SCALE UP THE BIOPOLYMER (PHB) FERMENTATION FROM SHAKE FLASKS TO 10L STIRRED TANK FERMENTOR

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"I hereby declare that I have read this thesis and in my opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)"

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I declare that this thesis entitled “Scale up the Biopolymer (PHB) Fermentation from Shake Flasks to 10L Stirred Tank Fermentor” is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : …….....

Name : GOH MEI FONG

Date : ……

To my beloved parents, brother and sisters

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ABSTRACT

Plastics have been an integral part of our life. However, disposal of these non-biodegradable (petrochemical derived) plastics poses a threat to our environment. In an effort to overcome these shortcomings, biochemical researchers and engineers have long been seeking to develop biodegradable plastics that are made from renewable resources. Polyhydroxybutyrates (PHB) are polymers that accumulate as carbon and energy in *Cupriavidus necator* and provide an alternative to petrochemical plastic because of their biodegradability properties. However, major problems in commercializing PHB is the high production cost due to expensive carbon substrates and tedious production procedures using pure cultures. Therefore, the applications of mixed cultures and cheap carbon sources have been explored. In this study, the biopolymer fermentor has to scale up from shake flask to 10L of stirred tank fermentor. This is to increase the mass production of PHB that produced by *Cupriavidus necator*. The biopolymer fermentor is scaled up is by fixing the "k_La". k_La value were derived by fitting the mass transfer equation to the data of dissolved oxygen tension (DOT) versus time on computer using Matlab method with two unknowns, namely k_La and the electrode mass transfer coefficient (ka_p) of oxygen. The stirred speed (rpm) and the air flow rate (A) in the 10L fermentor that produced the value of k_La found in the optimized conditions in shake flask was approximated by trail an error. Overall, scale up by using the method of constant volumetric oxygen transfer coefficient (k_La) in the 10L stirred tank fermentor will produce the same PHB production as in the shake flask.

ABSTRAK

Plastik adalah sebahagian daripada keperluan hidup kita. Namun demikian, sifat plastik (petrokimia tradisional) yang tidak boleh terurai menyebabkan alam sekitar terancam. Dalam usaha untuk mengatasi masalah ini, pengkajian biokimia dan jurutera telah lama mencari jalan untuk memperkembangkan plastik boleh terurai yang diperbuat daripada sumber boleh diperbaharui. Polyhydroxybutyrates (PHB) ialah polimer yang terkumpul sebagai karbon dan tenaga dalam *Cupriavidus necator* dan memberi satu alternatif kepada petrokimia plastik kerana sifat bio terurainya.Walau bagaimanapun, masalah utama untuk menkormersialkan PHB ialah kos pengeluarannya yang tinggi merujuk kepada karbon substrat yang mahal dan prosedur penyediaan kultur tulen yang rumit Oleh itu, aplikasi untuk mencampur kultur dan sumber karbon murah telah diekplotasi. Dalam kajian ini, fermentasi biopolimer ini telah diskala naik daripada kelalang goncang ke fermenter tangki teraduk berisipadu 10L. Ini adalah untuk menaikan jisim pengeluaran PHB yang dihasilkan oleh *Cupriavidus necator*. Fermentasi biopolimer ini diskala naik dengan menetapkan ”k_La”. Nilai-nilai k_La diterbitkan dengan memadankan persamaan permindahan jisim kapada data tekanan oksigen terlarut (DOT) menentang masa dengan komputer. Kaedah Matlab dengan dua pembolehubah iaitu k_La dan pekali permindahan jisim elektrod (ka_p) bagi oksigen telah digunakan. Kadar pengadukan (rpm) dan kadar alir udara (A) dalam fermenter 10L yang menghasilkan nilai k_La yang ditemui di dalam kelalang goncang yang teroptimum telah dianggarkan dengan kaedah cuba-cuba. Pada keseluruhannya, skala naik dalam fermenter tangki teraduk berisipadu 10L dengan menggunakan kaedah pekali pemindahan isipadu (k_La) oksigen tetap akan menghasilkan pengeluran PHB yang sama seperti dalam kelalang goncang.

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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

Global Environmental Pollution has become a serious issue nowadays. Environmental pollution is a term that refers to all the ways that human activity harms the natural environment. The major types of environmental pollution include air pollution, water pollution, soil pollution, noise pollution and pollution caused by solid waste and hazardous waste. The relationships among all the living and nonliving things in an environment make up an ecological system, called an ecosystem. All the ecosystems of the Earth are connected. Thus, pollution that seems to affect only one part of the environment may also affect other parts.

Use of biodegradable polymers as a part of recycling can be offered as a sound argument and a partly solution for the plastic waste problem. In an effort to overcome these shortcomings, biochemical researchers and engineers have long been seeking to develop biodegradable plastics that are made from renewable resources. The term biodegradable means that a substance is able to be broken down into simpler substances by the activities of living organisms, and therefore is unlikely to persist in the environment. The requirements range from 90 per cent to 60 per cent decomposition of the product within 60 to 180 days of being placed in a standard composting environment. The reason traditional plastics are not biodegradable is because their long polymer molecules are too large and too tightly bonded together to be broken apart and assimilated by decomposer organisms.

1.2 Problem Statement

Our whole world seems to be wrapped in plastic. Almost every product we buy most of the food we eat and many of the liquids we drink come encased in plastic. Since the development of plastic earlier this century, it has become a popular material used in a wide variety of ways. The problem comes when we no longer want these items and how we dispose of them, particularly the throwaway plastic material used in wrapping or packaging. Plastics are used because they are easy and cheap to make and they can last a long time. Unfortunately these same useful qualities can make plastic a huge pollution problem. The cheapness means plastic gets discarded easily and its long life means it survives in the environment for long periods where it can do great harm. Because plastic does not decompose, and requires high energy ultra-violet light to break down, the amount of plastic waste in our oceans is steadily increasing. The plastic rubbish found on beaches near urban areas tends to originate from use on land, such as packaging material used to wrap around other goods. On remote rural beaches the rubbish tends to have come from ships, such as fishing equipment used in the fishing industry. This plastic can affect marine wildlife in two important ways: by entangling creatures, and by being eaten.

Clearly see that the problem of plastic pollution is serious and requires further urgent study. Immediate action is also required such as:

- Reduction of the amount of plastic used in packaging which is usually immediately thrown away.
- Re-use of plastics should be encouraged. Plastic wrapping and bags should carry a warning label stating the dangers of plastic pollution, and shoppers should be encouraged to use their own bags, or recycled paper bags.

1.3 Objective

To scale up the biopolymer (PHB) fermentation from 500ml shake flask to 10L stirred tank fermentor.

1.4 Scopes of Research Work

The aims of this work were to study the mass production of biopolymer in 10L stirred tank fermentor and overcome the problems of fermentation at different scales. In addition, the relations of aeration rate and k_La value are studied and then the PHB yields are compared under the similar k_La values of both scales. This can be achieved by the following specific objectives:

- To study the effect of aeration on: oxygen transfer rate (OTR), dissolved oxygen tension (% DOT), biopolymer yield and dry cells weight (DCW).
- To scale up the biopolymer by fixing the "k_La" from shake flask to the 10L stirred tank fermentor.

The elucidated conditions will be used for fermentation in 10L stirred tank fermentor furthermore. The effect of aeration on OTR values during fermentation will also be investigated.

CHAPTER 2

LITERATURE REVIEW

Current worldwide dependence on fossil fuels for plastics manufacture (270 million metric tones of fossil fuels), the scarcity of space for disposal and growing environmental concerns for non-biodegradable synthetic plastics have fuelled research towards development of eco-friendly biopolymer materials (Grengross and Slater, 2000, Thompson, 2001). Considerable emphasis has been laid on the development of five different types of biopolymers which include fiber-reinforced composites, starch based materials, and plant produced polymers, microbially produced polymers and biologically based resins, coatings and adhesives (Kolybaba, 2004). Of these, maximum attention has been laid on the development of microbially produced polymers, polyhydroxyalkanoates (PHA), which are linear aliphatic polyesters composed of 3-hydroxy fatty acid monomers and polylactic acid (PLA).

2.1 Types of Biodegradable Plastics

Biodegradable plastic can be produced by several types.

1 Starch based plastics

Starch based plastics are mainly harvested from wheat, potatoes, rice and corn. Of these four starches, corn is the most commonly used and is the least expensive starch. Starch is a natural polymer. It is a white, granular carbohydrate produced by plants during photosynthesis and it serves as the plant's energy store. Cereal plants and tubers normally contain starch in large proportions. Starch can be

processed directly into a bioplastic but, because it is soluble in water, articles made from starch will swell and deform when exposed to moisture, limiting its use. This problem can be overcome by modifying the starch into a different polymer. First, starch is harvested from corn, wheat or potatoes, and then microorganisms transform it into lactic acid, a monomer. Finally, the lactic acid is chemically treated to cause the molecules of lactic acid to link up into long chains or polymers, which bond together to form a plastic called polyactide (PLA) However, because PLA is significantly more expensive then conventional plastics it has failed to win widespread consumer acceptance.

2 Bacteria based plastics

Another way of making biodegradable polymers involves getting bacteria to produce granules of a plastic called polyhydroxyalkanoate (PHA) inside their cells. Bacteria are simply grown in culture, and the plastic is then harvested. Going one step further, scientists have taken genes from this kind of bacteria and stitched them into corn plants, which then manufacture the plastic in their own cells.

3 Soy based plastics

Soy based plastics use another alternative material used for biodegradable plastics. Soybeans are composed of protein with limited amounts of fat and oil. Protein levels in soybeans range from 40-55%. The high amount of protein means that they must be properly plasticized when being formed into plastic materials and films. The films produced are normally used for food coatings, but more recently, freestanding plastics (used for bottles) have been formed from the plasticized soybeans.

2.2 What is PHB?

Poly ß-hydroxybutyric acid (PHB) is an intracellular microbial thermoplastic that is widely produced by bacteria. In terms of molecular weight, brittleness, stiffness, melting point and glass transition temperature, the PHB is comparable to some of more common petrochemical derived thermoplastics, such as polypropylene. PHB produced in nature in the presence of excess carbon by bacteria as storage granules providing food, energy and reducing power (Pfeffer, 1992; Salehizadeh and Van Loosdrecht, 2004). 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). However, the production cost of PHB is nine times higher in comparison to synthetic plastics as it involves production of biomass with expensive carbon sources (Serafim, 2004). This has limited the use of PHB to specialized areas like surgery and medicine. Efforts on cost reduction have been directed towards increase in PHB content by developing better bacterial strains and efficient fermentation and recovery systems (Lee, 1996; Wang and Lee, 1997; Choi, 1998).

2.3 Characteristics 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)).

2.4 General Applications of PHB

PHB can directly replace some more traditional, nonbiodegradable polymers. Wider use of PHB, primarily as polymer blends, is expected. Such blends will greatly increase the spectrum of possible applications by expending the range of available physical properties. PHB, in combination with other biocompatible and nontoxic polymers, would also have an enhanced scope in biomedical applications. Because PHB is resistant to water and ultraviolet radiation and it is impermeable to oxygen, it is especially suited to use as food packing. PHB is readily biodegraded in soil. Moreover, it can be processed by using the same technology that is currently used in making polyethylene or polypropylene components. PHB has been used in surgical structures and other uses are in development. (Kim, Ondrey and Kamiya, beeting big on biopolymers, Chemical Engineering 1998, 105(7), 43-7)

2.5 Liquid and Mass Rate Transfer

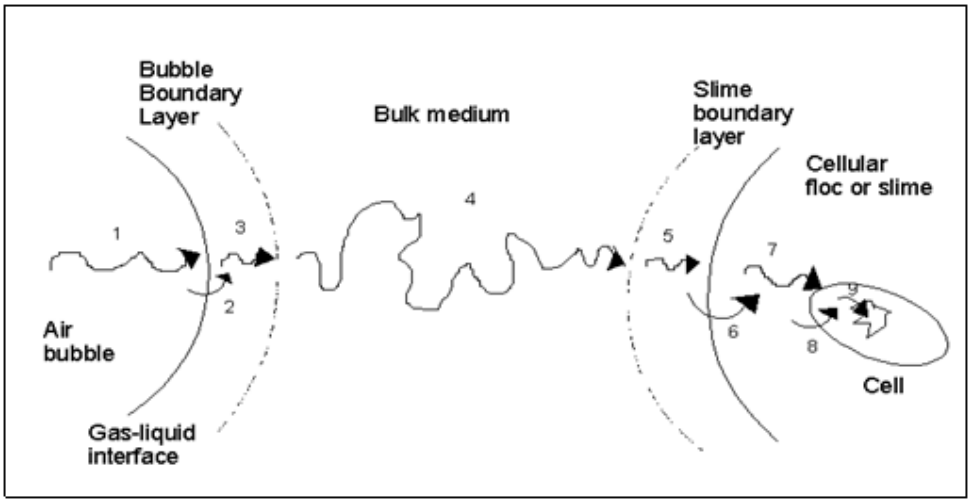

Figure 2.1 Oxygen moves from a bubble to an immobilized cell system

Figure 2.1 shown the oxygen mass transfer in a bioreactor. A more detailed understanding of the transport procedures of oxygen molecules from gas bubbles to bulk liquid to microorganisms is described by the “Two film model”.

- According to the two film model there are thin films on both sides of the gas/liquid boundary, which can be passed by diffusion only. A further film surrounds the microorganism.
- The way of an oxygen molecule is transferred from a gas bubble to a cell of a microorganism therefore is as follows:

  Firstly, the gas bubbles are crossing the gas film by diffusion. Then, it passes through the gas/liquid boundary into the liquid phase. After that is diffusion through the liquid film 1 (around the gas bubble). Next, it moves through the bulk liquid (fermentation broth). Finally, the gas bubbles are entering the liquid film 2 (around the microorganism) and cross the cell wall into the microorganism.

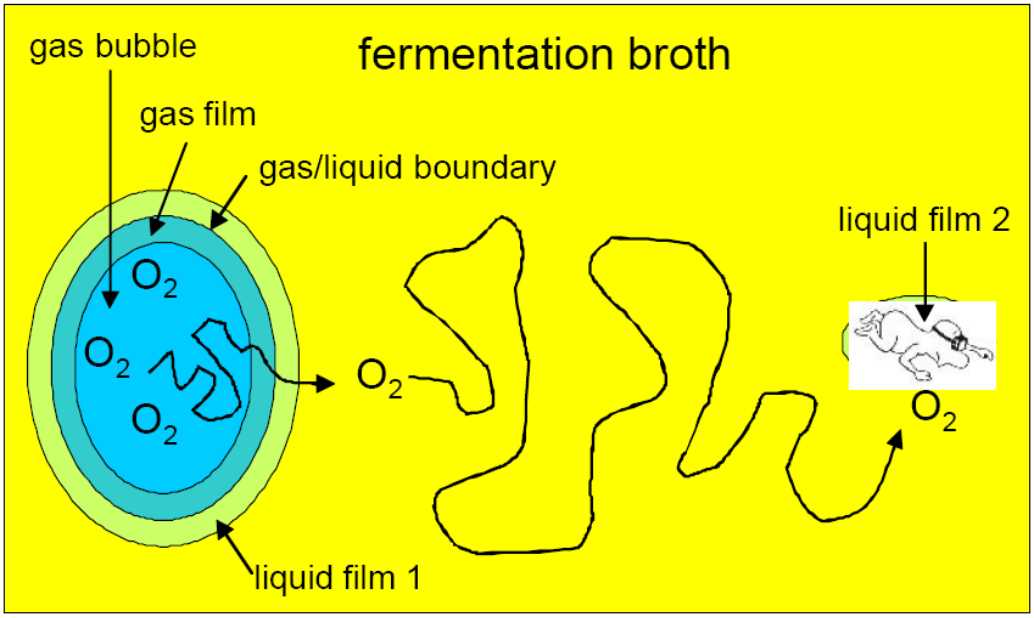

Figure 2.2 Two film model

2.6 Effect of Aeration and Agitation Rates on Oxygen Transfer Coefficient of (k_La) in Scale Up of Biopolymer.

Biopolymer synthesis generally occurs only when the microorganism is grown aerobically and usually under non-limited oxygen conditions, a polymer with higher molecular weight is produced (Sutherland, 1998). But the increased viscosity of broth formed a layer on cell surface and acts as a diffusion barrier, oxygen transfer to the cells becomes increasingly more difficult. The dissolved oxygen (DO) concentration becomes a limiting nutrient in processes of high oxygen demand (fast growing microorganisms, high biomass, and production of biopolymer) or when the rheological properties of broth offer a high resistance to the mass transfer, such as xanthan gum production (Casas, Santos, & Garcia-Ochoa, 2000; Lo, Hsu, Yang, & Min, 2001). The supply of oxygen (OTR) can be the controlling step in industrial bioprocesses, scale-up of aerobic biosynthesis systems (Al-Masry, 1999; Elibol & Ozer, 2000; Flores, Peres, & De La Torre, 1997; Gibbs & Seviour, 1996; Weuster-Botz, Hnnekes, & Hartbrich, 1998).

OTR is the most important parameter implied on the design and operation of aeration and agitation of bioreactors and in scale-up (Thiry & Cingolani, 2002; Wernersson & Tragardh, 1998). Efficiency of aeration depends on oxygen solubilization, diffusion rate into broths, and bioreactor capacity to satisfy the oxygen demand of microbial population. However, the DO in the broths is limited by its consumption rate on cells or the oxygen uptake rate (OUR), as well as by its OTR. The OTR could be affected by several factors, such as geometry and characteristics of the vessels, liquid properties (viscosity, superficial tension, etc.), the dissipated energy in the fluid, biocatalyst properties, concentration, and morphology of microorganisms.The OTR value depends on the air flow rate, the stirrer speed, mixing, etc. On the other hand, the OUR is limited by increase in viscosity resulting from polymeric property (Calik, Calik, & Ozdamar, 2000; Eickenbusch, Brunn, & Schumpe, 1995; Kobayashi, Okamoto, & Nishinari, 1994; Kwon, 1996).

Oxygen transfer can play an important role since it is often the limiting factor in order to obtain the appropriate volumetric oxygen transfer coefficient (k_La) that correlates with productivity in specific culture media (Montes, Catalan, & Galan, 1998; Tuffile & Pinho, 1970). There are many methods for k_La determination that have been reported by many authors and most k_La values are considerably affected by the geometry of the system. A dynamic biological method is widely used and involves physical oxygen absorption combined with oxygen consumption by a cell culture (Kouda, Yano, & Yoshinaga, 1997). The sulphite oxidation method is strongly discouraged and has come under severe criticism (Galaction, Cascaval, Oniscu, & Turnea, 2004). Because the reaction rate constant can vary in an unknown way, but be suitable in case of cell-free fermentation.

To reduce the complication of various variables and factors based on the theory of models and the principles of similarity, scaling-up for biopolymer production should be studied by consideration of the oxygen transfer parameters (Diaz & Acevedo, 1999; Nakayama, 1981; Winkler, 1983; Yuh-Lih & Wen-Teng, 2002). Fixing of kLa values has been commonly used criteria for scale-up of aerobic fermentations (Garcia-Ochoa, Gomez-Castro, & Santos, 2000; Gibbs & Seviour, 1996; Miura, 2003). The rationale of k_La values is to ensure a certain mass transfer capability that can cope with the oxygen demand of the culture and often serves to compare the efficiency of bioreactors and mixing devices as well as being an important scale-up factor.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Methods

3.1.1 DOT curves

The gassing out technique had been used to get the values of k_La and ka_p for the distilled water. The oxygen probe from 10L fermentor was dipped into the 500ml shake flask with 200ml distilled water. Then, nitrogen gas was bubbled into the distilled water until the DOT value become zero. Then, the shake flask was shaking on the orbital shaker at 200rpm at room temperature that is the optimum conditions for PHB. 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 fermentor 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 fermentor later.