OPTIMIZATION OF POLYHYDROXYBUTYRATE FERMENTATION IN SHAKE FLASKS USING OIL PALM TRUNK SAP MEDIUM AND SCALE UP TO 20 LITER STIRRED TANK FERMENTER

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ABSTRACT

The uses of synthetic plastic nowadays giving many bad effects to the environment. Inabilities of synthetic plastic to degrade completely and took hundreds years to degrade entirely make a problem especially to the mother nature. To overcome these problems, the researcher were came with the idea to form a biodegradable plastic to replace the existing plastic that can produce microbially and using a renewable source which was polyhydroxybutyrate (PHB). However, PHB have a problem to produce in a large scale because need a higher cost compared to the petrochemical based plastic. The carbon source for the PHB is expensive than the synthetic plastic. In this study Oil Palm Trunk Sap (OPTS) were used as the carbon source of the fermentation of PHB. Three parameter were studied to get the optimum value for the production of PHB there are fermentation temperature, agitation speed and the volume of oil palm trunk sap. Fermentation was done in 500mL shake flask that containing mineral salt medium, OPTS and inoculums. The fermentation process were done at 48 hours with different levels of parameters. Then the product were analysed for cell dry weight and PHB content. Then, for the scale up analysis, 1.5L inoculums, 2L carbon source where the mixture of glucose, fructose and sucrose were the same as OPTS sugar composition, 2L of MSM and 10.5L of distilled water made 15L working volume. The fermentation were done for 72 hours and sampling every 6 hours. Based on the result of cell dry weight, the optimum temperature, agitation speed and volume of oil palm trunk sap were 32.6 °C, 162.5 rpm and 38.8% while the highest predicted concentration of PHB and cell dry weight were 1.985 g/L and 1.401 g/L. The highest production of PHB and cell dry weight were at 72 hours for first run that are 0.419 g/L and 4.5 g/L respectively while in second run the highest production of PHB at 12 hour and 48 hours for cell dry weight that are 0.494 g/L and 4g/L respectively.

ABSTRAK

Kegunaan plastik sintetik kini memberi banyak kesan buruk kepada alam sekitar. Ketidakupayaan plastik sintetik untuk mengurai sepenuhnya dan mengambil beratus-ratus tahun untuk mengurai sepenuhnya memberi masalah terutamanya kepada alam semula jadi. Untuk mengatasi masalah ini, penyelidik telah datang dengan idea untuk membentuk satu plastik mesra alam untuk menggantikan plastik yang sedia ada yang boleh dihasilkan secara biologi dan menggunakan sumber yang boleh diperbaharui iaitu polyhydroxybutyrate (PHB). Walau bagaimanapun , PHB mempunyai masalah untuk dihasilkan dalam skala yang besar kerana memerlukan kos yang lebih tinggi berbanding dengan plastik berasaskan petrokimia . Sumber karbon untuk PHB adalah mahal daripada plastik sintetik. Dalam kajian ini Sap batang Kelapa Sawit telah digunakan sebagai sumber karbon penapaian PHB . Tiga parameter telah dikaji untuk mendapatkan nilai optimum bagi penghasilan PHB iaitu suhu penapaian , kelajuan pergolakan dan jumlah sap batang kelapa sawit . Penapaian dilakukan di dalam kelalang 500mL yang mengandungi campuran mineral,sap batang kelapa sawit dan inoculum. Proses penapaian dilakukan selama 48 jam dengan tahap yang berbeza parameter. Selepas itu, produk tersebut dianalisis untuk sel berat kering dan kandungan PHB . Kemudian, untuk analisis skala besar,1.5L inokulum ,2L sumber karbon di mana campuran glukosa , fruktosa dan sukrosa adalah sama seperti komposisi gula sap batang Kelapa Sawit, 2L campuran mineral dan 10.5L air suling untuk 15L jumlah kerja. Penapaian dilakukan selama 72 jam dan pensampelan setiap 6 jam. Berdasarkan kepada keputusan sel berat kering , suhu optimum , kelajuan pergolakan dan peratusan jumlah sap bantang Kelapa Sawit adalah 32.6°C, 162.5 rpm dan 38.8% manakala kepekatan tertinggi meramalkan PHB dan sel berat kering adalah 1.985 g/L dan 1.401 g/L. Pengeluaran paling tinggi PHB dan berat kering sel masing-masing pada 72 jam untuk jangka masa pertama yang 0.419 g/L dan 4.5 g/L manakala dalam jangka kedua penghasilan tertinggi bagi PHB pada 12 jam dan 48 jam untuk sel berat kering yang masing-masing 0.494 g/L dan 4g/L.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

PHB	Polyhydroxybutyrate
OPTS	Oil Palm Trunk Sap
PHA	Polyhydroxyalkanoate
PE	Polyethylene
PVC	Polyvinyl chloride
PP	Polypropylene
PHBV	Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)
k	number of parameter
α	Level of experiment
n	Number of experiment
KH_2PO_4	Potassium dihydrogen phosphate
$(NH_4)_2SO_4$	Ammonium Sulphate
$MgSO_4$	Magnesium Sulphate
K_2HPO_4,	Dipotassium hydrogen phosphate
NaOH	Sodium Hdroxide
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
°C	Degree Celcius
%	Percent
rpm	Agitation Speed
ANOVA	Analysis of Variance
L	Liter
CV	Coefficient of Variation

1 INTRODUCTION

1.1 Background of Study

In Malaysia, the plastic product industry grows rapidly over the past 4 years. Malaysia becomes the largest plastic producer in Asia with 1,550 manufacturers and employ over 99,100 people (Market Watch 2012, The Malaysian Plastic Industry). Malaysia has exports good worldwide including China, Hong Kong, Japan and Thailand. Plastic that exported from Malaysia mainly in a non-primary forms and several in a primary form like Polyethylene (PE) and Polyvinyl chloride (PVC). The high demands of plastics contribute to the development of the petrochemical sector in Malaysia in producing synthetic and petroleum based plastic.

However, plastic is unable to degrade completely and take hundreds years to degrade entirely. Because of this inabilities, it causes many problems to the environments. The plastic that throw in the garbage finally cause a landfills, because of fast development landfills site are limited to load all the garbage. A recent investigation shows the amount of Municipal Solid Waste in Kuala Lumpur shows plastic are 21% from total all solid waste and estimation of solid waste accretion are about 1.2 percent tons a year in 2001 and 3.317 tonnes per day or 1,210,705 tonnes a year in 2020 (Iwan et al., 2008). This huge amount of waste not only need a wide landfills site but also contribute the pollution.

Certain irresponsible people that easily littered everywhere will cause the drain clogged and this will contribute towards flood. Also, throw the plastic bag into the sea will harm the aquatic life. Incineration of plastic also not a good method to keep the environment free from plastic waste, because it produces a large amount of carbon dioxide and sometimes a toxic gas (Sinha et al., 2005).

There are many ways that can be taken to solve this problem. Government already launched "No Plastic Bag" and recycle campaign in order to minimize the using of plastic bag. On the other hand, in line with the progression of research and development

(R&D), a research to produce a biodegradable plastic to replace the existing plastic increase tremendously.

Biodegradable plastic can be defined as a plastic that have the ability to decompose in natural aerobic and anaerobic. Biodegradable polymer also as those that undergo microbially induced chain fission leading to the mineralization (Sinha et al, 2005). Biodegraable polymer can be made of biosource, synthesized by bacteria or from petroleum source. In this study, PHB is produce from biosource that is Oil Palm Trunk Sap (OPTS) and synthesized by bacteria (*Cupriavidus necator*).

1.2. Problem Statement

In this study, PHB is produce in 500mL shake flask for small scale and after that in 20L stirred tank fermenter for a large scale. However, PHB have a problem to produce in a large scale because of high production cost as it use expensive carbon source (Anshuman et al, 2006). To solve this problem, the optimum value for the carbon source (OPTS) in the growth media are needed to optimize the production of PHB by *Cupriavidus necator* and for the scale up process.

1.3.Objectives

The objective of this research are to optimize the PHB fermentation on shake flasks using oil palm trunk sap (OPTS) medium and scale up the process to 20L stirred tank fermenter.

1.4.Scope of this research

In this study, the scope of work are to find the value of variable that will optimize the production of PHB that will be scale up. There are three variables that will be tested in this study are:

1) Agitation speed (RPM)
2) Temperature (°C)
3) Volume percent of OPTS (%)

2 LITERATURE REVIEW

2.1 Introduction

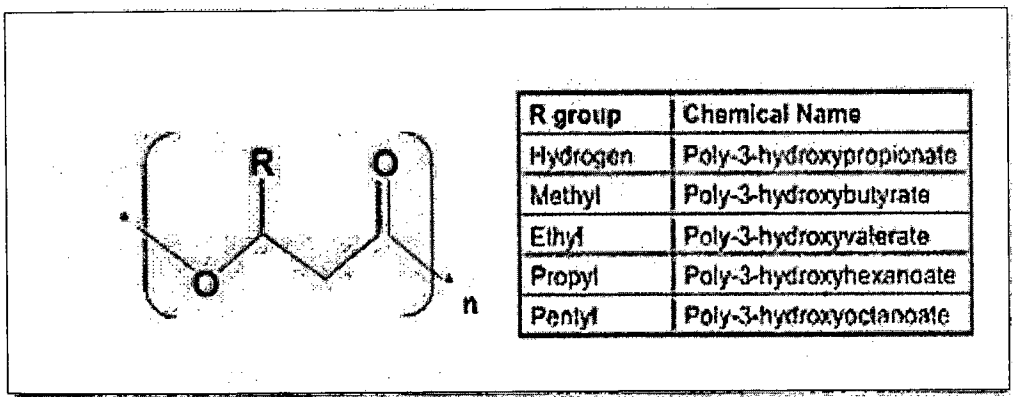
According to (Joel, 1995) the word plastic originated from the Greek word 'plastikos', which means 'able to molded into different shapes'. Plastic a long chain polymeric man made (Scott, 1999). With its stability, durability, plastic becomes synonym for materials that resistance to many environmental influences.

Synthetic plastic that widely used today, are made of from raw materials such as carbon, silicon, hydrogen, nitrogen, oxygen and chloride and the basic material are extract from non renewable source such as oil, coal and petroleum (Shimao, 2001). According to (Sabir, 2004) 30% plastic are used for packaging of product such as food, pharmaceutical, cosmetic, detergents and chemicals. Plastic that extensively used in packaging are polyethylene, polypropelyne (PP), polystyrene(PS), polyvinyl chloride (PVC), polyurethane (PUR),poly(ethylene terephthalate) (PET), and nylons.

Awareness of people about the impact of synthetic plastic to the environment, its open the way for the development of biodegradable plastic. According to (Lee et al., 1996) Bioplastic (Biopolymers) obtained from the growth of microorganism or from plants which are genetically-engineered to produce such polymers are likely to replace the existing plastic. The biodegradable plastics namely polyhydroxyalkanoates (PHA) and the most important are poly(3-hydroxybutyrate) (PHB).

2.2 *Polyhydroxyalkanoates (PHA)*

Recently, PHAs were the most investigated biopolymer as they a superior biodegradable polymer that can incorporate large number of different monomers. It have been found that about there were about 150 different hydroxylalkanoate units and the most common were PHB. PHA can be classified as scl-PHAs, mcl-PHAs and lcl-PHAs based on the length of the hydroxylalkanoic acid monomers (Steinbuchel & Pieper, 1992). Scl –PHAs were composed of C3 to C5 where PHB the first of PHAs studied extensively were in this group (Chandrashekharaiah, 2005). PHAs were non-toxic, biocompatible and biodegradable thermoplastic that can be produced from renewable resources. Also, PHAs have a high degree of polymerization, crystallinity, optically active, isolactic, piezoelectric and insoluble in water. This features make PHAs exhibit similarities with polypropylene the petrochemically based plastic (Chandrashekharaiah, 2005). The structure of PHA as shown as in Figure 2.1.



R group	Chemical Name
Hydrogen	Poly-3-hydroxypropionate
Methyl	Poly-3-hydroxybutyrate
Ethyl	Poly-3-hydroxyvalerate
Propyl	Poly-3-hydroxyhexanoate
Pentyl	Poly-3-hydroxyoctanoate

Figure 2 1: Structure of PHAs

2.3 *Polyhydroxybutyrate (PHB)*

In 1925, PHB was first discovered in *Bacillus Megatarium* by Lemoigne (Jackson & Srienc, 1994) The study from genus *Bacillus* at the end of 1950, suggest PHB function as an intracellular reserve for carbon and energy in these bacteria. After that, in 1974, the identification of hydroalkoanates (HA) beside 3-hydroxybutyrate (3HB) was reported by Wallen and Rohwedder like 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) (Sudesh et al., 2000). Polyhydroxybutyrate (PHB) is an intracellular microbial thermoplastic that is principle of polhydroalkoanates) that widely produced by many bacteria (Songri et al., 2009).

PHB belongs to the PHA group. Physical properties of PHB, it is a short chain PHA, a hard crystal and has a high melting point (Montaser et al., 2011), an aliphatic homopolymer of polyhydroxybutyric acid with a melting point of 179°C and high crystalline (80%). It can be degraded at the temperature above it melting point and 100% biodegradable and resistance to water (Hrabak, 1992). PHB produces naturally in the presence of excess carbon by bacteria as storage granules providing food, energy and reducing power (Khardenavis et al., 2007). PHB granules were act as energy reserves when nutrients such as nitrogen and phosphorus in limiting concentrations in the presence of excess carbon source. Previous study state that the presence of PHB in cell retarded the degradation of cellular components such as RNA and proteins during nutrient starvation (Anderson & Dawes, 1990).

PHB is organic polymer that has characteristics of being biodegradable and independent of fossil resources (Franz et al., 2011). PHB can degrade completely to water and carbon dioxide under anaerobic conditions and to methane under anaerobic conditions by microorganism in soil, sea, lake water and sewage. (Khanna et al., 2004). PHB becomes highly demand because its physical properties that have same as the petroleum based plastic that is, polypropylene (PP). PHB can replace PP because of it can produce by renewable source compared to PP that produce by non-renewable source.

According to (Yu et al., 2005), degradation of PHB can occur by one or several mechanism such as hydrolysis, thermal decomposition and chemical decomposition.

PHA are compostable over a wide range of temperatures, even at a maximum of around 60°C with moisture level at 55%. Previous studies shows that, about 85% of PHA degraded in 7 weeks (Johnstone, 1990 & Flechter, 1993). PHA also reported degraded within 254 days in aquatic environments at temperature not more than 6°C (Jhonstone, 1990). Brandl and Henselmann (1991) reported that PHB have different density from other conventional plastic materials. PHB have a high density which cause it not float, once it discarded, PHB will sink and degraded at the surface sediment by biogeochemical mechanisms.

In 1976, Imperial Chemical Industries (ICI) start the investigate on producing PHB from the carbohydrate feedstock in 1976. However, this project weas slowed down because of the high cost of production and insufficient demand. However, after few years, the awereness on importance of PHB, PHB copolymer were commercialized under the trade name BIOPOL® by Mosanto which owned the business from Zeneca Bioproducts, branch of ICI (Patnaik, 2006). The annual production of Biopol was about 10,000 tonnes (Lee, 1996) and this product successfully used for marketing various things.

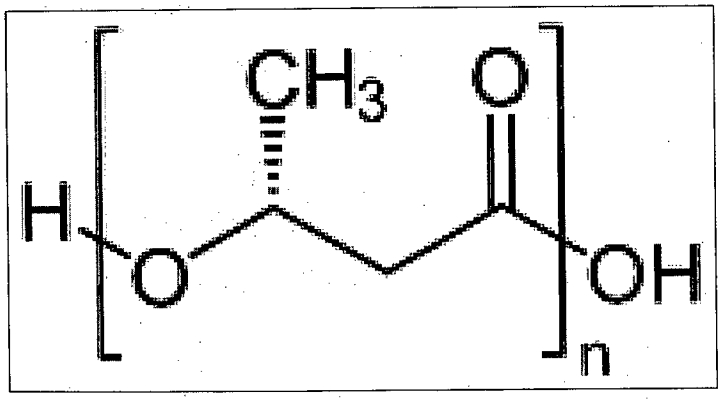


Figure 2 2 : Structure of PHB

2.4 *PHB Microbial Synthesis*

PHB productions begin in response to stress imposed on cells, usually by nitrogen or phosphorus limitation eventhough in the excess carbon source. In this condition, the cell do not grow or divided but divert their metabolites towards the biosynthesis of hydroxylalkyl-CoA (HA-coA). HA-coA is an activated monomeric precursor that was polymerized by the enzymatic action of PHA synthase to form PHA polyester. As it insoluble in water, PHA begin to form amorphous and spherical granules that fill the cell and force them to expand (Chandrashekharaiah, 2005).

The biosynthesis of PHB were consist of three reactions that catalysed by three different enzymes. The first reaction were the condensation of two acetylcoenzyme A (acetyl-CoA) molecules in to acetoacetyl-CoA by β-ketocylCoA thiolase (phbA). The second reaction is the reduction of acetoacetyl CoA to (R) -3- hydroxybutyryl-CoA by an NADPH dependent acetoacetyl-CoA dehydrogenase (phbB).lastly, the (R) -3-hydroxybutyryl-CoA monomers are polymerized in to PHB by P(3HB) polymerase or synthase, encoded by phbC (Huisman et al.,1989). The summary of the biosynthesis were as in Figure 2.3.

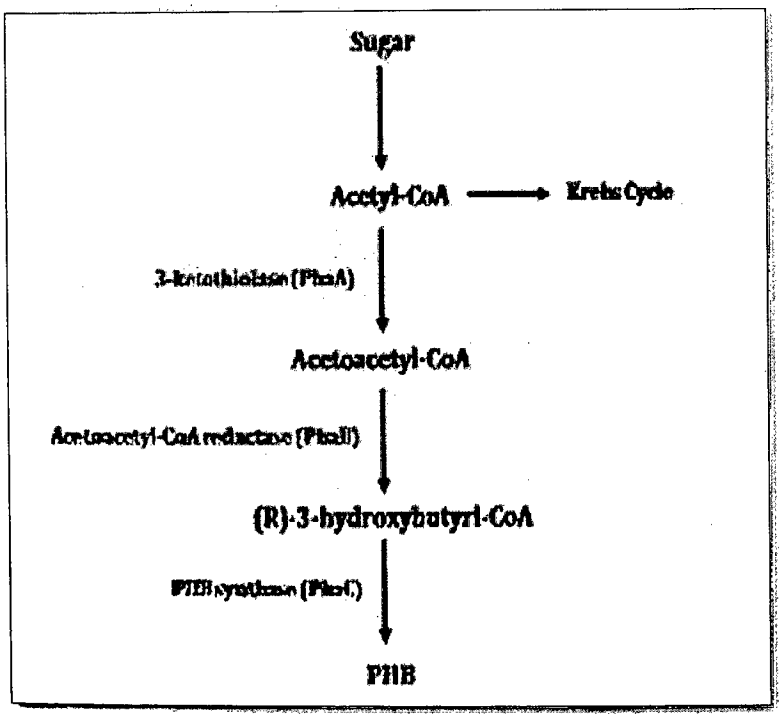


Figure 2 3: Microbial Synthesis of PHB

Cupriavidus necator (formerly known as *Ralstonia eutropha*) are suitable and widely used in production of PHB because of its potential to accumulate a high amount of PHB (Khanna et al., 2004). There are many carbon source can be utilized by *Cupriavidus necator* that are needed for the biosynthesis of PHB (Tohyama & Shimizu, 1999). Previous study shows that *Cupriavidus necator* capable to produce PHB homopolymer from even carbon numbered n-alkanoates, while odd n-alkanoates resulted in the accumulation of copolymer of 3HB and 3HV. *Cupriavidus necator* also can produced different types of PHA biopolymers depend on the carbon source. It synthesize scl-PHA (short length of PHA) mainly from sugar and some organic acid through the Entner-Doudoroff pathway and/or gluconeogenesis (Kaddor & Steinbuchel, 2011).

The efficiency of *Cupriavidus necator* showed that this strain can grow both lithoautotrophically on CO_2/H_2 mixtures or heterotrophically on sugars, glycerol, plant oil, biodiesel waste and volatile fatty acid (Cavalheiro et al., 2009). However, this strain unable to grow on the disaccharide lactose which is the main sugar contained in whey but previous research show that inserted *E.coli* lac operon in the chromosome of this strain can produce the PHB (Povolo et al., 2010). Byrom (1992) have reported that the strain that became the choice in ICI, UK were *Ralstonia eutropha sp*, as it produced an easily extracted PHA with high molecular weight.

Many literatures showed PHB also can be produce by many bacteria such as *Protomonas extroquens* (Suzuki. et al., 1988), *Candida utilitis* ATCC 8205 (Luong et al., 1987), and *Azetobacter vienelandii* (Wu. et al., 2001). *Alcaligenus latus* reported can store PHA up to 80 % under normal growth condition (Hrabak, 1992). *Pseudomonas sp.* had produced 66% of PHB on dry weight basis in methanol as sole carbon and energy source (Suzuki et al., 1986). Recombinant *E.coli* also capable produce about 80% PHB on molasses as the carbon source (Liu et al., 1998).

2.5 *Carbon Source for PHB Fermentation*

The major factor limiting the commercial use of PHA is the cost of its production where come from the substrate used and the downstream process. By using a cheaper carbon sources will reduce the cost. Agricultural waste such as beet cane molasses, corn syrup (Page, 1992), cheese whey (Yellore and Desai, 1998) with or without of ammonium sulphate supplement have been used as raw materials for PHA production.

In this study, OPTS is a feedstock that were used for the fermentation in production of PHB. Production of PHB in a large scale needs a high cost. Approaches that have been taken are by using renewable carbon resources that come from plants as a substrate for production of PHB. As we know OPTS are easily get in Malaysia, as Malaysia are the largest Oil Palm producer. On the other hand, OPTS is contain about 85.2 g/L of sugar that is glucose that become dominant sugar in all parts of trunk that is approximately about 86.9 %, 86.3% and 65.2 % in the inner,middle and outer parts respectively (Kosugi et al., 2010).

Eze and Ogan reported that oil palm sap collected in Nigeria, by tapping at the base of the inflourescene contained sucrose as the dominant sugar (10%, w/v) and glucose and fructose as minor sugar (< 1% w/v). The difference composition may because of the different species of and/or different cultivating condition.

Table 2 1: Free sugars contained in sap from felled oil palm trunk (Kosugi et al, 2010)

Free sugars	Arias		
	Center (A)	Middle (B)	Outer (C)
Arabinose	6.5	3.0	1.9
Galactose	0.9	0.8	1.0
Glucose	85.2	52.2	13.1
Xylose	0.7	0.8	1.4
Rhamnose	0.4	0.5	0.5
Fructose	4.1	3.1	2.1
Others	0.3	0.1	0.1
Total (g/L)	98	60.5	20

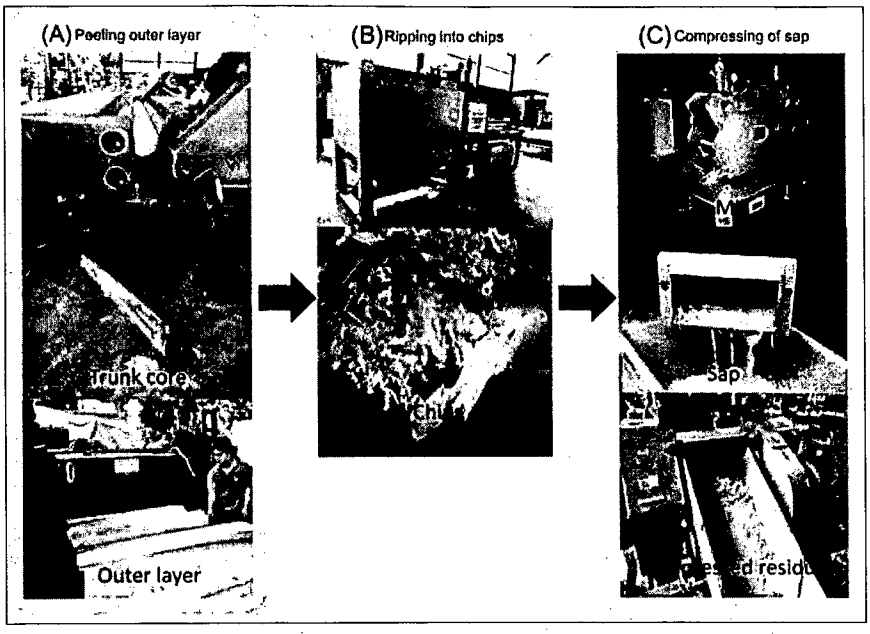


Figure 2 4: Flow chart of the compressing system for the palm trunks to get the sap Murata et al., 2012)

OPTS have a pH approximately 5.0 and specific gravity was 1.07. It was reported that the total amount of amino acids in the sap was 198.3 μg/ g, with serine, alanine, glutamic acid, and aspartic acid as the major amino acids. (Kosugi et al., 2010). The amino acid composition is similar as the sugar cane juice (Mee et al., 1979). As organic acid, OPTS contain abundant of citric, malic and maleic acid resulting the sap become acidic. Calcium, magnesium and chloride also contained in high concentration (Kosugi et al., 2010).

2.6 Application of PHB

The possible application of bacterial PHA is directly connected with their biological biodegradability, thermoplastic characteristic, piezoelelctric properties, and depolymerisation of PHB to monomeric D(-)-3-hydroxybutyric acid (Lafferty et al., 1988).

In medical and pharmaceutical, hydroxybutyric acid is a common intermediate metabolic compound in degradation of PHB so that, it was suitable, safe and biocompatible to be implanted to the animal tissue without toxic effect. PHA can be used as biodegradable carriers for long term dosage of drugs, medicine and hormones. They were used as the osteosynthetic materials in the simulation of bone growth owing

to their piezoelectric properties in bone plates, surgical sutures and blood vessel replace ments (Wang & Bakken, 1988).

In agricultural, PHA are biodegraded in soil. They can be used as biodegradble carriers for long term dosage of insecticides, herbacides, or fertilizers, seedling containers, biodegradable matrix for drugs release in veterinary medicine and tubing for crop irrigation. Then, it is not necessary to remove biodegradable items at the end of harvesting session (Lafferty, 1988).

As PHB have a tensile strength and flexibility properties, it is possible to used PHB for food packaging. (Lafferty, 1988). Initially PHA were used mainly in bags, containers and paper coatings. It also include the disposable items such as razors, utensils, diapers, feminine hygiene products and shampoo bottle (Oeding & Schlegel, 1973).

2.7 Advantages of PHB

PHB more commercially as biodegradable plastic material because its physical properties are mostly same as polypropylene (PP). PHB is biocompatible, non-toxic, low immunogenicity and suitable for medical application (Songsri et al., 2009). In addition, PHB also have similar degree of crystanility and melting point as PP. PHB however, more stiffer and brittle than PP and have better resistance to UV radiation but lower solvent resistance (Sinha et al., 2005).

2.8 Disadvantages of PHB

Thermoplastic PHB eventhough having biodgradable ability, however its very unstable and degrades at high temperature nears its melting point. and this cause PHB commercially limited. To solve this problem, researcher improve PHB by copolymer of PHB like poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with improved chemical (Liu et al., 2002). However, PHBV have limitations such as slow rate of crystallization, complex methodology and low elongation at break.

2.9 The Method of Factorial Experiments

Factorial design has been employed to determine the minimum number of experiments obtain an adequate model for the yield. 'k' variables at two levels (maximum and minimum) are used to form full two level factorial experiment or 2^k number of experiments. In this study 3 variables are tested, the minimum number of experiments ,N at 1 levels of each variable is 2^3=8. A linear regression equation for yields can be get from the results of factorial design (Venugopalan & Sathiyamoorthy, 2006).

2.10 The Method of Rotatable Composite Design by Quadratic Equation

Box and Wilson (1951) was first introduced this method and improved by Box and Hunter. This method used for fitting second order models. The total number of experiment required for this method are by using this formula, $2^k + 2k + n_o$ where k is the number of variables and n_o is replicate test at center. Recommended number of tests at center is for 3 variables. So the total number of test that required for this method are $2^3 + (2 \times 3) + 6 = 20$.

2.11 Summary

In conclusion, the production of PHB is possible by replacing the expensive carbon source to the renewable feedstock such as OPTS with the better strain of bacteria that can accumulate large amount of PHB.

3 MATERIALS AND METHODS

3.1 Background Of Study

This study was done to optimize the production of PHB in a 500mL shake flask with 200mL working volume using *Cupriavidus necator* as a microorganism and Oil Palm Trunk Sap (OPTS) as a feedstock. Then the process will be scale up to a 20L stirred tank fermenter. The variables were tested in order to get the optimum value to produce a maximum production of PHB. The variables that were tested are the agitation speed of incubator shaker (rpm), temperature (°C) and the volume percent of OPTS (%).

3.2 General Study of Procedure

The general step of this study are:

1. The Method of Factorial Experiment
2. Cultivation of Microbe
3. Inoculum development
4. Fermentation process
5. Cell Dry Weight Measurement
6. PHB Analysis
7. Scale up process

3.3 Material

3.3.1 Microorganism

The microorganism that used for this study is *Cupriavidus necator* CCUG 52238. This culture will be maintained on agar slant at 4°C and subcultures every 2 weeks to maintain their viability.

3.3.2 Chemicals

Chemicals are obtained mostly from Sigma Aldrich (peptone, glucose, yeast extract, nutrient broth, agar powder, Dipotassium hydrogen phosphate,K_2HPO_4, Potassium dihydrogen phosphate ,KH_2PO_4, Ammonium sulphate, $(NH_4)_2SO_4$ and Magnesium sulphate ,$MgSO_4$). Peptone, glucose, yeast extract, nutrient broth, agar powder are used to prepare agar medium and growth medium, while dipotassium hydrogen phosphate,K_2HPO_4, Potassium dihydrogen phosphate ,KH_2PO_4, Ammonium sulphate, $(NH_4)_2SO_4$ and Magnesium sulphate ,$MgSO_4$ are to prepare MSM medium.

3.3.3 OPTS

OPTS is a carbon source in the fermentation of PHB. OPTS was obtained from the Jerantut Estate, Pahang.

3.4 Mathematical Methodology

3.4.1 The Method of Factorial Experiment

The Method of Factorial Experiment was used to determine the effect when 3 variables (%Volume of OPTS, Temperature and Agitator speed) are changed run simultaneously from its lower level to its upper level. The equation that can be used are :

$$\text{Number of experiments} = 2^n$$

Where n = number of variables

So in this study, $2^3 = 8$. Then, the The Plan for the 2^3 Factorial Experiments table (Table 3.2) are constructed from the level of parameter in Table 3.1

Table 3 1 : Level Parameter of Experimental Design

Variables	$\alpha = -1$	$\alpha = 0$	$\alpha = +1$	Unit
Temperature	29.6	31.1	32.6	^0C
Agitation speed	108	138	168	RPM
Percent volume of Oil Palm Trunk Sap	28.8	33.8	38.8	%

Table 3 2: The Plan for the 2^3 Factorial Experiments

Number of experiment, n	Temperature, (°C)	Agitation Speed (RPM)	Percent Volume of OPTS (%)
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1

Table 3 3: The Plan of Replication at the Centre Point

Number of Experiment, n	Temperature (^{0}C)	Agitation speed (RPM)	Percent volume of OPTS(%)
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0
13	0	0	0

The Plan Replication at the Centre Point (**Table 3.3**) are used to require valid estimate of residual variance or also known as experimental error, this is to compare the experimental variable (Allen & Hopper, 1999).

3.4.2 The Method of Rotatable Composite Design by Quadratic Equation

The Method of Rotatable Composite Design gives the complimentary experimental points which can be tested to enable the area containing the maximum to be

approximated by a quadratic equation and the levels of the variables at the maximum point evaluated using matrix algebra.

Table 3 4: Level of Experimental for Rotatable Composite Design

Variable	α= -1.68	α= +1.68	Unit
Temperature	28.58	33.62	°C
Agitation Speed	87.6	188.4	RPM
Percent volume of OPTS	25.4	42.2	%

Table 3 5 : The Plan of 2^3 Rotatable Composite Design

Number of experiment, n	Temperature, (°C)	Agitation Speed (RPM)	Percent Volume of OPTS (%)
14	-1.68	0	0
15	1.68	0	0
16	0	-1.68	0
17	0	1.68	0
18	0	0	-1.68
19	0	0	1.68