SCALE UP OF BIOPOLYMER (PHB) FERMENTATION FROM SHAKE FLASK TO 10L STIRRED TANK FERMENTER AND OPTIMIZATION OF LEVELS OF VARIABLES AT 10L

MOHD HISHAFI BIN HUSIN

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Universiti Malaysia Pahang

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I declare that this thesis entitled “Scale-up of Biopolymer (PHB) fermentation from shake flask to 10L Stirred Tank Fermenter and optimization of level of variable at 10L” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature	:……
Name	: Mohd Hishafi Bin Husin
Date	: April 2009

Special Dedication to my family members,
my friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

In this research is about the fermentation process for PHB production by *Cupriavidus necator* using glucose as a substrate. The different parameters of glucose concentration and agitation speed were studied. There are two stages in this research. For the first stage, the scale-up process was done from shake flask to 10L fermenter based on constant volumetric mass transfer coefficient K_La. The shake flask agitation speed of 200 rpm and temperature 30°C were used as the basis of scale up. The result gives the PHB yield for shake flask and 10L as 60.12 mg/L and 60.19 mg/L respectively. From these results, it can be concluded that the first stage had achieved its objective. For the second stage, optimization of variables was done by using glucose concentration; 10g/L and 30g/L and agitation speed; 200 rpm and 260 rpm as the manipulated variables. It was observed that the PHB yield is affected by initial glucose concentration of glucose used and also by the agitation speed.

ABSTRAK

Di dalam kajian ini proses fermentasi untuk menghasilkan PHB dengan menggunakan *Cupriavidus necator* dan glukosa digunakan sebagai substrak. Ia melibatkan dua parameter yang berbeza iaitu kepekatan glukosa dan juga kadar pengadukan. Kajian ini melibatkan dua tahap. Tahap yang pertama ialah proses diskala naik daripada kelalang kon ke fermenter tangki teraduk berisipadu 10L yang berdasarkan pemindahan isipadu (k_La) oksigen yang tetap. Kadar pengadukan kelalang kon adalah 200 (rpm) dan suhu 30°C digunakan sebagai nilai asas dalam proses skala naik. Keputusan kajian untuk tahap yang pertama, PHB yang terhasil untuk kelalang kon dan fermenter tangki teraduk 10L adalah masing-masing 60.12 mg/L dan 60.19 mg/L. Berdasarkan nilai ini, satu kesimpulan boleh dibuat iaitu tahap satu telah tercapai objektifnya. Pada tahap yang kedua pula ialah proses mengoptimumkan parameter iaitu kepekatan glukosa; 10g/L dan 30g/L dan kadar pengadukan; 200 (rpm) and 260 (rpm) masing-masing digunakan sebagai pemboleh ubah yang boleh diubah. Berdasarkan pemerhatian keputusan kajian ini, kepekatan awal glukosa dan juga kelajuan kadar pengadukan memberi kesan terhadap penghasilan PHB.

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LIST OF ABBREVIATIONS

A	-	Air flow rate
C*	-	Saturated dissolved oxygen concentration.
DCW	-	Dry cell weight
DO	-	Dissolved oxygen
DOT	-	Dissolved oxygen tension
ka_p	-	Oxygen transfer coefficient (probe)
k_La	-	Oxygen transfer coefficient
NGY	-	Nutrient Glucose Yeast
OTR	-	Oxygen transfer rate
OUR	-	Oxygen uptake rate
PHB	-	Poly ß hydroxyl butyrate
rpm	-	Rotation per minute
t	-	Time
YR(t)	-	The value of dissolved oxygen from calculation (theory)

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Development of (polyethylene) plastic is begun since 1936 by American, British, and German companies. Plastic is a cheap, flexible, durable, and chemically resistant is now a days has became a big problems to our environment. Even though people know about these problems, they still used it everyday and almost every product of food or drink encase in plastic. The wide use of plastic makes it an important environmental issue. Though it can be recycled, most of the commercial plastic ends up in landfills and in the oceans. Plastic is not considered biodegradable, as it takes several centuries until it is efficiently degraded. Biodegradation is the process by which organic substances are broken down by the enzymes produced by living organisms. To counter this problem biochemical researcher produces PHB polymer that is more environmental friendly compare to polyethylene.

Poly β-hydroxybuyrate (PBH) is an intracellular microbial thermoplastic that provide carbon and energy reserves in several microorganism. PHB polymer is produced by microorganisms such as *Alcaligenes eutrophus or Basillus megaterium* and *Cupriavidus necator* under unfavourable conditions (Lee, 1996). Polyhydroxybutyrate has attracted much commercial interest as a plastic. These polymers have similar

properties to some of petrochemical-derived thermoplastics such as polyethylene in term of molecular weight, melting point, stiffness, brittleness and glass transition temperature (Steinbuchel & Funchtenbush, 1998). PHB polymer is resistant to water and ultraviolet radiation and also impermeable to oxygen. Therefore, it is suitable to replace the polyethylene for food and drink packaging. Wider used of these polymer can reduce land pollution because it is readily biodegradable in soil.

1.2 Problem Statement

The PHB polymer is produce by fermentation process in 500mL shake flask (small scale) and 10L stirred tank fermenter (large scale). In fermentation process, the important factor that must be considered is sterilization process. Sterilization is a process that effectively kills or eliminates transmissible agents such as fungi, bacteria and spore forms at a surface of equipment and biological culture medium. This factor is important to avoid the contamination of the equipment and biological culture medium by another microorganism. If the contaminations occur, it will affect the efficiency of production of PHB polymer and therefore it should be sterile carefully.

1.3 Objective of Research Project

- To scale-up biopolymer (PHB) fermentation from 500ml shake flask to 10 L stirred tank fermenter.
- Optimization of variable for the fermentation process at 10L stirred tank fermenter based on glucose concentration and agitation speed.

1.4 Scopes of Research Work

The scopes of this work are to scale up the production of biopolymer from shake flask to 10L stirred tank fermenter and optimization of level of variables at 10L stirred tank fermenter. It involves the following step:

- Determining the volumetric transfer coefficient (k_La) of oxygen of biopolymer in 500 ml shake flask.
- Obtaining a similar value of k_La in 10L stirred tank fermenter.
- Fermentation in 500 ml shake flask
- Fermentation in 10 L stirred tank fermenter.
- Optimization of the variables agitation speed and initial glucose concentration in 10L stirred tank fermenter.

CHAPTER 2

LITERATURE REVIEW

2.1 History

Polyhydroxybutyrate (PHB) is a polymer belonging to the polyesters class that was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne. PHB is produced by microorganisms apparently in response to conditions of physiological stress. The polymer is a primarily product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA.

2.2 Poly β-hydroxybuyrate (PHB)

2.2.1 What is PHB?

PHB and its copolymers with polyhydroxyvalerate (PHV) are melt-processable semi-crystalline thermoplastics made by biological fermentation from renewable carbohydrate feed stocks. They have been described as "the first example of a true thermoplastic from biotechnology" and are also biodegradable. Although quite stable under everyday conditions they degrade slowly when composted or in landfill sites.

PHB homopolymer is a stiff and rather brittle polymer of high crystallinity, whose mechanical properties are not unlike those of polystyrene, though it is less brittle and more temperature resistant. Hence, copolymers are preferred for general purposes. It is believed that the most likely area for the application of homopolymer is in the medical/biological fields.

2.2.2 Properties of PHB

PHB is water insoluble and relatively resistant to hydrolytic degradation. This differentiates PHB from most other currently available biodegradable plastics, which are either water soluble or moisture sensitive. PHB shows good oxygen permeability and also has good ultra-violet resistance but has poor resistance to acids and bases. Other properties of PHB are:

- Soluble in chloroform and other chlorinated hydrocarbons.
- Biocompatible and hence is suitable for medical applications.
- PHB has melting point 1750C., and glass transition temperature 150C.
- PHB has tensile strength 40 MPa which is close to that of polypropylene.
- It sinks in water while polypropylene floats. But sinking of PHB facilitates its anaerobic biodegradation in sediments.
- PHB is nontoxic.

2.2.3 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 (approximately 80%), a high number average molecular weight (approximately 105 • }106) and a high melting temperature (about 1750C) *(Dawes, 1990; Scandola, 1995; 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).*

2.2.4 Disadvantages of Poly-β-hydroxybutyrate (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 *(De Koning, 1995; Scandola, 1995; Fukui and Doi, 1997).*

2.2.5 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 & Funchtenbusch, 1998). PHB are considered strong candidates as they have very similar properties to synthetic polymers, but degrade completely to water and carbon dioxide under aerobic conditions (Lee, 1996).

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.

2.2.6 Characteristic of *Cupriavidus necator*

Cupriavidus necator was described by Makkar & Casida (1987) to accommodate a non-obligate bacterial predator of various Gram-negative and Gram-positive soil bacteria and fungi (Byrd, 1985; Sillman & Casida, 1986; Zeph & Casida, 1986). This organism shared with members of the genus Alcaligenes, which, at that time, comprised multiple species, including *Alcaligenes faecalis* (the type species), *Alcaligenes xylosoxidans* and allied species (now all classified in the genus Achromobacter; Yabuuchi et al., 1998) and *Alcaligenes eutrophus* (first reclassified in the genus Ralstonia (Yabuuchi, 1995) and recently transferred again, to the novel genus Wautersia (Vaneechoutte, 2004)).

2.3 Optimization

An objective function which we want to minimize or maximize. For instance, in a manufacturing process, we might want to maximize the profit or minimize the cost. In fitting experimental data to a user-defined model, we might minimize the total deviation of observed data from predictions based on the model. In designing an automobile panel, we might want to maximize the strength.

A set of unknowns or variables which affect the value of the objective function. In the manufacturing problem, the variables might include the amounts of different resources used or the time spent on each activity. In fitting-the-data problem, the unknowns are the parameters that define the model. In the panel design problem, the variables used define the shape and dimensions of the panel.

A set of constraints that allow the unknowns to take on certain values but exclude others. For the manufacturing problem, it does not make sense to spend a negative amount of time on any activity, so we constrain all the "time" variables to be non-negative. In the panel design problem, we would probably want to limit the weight of the product and to constrain its shape.

2.4 Medium Sterilization

Sterilization refers to any process that effectively kills or eliminates transmissible agents such as fungi, bacteria, viruses and spore forms from a surface, equipment, or biological culture medium. Sterilization should be used for instruments, surgical gloves and other items that come in direct contact with the blood stream or normally sterile tissues (Spaulding 1939). It can be achieved by high-pressure steam (autoclave), dry heat (oven), chemical sterilants (glutaraldehydes or formaldehyde solutions) or physical agents (radiation). Because sterilization is a process, not a single event, all components must be carried out correctly for sterilization to occur. For this experiment, all the medium, instrument and fermenter was sterile using high-pressure steam (autoclave).

Autoclaves commonly use steam heated to 121 °C or 134 °C. To achieve sterility, a holding time of at least 15 minutes at 121 °C or 3 minutes at 134 °C is required. Additional sterilizing time is usually required for liquids and instruments packed in layers of cloth, as they may take longer to reach the required temperature. After sterilization, autoclaved liquids must be cooled slowly to avoid boiling over when the pressure is released. Proper autoclave treatment will inactivate all fungi, bacteria, viruses and also bacterial spores, which can be quite resistant and the probability for the contamination nearly zero percent.

CHAPTER 3

METHODOLOGY

3.1 Experimental Method

3.1.1 DOT curves

The dynamic gassing out method is use to measure the change in dissolved oxygen in the distilled water that is the value of k_La. To determine the value of k_La, 500ml shake flask with 200ml distilled water was used as a solution. The solution was sparged with nitrogen until the DOT value become zero and then sparged with oxygen tube. After that, the value of DOT was determined using oxygen probe. Then, the shake flask was shaking on the orbital shaker at 200rpm at room temperature and at the same time stopwatch was started and the values of DOT are taken until it become constant.

The steps above were repeated by using 10L fermenter with 8L of distilled water to get the DOT curve that is almost the same as in the shake flask just now. These were done by trials and errors on the air flow rate and rpm. The air flow rate and rpm that produced the DOT curve almost same with in the shake flask will be used in the fermentation in 10L fermenter later.