NUTRITIONAL EVALUATION OF MEALWORMS FROM POLY(3-HYDROXYBUTYRATE) RECOVERY AND ITS POTENTIAL APPLICATION AS AQUACULTURE FEED INGREDIENT

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
%DM	Percentage of dry matter
±	Plus-minus
°C	Degree Celsius
α	Alpha
β	βeta
min	Minutes
ADC	Apparent digestibility coefficient
AOAC	Association of official analytical chemists
cm	Centimetre
CMC	Carboxy methyl cellulose
CME	Caprylic methyl ester
CoA	Coenzyme A
Cp	Crude protein
CDW	Cell dry weight
DSC	Differential scanning calorimeter
EC	Electrical conductivity
ECD	Efficiency of conversion of digested food
ECI	Efficiency of conversion of ingested food
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FBW	Final body weight
FCR	Feed conversion ratio
FI	Feed intake

FTIR	Fourier transform infra-red
g	Gram
g/L	Gram per litre
g/L/h	Gram per litre per hour
GC	Gas chromatography
GPC	Gel permeation chromatography
GSI	Gonadosomatic index
h	Hour
HCD	High cell density
HSI	Hepatosomatic index
IPF	Intraperitoneal fat
KDa	Kilo Dalton
kg	Kilogram
L	Litre
LDPE	Low density polyethylene
M	Molarity
MCL	Medium chain length
mL	Millilitre
MM	Mineral medium
MMC	Mixed microbial culture
M_n	Molecular number
mol%	Molar percentage
M_w	Molecular weight
MwM	Mealworm meal
NADPH	Nicotinamide adenine dinucleotide phosphate

NR	Nutrient rich
μm	Micrometre
μmol	Micromolar
kV	Kilo volt
PHAs	Polyhydroxyalkanoates
PLA	Poly (lactic acid)
PTFE	Poly(tetrafluoroethylene)
RH	Relative humidity
rpm	Rotation per minute
SGR	Specific growth rate
T_d	Degradation temperature
v/v	Volume per volume
w/v	Weight per volume
wt%	Weight percentage
N	Normality
n3	Omega 3
n6	Omega 6
MCL	Medium chain length
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PER	Protein efficiency ratio
PDI	Polydispersity index
PHAs	Polyhydroxyalkanoates
PO	Palm olein

PUFA	Polyunsaturated fatty acids
RH	Relative humidity
rpm	Rotation per minute
SCL	Short chain length
SCP	Single cell protein
SEM	Scanning electron microscopy
T_d	Degradation temperature
T_m	Melting temperature
UV	Ultraviolet
VSI	Viscerosomatic index
WG	Weight gain
3HA	3-hydroxyalkanoates

PENGELUARAN SERENTAK POLIHIDROKSIALKANOAT DAN ULAT TEPUNG YANG KAYA PROTEIN UNTUK FORMULASI MAKANAN AKUAKULTUR

ABSTRAK

Polihidroksialkanoat (PHA) adalah polimer asas biologi yang menarik untuk menggantikan beberapa plastik petrokimia. Sehingga kini, harga jualan PHA adalah tinggi kerana sebahagian besarnya disebabkan oleh proses hiliran yang mahal dalam pengeluaran PHA. Tambahan pula, bahagian sel bakteria yang berkhasiat yang tidak mempunyai PHA telah hilang semasa proses penulenan PHA. Ternakan bakteria dengan kepadatan sel tinggi untuk *Cupriavidus necator H16* (berat kering sel 161 g / L) dan kaedah penghasilan PHA secara biologi dengan menggunakan ulat tepung (*Tenebrio molitor)* telah dibangunkan. Sel-sel bakteria yang dibeku-keringkan samada yang dibasuh dan tidak dibasuh mengandungi 70 ± 2.0 wt% P (3HB) dan diberi makan kepada ulat tepung pada kadar 12.5% daripada jumlah berat badan (125 g sel / 1kg ulat). Sel-sel yang dibasuh lebih digemari kerana kandungan garam mineral yang lebih rendah dan dimakan setiap hari berbanding dengan pengambilan sel yang tidak dibasuh yang dimakan selang sehari. Penulenan pelet fecal dari sel-sel yang dibasuh dan tidak dibasuh adalah 82% dan 72% berat, masing-masing. Pelet fecal yang diperoleh secara biologi dibandingkan dengan pengekstrakan kloroform P (3HB), menunjukkan hasil yang sama. Ulat tepung yang diberi makan sel bakteria mempunyai 17% lebih protein dan 20% kurang kandungan lipid berbanding dengan yang diberi makanan dedak gandum (diet kawalan). Bagi menghasilkan jumlah sel yang mencukupi, sel dikultur dalam *fermenter* 100 L (60 L isipadu operasi) untuk makanan ternakan ulat tepung berskala besar. Ulat tepung yang diberi makan sel bakteria

(kandungan protein 75% dan lipid 10%) dibeku-keringkan dan dihancurkan untuk digunakan dalam formulasi lima jenis makanan kajian dengan kandungan nutrisi yang sama iaitu protein (35% protein kasar) dan lipid (8% lipid) dengan kadar penggunaan tepung ulat (MwM) dengan tahap kemasukan MwM pada 0 (diet kawalan), 25, 50, 75 dan 100% menggantikan tepung ikan. Semua makanan kajian diberi makan kepada tiga kumpulan tilapia hibrid merah (berat awal 4.69 ± 0.02 g) dan diberi makan dua kali sehari selama 54 hari. Ikan yang diberi makan MwM0 menunjukkan perbezaan ketara dengan berat badan akhir tertinggi (FBW) (41.41 g), pertambahan berat badan (WG) (787.72%), kadar tumbesaran specifik (SGR) (4.04%), pengambilan makanan (FI) (4.77 g) dan nisbah pertukaran makanan (FCR) yang rendah. Nisbah pencernaan protein (ADCP) secara beransur-ansur berkurangan dari 91.41% -83.64% apabila tepung ikan telah diganti dengan tepung ulat (MwM). Penambahan tepung ulat dalam diet kajian tidak memberi kesan negatif kepada hematokrit dan indeks organ dalaman. Malahan, ikan yang diberi makan dengan MwM100 mempunyai usus mikrovilli yang lebih panjang daripada yang diberi makan dengan MwM0. Menariknya, tiada perubahan histologi di hati tilapia hibrid merah pada diet kawalan dan diet kajian

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are interesting bio-based polymers to replace some petrochemical plastics. To date, the selling price of PHA is high due in part to the costly downstream processes in PHA production. Furthermore, the non-PHA part of the bacterial cells which is nutritive, is lost during the PHA recovery process. High cell density cultures of *Cupriavidus necator* H16 (161 g/L cell dry weight) and an improved biological recovery method of PHA by using mealworms (*Tenebrio molitor*), were established. Freeze-dried washed and unwashed cells containing 70 ± 2.0 wt% P(3HB) were fed to mealworms at 12.5% of their body weight (125 g of cell/ 1kg of mealworms). Washed cells was more palatable and consumed daily due to its lower content of mineral salts compared to the alternate days consumption of unwashed cells. The Purity of the recovered faecal pellets from washed and unwashed cells were 82 wt% and 72 wt%, respectively. The biologically recovered feacal pellets were compared with chloroform extracted P(3HB), revealing identical properties. Cell-fed mealworms had 17% more protein and 20% less lipid content compared to those fed with wheat bran (control diet). Sufficient amount of the cells was cultivated in 100 L fermenter (60 L working volume) for large scale mealworm feeding. The cell-fed mealworms (75% protein and 10% lipid content) were freeze-dried and pulverised for the formulation of five different isonitrogenous (35% crude protein) and isolipidic (8% lipid) diets at mealworm meal (MwM) inclusion levels of MwM0 (control diet), 25, 50, 75 or 100% replacement of fishmeal. All feeds were provided to triplicate

groups of red hybrid tilapia (initial weight of 4.69 ± 0.02 g) twice daily for 54 days. Fish fed MwM0 showed significantly the highest final body weight (FBW) (41.41 g), weight gained (WG) (787.72%), specific growth rate (SGR) (4.04%), feed intake (FI) (4.77 g) and lowest feed conversion ratio (FCR). The apparent digestibility coefficient of protein (ADCP) gradually reduced from 91.41%-83.64% when fishmeal was substituted with MwM.The addition of mealworm meal in the experimental diet had no adverse effects on the haematocrit and the internal organs indices. No histological changes were observed in the liver of tilapia from both the experimental and control diet. But tilapia fed with MwM100 had longer gut microvilli than those fed with MwM0. Mealworm meal inclusion led to a decrease in feed utilisation and growth performance of red hybrid tilapia, due to increasing content of chitin.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Increasing plastic waste accumulation is a significant challenge in our present world. Plastics are common pollutants in land and water bodies due to their recalcitrant to microbial degradation (Loo and Sudesh, 2007; Rodriguez-Perez et al., 2018). Plastic litters in the form of microplastics (microbeads and microfibers) released from cosmetic products and pieces of clothes, endanger marine biota (Cole et al 2011; Xanthos and Walker, 2017). Synthetic plastic pollution has been a growing concern, especially in the wake of sustainable development goals. Hence, the need for a more environmentally friendly material.

Bio-based plastics have similarity in properties to conventional plastics in addition to being environmentally friendly. Bio-based polymers include polylactic acid (PLA), polyhydroxyalkanoate (PHA), polybutylene succinate (PBS), polyglycolic acid (PGA), polyvinyl alcohol (PVA) (Babu et al., 2013). PHA is a microbial polyester of hydroxyalkanoate synthesized as an energy or carbon storage material. PHAs are based on monomer size classified into either short-chain-length (SCL) containing 3-5 carbon or medium-chain-length (MCL) having 6-14 carbon atoms. Poly(3-hydroxybutyrate) [P(3HB)] and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoates) [P(3HB-*co*-3HHx)] are the simplest forms of SCL and MCL PHA, respectively. PHA is produced by either batch, continuous or fed-batch fermentation strategies. Several bacterial cells such as *Alcaligenes latus*, *Bacillus megaterium*, *Cupriavidus necator*, cyanobacteria and archaea are capable of accumulating PHA in an excess supply of carbon sources but a limited amount of growth nutrients such as nitrogen, phosphorous, magnesium and sulfur (Kahar et al.,

2004). Plant oils, fatty acids, and sugars are the conventional carbon feedstocks used for PHA production (Loo et al., 2005; Koller et al., 2009; Wong et al., 2012; Zainab-L et al., 2018). Due to higher number of carbons per gram of oil, plant oils produce higher PHA yield and cell biomass of about 0.6-0.8 g compared to the 0.3-0.4 g of PHA per gram of sugar substrates (Akiyama et al., 2003; Chanprateep, 2010). Palm olein was used in this study to cultivate *C. necator* H16 to high cell density (HCD) in a fed-batch fermentation. Despite the remarkable success recorded in achieving HCD and PHA yield, the market price of PHA is high owing to the lack of a suitable downstream process. The conventional and most extensively studied solvent method of PHA extraction is effective in yielding high purity PHA with negligible reduction in molecular weight. However, the halogenated solvents, mostly chloroform are toxic, costly, and required in large volumes (Yu and Chen, 2006). Other methods of PHA recovery such as the chemical treatments, mechanical disruption, cell fragility, supercritical fluid, flotation, and gamma irradiation suffers a certain degree of drawbacks (Kunasundari and Sudesh, 2011). Though, the chemical method of recovery resulted in about 50% reduction in overall production cost, PHA of reduced molecular weight and lower purity were obtained (Yu, 2009; Anis et al., 2013).

The pursuit of a greener method of PHA recovery led to the discovery of a biological process involving the use of Sprague Dawley rats by researchers in USM (Kunasundari et al., 2013; Kunasundari et al., 2017). The concept of biological recovery was a breakthrough for the field of PHA. Although, the indigestibility of PHA in the gastrointestinal tract (GIT) of animals was earlier reported by Waslien and Calloway (1969). Kunasundari et al. (2013) reported a regular consumption of both the freeze-dried cells and commercial feed by the rats. But, the water intake of cell-fed rats was two-fold higher than those on commercial feed. Interestingly, the PHA content

of the recovered feacal pellet was about 90 wt%. However, rats were conceived unsuitable for large scale application due to the requirement for large space and human labour. Therefore, further attempts were made by the same research group to improve the process using mealworms (Murugan et al., 2016a; Ong et al., 2018a). Regrettably, the daily cell consumption was only at 5% of the mealworm body weight (50 g cells/kg mealworms) compared to the average 20-25% consumption of wheat bran (conventional feed) (250 g wheat bran/kg mealworms). In addition, the purity of the recovered PHA granules (feacal pellet) were very low. The PHA content of the freeze-dried cells was 55 wt% while that of the recovered feacal pellet was 59 wt%. In other words, only a 4% increase in PHA content was achieved. Based on observations, variations occurred in the cell consumption and appearance of the PHA granules with different batches of cells. It was, therefore, hypothesised that the low cell consumption might be due to the presence of residual mineral salts from the culture medium, which may likely be the reason the low cell consumptions by the mealworms.

Although, the search for insects was triggered by the growing world population and diminishing protein resources in most of the tropical countries (Van Huis, 2013; Van Huis et al., 2013). Mealworms, including other insects (black soldier fly, locusts, crickets) were recently promoted as the next generation of a sustainable source of protein in aviculture (Ramos-Elorduy et al., 2002), human nutrition (Ghaly and Alkoaik, 2009) and aquaculture (Ng et al., 2001). Aquaculture is a global fastest growing animal feed producing sector, anticipated to advance further in the future (Tacon et al., 2010). However, the expansion of fish-farming practices has been disrupting the marine ecosystem and socioeconomic well-being of the coastal communities. Consequently, the aquaculture industry is faced with the increasing cost of fishmeal. The aquaculture feeds represent 40% to 70% of the fish production cost

(Rana et al., 2009). The inclusion of soybean and other plants proteins into aquafeed as fishmeal replacement (Espe et al., 2006; Gatlin et al., 2007) were limited by the presence of anti-nutritional factors (Francis et al., 2001; Collins, 2014), decreased palatability and possible inflammation of the animal's digestive tract (Papatryphon and Soares, 2001; Merrifield et al., 2011). In comparison to fishmeal, plant feedstuffs lack taurine and hydroxyproline which are beneficial for fish health and growth (Aksnes et al., 2008; Pinto et al., 2013). Insects are part of the natural diet of both marine and freshwater fish (Howe et al., 2014; Whitley and Bollens, 2014). They are rich in amino acids, minerals, vitamins (Vvan Huis, 2013) and contain about 26 µmol/g of taurine and hydroxyproline (Pant and Agrawal, 1964; Bicker, 1992; Whitton et al., 1995). The successful substitution of fishmeal or soybean meal with mealworm meal in poultry diets were reported (Ramos-Elorduy et al., 2002; De Marco et al., 2015; Bovera et al., 2016). Similarly, in fish diet, such as European sea bass (*Dicentrarchus labrax* L.), rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis niloticus*) and common catfish (*Ameiurus melas*) (Ng et al., 2001; Sánchez-Muros et al., 2014; Belforti et al., 2015; Roncarati et al., 2015; Gasco et al., 2016). The fish growth performance differs due to differences in the nutritional composition of the insect meal and its digestibility (Finke, 2015). The farms from which the insects were sourced utilises different rearing substrates hence, the variations in the larval nutritional compositions. For instance, rainbow trout fed diet containing 25% and 50% level of fishmeal substitution with mealworm meal showed good growth performance (Gasco et al., 2014a) while 50% substitution level of mealworm meal brought about reduction in growth of European sea bass and poor feed conversion efficiency (Piccolo et al., 2014). From another study, fishes fed 50 to 100% of live mealworms had reduced feed intake (FI), weight gain (WG) and higher body lipid content (Ng et al., 2001).

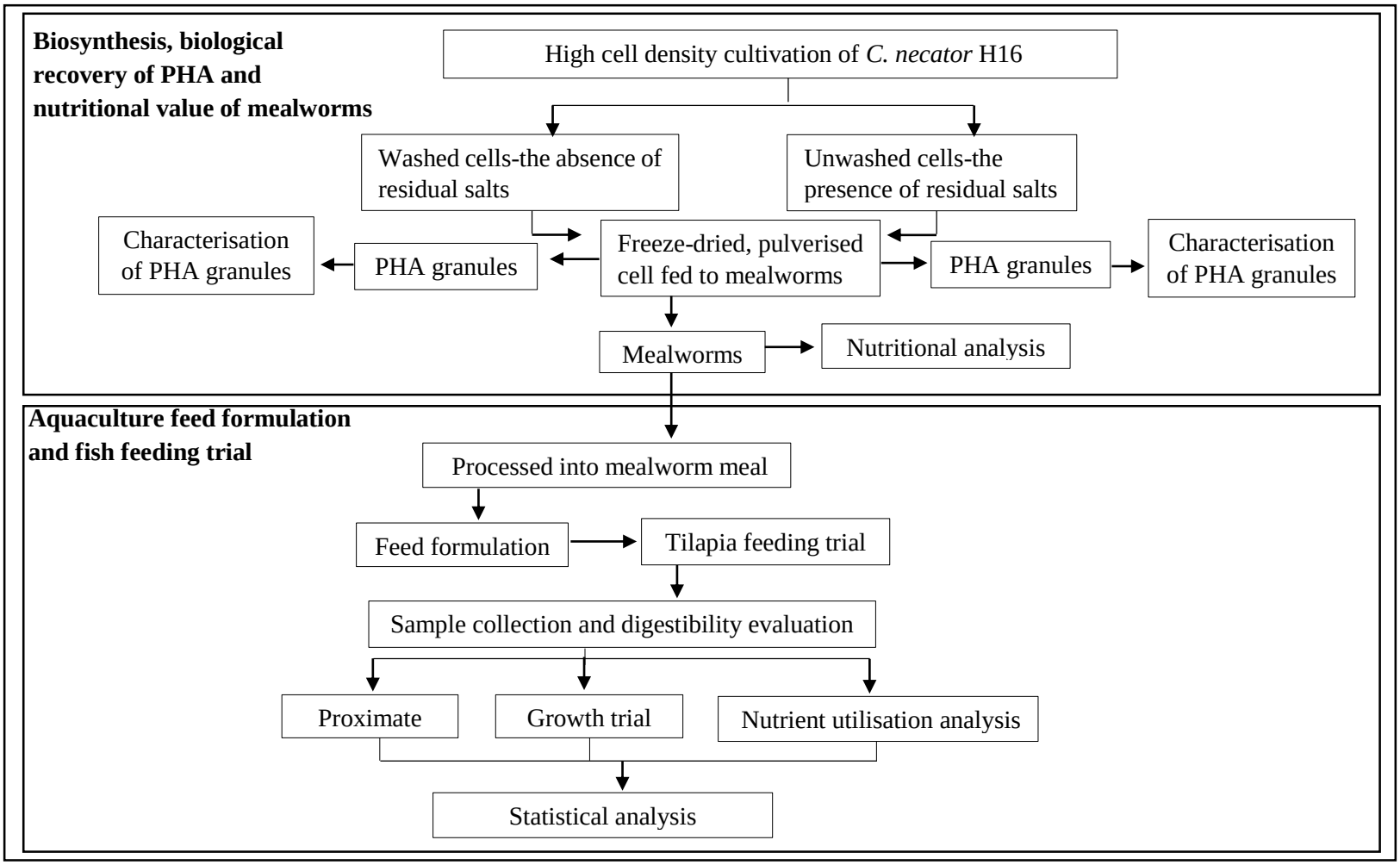
The cell-fed mealworms may have unique nutritional composition to fulfill the globally increasing demand for protein or serve as an interesting link in the animal feed chain and create flexibility in aquaculture feed formulations.

1.2 Objectives

The objectives of this study were:

1. To biosynthesise *Cupriavidus necator* biomass via high cell density
2. To improve the biological method of P(3HB) recovery and determine the nutritional value of the mealworms
3. To evaluate the value of cell-fed mealworms through analysis of growth performance, feed utilisation, and whole-body composition of the fish

The objectives were summarised in the flow chart below:



CHAPTER 2

LITERATURE REVIEW

2.1 Petroleum based plastics

Human day-to-day activities depend on the usage of plastics due to their low cost, durability, and versatility in terms of mechanical properties. Advancements in the field of science and technology have been increasing the production and utilisation of synthetic polymers globally, such that about 280 million tonnes of plastics are produced yearly (Shaw and Sahni, 2014). Global production of petroleum-based plastics has incredibly risen from 265 million tons in 2010 to 300 million tons in 2015 and expected to be 810 million tons by the year 2050 (Gourmelon, 2015). Approximately 30% of the plastics are used for packaging applications and only 2-3 million tonnes are used in agriculture (Shah et al., 2008; Leja and Lewandowicz, 2010). In the US, Australia and Germany, 20-25% of municipal solid waste such as coffee cup lids, plastic bottles and straws are made from synthetic polymers (Mohee and Unmar, 2007). Other major areas of plastic application include development of medical appliances, aerospace and building materials or even automobiles (Sigler, 2014). However, an unprecedented expansion in plastic production with increasing human population is alarming. Conventional plastics are recalcitrant to physical, chemical and microbial degradation, and its incineration causes hazardous gases emission to the atmosphere (Khanna and Srivastava, 2005). Mauritius, for example, has 19 high density polyethylene (HDPE) and 9 low density polyethylene (LDPE) producing companies for local utilisation and export respectively. According to report, about 1000 tonnes out of the 250-300 million tonnes of the plastic carry bags produced in Mauritius end up in landfills. Regrettably, the detrimental effect of oil-based plastics on our environment was only realised within the last 30 years.

Petroleum-based plastics only photodegrades into smaller pieces and ends up as pollutants on land and water bodies, particularly the Pacific, Atlantic, and Indian Oceans (Vert et al., 2002). The United Nations reported the presence of about 5-10 million tonnes of plastics in the North Pacific Ocean between Japan and California. A projection was made of about 200,000 plastic pieces per square kilometer in the North Atlantic Ocean and the Caribbean Sea (Gill, 2010). Over 500 billion and 35 million pieces of plastic bags and bottles used worldwide contaminates our beaches and oceans (Sigler, 2014). The degree of plastic litters carried to sea has become problematic. Plastic wastes enter the water bodies in whole of fragmented in the form of microplastics and microfibers. Large plastic items, such as discarded fishing rope and nets, can cause entanglement of mammals, and turtles. Over 270 species of turtles, mammals, fish, and seabirds have experienced restricted movement, starvation, or death (Sigler, 2014). Microplastics are light-weight and easily move to far distances in sea, or sedimentation to the seabed. Thousands of marine biotas are entangled and killed by litters from pieces of clothes and cosmetic products (Cole et al., 2011; Jambeck et al., 2015). Microplastics are mistaken for phytoplankton and eaten by fish and cetaceans. Upon consumption, plastic debris reduces stomach capacity and deter growth, cause internal injuries, and intestinal blockage (Plot and Georges, 2010). Debris of ropes, fishing line, nets, plastic bags, and Styrofoam have been removed from the digestive tracts of turtle. The turtle ingest floating plastic bags because of its resemblance to jelly fish, which consequently block the passage of food and eggs in female cloaca (Mascarenhas et al., 2004; Plot and Georges, 2010). Worldwide, no less than 86% of sea turtle species, 23% of marine mammal species, and 36% of seabird species are affected by plastic debris (Stamper et al., 2009). There is also a growing concern that plastics are acting as a faster medium for the introduction of foreign

species (mollusks, barnacles, and algae) compared to the natural means known for centuries (Sigler, 2014). In response to the aforementioned problems, the global attention shifted towards bio-based materials.

2.2 Bio-based and biodegradable plastics

Bio-based polymers are organic macromolecules synthesised through biological or chemical methods from renewable resources (natural carbon sources). The three classifications of bio-based polymers based on the process of synthesis are; the biosynthetic polymers such as polyhydroxyalkanoate (PHA) produced by microorganisms, the bio-chemosynthetic polymers [polylactic acid (PLA) and polybutylene succinate (PBS)] synthesised through chemical polymerisation of monomers derived from organic resources. The third group are the modified natural polymers from cellulose and starch. It is noteworthy that not all bio-based polymers are biodegradable polymers. For instance, bio polyethylene is bio-based but non-biodegradable (Sudesh and Iwata, 2008; Babu et al., 2013). A polymer is biodegradable when it undergoes deterioration and complete degradation by microorganisms in both aerobic and anaerobic processes. Amidst the various bio-based polymers**,** PHA is outstanding due to its complete biodegradability and compostability after use and does not contribute to landfill. Microorganisms secretes PHA depolymerases and hydrolases to completely degrade PHA to H_2O and CO_2 under aerobic condition or to H_2O and CH_3 in an anaerobic condition (Choi et al., 2004). The CO_2 released from PHA hydrolysis does not contribute to global warming because it originates from renewable carbon source for PHA biosynthesis and utilised back by plants during photosynthesis (Koller et al., 2010). Although, the degradation rate of a piece of PHA varies between few months (in anaerobic sewage) to years (in seawater),

depending on the environmental conditions and polymer composition (Madison and Huisman, 1999). These natural polyesters are therefore more preferred over petroleum-based plastics (Sudesh et al., 2000; Singh Saharan et al., 2014; Kourmentza et al., 2018).

2.3 Polyhydroxyalkanoate (PHA)

PHAs are hydroxyalkanoic acid (HA) monomers that are biosynthesised by microorganisms and stored as carbon and energy reserve materials. PHAs can be accumulated by over 100 types of microorganisms as energy storage materials in excess carbon supply but limiting essential nutrients such as nitrogen, oxygen, phosphorous, sulfur, or magnesium (Madison and Huisman, 1999). The storage of PHA enhances environmental persistence of the cell during starvation. These water-insoluble materials can be visualise directly by staining with Sudan Black B, Nile red and Nile blue (Ostle and Holt, 1982) or under phase-contrast light microscope owing to their high refractivity, or by using transmission electron microscopy (TEM) for more exceptional details (Doi et al., 1990; Sudesh et al., 2000). Due to the high molecular weight and low solubility of PHA, a bacterial cell is capable of accumulating about 8 to 12 PHA granules without affecting its osmotic pressure (Sudesh et al., 2000). PHA granules are usually coated with phospholipid and proteins (Pötter et al., 2002; Pötter and Steinbüchel, 2005) as shown in Figure 2.1.

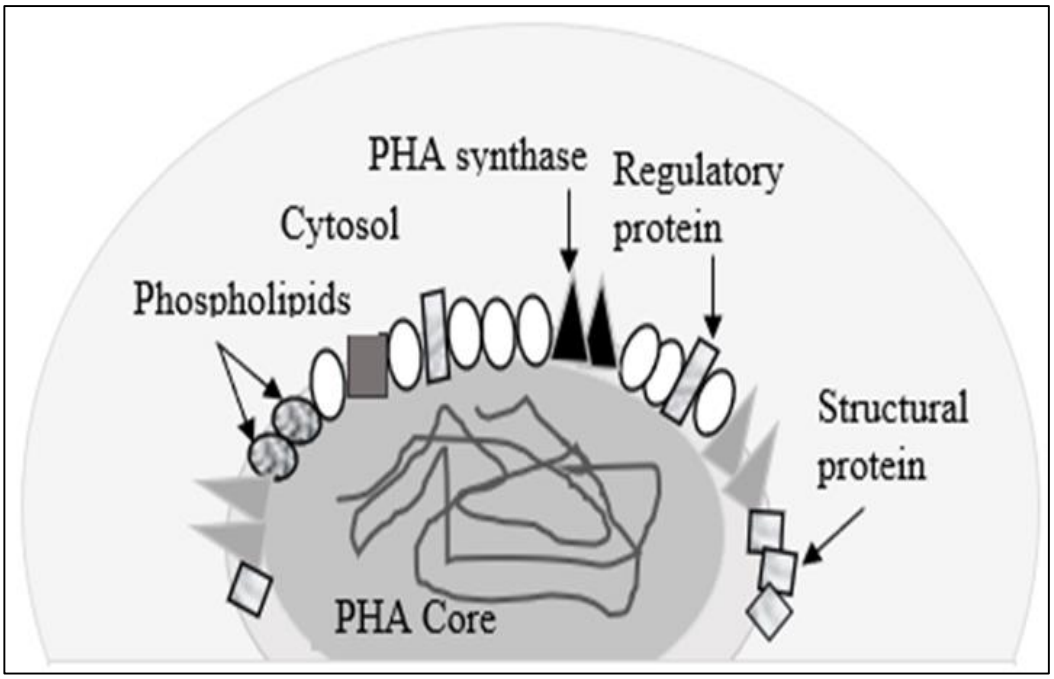


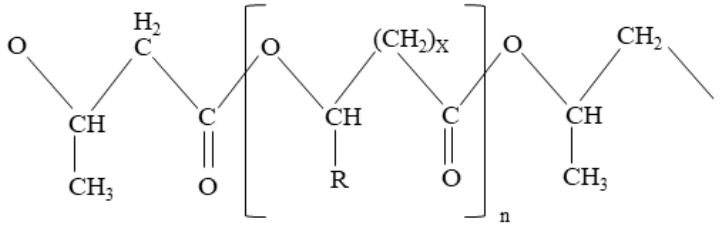
Figure 2.1: Schematic of a PHA granule

[Source: Modified from (Parlane et al., 2016)].

The size of a PHA granules varies between 200 and 500 nm in diameter depending on the Phasin protein. PHA exist in either amorphous or crystalline forms having a density of 1.18 g/cm^3 and 1.26 g/cm^3 respectively (Sudesh et al., 2000). PHA protects the cells against osmotic shock, desiccation, ultraviolet irradiation, oxidative stress, channels carbon compounds to metabolic pathways and regulates intracellular energy flow (Jendrossek, 2009). In addition, PHAs are biocompatible in human blood (Akaraonye et al., 2010; Rydz et al., 2015) due to the presence of 3-hydroxybutyric acid. Thus, utilised as medical devices such as a thread for knitting, syringes and for drugs delivery. Upon enzymatic or chemical hydrolysis, PHA releases monomers that can be converted into important commercial molecules such as 3-hydroxyacids, 3-hydroxyacids-esters, 3- hydroxyalkanols and acid lactones (Woodford, 2017).

There are over 150 monomer constituents of PHA classified into two, based on the number of carbon atoms. The SCL PHA contains 3-5 carbon atoms whereas, the

MCL PHA are made of 6-14 carbon atoms (Li et al., 2007). Examples of SCL PHA are P(3HB) and poly(3-hydroxyvalerate), [P(3HV)]. The MCL PHAs include P(3HB-*co*-3-HHx) and poly(3HB-*co*-3-hydroxyoctanoates). The first group is characterised by high crystallinity while the second category has elastomeric behaviours and adhesive properties (Solaiman et al., 2006). The melting temperature (T_m) of PHAs is in the range of 60-180°C (Sudesh et al., 2000) and the polymerisation degree (polydispersity index) about 2-4. The average molecular weight (M_w) ranges from 2×10^5 to 10×10^6 KDa depending on the type of PHA, the microorganism and its growth conditions (Chen, 2010). The chemical structure of PHAs and some of the identified monomers are shown in Figure 2.2. The most common range of PHA applications is in food packaging, agriculture, and medical and surgical devices such biodegradable carriers, non-woven patches, scaffolds for tissue engineering and implants (Lalan et al., 2001).



X=1	R= hydrogen	Poly (3-hydroxypropionate)
	R= methyl	Poly (3-hydroxybutyrate)
	R= ethyl	Poly (3-hydroxyvalerate)
	R= propyl	Poly (3-hydroxyhexanoate)
	R= pentyl	Poly (3-hydroxyoctanoate)
	R= nonyl	Poly (3-hydroxydodecanoate)
X=2	R= hydrogen	Poly (4-hydroxybutyrate)
	R= methyl	Poly (4-hydroxyvalerate)

Figure 2.2: The general structure of PHA
[Source: Modified from (Lee and Chang,1995a)].

2.3.1 Polyhydroxybutyrate [P(3HB)]

P(3HB), is the most extensive and well characterized simplest form of PHA discovered by Lemoigne from *Bacillus megaterium* (Lemoigne, 1926). It has M_w ranging from 1,000,000 to 3,000,000 Da depending on the microorganism and its growing conditions, the enzyme to substrate ratio, enzyme specificity and the intracellular concentration of the PHA synthase (PhaC) (Sudesh and Abe, 2010). P(3HB) has attracted commercial interest due to its thermo-plasticity and biodegradability (Angelini et al., 2016). Though, its applications are limited to packaging, agriculture, food and paint industry (Anderson and Dawes, 1990) because of poor mechanical properties, such as low elongation at break (1-15%), high brittleness and crystallinity (55-80%), high melting temperature (177°C) and low decomposition temperature (270°C) (Doi, 1995).

2.3.2 PHA Biosynthesis

The biosynthetic pathways for PHA occur in over 150 PHA-producing bacteria. To date, P(3HB) is the typical type of PHA synthesized through the action of three enzymes namely; PhaA, PhaB, and PhaC. The reaction (Figure 2.3) begins with the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA by PhaA (β-ketothiolase). This step can be inhibited when a high concentration of CoASH is released upon entry of acetyl-CoA into the TCA cycle. In the second step, acetoacetyl-CoA is converted to (*R*)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (PhaB). The final step is the polymerisation of (*R*)-3-hydroxybutyryl-CoA monomers into P(3HB) by PhaC. PhaC is the most important of the enzymes because it determines the type of PHA synthesised. Over 59 different PHA synthase, structural genes have been sequenced and cloned from different bacterial genera (Taguchi et al., 2002) and classified into four classes by primary

structures, substrate specificities and subunit compositions (Pötter and Steinbüchel, 2006). Each PHA synthase possesses a conserved region, an active site necessary for the polymerization reaction. Class I synthases represented by *C. necator* polymerize mainly SCL monomers [3HB and 3-hydroxyvalerate (3HV)] while class II by *Pseudomonas oleovorans* is more specific to MCL substrates such as CoA thioesters of (*R*)-3-hydroxy fatty acids of 6-14 carbons units (Pötter and Steinbüchel, 2005). Both classes I and II synthases have a single and common subunit (PhaC) of 60-70 kDa M_w. Class III synthases represented by *Allochromatium vinosum* is made up of two subunits, a 40 kDa M_w PhaC subunit with amino acid sequence similarity of 21-28% to classes I and II PHA synthase and PhaE subunit of 40 kDa having no resemblance to PHA synthases. Class III PHA synthase has a preference for CoA thioesters of (R)-3-hydroxy fatty acids of 3-5 carbon atoms. While, Class IV PHA synthases represented by *B. megaterium* contains PhaC and PhaR subunits of 40 kDa M_w (Antonio et al., 2000; Yuan et al., 2001).

Although the activity of PhaC in *C. necator* is towards the SCL-HA monomers, the incorporation of the monomers of 4 and 5-HA apart from the 3-HA, confirmed that the position of the oxidized carbons in the monomer is not a critical factor (Sudesh et al., 2000). For instance, P(3HB-*co*-3HV) copolymer was synthesized in *C. necator* H16 upon addition of propionic acids as an additional carbon source. P(3HB-*co*-3HV) biosynthesis is determined by the availability of 3-hydroxyvaleryl-CoA (3HV-CoA). The Propionic acid 3HV-CoA is first converted to propionyl-CoA as acetyl-CoA is generated from the TCA cycle. The condensation of propionyl-CoA and acetyl-CoA into 3-ketovaleryl-CoA and acetoacetyl-CoA respectively is facilitated by 3-ketothiolase. The products are then reduced to 3HV-CoA and 3HB-CoA by NADPH-

dependent acetoacetyl-CoA reductase. Finally, PhaC polymerizes both monomers to P(3HB-*co*-3HV) (Suriyamongkol et al., 2007).

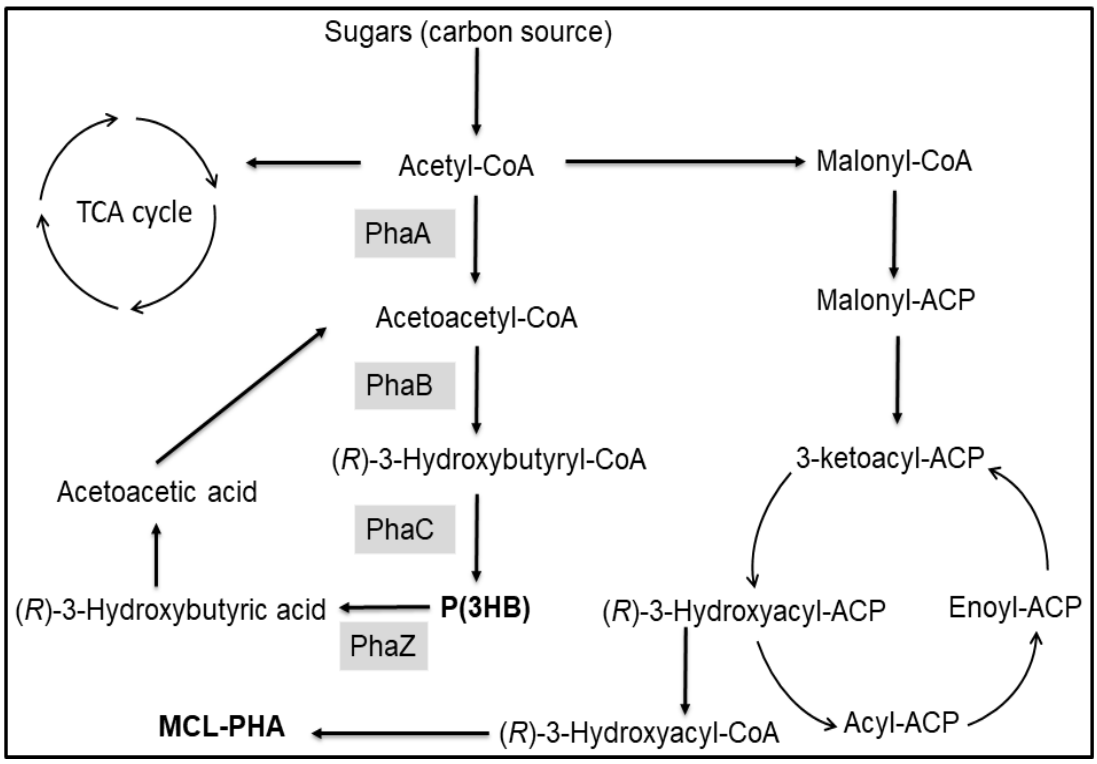


Figure 2.3: Biosynthesis pathways of P(3HB)/MCL-PHA and intracellular mobilization of accumulated P(3HB). PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaZ, PHA depolymerase

2.4 Carbon sources for PHA production

PHAs can be synthesised via the fermentative process (Anderson and Dawes, 1990) using a wide range of substrates such as plant (vegetable) oils, glucose, sucrose, molasses, by-products, whey glycerol and fatty acids (Sudesh, 2012). Vegetable oils are the most commonly used feedstocks by the polymer industries due to availability, low toxicity, comparatively low price, and biodegradability (Lu and Larock, 2009). Vegetable oil have shown great potential as source of carbon for PHA production, due to its higher carbon content compared to sugar; therefore, a more preferred substrate

in achieving higher cell biomass and PHA yield. Theoretically, a gram of oil produces about 0.6-0.8 g of PHA compared to only 0.3-0.4 g of PHA per gram of sugar substrates (Tsuge, 2002; Akiyama et al., 2003). The triacylglycerols (TAGs) composition of the oil is responsible for the higher carbon content of fat. Lipase secreting bacteria hydrolyses oil into monoglycerides, diglycerides, free fatty acids and glycerol (Jaeger et al., 1999; Brigham et al., 2010). According to Fukui and Doi (1998), plant oils for PHA biosynthesis is predicted to reduce the production cost of bacterial polyesters (Fukui and Doi, 1998). Various plant oils such as palm oil, rapeseed oil, frying oil, inedible jatropha oil, *Amygdalus pedunculata,* African elemi, bitter apple and desert date oils have been evaluated as carbon sources for PHA production by *C. necator* (Kahar et al., 2004; Loo et al., 2005; Obruca et al., 2010; Verlinden et al., 2011; Zainab-L et al., 2018).

2.5 PHA producing bacteria

Over 300 microorganisms ranging from photosynthetic bacteria, archaebacteria, to gram-negative and positive bacteria are capable of accumulating P(3HB) under unfavourable growth conditions (Madison and Huisman, 1999; Valdés-García et al., 2017). Though, not all are suitable for large scale production, certain bacteria such as *C. necator*, *B. megaterium*, *Azotobacter vinelandii*, *Alcaligen latus*, *Pseudomonas putida,* and recombinant *E. coli* have been extensively studied. *C. necator* H16 is a non-pathogenic, gram-negative soil bacterium formerly known as *Hydrogenomonas eutrophus, Alcaligenes eutrophus, Ralstonia eutropha,* and *Watersia eutropha.* This coccoid rod-shaped bacterium reproduces by binary fission and grows optimally at 30°C, pH range between 7 and 8 in an aerobic condition, or occasionally under anaerobic conditions. When cultivated on a nutrient agar plate for

two days at 30°C, the colonies of *C. necator* are off-white, smooth and convex with the entire edge having a diameter around 2-4 μm (Casida, 1988).

Bacterial selection for an industrial process is governed by several factors such as; bacterial safety and stability, growth rates, ability to utilise different carbon sources of low cost, achievable cell densities, PHA accumulation and contents, polymer extractability, molecular weights of the product and by-products occurrence (Kessler and Witholt, 2002). Both the wild-type and recombinant strains of *C. necator* are important organisms in biotechnology for their ability to utilise multitude of carbon sources for growth and biosynthesis of large quantities of both homopolymers and copolymers of PHA (Muhammadi et al., 2015). *C. necator* metabolises oils, fatty acid, lactate, fructose, acetate, succinate, and mannose for cell growth and P(3HB) accumulation but not glucose, glycerol, lactose, xylose, and rhamnose (Sharma et al., 2016). Earlier studies have reported PHA production using *R. eutropha* using plant oils including and its by-product as carbon sources (Fukui and Doi, 1998; Akiyama et al., 2003; Lee et al., 2008). *C. necator* secretes extracellular lipase into the culture medium containing oil to hydrolyse the triglycerides into fatty acids (Brigham et al., 2010). The fatty acids are then incorporated into the cells and metabolised to acetyl-CoA via the β-oxidation pathway for fatty acids. 3HB-CoA monomer is synthesized from two molecules of acetyl-CoA, via a two-step reaction catalysed by β-ketothiolase and NADPH-acetoacetyl-CoA reductase (Steinbüchel, 1996). As shown in Table 2.1, *C. necator* is capable of accumulating PHA up to 80% of the CDW (Lee, 1996; Lee and Choi, 1999). A CDW of 118-126 g/l containing 72-76 wt% P(3HB) was achieved using the wild-type strain. The inability of *C. necator* H16 to grow on glucose prevented its use for P(3HB) production on sugar-based biomass (Fukui et al., 2014). However, the recombinant strain utilised glucose and glycerol as carbon sources and

accumulated up to 80% and 70% (w/w) of P(3HB) respectively (Holmes, 1985). A recombinant PHA-negative mutant harboring the *Aeromonas caviae* PHA synthase gene, *phaCAc*, produced poly(3HB-*co*-5mol%3HHx) with a CDW and PHA content of 128-138 g/l and 71-74 wt%, respectively. At present, a Metabolix (USA) uses a glucose-utilising recombinant *C. necator* to produce and commercialize P(3HB-*co*-3HV) under the trade name of Biopol (Chai et al., 2013; Vigneswari et al., 2014).

Table 2.1: PHA accumulating bacterial stains and the carbon sources utilised

Bacteria	Substrates	Type of PHA	% PHA content	References
Burkholderia cepacian, DSM 50181	Glycerol	P(3HB)	31.3	(Zhu et al., 2010)
Bacillus megaterium QMB 1551	Gluconate	P(3HB)	46-85	(Liebergesell et al., 1994)
Alcaligenes eutrophus	Propionate	P(3HB)	26-36	(Park et al., 2014)
Pseudomonas putida	Terephthalic acid from polyethylene	mcl-PHA	27	(Kenny et al., 2008)
C. necator H16	Fructose + valerate	P(3HB-*co*-3HV)	86	(Du et al., 2001)
C. necator NCIB 10442	Palm oil, corn oil, olive oil, oleic acid	P(3HB), P(3HB-*co*-3HV)	3.9-40.7	(Fukui and Doi, 1998)
Wautersia eutropha Mutant	Palm kernel oil, crude palm oil, palm acid oil, and palm olein	P(3HB-*co*-3HHx)	87.0	(Loo et al., 2005)
C. necator Re2058/pCB113	Crude palm kernel oil and oil palm tree trunk sap	P(3HB-*co*-3HHx)	8-73	(Murugan et al., 2016b)
Wild and recombinant *C. necator*	Desert date oil, bitter apple oil, African elemi oil, and *Amygdalus pedunculata* oil	P(3HB), P(3HB-*co*-3HHx)	36-71	(Zainab-L et al., 2018)

2.6 High cell density (HCD) production of PHA

Amid the primary aims for the biotechnological process development is to maximize volumetric productivity (g/L/h) and achieve the highest density of products within a particular time. HCD cultivation targets microbial dry biomass concentrations higher than 100 g/L (Aragão et al., 1996). It was first initiated for yeasts in the production of single-cell protein, ethanol, and biomass (Riesenberg and Guthke, 1999). Other products such as antibiotics and polyhydroxyalkanoic acids were subsequently developed from fed-batch dense cultures of streptomycetes and methylotrophs respectively (Suzuki et al., 1987; Lee and Chang, 1995b). High cell density process favour PHA production, especially in terms of lower production costs and reduction of residual liquids compared to the low-cell-density methods (Brigham et al., 2011).

Over the past decade, several attempts were made to increase both biomass and PHA yields. A high PHA productivity cultures can arise from phosphate, nitrogen or other nutrient limitation. P(3HB) productivities of more than 1.0 g/L/h were observed when *C. necator* was grown in fed batch culture using sugar substrates (Kim et al., 1994; Ryu et al., 1997). Two stage culture systems have also been examined for maximisation of P(3HB) production by *C. necator*, where the initial stage served as cell growth, producing maximum biomass, and the second stage constituted P(3HB) accumulation. These two stage cultures exhibited a maximum productivity of 1.2 g/L/h with >70% PHB per CDW (Du et al., 2001). Typically, with PHA production in *R. eutropha* strains, nitrogen plays the role of limiting nutrient to trigger polymer biosynthesis and high productivity has been seen using phosphate limitation, also, with > 1.5 g PHB/L/h (Shang et al., 2003).

Presently, repeated batch, fed-batch, and continuous cultures are the dominant culture strategies used for efficient microbial fermentation. Fed-batch fermentation system increases the system productivity and reduces production costs, hence the most widely used approach in reaching HCD with the highest potential amount of P(3HB) (Akaraonye et al., 2010). In fed-batch fermentation, cells are grown in a bioreactor under a batch regime for a while, until near the end of the exponential growth phase. Nutrients are then fed into the bioreactor without the removal of culture broth until the anticipated volume is achieved. Nutrient addition improves the specific growth rate of the cells and prevents the production of by-products that may lead to premature cell death (Ienczak et al., 2013). PHA production in fed-batch fermentation with *C. necator* is non-growth-associated thus carried out in two phases; a growth phase in which the cells are grown to the required concentration, and a PHA accumulation phase (under nutrient limitation) wherein carbon sources are fed in excess. Carbon source limitation in the accumulation phase may lead to the degradation of the intracellular granules owing to PHA depolymerase enzyme activity (Aragão et al., 1996). A CDW of 111.7 g/L and 88 wt% P(3HB) were achieved from *Alcaligen latus* grown on sucrose under nitrogen limitation in a 6.6 L jar fermenter (Wang and Lee, 1997). A separate study reported 118-126 g/L CDW, 72-76 wt% P(3HB) in *C. necator* cultivated on soybean oil under phosphorous limitation in a 10 L fermenter (Kahar et al., 2004). PHA still holds a small fraction of the total global plastic market.

2.7 Challenges of industrial PHA production

Though, PHA has wide range of applications, its industrial production is hampered by high costs of carbon substrate and the downstream recovery (Leroy et al., 2012). In 2006, the price of P(3HB) was about € 10 per kg compared to € 1.15/kg

and € 1.47/kg for polyethylene and polypropylene respectively (Chanprateep, 2010). Industrial production and commercialisation of PHAs are still struggling to compete with conventional plastics in terms of price. Eventually, the production by fermentation depends on the cost of the substrate, the PHA yield per substrate and the efficiency of the downstream processing (Yamane et al., 1996). About 45% of the production cost arises from the use of high purity substrates and large quantities of solvents for polymer extraction (Choi and Lee, 1997; Kourmentza et al., 2017). These factors earlier prevented the operations of several biopolymer companies. The first PHA producing company in the United States (W. R. Grace) closed down in 1959 due to low production efficiency and lack of suitable purification systems. The P(3HB-*co*-3HV) commercialization by Imperial Chemical Industries Ltd, UK, also failed in 1970 and was sold to Monsanto in 1996, then bought over by Metabolix, Inc. PHA production was improved through the use of cheaper carbon sources such as waste streams, dairy waste, molasses, oil palm empty fruit bunch, sugarcane bagasse and bio-wastes (Silva et al., 2004; Kumar et al., 2009). For instance, a food and wastewater treatment plant successfully converted volatile fatty acids waste to 65 wt% PHA by sludge fermentation (Chen et al., 2013).

The need for sterile conditions affected the total economics of P(3HB) production. The pure culture was subsequently replaced with open-mixed microbial cultures (MMC) (Budde et al., 2011; Jeon et al., 2014). Production of PHA by MMC was cheaper as it does not require the sterilisation of bioreactor (Reis et al., 2003). Up to 89 wt% PHA content was achieved within 7.6 h fed-batch fermentation using MMC (Moita et al., 2014). A Similar result was reported from the cultivation of MMC on lactate (Jiang et al., 2011). The replacement of wild strain with recombinant strain or selection of a suitable substrate is another strategy to lessen the production cost

substantially. For instance, the Biopol™ selling price of P(3HB) synthesised from wild *A. eutrophus* was $ 6-16/kg compared to $ 0.25-0.5/kg for Polypropylene (PP) or Polyethylene (PE) (Choi and Lee, 1997; Khanna and Srivastava, 2005), but the price decreased to $ 4/kg when the strain was substituted with recombinant *E. coli*. (De Koning et al., 1997). These developments boosted the industrial production of PHA and companies like Mitsubishi Gas Chemical Company, Japan, Kaneka Corporation, Japan, Biomatera Inc. Canada, Biomer Inc. The production cost of P(3HB) and its copolymers is still higher that of conventional non-biodegradable plastic. Therefore, several attempts are being made to utilise inexpensive and renewable carbon sources to enable the commercialisation and widespread use of these polymers (Sudesh, 2012).

2.8 Commercialisation of PHA

The commercialisation of PHA under the trade name Biopol™ by Imperial Chemical Industries (ICI) dates back to 1980s. Commercialised bio-polymers (PHA, PLA, and starch-based thermoplastics) have demonstrated strong market growth with market analysis showing between 10% and 30% growth per annum. The markets for Bio-based polymer have been dominated by biodegradable food packaging and food service applications (De Jong et al.,2012). Currently, Germany, Brazil and industrial S.A., are producing PHA on a pilot scale with a production capacity of 1,000 to 50,000 tons per year, compared to 300,000 tons of polyethylene (Chanprateep, 2010). The selling price of the PHA was affected by the production capacity of the company. For instance, Mitsubishi Gas Chemical Company having 10,000 tons capacity sold P(3HB) at € 2.5-3.0/kg under the trade name BioGreen™ (Kosior et al., 2006). Other biopolymer companies such as Telles US having 50 tons capacity sold P(3HB) at € 3.0-5.0/kg whereas, Biomer Inc. Germany producing at 50,000 tons commercialised

at € 1.50/kg. PHA from octane was sold at about US $ 5-10/kg. The type of carbon substrate used also influences the selling price of PHA. For example, the selling price of PHAs produced using waste materials was about € 3.51/kg (Hazenberg and Witholt, 1997). Biomatera Inc. manufacture used agricultural residues fermentation to produce biopolymers for wide range of applications such as in creams and gels manufacturing for slow-release agents in the production of cosmetic, and tissue matrix regeneration (Chanprateep, 2010). In the current situation, it is hard for PHA to compete with the mass-produced petroleum-derived synthetic plastics, such as polyethylene and polypropylene, as their manufacturing prices are below 1 USD/kg, which is much lower than the cost of manufacturing PHA.

2.9 PHA recovery from microbial cells

The recovery of PHA plays a vital role in the overall production cost. PHA is an intracellular product, that requires cell pretreatment and extraction methods for its isolation and purification. The extraction of PHA granules is the major operation in PHA recovery. The downstream process accounted for about 30% of the PHA production cost. PHA recovery are composed of three steps, namely; pretreatment, extraction and purification. Pretreatment and purification steps could be added before and after each extraction step. The pretreatment step aims at improving the cell disruption process. It basically involves separating the cell biomass from the aqueous environment after fermentation, achieved by centrifugation or filtration. Followed by a drying step to get rid of water residue. Since moisture causes a decrease in the resulting PHA molecular weight. PHA extraction from bacterial cells requires initial rapture of the cell to remove the protein layer covering the granules, with subsequent