MIXED CULTURE POLYHYDROXYALKANAOTES (PHA) PRODUCTION USING CRUDE GLYCEROL AS CARBON SOURCE

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MIXED CULTURE
POLYHYDROXYALKANOATES (PHA) PRODUCTION
USING CRUDE GLYCEROL AS CARBON SOURCE

ABSTRACT

Polyhydroxyalkanoates (PHA) is a biopolymer that has the prospect of becoming a preferred renewable and environmentally friendly resource with various applications including in manufacturing and medicine. Intensive studies have been carried out to find the most practical and economical method for PHA production because current commercial production of PHA is stringent and extremely costly. The aim of this study is to enrich PHA-accumulators with high PHA production capabilities by evaluating the stability of the enrichment process and by observing the effect of organic loading rate (OLR) on the production potential. In this study, PHA-accumulators were cultivated using activated sludge with crude glycerol in a sequencing batch reactor (SBR). An aerobic dynamic feeding (ADF) strategy was employed for the enrichment process. This is then followed by production tests in a batch reactor to test for the maximum PHA production. This study successfully maintained a long-term enrichment operation where the PHA-accumulators produced a maximum PHA content of 80 wt % in biomass dry weight. A production yield of 0.7 mg C PHA/mg C substrate was obtained with a productivity ranging from 193 mg/(L·h) to 236 mg/(L·h) where the maximum production time is 6 hours. The increase of OLR was found to have insignificant effect on the final PHA production. However, it highlighted the robustness of the enriched PHA-accumulators and had significant effect on the biomass concentration. The increase of OLR from 360 mg C/(L·d) to 1000 mg C/(L·d) led to the increment of biomass concentration from less than 0.7 g/L to 2 g/L. An OLR of 1000 mg C/(L·d) was the

highest OLR feasible in this study and a higher OLR of 1250 mg C/(L·d) was found to be detrimental to the enrichment stage. The PHA-accumulators were found to accumulate a copolymer with 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) monomers at a HB:HV of 60:40. The PHA-accumulators were observed to be highly robust and the accumulation of copolymers is eminently a promising attribute. The feasibility of directly using waste substrate and mixed cultures for PHA production can help reduce the PHA production operational costs. This method also provides an option for resource recovery operation in converting waste to value-added products, paving the way for a more sustainable process.

Keywords: mixed culture, polyhydroxyalkanoates, crude glycerol, organic loading rate.

PENGHASILAN POLIHIDROKSIALKANOAT (PHA)
DENGAN KULTUR BAKTERIA CAMPURAN MENGGUNAKAN GLISEROL MENTAH SEBAGAI SUMBER KARBON

ABSTRAK

Polihidroksialkanoat (PHA) adalah biopolimer yang mempunyai prospek menjadi bahan mentah yang boleh diperbaharui dan mesra alam yang menarik dengan pelbagai aplikasi termasuk dalam pembuatan dan perubatan. Kajian intensif telah dijalankan untuk mencari kaedah yang paling praktikal dan ekonomi untuk pengeluaran PHA kerana pengeluaran komersial PHA yang sedia ada adalah sangat ketat dan sangat mahal. Tujuan kajian ini adalah untuk memperkayakan pengumpulan PHA dengan keupayaan pengeluaran PHA yang tinggi dengan menilai kestabilan proses pengayaan dan dengan memerhatikan kesan kadar loading organik (OLR) terhadap potensi pengeluaran. Dalam kajian ini, PHA-akumulator ditanam menggunakan enapcemar diaktifkan dengan gliserol mentah dalam reaktor kelompok penjujukan (SBR). Strategi pemakanan dinamik aerobik (ADF) digunakan untuk proses pengayaan. Ini kemudian diikuti dengan ujian pengeluaran dalam reaktor kumpulan untuk menguji pengeluaran PHA maksimum. Kajian ini berjaya mengekalkan operasi pengayaan jangka panjang di mana PHA-akumulator menghasilkan kandungan PHA maksimum sebanyak 80% berat dalam berat kering biojisim. Hasil pengeluaran 0.78 mg C PHA / mg C diperolehi dengan produktiviti dari 193 mg / (L · h) hingga 236 mg / (L · h) di mana waktu pengeluaran maksimum adalah 6 jam. Peningkatan OLR didapati tidak memberi kesan ke atas pengeluaran PHA akhir. Walau bagaimanapun, ia menonjolkan kekukuhan penumpuk PHA yang diperkaya dan mempunyai kesan yang ketara ke atas kepekatan biomas. Peningkatan OLR dari 360 mg C / (L · d) hingga 1000 mg C / (L · d) membawa kepada

penambahan kepekatan biomas daripada kurang daripada 0.7 g / L hingga 2 g / L. OLR 1000 mg C / (L · d) adalah OLR tertinggi yang boleh dilaksanakan dalam kajian ini dan OLR yang lebih tinggi iaitu 1250 mg C / (L · d) didapati merosakkan peringkat pengayaan. Pengumpul PHA didapati mengumpul kopolimer dengan monomer 3-hydroxybutyrate (3HB) dan 3-hidroksivalerat (3HV) pada HB: HV 60:40. Pengumpul PHA diperhatikan sangat teguh dan pengumpulan kopolimer adalah penemuan yang penting. Kebolehan menggunakan substrat bahan buangan secara terus dan kultur bakteria campuran untuk pengeluaran PHA dapat membantu mengurangkan kos operasi pengeluaran PHA. Kaedah ini juga menyediakan pilihan untuk operasi pemulihan sumber dalam menukar sisa kepada produk nilai tambah yang membuka jalan untuk proses yang lebih mampan.

Kata kunci: kultur bakteria campuran, polihidroksialkanoat, gliserol mentah, kadar loading organik

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List of Symbols and Abbreviations

3HB	:	3-hydroxybutyrate
3HHx	:	3-hydroxyhexanoate
3HOHH	:	3-hydroxyoxanoate-co-hydroxyhexanoate
3HV	:	3-hydroxyvalerate
ADF	:	Aerobic dynamic feeding
CG	:	Crude glycerol
DO	:	Dissolved oxygen
DO %	:	Dissolved oxygen saturation
DOC	:	Dissolved organic carbon
DF	:	Dilution factor
F/F	:	Feast / Famine
FID	:	Flame ionization detector
FISH	:	Fluorescence *in situ* hybridization
GC	:	Gas chromatography
HA	:	Hydroxyalkanoate
HRT	:	Hydraulic retention time
IC	:	Inorganic carbon
LDPE	:	Low density polyethylene
mcl-PHA	:	Medium chain length polyhydroxyalkanoate
MLSS	:	Mixed liquor suspended solids
MLVSS	:	Mixed liquor volatile suspended solids
PE	:	Polyethylene

P(3HB-co-HV)	:	Poly-3-hydroxybutyrate-co-hydroxyvalerate
PHA	:	Polyhydroxyalkanoate
PHB	:	Polyhydroxybutyrate
PHBHX	:	Polyhydroxybutyrate-co-hexanoate
PHBP	:	Polyhydroxybutyrate-co-propionate
PHP	:	Polyhydroxypropanoate
PLA	:	Poly-lactic acid
PP	:	Polypropylene
PS	:	Polystyrene
PTFE	:	Polytetrafluoroethylene
PVC	:	Polyvinyl chloride
q_s	:	Substrate uptake rate
q_{PHA}	:	PHA accumulation rate
SBR	:	Sequencing batch reactor
scl-PHA	:	Short chain length polyhydroxyalkanoate
SRT	:	Sludge retention time
SS	:	Suspended solids
rpm	:	Rotation per minute
TC	:	Total carbon
TOC	:	Total organic carbon
TSS	:	Total suspended solids
VSS	:	Volatile suspended solids
vvm	:	Air volume per reactor volume in one minute

W_0	:	Initial weight of crucible
W_{103}	:	Weight of crucible after heating in the oven at 103°C
W_{550}	:	Weight of crucible after heating in the furnace at 550°C
$Y_{O_2/S}$	:	Respiration yield
$Y_{PHA/S}$	:	PHA yield
$Y_{X/S}$	:	Biomass yield

CHAPTER 1: INTRODUCTION

1.1 Research background

Polyhydroxyalkanoates (PHA) is a biopolymer which is naturally produced by certain group of bacteria intracellularly as energy storage compounds. Since the discovery of PHA in 1925, it has been widely studied. One of the main motivations for PHA studies revolves around its potential of being a raw material for various applications from manufacturing to medicine since it shares physical and chemical characteristic with petro-chemical plastic (Madison & Huisman, 1999). PHA has additional advantages by being completely biodegradable in nature, renewable, and biocompatible making it a highly attractive resource (Yu et al., 2006). Research related to PHA is still intense because of these functionality and benefits that PHA has as a raw material.

The biggest competitor in the PHA industry is the conventional petro-chemical plastic with chemical and physical properties that provide high durability and versatility for wide range of applications. However, due to low degradation rate and non-biodegradable properties of plastic, it is now accumulating in the environment as pollutants and posing numerous environmental problems (Urtuvia et al., 2014). To be sustainable in the global market, PHA needs to be able to compete with other existing materials in terms of cost and capacity which is a major problem that the PHA production industry is facing. The biggest problem faced by the PHA industry in current commercial practice is the high production cost which is mainly contributed by the raw material used and the cultures used (Schmidt et al., 2016). Besides that, the strict operational procedure requiring aseptic techniques and high contamination risks also results in a complicated system (Wang et al., 2014).

To combat the aforementioned problems in PHA production and its commercialization, Rodriguez-Perez et al. (2018) have summarized key areas that are looked into. These areas revolves around reducing the operational cost, increase yield production, obtaining valuable and functional PHA polymers, and identifying the best PHA production process suitable for industrialization. Different carbon substrates from various sources are investigated to identify which substrates are most suitable as precursors for the production of PHA with high functionalities. Different type of microbial cultures including pure cultures, recombinant microorganisms, and mixed cultures have also been explored. To contribute to the expanding knowledge related to the study of PHA production, this study will focus on the use of waste substrate as the raw material and mixed cultures specifically activated sludge as the PHA accumulating organisms (henceforth named PHA-accumulators).

At the industrial scale, PHA production using pure cultures and pure substrates achieve PHA content up to 90% with cell densities reaching more than 150 g/L (Chen, 2009). This is not the case for mixed culture and waste substrate PHA production where the PHA content achieved can range from 40% to 70% with low cell densities. Examples of the waste substrates studied includes fermented molasses (Albuquerque et al., 2010), palm oil mill effluent (Lee et al., 2015), olive oil pomace (Waller et al., 2012), and crude glycerol (Moita et al., 2014). The problem when using waste substrate and mixed cultures is associated with the stability of the process specifically the cultivation stage and consequently the low PHA production efficiency. This study wants to address on the stability of the cultivation stage for the long-term enrichment of PHA-accumulators. Besides that, this study also plan on looking at how to increase the efficiency of the PHA production and what the production performance will be when the concentration of carbon loading is changed during the cultivation stage.

1.2 Research objectives

Based on the research problems described above, the following objectives have been outlined for this study:

i. To evaluate the long-term stability of the cultivation reactor for the enrichment of PHA-accumulators.
ii. To study the effect of organic loading rate (OLR) on the stability of the cultivation reactor and enriched culture.
iii. To evaluate the performance of the PHA production with crude glycerol as the substrate.

1.3 Structure of dissertation

There are five chapters in this dissertation and the content of each chapters are briefly described as below:

a) Chapter 1: Introduction

This chapter provides a brief overview on the development of PHA and the drawbacks that leads to the motivation and research question to carry out this study. It also includes the three objectives that will be investigated.

b) Chapter 2: Literature review

This chapter presents a review on the microbial polymer that exists and how PHA as one of the biopolymer can be used in various applications. It also includes details on commercial production of PHA, the development in studies to find alternative PHA production methods, and the bioreactor operation that is needed for this process.

c) Chapter 3: Methodology

This chapter has five sections where the first four sections will describe in detail the operations of the two bioreactor used in this study. The fifth section elaborates all the analytical methods used in the study.

d) Chapter 4: Results and discussions.

Chapter 4 discusses the long-term stability of the cultivation reactor, the effect of changing the organic loading rate (OLR) on the stability of the cultivation reactor, PHA accumulation, and lastly on the PHA production performance of the enriched sludge cultivated.

e) Chapter 5: Conclusions and recommendations

This chapter gives an overall conclusion that the study has achieved as well as the impact that it has in the discipline and to the society. Further recommendations for future investigations are also listed.

CHAPTER 2: LITERATURE REVIEW

2.1 Microbial growth response and storage response

Microbial growth is defined as the increase in number of cells which is achieved by cell division (Black, 2008). Growth occurs under optimum growth conditions involving both physical and nutritional factors. Physical factors include pH, temperature, moisture, osmotic pressure, and oxygen. On the other hand, nutritional factors include carbon source, nitrogen source, sulfur and phosphorus, and trace elements. Under ideal growth conditions where they are able to grow and multiply, the microbes are said to have a growth response. Microorganisms are known to have the ability to thrive in various extreme living conditions as well as in irregular growth environment. Depending on the changes in their environment, they are able to adapt by altering and regulating their metabolic process.

Besides controlling their growth and metabolic process, microbes also survive through a storage response (Campos et al., 2014). This occurs when microbes are in a non-ideal growth environment where one or more growth factors does not support their normal growth rate. For instance, a non-ideal growth condition can be when carbon source availability is sporadic. In order to adapt, microbes are able to convert the carbon source available to them into polymers to be stored inside their cells (Dawes, 1992). These stored polymers will act as energy reserves available for the microbes to metabolize as their energy source when the conditions permit. (Campos et al., 2014; Lee, 1996).

The storage response that occurred through intracellular polymerization by the microbes is beneficial for them. By converting the soluble carbon into insoluble molecules, the osmotic pressure of the cell is not affected and leakage of these newly formed energy reserves are also prevented (Dawes & Senior, 1973; Madison & Huisman, 1999). There are several polymers that are identified as microbial energy reserves such as poly-phosphate, poly-glucose, and poly-saccharide.

2.2 Microbial intracellular PHA

PHA is a type of storage polymer which gained popularity by having the potential of replacing conventional plastics that are petroleum based. PHA is known to have similar properties with polypropylene and polyethylene (Khanna & Srivastava, 2005) but more importantly have the additional advantage of being biodegradable (Akaraonye et al., 2010; Kleerebezem & van Loosdrecht, 2007). PHA in the cell is amorphous and occurs in discrete inclusions of 0.2 – 0.5 μm in diameter. Depending on the type of microorganisms and their growth condition, the molecular weight of the polymers can range between 200,000 – 3,000,000 Da.

PHA is a linear polymer composing of 3-hydroxy fatty acid monomers where the carboxyl group of one monomer forms an ester bond with the hydroxyl group of neighboring monomer. The polymer varies depending on its alkyl side chains which can be modified (Madison & Huisman, 1999). This leads to varying polymer that could randomly be a homopolymer (a polymer with one monomer) or a copolymer (a polymer with more than one monomer) depending on the type and proportion of the monomers. Figure 2.1 shows a representation of the generic PHA structure as well as a homopolymer and copolymers.

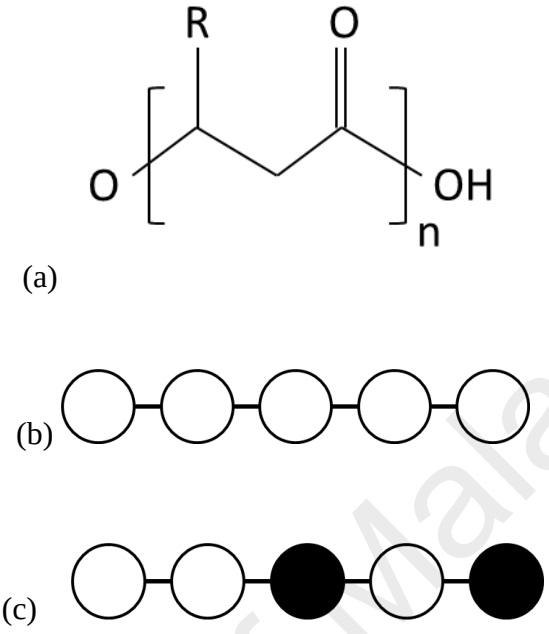

Figure 2.1: (a) PHA structure, 2.1 (b) homopolymer, and 2.1 (c) copolymers

The earliest and most common type of PHA found is a homopolymer called polyhydroxybutyrate (PHB). Another homopolymer found is polyhydroxyvalarate (PHV). Co-polymers that have been identified includes poly-3-hydroxybutyrate-co-valerate (PHBV) (Phukon et al., 2012), poly-3-hydroxybutyrate-co-3-hexanoate (PHBHx) (Chen et al., 2001), and poly-3-hydroxybutyrate-co-3-propionate (PHBP) (Shimamura et al., 1994). PHA can be categorized into short chain length PHA (scl-PHA) consisting of 3 to 5 carbon or medium chain length PHA (mcl-PHA) consisting of 6 to 14 carbons. According to Suriyamongkol et al. (2007), scl-PHAs have properties similar to conventional plastics while mcl-PHAs are generally regarded as elastomers and rubbers.

2.2.1 Metabolism of PHA

Since the discovery of PHA, researchers have been interested in the metabolic process of PHA production including the pathways, enzymes, and genes involved in the formation of PHA to further understand the production. Different species of bacteria would have a degree of variation in the metabolic pathways specific to their genetic coding. As an example, it is reported that different pathways are exhibited between *Ralstonia eutropha* (Anderson & Dawes, 1990) and *Pseudomonas oleovorans* (Lageveen et al., 1988).

Generally the polymerization of PHA has been outlined to have three major steps (Doi, 1990; Oeding & Schlegel, 1973). First is the condensation of two acetyl coenzyme (Acetyl-CoA) by the enzyme β-kethothiolase. The second reaction is the reduction of acetoacetyl-CoA to R-3-hydroxybutyryl-CoA by Acetoacetyl-reductase. Lastly, the R-hydroxybutyryl-CoA monomers are polymerized into PHB by the enzyme PHA synthase. PHA synthase enzyme has a broad substrate specificity (Sudesh et al., 2000) giving it the ability to synthesize numerous PHA polymer from various carbon sources.

One factor that determines the polymer formed is the carbon substrate available to the microbes (Santhanam & Sasidharan, 2010). Typically, carbon sources used for PHA production include sugars, vegetable oils, and fatty acids. As an example, Figure 2.2 provides an overview of how PHB can be formed from glucose, glycerol, and acetate. For glucose, it would go through the glycolysis process first where the end product is pyruvate. Pyruvate will then be turned into acetyl-coA through pyruvate decarboxylation before following the general PHB polymerization process as outlined earlier. Glycerol will also turn into pyruvate first before following the general PHB polymerization process. For acetate, it is directly transformed into acetyl-coA which then follows the polymerization steps to form PHB.

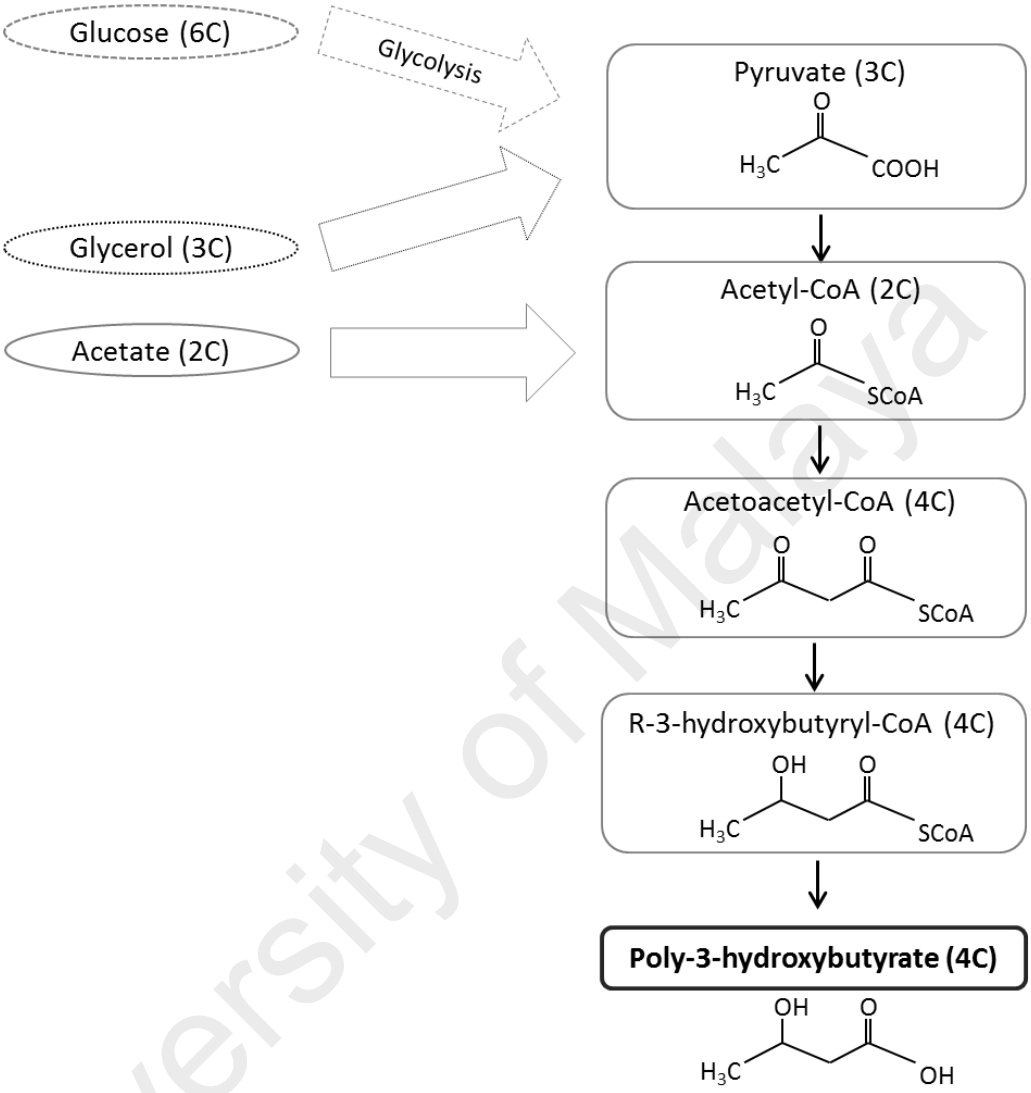

Figure 2.2: Overview of PHB metabolic pathway in microbial cells

2.3 Significant properties of PHA

PHA has several advantages over the conventional plastics that makes them a preferred alternatives. Those properties are elaborated further below along with some comparison to other types of plastics.

2.3.1 Thermoplastic properties

Plastic polymers are usually a thermoplastic which solidifies when cooled and becomes pliable when heated. The thermoplastic property is what makes these polymers flexible to be molded and shaped for their respective usage. An extensive review on the comparison of PHA properties with existing thermos-plastic has been reported by Laycock et al. (2014), Bugnicourt et al. (2014) and Shen et al. (2009). Example of these plastic polymers are poly-lactic acid (PLA), polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and polytetrafluoroethylene (PTFE). Important thermoplastic properties include thermal properties and mechanical properties. A PHA polymer is mostly compared to PP and PE which are excellent material used widely in food packaging, building material, and many more. This is because of their high melting temperatures, making them stable and long lasting. Applications of PP and PE are also highly dependent on their mechanical properties related to their strength and strain capabilities (Bugnicourt et al., 2014).

Figure 2.3 below shows comparison of the thermal properties and Figure 2.4 shows a comparison of the mechanical properties of different PHA compared to PP, PE, and PLA. When compared to PP and LDPE, PHA polymers PHB, PHV, PHOHH, and PHBV have higher melting temperature. For the glass transition temperature, the PHA polymers have a variety of temperature. PHA polymers will have different melting temperature and glass transition temperature depending on their structure and polymer composition. For their

mechanical properties, the Young's modulus and the elongation at break will be focused on. PP and LDPE are ductile materials that have high elongation at break point. Another advantage of PHA polymers are that they can be designed into copolymers to give the desired properties. For instance, the elongation of copolymers PHBV and PHBHx are higher than the homopolymer PHB and PHV. This characteristic can be taken into account during product design to fit the application better.

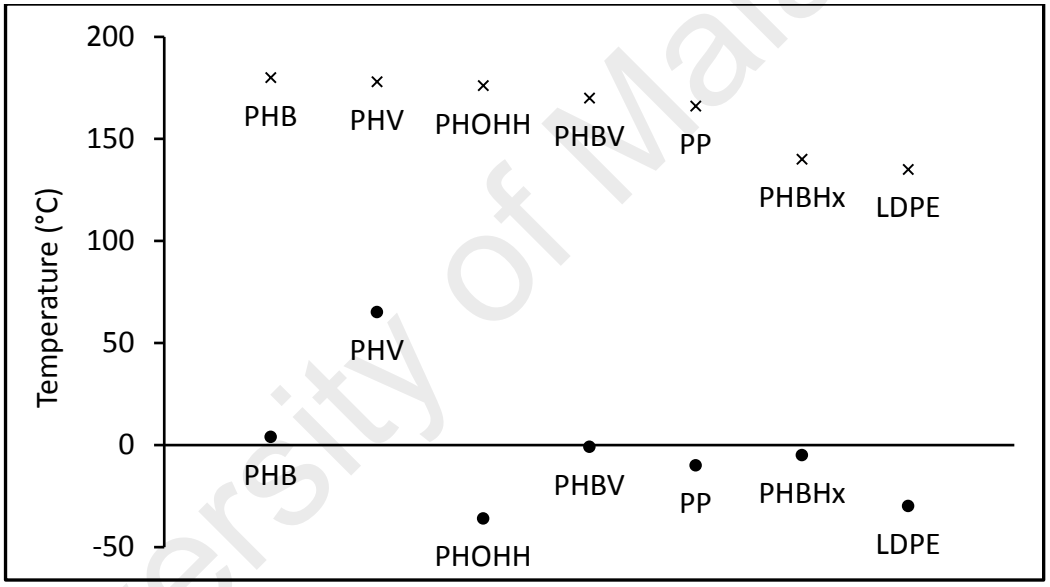

Figure 2.3: Thermal properties comparison between thermoplastic polymers where (×) is the melting temperature, and (•) is the glass transition temperature (Lacock et al., 2014)

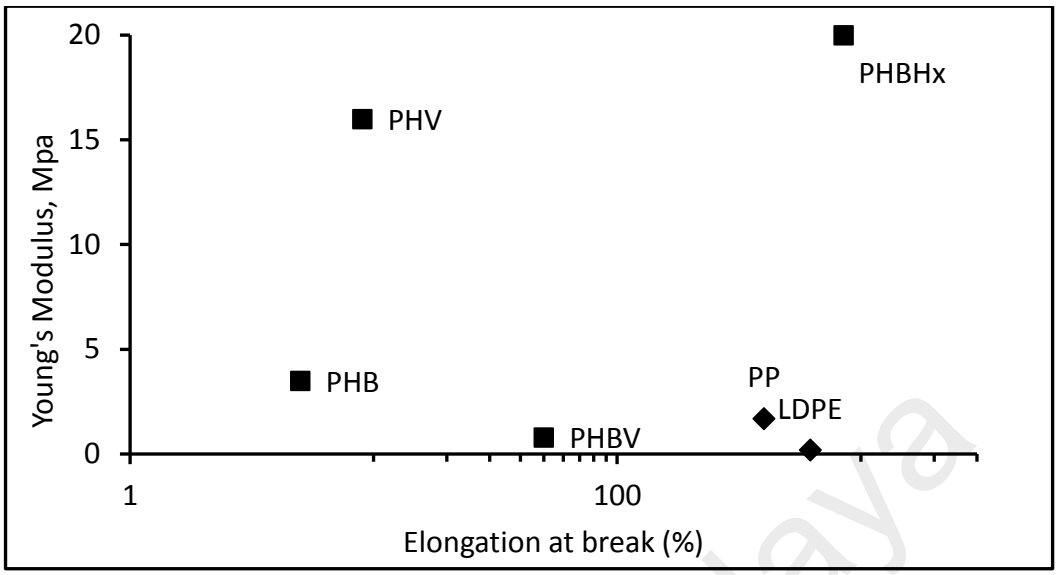

Figure 2.4: Mechanical properties comparison between thermoplastic polymers (Laycock et al., 2014)

2.3.2 Biodegradability of PHA

Biodegradable PHA as a renewable raw material makes it an attractive subject that is widely studied until today. As a storage polymer, it is degraded intracellularly by the accumulating organisms themselves when external carbon source is unavailable. York et al. (2003) reported that there could even be more than one intracellular depolymerase as seen in *Ralstonia eutropha* H16. PHA is also biodegradable in natural environments including soil (Mergaert et al., 1993), sea water (Doi et al., 1992), and lake water (Mergaert et al., 1995) by active enzymatic reaction. For example, studies have successfully isolated and purified functional depolymerase such as PHB depolymerase from *Alcaligens faecalis* T1 (Saito et al., 1989) and mcl-PHA depolymerase from *Streptomyces exfoliatus* K10 DSMZ 41693 (Martínez et al., 2015). With these depolymerase secreted into the environment, PHA material present can be degraded into the hydroxyalkanoate (HA) monomers, water, and

carbon dioxide under aerobic conditions while additional methane is produced if the degradation occurs under anaerobic conditions (Lee, 1996; Shah et al., 2008). Without any harmful by-products, the degradation of PHA makes it a compelling candidate for an environmental friendly material.

The biodegradability of PHA is not only influenced by the chemical structure and composition of the polymer that determines the polymer's crystallinity, melting temperature, side-chain length, and molecular weight. It is also influenced highly by the surface area, environmental pH, temperature, and the presence of natural organisms with biodegradation capabilities. For example, the biodegradability of PHA, PHB and copolymer PHBV have been investigated in a natural tropical coastal waters by Volova et al. (2010) and in a controlled environment by Shang et al. (2012). In the first study it was observed that there is no significant difference in the rate of biodegradation regardless of the different composition and crystalline properties and it was concluded that the rate of biodegradation depended on the shape and surface area of the polymer. However, in the later study it was observed that there was a difference in the rate of biodegradation that depended on the crystallinity of the polymer. With increasing HV fraction in the polymer, the crystallinity of the polymer decreased which improved the biodegradation rate. Along with other studies (Li et al., 2007; Numata et al., 2005) that also investigated the biodegradation of PHA polymers and copolymers, the rate of biodegradation did not follow the order of crystallinity but followed the order of side-chain length and molecular weight.

2.4 Applications of PHA

The novelty of PHA is due to its biodegradability, biocompatibility, and renewability (Urtuvia et al., 2014; Braunegg et al., 1998). With considerations to these properties, PHA can be applied in numerous sectors including manufacturing, medicine, pharmaceutical, and agriculture to name a few. One of the biggest motivations for PHA studies is for its application in the manufacturing industry. This potential is primarily due to their similar characteristic to petro-chemical plastics but as a more sustainable and environmentally friendly option (Bugnicourt et al., 2014). Along with the assortment of PHA polymer possibility, the PHA polymer can be designed and synthesized according to functions needed for the plastic thus increasing its appeal (Aldor & Keasling, 2003).

For example, the earliest application of PHA in the industry is BIOPOL™ produced by the company Imperial Chemical Industries, UK. It is a PHB based plastic having similar properties to PP. The polymer can be molded and injected making it suitable for producing everyday items such as shampoo bottles, disposable cutleries and cups, nappy linings, combs, and pens (Yogesh et al., 2012). It can also be used as packaging films for bags, containers, and paper coatings (Reddy et al., 2003). The application of PHA in the manufacturing industry is endless similar to the plastic products existing in the market.

Furthermore, PHA can be used as precursors for the production of fine chemicals such as antibiotics (Chiba & Nakai, 1985) and peptides (Liu et al., 2007). Studies have found that it can be also be esterified and used as biofuels (Gao et al., 2011). In agriculture, there has been reports on the use of PHA as coatings for fertilizers. By using PHA to coat pesticides and insecticides, the rate of which these compounds are released can be controlled based on the pest activity. Studies have found that PHA can also become a source of organic acids in

animal feeds. In the pharmaceutical industry, PHA has been identified as building blocks for various compounds (Ali & Jamil, 2016). For example, there has been reports of PHA application as cosmetic oil-blotting film and other skincare products.

Another promising application of PHA is in the medicine field. PHA are found to be suitable as biodegradable carriers where it has been studied for the use in drug delivery systems (DDS) (Shrivastav et al., 2013). PHAs are also suitable candidates for other biomedical application such as tissue regeneration, sutures, adhesion barriers, surgical mesh, and cardiovascular patch grafts (Wang et al., 2004; Volova et al., 2014; Martin & Williams, 2003, Ray & Kalia, 2017). An example of PHA application that is used in the medical field is the TephaFLEX absorbable suture prepared by using P4HB. It is produced by Tepha Inc. in Cambridge, MA and has been approved by the FDA in 2007 for market in the USA. These examples of PHA application shows that there is the need for PHA in these various industries. It is important to discover the right approach to producing it at a larger and more affordable scale.

2.5 Commercial production of PHA

2.5.1 Industrial production of PHA history

The commercialization of PHA has been going on since the late 1980s. Companies such as W. R. Grace Co. and Zeneca were the first few that ventured into PHA commercialization. Early PHA producers produced mainly PHB and PHV with production scale ranging from 300 t/a to 10000 t/a (Chen, 2009). By 1990s, PHA production has spread to Germany, China, Brazil, and Japan. Until early 2000s, most production is at pilot scale where the highest production capacity was 50,000 t/a, carried out by Metabolix, USA.

Figure 2.5 shows a summary of PHA production in the industry from the late 1980s where it first started up until 2010. Until present, the commercial growth of PHA is hindered because by employing pure cultures it is necessary to have a specific carbon feedstock, and aseptic conditions that results in high cost for substrate, expensive equipment, and high energy consumption (Bugnicourt et al., 2014; Choi & Lee, 1997; Salehizadeh & van Loosdrecht, 2004). Although PHA can be produced, the current cost and process conditions that requires stringent conditions have appeared to be reasons for pure culture PHA production to lack competitiveness in the polymer and material industry.

Currently in commercial PHA production, pure cultures are employed along with the use of pure substrates. The process is maintained aseptic to ensure that no contamination occurs to the culture that could lead to the productivity of the final product. In a report by Business Wire (2017), Technavio in their recent PHA global market report has announced six leading vendors from the year 2017 to 2021. These vendors are BioMatera, Bio-on, Danimer Scientific, TianAn Biological Materials, Tianjin GreenBio Materials, and Yield10 Bioscience. They also reported that although the bioplastic segment only holds a small share

of the global plastic market, it has a large growth opportunity. Vendors are using different manufacturing technologies which are patented to help increase their market shares.

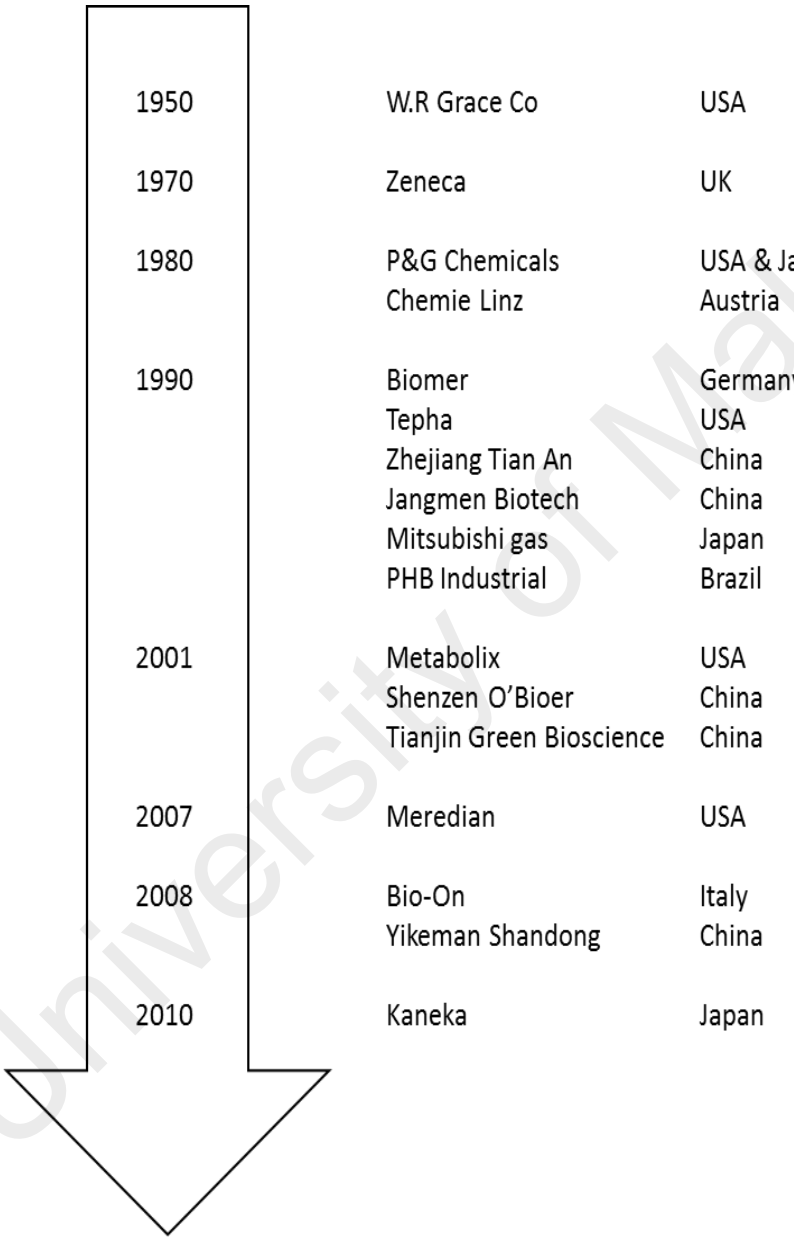

Figure 2.5: History of PHA production

2.5.2 PHA production in the industry

Before PHA production in the industry is carried out, there are certain steps to be taken at the process development stage as outlined in Figure 2.6. The first step to produce PHA is strain development which is to study and identify the strain involved for production. At this stage, microbiological strains are studied and developed to meet certain requirements. One, have a rapid and high growth, two to be able to produce controllable products, and three to determine the substrates needed. Examples of microbes that have been isolated with the ability to produce PHA are *Ralstonia eutropha* (Holmes, 1985), *Alcalligens latus* (Wang & Lee, 1997), *Azotobacter vinelandii* UWD (Page et al., 1992), *Cupriavidus necator* (Oliveira et al., 2007), and *Pseudomonas oleovorans* (Davis et al., 2013). Once the suitable strain is identified, a shake flask study at a laboratory scale is carried out to study the growth conditions as well as the most practical downstream processing. This is followed by a pilot scale study to assess the process development and optimize it. Lastly, a scale up that is stable with rapid production and high yield is required to be able to commercially manufacture and produce PHA.

In a production facility for PHA production, the general PHA production process is shown in Figure 2.7. The process starts with a fermentation process where PHA-accumulators store PHA in their cells. The cell cultures are provided with suitable substrates and grown on the necessary growth condition that has been previously identified. The cultures are than isolated before the downstream processing which starts with drying the cultures and followed by powdering them. This is followed by the necessary PHA extraction process to obtain the PHA from inside the cells. Once the PHA has been extracted, it will be dried and palletized for packaging.

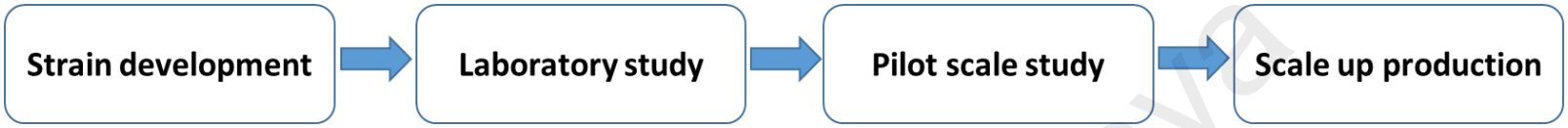

Figure 2.6: Process development sequence involved in PHA production prior to commercial production

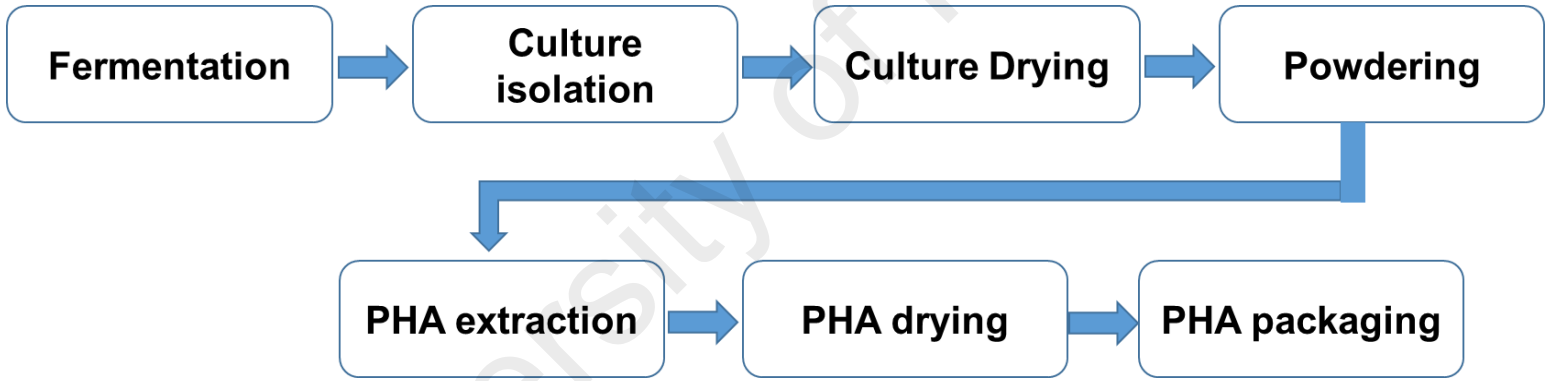

Figure 2.7: General PHA production process in a manufacturing facility

2.6 Alternative routes for PHA production

With the current status quo for commercial PHA production, the industry's growth is economically hindered. To overcome this and provide a healthier option, studies are still vigorous in order to find a cheap and practical method of producing PHA. The alternatives present for PHA production are based on the cultures and substrates.

2.6.1 Alternative cultures for PHA production

The use of pure cultures in commercial productions involves using specific strains and species of microorganisms that are known to produce high yields of PHA. Working with pure cultures demand the need for aseptic conditions in order to maintain their purity that leads to the high cost. In finding the right cultures to produce PHA, countless studies have been carried out to find alternatives to these pure cultures. There have been studies of producing PHA using transgenic plants as reported by Poirier et al. (1992) where the PHB observed is similar to the ones that are synthesized by microbes. Bohmert et al. (2000) also studied PHB production in transgenic plants and reported that the PHB accumulated was up to 4 % of the plant's fresh weight. However, it was also observed that PHB accumulation had negative effect on the plant's growth and metabolism. Similar to other reports (Bohmert-Tatarev et al., 2011; McQualter et al., 2014), it was also observed that the plant was stunted and the production of the plant's important metabolites like starch and biomass yield in leaves are reduced significantly.

With the advancement of technology, genetic engineering can be utilized for various applications including PHA production. Recombinant bacteria are engineered to not only have the ability to synthesize PHA but also to be able to produce specific novel polymer and copolymer desired by inserting the appropriate gene and providing the appropriate carbon

precursor (Park et al., 2012). As reported by Madison & Huisman (1999), natural PHA accumulators have long generation time, often hard to lyse, and contains pathways for PHA degradation all depending on their environment and need to adapt. The idea of a recombinant strain is to create a super strain that can be designed to give high product yield in short time. Some examples of successful strains engineered for the improvement of PHA accumulation are Recombinant *R. eutropha* (Insomphun et al., 2015), *Escherichia coli* CML3-1 (Pais et al., 2014), *and* recombinant *Cupriavidus necator* $H16_{AC}$ (Mifune et al., 2010).

Another option that is widely considered is the use of mixed cultures. Mixed cultures involve the use of multiple species of microorganisms naturally present in a specific environment. An example of an environment that have a mixed culture is the activated sludge system for biological wastewater treatment process. Naturally there exists a mixture of microbial population which has to cope with substrate availability fluctuations. In a study (Satoh et al., 1999), PHB and PHV along with some PHHx and PHP was found present in the activated sludge. Among the advantage of PHA production from activated sludge includes cost reduction, simpler facility construction, and material recovery from waste (Satoh et al., 1998). Use of mixed cultures results in a simpler process with no need for sterilization reduces the cost (Salehizadeh & van Loosdrecht, 2004). Studies have been carried out using mixed cultures for PHA accumulation with carbon sources such as pure lactate that achieved PHA accumulation up to 90 wt% (Jiang et al., 2011) while pure acetate achieved PHA accumulation up to 89 wt% (Johnson et al., 2009).

2.6.2 Alternative carbon sources for PHA production

In efforts to reduce the production cost, alternative carbon sources from agricultural and industrial waste and by-products (Divya et al., 2013) had been studied. By combining these

two alternatives, further cost reduction could be achieved. Nor Aslan et al. (2016) has outlined that suitable alternative carbon sources could be obtained from sugar rich feedstocks, industrial wastes and industrial wastewater, and lignocellulosic biomass.

To culture PHA-accumulators from mixed cultures, studies have been conducted using paper mill wastewater (Jiang et al., 2012), fermented molasses (Albuquerque et al., 2010), palm oil mill effluent (Lee et al., 2015), crude glycerol (Moita et al., 2014), and olive oil pomace (Waller et al., 2012). In all studies, PHA was successfully produced and the PHA content achieved ranged from 47 wt% to 77 wt%. This shows that further improvement is still needed to compete with pure carbon sources in producing high yield efficiency.

Molasses and corn-steep liquor (Purushothaman et al., 2001) have also been investigated. They have been used in culturing various *Azotobacter* strains. Wastes such as free-fatty acids from soy bean oil and waste frying oil were also studied for culturing various recombinant *Pseudomonas* strains (Fernández et al., 2005). Based on these studies using recombinant strains and waste substrates, the PHA yield obtained were between 29 wt% to 66 wt%. These studies show that culturing engineered strains for PHA production using waste substrates also leads to a relatively low PHA yield when compared to culturing pure cultures with specific pure carbons substrates.

With the growth of the biodiesel industry, there is now a surplus of crude glycerol (Yang et al., 2012). To manage the oversupply, various applications for crude glycerol have been employed including as a feasible feedstock option for PHA production. Besides being a feasible precursor for PHA production, crude glycerol is also an excellent choice because it can be directly used as the substrate for PHA production (Moita et al., 2014; Dobroth et al.,

2011). Moralejo-Garate et al. (2011) using glycerol achieved a maximum PHA production of 80 wt% with mixed cultures but the production stage was a lengthy 28 h fed-batch reaction. Dobroth et al. (2011) using crude glycerol achieved PHA content of 60 wt % but with a lengthy sludge retention time (SRT) and hydraulic retention time (HRT). Moita et al. (2014), also used crude glycerol with pre-enriched PHA-accumulators and found the final PHA production was only 47 wt%.

The challenge in culturing with waste substrates for PHA production could be the result of the different species present that may or may not have the efficiency of accumulating PHA. Even those with the capabilities of accumulating PHA, the low cell density results in low production efficiency. There are still many aspects of the processing conditions that need to be comprehended in order to improve this process in order for it to be suitable for the industry.

2.7 PHA production strategy using sequencing batch reactor (SBR)

In addition to the choice of microbial cultures and carbon source, a suitable bioreactor configuration has a significant impact on the productivity of PHA. In PHA production, the different types of bioreactors used are batch reactor, fed-batch rector, and sequencing batch reactor. Although most large scale PHA production operates using a fed-batch reactor there are numerous drawbacks to this process. Among the drawbacks discussed are irregular product quality between different batches and reduced productivity from the need to stop and revamp the reactor for the subsequent batch (Braunegg et al., 1995; Muhr et al., 2013).

In contrast to fed-batch reactors, a sequencing batch reactor (SBR) could achieve constant biomass concentration and PHA content once cyclic steady state is reached (Sun et al., 2007; Zinn, 2003). Due to ongoing cultivation, the microbial population can grow for an extended amount of time leading to a stable long-term population stability which results in high productivity and constant product quality. Senior et al. (1972) and Gräzer-Lampart et al. (1986) have compared fed-batch reactor and single-stage sequencing batch reactor and concluded that although the latter is more beneficial, it is undesirable for PHA production purposes because it cannot separate growth and PHA accumulation and may cause incomplete conversion of substrate.

Studies have investigated using a two-stage process for PHA production (Johnson et al., 2009; Moralejo-Gárate et al., 2013). In the first stage, the biomass in a sequencing batch reactor (SBR) to enrich the mixed culture with PHA accumulators and in the second stage uses a batch reactor PHA production optimization. For the multi-stage process, Renner et al. (1998) studied the feasibility and although they were successful, the process took up to 200 h which makes the process incredibly time consuming.

An important characteristic in PHA production at the cultivation stage is the aerobic dynamic feeding (ADF) strategy. Under conditions where microbes faces inconsistent substrate availability concentration there will be a difference in storage response and growth response (Beccari et al., 1998). Biomass can adapt to the environment by increasing growth rate if conditions are favorable for growth. But if conditions are not favorable they will adapt to a storage response by storing polymers instead of reproducing to gain better chances of surviving in the environment. This strategy is important in selecting the stronger PHA-accumulators over the weak PHA-accumulators and non PHA-accumulators from the mixed culture present (Chen et al., 2014).

As studied by Serafim et al. (2008) and Gobi & Vadivelu (2014), the ADF strategy will result in the presence of a feast and famine phase in a cycle. A feast phase starts when carbon substrate is provided and ends when the carbon substrate is depleted. During this phase, carbon will be consumed by the microbes for growth and storage. This phase will be followed by the famine phase where because no more carbon substrate is available, to survive, the microbes will have to rely on their polymer storage. Microbes without the capability to store any energy storage will not be able to survive the famine phase until the end of the cycle. For microbes that do have energy storage accumulated in their cells will be able to survive by hydrolyzing the stored polymers.

2.8 Summary of literature review

Based on the literature gathered, it is apparent that the study related to PHA production is vast and rapidly ongoing. The focus on finding the best practical and economical method of PHA production involves multiple aspects mainly the culture, the substrate and the operational method. For this study, the culture of interest is activated sludge as a mixed

culture. This is mainly to avoid the need for stringent operating conditions to reduce the cost and complexity of the process. As for the substrate, this study focuses on crude glycerol due to its availability and abundance as well as its potential to be converted into a value-added product. Numerous operating conditions and variables have been tested to find an optimum condition that can produce high PHA yield and efficiency. To contribute to the discussion in terms of production strategy, this study is inclined to use an SBR reactor with the ADF strategy. To study the stability of the cultivation stage, the variable that will be manipulated is the organic loading rate.

CHAPTER 3: MATERIALS AND METHODS

3.1 Two-stage PHA production

This study experimentally investigated the production of PHA by applying a two-stage PHA production process as shown in Figure 3.1. The first stage involved a sequencing batch reactor (SBR), hereafter referred to as the cultivation reactor. This was where the PHA-accumulators were enriched from the activated sludge with crude glycerol as the carbon source and nutrients to support growth. The second stage involved a batch reactor (BR), referred to as the PHA production reactor. This reactor was used to observe the maximum PHA production capabilities of the enriched sludge. The enriched sludge from the cultivation reactor was used as the inoculum and crude glycerol as the carbon source. In this reactor, nutrients were absent to promote storage of PHA rather than cell growth. The maximum PHA production was tested parallel to the cultivation reactor operation.

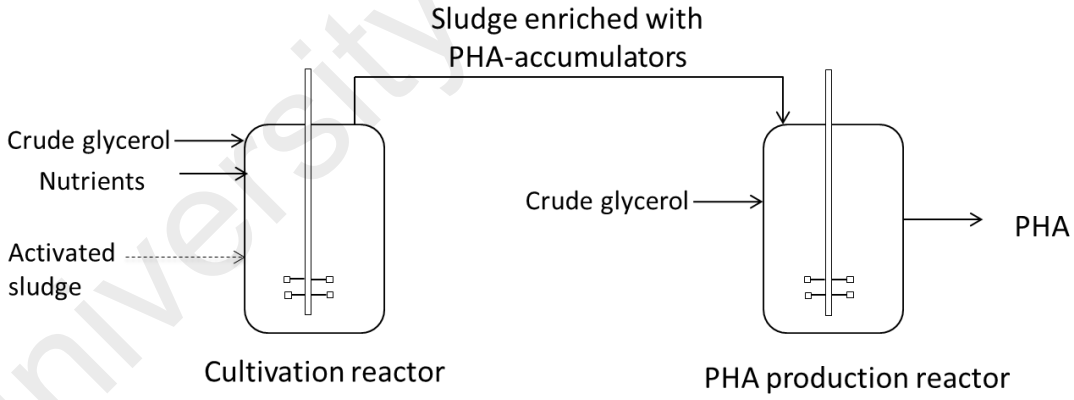

Figure 3.1: Two-stage PHA production process

3.2 Cultivation reactor to enrich PHA-accumulators

For the cultivation reactor a 3 L reactor (FS-01-A Series Fermentation System, Winpact, USA) was operated as an SBR. A schematic diagram of the cultivation reactor is shown in Figure 3.2 while Figure 3.3 presents the operation of SBR with the ADF strategy. One ADF cycle lasts for 24 hours and consists of (i) 12 minutes filling of 1.5 L feed solution made up of 750 mL of crude glycerol solution and 750 mL of nutrient solution, (ii) 23.6 h of reaction where feast and famine phases occur, and (iii) 12 minutes withdrawal of 1.5 L mixed liquor, with the agitator running. The withdrawal of effluent and addition of influent to the reactor was regulated using a weekly programmable electronic timer and a peristaltic pump (Easy-load Masterflex L/S, Cole Parmer, USA). After the withdrawal, the cycle repeats itself.

The influent of the feed for the cultivation reactor is made up of the crude glycerol solution and the nutrient solution. The crude glycerol solution and nutrient solution are separated in two different containers before they enter the reactor. Due to the nature of the solution, if it is stored in one container together, it would provide a medium for bacterial growth. This is avoided to prevent any contamination or change in both the solutions prior to adding it to the cultivation reactor. The effluent from the cultivation reactor would be a homogenous mixture of mixed liquor made up of the sludge and supernatant present in the reactor.

The volume of influent and effluent were both determined to be 1.5 L. Along with the cycle length of 24 hours, the solid retention time (SRT) and hydraulic retention time (HRT) were both 2 days. Assuming that the reactor's content is homogeneous,

$$SRT = \frac{V_R\ (L)\ \times X\ (\frac{g}{L})}{Q_W\ \left(\frac{L}{d}\right)\ \times\ X_R\ \left(\frac{g}{L}\right)} \qquad \text{Eq (3.1)}$$

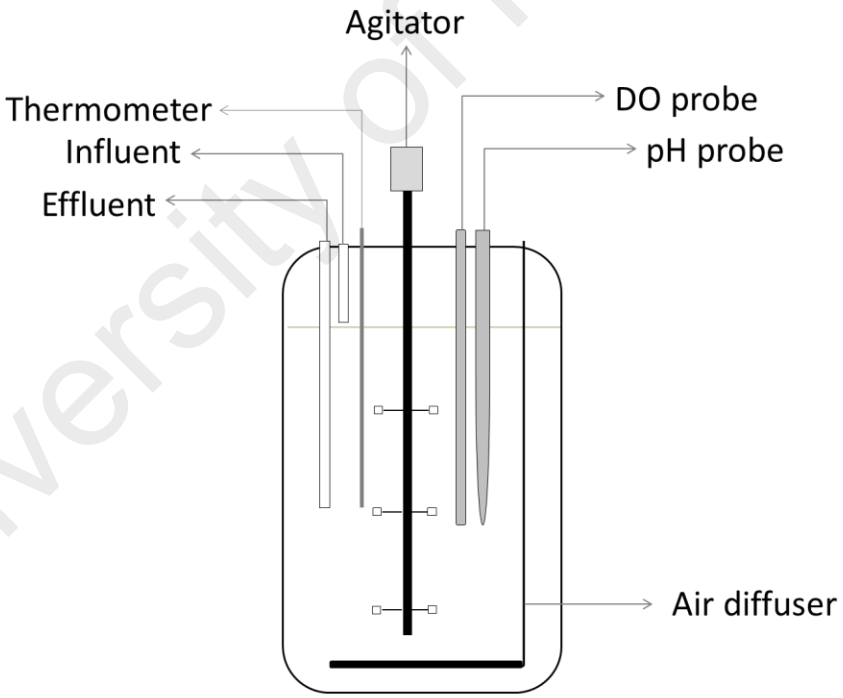

Figure 3.2: Schematic diagram of the cultivation reactor

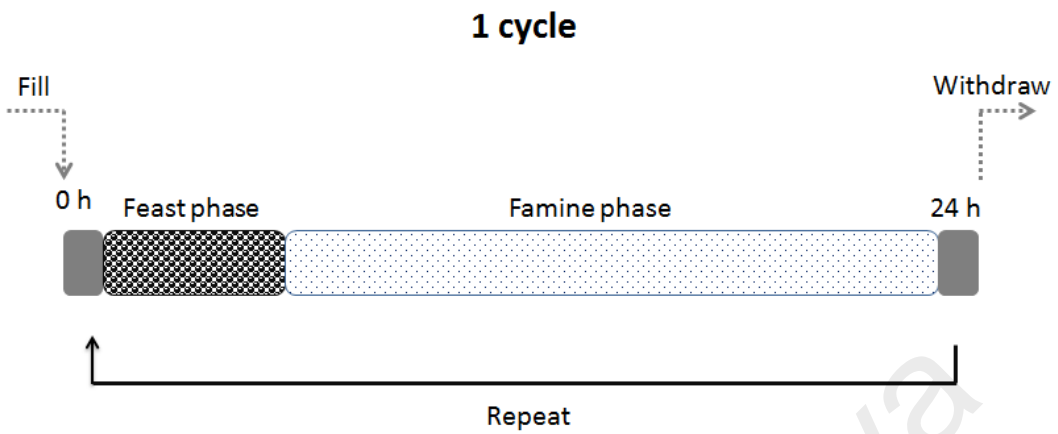

Figure 3.3: SBR process with ADF strategy

The details of the operating parameters are listed in Table 3.1. The organic loading rate (OLR) 360 mg C/ L·d was adopted from the works of Moita et al. (2014) to achieve a stable cultivation reactor. In a preliminary study, an OLR of 1000 mg C/ L·d resulted in unfavorable conditions in the cultivation reactor. Excessive foaming led to multiple occurrence of reactor overflow and high production of EPS made it difficult to separate between sludge and supernatant for analysis purposes. From the OLR, the volume of crude glycerol needed in each feed cycle was calculated. The necessary volume of crude glycerol will be diluted with tap water to achieve the desired OLR. The temperature of the cultivation reactor was not controlled throughout the whole cultivation period but maintained between 28 °C to 30 °C.

Table 3.1: Cultivation reactor operating conditions

Sequencing Batch Reactor (SBR)	Condition
Working volume (L)	3
pH	7 ± 0.2
Temperature (˚C)	28 – 30
Air supply (vvm)	1/3
Stirring (rpm)	200

The nutrient solution used in this experiment is made up of the components listed in Table 3.2. The basic major nutrient that is necessary for bacterial growth includes nitrogen, phosphorus, potassium, sulfur, and magnesium. Along with these major components, a small amount of trace elements are present in the nutrient solution to facilitate cellular growth. Also added in the solution is N-Allythiourea (ATU) to prevent the occurrence of nitrification.

The nutrient solution was prepared weekly where each nutrient component was weighed and diluted in RO water. Because the trace elements were only needed in small amount, a stock solution of the trace element was prepared as listed in Table 3.3 and stored at 4 °C. The exact volume of trace element needed will be measured and added in to the nutrient solution prepared for the week. The nutrient solution is adopted from Lee et al. (2014) and Ong et al. (2012) because of the similar sludge source used. Based on their work, the nutrients provided were suitable to support the growth of the activated sludge.

Table 3.2: Nutrient composition for the cultivation reactor

Nutrient composition	Concentration (in 1 L)
Potassium dihydrogen phosphate (KH_2PO_4)	1460 mg
Ammonium chloride (NH_4Cl)	1070 mg
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	660 mg
N-Allythiourea (ATU)	20 mg
Trace element solution	3.3 mL

Table 3.3: List of trace elements in nutrient feed for the cultivation reactor

Trace element composition	Concentration (mg/L)
Ethylenediamine tetraacetic acid (EDTA)	14400
Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$)	2160
Potassium iodide (KI)	260
Boric Acid (H_3BO_3)	220
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	220
Manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$)	170
Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	170
Sodium molybdate dehydrate ($Na_2MoO_4.2H_2O$)	80
Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$)	50

Before running the cultivation reactor, the sludge and the feed solution were prepared. The activated sludge was obtained from a local sewage treatment plant located in Kuala Lumpur, Malaysia. The activated sludge was left to settle before the supernatant was removed. The activated sludge was then stored at 4 °C if not ready to be used yet. Prior to use as the inoculum to start the cultivation reactor, the activated sludge was aerated overnight to re-activate the stored sludge. The suspended solids (SS) reading of the activated sludge was also determined before starting the cultivation process. For the carbon feed, crude glycerol used as the sole carbon source was obtained from KLK Bioenergy Sdn. Bhd. located in Shah Alam, Selangor, Malaysia. The crude glycerol stock solution has a purity of 88 % and is stored at 4 °C to avoid carbon degradation.

Table 3.4: Characteristics of the activated sludge

Activated sludge	
pH	6.65
TSS (mg/L)	6555
VSS (mg/L)	4885

Table 3.5: Characteristics of crude glycerol

Crude glycerol	
pH	5.6
tCOD (mg/L)	1402
sCOD (g/L)	1409
TOC (g/L)	235
Purity	88 %

3.3 Changing the organic loading rate

To study the effects of the organic loading rate (OLR) on the cultivation reactor, the OLR was changed gradually. To observe the robustness of the mixed culture in the enrichment stage, only the OLR was changed without terminating the cultivation reactor. The increment of OLR is shown in Table 3.6. The initial OLR in R1 was chosen based on Moita et al. (2014). The OLR 1000 mg C/ L·d in R2 was chosen to see how a sudden increase by threefold would affect the process. A further increase by 25% to 1250 mg C/ L·d was decided for R3 as a reasonable increment to further test the capabilities of the enriched sludge. The plan for R4 was to further increase the OLR by another 25% from R3 but due to the circumstances that will be discussed in the discussion chapter, the OLR for R4 was 1000 mg C/ L·d.

Table 3.6: The operating OLR for the different cultivation run

Cultivation reactor	R1	R2	R3	R4
OLR (mg C/ L·d)	360	1000	1250	1000

The OLR was increased at the beginning of a new cycle. The necessary carbon concentration was calculated and the feed was prepared accordingly beforehand. On the day when the new cultivation was to start, the feed with the higher OLR was ready to start as the next cycle starts. This is when the influent feed and nutrition solution is pumped in at 0h of the cycle and is then considered the next cultivation run. All other operating conditions and parameters are maintained as the previous run in order to evaluate OLR effect on the stability of the cultivation.

3.4 PHA-production reactor for maximum PHA production

To investigate the maximum PHA production, the PHA production reactor was set up as in Figure 3.4. The operating conditions of the production reactor are as listed in Table 3.5. Similar to the cultivation reactor, the pH was maintained at 7 ± 0.2 while the temperature was not controlled but maintained between 28 °C – 30 °C. SRT and HRT were irrelevant for this reactor as it is a batch reactor and no filling and withdrawal of solution were involved. To seed the reactor, the sludge in the effluent of the cultivation reactor was collected. The sludge was centrifuged to remove all the supernatant so only the enriched sludge which consists of the PHA-accumulators is used as the inoculum. The concentrations of the enriched sludge and the crude glycerol solution were prepared based on the F/M ratio desired. The PHA production reactor had a working volume of 1 L. 500 ml of water is used to dilute the necessary amount of crude glycerol while the remaining 500 mL is used to dilute the sludge collected.

Once the setup of the production reactor is ready, the inoculum is poured into the reactor first. The crude glycerol solution is added in last, and this will mark the start of the process or the 0 h. In PHA production, because the aim is to observe the maximum PHA storage capability, there is no need to monitor the feast and famine occurrence.

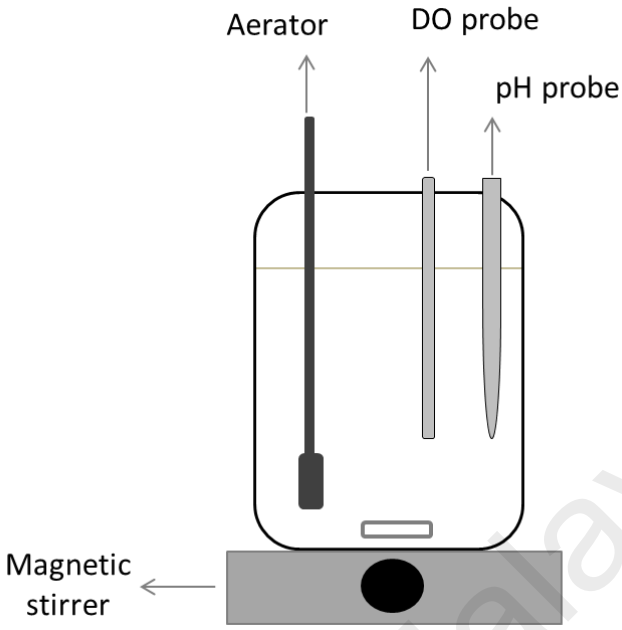

Figure 3.4: Schematic diagram of the PHA production reactor

Table 3.7: Operating conditions for the PHA production reactor

Batch Reactor (BR)	**Condition**
Working volume (L)	1
pH	7 ± 0.2
Temperature (˚C)	28 – 30
Air supply (vvm)	1
Stirring	360 rpm

3.5 Analytical methods

The analyses carried out to investigate the necessary parameters for the cultivation and the PHA production reactor are shown in Figure 3.5 along with the sampling points of each analysis. The DO data was logged continuously to observe the trend which was used for the determination of feast and famine phases. For the SS analysis, during the set up samples were taken at the start of the cycle and at the end of the cycle to observe the change in the biomass concentration. Once the reactor had stabilized, the SS analysis was carried out weekly for monitoring purposes. The DOC and PHA samples were both taken at the start of the cycle, the middle of feast phase, the end of feast phase, and lastly at the end of the cycle. The sampling point for both DOC and PHA was determined based on the feast and famine phases identified using the DO data. From these different points, the trend of the DOC consumption and PHA accumulation of a cycle can be evaluated. The details of each analysis are elaborated in the following sections.

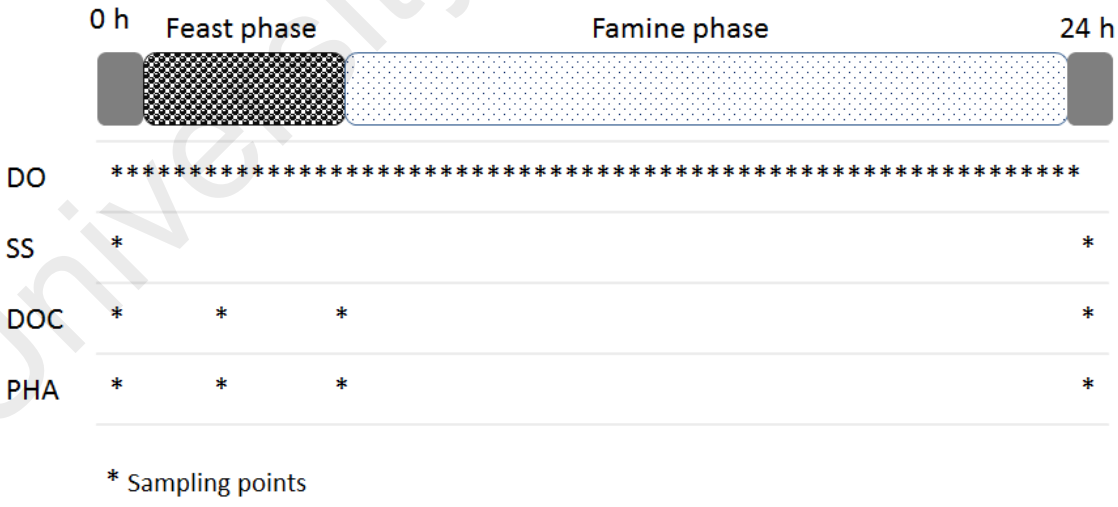

Figure 3.5: Sampling points for analytical measurements

3.5.1 Dissolved oxygen

Continuous monitoring of DO was done using a DO probe (Mettler Toledo, USA) inserted in the reactor. The probe attached to the Winpact FS-01 controller system enabled automatic data logging of the DO saturation percentage (DO %). DO % is extracted and a line graph is plotted to analyze the DO trend in order to determine the feast phase and famine phase period. The DO % is used as an indicator of feast and famine phases because it is a direct indicator of microbial activity. The DO % would drop to near zero when there is microbial activity while without microbial activity the DO % would be at 100 %. At the start of feast phase with the addition of the feed, the DO % would rapidly drop to near zero and maintain at near zero while the carbon is consumed. This period is the feast phase. Once the carbon has been consumed completely, the DO % would increase back to its saturation and this period is the famine phase. After the duration of the feast and famine phases was determined, sampling points for DOC and PHA analysis are also determined.

Maintenance for the DO probe was carried out weekly by taking it out and rinsing it with water to remove biofilms from the probe that could make the DO reading inaccurate. The electrolyte (0_2 – electrolyte, Mettler Toledo) in the probe is also checked regularly to ensure accurate DO measurements. If the electrolyte has dried up, the probe membrane is removed and the electrolyte is added in. Calibration of the DO probe was carried out periodically to ensure that the DO reading obtained is reliable. To calibrate the DO probe, the Winpact FS-01-A instruction manual was referred to.

3.5.2 Suspended solid analysis

As illustrated in Figure 3.8, the SS analysis was carried out using the standard APHA method (APHA, 1998) to obtain the total suspended solids (TSS) concentration and the volatile suspended solids (VSS) concentration. Before analysis was carried out with samples, the filter paper (0.47 mm microfiber filter discs, MGC, Sartorius, Germany) had to be pre-treated first to remove impurities present. The filter paper was placed in a crucible and dried in the oven at 103 °C for 1 hour. It is later placed in the furnace at 550 °C for 15 minutes and afterwards cooled down in an oven at 103 °C for 10 minutes before transferring it to a desiccator for another 15 minutes to cool further. The crucible with the filter paper is weighed to get the initial weight (W_o).

For the sample analysis, a fixed volume V (here 10 mL) of mixed liquor samples from the reactor were vacuum filtered through the filter paper. The filter paper with the filtered residue was oven-dried at 103 °C for one hour. The filter paper and crucible are allowed to cool first in the desiccator for 15 minutes before weighing (W_{103}). After weighing, the filter paper is transferred to the furnace at 550 °C for 15 minutes. To cool down the filter paper it is placed in an oven at 103 °C for 10 minutes before transferring to a desiccator for another 15 minutes to cool further. The final weight is then measured (W_{550}) before calculations were made. From the analysis, the TSS concentration and VSS concentration were obtained from the equation below,

$$\text{TSS (mg/L)} = \frac{W_{103}(\text{g}) - W_o(\text{g})}{\text{V(mL)}} \left|\frac{1000\ \text{mg}}{1\ \text{g}}\right| \frac{1000\ \text{mL}}{1\ \text{L}} \qquad \text{Eq (3.2)}$$

$$\text{VSS (mg/L)} = \frac{W_{103}(\text{g}) - W_{550}(\text{g})}{\text{V(mL)}} \left|\frac{1000\ \text{mg}}{1\ \text{g}}\right| \frac{1000\ \text{mL}}{1\ \text{L}} \qquad \text{Eq (3.3)}$$

To avoid measurement error, all SS analysis measurements are carried out in duplicates where two different samples are taken and the average value is calculated. When measuring the weight of the crucible and filter paper after heating, it has to be cooled down to avoid inaccurate measurement of the weight. As a safety measure, tongs are used to handle the hot crucible. Hot crucibles taken directly out of the furnace should not be placed immediately in the desiccator.

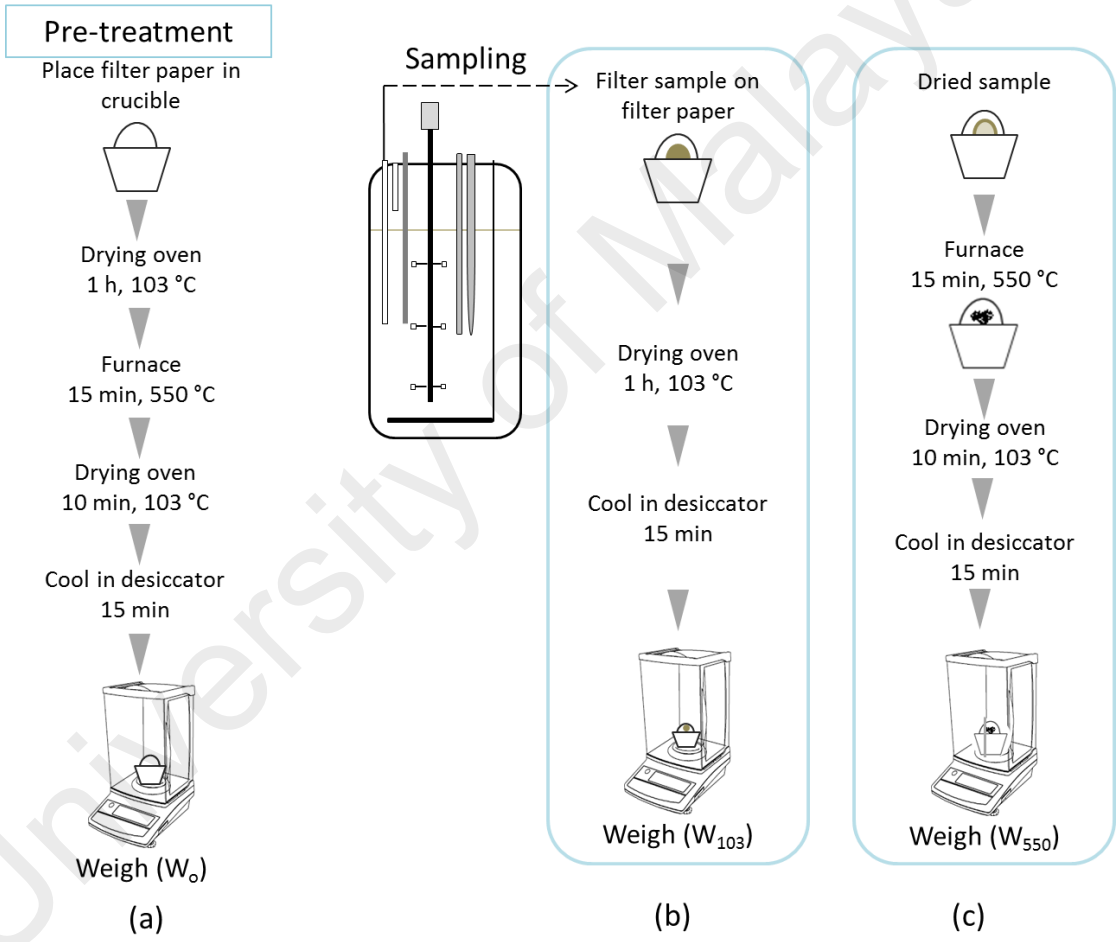

Figure 3.6: Suspended solids analysis procedure where (a) is the pre-treatment steps, (b) is to measure W_{103}, and (c) is to measure W_{550}.

3.5.3 Dissolved organic carbon

Dissolved organic carbon (DOC) analysis was carried out using a Total Organic Carbon Analyzer (TOC-V_{CSN}, Shimadzu, Japan). Before samples were analyzed, the reliability of the calibration curve of the TOC analyzer is checked by running a standard total carbon (TC) solution and a standard inorganic carbon (IC) solution. The standard solution stock for both TC standard and IC standard of 1000 mg/L is diluted to 100 mg/L. If the measurements of the standard solutions are accurate, the analysis of the samples is carried out. If the measurements of the standard solution are inaccurate, a new calibration curve has to be made. The calibration curve points used ranged between 10 mg/L to 500 mg/L.

As Figure 3.7 below illustrates for DOC analysis, the sample taken from the reactor was centrifuged first to separate the supernatant and sludge. Only the supernatant was used for the analysis where it was filtered through a 0.45 µm syringe filter into a TOC vial. The maximum detection limit of the TOC analyzer is 2500 mg/L for TC and 3000 mg/L for IC. To ensure that the sample concentration is within the detection limits of the TOC analyzer, samples from the start of the feast phase and the middle of the feast phase are diluted with ultrapure water with a necessary dilution factor (DF). If dilution was performed for a sample, the DOC concentration value calculated by the TOC analyzer should be multiplied by the DF to get the actual concentration.

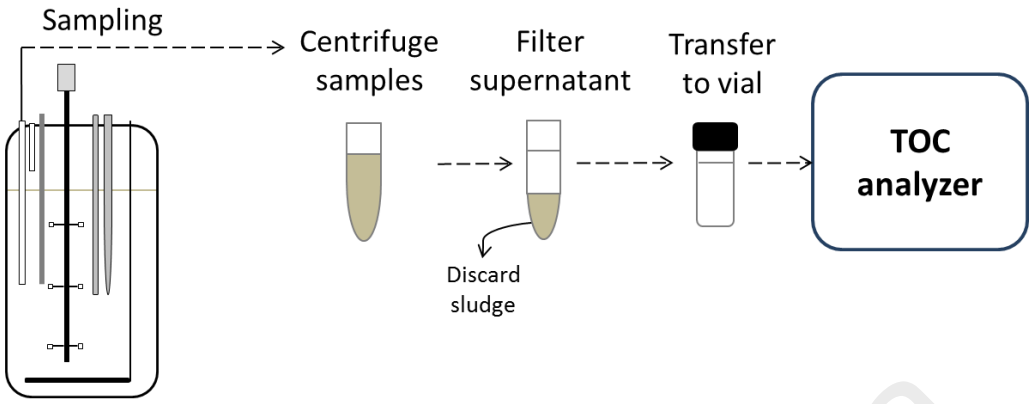

Figure 3.7: Dissolved organic carbon analysis procedure

3.5.4 PHA analysis

PHA analysis involves a series of steps where PHA is extracted from the cell by breaking the cell and dissolving PHA in a solvent. This is followed by recovery of the extracted PHA and lastly PHA concentration measurement. Duplicate samples of 10 mL mixed liquor from the reactor were collected in a glass vial for every sampling point. The mixed liquor is centrifuged at 3000 rpm for 5 minutes to separate the sludge. The supernatant is removed and the sludge sample is frozen at -20 °C overnight. The frozen samples are then freeze-dried (FDU-110, Eyela, Japan) before PHA extraction was carried out (Satoh et al., 1996).

For the PHA extraction procedure, acidified methanol had to be prepared beforehand. To prepare the acidified methanol, a container large enough to fit a 500 mL beaker is filled with water and ice. A 500 mL beaker was immersed into the iced water and was filled with 150 mL of methanol. To the methanol, 50 mg of Benzoic acid was added and the beaker was swirled. Sulfuric acid was then added slowly into the solution by letting it slide down the side of the beaker. The solution was then transferred into a 250 mL volumetric flask and more

methanol is added up to the 250 mL mark. This solution can be stored at 4 °C and prepared again once it is used up.

The freeze-dried sludge was crushed using a small spatula and 2 mL of chloroform and 2 mL of the acidified methanol is added to the vial. The vial was capped and the solution was mixed with a vortex before it was heated in the oven at 98 °C for 24 hours. The next day, the vials were taken out of the oven and let cool to room temperature. Once the vials are cool to touch, 1 mL of 14% ammonia solution is added. The solution is centrifuged at 3000 rpm for 5 minutes. After centrifugation, a bilayer will form. The bottom layer is of interest because it is the HA dissolved in chloroform. Using a Pasteur pipette, 1.5 mL of the bottom layer is pipetted out into a GC vial and 0.5 mL of ultrapure water is added to the solution. The GC vial is centrifuged at 3000 rpm for 5 minutes. After centrifugation, again a bilayer will form where the upper layer is water and the bottom layer is the dissolved HA. 1 mL of the bottom layer is then pipetted out and transferred to another GC vial and the solution is now ready for GC analysis.

There are three standards in this analysis which are benzoic acid, HB standard, and HV standard. Benzoic acid acts as an internal standard and is present in the acidified methanol solution that was added to the sample. The other two standards are 3HB (Sigma-Aldrich, USA) and 3HB-co-HV (Sigma-Aldrich, USA) containing 88 wt% 3HB and 12 wt% 3HV.

For GC analysis, a GC-2010 (Shimadzu, Japan) with a flame ionization detector (FID) equipped with an AOC-20S auto-sampler and AOC-20i auto-injector was used. The carrier gas used is Nitrogen gas (40 mL/min) with air (400 mL/min) and Hydrogen gas (30 mL/min)

for ignition of the detector. The column used was a capillary column (J&W, DB-Wax). The GC is linked to a computer with a GC solution software.

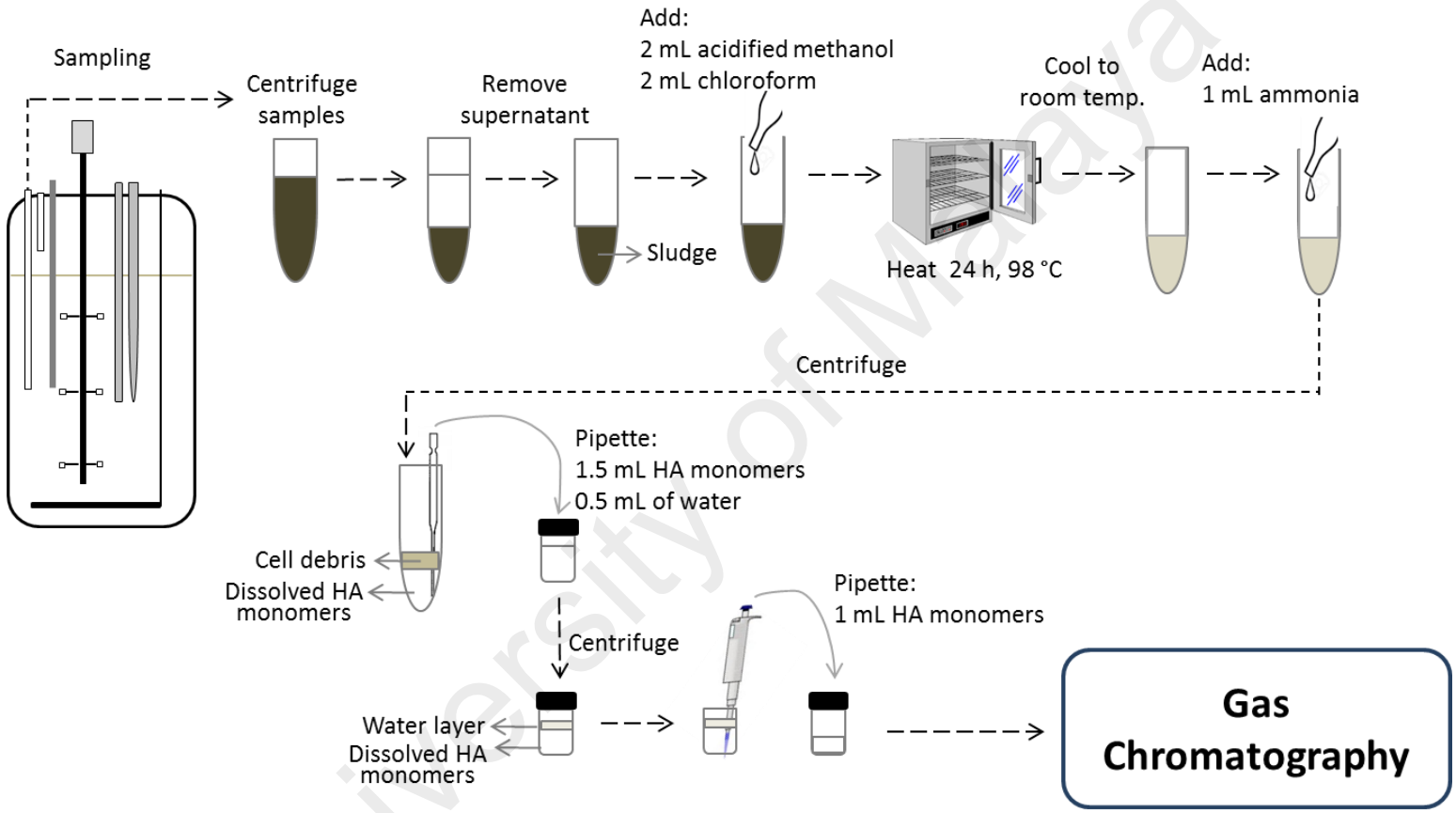

Figure 3.8: PHA analysis procedure

3.5.5 Microscopic analysis

All images were analyzed using a microscope (DM 2500, Leica Microsystems, Germany) fitted with a camera (DFC 310 FX, Leica, Germany). Phase contrast imaging was used to observe the morphologies of the microbes present and visualization of intracellular PHA using Nile Blue A staining (Ostle & Holt, 1982).

To prepare the slide, a small drop of sample is placed at the center of the slide. The droplet is then smeared to cover the slide. The sample is left overnight to air dry. Once dried, the sample was stained and because the stain is light sensitive, the procedure has to be done in a dimly-lit room. A small drop of the Nile Blue A solution is dropped onto the slide. The slide is tilted left to right to spread the stain over the sample. The stain is left to set for 15 minutes. Afterwards by using a squeeze bottle, water was squeezed out to the edge of the slide. Tip the slide sideways and let the water slide to remove any excess stain. Squeezing water directly at the sample may cause the sample to be lost. A drop of anti-fade agent (Gold antifade agent, Invitrogen, USA) was added to help retain the stain. The cover slip is carefully placed to cover the sample, to avoid trapping air bubbles. The slide is immediately observed under the microscope to ensure that the image observed is accurate.

To analyze and estimate the bacterial group present and their abundance in the reactor, fluorescence *in situ* hybridization (FISH) analysis was carried out. Samples from the cultivation reactor were fixed in 4% paraformaldehyde before the FISH analysis was performed (Daims et al., 2005). The probes used were EUBmix (EUB-338-I, EUB-338-II, and EUB-338-III) to target all bacteria (Amann et al., 1990, Daims et al., 1999), ALF968 to target *Alphaproteobacteria* (Neef et al., 1997), BET42a with competitor (GAM42a) targeting *Betaproteobacteria* (Manz et al., 1992) and GAM42a with competitor (BET42a) targeting

Gammaproteobacteria (Manz et al., 1992). The probes were labeled with 5(6)-carboxyflourescein-N-hydroxysuccinimide ester (FLUOS) or with the sulfoindocyanine dyes (Cy3) which was obtained from either Rikaken (Nagoya, Japan) or Nippon Gene (Tokyo, Japan). All samples were analyzed using a BX51 epifluorescence microscope (Olympus, Tokyo, Japan). For the FISH analysis, samples were homogenized by ultrasonic treatment prior to observation underneath the microscope. By using the Daime software (Daims et al., 2006), the biovolume values were calculated to represent the area of cells hybridized with the bacterial group probe as a percentage of the total area hybridized with the EUBmix probe. The mean values and the standard deviations were calculated based on 26-31 fields of view.

3.5.6 PHA performance evaluation

The PHA storage capacity and PHA volumetric productivity was evaluated based on the PHA content according to equation (1) and equation (2):

$$\text{PHA content, (wt\%)} = \frac{\text{PHA concentration (mg/L)}}{\text{Volatile suspended solids (mg/L)}} \times 100\% \qquad \text{Eq (3.4)}$$

$$\text{Volumetric productivity, (mg/L} \cdot \text{h)}$$

$$= \frac{\text{PHA concentration}}{\text{Mixed liquor volume} \times \text{production time}} \qquad \text{Eq (3.5)}$$

The specific substrate uptake rate ($-q_s$) and PHA production rate (q_{PHA}) were determined by using equation (3) and equation (4):

$$\text{Specific substrate uptake rate, } -q_s \left(\frac{\text{mgC S}}{\text{mgC X} \cdot \text{h}}\right)$$

$$= \frac{\text{Substrate concentration}_i - \text{Substrate concentration}_f}{\text{Biomass concentration}_f - \text{Biomass concentration}_i \times \text{h}} \quad \text{Eq (3.6)}$$

$$\text{PHA production rate, } q_{PHA} \left(\frac{mgC\ PHA}{mgC\ X \cdot h}\right)$$

$$= \frac{\text{PHA concentration}_i - \text{PHA concentration}_f}{(\text{Biomass concentration}_f - \text{Biomass concentration}_i) \times \text{h}} \quad \text{Eq (3.7)}$$

The carbon content of the biomass was calculated using the chemical formula $C_5H_7NO_2$ (Metcalf & Eddy, 2004). Three key overall process yields were determined in this work. The production yield on substrate ($Y_{PHA/s}$), biomass production yield on substrate ($Y_{x/s}$), and the respiration yield ($Y_{O_2/S}$) on substrate were calculated by using equation (5), (6), and (7):

$$\text{PHA yield, } Y_{PHA/S} \left(\frac{\text{mgC PHA}}{\text{mgC S}}\right)$$

$$= \frac{\text{PHA concentration}_f - \text{PHA concentration}_i}{(\text{Substrate concentration}_i - \text{Substrate concentration}_f)} \quad \text{Eq (3.8)}$$

Biomass yield, $Y_{X/S}$ $\left(\frac{\text{mgC X}}{\text{mgC S}}\right)$

$$= \frac{\text{Biomass concentration}_f - \text{Biomass concentration}_i}{(\text{Substrate concentration}_i - \text{Substrate concentration}_f)} \quad \text{Eq (3.9)}$$

Respiration yield, $Y_{O_2/S} = 1 - Y_{PHA/S} - Y_{X/S}$ Eq (3.10)

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Long term stability for the enrichment of PHA-accumulators

4.1.1 Monitoring of the cultivation reactor

Apart from carrying out the analytical methods to monitor the stability of the cultivation reactor, it is also important to note the general condition of the reactor. The reactor is observed for any signs of foaming and biofilm formation that could have effect on the analysis of the reactor. Daily maintenance of the reactor includes brushing the reactor and its walls to ensure a homogenous mixture. The probes are also cleaned to remove any biofilm or sludge that might have gotten stuck to it.

For analytical analysis, weekly monitoring of the dissolved organic carbon (DOC) was carried out to observe the uptake of the crude glycerol. In the beginning, hourly samples were taken from the beginning of the cycle (0 hour) to the 8^{th} hour of the cycle as well as the 24^{th} hour. As in Figure 4.1 below, it was observed that there is a significant gradual decrease in the DOC concentration up until the 6^{th} hour. From the 7^{th} hour onwards, there were no longer a significant change in the DOC concentration. From this observation along with previous research on PHA production conducted by Lee et al. (2015), the feast phase was determined to be 6 hours. From here, the sampling point for DOC analysis on the following weeks were taken at 0 h, 3 h, 6 h, and 24 h.

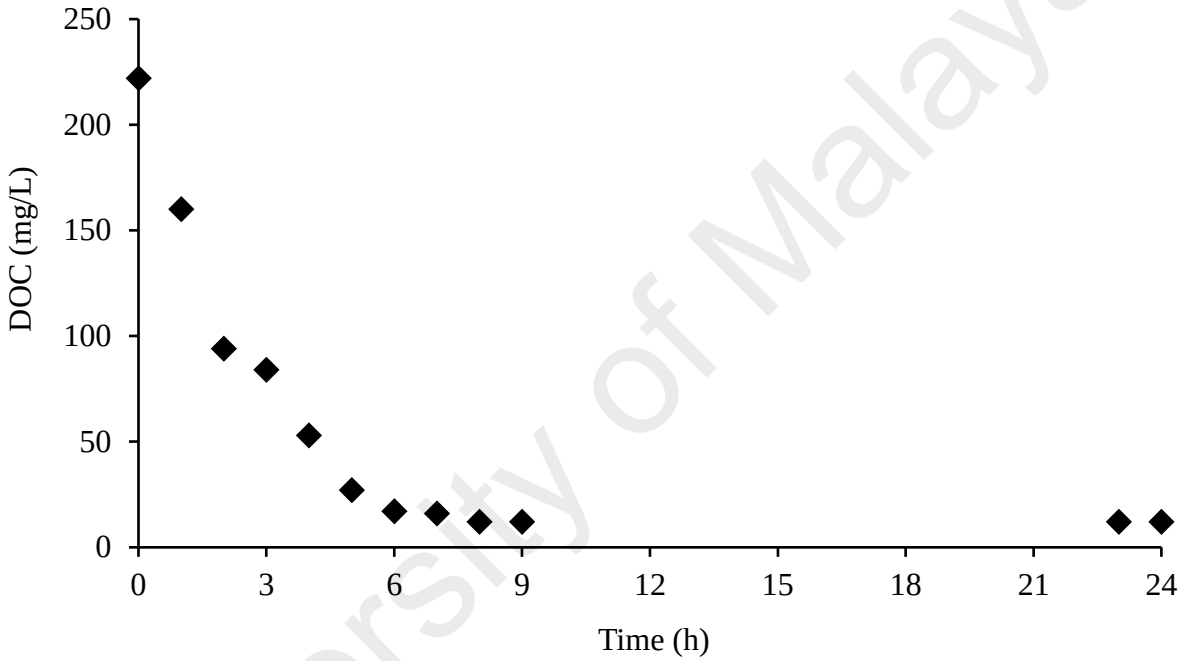

Figure 4.1: DOC concentration on day 3 of cultivation

While DOC analysis was carried out on a weekly basis, the monitoring of the dissolved oxygen (DO) was carried out on a daily basis. From the reactor control system, the DO data were automatically logged daily and available in real time. After comparing the DO data with the DOC data, it was found that the feast and famine phase were consistent in both sets of data as shown in the example below (Figure 4.2). It was decided that the DO data is reliable for the use of daily monitoring of the feast and famine phase. However, weekly DOC analysis were still carried out to check and confirm the reliability of the DO data obtained.

Another use of the DOC data is to check the initial concentration of the substrate present in the cultivation reactor at the beginning of the cycle. For this initial cultivation, the organic loading rate (OLR) set is 360 mg C/ L·d which means that at the start of the cycle, the DOC concentration should be 360 mg/L. However, it was observed that on every analysis, the initial DOC concentration would be less than 360 mg/L. The sampling point for 0 h takes place after the feeding pump completes the fill, which takes 12 minutes to happen. During the 12 minutes, it is believed that some consumption has already taken place leading to the lower DOC concentration at the point of sampling.

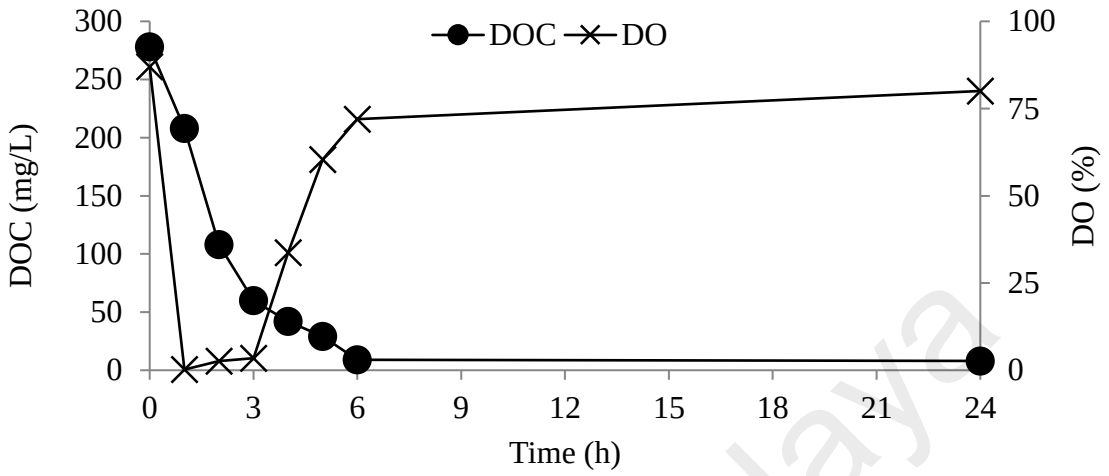

Figure 4.2: DOC and DO profile in one ADF cycle on week 21 of cultivation.

The feast and famine phase duration are important measurements that were used to determine the feast and famine ratio (F/F) (Valentino et al., 2014). Once the points for monitoring had been established, a complete cycle profile can be plotted consisting of the DOC concentration, DO saturation, and PHA concentration. This complete cycle profile is important as part of monitoring to give insight on the uptake of the carbon, the successful implementation of the ADF strategy, as well as the confirmation of PHA-accumulating capabilities of the enriched sludge. An example of this complete profile cycle is shown in Figure 4.3 below. At the beginning of the cycle, the concentration is near 360 mg/L. After 6 hours, the DOC concentration is near zero and remains so until the end of the cycle. This shows that there is complete uptake of the carbon by the 6th hour.

Corresponding to this, the DO saturation is seen to drop immediately to near zero as the cycle starts. This can be attributed to the aerobic condition where active uptake of the carbon is assisted by oxygen uptake. After the 6th hour where there are no longer carbon available, the DO gradually increases back to 100 % saturation. To confirm that the uptake of crude glycerol is transformed into PHA, the PHA concentration is also checked. The maximum PHA is observed at the 6th hour followed by the decline in PHA concentration which is expected as PHA is used to survive the remaining famine phase.

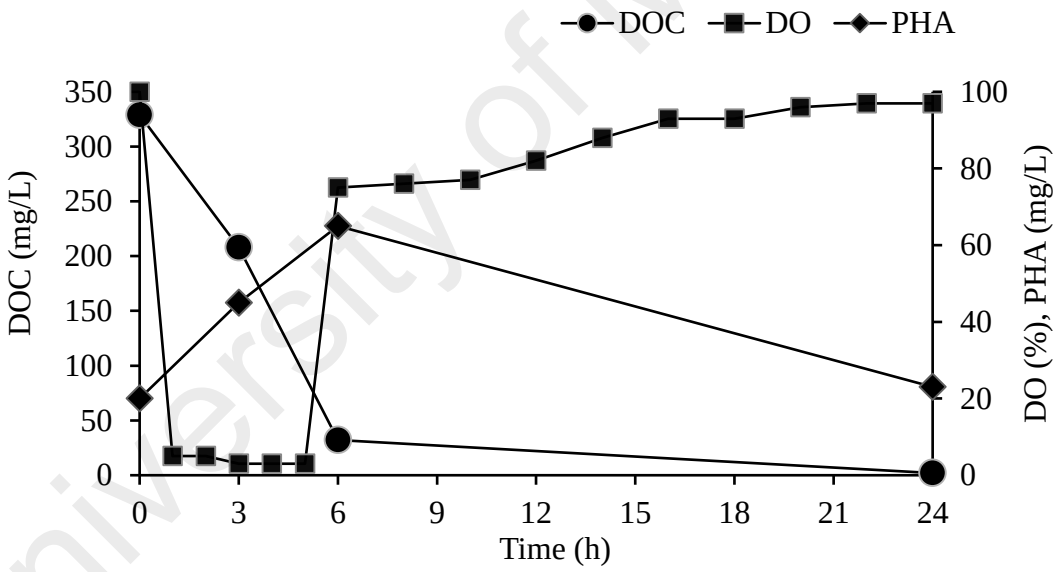

Figure 4.3 Cycle profile on cultivation day 36 of R1

4.1.2 Stability of the cultivation reactor

The first parameter used to evaluate the stability of the cultivation reactor is the feast and famine phase period. Once the cycle profile have been determined, the consistency was evaluated for stability. Based on Figure 4.3 (a), the fluctuation of the feast/famine ratio (F/F) seen in the first 8 weeks represents the acclimatization process that took place before steady state is achieved. In this study, an acclimatization period of 8 weeks was achieved. Reports of acclimatization stage in other studies with crude glycerol were not sufficient to compare. But in a study where activated sludge was fed with hardwood sulfite spent liquor in a long-term operation on PHA production was conducted, it took 34 weeks of acclimatization period (Queiros et al., 2015). It would be considered that this study achieved a short acclimatization stage since steady state was observed in a matter of two months.

After acclimatization, on average the feast phase stayed at 4 hours while the famine phase at 20 hours. During this steady state of cultivation run R1, the average F/F obtained was 0.2 which is an indication of a good storage response. This is supported by reports that an F/F ratio of less than 0.28 is favorable as faster storage is implied (Johnson et al., 2009; Dionisi et al., 2006). Only on a few occasions the feast phase was observed to be longer, as seen in weeks 30 and 36. It was identified that these longer feast phase occurred during reactor upsets where because of external problems, the reactor operation was interrupted. These interruptions were caused by one or more of the following; disruption in feeding time from broken timer, loss of pH control, and also power outage. These problems however highlighted that despite facing interruptions in operation, the reactor was able to recover quickly (usually within a day) after the operating conditions were restored. This is an important observation to show that the cultivation process can handle disturbances.

The second parameter in evaluating the stability of the cultivation reactor is based on the biomass concentration. This provides information on the abundance of biomass present in the mixture. In the first 6 weeks of cultivation run R1, the biomass concentration dropped from 1200 mg/L to 170 mg/L (Figure 4.3(b).) This observation is common and was expected. As Chen et al. (2017) explained, a remarkable dip in the early stage of cultivation happens and is attributed to the elimination of non PHA-accumulators. The concentration of the biomass would increase again before it reaches a steady state. This was observed in this study where after week 8, the biomass concentration increased and remained on average at 0.7 g/L. The biomass present now is considered to be strong PHA-accumulators as it had survived the acclimatization stage. The concentration was observed to be consistent for nearly a full year. Again comparison with other studies using crude glycerol could not be made as the information was not made available. A consistent biomass concentration was observed throughout the cyclic steady state of cultivation run R1. This is crucial in ensuring consistent and sufficient biomass supply for the production stage later.

The overall low biomass concentration throughout the cultivation stage was consistent with other reports (Martinez et al., 2015; Tamis et al., 2014). The problem is believed to be due to the combination of mixed culture with waste substrate as the carbon source. The high success rate of pure culture enrichment with pure substrates was the result of high specificity and efficiency of the two combination. Biomass concentration could easily exceed 150 g/L in pure culture fermentation. When waste substrate is used, impurities present, purity of carbon source, and the diluted nature of substrates from waste could be contributing factors that lead to low biomass concentration. For example, in cultivation using pure cultures, studies enriching *Cupriavidus necator* using fermented olive mill wastewater (Martinez et

al., 2015) and *Plasticicumulans acidivorans* using food industry wastewater (Tamis et al., 2014) both achieved biomass concentrationn of about 1.5 g/L which is low for pure culture cultivations. In a previous study where crude glycerol was used as the substrate in cultivation using mixed culture, they achieved 2.4 g/L of biomass (Moita et al., 2014). It was reported that their inoculum already had the affinity for PHA accumulation as it was pre-cultivated with bio-oil which was done in a separate study (Moita et al., 2013), resulting in the higher biomass concentration. Although the biomass concentration is slightly higher compared to this study, pre-cultivation with another substrate would result in additional operating systems that could be timely.

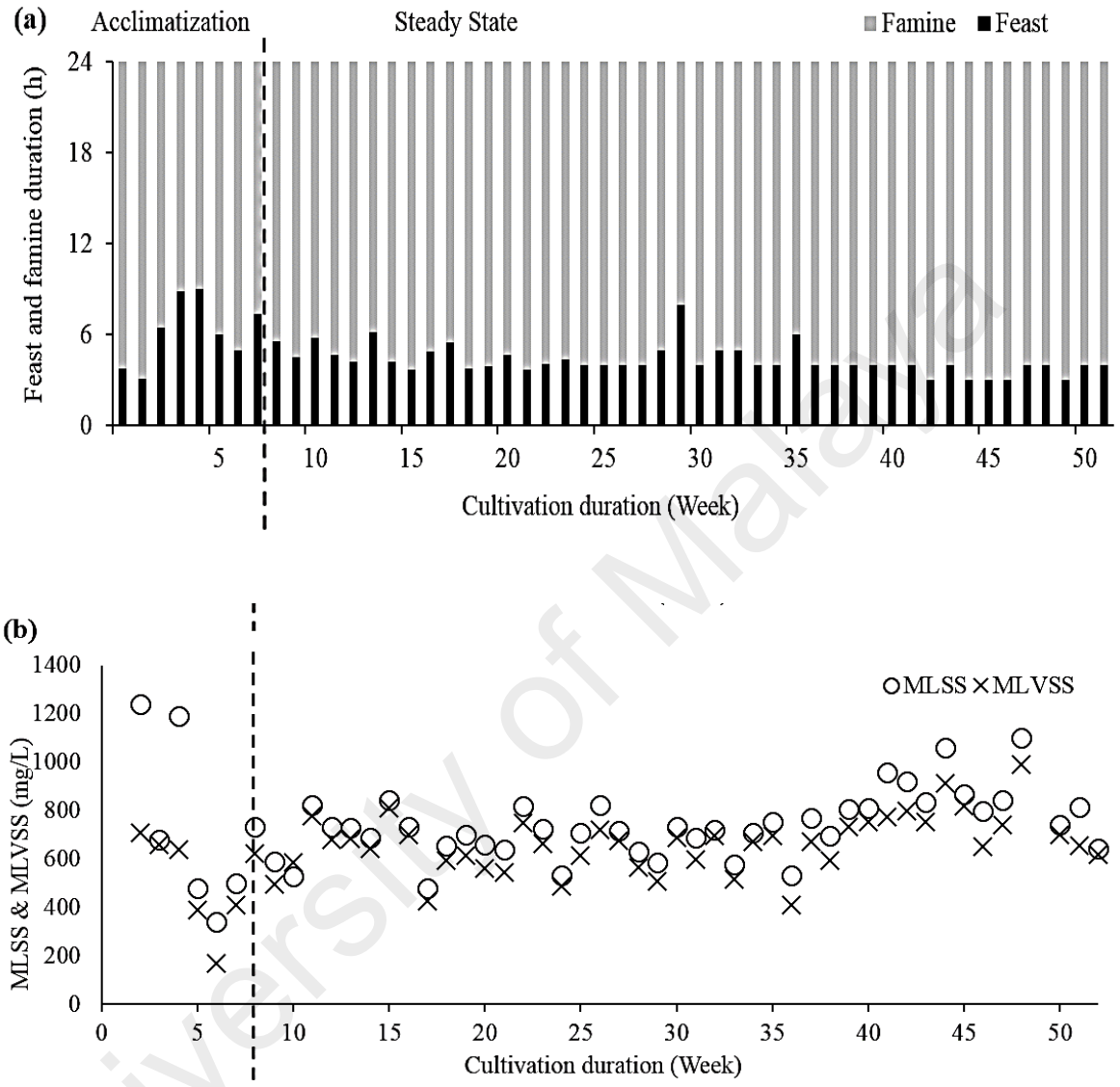

Figure 4.4: Stability of the cultivation reactor in cultivation run R1. (a) The feast and famine period duration, (b) the biomass concentration. Dashed lines indicate the acclimatization stage and the steady state of the cultivation run.

4.2 Effects of changing the organic loading rate (OLR) on the cultivation reactor

After the initial cultivation run R1, the OLR was further changed three more times gradually. Theoretically, increasing the OLR increases the supply of the carbon substrate which is expected to increase the final PHA produced. The result of the OLR change and the effects it had on the stability of the cultivation reactor were observed. To ease discussion in this section, the different cultivation runs R1, R2, R3, and R4 will be discussed together. As in R1, the stability in all the cultivation reactors are evaluated based on both the feast and famine phase and biomass concentration.

For R2 (with an OLR of 1000 mg C/ L·d), the feast and famine phase (as shown in Figure 4.5 (a)) showed that in 5 weeks the durations stayed on average at 5 hours and 19 hours respectively which were similar to cultivation run R1. The F/F ratio in cultivation run R2 was 0.26 which indicates a fast storage response was still occurring. This positive response to the increase of OLR was promising as it showed the potential and capability of the enriched PHA-accumulators to handle the sudden increase of OLR.

After 5 weeks of R2, the reactor moved on to R3 (with an OLR of 1250 mg C/ L·d). After two weeks of the OLR increase, the average feast phase was found to be 16 hours and the famine phase was merely 8 hours (Figure 4.5 (a)). The long feast phase and the short famine phase represents a failed ADF strategy. The long feast phase indicated that the enriched PHA-accumulators consumed the available carbon more slowly. With only 8 hours of famine phase before the next cycle starts, the sludge is not exposed to a lengthy famine phase that was the driving force for them to adopt the storage response. Without a long period of famine phase where the PHA stored would have been the source of energy, the start of a new cycle with carbon readily available diminishes the need to store PHA.

After 11 weeks of R3, it was concluded that the microbes present were unable to adapt to the higher OLR. This could be due to a number of reasons. One, there was an increase in the OLR but no change was made to the SRT and HRT of 2 days. With the same SRT and HRT as in R1 and R2 but a higher OLR, the sludge had insufficient time to complete an ADF cycle within 24 hours. Another possible reason could be due to the limited substrate uptake capabilities possessed by the PHA-accumulators present. The change from R1 to R2 was done after a year while the change of R2 to R3 was done only after 5 weeks. An acclimatization period was not observed or established before changes were made. Although R2 showed positive results, the change to a higher OLR in such short notice could have been premature where the enriched sludge present could still have been adapting and could not cope with another sudden surge in OLR. In hindsight, this experience is important in demonstrating the adaptiveness of the enriched PHA-accumulators to its external condition. Time plays a factor for the mixed cultures even those that are already enriched to develop their PHA-accumulating capabilities.

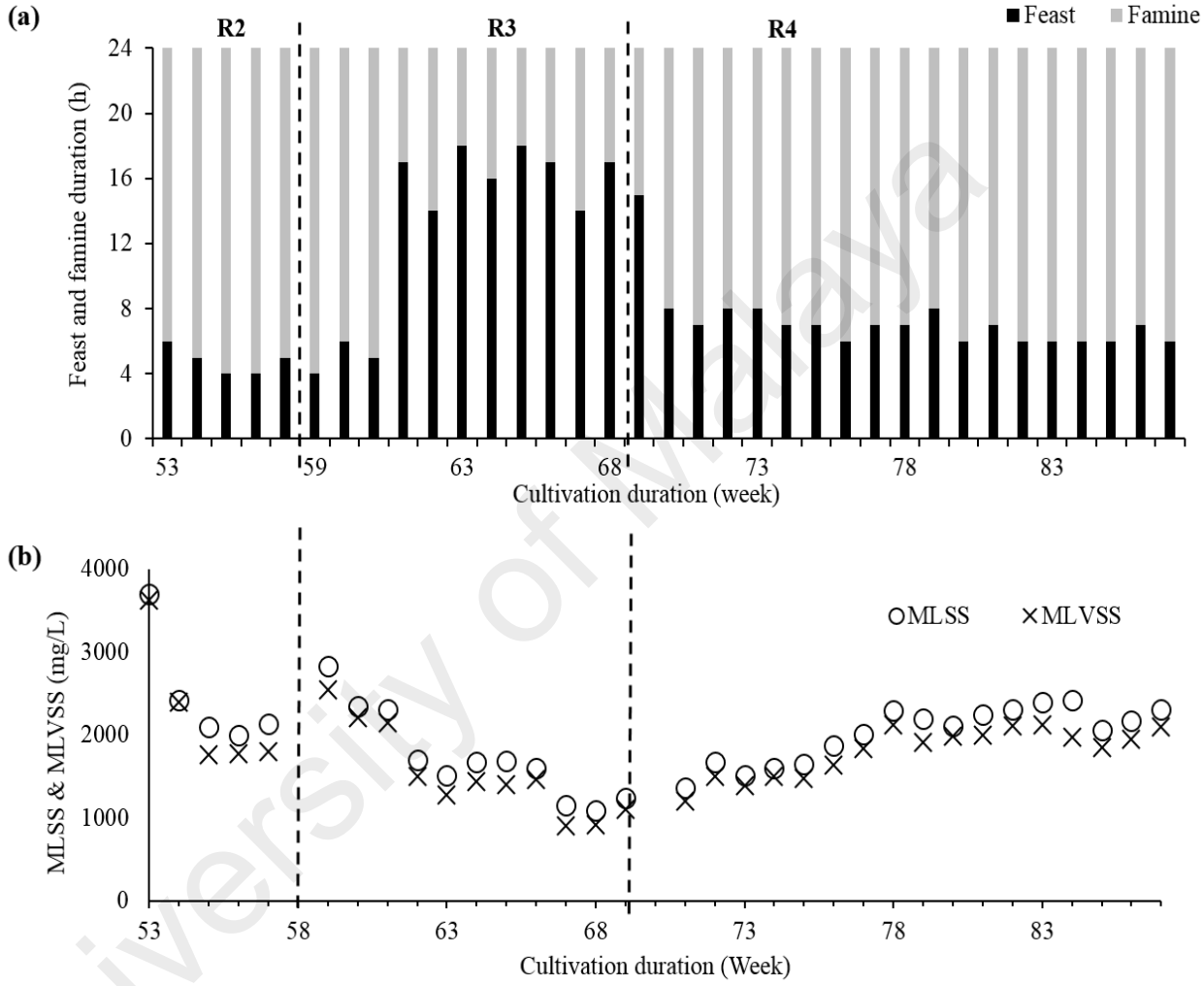

Figure 4.5: Stability of the cultivation reactor in cultivation runs R2, R3, and R4. (a) The feast and famine period duration, (b) the biomass concentration. Dashed lines indicate the acclimatization stage and the steady state of the cultivation run.

With the deterioration of R3, it was decided that instead of terminating the cultivation reactor, the OLR was to be reduced in the subsequent cultivation. This was a step taken to save and recover the cultivation reactor while keeping it running continuously. Cultivation run R4 ran with the exact same operating conditions as R2 (with an OLR of 1000 mg C/ L·d). This was based on the promising performance that was observed in R2. As seen in Figure 4.5 (a), within the first week, the feast and famine phase dropped to an average of 7 hours and 17 hours respectively. This indicates a positive response to the OLR supplied as well as a successful ADF strategy. This was expected because less carbon was being supplied, thus shortening the feast period and prolonging the famine period. This response demonstrated that the cultivation R2 was reproducible. More importantly, this response exhibited the recovery of the ADF strategy.

A stable feast and famine phase was seen throughout the next 15 weeks of the cultivation period. The F/F was averaging at 0.4, which is higher than the F/F obtained in cultivation runs R1, and R2. R4 had a higher carbon concentration compared to run R1 and has a slightly lower biomass concentration than run R2 both of which could have contributed to the higher F/F. Despite having a higher F/F ratio of 0.4, it is still recognized as an indication of the existence of storage response of the PHA-accumulators (Oliveira et al., 2017). The existence of a storage response is extremely important to indicate that the cultivation reactor is enriched with PHA-accumulators. From these different cultivation runs, it can be concluded that the F/F ratio is a preliminary indicator of whether or not storage response is achieved. Final PHA concentration analysis will still need to be carried out to confirm the amount of PHA stored.

This section focuses on the biomass concentration in evaluating the stability of the cultivation reactor (Figure 4.5 (b)). R2 was started using the enriched PHA-accumulators from cultivation at a higher concentration of 3.6 g/L. This is because it was expected that the biomass concentration would drop. The drop in biomass concentration from 3.6 g/L to 1.7 g/L as observed could be the result of further acclimatization to the new OLR. As discussed before, in adapting to the new OLR, some PHA-accumulators present could have a stronger ability to store PHA and those with weaker capabilities are washed out. By this stage, the majority of the biomass present should be considered PHA-accumulators, but because it is still a mixed culture system the diversity could still be high. This can also be an important reason why the acclimatization stage is very much important and need to be observed in ensuring a stable community of PHA-accumulator that have similar storage capacity. Despite the drop in concentration, there is still more biomass in R2 compared to R1 which is highly desirable.

The biomass concentration at the beginning of R3 also saw a decline initially. Although it did maintain on average at 1.2 g/L on the following weeks, in correlation with the feast and famine data for R3, the biomass present did not display the necessary PHA accumulation activity. Without PHA accumulation, this cultivation reactor is counterproductive. It could be assumed that with more carbon available and a failure in establishing the ADF strategy, the enriched sludge could transition from storage response to a growth response. However, the biomass concentration was observed to gradually decrease as the cultivation proceeds which was a worrying observation. This occurrence could be a result of two considerations. First, the OLR of 1250 mg C/ L·d together with the other operating condition could not have been optimum for a steady growth development. Second, as mentioned earlier, the sudden

change from R2 to R3 could have been too early. This did not allow the enriched sludge enough time to adapt and transition into the next OLR surge.

In an effort to restore the stability of the cultivation reactor, cultivation run R4 ran with an OLR of 1000 mg C/ L·d. It was found that the biomass concentration later recovered to an average of 2.0 g/L. It can be concluded that the biomass growth was able to maintain stability once again when the PHA-accumulators adapted to the current OLR with the operating condition. Despite the variation of biomass concentration throughout all the OLR change in the span of 8 months, it did raise slightly the biomass concentration. A significant revelation regarding the enriched sludge cultivated thus far is the robustness of the sludge. The sludge experienced different OLR in different time lengths, was exposed to a deterioration period, and was able to recover after restoration of the OLR. The final biomass concentration was found to be of similar concentration to other studies using mixed culture and waste substrate (Moita et al., 2014).

4.3 PHA accumulation by the enriched sludge

Microbial analysis specifically the Nile Blue A staining method was used as a quick method of confirmation for the accumulation of PHA intracellularly. Samples of the enriched PHA-accumulators were taken at the beginning of the feast phase, at the end of the feast phase, and at the end of the famine phase. These points are taken to comply with the ADF cycle profile.

For example, Figure 4.6 shows the staining from samples on week 50 from cultivation run R1. Image (a) shows the phase contrast image of the sludge while image (d) shows the stained image under fluorescent light. These two images respectively show that there are microbes

present and there is traces of PHA accumulation as indicated by the orange signals. Image (e) was the slide prepared using samples taken at the end of the feast phase showing the highest orange fluorescent intensity. This implies a higher content of PHA is present in the cell compared to the sample from the beginning of the feast phase. The end of the famine phase showed a weak orange intensity showing the decline of PHA (Image (f)). These observations confirm that there is accumulation of PHA during the feast phase and that the PHA stored was metabolized during the famine phase in order to survive external substrate unavailability.

To further analyze the community of microbes present, fluorescence *in situ* hybridization (FISH) method was used to estimate the abundance of *Alphaproteoacteria, Betaproteobacteria,* and *Gammaproteobacteria* in the sludge. In samples from week 50 of cultivation run R1, *Betaproteobacteria* was the most abundant (25.5 ± 14.5 %), followed by *Gammaproteobacteria,* and *Alphaproteobacteria* with, 17.7 ± 9.7 %, and 13.9 ± 9.2 % respectively.

However, cultivation run R4 samples from week 83 showed *Alphaproteobacteria* being the most abundant with 42.1 ± 10.6 %. This was followed by *Gammaproteobacteria* with 35.5 ± 9.9 %, and *Betaproteobacteria* with 7.2 ± 3.4 %. In this study, the OLR change could be the factor for the change in the abundance of proteobacteria between the two reactors. It is a common observation s that proteobacteria are heavily present in activated sludge that are able to accumulate PHA.

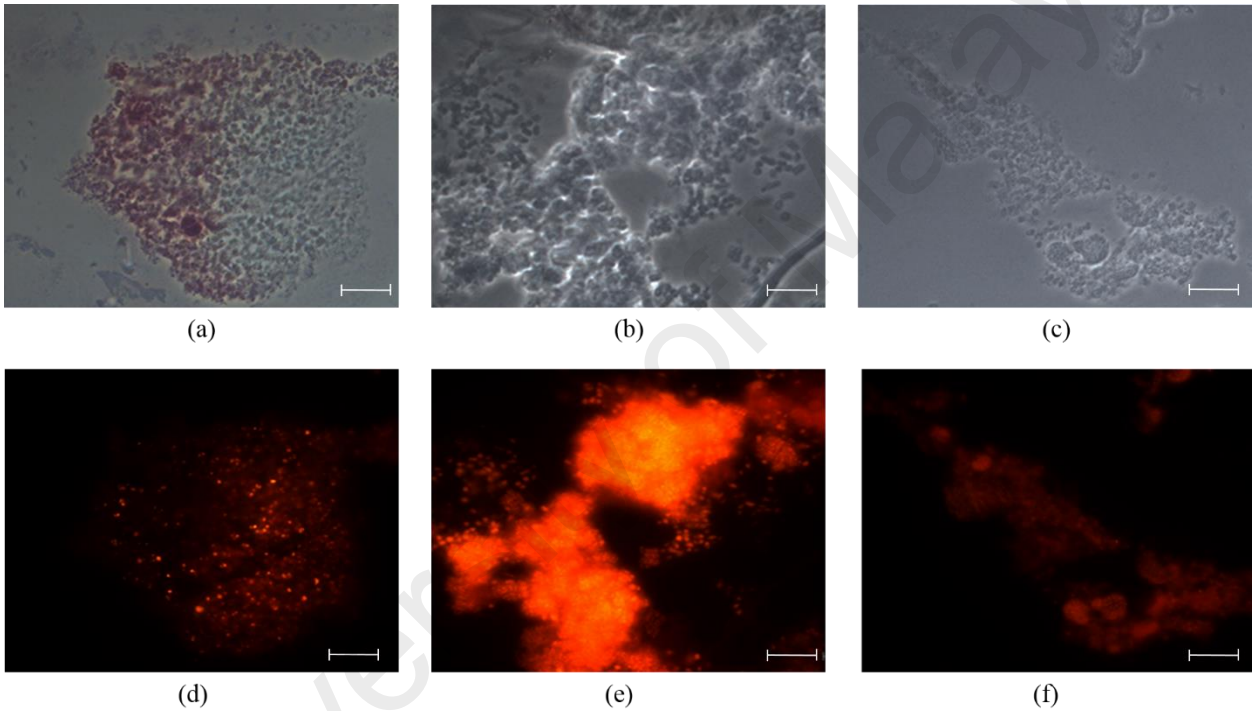

Figure 4.6: Microscopic images of the sludge taken from the cultivation R1 on week 24.
(a-c): Phase contrast images. (d-f): Nile Blue A staining images.
(a and d): Samples taken at the start of the feast phase. (b and e): Samples taken at the end of the feast phase. (c and f): samples taken at the end of the famine phase. 1 cm = 20 µm.

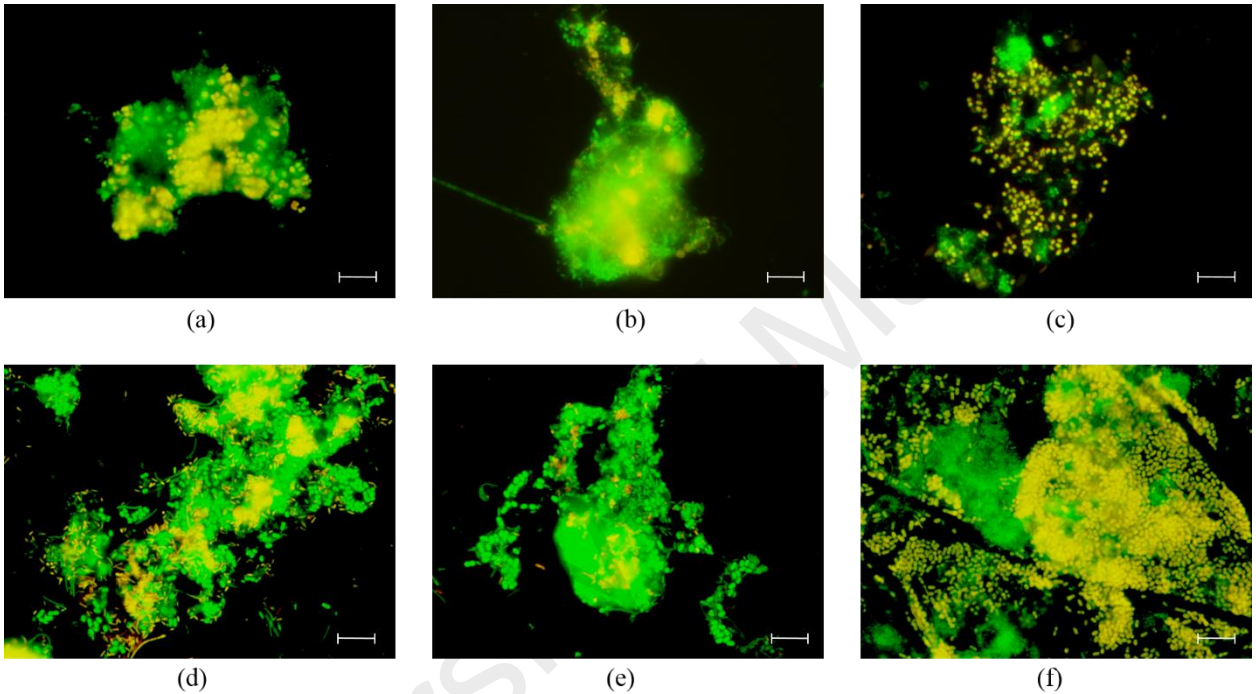

Figure 4.7: FISH images of sludge taken from R1 (week 50) and R4 (week 83).
(a-c): images from R1. (d-f): images from R4 where the green area represents microbes targeted with the probe EUBmix while the yellow area represents the specific group labeled with the Cy3-probe. (a and d): *Alphaproteobacteria*. (b and e): *Betaproteobacteria*. (c and f): *Gammaproteobacteria.* 1 cm = 10 µm.

4.4 PHA production performance evaluation

The second stage of PHA production is the production stage using the enriched PHA-accumulators from the cultivation reactor as the inoculum. The PHA-accumulators from the enrichment stage was ultimately tested for its PHA producing capabilities in batch tests. It is worth to note that different cultivation runs had different number of batch tests as a result of the different cultivation periods. Longer cultivation periods gave the opportunity for more production tests compared to the shorter cultivation periods. The activated sludge in this study prior to the enrichment process through the cultivation reactor only accumulated 10 wt% of PHA. This is consistent with another report that found activated sludge had PHA content of 9 wt% (Lee et al., 2015).

From the GC analysis, the monomers 3HB and 3HV were both identified during PHA quantification in the cultivation stage and in the production stage. This suggests that the PHA stored was the copolymer P(HB-co-HV). In contrast, studies on PHA production from mixed cultures using glycerol and crude glycerol accumulated only the homo-polymer PHB (Table 4.1). Before this work, P(HB-co-HV) from crude glycerol was only seen when using pure cultures. For example, in separate studies using crude glycerol, pure cultures of *Cupriavidus necator* and *Haloferax mediterranea* both accumulated P(HB-co-HV) with PHA content between 55 to 76 wt% of varying HB and HV composition (Garcia et al., 2013; Kachrimanidou et al., 2013; Herman-Krauss et al., 2013). Zhuge et al. (2013) have demonstrated that glycerol can be converted into propionyl-CoA which can act as a precursor for the accumulation of P(HB-co-HV) which proves that the production is indeed possible. (Kundu et al., 2014; Lu et al., 2009).

It is difficult to conclude how exactly a copolymer is produced in this study without investigating the specific metabolic route at which the polymer is formed. However, it has been discussed that the final polymer composition is not only influenced by the substrate as the precursor molecule. Other factors that influence type of polymer produced is the microbial population and its metabolic characteristics (Carvalho et al., 2014). The feeding regime was also found to be a factor that influence the final polymer composition. In a study conducted by Albuquerque et al. (2011), a continuous feeding as opposed to pulse-feeding of VFAs resulted in a higher HV composition in the final PHA produced. This is again demonstrated by Argiz et al. (2020) where they found that by changing the SBR operational cycle by adding a settling stage showed a rise in the HV fraction in the final PHA production. These examples provides explanation on how using the same substrate can still lead to different PHA composition when the operating conditions are manipulated.

Table 4.2 summarizes the maximum HB concentration, HV concentration, total PHA concentration and PHA content attained in this study. Focusing on the PHA concentration and PHA content, R1 and R2 was similar with 77 wt% and 78 wt% respectively. R1 had an OLR of 360 mg C/ L·d while R2 had an OLR of 1000 mg C/ L·d. The F/F ratio for R1 and R2 which are 0.20 and 0.26 are also similar. Further increase of OLR to 1250 mg C/ L·d in R3 however impeded the production capabilities, for which only 27 wt% of PHA was accumulated. This was associated with the declining stability of the cultivation reactor in the enrichment stage as indicated by the F/F ratio of 2.0. The feast phase exceeded the famine phase in the 24 h cycle and resulted in an absence of ADF strategy. This emphasizes the importance of the stability of the cultivation reactor in ensuring an efficient PHA production. Another notable observation is that the PHA content achieved from R3 is still higher than

PHA content found in the activated sludge (10 wt%) that was use as the seed inoculum used at the beginning of this study confirming the success of the enrichment stage.

From batch tests of R4, the PHA production was between 79 wt% and 80 wt%. This was similar to the PHA content achieved in R2 which was expected since it was a reproduction of R2. The high F/F observed in the cultivation stage may be a recovery stage after a failed ADF in R3. Although the OLR was reduced back to 1000 mg C/ L·d, the culture could be readjusting to the operating condition. It is an interesting avenue to consider in the future if with time the F/F would reduce further or there should be a change in other operating condition such as the SRT that could help improve this observation. The highest PHA content of 80 wt% obtained in this study is high compared to previous studies using crude glycerol as presented in Table 4.1. This can be the result of a successful stable cultivation reactor with the operating conditions used in this study. The activated sludge obtained locally could also be a determining factor where strong PHA-accumulators were able to be enriched using crude glycerol. Ultimately, the combination of suitable substrate, specific microbes present in the mixed culture, and an optimum cultivation reactor settings and operation dictates the final PHA production.

The total PHA concentration in R1, R2, and R3 are 1160 mg/L, 1263 mg/L, and 1417 mg/L respectively. The PHA concentration in R3 was found to be 390 mg/L which shows that even though the cultivation reactor did not exhibit a successful ADF strategy, there are still PHA-accumulators that exists. This is an important finding to note for two reasons. First, the cultivation stage managed to enrich strong PHA-accumulators that even in conditions where the ADF strategy is lost, when put in a production stage can still show signs of PHA producing capabilities albeit small. Second, based on the concentration found in R4, this

again shows how the PHA-accumulators present can recover their PHA producing capabilities.

The PHA production content throughout this whole study during stable state ranged between 77 wt % to 80 wt %. This is higher than the results obtained by Freches & Lemos (2017) where they studied the effect of OLR and cycle length using crude glycerol. In their study, they obtained PHA production yield of 0.44 g/g and a PHA content of 59 wt% with the optimum OLR to be at 50C mM. However, the PHA-accumulators that were used were already pre-acclimatized to crude glycerol. The SRT in this study was also shorter (2 days) compared to theirs (5 days). It has been reported that a shorter SRT would be sufficient for the supply of sludge for PHA production (Chua et al., 2003). Along with the formation of copolymer P(HB-co-HV) in this study, the results obtained are desirable. Pure HB have been found to be brittle which limits its application. The copolymer HB-co-HV are more malleable and less brittle making it a more desirable option for manufacturing into different products (Dias et al., 2006).

The volumetric productivity of the PHA production is listed in Table 4.3. The productivity of PHA production from R1, R2, and R3 ranged between 193 mg/L·h to 236 mg/L·h. The specific substrate uptake rates ($-q_s$) were similar in cultivations R1, R2, and R4, agreeing with the findings of Moita et al. (2014) when crude glycerol is used. The PHA accumulation rate (q_{PHA}) were likewise identical in cultivations R1, R2, and R4. From literature, the accumulation rate varied depending on the carbon used, concentration of carbon, and concentration of biomass (Chen et al, 2017). The PHA yield ($Y_{PHA/S}$) were consistent in this study but incomparable to other studies due to the carbon source. The values of biomass yield ($Y_{X/S}$) were constant throughout all four cultivations. Such low biomass yield could be

attributed to the nitrogen-limited condition in the production batches, which restricted biomass growth and favored the accumulation of PHA. The very small value (0.01) was found to be similar to other studies regardless of carbon source (Moralejo-Garate et al., 2013; Oliveira et al., 2017).

The low productivity from R3 (65 mg/L·h) can again be attributed to the failed ADF in the cultivation stage. By increasing the OLR and maintaining the SRT and HRT the cultures could not adapt and carry on with a storage response. As shown in Table 4.3, R3 has the lowest substrate uptake rate, lowest PHA accumulation rate, and subsequently lowest PHA production yield. As for the respiration yield, R3 showed high respiration yield at 0.69 compared to R1, R2, and R3 with only 0.29. The high respiration yield coupled with low substrate uptake rate and PHA accumulation rate can be an indicator of substrate inhibition. This observation was also shared by Freches & Lemos (2017). According to Moita et al. (2014), substrate inhibition can occur when additional substrate is added in excess. Instead of a storage response, a growth response is chosen by the culture present and this is translated into a high respiration yield and low substrate uptake rate.

Based on the results obtained in this study in relation to the increase of OLR in the cultivation reactor, the final PHA production does not significantly increase. However, this study has shown that the OLR does strongly affect the feast and famine phase which in turn provides the F/F. The F/F determines the extent of selective pressure imposed on the culture in the cultivation stage for the enrichment of PHA-accumulators. This has been highlighted before by Sepehri & Sarrafzadeh (2019) and Oliveira et al. (2017). The OLR still remains an important parameter that needs to be optimized as it also significantly influences the metabolic direction of the mixed culture. This study investigated a total of three different

OLR and found that the intermediate OLR of 1000 mgC/L·d was the most optimum together with the other operating conditions. As discussed by Fang et al. (2019), there is still room to investigate the highest OLR suitable for the enrichment of PHA-accumulators in order to produce high PHA productivity. They found in their study using rice winery wastewater, both experimental and mathematical modelling showed that moderate OLR are beneficial for the enrichment of PHA-accumulators.

Table 4.1: Comparison of PHA production from activated sludge and different substrates

Culture	Substrate	PHA content (wt%)	PHA composition (mol%)	References
Activated sludge	Crude glycerol	80	HB:HV = 60:40	This study
	Crude glycerol	47	HB = 100	Moita, et al. (2014).
	Glycerol	60	HB = 100	Dobroth et al. (2011)
	Crude glycerol	67	HB = 100	Moralejo-Garate et al. (2011)
	Palm oil mill effluent	64	HB:HV = 77:23	Lee, et al. (2015).
	Paper mill wastewater	77	Not reported	Jiang, et al. (2012).
	Sugarcane wastewater	61	Not reported	Chen et al. (2016)
	Hardwood sulfite spent liquor	4.6	HB:HV = 80:20	Queiros, et al. (2015).

Table 4.2: Maximum HB concentration, HV concentration, total PHA concentration, and PHA content attained in batch tests (at 6 hours).

Cultivation	OLR (mg C/(L·d))	F/F	3HB (mg/L)	3HV (mg/L)	Total PHA (mg/L)	PHA content (wt%)
R1	360	0.20	752	408	1160	77 (±2.5)
R2	1000	0.26	869	394	1263	78 (±3.0)
R3	1250	2.0	230	160	390	27
R4	1000	0.4	983	434	1417	80 (±1.5)

Table 4.3: Performance parameters of the PHA production.

	Volumetric productivity (mg /L·h)	$-q_s$ (mgC S/mgC X·h)	q_{PHA} (mgC PHA/mgC X·h)	$Y_{PHA/S}$ (mgC PHA/mgC S)	$Y_{X/S}$ (mgC X/mgC S)	$Y_{O2/S}$ (mgO_2/ mg S)
R1	193	0.24	0.17	0.7	0.01	0.29
R2	210	0.24	0.16	0.7	0.01	0.29
R3	65	0.06	0.06	0.3	0.01	0.69
R4	236	0.27	0.18	0.7	0.01	0.29

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study has explored a lab-scale process of PHA production using crude glycerol by activated sludge. Based on the results obtained from this study, several conclusions can be made.

i. This study managed to establish and maintain a long-term cultivation reactor that was stable and successful in enriching PHA-accumulators from activated sludge through ADF strategy.

ii. This study demonstrates a strategy to produce excellent PHA accumulation of 80 wt% as a result of using crude glycerol as substrate and activated sludge as the mixed culture at a maximum OLR of 1000 mg C/ (L·d).

iii. The maximum PHA production was achieved in 6 h with a productivity between 193 to 236 mg/ (L·h).

iv. The enriched sludge attained in this study were highly robust since it was able to preserve its PHA accumulating capabilities despite facing reactor upsets and multiple change in the operating conditions.

v. The identification of both HB and HV suggesting the production of the copolymer is a significant finding for the copolymer P(HB-co-HV) has better mechanical properties.

vi. The OLR change was found to not have a significant effect on the final PHA production and performance. However, it was able to highlight a strategy where the PHA production can be continuous and be restored when unforeseen upsets occur.

vii. The biomass concentration in the cultivation stage was improved significantly from 0.7 g/L to 2 g/L when the OLR was increased from 360 mg C/ (L·d) to 1000 mg C/ (L·d).

viii. Direct utilization of crude glycerol without prior treatment can be achieved together with activated sludge without the need for aseptic conditions the thus simplifying the process for PHA production.

5.2 Implication of this work

This work provides insight on the functionality of crude glycerol as a waste substance that could be converted into PHA a value added-product. The implications of this work are as follow;

i. PHA as a renewable and green alternative has multiple benefits towards the environment and the society directly by being a more environmentally friendly material.

ii. The conversion of waste substance into a beneficial product provides industries a direction to be more productive by managing their waste into another product.

iii. The PHA production industry can reduce their production cost as well as increase efficiency by eliminating the need for pre-treatment of substrate and further simplifying the process.

5.3 Recommendations for future work

There are several suggestions for that can be considered for further studies;

i. Study in detail other operating conditions of the cultivation reactor in relation to the OLR. For instance, the increase of OLR with an adjustment to the SRT and HRT.

ii. Evaluate further the cultivation period and its effect on the strength of the PHA accumulation capabilities. For example, in this study the cultivation period all had different cultivation lengths which made it difficult to determine whether the enriched sludge were fully acclimatized to the operating conditions.

iii. To look further into the structure and properties of the PHA accumulated and determine the best downstream processing method for the recovery process.

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LIST OF PUBLICATION AND PAPER PRESENTATION

Conference proceedings

Fauzi, A. H. M., Chua, A. S. M., Yeoh, H. K., & Yoon, L.W. (2015). Long-term stability of a mixed culture cultivation reactor for PHA-accumulating organisms using crude glycerol as substrate. In Proceedings of the 8th Regional Conference on Chemical Engineering (RCChE-2015), Hanoi University of Science and Technology, Vietnam, 30 Nov-1 Dec. (*Non-ISI/Non-SCOPUS*)

Journal article

Fauzi, A. H. M., Chua, A. S. M., Yoon, L.W., Nittami, T., & Yeoh H. K. (2019). Enrichment of PHA-accumulators for sustainable PHA production from crude glycerol. *Process Safety and Environmental Protection, 122*, 200-208. https://doi.org/10.1016/j.psep.2018.12.002 (*ISI-Indexed*)