treated by activated sludge process and PHA was formed inside the aerobic granules. Subsequently, the chemical structure and thermal properties of PHA synthesized was analysed. The PHA synthesized was proven to be P3(HB-co-HV) which consists of 3-hydroxybutyrate and 3-hydroxyvalerate monomers. Besides, its melting point was determined as 101.57°C and it lost about 30% of its initial weight throughout 8 weeks in biodegradation test. The tested samples exhibited similar melting point and weight loss. Overall, the PHA produced contained good processability and potential to substitute petroleum-based plastics. In this study, different activated sludge weights and aeration rates showed varying results in COD removal efficiency. The COD removal efficiency was increased from 51.91% until 76.31% when the activated sludge weight was increased from 200g to 400g. On the other hand, COD removal efficiency was promoted from 70.69% to 78.91% by increasing aeration rate from 1L/min to 3L/min. Hence, further study was carried out to investigate the effect of aeration rate of activated sludge weight on PHA yield produced from papermill wastewater. The PHA yield was increased from 19.17mg/g to 32.40mg/g as the activated sludge weight was increased from 200g to 400g. Conversely, the PHA yield were 26.40mg/g, 32.40g/mg and 28.90mg/g when the aeration rate was set as 1L/min, 2L/min and 3L/min respectively. Aeration rate showed a positive effect on the COD removal efficiency and MLSS of the sample, which is important in a wastewater treatment system.

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LIST OF SYMBOLS / ABBREVIATIONS

PHA	polyhydroxyalkanoate
PLA	polylactic acid
PBS	polybutylene succinate
PP	polypropylene
LDPE	low-density polyethylene
PHB	poly-3-hydroxybutyrate
PHBV	poly(hydroxybutyrate-*co*-hydroxyvalerate)
PHBHHx	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
HV	hydroxyvalerate
PET	polyethylene terephthalate
cP3HB	complexed PHB
SBR	sequencing batch reactor
COD	chemical oxygen demand
MLSS	mixed liquor suspended solids
FTIR	fourier transform infrared spectroscopy
KBr	potassium bromide
DSC	differential scanning calorimetry
DO	dissolved oxygen

CHAPTER 1

INTRODUCTION

1.1 Background

Recently, one of the major global concern that the world encountered is pollution, which including the contamination of air, land and water by various chemicals such as poisonous gases, waste materials and insecticides. Pollution has affected the balance of nature, destroyed many forms of wildlife and caused a variety of illness. Among the wastes that released to the environment uncontrollably, plastic is the major component that polluting the environment severely. According to HelpSaveNature (2018), plastic pollution is defined as the build-up of the non-biodegradable plastics in nature, as well as in water bodies such as rivers, oceans, canals and lakes (HelpSaveNature, 2018).

Plastic becomes the basic material that manufacturers used to produce everything, yet it also turns into the most harmful of trash dumped into the sea due to its properties of cannot be broken down naturally. Instead, it will remain in the seabed for over hundred years without decaying. The environmental impact that brought by the plastic is becoming worse when some of the marine animals like sea turtle and whale consume the plastic bag due to its appearance similarity to jelly fish. This will lead to the chocking and blockage of intestines of the marine animals and cause the infection in those animals.

Based on National Geographic (2018), a pilot whale was spotted in a southern Thailand canal which near the Malaysia border on 28th of May 2018. The pilot whale was found struggling, unable to swim or breathe. The veterinarians were tried to rescue

it and also provided the proper treatment to it, yet the whale threw up plenty of plastic packaging and died after few days. A necropsy unveiled that dozens of plastic bags clogging the whale's stomach which weighing 7.7kg in total. The waste that found in the whale was in the accumulation of 80 shopping bags and other plastic materials, which inhibiting it from ingesting nutritional food. This case is emblematic of a larger problem with plastic polluting the oceans.

As the human being enter the new millennium, the challenge for humankind is to transform existing economy into one that does not threaten or destroy the environment. Hence, the development of biopolymer is a feasible way to reduce the usage of non-degradable polymers.

1.2 Biopolymer

Biopolymer is the natural polymer that synthesized by living organisms. It is composed of many monomers, which is similar to normal synthetic polymer. However, comparing to synthetic polymer, biopolymer contains a higher structure complexity which could be developed out of primary, secondary and tertiary stages (Jogdand, 2007). On the other hand, the structure of synthetic polymer is less complex and more stochastic. There are lots of examples of biopolymer could be found within the human body, such as carbohydrates, lipids, proteins and nucleic acids, which are not derived from petroleum oil as synthetic petroleum-based polymer did.

Recently, the trend of application of biopolymer is increasing due to their biodegrading and other unique properties. The companies, such as Ecovative Design, Braskem, Mango Materials and Chinova Bioworks, have put more effort on research in the field of biomaterial to contribute towards the go green activity. This is due to the non-degradable plastics will harm the environment due to unorganized and improper disposal. Hence, the biodegradable biopolymer is more suitable to be used in manufacturing single-use products, such as straws, single-use utensils, packaging materials and others.

There are many types of biopolymer could be applied in industrial field, such as polylactide (PLA), polyhydroxyalkanoate (PHA) and polyglycolide (PGA). They could be used in building products, plastics, moulding materials and others applications. These biopolymers could be synthesized via fermentation of bacteria and extraction from genetically modified plants and biomass (Vroman and Tighzert, 2009). They share the similar properties to traditional petroleum-based polymers, which are versatile to wide range of mechanical, physical and chemical properties, chemical resistance and easy to fabricate.

The increasement on usage of biopolymers could reduce the dependence of fossil fuel. Fossil fuel is used in large scale as plastic materials due to the high demand in manufacturing commercial product. It is a non-renewable and limited resource, yet the consume rate of fossil fuel is far higher than the rate that they can be restored by natural cycles. Hence, biopolymers are becoming competitive when the fossil fuel availability becomes lower and price becomes higher than biopolymers' feedstocks, such as corn and bacteria.

1.3 Thermal Properties

Thermal properties are very important in defining a material's performance when the material was exposed to a heat source. The heat energy supplied will affect the potential energy of the elemental atoms of a sample and hence influence its properties. Most of the properties will be manipulated by the temperature, such as mechanical hardness, magnetic and electrical properties (Buck and Rudtsch, 2011). So, the thermal properties are related to the physical properties of a matter related to the application of heat energy. The essential thermal properties include heat capacity, thermal conductivity, thermal expansion and thermal stress.

Heat capacity of a sample is known as the amount of heat needed to produce a unit temperature rise. The temperature can be used to measure the potential energy, which is converted from heat energy, stored in a sample. Hence, heat capacity is a property that is indicative of a materials ability to absorb heat from the external

surroundings (Kailas, 2009). For thermal expansion, it is defined as the expansion or contraction of a material in length, shape or volume due to the variation of temperature.

On the other hand, thermal conductivity is known as the rate of heat transfer through a unit of thickness of material from high temperature region to low temperature region per unit area per temperature difference (Bahrami, 2011). It can be used to test a material whether is a good or poor heat conductor. Last but not least, thermal stress is the stress encountered by a material owing to the expansion or contraction which caused by the variation of temperature.

These thermal properties could further understand the glass transition temperature, melting temperature and thermo-degradation temperature of a material, which are usually tested and measured to identify the optimum temperature which a polymer could be manufactured and applied.

1.4 Biodegradation

Biodegradation is defined as the decomposition of materials via microorganisms into simpler chemical structures. The organic substance is broken down into smaller compounds by enzymes that synthesized by bacteria, fungi or yeast, finally introducing the molecules into the environment.

Biodegradation acts as the most important mechanism in removing the chemicals from the environment. It is a nature's waste management and recycling system which could disintegrate all organic matters into crude oil and basic chemical compounds, and hence ensuring the cleanliness of Earth (Bio-Tec Environmental, 2013). Thus, it is very important that the polymers have the biodegradation properties to assure the elimination of the waste. Biodegradable polymers are identified as polymers that composed of monomers connected to one another through functional groups and have unstable links in the backbone.

The mechanisms for polymer biodegradations are enzymatic degradation and hydrolysis, which also known as bioerosion (Malla Reddy College of Pharmacy, 2014). The bioerosion is classified into two types, which are bulk erosion and surface erosion. For bulk erosion, the hydrolysis takes place throughout the whole body of the polymer. On the other hand, surface erosion is the erosion that takes place only at the outer surface of polymer, and the inner part of the polymer remains unchanged. Furthermore, enzymatic degradation is known as the degradation that mediated by water, enzymes and microorganisms, and eventually decomposed into simpler substances.

1.5 Problem Statement

Growth of petroleum based plastic production and consumption have created severe negative impacts on the environment and society. The plastic's durability and resistance to decomposition will cause the plastic hard to be broken down by microorganisms. This will lead to the destruction of natural environment as a consequence of improper disposal of non-degradable plastics. Hence, the research in chemical alternatives is important in reducing non-renewable energy consumption and petroleum-based plastic demand.

Biopolymers have been studied extensively with the intention of substituting the petroleum-based polymers and hence reducing the petroleum-based polymers consumption. Polyhydroxyalkanoates (PHA), polylactic acid (PLA) and polybutylene succinate (PBS) are three types of well-known biopolymers for their superior properties (Chen, 2010). These biopolymers can be synthesized via bacterial transformation and they emerge as future green polymers. This is due to they are expected to have the similar thermal and mechanical properties with conventional plastics, such as polypropylene (PP) and low-density polyethylene (LDPE) (Kourmentza, 2017). Biopolymer could be produced through several ways. Some biopolymers are obtained from fermentative processes by using natural raw ingredients, such as rice, corn, sugar, wheat and potatoes. However, they are costly and require ample consumption per unit of biopolymer produced. Instead of using the costly materials, recent development points to the production of biopolymer from less

valuable feedstock, such as food industry and agricultural wastes, especially PHAs. PHA can be produced from the papermill wastewater and it is a type of renewable, biodegradable, and bio-based polymers, in the form of polyesters. However, the characterization of PHA is still incomplete.

PHA have the most diversity in terms of structure, resulting in the most inconsistent thermal properties, which including melting temperature, glass-transition temperature and thermo-degradation temperature. Therefore, the aim of the present study is to review the thermal and biodegradation properties of PHA which produced from papermill wastewater.

1.6 Objectives

This thesis is to study and investigate the characterization and properties of PHA that produced from papermill wastewater. The objectives of this research include:

i. To synthesize polyhydroxyalkanoate using activated sludge and paper mill wastewater.
ii. To quantify the weight, thermal properties and biodegradability of PHA produced from papermill wastewater.
iii. To study the effect of aeration rate and activated sludge content on PHA yield.

CHAPTER 2

LITERATURE REVIEW

2.1 Structure of Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHA) are classified as optically active natural polyesters which constituted of (R)-3-hydroxyalkanoate ((R)-3HA) monomer units, as shown in Figure 2.1. These PHA were first discovered in 1925 by the French microbiologist Maurice Lemoigne and they are classified as polyesters which are known with their superior renewability, biodegradability, hydrophobicity, biocompatibility, and impermeability to gas.

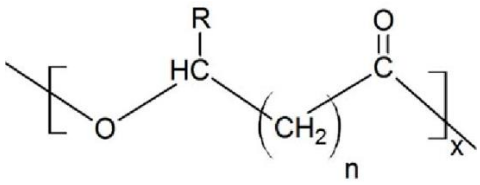


Figure 2.1: Structure of Polyhydroxyalkanoates (PHA) (Lee, 1996a).

Initially, they are produced as intracellular biopolymers that act as the energy storage granules of bacteria, as shown in Figure 2.2. They could be observed under the phase contrast light microscope due to the refractivity of PHA is relatively high. The PHA within the bacteria are visualized as electron-dense substances yet thin sections of PHA-accumulating bacteria are observed through transmission electron microscopy (Sudesh, Abe and Doi, 2000).

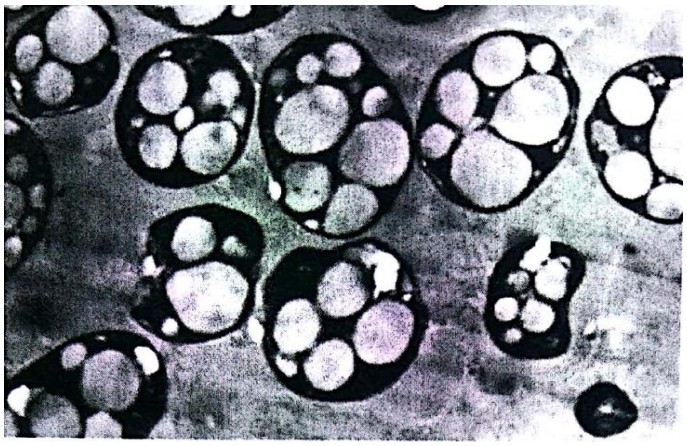


Figure 2.2: Observation of PHA inside Bacterial Cells through Scanning Electron Microscopy (Yu, 2009).

While the carbon is in excess condition and nutrient stress occurred, PHA will be formed in the state of intracellular compound. They are known as an output of carbon assimilation and they will be utilized by bacteria as a form of energy source molecule to be metabolized while the alternative energy sources are absent. The synthesis of PHA could be maximized to 90% of some species' dry mass as polymer under certain specific fermentation conditions.

In addition, PHA have similar properties to synthetic plastics. Due to their biodegradability, they are attractive as potential alternatives for nondegradable petroleum-based polymers. Apart from their biodegradability, they can also be used as biomaterials for implant purposes owing to their biocompatibility (Robertson, 2012). Besides, due to their water-insolubility and hydrolytic degradation resistant, PHA are superior to other trendy obtainable biopolymers that are water-soluble and moisture-sensitive. However, PHA are soluble in chloroform or other chlorinated hydrocarbons. They also manifest good oxygen permeability and ultraviolet resistance, but poor acids and alkali resistance (Kumbar, Laurencin and Deng, 2014). PHA also possess good settleability that may facilitate the task of anaerobic biodegradation in sediments.

Among so many types of PHA, the most common type of polyhydroxyalkanoate is in the form of poly-3-hydroxybutyrate (PHB), yet other polymers of this family are synthesized by various of organisms. To improve and enhance the properties and productivity of PHAs as environmental-friendly

biodegradable thermoplastics, many experiments and researches have been carried out, such as the large-scale production of polyhydroxybutyrate (PHB), co-polyesters of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), co-polyesters of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx), and medium-chain-length (mcl) PHA. The physical properties of various PHAs are shown in Table 2.1. On the other hand, PHAs have a broad spectrum of monomers that provide PHAs with variable and distinct properties, as shown in Figure 2.3 (Han, 2014).

Table 2.1: Physical Properties of Various PHAs In Comparison with Conventional Plastics (Chang, et al., 2014).

Samples	T_m (°C)	T_g (°C)	Tensile strength (Mpa)	Elongation at break (%)
PHB	177	4	43	5
P(HB-*co*-10% HV)	150	-	25	20
P(HB-*co*-20% HV)	135	-	20	100
P(HB-*co*-10% HHx)	127	1	21	400
P(HB-*co*-17% HHx)	120	-2	20	850
Polypropylene	170	-	34	400
Polystrene	110	-	50	-

3-Hydroxy Acid	3-Hydroxy Acid (Unsaturated)	3-Hydroxy Acid (Branched)	3-Hydroxy Acid (Substituted Side Chain)	Other Than 3-Hydroxy Acid	Aromatic Side Chain	Other Functional Groups
Butyanoic	4-Hexenoic	2,6-Dimethyl-5-heptenoic	7-Fluoroheptanoic	4-Hydroxybutanoic	Dimethyl esters of 3,-6-epoxy-7-nonenoic acid	3-Hydroxy-7-oxooctanoate
Pentanoic	5-Hexenoic	7-Cyanoheptanoic	9-Fluoroheptanoic	4-Hydroxyhexanoic	3-Hydroxyphenylhexanoic	3-Hydroxy-5-oxohexanoate
Hexanoic	6-Heptenoic	5-Methylhexanoic	6-Chlorohexanoic	4-Hydroxyoctanoic	3-Hydroxyphenylheptanoic	8-Acetoxy-3-hydroxyoctanoate
Heptanoic	6-Octenoic	4-Methyloctanoic	8-Chlorooctanoic	5-Hydroxyheptanoic	3-Hydroxyphenyloctanoic	6-Acetoxy-3-hydroxyhexanoate
Octanoic	7-Octenoic	5-Methyloctanoic	6-Bromohexanoic	5-Hydroxyhexanoic	3-Hydroxy-6-pmethylphenoxyhexanoate	
Nonanoic	8-Nonenoic	6-Methyloctanoic	8-Bromooctanoic	4-Hydroxyhexanoic		
Decanoic	9-Decenoic	6-Methylnonanoic	11-Bromoundecanoic	2-Hydroxydodecanoic		
Undecanoic	10-Undecenoic	7-Methylnonanoic	7-Cyanoheptanoic			
Dodecanoic	6-Dodecenoic	8-Methylnonanoic	9-Cyanononanoic			
7-cyanoheptanoic	5-Tetradecenoic	7-Methyldecanoic	12-Hydroxydodecanoic			

Figure 2.3: The Monomers in Polyhydroxyalkanoates (PHAs) (Han, 2014).

Polyhydroxybutyrate (PHB) is classified as a short-chain-length PHA (scl PHA) due to its monomers just involving 4 - 5 carbon atoms. By altering the growth medium, a poly[hydroxybutyrate-*co*-hydroxyvalerate] (PHBV) which is a random copolymer that containing both 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) is received (Lu et al., 2009). The flexibility, tensile strength and melting point of PHBV could be altered and similar to either PP or LDPE by manipulating their HV content, which are low HV and high HV respectively. PHBV possesses good chemical and moisture resistance as well as good O_2 and aroma barrier properties. There are several ways to further improve the mechanical properties and biocompatibility of PHA, including blending PHA with other polymers, enhancing the surface or joining them with inorganic substances, hence allowing them to be applied in different applications.

Even though the production cost of PHA is relatively high compared to petroleum-based plastic, the effort made in developing the PHA production processes, such as fermentation, extraction and purification technologies. In addition, the researchers also focus on the improvement of bacterial strains to reduce the price of PHA and to ensure they could compete with other biodegradable polymers such as PLA and aliphatic polyesters. This study may enhance the efficiency of fermentation

technologies and reduce the production cost by utilizing cheap carbon, and thus increases the feasibility of PHA production.

Due to almost half of production cost of PHA depends on the cost of the carbon source, considerable interest in the use of cheap carbon substrates for PHA production are aroused. Potential substrates include palm oil mill effluent, papermill wastewater, agricultural wastewater, wastewater from olive mills, molasses, corn steep liquor, starchy wastewater and palm oil mill effluent.

2.2 Types of PHAs

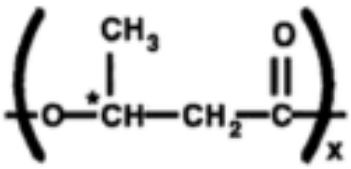
The bacteria and fungi in nature are able to synthesize different types of PHA based on the types of carbon sources available and the biochemical reactions that are occurring within the cell. Much effort has been made to determine types of monomers that could be incorporated with PHA synthase since monomers other than R-3-hydroxybutyrate (3HB) were identified in environmental samples (Bastioli, 2005). It is now attainable to produce PHA homopolymers and copolymers that consist of certain monomer composition.

2.2.1 Poly[R-3-Hydroxybutyrate] (P[HB])

Poly(3-hydroxybutyrate) (PHB) is defined as a linear polyester of 3-hydroxybutyric acid and it is the most common type of PHA. Its crystallinity is high and it could be synthesized commercially via fermentation of glucose by the bacterium *Alcaligenes eutrophus* (Sastri, 2010). It also contains of several unique properties, such as biodegradability and biocompatibility, which could be applied in biomedical field. There is only one type of optical active form of PHB synthesized due to the PHB is synthesized through bacteria, and hence the structure is absolutely perfect. This would promote the P3HB achieve the degrees of crystallinity of more than 95%.

PHB could be degraded through hydrolysis process and one of its main characteristics is that it has the same mechanical properties with polyethylene terephthalate (PET). It has a glass transition temperature of about 4 °C, yet it is less hydrophobic than PET and hence it is good in water absorption (Hill, 2005). The biodegradability of PHB within human body and soil causing it becomes an attractive degradable packing material.

Although possessing similar physical properties to polypropylene, the PHB homopolymer synthesized by microorganisms is relatively higher in terms of brittleness and thermally unsalability compared to polypropylene. The brittleness is caused by the formation of large crystalline domains in the form of spherulites. Due to the outstanding purity of PHB, the huge spherulites are formed within the biologically synthesised PHB. However, the brittleness of PHB could be reduced by applying the optimum processing conditions, and hence produce the functional ductile film. The structure of PHB was shown in Figure 2.4.



R-3HB

x = 120-200: low molecular weight P[3HB]

x = 1,000-20,000: high molecular weight P[3HB]

x ~ 100,000: ultrahigh molecular weight P[3HB]

Figure 2.4: Chemical Structure of Poly [R-3-Hydroxybutyrate] (PHB) (Bastioli, 2005).

The low molecular weight PHB, also defined as complexed PHB (cP3HB), act as a ubiquitous cell constituent that exists in eubacteria, archaebacteria, and eukaryotes (Reusch, Hiske and Sadoff, 1986). Recent studies have also proved the existence of cP3HB in human bodies. The weight of low molecular weight cP3HB, which

comprises about 120-200 3HB units, is around 12,000 Da. The cP3HB could be classified into chloroform-soluble and chloroform-insoluble types, by depending on their macromolecules structure.

In spite of the low molecular weight cP3HB, microbial cell cytoplasm will produce and build up high molecular weight P3HB in the form of water-insoluble inclusion bodies. Invariably, it plays a role in storing carbon and energy for the microorganisms. The molecular weight of this storage P3HB is normally within the range of 200,000 to 3,000,000 Da and the types of microorganism and the growth condition may affect the value. The high molecular weight P3HB had gained a lot of interests in the 1960s and 1970s due to its thermoplastic property. Due to its popularity, many studies have been done on it to identify its physical properties and discover its potential utilizations.

As of now, the ultra-high molecular weight P3HB has achieved the desired production due to the advancement of fermentation technology by applying a recombinant *Escherichia coli* cultivated under specific conditions. It seems to reveal some improved characteristics compared to high molecular weight P3HB which has high brittleness (de Graaf and Janssen, 2000). Its mechanical properties could be persisted for six months at room temperature even though it is in stretched and annealed form. On the other hand, the complete degradation of ultra-high molecular weight P3HB at 25 °C in a natural freshwater river within the period of three weeks showed that it possesses good biodegradability.

2.2.2 Poly[R-3-Hydroxybutyrate-*co*-R-3-Hydroxyvalerate] (PHBV)

P3HB is restricted by its downside, such as brittleness, firmness and crystalline nature. Hence, the monomers are introduced into the polymer chain of P3HB to obtain a copolymer with better properties. The extensive studies on improving the properties of P3HB has prompted the creation of 3-hydroxybutyrate and 3-hydroxyvalerate copolymer, which is known as poly(R-3-hydroxybutyrate-*co*-R-3-hydroxyvalerate) (PHBV).

PHBV receives a lot of attention from researchers for extent studies due to its properties and biodegradability (Singh, et al., 2008). It could be synthesized by the same fermentation process as PHB production process, by supplying propionic acid and glucose as carbon source. The propionic acid which is discovered in the sustenance of bacteria plays a role in manipulating the concentration of hydroxyvalerate (HV) monomers in the copolymer. The melting temperature, glass transition temperature and crystallinity decrease with the elevating HV content, and hence enhancing the processing and toughness of PHBV (Brunel, et al., 2014). The research shows that the melting point was reduced considerably while the HV content in PHBV increases from 0 to 50% (Wang, et al., 2013). The chemical structure of PHBV was shown in Figure 2.5.

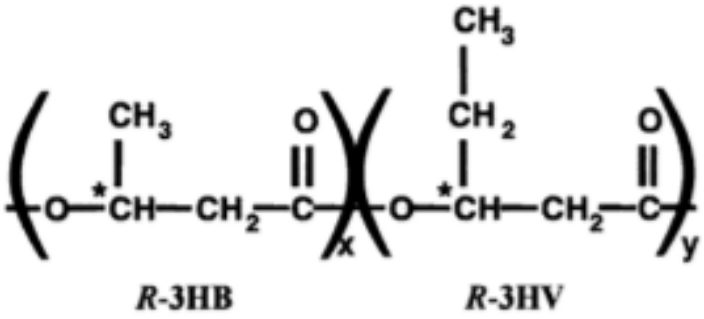


Figure 2.5: Chemical Structure of Poly[R-3-Hydroxybutyrate-*co*-R-3-Hydroxyvalerate] (PHBV) (Bastioli, 2005).

In comparison to PHB homopolymer, the PHBV copolymer possesses better physical properties in terms of toughness, impact resistance, flexibility and other properties that might enhance the processability. Even though a variety types of carbon sources could synthesize PHBV, the major barrier restricting the economical production of PHBV is the cost of the feedstock, which occupies 28–50% of the total production cost during microbial fermentation (Wong, et al., 2012). Thus, the manipulation of substrate composition is very important to minimize the production cost and optimize the production of PHBV.

2.2.3 Poly[3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate] (PHBHHx)

Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) is also categorized as one of the PHA species. The arrangement of co-polymer is random and its chemical structure contains 3-hydroxybutyrate and 3- hydroxyhexanoate monomers, as shown in Figure 2.6.

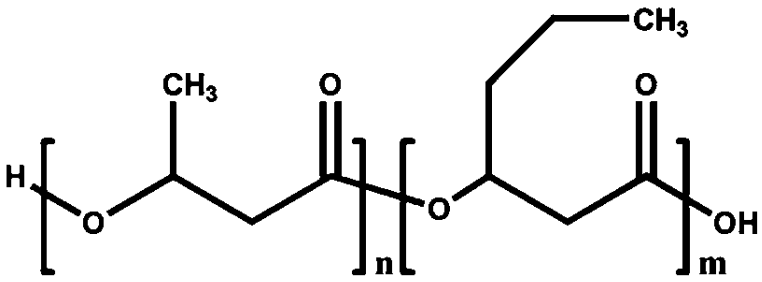


Figure 2.6: Chemical Structure of PHBHHx (Hsu, Hung and Chen, 2016).

PHBHHx acts as an important material within the biomedical field. Due to its superior mechanical, physical and chemical properties, it is likely practical for a wide range of biomaterials applications. A lot of studies showed that the biocompatibility of PHBHHx is relatively high and it is applicable for a wide range of body cell types such as fibroblasts, smooth muscle cells, chondrocytes, osteoblasts, and bone marrow cells (Chang, et al., 2014). The PHBHHx products will not release any toxic substances and will provide some nutrients during the degradation process. The studies also proved that HB will ease neuronal survival and differentiation.

A study was carried out in vivo to observe the degradation of PHBHHX and PHBHHx/polyethylene glycol (PEG) blend by implanting both polymers in rabbits for six months. The results showed that the rate of weight loss of PHBHHx is relatively higher than PHB but lower than PLA. On the other hand, the degradation of PHBHHx was highly enhanced by blending with PEG, yet the blends give rise to strong tissue response. Hence, PHBHHx is still the preferred biodegradable plastic that could be used for in vivo applications.

2.3 Thermal Properties of PHA

Melting temperature (T_m) and glass transition temperature (T_g) defined as the basic thermodynamic properties for the polymers. The Differential Scanning Calorimetry (DSC) could be applied to measure these properties. Melting point (T_m) acts as the temperature to determine the transition temperature between solid and liquid phases (Karelson and Dobchev, 2016). It has been applied to determine the purity of a compound and also estimate the physical properties of a substance in terms of aqueous solubility and liquid viscosity. On the other hand, glass transition temperature is referred as the temperature that identify the state of polymers either in glassy or crystalline state (Ebnesajjad, 2016). It is a major parameter when considering polymers for a specific application. The materials would be elastic when the temperature is above their T_g, yet the properties of a material changes intensely and movability decreases significantly as the temperature falls below T_g. Glass transition temperature is normally related to fully or partially amorphous polymers. There is no an actual value for T_g due to the value of the glass transition temperature will be manipulated by strain rate and heating or cooling rate. Both melting and glass transition temperature are important for PHA to determine their processability and mechanical properties.

The material properties of PHA might be altered by the composition of the PHA during the biosynthesis. The minor difference between the decomposition temperature and the high melting temperature will enhance the processability for melt extrusion technology. The melting temperature and glass transition temperature of different composition of PHA was shown in Figure 2.7.

	PHB	Poly (3HB-*co*-3% 3HV)	Poly (3HB-*co*-20% 3HV)	Poly (4HB)	Poly (3HB-*co*-3% 4HB	Poly (3HB-*co*-16% 4HB)	Poly (3HB-*co*-64% 4HB	Poly (3HO-*co*-12% 3HH)
Melting temperature (°C)	177	170	145	60	166	152	50	61
Glass-transition temperature (°C)	4	–	–1	–50	–	–8	–	–35
Tensile strength (MPa)	40	38	32	104	28	26	17	9
Young's modulus (GPa)	3.5	2.9	1.2	149	–	ND	30	0.008
Elongation at break (%)	6	–	50	1,000	45	444	591	380

PHB poly[(*R*)-3-hydroxybutyrate], *3HB* 3-hydroxybutyrate, *3HV* 3-hydroxyvalerate, *4HB* 4-hydroxybutyrate, *3HH* 3-hydroxyhexanoate, *3HO* 3-hydroxyoctanoate, *ND* not determined

Figure 2.7: Characteristics of Representative PHAs (Koller, et al., 2009).

The melting temperature of PHA locates between of 50°C and 177°C, yet the glass transition temperature is within the range of -50°C to 4°C. For PHB, it contains of high melting point and tensile which is enough to compare with petroleum-based polymers, such as polypropylene. However, the application of pure PHB is restricted because of its narrow processing window and inherent fragility which showing low strain at break. The elongation at break is also highly distinct between PHB and PP, which are 5% and 400% respectively. On the other hand, the high brittleness of PHBV is proved by the low elongation at break of PHBV which is less than 15%. The modulus and fracture stress of PHBV are 1.2 GPa and 25 MPa (El-Hadi, et al., 2002).

According to Bugnicourt, et al. (2014), there are a few factors that affecting the brittleness of PHB and PHBV, which including:

- The secondary crystallization of the amorphous phase will occur throughout storage at room temperature.
- The T_g of PHB is almost similar to room temperature.
- Inter-spherulitic cracks happen in large spherulites due to low nucleation density of PHB.

PHB that is obtained naturally can initiate crystallization more easily because it has no impurities to act as heterogeneous nuclei (Di Lorenzo, et al., 2001). Without heterogeneous nuclei that can promote the onset of crystallization, the crystallization kinetics is slow because the crystallization begins from homogeneous nuclei. As a

result of the slow crystallization kinetics of PHB, According to Androsch (2008), the crystallinity level can be manipulated to influence the rigid amorphous chains coupled with the crystals owing to slow crystallization kinetics of PHB. A nucleating agent may be added to promote the amount of small spherulites. Based on the research, high molecular weight PHB, which is around 500 kDa, has almost double rigid amorphous fraction than low molecular weight PHB that weighted as 5 kDa, despite crystallinity. Due to the strengthened covalent coupling of crystals and amorphous structure, the rigid amorphous section will be increased (Androsch, 2008). Rigid amorphous fraction (RAF) only exists in semi-crystalline polymers and it presents at the interface of crystal and amorphous regions which caused by the immobilization of a polymer chain due to crystal. Molecule segments in the amorphous phase will show a restricted mobility if they form covalent bonds with crystalline phase.

The glass transition temperature of PHB would be reduced due to the enhancement of molecular motion by adding plasticizers. It is very important to lower the glass transition temperature than testing temperature to attain high elongation at break and high flexibility for formulated PHB. Besides, the impact strength could be manipulated by the glass transition temperature as well as morphology. The cooling rate and nucleation density of PHB could influence the nucleation rate and spherulite size of the blends too. For example, quenching will enhance the crystallization rate due to the formation of small spherulites and restriction of crystallinity. This is known as the basic requirement to achieve fundamental mechanical properties.

In addition, PHB will be thermally degraded at any temperature slightly above its melting point. A critical decomposition of PHB will be initiated very shortly by exposing to temperature near 180°C. The degradation will be accompanied by emission of the degraded products in terms of olefinic and carboxylic acid substances through the random chain scission reaction. The microbially induced chain scission is defined as the main reaction for biodegradation which cause a rapid decrement of molecular weight (Mohanty, Misra and Drzal, 2002).

The weak resistance of PHB against thermal degradation has been the major problem when processing PHB. To overcome this issue, the lubricant will be applied during the processing of PHB to prevent the degradation of chains and to ensure the

material could be processed within the range of 170 – 180°C. This might cause a decrease in molecular weight and decrement of melt viscosity. Consequently, the crystallization time will be longer due to the crystallization temperature alters to lower values.

Besides applying lubricant during process, the blending of PHB could solve the problem too. The production of PHB derivatives through the biosynthesis of co-polyesters containing PHB units with other 3-hydroxyalkanoates units, such as poly (3-hydroxybutyrate-*co*-hydroxyvalerate) (PHBV) or poly (3-hydroxy butyrate-*co*-3-hydroxyhexanoate) (PHBHHx), with different molar ratios of hydroxycarboxylic acids could enhance the fundamental properties of PHB. This approach has been further studied extensively due to its effectiveness on improving mechanical properties as well as lowering the melting point. Aside from producing co-polymers, addition of plasticizers in terms of other biodegradable polymers is also a feasible way in enhancing the physical and thermal properties of polymers. The reduction of production cost and easier processability could be achieved by reducing the processing temperature. Hence, blending of PHB with plasticizers and nucleation agents is normally carried out to reduce the glass transition temperature and crystallinity by forming countless, fine and imperfect crystallites.

2.4 Biodegradation Process

Biodegradable bioplastics refers to the plastics that fully degraded by microorganisms, without releasing any harmful substances. The term “biodegradable” is known as the ability of a substance being decomposed naturally into biogases and biomass as a result of exposing to a microbial environment and humidity (Jain, Kosta and Tiwari, 2010). PHA are a type of classic biodegradable plastics due to their remarkable properties which could be competed with conventional plastics. The biosynthesis and biodegradation cycle of PHA was shown in Figure 2.8.

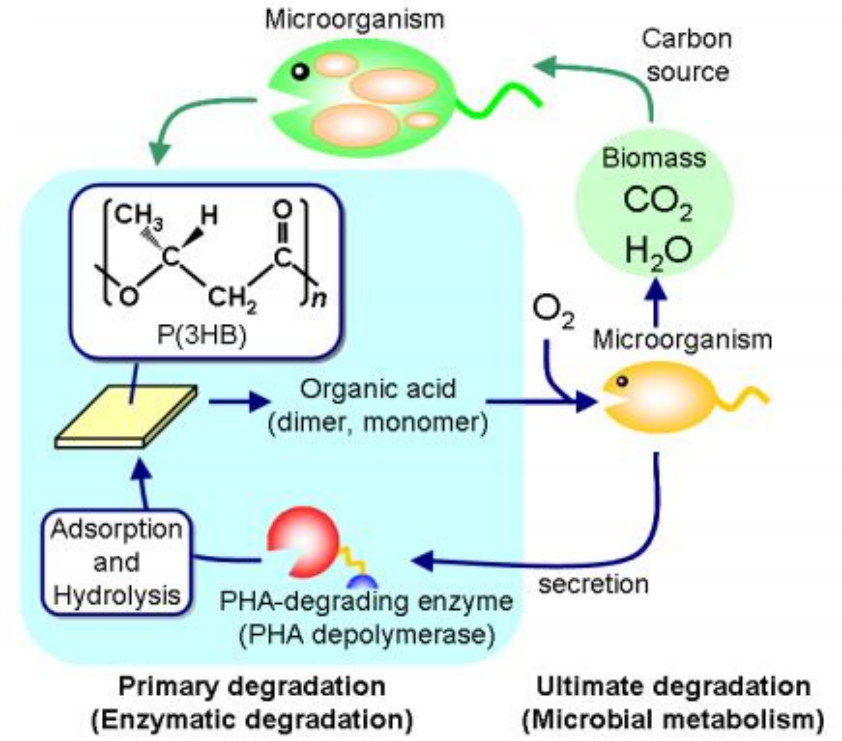


Figure 2.8: Production and Decomposition Pathway of PHA in a Nature (Numata, Abe and Iwata, 2009).

PHA could be degraded by PHA-degrading enzymes that exist in natural microorganisms, such as PHA hydrolases and PHA depolymerases. Subsequently, these degradation products will be metabolized by the microorganisms into water and carbon dioxide. Then, water and carbon dioxide will be consumed by microorganisms again and PHA are synthesized by the microorganisms as intracellular energy and carbon storage granules. The reactivity of these enzymes will be manipulated and controlled by the environmental conditions and composition of polymer.

The types of PHA depolymerases could be classified into two types based on their degradation methods, which are extracellular degradation and intracellular degradation. Extracellular degradation is defined as the disintegration of exogenous energy reservoir by other microorganisms. The major source of the exogeneous carbon source is PHA that released by accumulating cells after death. The extracellular depolymerases will be secreted out by microorganisms in terms of bacteria and fungi to break down the extracellular PHA into water-soluble components. Conversely, intracellular degradation is defined as the active breakdown of an endogenous storage

source by the accumulating bacterium itself. This will occur in the condition of nutrient depletion and intracellular PHA depolymerases will be synthesized to catalyse the intracellular decomposition of PHA.

Both PHA would be disintegrated into carbon dioxide and water or methane by a range of ubiquitous microorganisms that exist in nature. The superior biodegradability came as a surprise given the inertness of the water-insoluble, hydrophobic and partially crystalline structure. The distinction between extracellular and intercellular degradation is important due to the biophysical states of PHA will be vary according to the location of PHA in terms of in vivo and outside of the cell. The intracellular PHA which consists of high molecular-weight is in the amorphous state, whereas the extracellular PHA is normally in partially crystalline state (Kynadi and Suchithra, 2014).

There are a few factors that could influence the biodegradation rate, in terms of temperature, moisture, molecular weight of polymer, pH, the exposed area and microbial activity of the environment (Boopathy, 2000). PHA could be degraded over a broad range of temperatures, even at a maximum of around 60 °C with moisture level of 55%. Besides, in the case of PHA, its polymer composition and crystallinity will affect the rate of biodegradation too (Lee, 1996b). A variety of researches show that degradation rate of copolymers containing PHB monomer units is higher than either pure PHB or 3HB-*co*-3HV copolymers. There are few studies show that around 85% of the PHA samples were degraded within 7 weeks in composting trials, and PHA-coated paper was disintegrated and absorbed into the compost. The Figure 2.9 illustrates the biodegradability of PHA bottles in the soil within two months.

Figure 2.9: Biodegradability of PHA Bottles in Soil within Two Months (Van Der Hoeven, 2016).

Several authors had carried out the experiment of biodegradation analysis of film specimens from PHB and PHBV in several environments, which are sea water of South China Sea, soils from Vietnam and soils from Russia (Boyandin, et al., 2012). As a result, the rate of weight loss of PHBV and PHB are almost similar in sea water. However, PHB degrades faster than PHBV in tropical soil. In the case of Siberian soil, the degradation rate of the PHBV was higher than the PHBV of tropical soil. This could be proved by the decrement of PHA specimens' molecular mass during biodegradation. The biodegradation of PHA specimens in Vietnam was shown in Figure 2.10.

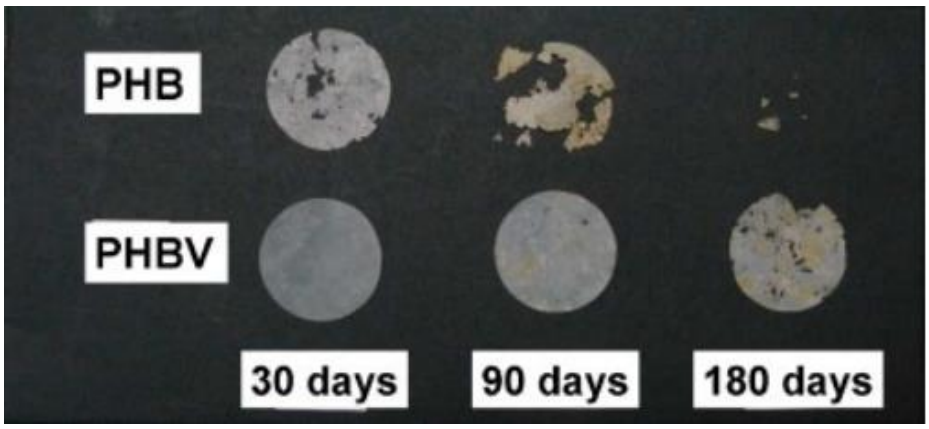


Figure 2.10: Biodegradation of PHA Specimens in Vietnam Soil (Boyandin, et al., 2012).

Adding to its virtues, the degradation product components of PHA that released to the nature are non-toxic in nature. It possesses of good biocompatibility and they have no negative effects in living organisms. This could be proved by PHB did not influence cell metabolism or cell growth, which measured as lactate production and glucose consumption, during the exposure of compressed PHB tablets to mouse fibroblast cells in culture. In addition, the study also shows that monomers of PHA may not be toxic, yet they could release some nutrients to the organisms. The polymer would be hydrolysed within the mammals, but in a slightly slow rate. The advantages of PHA, in terms of biodegradability without toxicity and thermo-processability make it becomes aesthetically pleasing as biomaterials for applications in both conventional medical devices and tissue engineering.

CHAPTER 3

METHODOLOGY

3.1 Process Flowchart

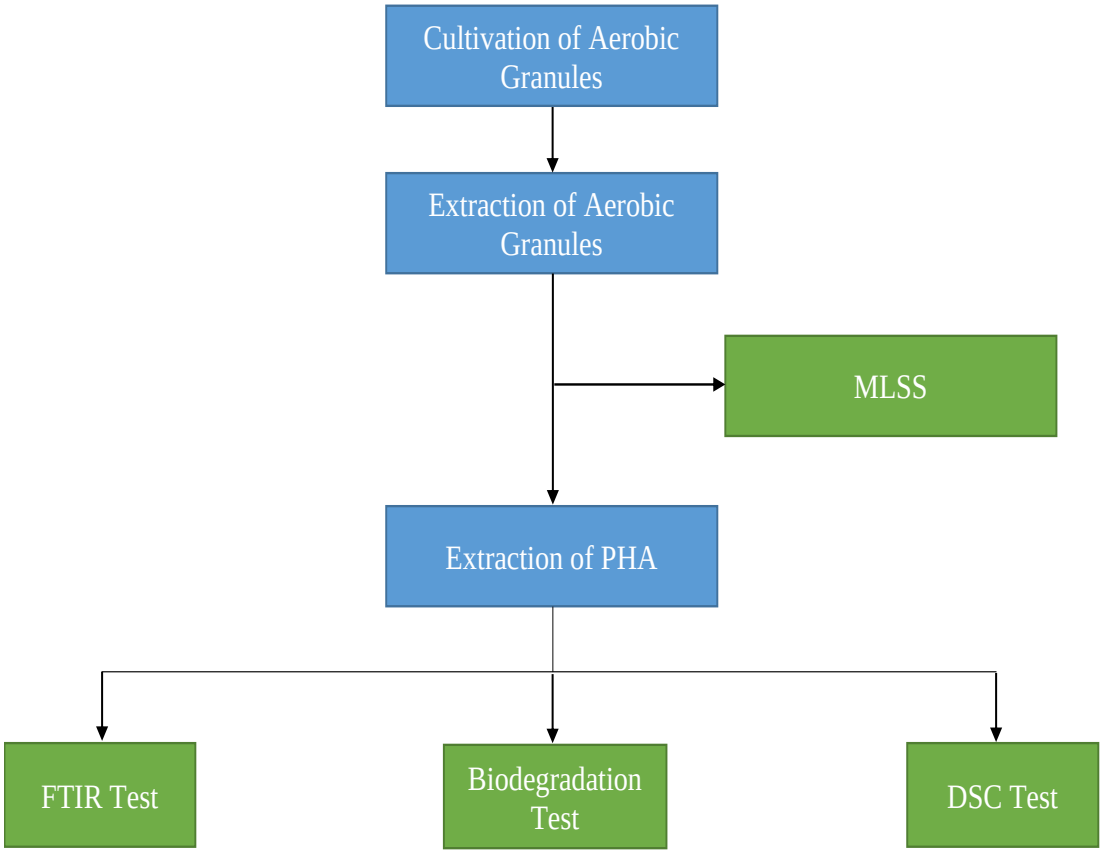


Figure 3.1: Process Flowchart of Experiment.

Initially, the papermill wastewater was treated by activated sludge process to cultivate the aerobic granules and promote the production of PHA within aerobic granules. Then, the aerobic granules were extracted from the reactor in the interval of 4 days due to the hydraulic retention time was set as 4 day. 10 mL of wastewater was withdrawn from reactor to carry out MLSS test. On the other hand, 1g of sludge was removed from reactor to perform extraction of PHA. The extracted PHA was characterized by undergoing few tests, in terms of FTIR test, biodegradation test and DSC test.

3.2 Sequencing Batch Reactor

An aerobic sequencing batch reactor (SBR) with a working volume of 2L was set up to culture the activated sludge. The wastewater could be treated by applying the activated sludge process. It was operated under atmospheric pressure and at the room temperature. The cylindrical reactor consisted of air flow meter at the bottom of the reactor to control the aeration rate to the effluent within the reactor. The operation of the SBR was carried out based on 24 hours batch cycle, which consisting of feeding phase, reaction phase, settling phase and decanting phase.

During the feeding phase, the air pump was switched off and 500mL of raw wastewater was introduced into the reactor to mix with the biomass for reaction. Subsequently, the air pump was switched on and aeration was provided at the reaction phase to promote the reaction. For settling phase, the air pump was switched off again to allow the sedimentation of suspended solids and a clear supernatant was left at the upper part of SBR. Once the settle phase was completed, 500mL of supernatant was withdrawn from the upper valve of SBR during decanting phase.

The period of feeding phase and aeration phase had been fixed for 5 minutes and 1420 minutes respectively. Conversely, the settling phase and decanting phase were manipulated throughout the experiment. Initially, the settling phase and decanting phase were set for 5 minutes and 10 minutes separately for each parameter. However, the time taken for settling phase was decreased gradually in terms of 3 minutes, 1.5 minutes and 1 minute due to the settling rate of aerobic granules increased significantly as the experiment carried out.

3.3 Wastewater and Aerobic Granules

Papermill wastewater and aerobic activated sludge were employed in this experiment. Both papermill wastewater and activated sludge were obtained from Muda Paper Mills Sdn. Bhd., Simpang Ampat, Penang. They were kept inside the fridge at approximately 4°C. The The collected activated sludge was rich with unwanted liquid. To obtain a

more concentrate activated sludge, the sludge was allowed to settle for few minutes and the supernatant was removed. The initial pH value and COD of papermill wastewater was measured and recorded.

3.4 Cultivation of Aerobic Granules

During the preparation stage, 1.5L of papermill wastewater was measured and introduced into the SBR. After that, 200g of activated sludge was weighed and mixed into papermill wastewater, as shown in Figure 3.2. The reactor was operated at a flow rate of 2L/min. Eventually, the batch cycle was begun with reaction phase until decanting phase. Each parameter was operated for 12 days and the experiment was repeated with different activated sludge weight and aeration rate.

Figure 3.2: Sequencing Batch Reactor Was Filled with Papermill Wastewater and Activated Sludge During the Preparation Stage.

Throughout the 12 days, the experiment was classified into non-full cycle and full cycle states. Full cycle was performed in the interval of 4 days. The chemical oxygen demand (COD) test was carried out at feeding phase and decanting phase daily. However, for full cycle day, the COD test was also performed every hour for 6 hours. Besides, the extraction of PHA and MLSS test were carried out on the full cycle day too.

3.5 Effect of Activated Sludge Weight and Aeration Rate

To identify the effect of content of activated sludge and aeration rate on aerobic granules and PHA, the whole set of experiments was repeated with 200g, 300g and 400g of activated sludge. Besides, the effect of aeration rate on aerobic granules and PHA was also studied in this project. Hence, the experiment was repeated with 1.0L/min, 2.0L/min and 3.0L/min of aeration rates too. At the beginning, different activated sludge weights were operated under the same aeration rate, which was 2.0L/min. After comparing the results, the weight that obtained the best performance was used to act as the constant variable and the experiment was repeated with 1.0L/min and 3.0L/min aeration rates.

3.6 Extraction of Polyhydroxyalkanoate (PHA)

According to Gobi and Vadivelu. (2014), sodium hypochlorite-chloroform method was the optimum way to extract PHA. Firstly, around 1g of aerobic granules were withdrawn from the SBR during the end of feast phase. The aerobic granules were mixed with 12.5mL of sodium hypochlorite and 12.5mL of chloroform in a 50mL centrifuge tube. Sodium hypochlorite and chloroform were used for cell digestion step and PHA dissolving step respectively (Gobi and Vadivelu, 2015). The role of sodium hypochlorite was to break the microbial cells by disintegrating the cell wall and dissolve all other cell constituents except PHA. Conversely, chloroform was used to dissolve the PHA that exposed to the mixture.

Then, vortex shaker was utilized to shake the centrifuge tube for around 2 minutes. The vigorous shake of mixture was allowed to disrupt the aerobic granules and cause cell lysis. Subsequently, the mixture was incubated in a water bath shaker at 37°C for 90 minutes. The mixture was then centrifuged at 4500rpm for 30 minutes and three separate phases were obtained after centrifugation. The upper layer was the sodium hypochlorite solution, the middle layer contained the cell debris and the bottom layer consisted of PHA-enriched chloroform. To obtain the PHA-enriched solvent, the upper layer and middle layer of the mixture were removed by pipette and simple filtration method respectively.

An empty 100mL beaker was weighed and the filtered solvent was transferred into the beaker. Then, the titration of ice-cold methanol (30% (v/v)) was carried out by using a burette to precipitate the PHA. During the titration, the beaker was swirled moderately to enhance the reaction. Finally, the solution was left at the fume hood for few days to vaporize the unwanted liquid. The precipitated PHA was formed as a dry solid layer at the bottom of the beaker. The weight of the beaker was measured again to calculate the weight of PHA obtained. The Equation 3.1 was used to calculate the content of PHA within the aerobic granules collected.

$$PHA\ content = \frac{weight\ of\ PHA\ (mg)}{weight\ of\ aerobic\ granules\ collected\ (g)} \quad (3.1)$$

3.7 Analysis Methods

3.7.1 Chemical Oxygen Demand (COD) Measurement

The COD test was carried out by using the HACH 21259 High Range (20 – 1500 mg/L) COD vial based on APHA standard method. Firstly, a COD digital reactor was preheated to 150°C. Then, 2.00mL of sample was filtered through a 0.22 μm syringe filter to remove the suspended solids inside the sample. The filtered sample was pipetted into the COD vial and the content of COD vial became hot due to the exothermic reaction. The COD vial was inverted immediately for few times to mix

well. In addition, a blank sample was prepared by inserting 2.00mL of distilled water into another vial. This blank sample was used to act as a reference when measuring the COD concentration. Then, both vials were digested in the preheated COD digital reactor at 150°C for 2 hours, as shown in Figure 3.3.

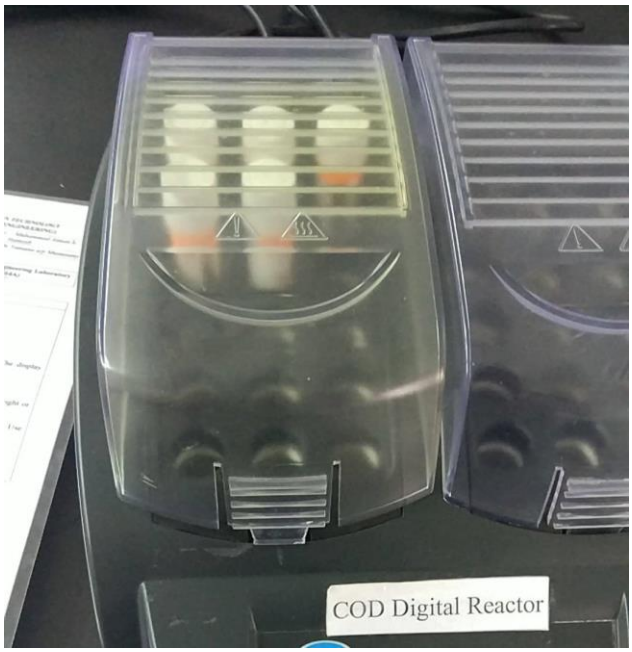


Figure 3.3: The Vials Were Heated in COD Digital Reactor.

Afterwards, the vials were kept in a dark space to avoid the exposure to UV light and to be cooled down to room temperature. Eventually, the COD vials were measured by using UV-VIS spectrophotometer after they were cooled. As mentioned above, the COD test was carried out at feeding phase and decanting phase daily. Furthermore, it was also executed every hour for 6 hours during the full cycle day.

3.7.2 Mixed Liquor Suspended Solids (MLSS)

MLSS was used to measure the concentration of suspended solids in a sample of mixed liquor. Mixed liquor was defined as the mixture of activated sludge and raw wastewater that treated by activated sludge process within an aeration basin. Firstly, a sample was withdrawn and measured to a volume of 10 mL

from the SBR effluent during reaction phase. After that, a glass-fibre filter paper was weighed and the sample was filtered through the filter disk by using multichannel manifold filter. The filter paper was placed with correct position in the apparatus and the vacuum suction was applied continuously until all liquid contents were removed. Subsequently, the filter paper was removed with care from the filter and transferred to an evaporating dish, as shown in Figure 3.4.

Figure 3.4: Glass-Fibre Filter Paper Was Placed Inside Evaporating Dish.

Eventually, the filter paper was dried in an oven at the range of 60 – 80°C for 24 hours to remove the moisture from filter paper. The filter paper was removed from the evaporating dish and it weight was measured after the drying process was done. The MLSS could be calculated by using Equation 3.2.

$$MLSS = (weight\ of\ filter\ paper\ and\ dried\ suspended\ solids - mass\ of\ empty\ filter\ paper) \times \frac{1000mg}{g} \times \frac{1}{10mL} \times \frac{1000mL}{1L} \quad (3.2)$$

3.7.3 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis was carried out to identify the organic and polymeric components that existed within the sample. The infrared light was applied to scan test samples and observe chemical properties. The material's molecular composition could be determined by analysing the sample's absorbance of the infrared light's energy at various wavelengths. Firstly, the sample was blended with potassium bromide (KBr) powders where the ratio of sample to KBr was set at 1:10. Then, the blended powders was transferred into a cast and they were compressed under a high pressure to form a thin slice sample. The sample should be thin enough for the infrared light to transmit through. Subsequently, the thin slice sample transferred to the spectrometer to carry out the FTIR analysis. The FTIR spectrometer was operated at 27°C from 400 to 4000cm^{-1} range.

3.7.4 Differential Scanning Calorimetry (DSC) Test

DSC analysis was carried out to determine melting temperature, glass transition temperature and latent heat of melting of PHA. The calorimeter contained one sample cell and one reference cell. The weight of PHA used was weighed and it was referred as sample cell. Conversely, the reference cell was remained empty. Subsequently, both samples were exposed to a temperature profile over 20 to 200°C. The temperature was ramped at a heating rate of 10°C/min with the utilization of nitrogen gas. The heat flow during the isothermal crystallization was recorded as a function of time.

3.7.5 Biodegradation Test

The biodegradation test on PHA was carried out based on soil burial method. The test was carried out at E209A Project Lab, Universiti Tunku Abdul Rahman (UTAR). Initially, the weight of concavus pan was measured. Then, 30mg of PHA was weighed and it was covered in the BABA Organic Potting Mix soil at a depth of 5mm. The soil was purchased from Tesco. Subsequently, the total weight of the sample was measured again to determine the weight of soil used. One drop of water was added to the sample daily to provide the moisture that required by biodegradation reaction. The sample was weighed weekly for 7 weeks to calculate the weight loss of PHA.