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OPTIMIZATION OF POLYHYDROXYALKANOATE (PHA) PRODUCTION BY *Burkholderia cepacia* BPT1213 USING WASTE GLYCEROL AS THE SOLE CARBON SOURCE

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OPTIMIZATION OF POLYHYDROXYALKANOATE (PHA) PRODUCTION BY *Burkholderia cepacia* BPT1213 USING WASTE GLYCEROL AS THE SOLE CARBON SOURCE

by

NOR FAUZIAH BINTI MOHD ZAIN

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NOR FAUZIAH BINTI MOHD ZAIN
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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
&	And
=	Equal
+	Plus
-	Minus
±	Plus-minus
×	Times / Multiply
$CaCl_2$	Calcium chloride
CH	Methine group
CH_2	Methylene group
CH_3	Methyl group
H_2O	Water
HCl	Hydrochloric acid
$CoSO_4$	Cobalt sulphate
$CuCl_2$	Copper (II) chloride
$FeSO_4$	Iron sulphate
KBr	Potassium bromide
KH_2PO_4	Potassium dihydrogen phosphate
$MgSO_4$	Magnesium sulphate
$MnCl_2$	Manganese chloride
$NaCl$	Sodium chloride
$NaOH$	Sodium hydroxide

$(NH_4)_2SO_4$	Ammonium sulphate
$ZnSO_4$	Zinc sulphate

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
A_{PHA}	Area of PHA peak
A_{IS}	Area of internal standard
ATP	Adenosine triphosphate
CCD	Central Composite Design
CDW	Cell dry weight
CME	Caprylic methyl ester
C/N	Carbon to nitrogen
CoA	Coenzyme A
DSC	Differential scanning calorimetry
g	Gram
GC	Gas chromatography
h	Hour
Kg	Kilogram
kPa	kilopascal
L	Litre
M	Molar
mg	Milligram
mm	Millimetre
MSM	Mineral salt medium
mL	Millilitre
NR	Nutrient rich
OD	Optical density
P(3HB)	Poly(3-hydroxybutyrate)

PHA	Polyhydroxylakanoate
PLA	Polylactic acid
rpm	Revolution per minute
sp.	Species
TEM	Transmission electron microscope
v	Volume
v/v	Volume/volume
vvm	Volume/volume/minute
w/w	weight/weight

PENGOPTIMUMAN PENGHASILAN POLIHIDROSIALKANOAT (PHA) OLEH *Burkholderia cepacia* BPT1213 MENGGUNAKAN GLISEROL SISA SEBAGAI SUMBER KARBON TUNGGAL

ABSTRAK

Polihidrosialkanoat (PHA) diperkatakan sebagai alternatif mesra alam kepada plastik berasaskan petrokimia yang tahan lasak. Berikutan isu kos penghasilan yang tinggi, gliserol sisa yang terhasil daripada industri biodiesel berpotensi digunakan sebagai sumber karbon tunggal yang murah untuk sintesis PHA oleh mikroorganisma. Objektif kajian ini adalah untuk mengoptimumkan dan meningkatkan skala penghasilan PHA oleh pencilan tempatan, *Burkholderia cepacia* BPT1213 daripada sistem kelalang goncang kepada sistem bioreaktor 2.5 L. Sepanjang kajian ini, pencilan ini ditumbuh dalam medium garam mineral (MSM) yang ditambah dengan 2% (v/v) gliserol sisa (86.70% tulen) yang merupakan produk sampingan penghasilan biodiesel daripada minyak sawit sebagai sumber karbon tunggal. Keputusan menunjukkan, di dalam kultur kelalang goncang, pertumbuhan pencilan ini boleh menghasilkan 1.33 g/L berat kering sel dengan 22.21% kandungan PHA, justeru menghasilkan 0.30 g/L kepekatan PHA keseluruhan sebelum pengoptimuman. Satu reka bentuk ujikaji faktorial yang telah dijalankan menunjukkan semua parameter; kepekatan kalium dihidrogen fosfat (KH_2PO_4), kepekatan dinatrium hidrogen fosfat ($Na_2HPO_4 \cdot 2H_2O$), nisbah karbon kepada nitrogen (C/N), pH awal medium kultur, dan suhu dengan ketara mempengaruhi pertumbuhan (berat sel kering) dan kandungan PHA. Kaedah Sambutan Permukaan (RSM) menggunakan Reka Bentuk Komposit Pusat (CCD) untuk pengoptimuman semua parameter telah mencadangkan keadaan optimum pada 2.5 g/L KH_2PO_4, 4.5 g/L $Na_2HPO_4 \cdot 2H_2O$, 30 (g/g) nisbah C/N, pH awal medium 8.5 dan suhu 37 ˚C dengan jangkaan berat kering sel 3.43 g/L dan 45.71%

kandungan PHA menyumbang kepada 1.57 g/L.kepekatan PHA keseluruhan. Eksperimen pengesahan telah menghasilkan 3.60 g/L berat kering sel dengan 48.08% kandungan PHA menyumbang kepada 1.73 g/L kepekatan PHA keseluruhan (meningkat 5.77 kali ganda). Fermentasi telah dinaikkan skala kepada bioreaktor 2.5 L dengan 70% (v/v) isipadu kerja menggunakan keadaan teroptimum dalam sistem kelalang goncang. Bekalan oksigen dikawal oleh kadar pengudaraan dan kawalan kelajuan agitasi. Sebelum pengoptimuman, *B. cepacia* BPT1213 menghasilkan 2.27 g/L berat kering sel dan 51.20% kandungan PHA, dengan 1.16 g/L kepekatan PHA keseluruhan menggunakan kadar pengudaraan 1 vvm pada kelajuan agitasi 200 rpm. Reka bentuk CCD telah dijalan untuk mengoptimumkan kadar pengudaraan, kelajuan agitasi dan masa fermentasi. Titik optimum untuk penghasilan maksimum berat kering sel dan kandungan PHA telah dicadangkan pada 1.5 vvm kadar pengudaraan dan 300 rpm kadar agitasi selama 72 jam fermentasi dengan nilai jangkaan 5.08 g/L berat kering sel, 66.07% (g/g) kandungan PHA dan 3.35 g/L kepekatan PHA keseluruhan. Eksperimen pengesahan menghasilkan 5.63 g/L berat kering sel dengan 64.00% kandungan PHA, menghasilkan kepekatan PHA keseluruhan 3.60 g/L, memberi peningkatan 1.56, 1.33 dan 2.08 kali ganda masing-masing berbanding penghasilan dalam kultur kelalang goncang pada keadaan teroptimum. Perbandingan struktur molekul menggunakan analisis NMR, dan kumpulan berfungsi menggunakan FT-IR telah membuktikan polimer yang terhasil oleh *B. cepacia* BPT1213 adalah jenis P(3HB). Suhu peleburan, suhu penghabluran dan suhu peralihan kaca masing-masing pada 173.45 ˚C, 79.97 ˚C dan 8.08 ˚C yang menghampiri ciri termal polimer P(3HB) menyokong dapatan ini. Kesimpulannya, pendekatan reka bentuk ujikaji faktorial dan Kaedah Sambutan Permukaan (RSM) berjaya meningkatkan penghasilan PHA oleh *B.cepacia* BPT1213 menggunakan gliserol sisa sebagai sumber karbon tunggal.

OPTIMIZATION OF POLYHYDROXYALKANOATE (PHA) PRODUCTION BY *Burkholderia cepacia* BPT1213 USING WASTE GLYCEROL AS THE SOLE CARBON SOURCE

ABSTRACT

Polyhydroxyalkanoate (PHA) is touted as an environmental friendly alternative to recalcitrant petrochemical derived plastics. Due to high production cost issue, waste glycerol produced from biodiesel industry has potential as cheaper carbon source for PHA synthesis by microorganisms. The objectives of this study were to optimize and scale up the production of PHA by a local isolate, *Burkholderia cepacia* BPT1213, from the shake flask system to a 2.5 L bioreactor system. Throughout this study, the strain was grown in minimal salt medium (MSM) supplemented with 2% (v/v) of waste glycerol (86.70% purity), a by-product of the production biodiesel from palm oil as the sole carbon source. Results showed that, in shake flask culture, the growth of the strain can build up to 1.33 g/L cell dry weight (CDW) with 22.21% of PHA content, thus giving 0.30 g/L total PHA concentration before optimization. A factorial design experiment showed that all parameters potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate anhydrous ($Na_2HPO_4 \cdot 2H_2O$), carbon-to-nitrogen ratio (C/N), initial pH of medium, and temperature significantly affected the growth (cell dry weight, CDW) and PHA content. Response Surface Methodology (RSM) using Central Composite Design (CCD) was then applied to optimize these parameters. The optimum suggested conditions were at 2.5 g/L KH_2PO_4, 4.5 g/L $Na_2HPO_4 \cdot 2H_2O$, 30 (g/g) C/N ratio, initial medium pH of 8.5 and 37 ˚C cultivation temperature, with a predicted CDW of 3.43 g/L and PHA content of 45.71% contributing to 1.57 g/L total PHA concentration. The verification experiment resulted in 3.60 g/L of CDW with 48.08% of PHA content contributing to 1.73 g/L total PHA concentration (5.77 folds improved). Fermentation was scaled up in a bioreactor with 70% (v/v) working volume using optimized conditions from the shake flask system. Oxygen supply was controlled by the aeration and agitation speed controls. Before optimization, *B. cepacia* BPT1231 gained 2.27 g/L and 51.20% of CDW and PHA content respectively, with 1.16 g/L total PHA concentration using an aeration rate of 1.0 vvm at 200 rpm agitation speed. The CCD was carried out to optimize the aeration rate, agitation speed and fermentation time. The suggested optimum points for maximum production of CDW and PHA content were 1.5 vvm of aeration and 300 rpm of agitation for 72 h of fermentation period with predicted values of 5.08 g/L CDW and 66.07% PHA content giving 3.35 g/L total PHA concentration. The verification experiment gave 5.63 g/L of CDW with 64.00% of PHA content producing a total of 3.60 g/L total PHA concentration respectively, giving a 1.56, 1.33 and 2.08 fold increases compared to the values obtained from shake flask cultures at optimized condition. The determination of molecular structure using NMR analysis and functional group using FT-IR, proved that the polymer produced by *B. cepacia* BPT1213 was P(3HB). Its melting temperature (T_m), crystallization temperature (T_c) and glass transition temperature (T_g) of 173.45 ˚C, 79.97˚C, and 8.08 ˚C respectively, which were close to the thermal properties of the P(3HB) in previous studies supported the finding. In conclusion, the statistical approach using factorial design and Response Surface Methodology have succeeded in increasing the production of PHA by *B. cepacia* BPT1213 using waste glycerol as the sole carbon source.

CHAPTER 1 : INTRODUCTION

1.1 Introduction

Synthetic plastic is widely used since it was first introduced in the 1950's (Loo & Sudesh, 2007). About 1.5 million tone of plastics was used in the early of its discovery, increasing to 245 million tons in 2008 and has kept increasing at 9% per annum globally. In Malaysia, the population growth at a rate of 2.4% or 600,000 per annum since 1994 is reciprocated by an increase in the municipal solid waste (MSW) generation. It was reported that about 0.5–0.8 kg/person/day amount of MSW was generated in 2003, a figure that has since increased to 1.7 kg/person/day in major cities in Malaysia (Kathirvale *et al*., 2003). This quantity was estimated to have increased to 31,000 tons in coming to 2020 (Sin, *et al*., 2013).

Commonly, the conventional plastics are derived from petroleum-based polymers. These plastics are chemically formed by large and tightly bond molecules, making them difficult to undergo chemical degradation and microbial decomposition. Therefore, they have a very long lifespan because of their excellent properties which are durable and highly resistant to the environment (Loo & Sudesh, 2007). Normally, single-use application of plastic material especially from food packaging and medical purpose results in the bulking accumulation as solid wastes after being discarded. Since the plastics take years to be degraded, they remain as pollutants and are in abundance in the landfill sites or ecosystem, compounded by the increasing human population. Besides, the versatility of their properties can fit a lot of applications, making the use plastic difficult to be controlled.

Awareness of this problem is reflected by the response from many institutes that have been galvanized into action to solve the problem. These include the 3R (reuse, reduce and recycle) campaign to reduce plastic use in supermarket, increase use of biodegradable plastic or bags and a lot more awareness campaigns from environmental activists, government and non-governmental organizations (NGOs) to protect the nature (Manaf *et al*., 2009; Sin *et al*., 2013). The awareness also shows in research areas which are increasingly focused on the development of alternative materials to replace the conventional plastic. These materials are suggested to have similar properties with current conventional synthetic plastic but with biodegradable and environmental friendly characteristics (Albuquerque *et al*., 2010; Arshad *et al*., 2007, (Salehizadeh & Van Loosdrecht, 2004).

Polyhydroxyalkanoates (PHAs) become the best candidate to replace the conventional chemically synthesized polymers because apart from being fully biodegradable, they are of biological origin and are produced from renewable sources, thus allowing for a lower environmental impact. Furthermore, the biosynthesis occurs naturally through fermentation of bacteria. The PHA polymers have similar characteristics as that of the commercially used conventional plastic (Lageveen *et al*., 1988). Their thermoplastic properties give them potential to replace conventional polyolefin (Albuquerque *et al*., 2010; Arshad *et al*., 2007). PHAs also have numerous applications in medicine, pharmacy (implants, covering of pharmaceuticals), and packaging (Lemos *et al*., 1998).

The biodiesel industry is also getting attention worldwide including in Malaysia due to the consideration of world petroleum crisis, environmental issues, and the concerns about petroleum resources dwindling in future. The result of the increase in biodiesel demand has been the increase in production of crude glycerol which is the co-product (Chatzifragkou & Papanikolaou, 2012). As the biodiesel industry is available in Malaysia, the waste glycerol from palm oil based biodiesel industry can be used as a cheaper carbon source which is continually available for PHA production feedstock.

Optimization of fermentations has long been used to enhance the yield and productivities of many bioprocesses (Khanna & Srivastava, 2005). Response Surface Methodology (RSM) is a group of mathematical and statistical techniques useful for modelling and analysis of problems. When several variables are influenced by another variable, RSM is applicable to get the best result by optimizing the levels of the variables by consider the interaction of variables. This will reduce the number of fermentations, time, labour, and consumption of materials for testing each factor individually (Grothe *et al*., 1999; Shahhosseini, 2004). Several factors that are usually considered during PHA production fermentation include temperature, pH, carbon-to-nitrogen ratio in the feed, concentration of substrates, concentration of trace elements, ionic strength (IS), agitation intensity, and dissolved oxygen (Grothe *et al.*, 1999).

1.2 Research background and objectives

Even though PHA has emerged as the most interesting alternative to replace the petrochemical based plastic, the commercialization of PHA faces some constraints especially in production cost. Therefore, the study for optimization of the media formulation, environment and selection of the best bacteria strain must be carried out to provide better solutions. Suhaila (2014) had successfully optimized the fermentation condition for *B. cepacia* BPT1213 in shake flask study using pure glycerol. Under the influence of initial pH (9) for the culture media, temperature (37

˚C) and inoculum size (4% (v/v)) in mineral salt medium (MSM) content with 2% (v/v) of laboratory grade glycerol (99.5%), *B. cepacia* BPT1213 able to yield CDW up to 3.94 g/L and 78.34% (w/w) of PHA content.

This study was aimed to optimize the fermentation conditions using statistical approach, for production of PHA by *B. cepacia* BPT1213 in both shake flask, and scale-up to 2.5 L bioreactor, using waste glycerol as the sole carbon source. The objectives of this study were:

a) To screen the environmental factors that affect *B. cepacia* BPT1213 growth and PHA production using Factorial Design approach.
b) To optimize the growth conditions for PHA production by *B. cepacia* BPT1213 in shake flask culture using Response Surface Methodology (RSM).
c) To optimize the growth conditions for PHA production by *B. cepacia* BPT1213 in 2.5 L bioreactor using Response Surface Methodology (RSM).
d) To characterize the PHA polymer properties produced by *B. cepacia* BPT1213.

CHAPTER 2 : LITERATURE REVIEW

2.1 Biopolymer

Biopolymers are special polymers that can be present naturally or synthesized by living organisms. They can be from bio-based origin and renewable or non-renewable sources. In addition, a bio-based polymer can also be either biodegradable or non-biodegradable whereas biodegradable polymer can be bio-based or non-bio-based (Babu, *et al.*, 2013). Bio-polymer can be synthesized from renewable monomer resources to become polymer through bio-chemosynthesis, chemical polymerization and biosynthesis (Avérous & Pollet, 2012a; Babu *et al.*, 2013).

Bio-based plastic can be derived from modification of natural bio-based polymers such as starch, protein and cellulose commonly obtained from plant tissues or agro-resources. This natural polymer is extracted and separated before being partially altered to meet the bioplastic properties. Corns, potatoes, tapioca and others starchy plant are the common natural sources for natural biopolymer but the limitation is that they are edible sources, and therefore presents ethical issues when utilised for non-food purpose (Avérous & Pollet, 2012a; Babu *et al.*, 2013).

Bioplastics from bio-based monomers also can be synthetically produced through conventional chemistry followed by polymerization (Avérous & Pollet, 2012b; Babu *et al.*, 2013), using the same technology as the conventional chemical plastic production. At the same time, the produced bio-based plastic is also promising similar properties as the conventional chemical plastic. For example, polylactic acid (PLA) is a polymer of lactic acid produced by modification or microbial fermentation of starch or sugar from corn. Pure PLA is biodegradable polymer when it is exposed to carbon dioxide, water and methane environment (Wang *et al*., 2001; Yew *et al*., 2005). Even though crystalline PLA is virtually non-biodegradable like polyhioester and cellulose ester derivatives, the modification of molecular characteristic of PLA makes it biodegradable at various conditions include hydrolytically degradable by enzymatic reaction (Sudesh & Iwata, 2008; Yew *et al*., 2005).

Another type of bio-based plastic can be produced through the biosynthesis of polymers by microorganisms especially by bacteria through fermentation of sugar, lipids and other carbon sources. One polymer, known as polyhydroxyalkanoate (PHA) (Avérous & Pollet, 2012b; Babu, *et al*. 2013) has been getting a lot of attention due to its similar properties and characteristics compared to conventional plastic. Moreover, it can be derived from a renewable bio-based origin and is biodegradable, making it an ideal type of bioplastic (Arshad *et al*., 2007; Chee *et al*., 2010b).

The best bioplastics, then, should be derived from renewable biological origin (bio-based) and be biodegradable at its end life. Examples of bioplastics and their degradability are shown in Figure 2.1 (Tokiwa *et al*., 2009; Rujnić-Sokele & Pilipović, 2017). Some of petroleum based plastic such as polycaprolactone (PCL), and poly(butylene succinate) (PBS) are considered as biodegradable since they can be degraded by microorganisms. Even tough bio-based plastics such polyethylene (PE) and Nylon 11 (NY11) can be produced from biomass or renewable resources, but they are non-biodegradable. In contrast, PHA, PLA and starch blends are produced from biomass or renewable resources, and at the same time they are biodegradable. In other instance, the biodegradability characteristic of bio-based acetyl cellulose (AcC) will be reduced as the substitution ratios of acetyl increased (Tokiwa *et al*.,2009).

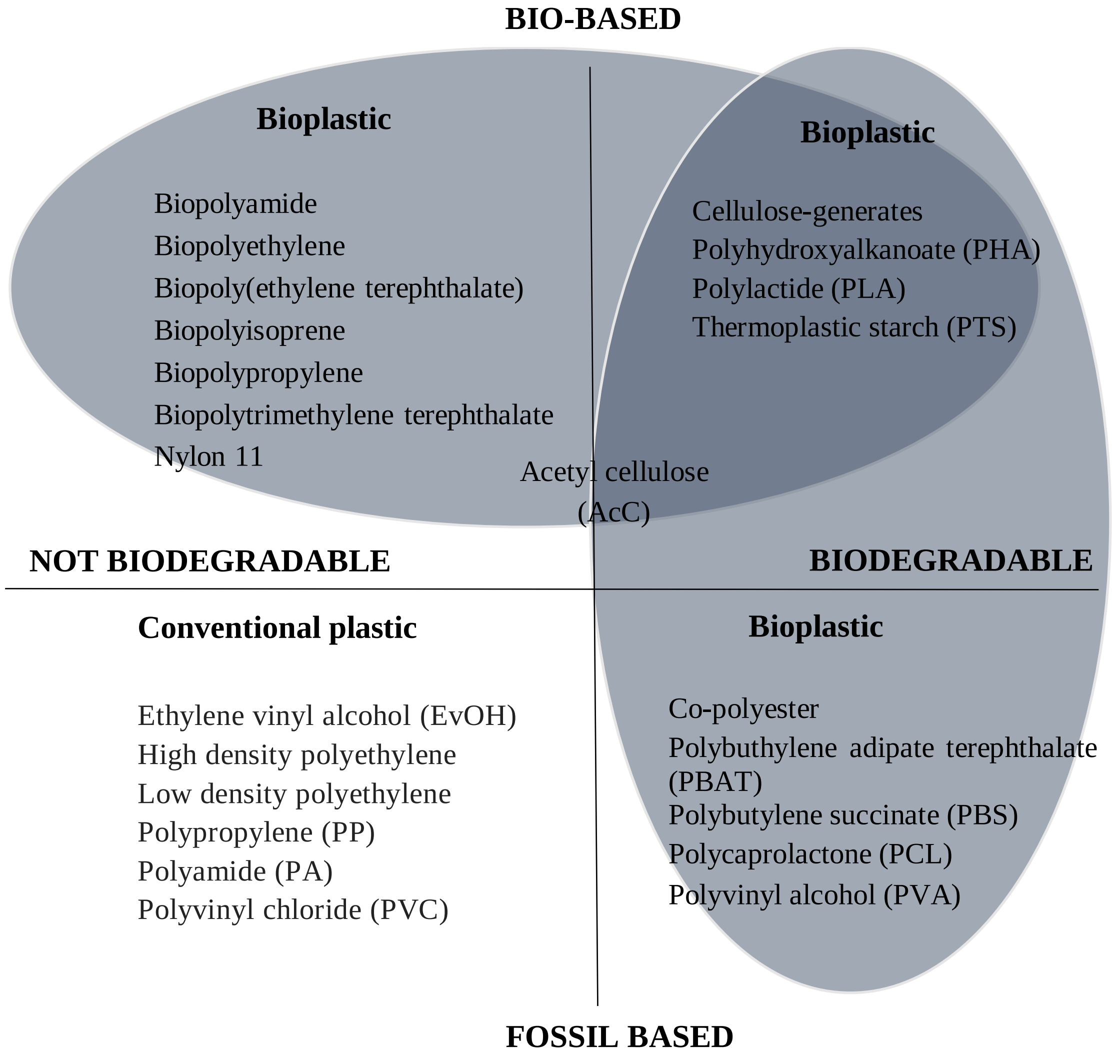


Figure 2.1: Classification of plastics. Bioplastics encompasses of biodegradable plastics and bio-based plastics (Tokiwa *et al*., 2009; Rujnić-Sokele & Pilipović, 2017)

2.2 Polyhydroxyalkanoate (PHA)

Polyhydroxyalkanoate (PHA) is a natural intracellular polyester of hydroxyl fatty acid that can be produced by various types of bacteria. This biomaterial is usually accumulated as in the cytoplasm of bacteria (Barnard & Sanders, 1989, Sudesh, *et al.*, 2000). PHA synthesis occurs when the excess carbon source is available in a restricted growth condition for microbial growth such as nutrient depletion and oxygen limitation. At least one of the nutrients such as nitrogen, phosphorus, sulphur or oxygen must be present in limited concentration (Anderson & Dawes, 1990; Lee, 1996b; Poirier *et al.*, 1995). A PHA producer is able to preserve this excess carbon source in the form of PHA granule as their carbon reservoir and energy storage by a special enzymatic pathway (Amirul *et al.*, 2008). This enables them to survive during the stress phase caused by the lack of nutrient and in unfavorable conditions for growth. PHA only then can be used back as an energy source after the nutrient starvation period is recovered (Chee *et al.*, 2010b).

2.3 History of PHA

PHA was first discovered by a French scientist, Lemoigne at the Institute Pasteur in 1923. He observed the formation of 3-hydroxybutric acid in an aerobic spore-forming *Bacillus* sp. when it was grown in anaerobic condition. The material was confirmed as 3-hydroxybutric acid in his further investigation in 1927 after he successfully extracted it from *Bacillus* using chloroform (Macrae & Wilkinson, 1958). However, the study of this material was restricted by limited methods for P(3HB) detection and qualification. Only in the 1950s was the function of this material recognized through the understanding of microbial physiology and metabolism (Doudoroff, *et al.*, 1959; Williamson & Wilkinson, 1958). Macrae & Wilkinson, (1958) found that PHA accumulation was increased and promoted by the limitation of nitrogen in growth media. Following its discovery, various aspects of this material has been studied in the last century, including the synthesis and breakdown enzymes, the formation pathways in bacteria, the assay involved in PHA study, and their chemical and physical properties (Lundgren *et al*., 1964). The timeline of the various aspects of PHA study was illustrated by Sudesh *et al*., (2000) (Figure 2.2).

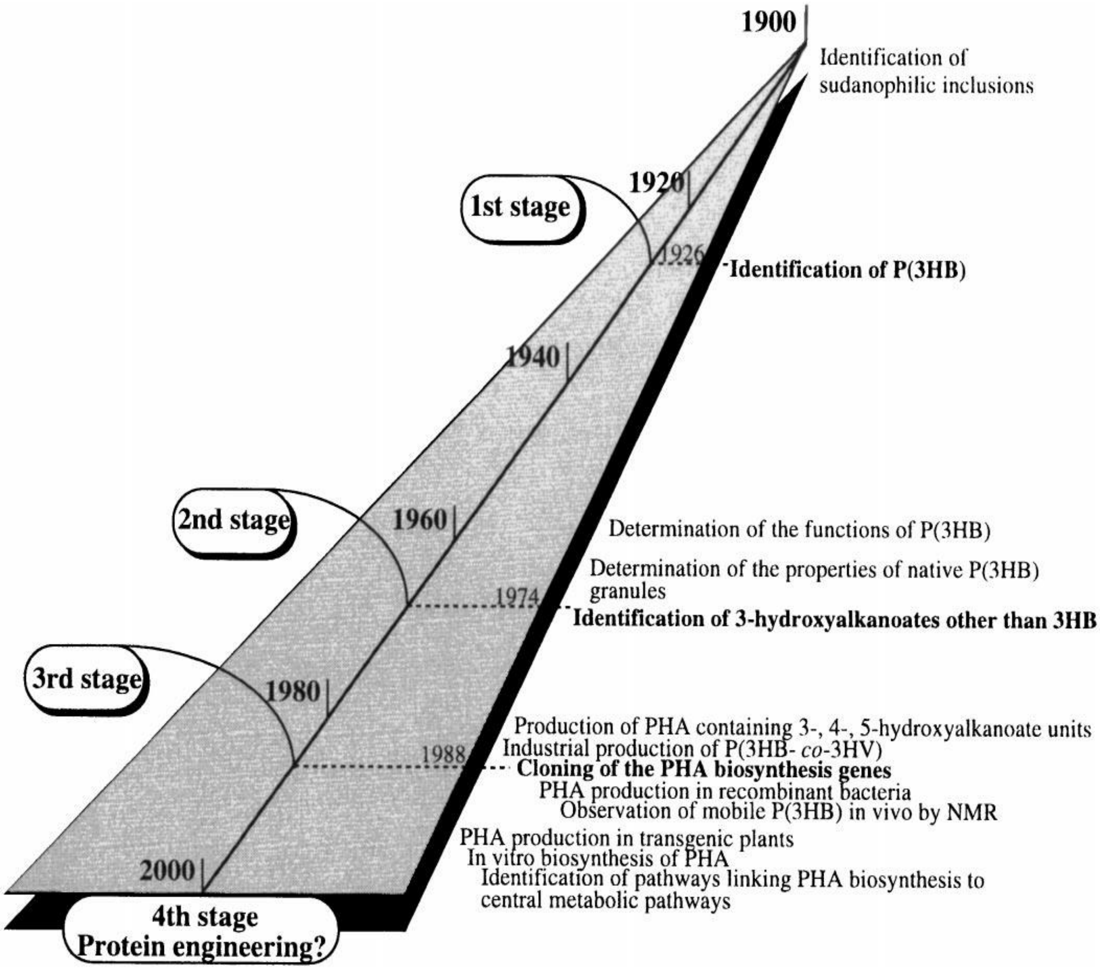
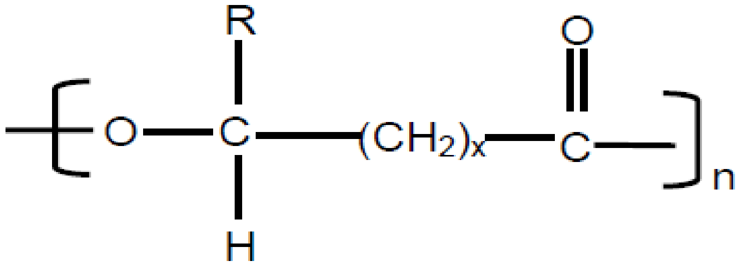


Figure 2.2: Milestones of the PHA research and development until the twenty first century (Sudesh *et al*., 2000)

The commercial production of poly(3-hydroxybutyrate) (P(3HB)), a type of PHA, faced many failures since it was explored in the early 1960s. Baptist and Werber at WR Grace & Co. (U.S.A) have obtained the patent for P(3HB) production and isolation but the fermentation yield was low and required expensive solvent extraction method. The cost hampered their effort and production was stopped (Philip *et al*., 2007). In the 1970s, the world oil crisis forced PHA to be was re-evaluated as a petrochemical based plastic substitution. ICI (UK) succeeded in optimising conditions for P(3HB) production by *Alcaligenes latus.* PHA content was increased up to 70% of its dry cell weight. However, once again the commercialization failed to take off due to the brittle and poor mechanical properties of the P(3HB). Finally, the invention of a novel polymer, BIOPOL (a copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate) had improved properties, such as lower crystallinity and more elasticity than P(3HB) (Philip *et al*., 2007).

2.4 Properties of PHA

The general structure of PHA is shown in Figure 2.3. The PHA monomer known as 3-*R*-hydroxyalkanoic acid (HA) is built up of carbon, oxygen and hydrogen (Loo & Sudesh, 2007). PHA can be found in straight, branched, saturated, unsaturated and aromatic chains. 'R' refers to the functional group (Table 2.1) (e.g., halogens, carboxyl, hydroxyl, epoxyl, phenoxy, nitrophenoxy, thiophenoxy, and methylester groups) in the side chains that contributes to the variation of PHA properties. Both the length of these side chains and their functional groups affect some chemical and physical properties of the bioplastic, e.g., melting point, glass transition temperature, and crystallinity (Eggink *et al*., 1995).



$$\left[O - \overset{R}{\underset{H}{C}} - (CH_2)_x - \overset{O}{\overset{\|}{C}} - \right]_n \quad = 100\text{-}30\ 000$$

Figure 2.3: General structural of PHA (Lee,1996a)

n and x = refer to the number of each repeating unit in the copolymer
R = functional group at the side chain

Table 2.1: The functional groups in the side chain of PHA and type of monomer formed (Lee,1996a)

	R group		**Type of monomer**	
x=1	Hydrogen	(H)	3-hydroxy propionate	(3HP)
	methyl	(CH_3)	3-hydroxybuytrate	(3HB)
	ethyl	(C_2H_5)	3-hydroxyvalerate	(3HV)
	propyl	(C_3H_7)	3-hydroxyhexanoatem	(3HHx)
	pentyl	(C_5H_1)	3-hydroxyoctanoate	(3HO)
	nonyl	(C_9H_{19})	3-hydroxydodecanoate	(3HDD)
x = 2	Hydrogen	(H)	4-hydroxybutyrate	(4HB)
x = 3	hydrogen	(H)	5-hydroxyvalerate	(5HV)
	methyl		5-hydroxyhexanoate	
x = 4	hexyl		6-hydroxydodecanoate	

2.4.1 Chemical properties of PHA

PHA can be found in wide range of chemical composition that depends on several factors. The use of different carbon sources fed in the PHA fermentation lead to the variation of the PHA monomer composition. Substrates specificity is different, depending on the PHA enzymatic and metabolic pathways that are available in the PHA producer (Loo & Sudesh, 2007). Generally, the PHA can be classified according to the size of monomer (length of the monomer or carbon number), type of polymer and the chemical nature of the monomers as explained by Loo & Sudesh, (2007) in Table 2.2.

Based on the physiological and enzymatic studies, there are two major classes of PHA that are affected by the number of carbon in the PHA monomer (Haywood *et al.*, 1989). Short-chain-length (SCL) polymers are composed of monomers containing 3 to 5 carbon atoms that might contain 3HB or 3HV. The medium-chain-length PHA (MCL-PHA) polymers are composed of 6 to 14 carbon atoms monomers which are longer than 3HV (Lee, 1996b; Loo & Sudesh, 2007). The molecular weight and monomer composition of polymer depend on specific PHA synthase enzymes and specific carbon substrates utilization by the microorganism (Anderson & Dawes, 1990; Sim *et al.*, 1997). Some of PHA producers can synthesize both SCL-PHA and MCL-PHA either as a separated unit or mixed repeating unit of PHA known as hybrid SCL-PHA and MCL-PHA polymer (Kato *et al.*, 1996; Lee *et al.*, 1995; Fukui *et al.*, 1997; Kim & Lenz, 2001).

Table 2.2: Classification of microbial PHA according to different criteria (Loo & Sudesh, 2007)

Classification	Criteria	Example
Biosynthesis origin	1) Natural PHA: Microbial PHA that are synthesized naturally from various substrates	Poly(3-hydroxybutyrate) (P(3HB))
	2) Semisynthetic PHA: Biosynthesis of PHA is enhanced by addition of unusual precursors (e.g., 3-mercaptopropionic acid)	3-poly(3-hydroxybutyrate-co-mercaptopropionic (P(3HB-co-3MP)
Monomer size	Depends on the number of carbon atom in monomer 1) Short chain-length PHA (SCL-PHA): contains 3-5 carbon atoms	
	2) Medium chain-length PHA (SCL-PHA): contains 6-14 carbon atom	
Number of different monomers in PHA	1) Homopolymer: Built up by polymerization of monomeric unit that are linked together through ester bonds to the carboxylic group of the next monomer	P(3HB)
	2) Heteropolymer: Two or more monomer units are linked together known as copolymer	P(3HB-co-4HB)
Chemical nature of monomer	1) PHA containing aliphatic fatty acid	P(3HB)
	2) PHA containing aromatic fatty acid	
	3) PHA containing both aliphatic and aromatic fatty acids	P(3HB-co-3MP)

2.4.2 Physical and mechanical properties of PHA

A wide range of PHA can be synthesized by different types of microorganism, either in the form of homopolyesters, copolyesters, or polyester blends (Amirul *et al*., 2008). Consequently, the existence of the different types of PHA caused the dissimilarity of their properties depending on the size of the granules, the monomer composition, macromolecular structure and physicochemical properties. The type and relative proportion of hydroxyalkanoate monomers is the key for PHA thermoplasticity, biodegradability and biocompatibility. Therefore, they present a broad range of structural, thermal and mechanical properties (Lemos *et al*., 1998; Valappil *et al*., 2007; Albuquerque *et al*., 2010).

The most well-known in PHA family being produced by microorganism, P(3HB) has poor properties such as being highly crystalline, stiff and brittle depending on its molecular weight (Iwata, 2005; Zhu *et al*., 2013). A comparison of several homopolymers and copolymers are shown in Table 2.3 (Zhu *et al*., 2013). Sudesh *et al*. (2000) reported that the P(3HB) polymer elasticity expressed by Young's Modulus was 3.5 GPa, while that of polypropylene was 1.7 GPa. They have similar tensile strength and high melting point, where that of P(3HB) homopolymer is 43 Mpa and 177 ˚C and that of polypropylene is 38 Mpa and melting temperature at 176 ˚C respectively. However, the elongation break percentage of P(3HB) homopolymer (5%) was much poorer than that of polypropylene (400%), making it stiffer, brittle and less flexible.

Table 2.3: Comparison of physical and mechanical properties of PHAs and petroleum-derived plastics from previous studies (Tsuge, 2002; Zhu *et al.*, 2013)

Polymer types	**Crystallinity (%)**	**Melting temperature, T_m (˚C)**	**Glass transition temperature, T_g (˚C)**	**Tensile strength (Mpa)**	**Elongation to break (%)**	**Reference**
P(3HB)	60	177	4	43	5	Tsuge, 2002
P(3HV)	N.A	130	-16	31	14	Yamane *et al.*, 1996
P(4HB)	25	54	-48	104	1000	Spyros & Marchessault, 1996
P(3HB-co-20%HV)	56	145	-1	20	50	Tsuge, 2002
P(3HB-co-84%HV)	N.A	N.A	N.A	20	35	Yamane *et al.*, 1996
P(3HB-co-16%4HB)	45	150	-7	26	444	Tsuge, 2002
P(3HB-co-10%HHx)	34	127	-1	21	400	Tsuge, 2002
PLA	56	170	57	41	4	MacDonald, *et al.*, 1996; Martin *et al.*, 2002
Polypropylene	50-70	176	-10	38	400	Tsuge, 2002
Polyethylene (LDPE)	20-50	130	-38	10	620	Tsuge, 2002

(N.A) symbol means data is not available

The formation of copolymer between 3HB and other types of polymers can improve on its undesirable properties. All the copolymers shown in Table 2.3 showed reduction in crystallinity compared to P(3HB) homopolymer (Tsuge, 2002; Zhu *et al.*, 2013). It shows that P(3HB) homopolymer has better tensile strength (43 MPa) with poor elongation break (5%) compared to P(3HV) homopolymer which has lower tensile strength (43 MPa) but better elongation break at 14%. The better elongation break in P(3HV) gives it less stiffness and higher flexibility than P(3HB) (Poirier *et al.*, 1995). The combination of P(4HB) and P(3HB) as copolymers have improved the elongation breaks (1000 %). The copolymers P(3HB-co-16%4HB) and P(3HB-co-10%HHx) have greatly improved elongation breaks (444 and 400%) making them less stiff, but have much higher elasticity and flexibility. The copolymer P(3HB) with different mole fractions of 3(HV) resulted in different physical and mechanical properties of the PHA polymer (Table 2.3 and Table 2.4). The increase of 3HV mole fraction reduces the melting temperature, glass transition temperature and tensile strength. The various properties make them fit the different applications include food packaging, pharmaceutical industry and others (Loo & Sudesh, 2007).

PHA produced by two recombinant *Escherichia coli* may have different relative molecular mass, glass transition temperature, melting temperature and percentage of crystallinity (De Almeida, *et al.*, 2010). These variation of physical properties are due to the presence of different types of genes that catalyze polymer biosynthesis, the different types of carbon source, and agitation speeds applied to the culture. Zhu *et al.*, (2010) concluded that an increase of substrate (glycerol) concentration ranging 3-9% fed to *Burkholderia cepacia* ATCC 17759 not only caused a reduction of biomass weight and PHA content, but also the polymer molecular weight.

Table 2.4: Physical and mechanical properties of P(3HB-*co*-3HV) (Loo & Sudesh, 2007)

Mol fraction (mol%)		**Melting temperature, T_m (˚C)**	**Glass transition temperature, T_g (˚C)**	**Young's modulus (GPa)**	**Tensile strength (Mpa)**
3HB	3HV				
100	0	179	10	3.5	40
97	3	170	8	2.9	38
91	9	162	6	1.9	37
86	14	150	4	1.5	35
80	20	145	-1	1.2	32
75	25	137	-6	0.7	20

2.5 Applications of PHA

With the similar characteristic and properties as many conventional petrochemical derived plastics, PHA has numerous applications covering the medical, pharmaceutical (implants, covering of pharmaceuticals), and packaging sectors (Lemos *et al.*, 1998). For example, PHA type P(3HB-3HHx) by *Aeromonas hydrophila* was produced in industrial scale and has a wide application in the manufacture of flushable nonwoven binder, flexible packaging, synthetic paper and medical devices (Chen, *et al.*, 2001; Philip *et al.*, 2007).

In the packaging industry, PHA has comparable or better properties to existing packaging material. For example, PHA has good moisture vapour barrier properties similar to polyethylene and polypropylene. Copolymers of the PHA, poly(hydroxybutyric acid) (PHB) and poly(hydroxyvaleric acid) (PHV) served as better materials for food packaging because they have lower oxygen permeability than polyethylene and polypropylene. This reduces the amount of antioxidant added to this copolymer (Albuquerque *et al.*, 2010). Besides, high surface energy in PHA makes it suitable for inks and dyes printing on it. Packaging material from PHA also has high UV resistance as well as oxygen resistance compared to ethyl vinyl alcohol (EVOH).

PHA is also applicable as packaging material for food packaging, bags, wrapping films and household containers (Yunus *et al.*, 2008).

The excellent properties of PHA make it widely applicable in the medical and pharmaceutical field, regardless the production cost (Chen, *et al.*, 2009). The implementation of D-(3)-hydroxybutyrate was concluded as non-toxic to mammalian tissues when the degraded products were highly detected in human plasma (Yunus *et al.*, 2008; Ojumu *et al.*, 2004). In the surgical application, P(3HB) can be used for surgical pins, bone replacement, sutures, swabs, wound dressing and blood vessel replacement materials (Yunus *et al.*, 2008). P(3HB-3HHx) and its PHB blends are useful to build up the three-dimensional structure of cartilage, osteoblast, and fibroblast (Chen *et al.*, 2005). Their piezoelectric properties enabled them to be used as osteo synthetic materials in simulation of bone growth (Senior & Dawes, 1971; Oeding & Schlegel, 1973). PHA as a biodegradable carrier for long term dosage and slow release of drugs and hormones inside the body was also well studied (Shrivastav *et al.*, 2013).

PHAs also play important roles in several industrial fields. A small amount of PHA added to latex create a water-resistant surface and are used to coat paper or cardboard hence reducing the production cost to replace non-biodegradable aluminium. In the agriculture field, PHA provides a continuous advantage in the form of biodegradable agriculture film used for urea fertilizers, herbicides and insecticides slow release. The consumption of PHA that degraded anaerobically in the soil by the bacteria will enhance nitrogen fixation in plants (Philip *et al.*, 2007). Sudesh and co-workers (2007) concluded that the oil absorbance properties of PHA make it applicable as cosmetic oil-blotting film, kitchen utensils and plastic cutleries. Other than that, the

P(3HB) film was found effective as dye removal as explained by Sridewi, *et al*. (2011a) and Sridewi, *et al*. (2011b), and is forecasted to be applicable in industrial waste water treatment in the future.

2.6 Bacteria as a PHA Producer

A diverse class of microbes beyond 300 different microorganisms are able to produce over 150 different monomer units as constituents of PHA (Salehizadeh & Van Loosdrecht, 2004; Amirul *et al*., 2008). The synthesized PHA monomers come with different chemical structures either in a straight, branched, saturated, unsaturated and also aromatic structures (Witholt & Kessler, 1999; Chee *et al*., 2010b). PHA producers can be either Gram positive (Williamson & Wilkinson, 1958; Findlay & White, 1983) or Gram negative bacteria (Forsyth *et al*., 1958), an archaebacteria (Doi, 1990), photosynthetic bacteria (Hashimoto *et al*., 1993; Hassan *et al*., 1996; Hassan *et al*., 1997, Hassan *et al*.,1998) or cyanobacteria (Jensen & Sicko, 1971; Jau *et al*., 2005) in both aerobic and anaerobic conditions (Chee *et al*., 2010b).

Besides wild isolated bacteria, a genetically modified PHA producer also contributes to PHA production. Together with pure cultures *Alcaligenes latus, Azotobacter vinelandii* and *Pseudomonas oleovorans*, the recombinant *Alcaligenes eutrophus* and recombinant *Escherichia coli* also have been used in PHA industry (Salehizadeh & Van Loosdrecht, 2004). The engineered microorganisms modified for PHA production give several advantage including the substrates versatility such as xylose, glucose and fat (Chee *et al*., 2010b). A recombinant *E. coli* can produce PHA in a shorter time of 24 h compared to 3 days generally taken by wild types. The genetically engineered bacteria can be made capable of controlling the polydispersity index by modeling the PHA synthase activity. Besides, modified microorganism can avoid natural degradation mechanism that is normally present in native PHA-producing bacteria which may cause difficulty in PHA recovery (Lee *et al.,* 1995; Kusaka *et al*., 1997; Sim *et al*., 1997; Sudesh *et al*., 2000).

2.7 *Burkholderia cepacia* as PHA producer

Burkholderia cepacia strain is a Gram negative bacteria which can be found naturally around the plant roots in watered area such as moist soil and river sediment (Govan & Deretic, 1996). It was first isolated by William Burkholder and was initially categorized as *Pseudomonas* because of the phenotypic similarity (Mahenthiralingam, *et al.,* 2008). It was later reclassified as a new genus, and renamed *B. cepacia* after molecular taxonomic analysis showed it to be sufficiently different from *P. aeruginosa* (Yabuuchi *et al*., 1992).

Many researchers have shown that *B. cepacia* was able to produce high intracellular concentrations of PHAs under nutritional limitation in the presence of various carbon sources such as glycerol, levulinic acid, glucose, xylose and lactose. Zhu *et al.,* (2010) reported that *B. cepacia* mostly synthesized 3HB and the continuous feeding of levulinic acid as co-substrate to glycerol leads to the production of P(3HB-co-3HV) co-polymer by *B.cepacia* (Zhu *et al*., 2013).

2.8 Renewable carbon source for PHA production

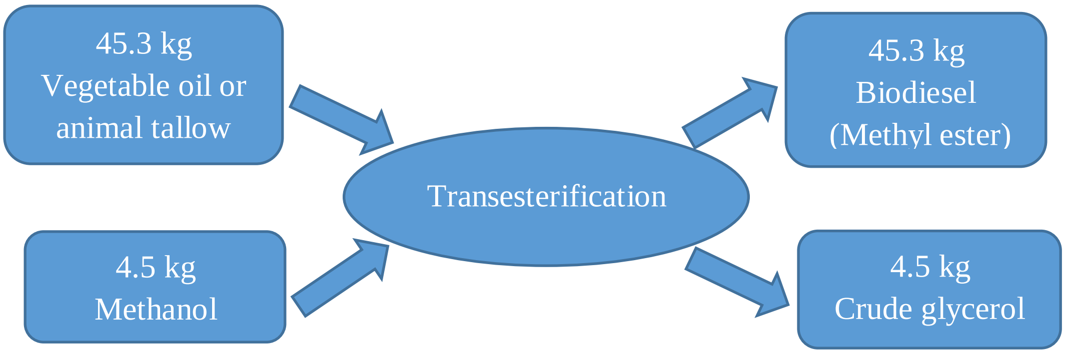
Despite its many applications, the broad application of PHA is still held back by its high cost production. The main problems restricting PHA bulk production for commercialization since 1980's are the costs of raw material and solvent for recovery. The high cost hampered its competitiveness against the petroleum-based plastic such as polyethylene and polypropylene with lower commodity market price of around US$1 per kg. The price suggested production cost should be US$3-5 per kg to make PHA commercially viable (Choi & Lee, 1997). Consequently, extensive effort has been devoted to reduce labor cost by the development of better bacterial strains, fermentation, cheaper carbon sources and a more efficient extraction process (Lee, 1996b; Grothe *et al*., 1999).

The cost of raw material contributes up to 48% of the total production and 70-80% of the raw material cost was due to the carbon source cost (Choi and Lee, 1997). Earlier, the first-generation technologies mainly focused on edible resources such as corn, starch, rice, etc. to produce bio-based polymers (Babu *et al*., 2013). To avoid the ethics debate, researchers have changed the PHA production pattern by using more green and sustainable material and technology to reduce the cost. A simple renewable carbon source such as cellulosic materials, vegetable oils, organic waste, municipal solid waste, and fatty acids can be the feedstock depending on the specific PHA required (Chee *et al*., 2010b).

2.9 Waste glycerol from biodiesel industry

2.9.1 Biodiesel production

Biodiesel is defined as the monoalkyl esters of fatty acids or fatty acid methyl esters (FAME) derived from vegetable oil or animal fat after a trans-esterification process in addition of alcohol (methanol or ethanol) and acid or alkaline catalyst, producing glycerol as a by-product (Figure 2.4) (Zhou *et al.*, 2008). Commonly, three wastes produced from the biodiesel process are composed of glycerol, methanol and wastewater (Chatzifragkou & Papanikolaou, 2012; Yusuf *et al.*, 2011).



(Catalyst)

$$\left.\begin{array}{lcl} \textbf{Triglycerides} + CH_3OH & \rightleftharpoons & \textbf{Diglycerides} + R_1COOH_3 \\ \textbf{Diglycerides} + CH_3OH & \rightleftharpoons & \textbf{Monoglycerides} + R_2COOH_3 \\ \textbf{Monoglycerides} + CH_3OH & \rightleftharpoons & \textbf{Glycerol} + R_3COOH_3 \end{array}\right\} \textbf{Biodiesel}$$

$$\textbf{Triglycerides} + 3\,CH_3OH \rightleftharpoons \textbf{Glycerol + Methyl ester (Biodiesel)}$$

*Where R_1, R_2, R_3 are hydrocarbon chain containing 15 to 21 carbon atoms

Figure 2.4: Formation of waste glycerol during transesterification (Zhou, *et al.*,2008)

In step 1, methanol produces diglycerides and methyl ester by reacting with triglycerides. Next, diglycerides react with methanol again to form monoglycerides and methyl ester. In final step, monoglycerides react with methanol. Producing glycerol and methyl ester. Every three mol of methanol generate one mole of waste glycerol

2.9.2 World biodiesel trend

Biodiesel and bio-ethanol is a form of energy to replace petroleum products with similar quality and can be used in existing power generators or engines (Yusuf *et al*., 2011). Even though solar energy offers renewable resource ever, it is constrained by weather conditions of a place. In addition to this, it also requires modification of power generation to replace petroleum energy generators for use on vehicles or machine (Abdullah, *et al*., 2009; Lam *et al*., 2009). The biodiesel industries are rapidly getting attention as global trend due to environmental concern. It is a renewable, sustainable, environmentally friendly and nontoxic energy source which is better than the harmful fossil fuel (Yusuf *et al*., 2011).

Various types of animal and plant based oils as well as oils produced by microbes can be used as a feedstock for biodiesel production. Non-edible type of oils or fats are the most preferred starting material. Animal-derived oil or fat has limited application in biodiesel production due to its high melting point. Special organic solvent treatment with appropriate tool is required to dissolve the solid fat and solvent recovery which should be avoided due to the development cost (Tashtoush *et al*., 2004; Al-Zuhair, 2007; Chatzifragkou & Papanikolaou, 2012). Vegetable oils such as jatropha, palm, rapeseed and soybean are widely used in biodiesel production (Demirbaş & Kara, 2006). Soybean oils are most preferable as starting material in the USA (Reijnders & Huijbregts, 2008; Qi *et al*., 2009; Yusuf *et al*., 2011) while rapeseed oil is mainly used in the biodiesel industry in the European region (Abdullah *et al*., 2009; Georgogianni *et al*., 2009; Shi & Bao, 2008). The use of waste oil has also been adopted, but faces some challenges due to the presence of impurities like water and fatty acid that may disrupt the biodiesel production process. The worth of the purification cost must be considered against the profit (Chatzifragkou & Papanikolaou, 2012).

2.9.3 Malaysia palm oils and biodiesel industries

Malaysia is second largest palm oil producer after Indonesia with a total of 5 million hectares of palm plantation as reported in 2011 (Malaysian Palm Oil Board (MPOB, 2011). Malaysia palm oil industries have faced great criticisms from NGOs around the world due to deforestation and wildlife habitat issues, especially the orangutan because the animal is indeed threatened with extinction (Tan *et al.*, 2009). In addition, the nutritional contents of palm oil products are in dispute. However, many researches have proved that the palm oil appears to contain a good variety of food sources. Palm oil biodiesel has also become a controversial issue as it is an edible feed stock. Despite this, thus far, the oil palms are contributing to a sustainable economy in the food chain and other lipid oleo industry worldwide (Lam *et al*., 2009).

Malaysia started its biodiesel program since 2006. In 2013, it was reported that 113,000 tons of palm biodiesel (B5 type; blend of 5% biodiesel in petrol based diesel) per year has been used in central Malaysia region. This was followed by the implementation of the B5 biodiesel mandate in the southern region, where 415 fueling stations were selling the B5 biodiesel (Kotrba, 2013). Then, in Mac 2014, the northern and eastern regions completed the biodiesel program in peninsular Malaysia, with 478 stations in Pahang, Terengganu and Kelantan (eastern region), and 789 stations in Perak, Kedah, Penang and Perlis (northern region) selling the palm biodiesel B5.

Approximately 100,000 tons of biodiesel per annum has been used, and thus contributed to the reduction of petroleum diesel consumption of about 115.7 million