INOCULUM DEVELOPMENT IN 2L STIRRED TANK FERMENTER FOR PRODUCTION OF PHB BIOPOLYMER IN A 20 L STIRRED TANK FERMENTATION ON A MEDIUM OF OIL PALM FROND JUICE (OPFJ)

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ABSTRACT

Polyhydroxybutyrate (PHB) is a biopolymer plastic as intracellular product of bacteria which can be degradable naturally. The purpose of this experiment is to scale up the production of PHB biopolymer by *Cupriavidus Necator* using oil palm frond juice (OPFJ) as carbon source. The process will involve fermentation process, the analysis on cell dry weight and concentration of PHB. The fermentation will be done in the 2 L stirred tank fermenter model Sartorius with the combination of 450 ml oil palm frond juice, 150 ml inoculum development 2, and 900 ml mineral salt medium (MSM) by manipulating the agitation speed and fixed the aeration rate at 1.5 L/min. Next, the cell dry weight and PHB will be analyse. From the result of DOT curve, 200 rpm in fermenter shows the same pattern as in 220 rpm in flask. 200 rpm is used in 2L fermentation. Highest PHB yield for fermentation in 2 L was 2.0650 g/L and highest cell dry weight was 5.7 g/L. While for cascade fermentation highest cell dry weight was 9.5 g/L and highest PHB content was 5.42 g/L.

PEMBANGUNAN INOKULUM DALAM TANGKI 2L FERMENTER UNTUK PENGELUARAN PHB BIOPOLIMER DALAM TANGKI 20L FERMENTER DENGAN MENGGUNAKAN JUS PELEPAH KELAPA SAWIT (JPKS)

ABSTRAK

Polyhydroxybutyrate (PHB) adalah plastic biopolymer sebagai produk intrasel bakteria yang boleh terurai secara semulajadi. Tujuan eksperimen ini adalah untuk skala pengeluaran PHB biopolymer oleh *Cupriavidus Necator* dengan menggunakan jus pelepah kelapa sawit sebagai sumber karbon. Proses ini akan melibatkan proses penapaian, analisis berat sel kering dan kepekatan PHB. Penapaian akan dilakukan di 2 liter fermenter model Sartorius dengan kombinasi 450 ml jus pelepah kelapa sawit, 150 ml pembangunan inokulum 2, dan 900 ml medium garam mineral (MSM) dengan memanipulasi kelajuan pergolakan dan menetapkan kadar pengudaraan pada 1.5 L / min. Seterusnya, berat sel kering dan PHB akan dianalisis. Dari hasil ujian yang dijalankan DOT, 200 rpm dalam fermenter menunjukkan corak yang sama seperti dalam 220 rpm kelalang. 200 rpm digunakan dalam penapaian 2L. Tertinggi hasil PHB adalah 2.0650 g / L dan berat sel kering tertinggi ialah 5.7 g / L. Manakala untuk penapaian lata (cascade), berat sel kering tertinggi ialah 9.5 g/L dan kandungan tertinggi PHB ialah 5.42 g/L.

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LIST OF SYMBOLS

KH_2PO_4	Monopotassium phosphate
K_2HPO_4	Potassium phosphate
$(NH_4)_2SO_4$	Ammonium sulfate
$MgSO_4.7H_2O$	Magnesium sulfate
$NiCl_2.6H_2O$	Nickel(II) chloride hexahydrate
$CuCl_2.2H_2O$	Copper(II) chloride
$ZnSO_4.7H_2O$	Zinc sulfate
$MnCl_2.4H_2O$	Manganese(II) Chloride Tetrahydrate
$Na_2MoO_4.2H_2O$	Sodium Molybdate (VI) dihydrate
$CuSO_4.5H_2O$	Copper (II) sulfatepentahydrate
$CrCl_3.6H_2O$	Chromium(III) Chloride Hexahydrate

LIST OF ABBREVIATIONS

PHB	Polyhydroxybutyrate
k_La	Mass transfer coefficient
PHA	Polyhydroxyalkanoates
OTR	Oxygen transfer rate
OPF	Oil palm frond
DOT	Dissolved oxygen tension
DO	Dissolved oxygen
RPM	Revolution per minute
°C	Degree Celsius
Min	Minute
Hr	Hour

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Nowadays with the advances in technology and the increase of global population, the plastic materials have found wide applications in every aspect of life and industries. However, most conventional plastics such as polyethylene, polypropylene, polystyrene, poly (vinyl chloride) and poly (ethylene terephthalate), are non-biodegradable, and their increasing accumulation in the environment has been a threat to the planet. To overcome all these problems, some steps have been undertaken. The first strategy involved production of plastics with high degree of degradability.

Modern world started utilizing plastics because of its cost and properties suitable for the usage in various areas. The plastics are obtained either from fossil fuels or from natural resources. The utilization of fossil fuels has increased tremendously in many forms driving the depletion of them as the fuels are non-renewable. The degradation of fuel derived plastics is also happening at very low

pace endangering the environment. The need of plastics which can overcome the environment problems is driving the search for bio based polymers which are biodegradable.

The problem concerning solid waste management and global environment have formed significant interest in the development of biodegradable plastic in recent times. The intrinsic qualities of durability and resistance to degradation over the last two decades have been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials (Poirier *et al.*, 1999). There is an urgent need to address the problem of improving productivity and yield of Poly-β-hydroxybutyrate (PHB) production through fermentation so that it can provide a viable alternative and economically compared to the production of conventional plastic material.

Poly-β-hydroxybutyrate (PHB) is the most prominent member of the family of PHAs, and it has many potential uses either as a homopolymer or as a copolymer, with polyhydroxyvalerate being a common component. Poly-β-hydroxybutyrate (PHB) is an intracellular storage compound, which provides a reserve of carbon and energy in several kinds of microorganism (Khosravi and Vasheghani, 2005). PHB, which is a biodegradable and biocompatible thermoplastic compound, has broadly similar physical properties to poly (propylene). It has many applications in medicine, veterinary practice, tissue engineering materials, food packaging and agriculture due to its biodegradability (Bucci *et al*., 2005; Chen and Wu, 2005).

1.2 Problem Statement

The PHB polymer is produce by fermentation process in 500mL shake flask (small scale) and 2L stirred tank fermenter (large scale). Usually, the productivity of the desired product is high in small scale, and will be gradually reduced as the scale is enlarged because of the complexity of fermentation process. This will affect the efficiency of industrial fermentation process.

During fermentation, another factor that is very important is the sterilization process. When in small scale, the effect of sterilization is minimized because of the shorter time exposure towards the high temperature and pressure. However, when in larger scale the exposure is longer so the productivity is reduced significantly. So, it is important to study scale up in fermentation process and adopt suitable strategy of scaling up in order to increase the productivity of the desired product on the industrial level.

1.3 Research Objective

1. To scale-up biopolymer (PHB) fermentation from 2L to 20L stirred tank fermenter.
2. To study the effect of dissolved oxygen to the production of PHB in 2L fermenter.

1.4 Scopes of Study

The scopes of this work are to scale up the production of biopolymer from 2L to 20L stirred tank fermenter. It involves the following step:

i. Obtaining a similar value of k_La (as in 500ml flask) in 2L stirred tank fermenter and determines the agitation speed.
ii. Fermentation in 2L stirred tank fermenter.
iii. Cascade fermentation in 2L stirred tank fermenter.
iv. Analysis of PHB content and cell dry weight.

1.5 Significance of Study

The problem concerning solid waste management and global environment have formed significant interest in the development of biodegradable plastic in recent times. There is an urgent need to address the problem of improving productivity and yield of Poly-β-hydroxybutyrate (PHB) production through fermentation so that it can provide a viable alternative and economically compared to the production of conventional plastic material. So it is important to study scale up in fermentation process and adopt suitable strategy of scaling up in order to increase the productivity of the desired product on the industrial level.

CHAPTER 2

LITERATURE REVIEW

2.1 What is PHB?

Polyhydroxybutyrate (PHB) is a biopolymer that can be used as a biodegradable thermoplastic material for waste management strategies and biocompatibility in the medical devices. PHB is a lipid-like polymer of 3-hydroxybutyrate, is a representative member of polyhydroxyalkanoates (PHAs) formed in many bacteria.PHB is produced intracellularly by microorganisms (like *Ralstonia Eutrophus* or *Bacillus Megaterium*) apparently in response to conditions of physiological stress. The polymer is primarily a product of carbon assimilation and is employed by micro-organisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. They accumulate as a carbon and energy reserves under unbalanced (unfavorable) growth conditions, such as nutrient limitation (Wang and Yu, 2000). PHB is the most common and best-characterized PHA stored by bacteria. Other frequently stored PHAs are polyhydroxyvalerate or polyhydroxyhexanoate. Their presence and relative

proportions depend on the type of carbon substrate used by the microorganism. Bacteria that have been shown to efficiently produce PHB include *Alcaligenes Eutrophus, Alcaligenes Latus, Azotobacter Vinelandii*, and recombinant *Escherichia Coli.*

2.2 Advantages of Poly-β-hydroxybutyrate (PHB)

The biopolymer is a biodegradable and biocompatible thermoplastic with an isotactic structure (Isotatactic refers to those polymers formed by branched monomers that have the characteristics of having the entire branch group on the same side of the polymeric chain), a high degree of crystallinity and a high melting temperature (about 1750C) (Madison and Huisman, 1999).

Production of organic polymeric materials is currently one of the principal areas of PHB is a thermoplastic material that has attracted much attention due to such properties as biocompatibility and biodegradability.

Microorganisms in nature are able to degrade PHA using their enzymes such as PHA hydrolase and PHA depolymerases (Jendrossek and Handrick, 2002; Choi *et al.,* 2004).The activities of these enzymes may vary and depend on the composition of the polymer and the environmental conditions. The degradation rate of a piece of PHB is typically in the order of a few months (in anaerobic sewage) to years (in seawater) *(*Madison and Huisman, 1999).Ultraviolet light can accelerate the degradation of PHAs (Shangguan *et al.*, 2006).

PHAs have been proved biocompatible, which means they have no toxic effects in living organisms (Volova *et al.,* 2003). Within mammals, the polymer is

hydrolysed only slowly. After a 6 months period of implantation in mice, the mass loss was less than 1.6% (w/w) (Pouton and Akhtar, 1996).

2.3 Disadvantages of PHB

There are drawbacks of using PHB as a plastic material such as its tendency to be brittle. When it was spun into fibers it behaves as a hard-elastic material (Antipov *et al.,* 2006). This problem could be solved by using by synthesis of copolymers of 3-hydroxybutyrate and other hydroalkanoates with a relatively low molecular weight and melting point (Fukui and Doi, 1997).

2.4 Application of PHB

PHB is more biodegradable and biocompatible making the product is more suitable for wider applications (Lee, 1996). The major obstacle to the wide acceptance of PHB in market is its high price, which is more than 10 times higher than price of synthetic one. It is estimated that the high production cost of PHB is mainly attribute to the carbonaceous raw material (> 45%) an recovery/purification process (>26%) (Steinbuchel and Funchtenbusch, 1998).

In addition to its potential as plastic material PHA is useful source of stereo regular compounds which can serve as chiral precursors for the chemical synthesis of optically active compounds, particularly in synthesis of some drugs or insect pheromones. For example can be readily hydrolysed to R-3-hydroxybutyric acid and

is used in the synthesis of Merck's anti-glaucoma drug truspot. Another opportunity of PHB is for food manufacturing and also in food service and packaging industry.

2.5 Introduction to Scaling Up

Scale up is the process whereby small scale production (several culture dishes) is transformed to a large scale production (a reactor of several liters). In other words, scale up is to perform an experiment in bulk, after the optimal conditions have been determined by a screening experiment. Both definitions referred to a process in which the data from an experimental scale operation is used in a larger scale unit for larger production.

The purpose of scaling up is to obtain the same product per volume in both small scale and large scale at the same time. The basis of constant mass transfer coefficient (k_La) of oxygen is used in order to scale up. During scale up, three major factors should be considered to eliminate problem that will arise which are inoculum's development, medium sterilization and aeration.

2.6 Factors affecting mass transfer coefficient (k_La) and oxygen transfer rate (OTR)

The value of the volumetric mass transfer coefficient k_La depends among other factors. First factor is medium viscosity which when viscosity increased, the k_La decrease. Second factor is degree of mixing. Increased mixing caused k_La to

increase. Third factor is velocity increase which reduces the k_La and vice versa. Finally, antifoaming agents decrease k_La substantially. The following methods are frequently used to increase the oxygen transfer rate (OTR). First is to increase the agitator speed and second is to increase the aeration rate.

2.7 Microorganism

The microorganism that is used to produce PHB in this study is *Cupriavidus Necator* CCUG52238. The reason for choosing this microorganism is because it had been found out that *Alcaligenes Eutrophus* also known as *C. Necator* is the primer PHB producer (Doi *et al.*, 1987).

Ralstonia Europha (formerly *Alcaligenes Eutrophus*) is the most extensively studied bacterium in both basic and applied research on the formation of PHAs. This species can accumulate PHAs up to 80% (wt.) of dry cell mass using various carbon sources including carbohydrates, alcohols and organic acids (Anderson and Dawes, 1990).

Alcaligenes Eutrophus can use inexpensive carbon sources, which is important in industrial scale production. The organisms show differences in their growth and polymer production conditions but they were chosen because of their high polymer production capacity. Another criterion for the selection is the ease of separation of the polymer from the cells.

2.8 Oil Palm

The average economic life-span of the oil palm is 25 years. A marked increase in the cultivation of oil palm began in 1960 (Kamaruddin *et al.*, 1991), so that the year 1990 onwards will see a peak in replanting. This will be a good opportunity to harness the ligno-cellulosic biomass or by-products of the oil palm, including the fronds. Oil palm fronds are available daily throughout the year when the palms are pruned during the harvesting of fresh fruit bunches for the production of oil.

Zahari (2012) reported that pressed juice from oil palm frond (OPF) contained renewable sugars such as glucose, sucrose and fructose. By using a simple sugarcane press, 50% (wt/wt) of OPF juice was obtained from fresh OPF. The glucose content in the juice was 53.95+/-2.86g/l, which accounts for 70% of the total free sugar. The cell dry mass in shake flask experiment reached 8.42g/l, with 32wt% of P(3HB) at 30% (v/v) of OPF juice, comparable with using technical grade sugars (Zahari *et al.*, 2012)

Oil palm frond (OPF) juice was used in this experiment as substrate. OPF juice was chosen because it is a good substrate for the production of PHB from *Cupriavidus Necator* (CCUG52238), with better yield of product formation in comparison to technical grade sugars (Zahari *et al.*, 2012). This is because the presence of minerals and nutrients in the OPF juice which are essential for bacterial growth during fermentation. Apart from contributing to higher product formation and microbial growth, the use of OPF juice is advantageous compared to the other lignocellulose based sugars due to the ease in its processing wherein no harsh

pretreatment steps and enzymatic treatment will be needed in order to obtain the sugars (Zahari *et al.*, 2012).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Research Methodology

3.1.1 Mathematical Method

3.1.1.1 Dissolved Oxygen Tension (DOT) Curve

Determination of the values of k_La for the gassing out technique, 2L stirred tank fermenter is used. The fermenter was filled with 1.5L of distilled water. Then, calibration of DO probe was done. Nitrogen was purged into the bioreactor to get zero DOT value. After get zero DOT value, the air was allowed to enter the bioreactor at 1.5L/min meanwhile, the agitator speed was set at 100, 150, 200, 250, and 300.

3.1.2 Experimental Method

The experimental method involve in 6 stages:

1. Medium preparation
2. Regeneration of bacteria
3. Inoculums development 1
4. Inoculums development 2
5. Fermentation in 2L fermenter
6. Cascade fermentation in 2 L fermenter

3.1.2.1 Medium Preparation

3.1.2.1.1 Nutrient Agar Medium

Nutrient agar medium was heated in a beaker with continuous stirring on laboratory hot plate to prevent the agar from hardening. The agar medium was autoclave for 20 minute at 121°C. After autoclave, while it still warm poured 15-20 mL into empty petri plate. The experiment was done in laminar air flow to avoid any contamination. Aseptic technique was applied during the experiment. The nutrient agar was allowed to harden for a few minute. In order to ensure the agar was hardened, the petri was flip upside down. The agar plate was stored in freezer in inverted position to avoid water drop on agar. The plate was sealed with parafilm. This agar plate can be stored for a long time as long as it is not contaminate.

Table 3.1 Nutrient agar medium

Chemicals	Amount (g/L)
Peptone	5
Glucose	10
Yeast extract	3
Agar	15
Nutrient broth	8
Aqueduct	Added until total volume= 1L

(Source: Zahari *et al.*, 2012)

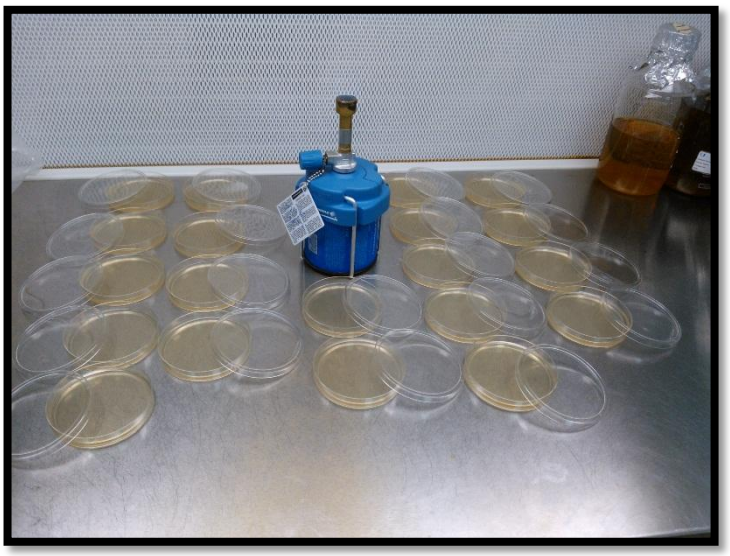

Figure 3.1 Agar plate