OPTIMISATION OF EMULSIFIED LIQUID CULTURE SYSTEM FOR BACTERIAL POLY-3-HYDROXYALKANOATE PRODUCTION

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ABSTRACT

The use of polyhydroxyalkanoates (PHAs) as a polymer in various industries, including packaging and medicine, is receiving significant attention due to their biodegradability and biocompatibility. However, the high cost of production from carbon sources remains a major obstacle to the widespread use of PHAs as alternative polymers. Consequently, there has been a search for cost-effective substrates that can yield high productivity. One such substrate is cooking oil, which serves as an energy and carbon source. However, using an immiscible substrate like oil presents unique challenges because the fermentation liquid consists of a heterogeneous multiphase system that makes it difficult for bacterial to grow. Therefore, this study aimed to optimize the PHA production using the Box-Behnken method with oil, inoculums, and surfactants as the variables. The amount of PHA produced was comparable to the control experiments using a pure, single-type fatty acid as the sole carbon and energy substrate. The addition of surfactant (Tween-80) to the mixture of liquid medium and oil facilitated the emulsification of the immiscible substrates, thereby promoting bacterial growth. The optimal conditions for PHA production were determined as follows: biomass of 0.375 ml, oil of 1.25 g, and surfactant of 0.125 ml per 100 ml of medium. Furthermore, the molecular weight and chemical characteristics of the extracted PHA were analyzed. The study demonstrated the effectiveness of surfactants in achieving favourable cellular growth while simultaneously enhancing PHA production.

Keywords: Polyhydroxyalkanoate, Surfactant, Tween-80, Emulsification, Microorganisms

PENGOPTIMUMAN SISTEM BUDAYA CECAIR TEREMUSI UNTUK PENGELUARAN POLI-3-HIDROKSYALKANOAT BAKTERIA

ABSTRAK

Penggunaan polyhydroxyalkanoates (PHAs) sebagai polimer dalam pelbagai industri, termasuk pembungkusan dan perubatan, mendapat perhatian yang ketara kerana biodegradasi dan biokompatibilitinya. Walau bagaimanapun, kos pengeluaran yang tinggi daripada sumber karbon kekal sebagai penghalang utama kepada penggunaan PHA secara meluas sebagai polimer alternatif. Akibatnya, terdapat pencarian untuk substrat kos efektif yang boleh menghasilkan produktiviti yang tinggi. Salah satu substrat tersebut ialah minyak masak, yang berfungsi sebagai sumber tenaga dan karbon. Walau bagaimanapun, menggunakan substrat tidak bercampur seperti minyak memberikan cabaran yang unik kerana cecair penapaian terdiri daripada sistem berbilang fasa heterogen yang menyukarkan pembiakan bakteria. Oleh itu, kajian ini bertujuan untuk mengoptimumkan pengeluaran PHA menggunakan kaedah Box-Behnken dengan pembolehubah minyak, inokulum dan surfaktan. Jumlah PHA yang dihasilkan adalah setanding dengan eksperimen kawalan menggunakan asid lemak tulen jenis tunggal sebagai karbon tunggal dan substrat tenaga. Penambahan surfaktan (Tween-80) ke dalam campuran medium cecair dan minyak memudahkan pengemulsi substrat tidak bercampur, dengan itu menggalakkan pertumbuhan bakteria. Keadaan optimum untuk pengeluaran PHA ditentukan seperti berikut: biojisim 0.375 ml, minyak 1.25 g, dan surfaktan 0.125 ml setiap 100 ml medium. Tambahan pula, berat molekul dan ciri kimia PHA yang diekstrak telah dianalisis. Kajian itu menunjukkan keberkesanan surfaktan dalam mencapai pertumbuhan selular yang menggalakkan dan pada masa yang sama meningkatkan pengeluaran PHA.

Kata kunci: Polihidroksialkanoat, Surfaktan, Tween-80, Pengemulsian, Mikroorganisma

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LIST OF SYMBOLS AND ABBREVIATIONS

°*C*	degree Celsius
M_n	number-average
M_w	weight-average
ANOVA	Analysis of Variance
C:N	Carbon to Nitrogen ratio
Da	Dalton
DCW	Dry cell weight
DF	Dilution factor
DMSO	Dimethyl sulfoxide
FTIR	Fourier-transform infrared spectroscopy
g	Grams
GCMS	Gas chromatography mass spectrometry
GPC	Gel permeation chromatography
HA	Hydroxyalkanoate
HMDS	Hexamethyldisilazane
IS	Internal standard
K	Potassium
ml	milliliters
MMC	Mixed microbial culture
MT	Minimal trace elements
N	Nitrogen
NB	Nutrient broth
NMR	Nuclear magnetic resonance
OD	Optical density
P	Phosphorus

P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-valerate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HHx-*co*-3HO)	Poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate)
P(4HB)	Poly(4-hydroxybutyrate)
P. putida Bet001	*Pseudomonas putida* Bet001
P3HB	Poly-3-hydroxybutyrate
PDI	Polydispersity index (M_w/M_n)
PHA	Polyhydroxyalkanoates
PHA_{LCL}	Long-chain-length PHA
PHA_{MCL}	Medium-chain-length PHA
PHA_{SCL}	Short-chain-length PHA
PHB/P(3HB)	Poly(3-hydroxybutyrate)
PHBV	Polyhydroxybutyrate-*co*-valerate
PHD	Poly(3-hydroxydecanoate)
PHDD	Poly(3-hydroxydodecanoate)
PHHp	Poly(3-hydroxyheptanoate)
PHHx	Poly(3-hydroxyhexanoate)
PHN	Poly(3-hydroxynonanoate)
PHO	Poly(3-hydroxyoctanoate)
PHPp	Poly(3-hydroxypropionate)
PHV	Poly(3-hydroxyvalerate)
PLA	polylactic acid
PMSF	Phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
sp.	Species

CHAPTER 1: INTRODUCTION

1.1 Petroleum-based plastic challenge

Environmental pollution is a global challenge that affects countries of all sizes, including those that contribute less to global heating. The primary root of environmental pollution includes the release of greenhouse gases from heating fossil propellants like petroleum, coal and natural gas, as well as from solid waste and trees. Another significant cause is the release of non-biodegradable substances into land and water bodies, which harms organisms in terrestrial and aquatic habitats. Aquatic organisms have been found to contain foreign substances in their bodies (Law, 2017), with petroleum-based plastic being a major contributor to air and water pollution (Law, 2017).

These plastics can persist in the environment for up to 300 years without degrading (Ali et al., 2020). While polymers like polyethylene are useful in packaging, plumbing, and piping, they are not typically recyclable and emit harmful substances during production, posing risks to both humans and the environment. In 2019, plastic production resulted in the release of approximately 900 million metric tons of environmental toxic gases, corresponding to emissions from about 190 units of 500MW power plants powered with coal (Cabernard et al., 2022).

The plastic challenge has prompted calls for the development of bio-plastics that are environmentally friendly and sustainable. Bio-plastics are polymeric materials made from biomass (Keshavarz and Roy, 2010), which can be derived from natural polymers like carbohydrates, proteins, and lipids (Keshavarz and Roy, 2010). Unlike petroleum-based plastics, bio-plastics are biodegradable and have a favorable life cycle assessment for the ecosystem. However, despite their significant potential, research on bio-plastics is still ongoing, and their commercial viability is currently considered less economical.

1.2 Microbial polyhydroxyalkanoates (PHAs) as the alternative

PHAs are biodegradable and eco-friendly polyester groups that are stored as materials by various species of bacteria (Muthu Kumar, 2017). These bacteria accumulate PHAs in their cytoplasm like insoluble inclusions when they synthesize them (Muthu Kumar, 2017). The accumulation of PHA in microorganisms typically occurs under conditions that limit their growth, in addition having carbon sources in the bacteria growth medium is important. Some of these growth-limiting environments involve limiting access to nutrient such as nitrogen, sulfur, phosphorus, oxygen, and potassium.

PHAs are usually produced through bacterial fermentation of carbon sources like sugar and oil (Park et al., 2012). Because of their high compatibility, PHAs have a broad collection of potential uses in various industries. In the medical field, they can play an essential role in artificial organ construction, tissue regeneration and repair, drug delivery, and other therapeutic applications (Chen and Wang, 2013). According to Visakh, 2014, there is a wide variation in bacterial PHAs, with over 150 different monomers, including mercaptoalkanoic and hydroxyalkanoic acids. The molecular weight of the biopolymer produced can range from 50,000 to 1 million Daltons (Keshavarz and Roy, 2010).

Cheap sources of carbon including, corn starch, animal fat, alkanols, cane molasses, plant oils and beet can be used in PHA production (Peña et al., 2014). Hydrolysate of wood waste has also been reported as an excellent carbon source for the production of PHA (Muthu Kumar, 2017). Additionally, there is growing interest in using used cooking oils as carbon sources in PHA production due to their abundance in waste disposal (Favaro et al., 2018). Nitrogen sources are also important macro-nutrients that can be manipulated to optimize PHA production. For example, the

adjustment of carbon-to-nitrogen ratio (C:N) weight to 76% increased poly-3-hydroxybutyrate (P3HB) production to over 70%, highlighting the importance of nitrogen in PHA production (Khanna and Srivastava, 2005). Another study that varied the C:N ratio and the sequence of their supply found that the yield and properties of PHA produced varied. The study reported that a high C:N ratio, especially during the accumulation phase, creates favorable conditions for the microbes, resulting in a wider range of PHA yield (Ntaikou et al., 2019).

The optimization of the content of the growth medium will be a viable approach to minimize the cost for producing PHA and make it more economically viable. In addition to PHA yield, the cost of the substrate used largely determines the final selling price (Muthu Kumar, 2017). Several studies have suggested ways to minimize the cost of PHA production, including using an open and weak aerating system, recycling the wastewater used, and applying multiple fissions to enable fast growth (Chen et al., 2020). This current study made use of an inexpensive carbon source specifically the refined cooking oil. We also investigated using of surfactants in the growth medium to ensure high oil-water miscibility.

1.3 Problem statement

Petroleum-based plastics contribute significantly to global pollution, both in water bodies and on land. The adverse effects of these plastics are apparent, leading to the necessity for the manufacturing of sustainable, cost-effective, and environmentally-safe alternatives.

An oil-based substrate can serve as an abundant and economical carbon feedstock for the fermentation process of eco-friendly plastic alternatives like PHA.

However, the immiscibility of the oil-based carbon substrate often limits its accessibility to metabolizing cells for optimal growth and PHA production. To address this limitation, the hypothesis is that the use of a non-toxic and eco-friendly surfactant can emulsify the oil into droplets, making it more accessible to microbial attack. This approach eliminates the need for saponification, which is commonly used to enhance oil solubility in an aqueous environment. Saponification involves additional processes and expenses, often resulting in environmental and energy costs due to the requirement of strong alkali and heating components. Moreover, saponified oil possesses strong alkaline properties, leading to storage complications and the need for pH adjustment before using it as a carbon substrate in fermentation.

1.4 Research objectives

The rationale of the study is to develop a simple but inexpensive culture system based on triglyceride-emulsified liquid medium for microbial PHA fermentation. Inoculum, oil and surfactant are believed to be the important parameters in the fermentative PHA production. Thus, the current study intends to achieve the following objectives:

1. To study the effects of variation of biomass, oil, and surfactant (Tween-80) amounts on the PHA production;
2. To optimize the variation of biomass, oil, and surfactant (Tween-80) using Box-Behnken design;
3. To investigate the hydrophobicity, melting point and biodegradability of the produced PHA.

CHAPTER 2: LITERATURE REVIEW

2.1 Brief background of polyhydroxylalkonates (PHAs)

The presence of granules stored by microorganisms under specific conditions has been known since the 17th century. In 1881, Martinus Willem Beijerinck, a Dutch microbiologist, was the first to report on these granules, later known as PHA (Palmeiro-Sánchez et al., 2022). Beijerinck observed lipid-like granules in the bacterium *Azotobacter chroococcum* while studying nitrogen fixation (Palmeiro-Sánchez et al., 2022). However, little attention was given to this discovery until 1927, when Maurice Lemoigne, a French researcher considered the father of PHAs, discovered poly-3-hydroxybutyrate (PHB) which is considered the first affiliate in the PHA family, while working with the bacterium *Bacillus megaterium* (Palmeiro-Sánchez et al., 2022). Lemoigne determined the empirical formula of the compound to be (C4H6O2)n and studied its chemical composition, confirming it as one of the classes of PHAs found in nature. Later on, microbiologists began using the presence or absence of PHB as a taxonomic indicator for identifying bacteria. During this time, PHA received little attention due to the perception of abundant and cheap fossil fuel. However, after the oil crisis of the 1970s, interest shifted toward bio-plastics as a potential replacement to petroleum-based polymers (Palmeiro-Sánchez et al., 2022). Since then, researchers have re-engaged with PHA research, leading to numerous exciting discoveries in various sectors such as household, industrial, and medicine.

In 1982, it was discovered that poly-3-hydroxybutyrate (PHB) shared similar properties with synthetic plastic polypropylene. That same year, the bio-compatibility and biodegradability of P3HB were published (Palmeiro-Sánchez et al., 2022). However, PHA research has primarily remained academic, and more work needs to be done to gain acceptance in the industry. The number of publications on PHAs has increased

from around ten research works in the 1990s to approximately two hundred in the 2010s (Palmeiro-Sánchez et al., 2022). Numerous symposiums and conferences have focused on bio-plastics, particularly PHAs. The formation of the Biodegradable Plastics Association aimed to promote the interests of biodegradability, including PHAs. Journals like the Journal of Polymers and the Environment have published works on bio-based polymers, including PHAs. Furthermore, international companies like Imperial Chemical Industries have taken the lead in researching PHAs and their sub-units in Europe and North America. The company initiated PHB research in 1975 and later used the sub-unit polyhydroxybutyrate-co-valerate (PHBV) for commercial packaging. To assess the potential of the PHA market, several companies, such as Monsanto and Metabolix, have acquired the rights to own the PHA polymers. The names of some companies involved in PHA and its sub-units are listed in Table 2.1.

Table 2.1: Examples of companies involved in PHA production

Company	Country of origin	Sub-unit of PHA	Year of Research	Industrial Usage
Imperial chemical Industries	United Kingdom	PHBV	1980s	Packaging
Chemie Linz	Austria	PHB	1980s	Drug delivery and packaging
Mitsubishi	Japan	PHB	1990s	Packaging
BTF GmbH	Austria	PHB	1990s	Drug delivery and packaging
Monsanto	United States	PHB, PHBV	1990S	Raw Materials
Tepha, Inc.	United States	Several sub-units of PHA	1990s until present	Medical devices

PHAs are discrete cellular inclusions that range in diameter from 0.2 to 0.5 μm. They are found in the cytoplasm of cells and are usually imaged using a phase contrast light microscope because of the refractive properties (Sudesh et al., 2000). When examined under a transmission electron microscope, PHA inclusions in thin sections of bacteria that produce PHA are seen as electron-dense bodies, as shown in Figure 2.1. Popular stains especially basic oxazine dye including Nile blue A, Sudan black B, and Nile red produce good stain result for studying native PHA inclusions. While staining methods can indicate the presence of PHAs, the use of analytical chemistry such as gas chromatography (GC), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) are essential to establish their monomeric compositions. This study utilizes both GC and NMR techniques to identify the PHA.

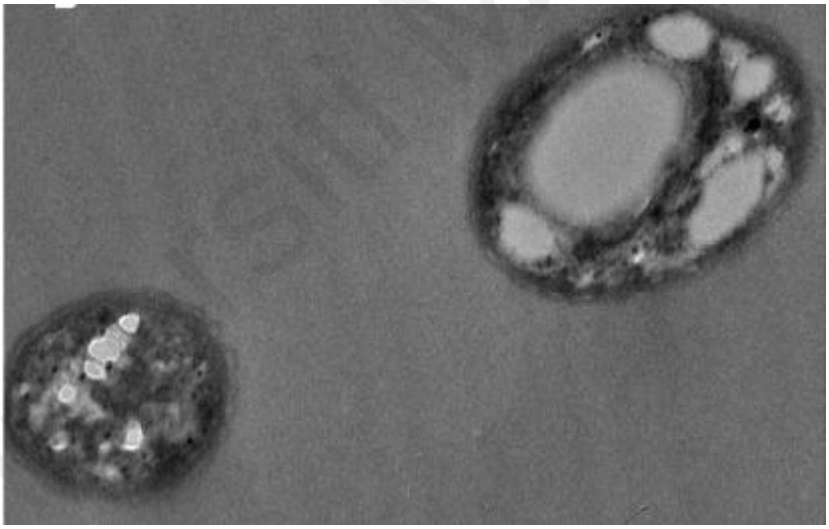


Figure 2.1: Transmission electron micrograph of intracellular accumulated discrete granules of PHA in *P. putida* BET001

(Source: Gumel et al., 2012)

2.2 Structural and physicochemical characteristics of PHAs

PHAs are polyesters that have side chains and (R)-3-hydroxy fatty acids with hydroxyl groups located on the 4- or 5-position. PHAs are divided into three when categorized on the length of chain of monomers of fatty acid:

(a) Short-chain-length polyhydroxyalkanoic acids (PHA_{SCL}) have an alkyl side chain and synthesized by bacterial species like *Cuprivadus necator, Alcaligenes latus, and Ralstonia eutropha*. PHA_{SCL} contain between 3 to 5 carbon atoms. Examples consist of poly-3-hydroxybutyrate (P3HB), poly(3-hydroxybutyrate-co-4-hydroxyvalerate) P(3HB-co-4-HV), poly(3-hydroxybutyrate-co-3-hydroxyvalerate), and P(3HB-co-3HV), poly-4-hydroxybutyrate (P4HB).

(b) Medium-chain-length polyhydroxyalkanoic acids (PHA_{MCL}) have multiple alkyl side chains and synthesized by bacterial species, especially *Pseudomonas syringae sensu stricto*, such as *Pseudomonas mendocina, Pseudomonas oleovorans, and Pseudomonas putida*. PHA_{MCL} contain between 6 to 14 have carbon atoms. Examples consist of poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate) P(3HHx-co-3HO), poly 3-hydroxyoctanoate P(3HO) and poly 3-hydroxyhexanoate P(3HHx).

(c) Long-chain-length polyhydroxyalknoic acids (PHA_{LCL}) are derived from long chain fatty acids with at least 15 carbon atoms. Less is known about this class of PHA.

There is growing interest in the intracellular depolymerization of PHA in PHA_{SCL} producers compared to PHA_{MCL} and PHA_{LCL}. PHA_{SCL} especially P(3HB-co-3HV) and P(3HB) are crystalline, fragile, and stiff polymers with low glass transition temperatures and high melting points and. Alternatively, P(4HB), another member of PHA_{SCL} family, is a tough, flexible thermoplastic polyester with a comparatively simple composition. In comparison to polypropylene and polystyrene, PHA_{SCL} have higher

tensile strength, while PHA_{MCL} are thermoplastic elastomers with high elongation to break but low crystallinity and tensile strength. They have higher glass transition temperature but lower melting points when compared to PHA_{SCL} and polypropylene.

The PHAs physical and chemical properties especially molecular structure and monomer composition vary, often depending on the organism from which they are produced and the source of carbon used in growing them. Over the years, more than 150 monomers of PHA have been discovered, which can combine to produce PHAs with different physicochemical characteristics. In Figure 2.2, the chemical structures of some PHAs are shown.

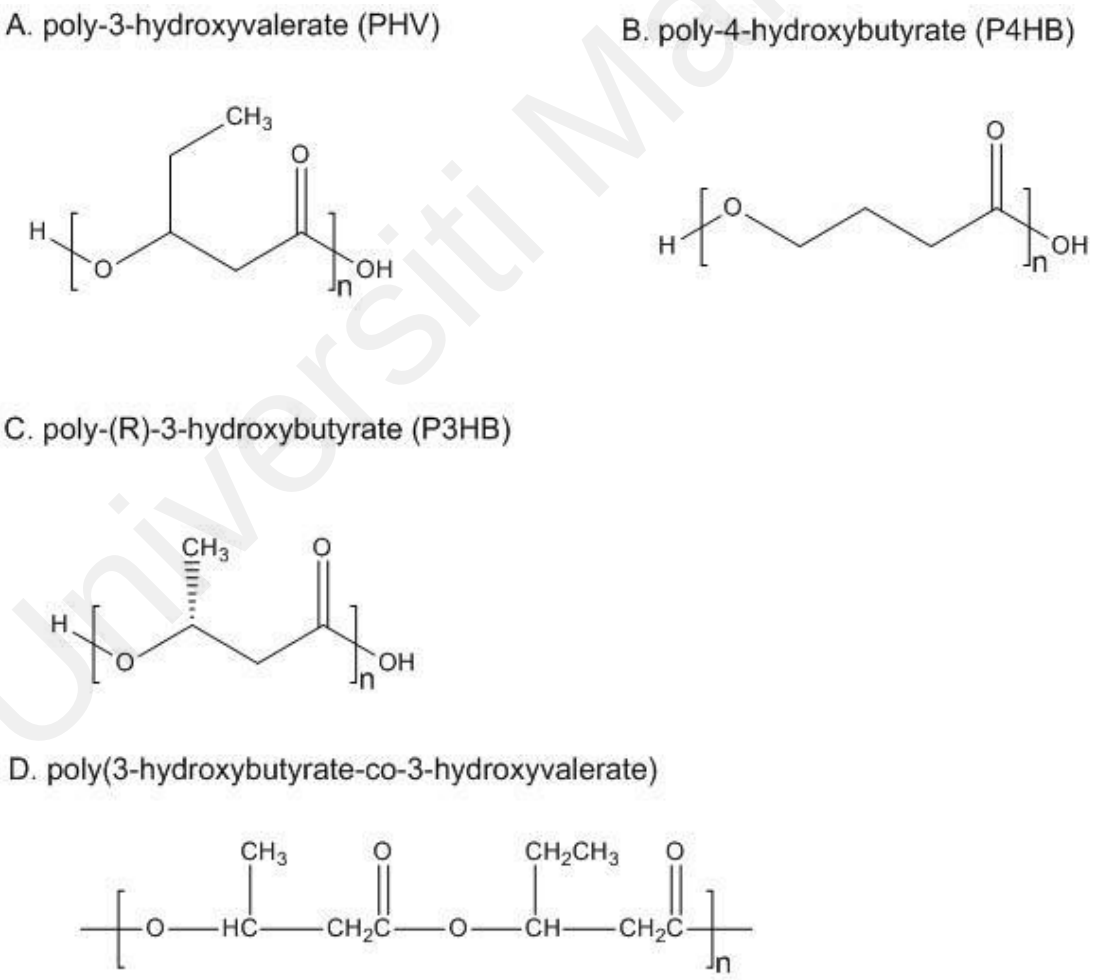


Figure 2.2 Chemical structures of PHA monomers

Crystallinity and chemical structure are important factors that determine the mechanical characteristics of PHAs (Visakh, 2014). For instance, an increase in side chains inside a polymer chain of PHA_{MCL} will affect the crystallization ability, leading to distinct features in the crystallinity of PHA_{MCL} (Visakh, 2014). In addition, the composition of the monomer and the chemical structure of the polymer affect the physical characteristics of PHAs (Visakh, 2014). Some of these factors include the chemical nature of the pendant groups, the extent of the pendant groups extending from the polymer backbone, and the distance connecting the ester linkages in the polymer (Visakh, 2014).

Scientists have conducted experiments involving the combination of PHAs and synthetic plastics. In a study where PHBV was mixed with polylactic acid (PLA), the resulting blend exhibited improved elongation at break, flexural strength and elasticity modulus. On the other hand, there was no effect on the tensile strength (Visakh, 2014). Similarly, another study found that blending PHB and PLA resulted in improved mechanical properties. In contrast, the addition of polyvinyl acetate (PVA) to a mixture of poly-*cis*-1,4-isoprene (PIP) and PHB enhanced strength of impact and tensile characteristics when compared to the blend of PHB/PIP. Combining synthetic plastics or starch with PHAs produces outstanding wrapping films. For example, blending thermoplastic starch with PHA yielded a product with superior hydrolytic and UV stability (Visakh, 2014).

2.3 Towards sustainable production of PHA

The last decade has seen significant efforts in improving PHA production as a probable replacement for petroleum-based plastics, which continue to damage the ecosystem (Koller, 2017). The goal has been to make PHA comparable with petroleum-based plastics in relations to affordability and value (Koller, 2017). Strategies for enhancing the quality of PHA-based materials include creating composites with compatible materials, optimizing microbial feeding strategies during the bioprocess, and blending PHA with other polymers (Kourmentza et al., 2017). Additionally, sustainability and reproducibility are crucial in every stage of producing PHA (Kourmentza et al., 2017).

Advancements in biotechnology have made it possible to produce PHA using either pure bacteria culture or mixed microbial culture (Kourmentza et al., 2017). In pure culture fermentation, growth-associated producers like *Alcaligenes latus* or *Paracoccus denitrificans* are used when the raw materials are rich in nutrients and carbon source. However, non-growth associated bacteria like *Cupriavidus necator* are used when the feedstock are deficient in important nutrients for growth. To be considered a PHA-producing microorganism, bacteria must possess specific characteristics (Kourmentza et al., 2017).

There are few microorganisms that have the ability to produce PHAs quickly by saccharifying cellulose (Kourmentza et al., 2017). Microorganisms isolated from contaminated sites are known for their ability to combine PHA production with bioremediation of toxic pollutants. Bacteria isolated from hypersaline environments have also shown promising PHA production capabilities. Furthermore, advancements in genetic engineering have enabled the development of genetically-modified bacteria, which can create more cellular space for PHA accumulation and enhance efficiency

(Palmeiro-Sánchez et al., 2022). Previous research have shown that genetic engineering can improve the efficiency of PHA production by eliminating genes responsible for producing side products like exopolymers (Kourmentza et al., 2017). Recombinant *Escherichia coli* are known for their ability for the manufacturing of 1.1 % PHA from xylan, it was further improved to 30.3 weight% and 40.4 weight% with the addition of arabinose and xylose, respectively (Salamanca-Cardona et al., 2014). In a genetically modified study, *Alcaligenes eutrophus* was able to accumulate about 90 weight% of PHB, while the wild type of *E. coli* could only accumulate about 70 weight%. Another study found that recombinant *E. coli* could accumulate 95 weight% of PHB, making it suitable for PHB production (Palmeiro-Sánchez et al., 2022).

Halophilic bacteria have also been extensively used for genetic manipulation due to their ability to produce PHA under continuous mode and unsterile environments (Yue et al., 2014). This has led to the advancement of a hyper-producing strain of the bacteria (Kourmentza et al., 2017). For instance, when wild-type and recombinant strains of *Halomonas campaniensis* LS1 were grown on wastes sourced from the kitchen with the addition of 26.7 g/L NaCl, at pH and temperature of 10 and 37 °C incessantly for 65 days devoid of contaminant, the recombinant strain synthesized about 70 % more PHB compared to the wild-type strain, with PHA representing about 30 % of the cell dry weight (Yue et al., 2014).

Genetic engineering of bacteria morphology, particularly increasing cell size, has also been reported (Kourmentza et al., 2017). Bacteria are known to have the capacity to accumulate other biopolymers such as glycogen, polyphosphates, proteins and sulfur inside their cells, which limits cell cytoplasm (Kourmentza et al., 2017). In a study aiming to increase cellular size, when the actin-like protein gene *mreB* was either deleted or inhibited in the recombinant *E. coli*, it led to a 100% increase in PHB

accumulation (Jiang et al., 2015). The manipulation of granule-associated proteins of the PHA producing bacteria can also boost the PHA granule size, making separation better (Pfeiffer et al., 2012).

Additionally, the preference of carbon substrate is a significant feature in PHA production. The optimization of the parameters involved in cultivation to enhance carbon inflow towards PHA biosynthesis can increase PHA efficiency (Kourmentza et al., 2017). Traditionally, complex polysaccharides like alginate, agar, β-glucan, cellulose, xylan, chitin, pectin, pullulan, laminarin, and starch have been used as sources of carbon (Kourmentza et al., 2017). However, recent studies have identified cheaper and more efficient carbon sources, such as biomass residues from foods like cassava starch, corn starch, sweet potato, and wheat starch (Sheu et al., 2009; Muthu Kumar, 2017). Recently, scientists have been using waste sources that contain carbon to both generate carbon mass and clean up the environment. For instance, in one study, they used the highly polluting waste olive mill wastewater as the exclusive source of carbon for the production of PHA. With a medium making up 15 % of the wastewater, the study obtained 43 % of PHBV/CDW (Alsafadi and Al-Mashaqbeh, 2017). Another study utilized used cooking oil as the source of carbon to produce PHB, *C. necator* DSM 428 was cultivated inside the batch reactors, and the granules of PHB produced from the biomass were purified using surfactants such as EDTA, SDS and Alcalase in the aqueous medium. They concluded by showing that a recovery of over 90 % of the PHB granules was obtained (Martino et al., 2014).

2.4 Challenges of conventional PHA production

PHA biopolymer production is considered expensive compared to other contemporary plastics. For instance, the cost of polymers like polypropylene is about USD 0.87/lb, whereas PHA biopolymer costs about USD 2.75/lb (Kourmentza et al., 2017). The exorbitant production cost of PHA is due to several factors including:

- The expensive ultra-pure substrates, such as glucose, used in the production process;
- The production being done in discontinuous batch and fed-batch cultivation modes;
- The significant quantity of labour and solvents required for downstream processing (Kourmentza et al., 2017).

Although the addition of 3-hydroxyvalerate (HV) to PHB in the production of PHBV results in a more flexible and resistant polymer with enhanced impact strength, elongation modulus, and tensile strength, it is still expensive and cost-prohibitive compared to other polymers (Visakh, 2014).

The use of high purity substrate accounts for approximately 45 % in the cost of PHA production (Kourmentza et al., 2015). To reduce this cost, researchers are investigating the use of renewable feedstock and the development of novel bioprocesses for recycling waste streams and by-products (Kourmentza et al., 2015; 2017). Additionally, government policies are being implemented to support and promote the use of biodegradable waste management (Kourmentza et al., 2017).

Addressing major cultivation and operating issues is crucial so as to enhance yield of PHA produce and reduce total cost of PHA production.

pH plays a noteworthy function in PHA productivity and the composition of its monomers in the fermentation process (Saharan et al., 2014). Research on the effects of pH on acidogenic fermentation has shown that a starting pH of 9 (alkaline) can result in the production of a substantial quantity of volatile fatty acids (Chen et al., 2013). Studies on the pH range have also indicated its impact on the polymer composition formed during batch fermentation, particularly in relation to HV content (Saharan et al., 2014). When a mixed microbial culture shifted from a sequencing batch reactor with a pH of 8.5 to batch reactors with a pH of 9.5, the PHA accumulated had a higher HV monomer content without any reduction in overall PHA production. Specifically, the HV content amplified from 10 to 30 % mol as the pH increased from 5.5 to 9.5 in that order (Dionisi et al., 2005). This study highlighted how microorganisms maintain the cytoplasmic pH necessary for optimal cell functioning in response to changes in the external environment's pH. Operational conditions at pH 7 resulted in 25 % PHB accumulation compared to 8. 5% at pH 9 and 15 % at pH 6 (Venkata Mohan et al., 2010). In the study, a mixture of propionic and acetic acids in various fractions was added as the source of carbon to enhance the culture with an organic load of 8.5 g COD/L.day at pH ranging from 7.7 to 9.5. The enhanced culture was reported to have increased production yields and rates (389 mg PHA/g of non-polymer biomass) in a sequencing batch reactor maintained at pH 7.5, *Lampropedia hyalina* was used as the main bacterial species in the study (Villano et al., 2010).

Furthermore, the PHA composition and production vary based on the feeding scheme used, which can also influences the composition of monomers in the produced PHA. A study reported that with the use of ^{13}C-NMR spectroscopy, the chemical composition and microstructure of the copolymer P(3HB-co-HV) produced were extensively influenced by the feeding regimen (Ivanova et al., 2009). Feeding with acetate as the source of carbon under aerobic conditions in a sequencing batch reactor

had no consequence on the composition of the microbial. However, the substrate utilization rate was higher with pulse feeding mode compared to continuous feeding mode (Ciggin et al., 2012). Conversely, continuous feeding mode led to the accumulation of PHA up to 64.5 % in a sequencing batch reactor using activated sludge collected from wastewater treatment plant and food wastes (Chen et al., 2013). Albuquerque et al. (2011) showed that an 8% increment in HV content of PHA when fermented molasses was used as the source of volatile fatty acids and a continuous feeding regime was employed. This was in contrast to the commonly used pulse mode feeding. Another study found a 4.8-fold increment when whey was intermittently fed with ammonium sulfate in a fed-batch culture of *Methylobacterium sp.* ZP24 under limited source of oxygen (Nath et al., 2008). The production of PHA requires careful planning of the feeding strategy. Periodic feeding of a carbon substrate consisting of propionic lactic and acetic acids at a rate of 8.5 g COD/L.day every 2 hours per day, led to both the P(3HB-co-3HV) co-polymer and the PHB homopolymer production from activated sludge enriched in a sequencing batch reactor under selective pressure (Dionisi et al., 2004). Increasing the organic loading rate to 12.75 g COD/L.day led to a microbial population with high storage capacity and yield. Periodic feeding of a mixture of lactic, propionic and acetic acids, at a frequency of every two hours with a dilution rate of one per day, produced a copolymer P(3HB-co-3HV) with 18% HV content. The dominant bacterial genera under these conditions were *Methylobacteriaceae Flavobacterium sp*., *Candidatus Meganema perideroedes*, and *Thauera sp.* (Dionisi et al., 2005). *Cupriavidus necator* DSM 545, when pulse-fed with soybean oil rich in fatty acids, produced 81 % PHB under growth-limited conditions with depletion of calcium, iron, copper and phosphorus (da Cruz Pradella et al., 2012). PHB production by *P. putida* was improved in multi-stage fed-batch cultivation when pre-grown cells in

glucose were transferred to medium containing octanoate under nitrogen- and oxygen-limiting conditions (Kim et al., 1997).

The C:N ratio is also a critical variable in PHA production. The highest accumulation of PHB was observed when a high C:N ratio was used, while the absence of carbon in the medium resulted in PHB degradation (Palmeiro-Sánchez et al., 2022). Maximum PHA production was achieved with an optimal C:N ratio of 25 (Mokhtarani et al., 2012). In a batch culture of *A. latus* ATCC 29713 with a C:N ratio of 28.3 using sucrose as the source of carbon and ammonium sulphate as the source of nitrogen, at pH 6.5, and temperature of 33 °C, the production of PHB increased by 1.8-fold (Grothe et al., 1999). Additionally, the design of cultural conditions plays a crucial role in enhancing PHA production by providing suitable nutrients. The response surface methodology, specifically the Box-Behnken design, is commonly used to optimize cultural conditions for maximum production of PHA. In a study using glucose as the carbon source, KH_2PO_4 and Na_2HPO_4 as the phosphorus source, and ammonium sulfate as the nitrogen source, cultivation parameters yielded a highest production of 1.45 g/L in *B. megaterium* SW1-2 with a 75 % validity, which was validated through a verification experiment (Berekaa and Al Thawadi, 2012). The various challenges facing the production are summarized in Table 2.2

Table 2.2: Challenges facing PHA Production

Challenges	
Technological	Inconsistent PHA properties
	Fermentation process and Bioreactor setup
	Downstream processing and recovery
Economical	High cost of production
	Cost of substrate
	Expensive bioplastic
Biological	Efficient low PHA accumulation
	Inability to grow a high cell density
	Identifying new microbes

2.5 Variation of carbon source

Carbon sources play a vital role in PHA production, falling into three main classes: simple sugars (monosaccharides), triacylglycerol, and hydrocarbons. The majority of microorganisms that produce PHA rely on simple sugars as their source of carbon. Triacylglycerol has been identified as a carbon source for a few microorganisms, while hydrocarbons are commonly used by *Pseudomonas* species (Jiang et al., 2016). Interestingly, different bacteria can produce PHAs with varying compositions using the same substrate. For example, *Pseudomonas sp.* can utilize glucose and other simple sugars to produce PHA_{MCL} like poly-3-hydroxylhexanoate (P3HHx) (Sun et al., 2015;

Jiang et al., 2016), whereas *R. eutropha* only synthesizes poly-3-hydroxybutyrate (P3HB) using glucose (Jiang et al., 2016).

Various types of sugar molecules have different effects on PHA production. Sucrose, a disaccharide found in sugar cane and sugar beet, has been extensively used as a source of carbon for producing PHAs. However, *R. eutropha* cannot utilize sucrose directly (Park et al., 2015). Wild-type bacterial strains that are known to synthesize PHAs from sucrose include *Azotobacter vinelandii*, *A. latus*, and *Hydrogenophaga pseudoflava* (Grothe et al., 1999; Tanamool et al., 2010). According to Grothe et al. (1999), optimized culture conditions for producing PHAs using sucrose include temperatures of 25-37 °C, ammonium chloride or sulfate as the nitrogen source, and a pH of 6.5. Under these conditions, PHA production reached 63 % of the CDW. Recombinant microorganisms have the ability to produce even greater amounts of PHA compared to wild strains. Additionally, they can modify the polymer composition as needed. For example, recombinant *C. necator* produced PHA from whey at 80 % of the cell dry mass relative to the wild-type strain, which only reached 30 %. Genetically engineered *Rhodospirillum rubrum* was capable of producing a wide range of PHBV copolymers using different carbon sources, whereas the wild-type strain could only produce PHB homopolymer (Palmeiro-Sánchez et al., 2022).

Another disaccharide, lactose, which consists of galactose and glucose, is also an inexpensive carbon source. It can be obtained from whey (Jiang et al., 2016). Microorganisms that are known to directly produce PHAs using whey include *H. pseudoflava* DSM 1034 and *Sinorhizobium meliloti* 41 (Povolo et al., 2013). Other microorganisms include recombinant *E. coli*, *Methylobacteria sp.* ZP24, *Thermos thermophilus* HB8, and *Bacillus megaterium* (Pandian et al., 2010). A study comparing the fermentation of arabinose and xylose to glucose using *P. pseudoflava* found that the

conversion rate of arabinose and xylose substrate to PHA was 0.17-0.19 g PHA/g substrate, while glucose substrate had a conversion rate of about 0.4 g PHA/g substrate. Additionally, the production of PHA was about five times slower when using xylose and arabinose compared to glucose (Bertrand et al., 1990). The study also reported that the molecular weight of the P3HB produced from xylose fermentation was similar to that of glucose, while arabinose fermentation yielded PHB with a lower molecular weight compared to glucose fermentation. The researchers concluded that these differences in molecular weight did not considerably influence the mechanical performance of the PHA materials (Bertrand et al., 1990).

Another inexpensive and sustainable source of carbon for PHA production is triglyceride, specifically waste frying oil. After undergoing several chemical reactions during industrial use, these oils are often considered as waste. Several studies have documented the use of waste frying oil as a source of carbon for producing PHA. For example, propanol and waste rapeseed oil were used as co-substrates with *R. eutropha* to synthesize P(3HB-co-3HV) (Obruca et al., 2010). Comparable study was conducted using waste rapeseed oil with the same strain to produce PHAs (Verlinden et al., 2011). Both studies reported that the presence of other nutrients in the waste frying oil improved the PHA production. Another study conducted a 5 L batch fermentation using R. eutropha as the microorganism and reported a productivity of 0.14 g PHB L/h and a yield of 0.14 g PHB/g oil waste (Morais et al., 2014). This study focused on optimizing the conditions for PHA production by varying factors while using fresh cooking oil as the carbon source.

2.6 Microbial community of PHA producer

Microbes, including microbial organisms, are known to proliferate through binary fission (Chen et al., 2020). These organisms live, reproduce, and synthesize new products by extracting nutrients from their immediate environment. While the growth period in microbes consists of lag, log, stationary, and death phases, it is during the stationary phase, when cell growth remains constant, that polymers such as PHA are formed. In this phase, the limitation of oxygen and/or other nutrients, coupled with the accumulation of toxic by-products, often leads to PHA production when a carbon source is added. Studies have shown that altering the pattern of cell division and growth can enhance PHA production (Chen et al., 2020).

PHA producers can be categorized based on their ecological niches, such as halophilic bacteria, photosynthetic bacteria, plant root growth-promoting rhizobium and hydrocarbon degraders.

Hydrocarbon degraders are bacterial strains belonging to genera like *Pseudomonas, Acinetobacter, Sphingobacterium, Brochothrix, Caulobacter, Ralstonia, Burkholderia, and Yokenella*. These bacteria have been found to produce PHA (Dalal et al., 2010). They accumulate PHA while degrading oil, particularly in contaminated sites. These organisms are able to do so because oil-contaminated sites contain higher amounts of carbon (84 %) and lower amounts of nitrogen (>1 %), which create favorable conditions for PHA production in cells (Atlas, 1995). A study reported that *R. eutropha* JMP 134 can produce PHB up to 50 % of its cell dry weight when exposed to growth-inhibiting substances like phenol or sodium benzoate as a carbon source under nutrient-limiting conditions. This demonstrates the ability of hydrocarbon degraders to produce significant amounts of PHA (Maskow and Babel, 2000). Halophilic bacteria are able to survive in high salinity environments, which make them ideal candidates for

PHA production. This is because the high salt concentration prevents cross contamination and allows for inexpensive extraction methods (Palmeiro-Sánchez et al., 2022). For example, extremely halophilic archaebacteria from the *Halobacteriaceae* family have been found to produce PHB under nutrient-limited conditions with abundant carbon sources (Saharan et al., 2014). *Haloferax mediterranei*, another halophilic organism, is capable of producing 60 to 65 % PHA of its cell dry weight when grown in phosphate limiting conditions with glucose or sucrose as the source of carbon (Garcia Lillo and Rodriguez-Valera, 1990). Similarly, the moderate halophile *Halomonas boliviensis* LC1 produces a higher amount of PHB at 56 % cell dry weight when grown on starch hydrolysate as a substrate. Furthermore, it can produce high amounts of PHB, around 88 % cell dry weight, when excess butyric acid and sodium acetate are used as carbon sources under nutrient-limited conditions (yeast extract 0.1 % w/v) during the stationary phase (Quillaguam´an et al., 2006).

Photosynthetic prokaryotes, especially cyanobacteria, also have the ability to produce and store PHA. Cyanobacteria such as *Synechococcus sp.* MA19, *Nostoc muscorum*, and *Spirulina platensis* have been reported to produce PHB under phosphate limited conditions (Nishioka et al., 2001; Panda et al., 2005). Research has shown that pre-grown cells of *Synechocystis sp*. PCC 6803 produce about 29 % PHA by cell dry weight under phosphorus-deficient conditions with the addition of 0.4 % acetate using glucose as the major carbon source (Panda et al., 2006). Moreover, using cyanobacteria as PHA producers with energy from sunlight may reduce the cost of PHA production while capturing CO_2 from the atmosphere for carbon conversion (Wu et al., 2002).

The soil in the vicinity of plant roots, known as the rhizosphere, is known to house microorganisms that enhance root and plant growth by producing extracellular metabolites (Saharan et al., 2011). Various plant growth-promoting rhizobacteria,

including *Burkholderia terricola*, *Lysobacter gummosus*, *P. extremaustralis*, *P. brassicacearum*, and *P. orientalis*, have been identified as PHA producers using molecular sequencing techniques that target the polyhydroxyalkanoate synthase (*phaC*) gene (Gasser et al., 2009). *Streptomyces* is a type of aerobic, gram-positive filamentous bacteria that is well-known for producing important metabolites, including antibiotics. It has been found that *Streptomyces* can produce PHA intracellularly in a granular form. These granules then function as a source of carbon units for the synthesis of antibiotics and sporulation (Saharan et al., 2014).

Various strains of *Streptomyces*, such as *S. aureofaciens* 84/25, *S. griseus*, *S. olivaceous*, *S. fradiae*, *S. parvus*, *S. albus*, and many others, have been reported to produce PHB when provided with glucose as the carbon source (Verma et al., 2003; Saharan et al., 2014). For example, when *S. griseorubiginosus* was supplemented with nitrogen and an increased carbon source, it showed a positive effect on PHB production. In fact, this microorganism was able to produce up to 9.5 % of mycelial dry mass of PHB during the early stationary phase (Saharan et al., 2014). The PHB produced by different strains of *Streptomyces* has been found to play a significant role in antibiotic production.

2.7 Biodegradation of PHA

The practical advantage of PHA over petroleum-based plastics, including other bioplastics, is its high biodegradability. PHA degrades at different rates depending on the specific monomers. In a microbiologically active environment, PHBV usually degrades within five to six weeks, releasing water and carbon dioxide in aerobic conditions and methane in anaerobic conditions (Visakh, 2014). Studies have shown

that degradation is faster in anaerobic environments compared to aerobic environments (Visakh, 2014).

The enzymatic degradation of PHA within cells occurs rapidly, with microorganisms able to reduce their cellular weight by ten times in just a few hours (Palmeiro-Sánchez et al., 2022). When PHAs are extracted as granules, biodegradation can occur within hours. However, chemical processing can result in longer biodegradation times, ranging from days to weeks (Palmeiro-Sánchez et al., 2022). For example, a PHB granule with a diameter of 75 μm would take about a week to biodegrade in anaerobic conditions, but around 90 days when in soil (Palmeiro-Sánchez et al., 2022). In natural environments such as soil, freshwater, and seawater, PHAs can take approximately six months to biodegrade (Palmeiro-Sánchez et al., 2022).

Numerous studies have focused on the controlled degradation of PHAs using various approaches, including enzymatic hydrolysis (Renard et al., 2004), thermal degradation (Ariffin et al., 2008), microwave-assisted degradation (Ramier et al., 2012), and alkaline- and acid-catalyzed hydrolysis (Yu et al., 2005). Acid-catalyzed hydrolysis produces linear oligomers with a hydroxyl group at one end and a carboxyl group at the other end, along with small amounts of cyclic structures (Ishak, 2014). Alkaline conditions result in the formation of oligomers containing a hydroxyl end group and an ester end group. There is a distinction between the degradation of PHA_{MCL} and PHA_{SCL} in thermal processes. PHA_{SCL} primarily undergoes a random chain scission reaction with a six-member cyclic transition state, resulting in oligomers containing an unsaturated crotonic end group. On the other hand, PHA_{MCL} involves hydrolysis of ester linkages, leading to the subsequent dehydration of hydroxyl terminal groups and the production of alkenoic acids as end products (Chan Sin et al., 2010).

Both PHA_{MCL} and PHA_{SCL} can be degraded by various bacteria and fungi under anaerobic and aerobic conditions. Popular bacteria that degrade PHA belong to the *Pseudomonas*, *Bacillus*, and *Burkholderia* genera, while fungi from the *Ascomycota* and *Zygomycotina* genera are more effective PHA degraders than bacteria (Kim and Rhee, 2003; Dalton et al., 2022).

PHA depolymerases are enzymes involved in the extracellular biodegradation of PHA. They break down the polymer into shorter chains through hydrolytic depolymerization, forming oligomers that further degrade into trimer and dimer units. This breakdown can occur through the action of PHA polymerase, as well as lipases and hydrolases (Dalton et al., 2022; Meereboer et al., 2020). PHB, a common PHA, is degraded in this manner.

The enzymatic hydrolysis of PHAs is a two-step process, even though it involves three domains for enzyme attachment to the PHB surface (Dalton et al., 2022). The first step is the absorption of enzymes onto the PHB surface, followed by the cleavage of PHA bonds induced by the absorbed enzymes (Dalton et al., 2022). This process is more favorable for PHAs with lower crystallinity and amorphous surface crystals, as the hydrolytic enzymes can access the less ordered structure more easily (Dalton et al., 2022).

The end products and efficiency of enzymatic degradation depend on the type of PHA. The physical and chemical properties of PHA, such as crystallinity, copolymeric structure, and polymer types, influence the biodegradation rate and efficiency (Meereboer et al., 2020). For example, PHBV has a greater amorphous region compared to PHB due to the presence of 3-hydroxyvalerate, allowing for easier access of the enzyme catalytic domain and cleavage of the PHA (Dalton et al., 2022). Other factors that determine the biodegradability of PHAs include unknown enzymes, PHA side chain

length and frequency, environmental conditions (e.g., water temperature), and molecular weight (Dalton et al., 2022).

2.8 The role of surfactant in PHA production

Surfactants are an important agent in PHA production. The mechanism of action of most surfactant involves incorporating into the lipid bilayer of the cell membrane, which causes the cell volume to increase. Eventually, this leads to the membrane breaking and the production of large micelles containing surfactant and phospholipids, resulting in the synthesis of PHA (Pagliano et al., 2021). Additionally, surfactants have the ability to solubilize proteins and other cellular materials, aiding in the disruption of the cell membrane (Pagliano et al., 2021). As additives for cellular lysis, surfactants can be directly added to cultures, bypassing the need for biomass drying. However, this method can affect the properties of the extracted PHA, potentially leading to PHA degradation and a decrease in molecular weight (Pagliano et al., 2021).

Most studies indicate that combining surfactants with chemicals like NaOH, NaClO, and chelating agents can enhance the purity and recovery yield of the PHA extracted (Mannina et al., 2019).

There are different types of surfactants that can be grouped as follows:

1. Anionic surfactants, such as sodium dodecyl sulfate (SDS) or alkylbenzene sulfonates (LAS), are the most commonly used. They are often tested alone or in combination with chemical or thermal pre-treatments. SDS, in particular, is known to produce inconsistent results in terms of recovery, purity, molecular weight, and polydispersity index of the PHA, making it difficult to determine its true effect on these parameters (Pagliano et al., 2021).

2. Cationic surfactants, such as benzalkonium chlorides (BAC), hexadecyltrimethylammonium bromide (CTAB), or palmitoyl carnitine are less commonly used compared to anionic surfactants. They tend to decrease the molecular weight of the recovered polymer, although the purity and recovery remain similar (Pagliano et al., 2021).

3. Non-ionic surfactants, such as Triton X-100 or Tween-20, fall between the previous two categories. They are known to perform excellently in relations to polymer recovery, polymer purity, and molecular weight of the recovered polymer (Pagliano et al., 2021).

In mixed microbial cultures (MMC), surfactants have been reported as an effective method for extracting PHA with good purity and increased yields, especially when combined with specific pre-treatments (Pagliano et al., 2021). For example, when applied alone, SDS or ammonium laurate resulted in PHA purity values of 40-60 %. However, when pre-treatments like NaClO or freeze-drying were employed before adding the surfactant, the purity value increased to 90 %. It is important to note that surfactants play an important role on the molecular weight of PHAs, which is around 0.1 MDa (Mannina et al., 2019). Furthermore, a high surfactant-to-biomass ratio is typically necessary to achieve a high PHA yield, which can result in a large amount of wastewater that needs to be treated (Jacquel et al., 2008). To address this concern, recyclable surfactants such as Switchable Anionic Surfactant (SAS) have been used in both single strains and MMC (Mannina et al., 2019).

CHAPTER 3: METHODOLOGY

3.1 Materials Used in the Study

3.1.1 Microorganism

This study obtained *P. putida* Bet001, a bacterial strain, from the Bioprocess and Enzyme Technology Laboratory situated at the Institute of Biological Science (ISB), Universiti Malaya. The freeze-dried *P. putida* Bet001 was reconstituted using nutrient broth. A 3 % (v/v) stock inoculum of the bacteria (*P. putida* Bet001) was initiated aseptically into a rich medium and incubated in a shaker incubator at 30 °C and 250 rpm for 24 hours. The biomass was harvested from 3 ml of culture broth by centrifugation at 4 °C and 9000 ×g for 10 minutes. The biomass pellet recovered from the centrifugation was resuspended in a 0.9 % w/v normal saline solution after which it was aseptically seeded into the E2 medium. The flowchart for the research is shown in Figure 3.1.

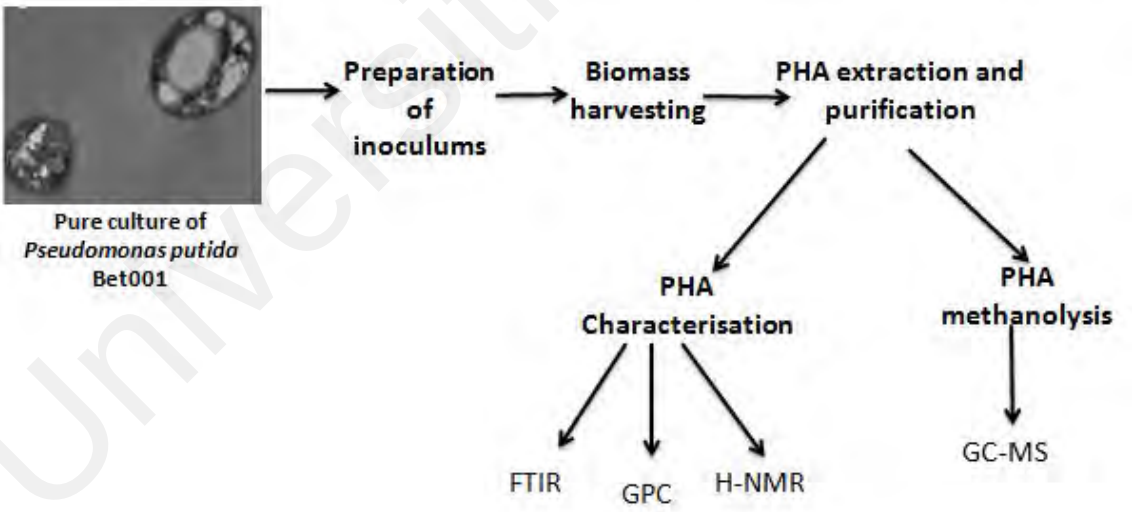


Figure 3.1 Research flowchart of the study

3.1.2 Nutrient broth

50 ml of the nutrient broth was prepared using a 100 ml conical flask. The nutrient broth powder (0.4 g) was dissolved further in 50 ml of distilled water before been autoclaved.

3.1.3 Rich medium

The preparation of the rich medium was performed by fully dissolving all the component chemicals (see Table 3.1) in a pre-determined volume of distilled water.

Table 3.1 Preparation of rich medium

Component	Mass (g) per litre of distilled water
Yeast extract	5.0
Nutrient broth	7.5
Ammonium sulphate	2.5

3.1.4 E2 medium and mineral supplementation

The E2 medium (Table 3.2a) was prepared in three stages. In the first stage, two different phosphate salts and an ammonium salt were dissolved in distilled water. The second stage involved adding a pre-determined volume of oil and surfactant, followed by autoclaving. Finally, in the third stage, a sterilized solution containing the trace elements (Table 3.2b) and $MgSO_4.7H_2O$ was added aseptically before the cultivation began (Lageveen et al., 1988).

This step was meant to prevent the precipitation of E2 medium components caused by the high temperature and pressure during autoclaving. The E2 medium is a nitrogen source-limited preparation that promotes the accumulation of PHA by *P. putida* Bet001.

Table 3.2a Preparation of E2 medium

Component	Mass (g) per 100mL of distilled water
K_2HPO_4	0.57
KH_2PO_4	0.37
$NaNH_4HPO_4.4H_2O$	0.35
0.1M $MgSO_4.7H_2O$	1ml
Trace elements (Table 3.2b)	0.1ml

Table 3.2b Trace elements composition

Component	Mass (g) per 500mL of 1N HCL
$FeSO_4.7H_2O$	1.39
$MnCl_2.4H_2O$	0.99
$CoSO_4.7H_2O$	1.41
$CaCl_2.2H_2O$	0.74
$CuCl_2.2H_2O$	0.08
$ZnSO_4.7H_2O$	0.14

3.1.5 Cooking oil

Two kilograms of pure cooking oil made from palm seed at a local supermarket in Kuala Lumpur were obtained for this study. The brand name of the oil is Buruh, produced by Lam Soon Edible Oils Sdn. Bhd. located in Shah Alam, Selangor, Malaysia. The composition of the cooking oil can be found in Table 3.3.

Table 3.3 Composition of Buruh refined cooking oil

Component	Per 100 g
Energy	900 Kcal
Carbohydrate	0 g
Protein	0 g
Total fat	100 g
Mono-unsaturated fat	45 g
Poly-unsaturated fat	12 g
Saturated fat	43 g
Trans fatty acid	0 g
Cholesterol	0 g
Vitamin E	60 mg
Sodium	0 mg

3.1.6 Components of emulsified liquid culture system

The emulsified liquid culture system consists of biomass, vegetable oil, E2 medium, and surfactant (Tween 80).

To produce PHA, 100mL of E2 medium is combined with different quantities of inoculum, oil, and surfactants in a 250 mL baffled Erlenmeyer flask.

3.1.7 Sterilization

Throughout this study, for sterilization purposes, Tomy SS-325 autoclave (Osaka, Japan) was utilized. A temperature and pressure used was set for 121 °C and 103 kPa respectively for duration of 15 minutes.

3.1.8 Shaker-incubator setup

Shake-flask cultivations were conducted with the Hotech Model 721 orbital shaker and the Hotech Model 624 low-temp incubators manufactured by Hotech Instruments Corporation (Taipei, Taiwan). The cultures were incubated at 25 °C ± 0.2 for the cell-growth phase and at 30 °C ± 0.2 for the PHA-accumulation phase. The shaking speed remained constant at 200 rpm throughout both phases.

3.1.9 Centrifugation

Centrifugation was performed with the help of the Thermo-Line MLX-210 mini centrifuge (Beijing, China) for liquid volumes of 1.0 ml, the Hettich Zentrifugen EBA 20S bench-top centrifuge (Tuttlingen, Germany) for liquid volumes of 10.0 ml, and the Thermo Scientific Sorval RC-5C Plus ultracentrifuge (Massachusetts, USA) for liquid volumes of 50.0 ml.

3.1.10 Analytical instruments

i. Gas Chromatography (GC)

The quantification and identification of PHA monomers were conducted with the Thermo Scientific Trace GC Ultra. The GC was equipped with a fused silica capillary column (30 m × 0.32 mm ID) known as the Thermo Scientific TG-5MS

(Massachusetts, USA). A flame ionization detector with splitless injection was employed. The inert gas, Helium was used as the transporter gas at a carrier rate of 2.734 ml/min. The temperature of injector was 200 °C, while the temperature of the detector was 280 °C. The oven ramping temperatures were set to increase from 50 to 280 °C at a rate of 10 °C/min.

ii. Proton Nuclear Magnetic Resonance (^{1}H-NMR)

The chemical structure of the produced PHA_{MCL} samples was studied using the Proton NMR (1H-NMR). The spectroscopy of the ^{1}H-NMR was recorded using a Fourier Transform NMR spectrometer (JEOL JNM-LA 400) set at a frequency of 400 MHz, and temperature of 25 °C. The PHA sample was dissolved in deuterated chloroform ($CDCl_3$) at a concentration of 5.0 mg/ml, with tetramethylsilane (TMS) been the internal reference.

iii. Fourier Transform Infrared spectroscopy (FTIR)

FTIR analysis was performed using a Perkin-Elmer FTIR RX spectrometer. The control and emulsified PHA samples were cast onto NaCl FTIR cells as thin films. The spectra were recorded between 4600 and 4000 cm, with a resolution of 4 cm and 10 scans taken at room temperature.

iv. Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC) was utilized to derive the average molecular weight and distribution of both control and emulsified PHA samples. A Waters Styrogel HR column was utilized in the GPC. The eluent used was tetrahydrofuran, set at a flow rate of 1 ml/min and a temperature of 40 °C. Before injection into the machine, a 2.0 mg/ml of the sample solution was filtered through a 0.45 µm filter paper, with 1.0 ml of the solution being filtered. For each polymer sample,

100 µl of the filtrate was injected. To calibrate the instrument, monodisperse polystyrene (PS) standards were used.

3.1.11 Analytical solutions

i. Acidified methanol

The acidified methanol solution was prepared for PHA methanolysis. A mixture of 85 ml of cold methanol with 15 ml of concentrated sulphuric acid was created and stored at 4 °C until ready to use.

ii. 3-hydroxyalkanoic methyl ester standards

Methylated 3-hydroxyalkanoate standards (Table 3.4) were prepared as solutions for identifying and quantifying PHA monomers using GC. These solutions were prepared with an initial stock concentration of 10,000 parts per million (PPM) using dichloromethane as the solvent.

Table 3.4 PHA monomer standards

Standards	Suppliers
Methyl 3-hydroxybutyrate	Aldrich (Germany)
Methyl 3-hydroxyvalerate	Aldrich (Switzerland)
Methyl 3-hydroxyhexanoate	SAFC (U.S.A)
Methyl 3-hydroxyoctanoate	LARODAN (Sweden)
Methyl 3-hydroxydecanoate	LARODAN (Sweden)
Methyl 3-hydroxydodecanoate	LARODAN (Sweden)
Methyl 3-hydroxytetradecanoate	LARODAN (Sweden)
Methyl 3-hydroxyhexadecanoate	LARODAN (Sweden)

3.2 Preparation of stock culture

The pure culture of *P. putida* Bet001 was stored at -20 °C in a glycerol solution. To prepare the stock, 0.5 ml of sterile rich medium was mixed with 1 ml of glycerol solution in Fischer Scientific Eppendorf tube (Massachusetts, USA). Multiple aliquots were created to guarantee availability throughout the study period.

3.3 Preparation of inoculum for PHA cultivation

The frozen glycerol culture stock was thawed to room temperature, and then poured into sterile nutrient broth using aseptic techniques. The nutrient broth was incubated at 30 °C for 24 hours.

To initiate cell growth, 10 ml of the nutrient broth culture was used to inoculate a 200 ml rich medium, before it was further incubated for 24 hours. The cells were subsequently harvested by centrifugation at 8000 rpm for 3 minutes at 4 °C. A predetermined volume of the biomass was measured in centrifuge tubes for PHA production.

The biomass was transferred to the E2 medium for the PHA accumulation phase under nitrogen-limited conditions. The cell culture was incubated in a shaker incubator at 30 °C for 48 hours, with the addition of oil and surfactant.

3.4 Biomass harvesting

After incubating in E2 medium for 48 hours, the cells were harvested. This was done by centrifuging them inside a 4 °C centrifuge at 8000 rpm for 3 minutes. Once harvested, the cells were rinsed thrice with normal saline solution that had a concentration of 0.9 % (*w/v*). After rinsing, the cells were oven-dried at a temperature of 70 °C. The weight of each sample was then recorded.

3.5 PHA extraction and purification

For PHA extraction, the dried cells were transferred into heat resistant round-bottom glass tubes and refluxed in 3.0 ml of n-hexane at 70 °C for 2 hours. The resultant liquid solution was collected using a Pasteur pipette. The remnant dried cells were then subjected to another cycle of solvent reflux with 3.0 ml of n-hexane for 2 hours to ascertain maximum extraction of PHA from the cells. The filtrates from both cycles were combined and concentrated by allowing the solvents to evaporate in the fume hood.

For PHA purification, the concentrated polymer extract was mixed with cold methanol in a thick glass tube at a ratio of 1:7 to precipitate the PHA. 30 minutes later, the precipitated PHA was collected by centrifugation at 2500 rpm set at duration of 10 minutes. The solvent mixture was carefully emptied, ensuring that there is PHA at the bottom of the tube. Further purification was performed by redissolving the PHAs in a small fraction of acetone and further precipitation in the excess methanol. This purification step was repeated several times to obtain a highly purified PHA.

3.6 PHA methanolysis

PHA methanolysis was performed to convert the PHA into a mixture of monomers for qualitative characterization by GC-MS analysis. To do this, 0.05 g of PHA was diluted in 3.0 ml of filtered dichloromethane in a screw-cap tube sealed with PTFE tape. Then, 3.0 ml of acidified methanol (containing 15% (v/v) concentrated sulfuric acid) was added to the polymer solution. Methanolysis was carried out at 100 °C for 140 minutes using a Wisetherm HB-48 heating block with continuous shaking. The mixture was allowed to cool at room temperature after which 3.0 ml of distilled water was added. Vigorous vortexing allowed the mixture to separate into two layers. The organic phase at the bottom of the tube was gently recovered and transferred into a small screw-cap vial containing sodium sulfate anhydrous to remove any trapped moisture. A fraction of the organic phase was diluted with dichloromethane when necessary, before they are used for GC analysis.

3.7 PHA characterisation

The monomeric composition of the PHA produced was studied using GC-MS analysis, following the protocol reported by Gumel et al., (2012). The analysis used a sample of PHA in the form of 3-hydroxyalkanoic acid methyl esters. A methanolyzed sample (1 µl) was injected into the GC at a split ratio of 1:50. The injection temperature was set at 280 °C, while the oven and column temperatures were set up as follows: 40 °C for 1 minute, then increased to 120 °C at a ramping rate of 15 °C/min, held for 2 minutes, and finally increased to 250 °C at a ramping rate of 10 °C/min, held for 15 minutes. The carrier gas used was the inert gas helium, with a flow rate of 48.3 ml/min and a pressure of 0.41 bar. Mass spectra were acquired at a scan speed of 1250 using electron impact energy of 70 eV at ion-source and interface temperatures of 200 °C and

280 °C, respectively. Standard monomers of methyl hydroxyalkanoates were used as references for peak retention time and ionization mass determination.

FTIR analysis was used to study the structural analysis of the PHA produced. In this analysis, the PHA was diluted in chloroform and cast onto sodium chloride (NaCl) pellets. After the complete evaporation of the solvent, the FTIR spectra were recorded using a FTI Perkin Elmer spectrometer, with a wave number range of 500 to 4000 cm.

The molecular weight of the PHA produced was studied using gel permeation chromatography (GPC). GPC is a kind of liquid chromatography that utilizes solid stationary and liquid mobile phases. The GPC system used a Waters Styrogel HR column and relied on the size of the polymer molecules in solution for separation. Approximately 5 mg of the PHA was diluted in 2 ml of tetrahydrofuran (THF) as the eluent and injected into the column at 40 °C with a flow rate of 1 ml/min. Prior to injection, a 2.0 mg/ml sample solution was filtered through a 0.45 μm filter. A 100 μl portion of the filtrate was injected for each polymer sample. The GPC machine was calibrated using monodisperse polystyrene (PS) standards.

The chemical structure of the produced PHA was studied using proton NMR (^{1}H-NMR) spectroscopy. The ^{1}H-NMR spectra were recorded on a JEOL JNM-LA 400 FT NMR spectrometer at 400 MHz and 25 °C. The spectra were obtained via a solution of PHA in $CdCl_3$ at a concentration of 2 mg/ml. The internal reference used in the study was Tetramethylsilane (TMS).

3.8 Box-Behnken optimization

The Box-Behnken design was created by George Box and Donald Behnken in the 1960's. It is highly regarded as a powerful and efficient methodology for response surface optimization (Karmoker et al., 2019). In this study, we utilized the Box-Behnken design to enhance the culture conditions and factors involved in the PHA production, aiming to better comprehend their relationship.

By employing the Box-Behnken design (BBD), we conducted an optimization process for the three chosen independent variables: biomass, oil, and surfactant quantities. Table 3.5 provides the range of these uncoded independent variables and their corresponding levels. The determination of this range was based on prior exploratory work, which unfortunately has not been published.

Table 3.5 The experimental points in the Box-Behnken design

Independent	Levels (per 100 ml innoculum)		
	Lowest	Middle	Highest
Biomass, ml (X_1)	0.25	0.375	0.5
Oil, g (X_2)	0.5	1.25	2
Surfactant, ml (X_3)	0	0.125	0.25

CHAPTER 4: RESULTS AND DISCUSSION

4.1 PHA production parameters

The usage of inorganic and organic oil for the production of PHA is widely accepted due to its cost-effectiveness. Various types of oils, including plant and animal oils including palm oil, rapeseed oil, vegetable oil, jatropha oil, animal fats, and fish oil, have been tested for PHA biosynthesis (Surendra et al., 2020). However, improper waste management can be a burden when disposing of the waste generated from many of these oils.

This study demonstrates the potential of using cooking oil for PHA production, especially when combined with an effective surfactant. The surfactant used in this study was Tween-80, which enables emulsification in the mixture of cooking oil and aqueous medium. Other surfactants, including gum Arabic, Tween-20, Tween-80, and egg white, have also been reported in the production of PHA (Thinagaran and Sudesh, 2019). The addition of a surfactant enhances the reproducibility of the experiment, particularly when plant oils are used (Thinagaran and Sudesh, 2019; Budde et al., 2011). While previous studies have used Tween-80, this study provides strong evidence supporting its use in facilitating the accessibility of aqueous-immiscible carbon sources to metabolizing cells, ultimately leading to increased PHA biosynthesis after optimization.

4.1.1 Box-Behnken design

Table 4.1 presents the randomized run order, generated with Minitab® 16 software, for the optimization of the three independent variables and their equivalent response variables, including replicates. Based on the results, the 2-D contour plots and 3-D response surfaces (Figures 4.1 a-f) were used to examine the effects of the

independent variables and their relationships on PHA yield. These plots demonstrate the roles of the two factors on the response; however, the third factor was put constant at the mid level. The 2-D plot represents an elliptical contour plot that shows an important interactive effect between the variables, whereas the 3-D plot represents a circular contour plot that shows non-significant interactive effect between the variables (Suryawanshi et al., 2020).

Figures 4.1 (a) and (b) depict the 2-D contour and 3-D surface plots at different biomass-to-oil ratios along with PHA yield. The maximum PHA yield, at 30 %, can be attained when the biomass is above 0.25 ml and the oil is above 1.5 g per 100 ml of medium. The surfactant was fixed at 0.125 ml. In this study, the 100 ml inoculum contained 0.3 g of cell biomass.

The 2-D contour plots together with the 3-D response surface seen in Figures 4.1 (c) and (d) illustrate the surfactant-to-oil ratio and PHA yield at a biomass of 0.375 ml. It was determined that the maximum percentage of PHA, at 24 %, was achieved with 1.25 g of oil and 0.12 ml of surfactant per 100 ml of medium. This combination yielded approximately 24 % of PHA.

The 2-D contour together with the 3-D response surface plots based on the biomass-to-surfactant ratio and PHA yield at 1.25 g of oil are displayed in Figures 4.1 (e) and (f). These plots reveal that the maximum percentage of PHA can be attained when the surfactant is 0.25 ml and the biomass is 0.50 ml, resulting in a yield of about 30 % of PHA.

From the contour and 3-D plots, it is evident that to achieve a PHA yield above 20%, the initial ranges of biomass, surfactant, and oil should be 0.25 to 0.40 ml, 0.05 to 0.20 ml, and 0.5 to 2.0 g, respectively.

Figures 4.1 a-f demonstrate that all three parameters play significant roles in obtaining a high PHA yield from biomass, and there are important relationships among the independent variables that influence the PHA yield ($p<0.05$). Using Minitab® 16 software, the optimal conditions for PHA production were determined: biomass of 0.375 ml, surfactant of 0.125 ml, and oil of 1.25 g per 100 ml of medium.

Findings from this current study robustly support the hypothesis for the use of non-toxic, eco-friendly surfactants like Tween-80 in the aqueous fermentation system of PHA. In addition to promoting cell growth and PHA content by improving the accessibility of the immiscible oil substrate to the metabolizing cells, the use of surfactants eliminates the need to saponify the oil, which is a common practice to enhance oil solubility in the aqueous medium. Saponification involves additional costs and a chemical-intensive process that includes heating and the use of strong alkali. Furthermore, saponified oil leads to a strongly basic pH in the aqueous medium formulation, requiring neutralization with a strong acid prior to inoculating the medium with microbial culture.

The results in Table 4.1 indicate that excluding the surfactant from the medium reduces the likelihood of PHA production by 50 %. When the surfactant is included in the medium at varying amounts, different percentages of PHA accumulation and yield are obtained.

Table 4.1: Randomised run of the variables and their responses

Std Order	Run Order	Biomass (ml) X_1	Oil (g) X_2	Surfactant (ml) X_3	PHA (%)
10	1	0.375	2	0	0
29	2	0.375	1.25	0.125	11
44	3	0.375	1.25	0.125	44
14	4	0.375	1.25	0.125	13
7	5	0.25	1.25	0.25	7
25	6	0.375	2	0	9
35	7	0.25	1.25	0	0
42	8	0.375	2	0.25	8
28	9	0.375	1.25	0.125	18
30	10	0.375	1.25	0.125	11
41	11	0.375	0.5	0.25	13
45	12	0.375	1.25	0.125	13
13	13	0.375	1.25	0.125	60
38	14	0.5	1.25	0.25	7
17	15	0.5	0.5	0.125	13
5	16	0.25	1.25	0	0
21	17	0.5	1.25	0	8
24	18	0.375	0.5	0	20
33	19	0.25	2	0.125	22
1	20	0.25	0.5	0.125	30
26	21	0.375	0.5	0.25	14
11	22	0.375	0.5	0.25	13
15	23	0.375	1.25	0.125	20
32	24	0.5	0.5	0.125	25
23	25	0.5	1.25	0.25	6
36	26	0.5	1.25	0	10
6	27	0.5	1.25	0	0
43	28	0.375	1.25	0.125	11
8	29	0.5	1.25	0.25	7
3	30	0.25	2	0.125	40

34	31	0.5	2	0.125	21
2	32	0.5	0.5	0.125	14
40	33	0.375	2	0	0
9	34	0.375	0.5	0	17
22	35	0.25	1.25	0.25	9
12	36	0.375	2	0.25	8
19	37	0.5	2	0.125	6
16	38	0.25	0.5	0.125	43
37	39	0.25	1.25	0.25	8
4	40	0.5	2	0.125	7
18	41	0.25	2	0.125	25
20	42	0.25	1.25	0	0
39	43	0.375	0.5	0	33
27	44	0.375	2	0.25	9
31	45	0.25	0.5	0.125	17

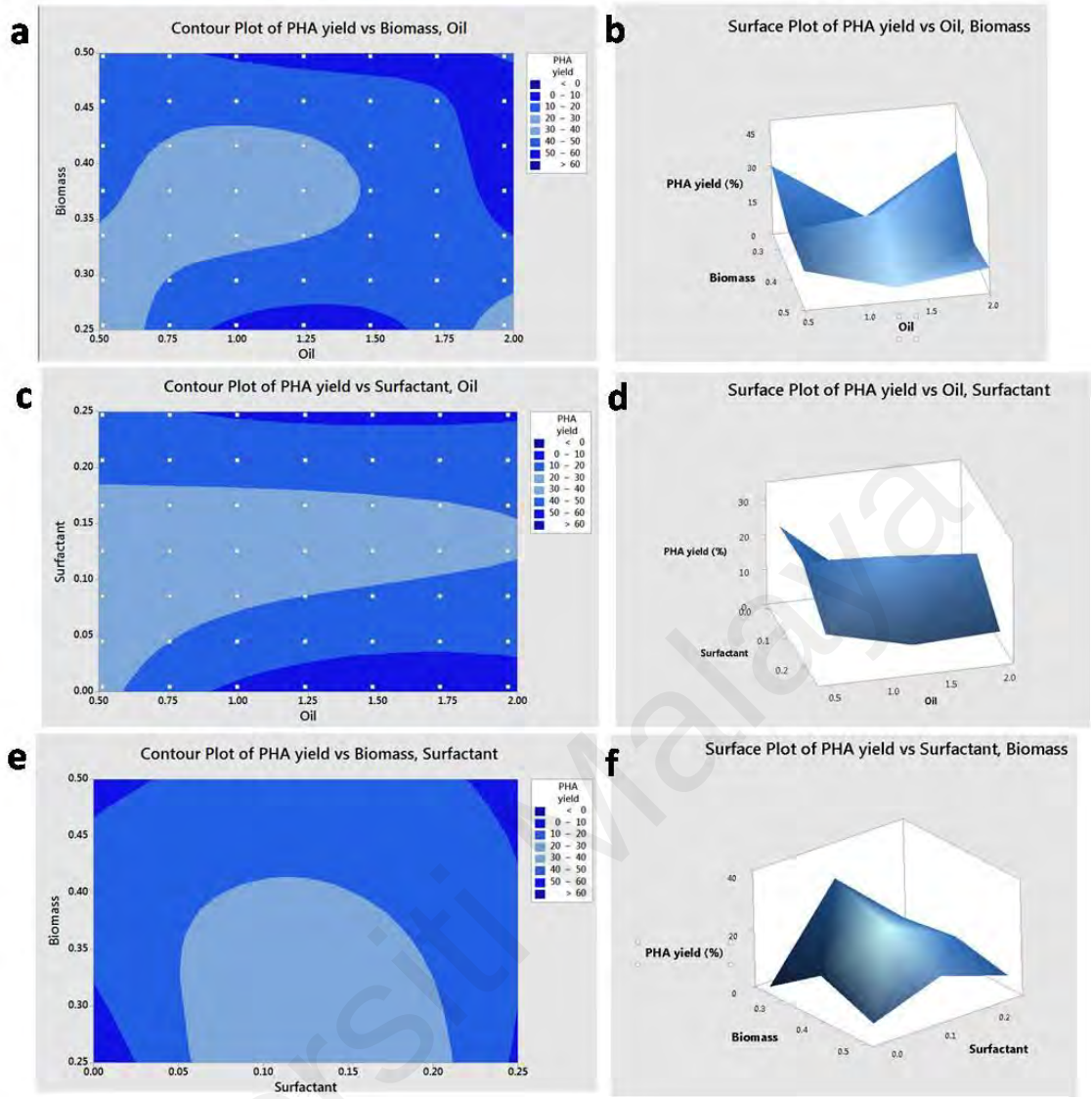


Figure 4.1 Contour and surface plots showing the effect of biomass (X_1), oil (X_2), and surfactant (X_3) on the percentage of PHA in cell biomass.

4.1.2 Statistical analysis and model fitting

The following second-order polynomial equation relates the response variable (yield of PHA) and the test variables, based on the analysis of the experimental data using multiple regressions.

$$Y = -72.1 + (537\ X_1) - (5.7\ X_2) + (263\ X_3) - (655\ X_1^2) + (2.40\ X_2^2) - (1210\ X_3^2) - (39.1\ X_1X_2) - (123\ X_1X_3) + (55.1\ X_2X_3)$$

Y represents the percentage of PHA in biomass (%), while X_1, X_2, and X_3 represent the independent variables for biomass, oil and surfactant amount respectively.

The model was analyzed using analysis of variance (ANOVA) to conduct statistical testing. The determination coefficient (R^2 = 58.89) showed that no more than 41.11 % of the sum total of the variations were unexplained from the model. Meanwhile, the adjusted determination coefficient (adjusted R^2 = 49.58) suggested that the model's ability to explain the results was only 50 %, which is not considered high. The model's significance was supported by a high F-value (F = 32.03) and low p-value ($p < 0.05$). Additionally, the model's lack-of-fit was tested and discovered to be significant with an F-value of 2.98 and a p-value of 0.046 (since $p < 0.05$). This lack-of-fit is also reflected in the values of R^2 and adjusted R^2 for the second-order model.

4.1.3 Verification of the predictive model

In the study, the polynomial model's predictive strength was assessed by testing it on the optimal combination and one randomly chosen non-optimal combination. Table 4.2 displays the two experimental combinations, together with the yields of the actual and predicted PHA. This model has successfully predicted the actual outcomes in the emulsified liquid culture PHA production system.

Table 4.2 Comparison between predicted and actual responses

Biomass (g)	Oil (g)	Surfactant (ml)	PHA (%)	
			Predicted	Actual
0.25	1.25	0.25	5.3	7
0.25	0.5	0.125	27	30

4.2 PHA characterization

4.2.1 Gel permeation chromatography (GPC) analysis

The average molecular weight and distribution of PHA synthesized using vegetable oil (designated as PHA-VE) as the carbon source was determined through GPC analysis. Table 4.3 compares the PHA molecular weight with that of PHA produced using lauric acid (designated as PHA-LA). The results revealed no significant difference in the number-average (*Mn*) and weight-average (*Mw*) values between the two types of PHA. In a study by Thinagaran and Sudesh (2019), it was reported that saponifying palm oil into free fatty acids surfactants resulted in a higher amount of readily available carbon source for the bacteria strain. This, in turn, led to increased cell biomass, PHA yield, and molecular weight compared to non-saponified palm oil. The findings are consistent with the results of Mozejko and Ciesielski (2013), who observed that there was 43 % increase when saponified waste palm oil was used in PHA production. Riedel et al. (2015) also reported the bio-production of PHA using waste tallow and animal fats and found that the substrate concentration had an impact on the molecular weight of the produced PHA.

Table 4.3 Molecular weight of the PHA samples

Samples	Molecular weight ($\times 10^5$g mol^{-1})		
	M_nM_w		PDI (M_w/M_n)
PHA-VE	0.07	0.09	1.20
PHA-LA	0.44	0.88	1.79

4.2.2 ^{1}H-NMR Spectroscopy

Figures 4.2 (a) and (b) show the ^{1}HNMR spectra of PHA-VE and PHA-LA, correspondingly. Compared to PHA-LA (used as a reference) (Ishak et al., 2014), PHA-VE exhibits similar peaks at 0.91, 1.27, 1.6, 2.5, and 5.2 ppm. The peak at 0.9 ppm is allocated to the methyl proton, while the peak at 1.27 ppm is assigned to the methylene proton, both of which are within the side chain of the polymers. The chemical shift seen at 1.6 ppm is can be ascribed to the methylene protons adjoining to the β-carbon in the side chains of the 3-hydroxyalkanoates (3HA) copolymer. In addition, the multiplet peaks and triplet peaks at 2.5 and 5.2 ppm, are attributed to the methylene and methine protons of the α- and β-carbon respectively. Similar results have been reported by others (Razaif-Mazinah et al., 2016; Gumel et al., 2012). However, additional peaks were observed in the PHA-VE spectrum at 2.0, 5.3, and 2.3 ppm, representing the occurrence of double bonds in the side chain of the PHA produced (Chan Sin et al., 2010). Furthermore, there were no traces of unknown peaks, indicating the absence of possible contaminants in the spectrum.

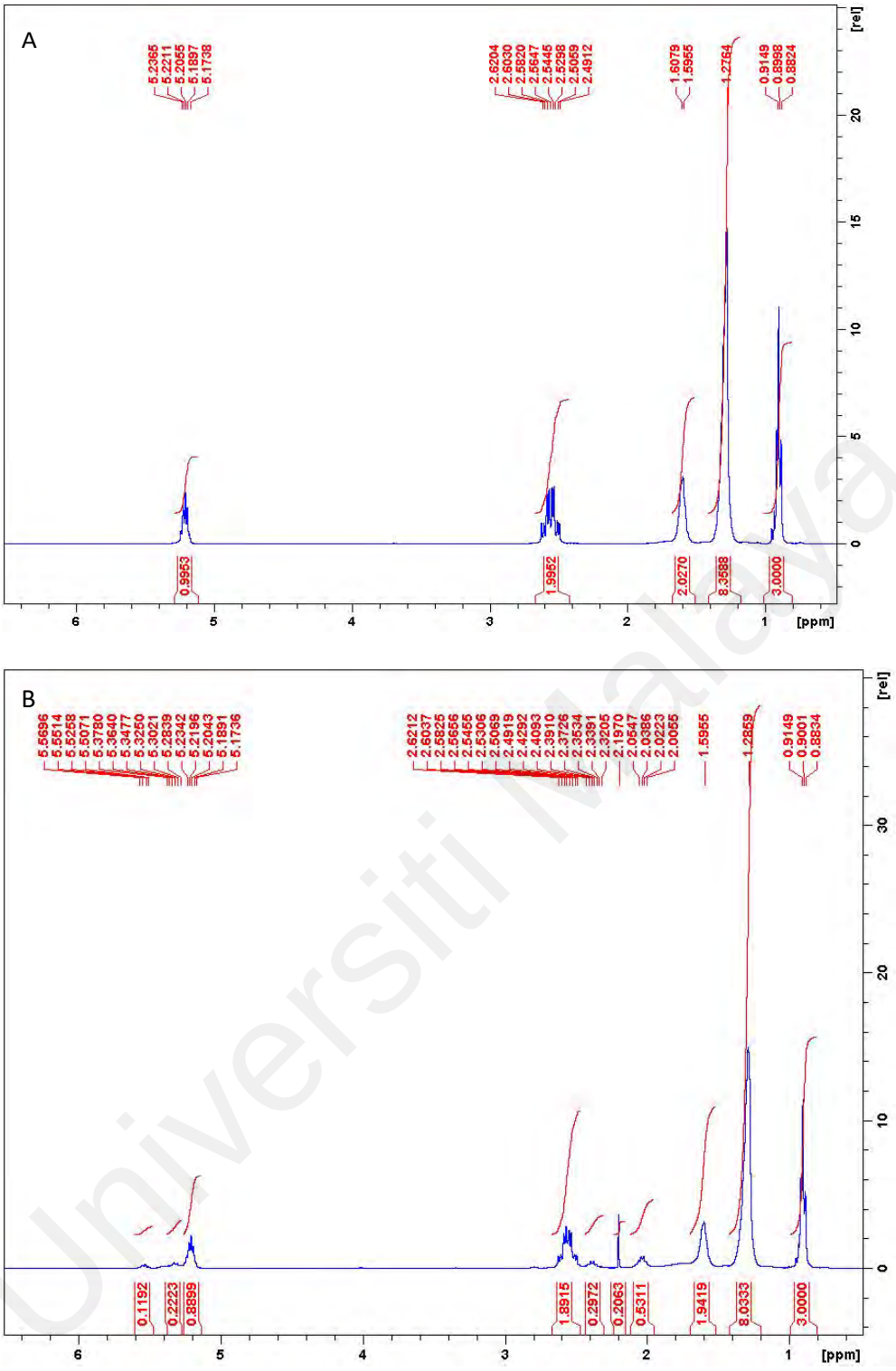


Figure 4.2 ^{1}H-NMR spectra of (a) PHA-VE and (b) PHA-LA

4.2.3 FTIR Spectroscopy

Figures 4.3 (a) and (b) illustrate the FTIR spectra of PHA-VE and PHA-LA. All spectra showed similar pattern to previous reports where there are identical resemblances in the control (PHA-LA) and PHA-VE (Guo et al., 2012; Ishak 2014). The spectrum of the isolated PHA_{MCL} showed ten absorption bands which are assigned as the following:

a at 2925 cm attributed to –CH stretch bond

b at 2847 cm attributed to CH bond with aldehyde stretch

c at 1729 cm attributed to C=O bond

d is 1464 cm attributed to CH_2 bond

e is 1379 cm attributed to CH_3 bond

f at 1325 cm attributed to C-O bond

g at 1170 cm attributed to C-O-C bond

h is at 1100 cm attributed to C-C bond

i is at 975 cm attributed to CH bond with disubstituted-E bend

j is at 727 cm attributed to CH_2 bond

k is 602 cm attributed to CH bond with acetylenic bend

The results did not indicate any significant variation in the molecular structure of the produced PHA using emulsified vegetable oil and free fatty acids. However, the presence of a double bond on the PHA side-chain is attributed to the peak observed in the PHA-VE spectrum, as indicated by the arrow.

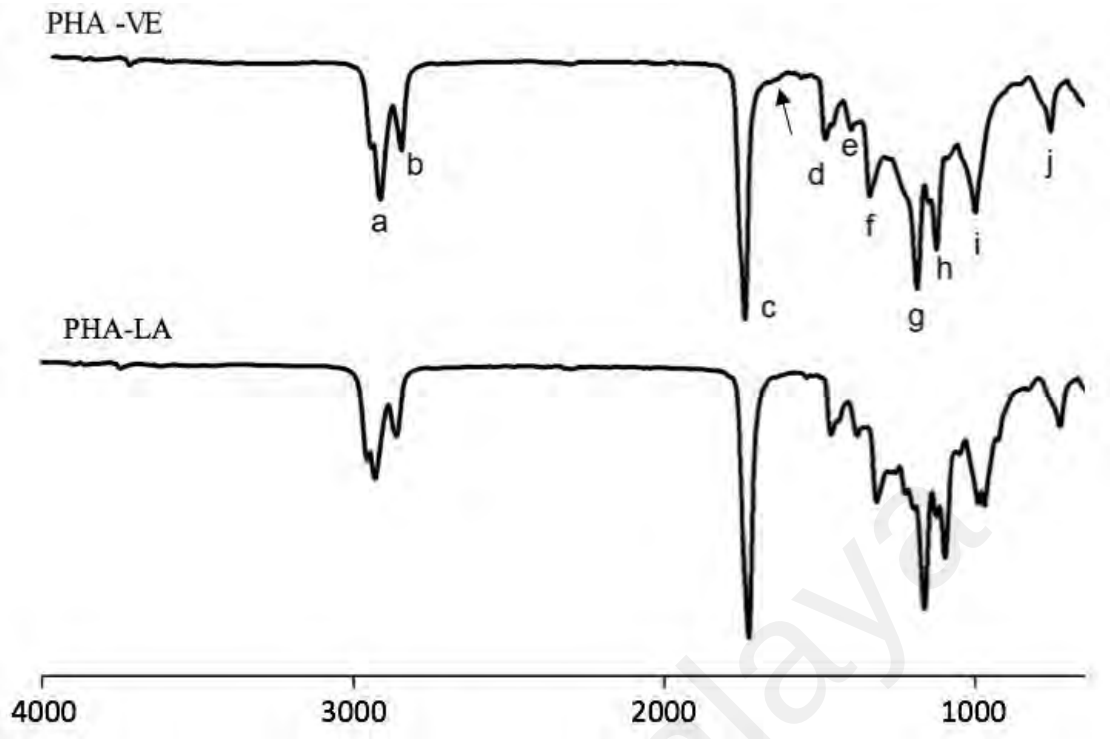


Figure 4.3 FTIR spectra of PHA-VE and PHA-LA

4.2.4 GCMS Analysis

The monomeric composition of the produced PHA using emulsified vegetable oil was determined through GCMS analysis. Figure 4.4 displays the chromatogram, which reveals four major peaks corresponding to the four major monomers found in medium-chain-length PHA. These monomers have been reported previously by Anis (2017). Figure 4.6 presents the analysis of ion fragments from the mass spectrometry, which confirms the presence of methyl 3-hydroxyoctanoate, methyl 3-hydroxy-decanoate, methyl 3-hydroxytetradecanoate, and methyl 3-hydroxyhexadecanoate. Table 4.4 provides the peak report for these monomers.

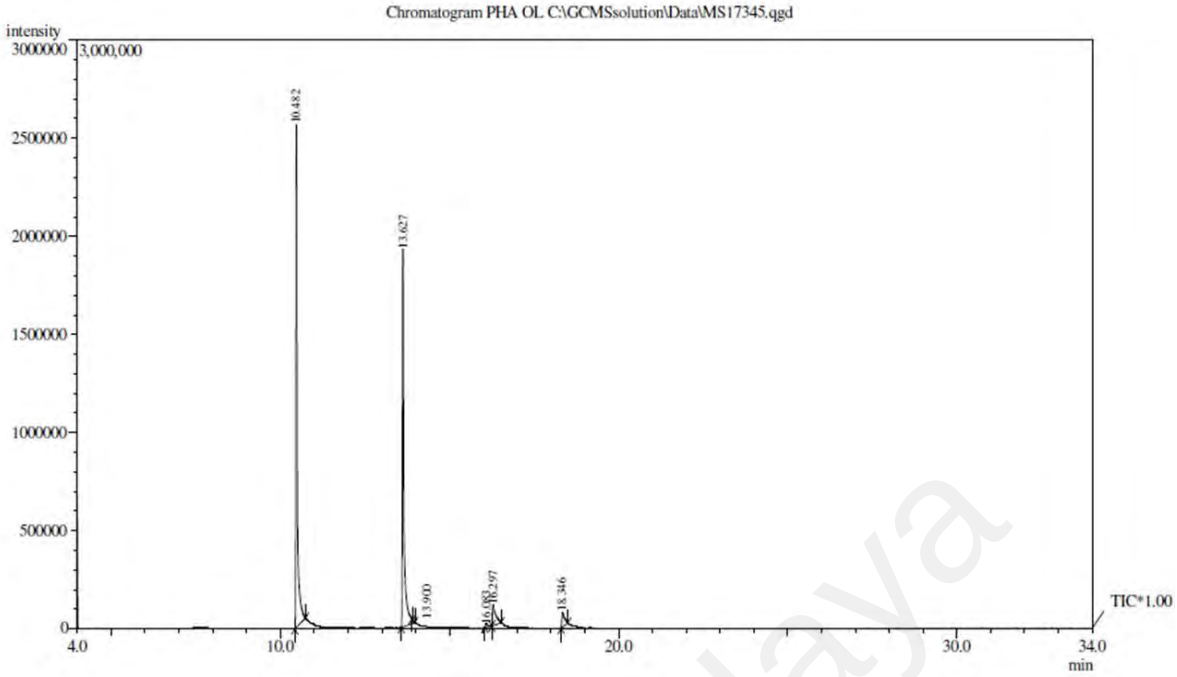


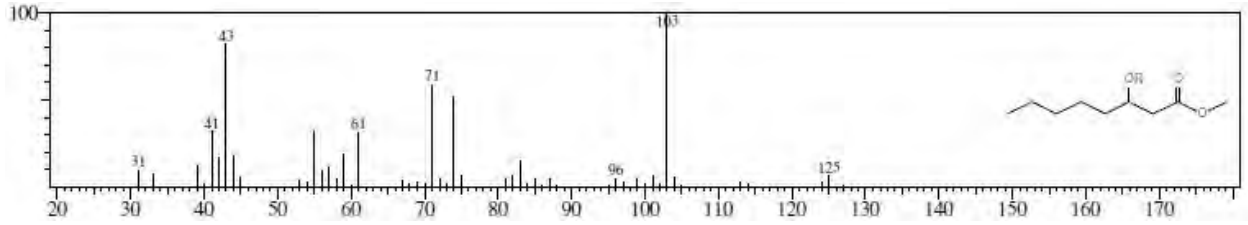
Figure 4.4 Gas chromatogram of methyl 3-hydroalkanoates of PHA-VE

Table 4.4: Peak report of the monomers

Peak #	Retention Time (min)	Area (A)	Area %	Height (H)	Height %	A/H
1	10.482	7060437	48.89	2560302	54.66	276
2	13.627	5936831	41.95	1926042	41.12	3.08
3	13.900	8	0.00	2504	0.05	0.00
4	16.083	64282	0.45	14896	0.32	4.32

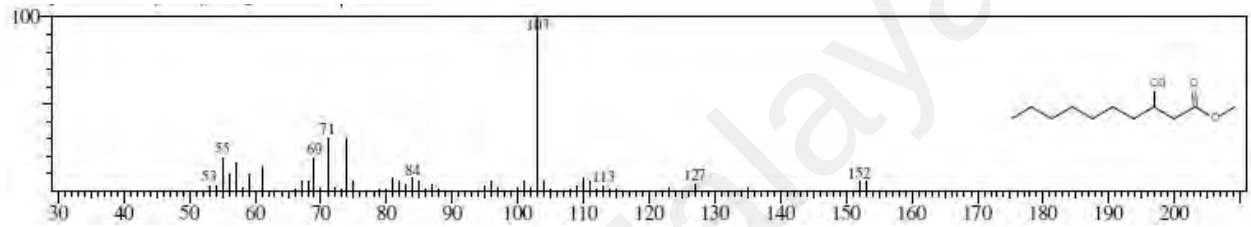
a) Methyl 3-hydroxyoctanoate

Formula: $C_9H_{18}O_3$ CAS: 7367-87-5 Molecular Weight: 174



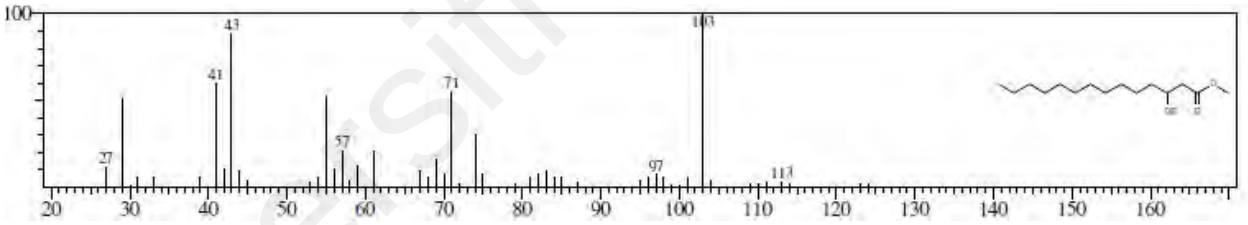
b) Methyl 3-hydroxydecanoate

Formula: $C_{11}H_{22}O_3$ CAS: 0-00-0 Molecular Weight: 202



c) Methyl 3-hydroxytetradecanoate

Formula: $C_{15}H_{30}O_3$ CAS: 55682-83-2 Molecular Weight: 258



d) Methyl 3-hydroxyhexadecanoate

Formula: $C_{17}H_{34}O_3$ CAS: 51883-36-4 Molecular Weight: 286

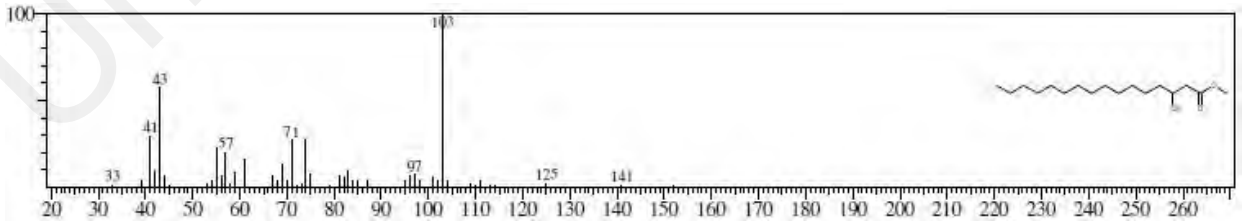


Figure 4.6 Four major monomers of PHA-VE samples identified as a) methyl 3-hydroxyoctanoate; b) methyl 3-hydroxydecanoate; c) methyl 3-hydroxytetradecanoate; d) methyl 3-hydroxyhexadecanoate (NIST11 library)

CHAPTER FIVE

CONCLUSION

5.0 CONCLUSION

This study made use of *Pseudomonas putida* Bet001 as the model organism to investigate the bioproduction of PHA_{MCL}.

Results from this study showed that this strain of bacterial exhibited a strong capability to produce PHA_{MCL} when provided with cooking oil. This validated the first objective of the study showing the potency of the microorganism to produce PHA.

With the Box-Behnken design, the study was able to obtain a high percentage of PHA yield. This was achieved under optimal cultivation conditions, which consisted of 0.375 ml biomass, 1.25 g oil, and 0.125 g surfactant per 100 ml of medium.

Additionally, the bacteria produced four different types of monomers: methyl 3-hydroxyoctanoate, methyl 3-hydroxy-decanoate, methyl 3-hydroxytetradecanoate, and methyl 3-hydroxyhexadecanoate which provide a better understanding of the type of PHA produce validating the third objective of the study.

Lastly, results from this study contribute to our understanding of the importance of using surfactants to maximize PHA production. The surfactant aids in emulsifying the bulk oil into smaller droplets, thereby increasing the accessibility of the carbon source for bacterial consumption.

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