SCALE UP OF BIOPOLYMER (PHB) FERMENTATION FROM 500 mL SHAKE FLASKS TO 2L STIRRED TANK FERMENTOR

AIMI SALMA BINTI AWALLUDIN

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

DECLARATION OF THESIS / UNDERGRADUATE PROJECT PAPER AND COPYRIGHT

Author's full name : Aimi Salma Binti Awalludin

Date of birth : 5th February 1986

Title : Scale up of biopolymer (PHB) fermentation from 500mL shake flasks to 2L stirred tank fermentor.

Academic Session : 2008/2009

I declare that this thesis is classified as :

☐	**CONFIDENTIAL**	(Contains confidential information under the Official Secret Act 1972)*
☐	**RESTRICTED**	(Contains restricted information as specified by the organization where research was done)*
√	**OPEN ACCESS**	I agree that my thesis to be published as online open access (full text)

I acknowledged that Universiti Malaysia Pahang reserves the right as follows:

1. The thesis is the property of Universiti Malaysia Pahang.
2. The Library of Universiti Malaysia Pahang has the right to make copies for the purpose of research only.
3. The Library has the right to make copies of the thesis for academic exchange.

Certified by :

SIGNATURE	**SIGNATURE OF SUPERVISOR**
860205–14–5378	PROF IR DR. JAILANI BIN SALIHON
(NEW IC NO. /~~PASSPORT NO~~.)	**NAME OF SUPERVISOR**
Date :	Date :

NOTES : * If the thesis is CONFIDENTAL or RESTRICTED, please attach with the letter from the organization with period and reasons for confidentiality or restriction.

"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)"

Signature : .....

Name of Supervisor : PROF IR DR. JAILANI BIN SALIHON

Date : ……

SCALE UP OF BIOPOLYMER (PHB) FERMENTATION FROM 500 mL SHAKE FLASKS TO 2L STIRRED TANK FERMENTOR

AIMI SALMA BINTI AWALLUDIN

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

APRIL 2009

I declare that this thesis entitled “Scale up of Biopolymer (PHB) Fermentation from 500 mL Shake Flasks to 2L 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 : AIMI SALMA BINTI AWALLUDIN
Date : ……

To my beloved parents, brothers and sisters

ACKNOWLEDGEMENT

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. I am deeply obliged to my supervisor Prof. Ir Dr Jailani Bin Salihon whose help, stimulating suggestions, and guided me for the direction of my thesis from the beginning.

A greatest appreciation and gratitude to those who helped me throughout this process, especially Miss Azrina Bt Mat Isa for all the guidance and motivation. Not to forget, Mr. Zainal Bin Giman, for his cooperation while handling the equipments.

I am grateful for all the lecturers of the Faculty of Chemical and Natural Resources Engineering in Universiti Malaysia Pahang (UMP) for always providing a helping hand especially Mr. Rozaimi Bin. Abu Samah.

Next, I would like to give my special thanks to Izwan Bin Abdul Rahman who's been very supportive during the ups and downs as I pursued my graduate degree. Also, I am very thankful to all my fellow friends especially Asyuaraa Sukmarsari Binti Aida Rimi, Nur Husna Binti Baharuddin and Muna Suhaili Binti Zakaria, who's always; accompany me during my data collection.

I am as ever, especially indebted to my family for their love and support throughout my life and to God, who made all things possible.

ABSTRACT

This study is about the scale up of biopolymer fermentation from 500 mL shake flasks to 2L fermentor. The purpose of this project is to determine air flow rate at 2L fermentor which gives the same k_La and ka_p as produced in shake flasks. The rationale of using the k_La value is to ensure a certain mass transfer capability in order to cope with the oxygen demand of the culture, thus being an important scale-up factor. Both values were derived by fitting the dissolved oxygen tension (DOT) versus time data into The Fibonacci Min Search (Fminsearch) method. Two values, which are 0.2960 for volumetric mass transfer coefficient for oxygen (k_La) and 0.0220 for the electrode mass transfer coefficient (ka_p) were obtained at small scale. These values are required to be duplicated in the larger scale. It was done by using a fixed agitation rate of 200 rpm and a manipulated aeration rate which is 1L/min,1.5L/min,2L/min and 1.75L/min. The most comparable k_La and ka_p values obtained from the trial are at 1.75L/min. This aeration rate will be used in the 2L fermentor in order to investigate the production of poly-β-hydroxybutyrate (PHB). Fermentation is run at both scales to compare the glucose, biomass and PHB profile. From the experiment and calculation, the maximum concentration of PHB is achieve at the 36^{th} hours, which is 1.415g/L for 500 mL shake flasks, and 2.17g/L for 2L fermentor. The cell dry mass obtained at the optimum harvesting time is 6.065 g/L for both scales.

ABSTRAK

Kajian ini adalah mengenai menskala naik fermentasi biopolimer dari 500 mL kelalang goncang ke 2L tangki teraduk. Tujuan utama projek ini adalah untuk mencari kadar aliran udara pada skala 2L tangki teraduk yang memberi nilai k_La dan ka_p yang sama sebagaimana terhasil dari kelalang goncang. Rasionalnya, pengunaan nilai k_La adalah untuk memastikan kemampuan pekali pemindahan jisim yang berupaya memenuhi keperluan oxygen kultur lantas menjadi faktor penting untuk menskala naik. Kedua-dua nilai dihasilkan dengan memasukkan data tekanan oksigen terlarut (DOT) menentang masa pada kaedah The Fibonacci Min Search (Fminsearch). Dua nilai iaitu 0.2960 untuk pekali pemindahan jisim bagi oksigen dan 0.0220 bagi pekali pemindahan jisim bagi elektrod terhasil pada skala kecil. Nilai-nilai ini adalah perlu untuk diduplikasi pada skala yang lebih besar. Ia dihasilkan dengan menggunakan kadar pengadukan(rpm) yang tetap iaitu 200 dan kadar aliran udara yang dimanipulasikan iaitu 1L/min,1.5L/min,2L/min and 1.75L/min. Nilai k_La dan ka_p terhampir didapati dari cubaan adalah pada kadar aliran udara 1.75L/min. Kadar aliran udara ini akan digunakan pada 2L tangki teraduk untuk mengkaji penghasilan poly-β-hydroxybutyrate (PHB). Fermentasi dilakukan pada kedua skala untuk membandingkan profil gula, jisim kering sel dan PHB. Menerusi eksperimen dan pengiraan, nilai tertinggi bagi kepekatan PHB didapati pada jam ke 36, adalah 1.415g/L untuk 500 mL kelalang goncang, dan 2.17g/L bagi 2L tangki teraduk. Berat kering sel terhasil pada masa tuaian optimum adalah 6.065 g/L pada kedua-dua skala.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

C*	-	Dissolved oxygen concentration.
DCW	-	Dry cell weight
DO	-	Dissolved oxygen
DOT	-	Dissolved oxygen tension
ka_p	-	Electrode mass transfer coefficient
k_La	-	Volumetric mass transfer coefficient for oxygen
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)

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

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.

On the lab scale, fermenting of PHB had brought about promises to the mass production of biodegradable plastic. However, there are still issues and obstacles that require research to be carried out before such becomes a reality. Badly needed are viable solutions to the production of biodegradable plastic for the use of mankind today.

1.2 Problem Statement

Environmental concerns are the biggest threat to the conventional plastics industry today. Among the issues are the releases of greenhouse gas, toxic pollutants, and non-biodegradable landfill impact. This is the result of the irresponsible disposal of petroleum and petroleum-based plastics.

Because of the environmental problem cause by polymer, numbers of research are done to find an alternative ways to reduce the use of conventional plastic. One of the approaches is by producing biodegradable plastic or biopolymer.

Simultaneously, several factors inhibit the large scale of biopolymer production and commercialization. These include the high cost of production in terms of media substrate raw material such as glucose, the extraction method, and the market price for PHB based plastic, are higher than polymer from petrochemical product.

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. In scale up process, besides the development of inoculums and medium sterilization, the aeration and agitation presence in culture are also some of the arising problem.

Ideally, oxygen transfer rate should be measured and the basis of constant volumetric transfer coefficient for oxygen (k_La) is used in order to scale up. Another parameter that also contributes towards the obtaining of the aeration rate needed, would be the electrode mass transfer coefficient (ka_p). In scale up process, both parameters should be in positive value. The problem arise when previous research dealing with this process obtaining the negative ka_p.

1.3 Objective

To scale up the biopolymer (PHB) fermentation from 500mL shake flask to 2L stirred tank fermentor.

1.4 Scopes of the Research Work

In this study, the scopes of research are focusing on several aspects which are:

1. To determine air flow rate at 2L fermentor which gives the same k_La and kap produce by shake flask with a positive value.
2. To scale up the fermentation at 2L and comparing the glucose, biomass and PHB profile.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Plastic Industry

2.1.1 Background of plastic

Plastic literally means "changeable" and it refers to any natural and synthetic materials that can be shaped when soft and then hardened. Today, the plastic industry is heavily integrated with the oil industry. In fact a popular view is that it would not be able to produce plastics if oil were not available. This is very different from the situation 40-50 years ago when the plastic industry was being described as a 'scavenger of raw material' (Brydson, 1999).

However, conventional plastic produced from the petroleum based sources are causing multitude problems and concerns because such products cannot easily degrade. It was cited that non-degradable plastics accumulate in the environment at a rate of more than 25 million tones per year (Lee, 1996). Newer biodegradable plastic production with the aid of microorganisms is thus urgently needed.

2.1.2 Development Biodegradable Polymer (Biopolymer)

Biopolymers are polymers that can be synthesized from living organism. Examples of input materials that can be used to produce biopolymer are starch, sugar, cellulose or other synthetic materials.

Biopolymers may be defined as products which are based on renewable agricultural or biomass feedstock, capable of behaving like conventional plastics in production and utilization, but degradable through microbial processes upon disposal. It is this progressive development of biopolymers which has led to a surging interest of a plastic and composite industry based on biological materials (Mohanty *et al*, 2003).

Some of the biodegradable plastic materials under development include poly-hydroxyl-alkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides, and copolymers and blends of starch and polypropylene (Lee *et al*, 1996).

2.1.3 Rational behind Biopolymer

Recently, a large scale production of Poly-β-hydroxybutyrate (PHB) by bacteria has become a subject of increasing interest. PHB is a useful biodegradable polymer which can be used as a thermoplastic (Byrom, 1987; Holmes, 1985; Doi 1990).

Biopolymers are possible alternatives to the traditional, non-biodegradable petrochemical derived polymers. In terms of molecular weight, brittleness, stiffness and glass transition temperature, the PHB homopolymer is comparable to some of the more common petrochemical-derived thermoplastics, such as polypropylene (Barham, 1990).

2.2 Poly-β-hydroxybutyrate (PHB)

2.2.1 Introduction

Poly-β-hydroxybutyrate (PHB) belongs to the class of biodegradable plastics PHAs. PHB was first among the family of PHAs to be detected by Lemoigne in 1926 as a constituent of bacterium *Bacillus megaterium* (Lemoigne, 1926). Approximately 150 different hydroxyalkanoic acids are at present known as constituents of these bacterial storage polyesters (Steinbüchel and Valentin 1995).

Polyhydroxyalkanoates (PHAs), a family of bacterial polyesters, are formed and accumulated by various bacterial species under unbalanced growth conditions. PHAs have thermomechanical properties similar to synthetic polymers such as polypropylene, but are truly biodegradable in the environment (Lee *et al*, 1996).

The molecular structure of PHB are describes in Figure 2.1. PHB act as an energy storage facility, and are developed when the bacteria's surroundings include excess carbon, and a deficiency of another nutrient.

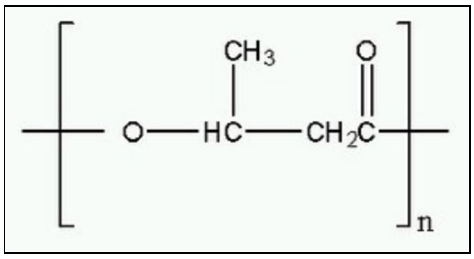


Figure 2.1: Structure of PHB

2.2.2 Synthesis route / Production of PHB

PHB are produced by many genera of bacteria as inclusion bodies to serve as carbon source and electron sink. PHB is synthesized from acetyl-CoA produce by the bacteria in sequential action of three enzymes. 3-ketothiolase (phbA gene) catalyses the formation of a carbon-carbon bond by condensation of two acetyl-CoA (Masamune *et al*, 1989)

NADPH dependent acetoacetyl-CoA reductase (phbB gene) catalyses the stereoselective reduction of acetoacetyl-CoA formed in the first reaction to R-3-hydroxybutyryl CoA. The third reaction of this pathway is catalyzed by the enzyme PHB synthase (phbC gene) that catalyzes the polymerization of R-3- hydroxybutyryl-CoA to form PHB. The EC number is yet to be assigned to PHA synthase (Steinbüchel and Schlegel 1991, Belova *et al*. 1997). Figure 2.2 showed the biosynthetic pathway of PHB from acetyl-CoA

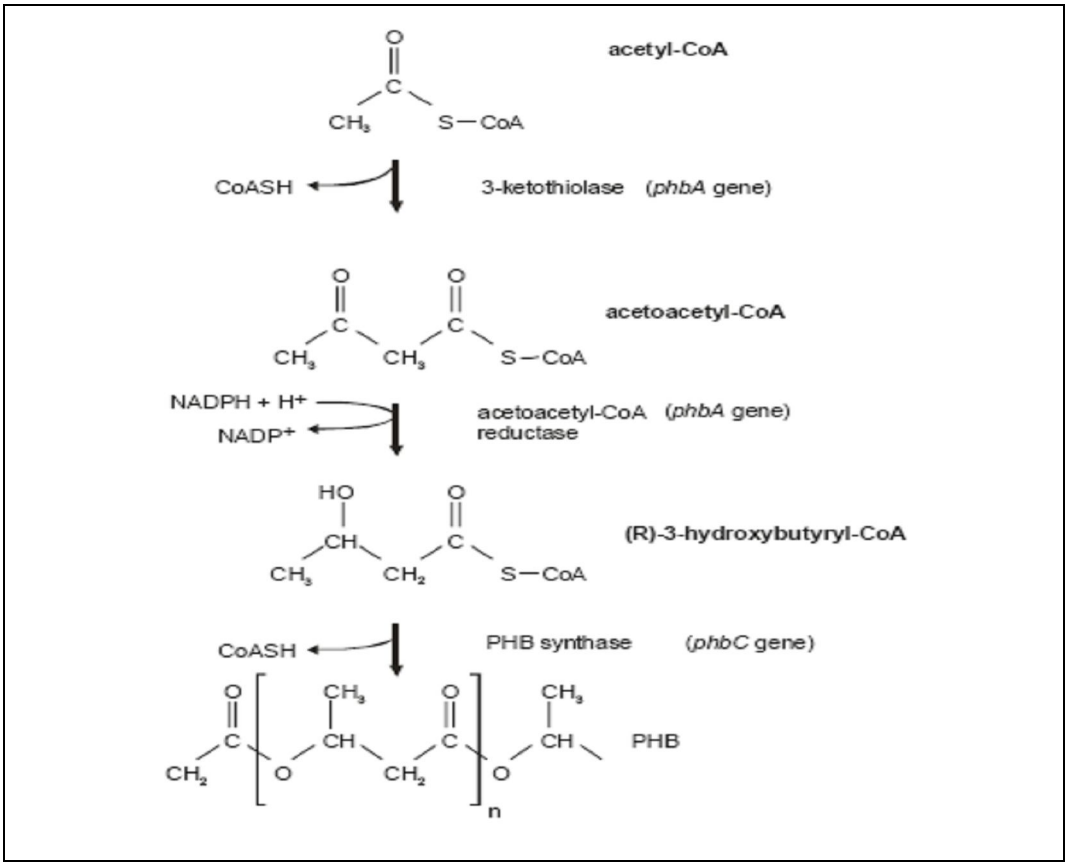


Figure 2.2: Biosynthetic pathway of PHB from acetyl-CoA (Taguchi et al.)

2.2.3 Application of PHB

Economic and technological barriers are the main concerns regarding large-scale microbial production of PHAs and poly-β-hydroxybutyrate (PHB). Byrom cited that large scale production of poly-β-hydroxybutyrate(PHB) by bacteria has become a subject of increasing interest (Byrom, 1897).

Applications focus in particular on packaging such as containers and films (Bucci and Tavares, 2005). It is also processes into toners for printing applications and adhesives for coating applications (Madison and Huisman, 1999*)*.

PHB could replace some of the more traditional, non biodegradable polymers. Polymer blends is expected to be more widely accepted. It is cited that such blends will greatly increase the spectrum of possible applications by expanding the range of available physical properties. PHB in combination with other biocompatible and nontoxic polymers would also have an enhanced scope in biomedical applications (Christi *et al*, 1999).

2.2.4 Advantages of PHB

The viability of microbial large-scale production of polyhydroxyalkanoates (PHAs) is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics. A shift emphasis in biomaterials engineering in recent years has moved the focus of attention from materials that will remain completely stable in the biological environment, to materials that will, in some way, alter their properties or biodegrade. poly-β-hydroxybutyrate (PHB) is polyester made by micro-organisms and is fully biodegradable.

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 sea water (Madison and Huisman, 1999). Yet, ultraviolet light can accelerate the degradation of PHAs (Shangguan *et al*, 2006).

The main advantage in the medical field is that PHB is a biodegradable plastic which can be inserted into the human body and does not have to be removed again. It is also biocompatible as it is a product of cell metabolism and also 3-hydroxybutyric acid, the product of degradation which is normally present in blood concentrations between 0.3 and 1.3 $mmoll^{-1}$ (Zinn *et al*, 2001).

2.2.5 Disadvantages of PHB

There are some of disadvantages of using PHB as a plastic material since its tendency to be brittle. Apart from brittleness, price is also another drawback. The high price of commercial grade PHB- about 15-fold greater than comparable synthetic plastic limits its use to specialist niches. For example, Biopol, a copolymer of β-hydroxybutyric acid and of β-hydroxyvakeric acid produced by Ralstonia eutropha, sell about 17 times the price of synthetic plastic(Braunegg *et al*, 1998).

2.3 Fermentation of BioPolymer

2.3.1 Introduction to Fermentation

In its broadest sense, fermentation refers to any process by which large organic molecules are broken down to simpler molecules as the result of the action of microorganisms. The most familiar type of fermentation is the process by which sugars and starches are converted to alcohol by enzymes in yeasts. Normally, the

fermentor volume is usually filled to only 70–80% of its total capacity, to leave head space above the fermentation broth (Bailey and Ollis, 1986).

Fermentation is the use of microorganisms to break down organic substances in the absence of oxygen. Today, fermentation can be carried out with genetically engineered microorganisms, specially designed for the conditions under which fermentation takes place, and for the specific substance that is being broken down by the microorganisms. In this study, fermentation is the process used to produce PHB.

2.3.2 Microorganism

For fermentation to take place, the microorganism that is used to produce PHB in this study is *Cupriavidus necator* (also known as *Ralstonia eutropha* or *Alcaligenes eutrophus*). The reason for choosing this microorganism is because it had been found out that *Alcaligenes eutrophus* is the prime 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.

2.4 Scale up of Biopolymer Fermentation

2.4.1 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 volumetric 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.

2.4.2 Important parameter in scale up processes

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) The supply of oxygen (OTR) can be the controlling step in industrial bioprocesses, scale-up of aerobic biosynthesis systems (Al-Masry, 1999, Elibol and Ozer, 2000).

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 (Çalik *et al.*, 2000). 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 *et al.*, 1998, Tuffile and Pinho, 1970).

Fixing of k_La values has been commonly used criteria for scale-up of aerobic fermentations (Garćia-Ochoa *et al*., 2000, Gibbs and Seviour, 1996 and Miura *et al*., 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

METHODOLOGY

3.1 Mathematical Method

3.1.1 Dissolved Oxygen Tension (DOT) Curve

Determination of the values of k_La and ka_p for the distilled water involved the gassing out technique. 2L fermentor oxygen probe are dipped into the 500ml conical flask filled with 200ml distilled water. In the intention of achieving the zero value of DOT, the nitrogen was bubbled into the distilled water. Next, the shake flask is placed on the orbital shaker at 200rpm at room temperature which will be the optimum conditions for PHB. At once, the values of DOT are taken at every one minute until it become constant. A DOT versus time curve are developed from the data obtained. Steps are repeated using 2L fermentor in obtaining identical percent of DOT. It is achieved by trials and errors by varying the air flow rate while maintaining the same rotational speed which is 200rpm. The air flow rate obtained which gives the similar value of k_La and ka_p will be use at larger scale.

3.1.2 k_La and ka_p determination

Determination of two unknown, namely volumetric liquid mass transfer coefficient for oxygen (k_La) and the electrode mass transfer coefficient (ka_p) are obtained by using MATLAB software. The Fibonacci Min Search (Fminsearch) method was selected to obtain both values k_La and ka_p. The values are derived by fitting the mass transfer equation with the data of dissolved oxygen tension (DOT) versus time. It involves the equation of;

$$YR(t) = C^*[((ka_p.\exp(-k_La.t)/(ka_p - k_La))- (k_La.\exp(-ka_p.t)/(k_La - k_{ap}))]$$

*taken from scaling up fermentation process based on constant volumetric transfer coefficient (k_La) of oxygen by Ahmad Jaril Asis, Zulaikha Paidi, Michael A. Winkler and Jailani Salihon.

The equation are applied in Matlab software (Fminsearch) that will give the results of two positive variables involved which are k_La and ka_p.

Strictly, the value of C* should have been in concentration terms, and the values of YR(t) should also have been in concentration terms. In this thesis, the values of YR(t) are left as DOT values, while the value of C* is set at 100, which is the DOT at maximum concentration.

3.2 Experimental Method

In this experimental method, the procedures are divided into 3 stages: which are;

1. Regeneration of the bacteria
2. Inoculums development
3. Fermentation in 2L fermentor

3.2.1 Regeneration of the bacteria

Regeneration of the bacteria is done every two weeks. The culture is maintained at slanting agar which are prepared with the following composition;

Table 3.1: NGY agar medium composition

Chemicals	Amount (g/L)
Peptone	5
Glucose	10
Yeast extract	3
Beef extract	0.3
Agar	30
Aqueduct	Added until total volume= 1L

The solutions are heated and on the same time are stirred on laboratory hot plate until its boil. About 10 ml of the hot agar solution are poured into each universal bottle. The bottles are sealed with aluminum foil for sterilization process. The process is made in autoclave for 30 minutes at 121ºC. The bottles are placed in incline position so that the agar will set with inclined surface in the tubes and placed in sterile incubator for one night. Next, the bacteria are transferred from the old slant to the new slant in sterile laminar air flow hood with the following procedure:

i. Firstly, the metal loop is heated until burning red and placed in a beaker for cooling purpose.
ii. The old slant containing bacteria are opened.
iii. Then, one loop full of bacteria is scrapped and quickly transferred it to the new slant by slightly scratch the agar surface.

The slants are placed in the sterile incubator at room temperature for about 24 hours until the bacteria seem to grow. Then it is kept in the refrigerator at 4ºC for long time maintenance.

3.2.2 Inoculum Development

The development of inoculums consists of two parts which are starter 1 and starter 2.

Starter 1:

The medium for NGY broth are prepared with the following composition;

Table 3.2: NGY broth

Chemicals	Amount (g/L)
Peptone	5
Glucose	10
Yeast extract	3
Beef extract	0.3
Aqueduct (water)	Added until total volume= 1L

Firstly, 10 ml of NGY broth are filled in 100 ml conical flask and the flasks are closed with sterile cotton. It is placed in autoclave for 30 minutes at 121ºC for sterilization process. Next, it is let to stand in sterile incubator for 24 hours at room temperature. One loop of the bacteria is scrapped from the slant and put it into the incubated medium. Transfer should be conducted in sterile laminar air flow hood. Then, it is incubated for 24 hours before moving the content for further development.

Starter 2:

Table 3.3: Mineral Salts Medium (Ramsay Medium)

Composition	g/L
Glucose	20.0
Peptone	5.0
$(NH_4)_2.SO_4$	2.0
Na_2HPO_4	6.7
KH_2PO_4	1.5
$MgSO_4$	0.2
Aqueduct	To make total volume 1L

It starts with the addition of 90 ml of Ramsay medium in 500 ml conical flask and the inlet are plugged with sterile cotton. It is sterilized in autoclave for 30

minutes at 121ºC. After sterilization, the flask are let to stand for 24 hours in sterilize incubator. Lastly, starter 1 was poured into the flask and placed in incubator shaker for 24 hours.

3.2.3 Fermentation in 2L fermentor

Initially, the bioreactor is cleaned thoroughly before using. Then, 1440 ml of Ramsay medium are filled into the bioreactor and sterilize it for 30 minutes at 121˚C. Preparation of 0.5 M NaOH and 0.5 M HCl are made for pH control. Si solution is prepared for antifoaming (dilute 50x from the original solution). The bioreactor are turned on and supplied with 1.75 L/min aeration rate for compressed air. Starter 2 is flow into the bioreactor through the automatic input. The bioreactor is run and samples are withdrawn through the sampling line every 6 hours.

3.3 Method of analysis

3.3.1 Glucose analysis

Glucose analysis is performed by using Dinitrosalycilic method (DNS method). Firstly,1 ml of supernatant and 1 ml DNS reagent are mix evenly. A blank are also prepared by mixing of 1ml medium without glucose and 1 ml of DNS reagent. The mixtures are soaked in water bath for 10 minutes at 100^{o}C. The samples are cooled under running water. Then the samples including the blank are being diluted with deionized water and evenly mixed. Finally, the absorbance obtained by using uv-vis at 540nm.

3.3.1 Cell Dry Mass

Firstly, 10mL of culture sample are centrifuged at 6000rpm, 10 minutes, and 4ºC. After centrifugation, the supernatant and the pellets are separated. The supernatant is used for glucose analysis. The pellets are washed and recovered using DI water. Then the samples including are evenly mixed using vortex. Finally, the absorbance obtained by using uv-vis at 600nm.

3.3.2 PHB harvesting

Evaluation of PHB content is done by gravimetric. Firstly, 20 mL of Sodium dodecyl sulphate solution (1% w/v, pH 10) are added into the biomass pellet that are obtained from the centrifugation process as describe in cell dry mass measurement. It is then incubated at 200 rpm, 37°C for 60 minutes. Next, further lysis of the cell is recovered from the centrifugation process using sodium hypochlorite solution (5.64% w/v) that has been diluted using 20 mL. Centrifuge the pellet at 6000 rpm for 4 minutes at 25^{0}C. Wash it with 20 mL deionized water to separate cell and PHB. Vortex the remaining pellet and pour it into a aluminum dishes. Dry the pellet that already obtained from the centrifugation process at 90°C for 24 hours to constant weight.

CHAPTER 4

RESULTS AND DISCUSSIONS

In this project, there are two major parts that are being considered. Firstly, is the determination of k_La and ka_p values and followed by the fermentation process itself. Knowing that there are challenges in scaling up, certain parameters are investigated. This includes the aeration and agitation used during the fermentation process. The volumetric liquid mass transfer coefficient (k_La) is a useful parameter to characterise bioreactors capacity for aeration. Simultaneously, the electrode mass transfer coefficient ($ka_{p)}$ value enabled the mass transfer coefficients to be determined accurately over the whole surface of the electrode under the conditions of gas sparging. Both values obtained will help the reactor design, optimisation of technologies and scaling up or scaling down processes.

4.1 Determination of k_La and ka_p values.

The determination of the k_La and ka_p values is essential in order to create the same aeration efficiency at both scales. Furthermore, it is also to quantify the effects of operating variable on the oxygen provided. Ideally, oxygen transfer rates should be measured in biological reactors which include the nutrient broth and cell population of interest. As this process requires all the account for inoculums and medium preparation, environmental control for the cell culture, and prevention of contamination, it is inconvenient to conduct mass transfer experiments. However, finding these values in this experiment, using distilled water is an alternative to replace the fermentation medium. This is because; since water is the major component of the fermentation media and it is also cost effective. Initially, the flasks are placed on an orbital shaker and agitation and aeration are fixed at certain value to produce the k_La. At 500 mL shake flasks, 200 rotations per minute for agitation speed and 1 L/min air flow rate for aeration were being used. Readings were gathered and a 'Dissolved Oxygen Tension Versus Time Graph' (Figure 4.1) was plotted. k_La and ka_p values were derived by fitting the mass transfer equation to the data of dissolved oxygen tension (DOT) versus time using Fmin search method from Matlab. Command window for k_La determination in 500 mL shake flasks will be shown in Appendix B. Two values, which are 0.2960 for liquid mass transfer coefficient (k_La) and 0.0220 for the electrode mass transfer coefficient (ka_p) were obtained. These values are required to be duplicated in the large scale.

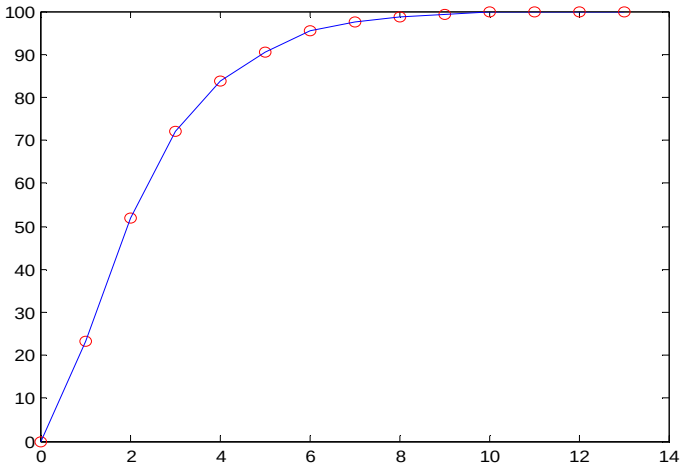


Figure 4. 1: DOT versus time curve for 500 ml shake flask

4.2 DOT versus Time Curves

The values of k_La and ka_p obtained from the small scale are used as a basis to find the same or most similar values in 2L fermentor. It is done by trial and error on the fixed agitation speed and a manipulated value of compressed air flow rates which are 1L/min for first trial, 1.5L/min for second trial, 2L/min for third trial and 1.75 L/min for fourth trial. After the entire trials (Figure 4.2), the third and fourth trial produced nearly the same pattern as in the 500mL shake flask above. In all the trials, the agitation rate cannot be set too much higher because the shear rate can cause damage to the cells growing inside the 2L fermentor. Thus; we remain the same values as in 500mL shake flasks and the compressed air flow rate needs to adjust to achieve the same DOT as in the 500mL shake flask. Looking at the trends of a DOT versus time curves, the values will increase dramatically to a maximum level and then keep constant afterwards.

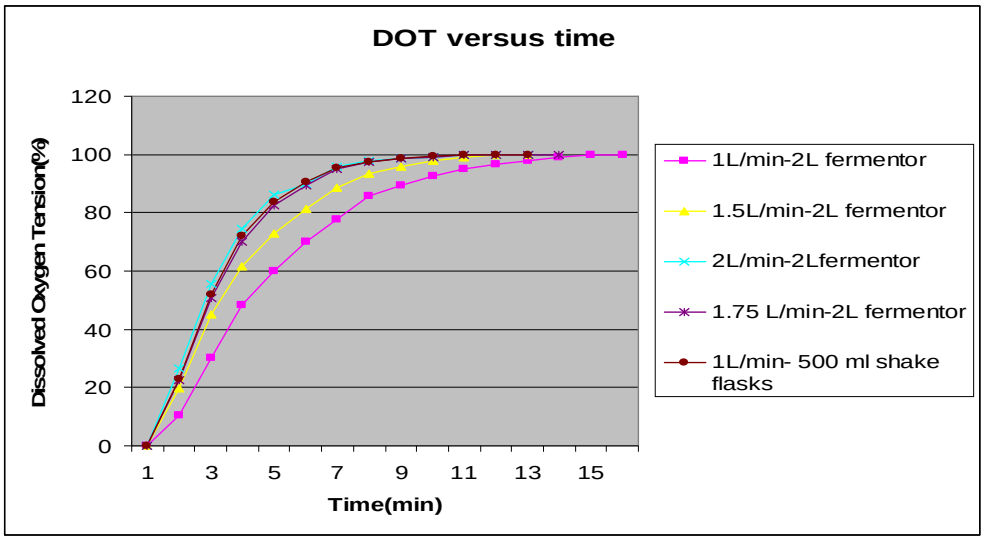


Figure 4. 2: Comparison for DOT curves

4.3 MATLAB Fitted Curves for 500ml shake flask and 2L fermentor.

All the trials data gathered from the k_La determination are fitted in the MATLAB software. The Fibonacci Min Search method was selected to calculate both k_La and ka_p values. With the mass transfer equation

$$YR(t) = C^*\{[(ka_p.\exp(-k_La.t))/(ka_p - k_La)] – [(k_La.\exp(-ka_p.t))/(k_La - ka_p)]\}$$

Equation was taken from Ahmad Jaril Asis, Zulaikha Paidi, Michael A. Winkler and Jailani Salihon from Jurnal Kejuruteraan 2 (1990) 179-195.). As stated before, the use of YR(t) and C* should have been in concentration terms. However, percentage data obtained from the DOT versus time graph are used to replaced the value of dissolved oxygen concentration (YR.(t)) and 100% are used for C* which will be the saturated dissolved oxygen concentration.

From the DOT and time data, the k_La and ka_p values for 500ml shake flask were 0.2960 min^{-1} and 0.0220 min^{-1}. However, referring to the Table 4.1, the closest values for 2L fermentor were 0.2763 min^{-1} and 0.0248 min^{-1} obtained from the fourth trial. The 500ml shake flask fitted curves and the 2L fermentor fitted curves shown the comparisons between the experimental curve and the MATLAB fixed curve. For the k_La, the error is 6.94%, however for the ka_p, the error is 12.72%

Table 4.1: Values of volumetric mass transfer coeefficient(k_La) and electrode mass transfer coefficient(ka_p)

2L fermentor trials	k_La	Percentage of error (%)	ka_p	Percentage of error (%)
Trial 1	0.1514	49.01	0.0225	2.27
Trial 2	0.1970	33.65	0.0348	58.18
Trial 3	0.3300	11.15	0.0193	12.27
Trial 4	0.2763	6.94	0.0248	12.72

4.3 Glucose Analysis

The rate at which the carbon source is metabolized can often influence the growth of biomass and the production of primary metabolites which will be the PHB. In this experiment, DNS method is used as a method to analyse glucose uptake from *Cupriavidus necator*. First of all, a glucose concentration standard curve are prepared and the straight line with equation of $y = 1.1361x - 0.0264$ was obtained. y represented the optical density of the glucose samples taken for every six hours; while x represented the remaining glucose concentration inside the medium. The glucose concentration was then multiplied by dilution factor depending on the dilution made.

Referring to Figure 4.3, the glucose concentrations were declined from the beginning sixth hours until the 72^{nd} hours for both scale. For the 500ml shake flask fermentation, the glucose concentration declined slightly from sixth to 30^{th} hours, but showing a swift declined from 30^{th} until 72^{nd} hours. Conversely for the 2L bioreactor fermentation, the glucose concentration declined greatly from the initial to first 6 hours, but then declined slightly from 6^{th} to 72^{nd} hours. Looking at the trend of the curves, for small scale fermentation, glucose is utilized almost completely by the microorganisms. Differ in 2L fermentor, at the end of the process, glucose is still present and the most uptake of glucose in the fermentation process is at the beginning. It is important to make sure that sufficient amount of glucose for the growth of bacteria is supplied at both scales.

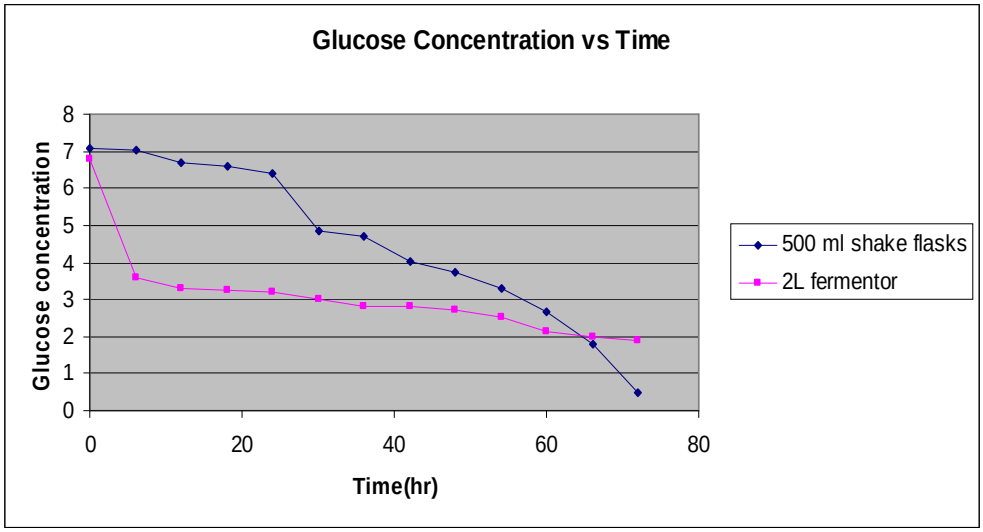


Figure 4. 3: Comparison of graph for glucose analysis

4.4 Dry Cell Weight Analysis

Dry cell weight analysis was done to observe the growth profile of the bacteria. The net weight of dry cells produced was in a sample of 10ml.The net weights were multiplied then by conversion factor to get the dry cell weight in gram per 1L. Based on figure 4.4, dry cells weights were increased from the beginning until the maximum of 48th hours for both 500ml shake flasks and 2L bioreactor fermentations. Then, it was slightly decreased for 2L fermentor but showed a swift decline for 500mL shake flasks.

Comparing both graph produced, it shows that 500ml shake flasks growth is slower at the beginning of the fermentation process. This is because; the culture may become oxygen limited because sufficient oxygen cannot be made inside the flasks. This situation hold opposing views for 2L fermentor since it contain a better aeration system thus shortened the lag phase.

Numerous factor affecting culture performance including concentration of substrates, pH, temperature, carbon-to-nitrogen ratio, agitation and aeration rate (Grothe, 1999). It this projects, it shows that nitrogen sources tend to slow the growth. Microorganisms utilize di-ammonium sulphate as the inorganic nitrogen sources. This salt will usually produce acid condition as the ammonium ion is utilized and the free acid is liberated (Morton and MacMillan, 1954). The optimum pH for the fermentation is at 7.0. The growth inside 500 mL shake flasks start to undergo death phase since in the small scale, there are no pH control

The maximum dry cell weight for the 500ml shake flask fermentation is 7.6704g/L at 48th hours. However, 6.5951g/L at 48th hours are obtained at 2L fermentor. Comparing the dry cell weight and PHB profile, the highest concentration produced for dry weight cell do not guarantee the maximum production of the PHB. This is due to the restriction whereby the production of PHB itself is a response to the limitation of an essential nutrient which occurred to be nitrogen.

Below is a calculation for biomass yield coefficient on glucose for both scales;

The highest biomass yield coefficient on glucose for 500mL shake flask

$Y_{(x/s)}$ = (dry cell produced / unit mass of glucose consumed)

= (7.670424 g cells / 1L medium) / (20-3.751078g glucose / 1 L medium)

= 0.4721 g cells / g glucose

The highest biomass yield coefficient on glucose for 2L fermentor

$Y_{(x/s)}$ = (dry cell produced / unit mass of glucose consumed)

= (6.595098g cells / 1L medium) / (20-2.709269g glucose / 1L medium)

= 0.3811 g cells / g glucose

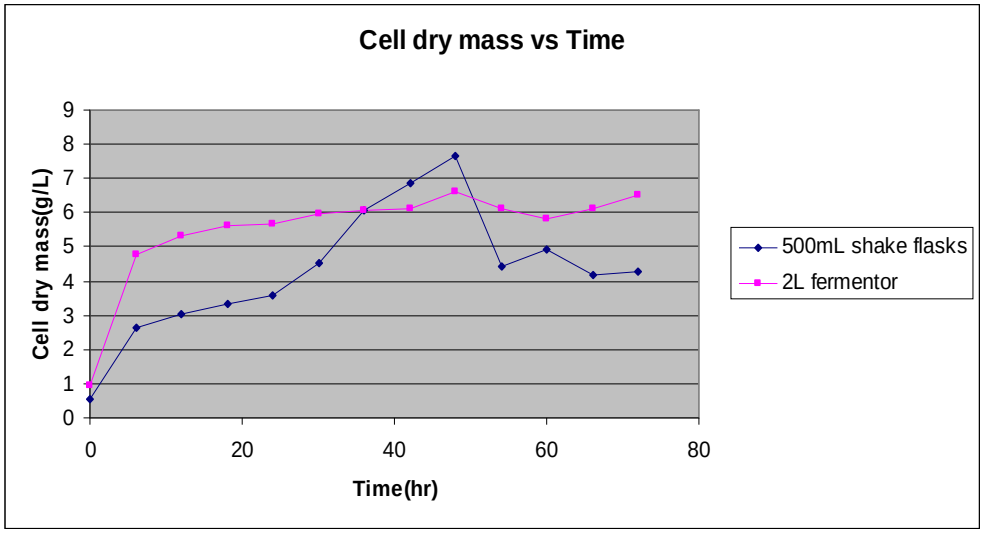


Figure 4. 4:Comparison for cell dry weight analysis

4.5 PHB Analysis

The PHB analysis method was evaluated by the gravimetric method. By definition, this method includes all methods of analysis in which the final stage of the analysis involves weighing. The net weight of PHB produced was in a sample of 20ml. The net weights were simply multiple by conversion factor which are 50 to get the concentration of PHB in gram per 1L. During the analysis, sodium dodecyl sulphate solution was used for lysis the bacteria cells wall and sodium hypochlorite solution was used to promote further lysis and to extract the PHB from the bacteria.

From Figure 4.5, it is clearly shown that the PHB concentrations were increased from beginning until the maximum of 36th hours, and then decreased greatly after that until 72nd hours. Similar trend shows for 2L bioreactor where the PHB also increased and achieved maximum concentration at 36th hours but fluctuate until 72nd hours.

Comparing result for both scale shows that, PHB concentration are higher at larger scale although cell dry mass are at the same value. This show that at larger scale, fermentation process are having a better aeration system compare to small scale. As mention earlier, *Cupriavidus necator* accumulates PHB inside the cell in response to the limitation of an essential nutrient. But a restriction towards nutrient like nitrogen will limit the growth of bacteria.

The maximum PHB concentration for the 500ml shake flask fermentation is 1.415g/L, however for the 2L bioreactor fermentation is 2.37g/L at both 36th hours. This explains that the 36th hours are the optimum time to harvest PHB since it will produce the maximum yield of product or biomass per gram substrates used. At the final stages of the profile, no appearance of PHB observed. This is a result whereby the bacteria which store energy in the lipid poly-β-hydroxybutyrate (PHB), could utilise back the storage to support reproduction in the absence of carbon source.

Below is the calculation for maximum yield of PHB for both scales;

The highest PHB yield coefficient on dry cell weight for 500ml shake flask

$Y_{(P/X)}$ = (PHB obtained / unit dry cell weight)

= (1.415 g PHB / 1L medium) / (6.065 g dry cell / 1L medium)

= 0.2333 g PHB / g dry cell

The highest PHB yield coefficient on dry cell weight for 2L fermentor

$Y_{(P/X)}$ = (PHB obtained / unit dry cell weight)

= (2.17 g PHB / 1L medium) / (6.065 g dry cell / 1L medium)

= 0.3578 g PHB / g dry cell

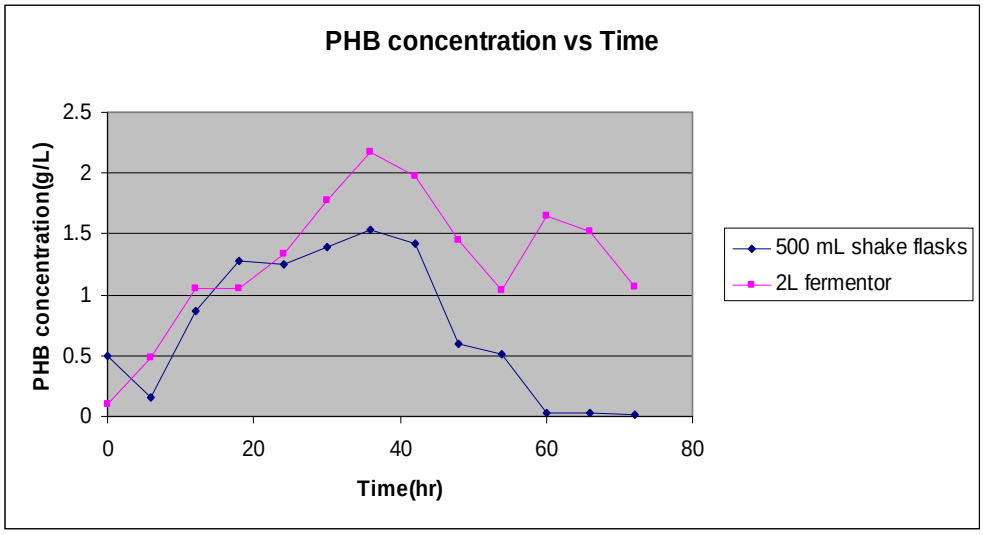


Figure 4. 5:Comparison for PHB analysis

CONCLUSION

In conclusion, oxygen transfer play an important role since it is often turn out to be the limiting factor to obtain the volumetric oxygen transfer coefficient (k_La) that correlates with productivity in specific culture media (Montes *et al.*, 1998). Therefore, in this project, determination of k_La is crucial in order to make sure that scale up process reach its goal. k_La and ka_p are determined at 500 mL shake flask with 200 rotation per minute for agitation speed and 1L/min for aeration rate. By trial and error method, 1.75L/min for aeration rate are obtained at larger scale with fixed agitation speed. Table 5.1 show the nearest value for k_La and ka_p achieved. For the k_La value, the error is 6.94%, however for the ka_p, the error is 12.72%

Table 5.1: Values of k_La and ka_p for 500 mL shake flasks and 2L fermentor

Scale	Agitation speed (rpm)	Aeration rate (L/min)	k_La	ka_p
500 mL shake flasks	200	1	0.2960	0.0220
2L fermentor	200	1.75	0.2763	0.0248

Results show that cell dry weight in 2 L fermentor is comparable to the cell dry weight in 500 mL shake flasks harvest at the optimum time of the production of PHB. Therefore, this situation differ where the yield of PHB are higher at 2L fermentor since it deals with a enhanced aeration system and presence control of pH.

Table 5.2: Comparison for PHB and dry cell weight at 36^{th} hours

Result	Shake flask	2L fermentor
PHB Concentration(g/L)	1.415	2.370
Dried cell weight (g/L)	6.065	6.065

RECOMMENDATIONS

Scaling up process of PHB is very crucial for current and future benefits. As a result, by doing this research, it can improve the knowledge of scaling up for future references. Several recommendations that should be practiced for future study are:

1. Precaution should be made after the sterilization, whereby all connection such as wires and probe should be connected for fermentor and leave for 6 hours for the polarization of probe before placed in the inoculums. This is to make sure that the reading from the entire probe is stable and accurate.
2. A better extraction method for PHB harvesting is needed since SDS solution is carcinogenic. Thus, for larger scale, NaOH digestion method can be efficiently used since it is inexpensive and environmental friendly, high degree of purity (>98%) of PHB can be obtain and no degradation of PHB during extraction.

REFERENCES

Ahmad Jaril Asis, Zulaikha Paidi, Michael A. Winkler and Jailani Salihon**,** (1990), Scale-up of a Fermentation Process Based on Constant Volumetric Transfer Coefficent (k_La) of Oxygen, Jurnal Kejuruteraan II, 179-193.

Al-Masry, W. A. (1999). Effect of scale-up on average shear rates for aerated non-newtonian liquids in external loop airlift reactors. *Biotechnology and Bioengineering*, 62(4), 494–498.

Anderson AJ, Dawes EA (1990). *Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates*. Microbial Rev 54:450-472

Anderson, A., and E. A. Dawes (1990). *Occurrence, metabolism, metabolic role,and industrial uses of bacterial polyhydroxyalkanoates*. Microbiol. Rev. 54:450–472.

Barham PJ.(1990) *Physical properties of poly(hydroxybutyrate) and poly(hydroxybutyrate-co-hydroxyvalerate)*. In:Dawes EA, editor. Novel biodegradable microbial polymers. Dordrecht: Kluwer p:81-96.

Braunegg G, Lefebvre G, Genser KL.(1998) Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects. J Biotechnol;52::27-58

Braunegg G, Lefebvre G, Genser KL.(1998) Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects. J Biotechnol;52::27-58

Byrom D (1987). Polymer synthesis by microorganisms: technology and economics.Trends biotechnology 5:246-250

Calik and Ozdamar, T. H. (2000). Oxygen-transfer strategy and its regulation effects in serine alkaline protease production by *Bacillus licheniformis*. *Biotechnology and Bioengineering*, 69(3), 301–311.

Chisti Y, Moo-Young M (1996). *Bioprocess intensification trough bioreactor engineering.* Trans Inst Chem Eng ;74A:575-83

Elibol, M., and Ozer, D. (2000). Influence of oxygen transfer on lipase production by Rhizopus arrhizus. *Process Biochemistry*, 36, 325–329.

Elibol, M., and Ozer, D. (2000). Influence of oxygen transfer on lipase production by Rhizopus arrhizus. *Process Biochemistry*, 36, 325–329.

Enrico Grothe, Murray Moo-Young, Yusuf Chisti. (1999) *Fermentation optimization for the production of poly(β-hydroxybutyric acid) microbial thermoplastic.*Enzyme & Microbial Technology 25 132-141.

Fusun T, Zeynep F (2000). *Biosynthesis of PHB and its copolymer and their use in control drug release.* Turk. J. med Sci. 30:535-541.

Holmes, PA (1985) Applications of PHB-a microbially produces biodegradable thermoplastic. Phys. Technol ;16, 32-36

Lee, S.Y., (1996). Plastic bacteria, Progress and prospects for polyhydroxyalkanoate production in bacteria. *Trends Biotechnology*. 14, 431–438.

M.Lemoigne (1926) Products of dehydration and of polymerization of β-hydroxybutyric acid, Bull. Soc. Chem. Biol. 8 770-782

Miura, S., Arimura, T., Hoshino, M., Kojima, M., Dwiarti, L., and Okabe, M. (F). Optimization and scale-up of L-lactic acid fermentation by mutant strain *Rhizopus* sp. MK-96-1196 in airlift bioreactors. *Journal of Bioscience and Bioengineering*, 96(1), 65–69.

Montes, F. J., Catalan, J., & Galan, M. A. (1998). Prediction of k_La in yeast broths. *Process Biochemistry*, 34, 549–555.

Poirier Y. (1999) Green chemistry yields a better plastic. Nat Biotechnol 17:960–1

Ramsay BA, Lomaliza K, Chavarie C, Ramsay JA (1990). *Production of poly-bhydroxybutyric–co-b-hydroxyvaleric acids.* Applied. Environment Microbiology 56: 2093-2098.

Salehizadeh, H., Van Loosdrecht, M.C.M., 2004. Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. *Biotechnology Advertisement* 22, 261–279.

Sang Yup Lee, Jong-il Choi, Kyuboem Han, and Ji Yong Song (1999) Removal of Endotoxin during Purification of Poly(3-Hydroxybutyrate) from Gram-Negative Bacteria Appl Environ Microbiol; 65(6): 2762–2764.

Steinbuchel A, DoiY. (2001) Biopolymers: polyesters II—properties and chemical synthesis. Wiley-VCH;.

Sutherland, I. W. (1998). *Novel and established applications of microbial polysaccharides. Trade in Biotechnology, 16, 41-46.*

Tuffile, C. M. and Pinho, F. (1990).Determination of oxygen transfer coefficients in viscous Streptomycetes fermentations. *Biotechnology and Bioengineering*, 12, 849–871.

APPENDIX A

DOT (%) and Concentration Data for 500ml Shake Flask

Trial 1

1. Rpm 200
2. Air Flow rate = 1L/min

Time(min)	1st	Replicate	Average
0	0	0	0
1	23.1	23.2	23.15
2	52.7	51.1	51.90
3	72.4	71.9	71.15
4	84.4	83.2	83.80
5	91.2	89.8	90.50
6	95.6	95.1	95.35
7	97.8	97.1	97.45
8	99.1	98.4	98.75
9	99.8	99.0	99.40
10	99.9	99.6	99.75
11	100	99.9	99.95
12	100	100	100
13	100	100	100

APPENDIX B

MATLAB Command Window for 500 mL shake flasks

- Rpm=200
- Air Flow rate= 1L/min

```
>> t=(0:1:13);
>> y=[0 23.15 51.90 72.15 83.80 90.50 95.35 97.45 98.75 99.40 99.75 99.95 100
100]';
>> plot(t,y,'ro');hold on;h=plot(t,y,'b');hold off;
>> title('DOT(%)vs time(min)');ylim([0 100])
>> type fitfun

function err = fitfun(lambda,t,y)
%FITFUN Used by FITDEMO.
%   FITFUN(lambda,t,y) returns the error between the data and the values
%   computed by the current function of lambda.
%
%   FITFUN assumes a function of the form
%
%     y =  c(1)*exp(-lambda(1)*t) + ... + c(n)*exp(-lambda(n)*t)
%
%   with n linear parameters and n nonlinear parameters.

%   Copyright 1984-2004 The MathWorks, Inc.
%   $Revision: 5.8.4.1 $  $Date: 2004/11/29 23:30:50 $

A = zeros(length(t),length(lambda));
for j = 1:length(lambda)
   A(:,j) = exp(-lambda(j)*t);
end
c = A\y;
z = A*c;
err = norm(z-y);


>> start=[1;0];
>> outputFcn=@(x,optimvalues,state)fitoutputfun(x,optimvalues,state,t,y,h);
>> options=optimset('OutputFcn',outputFcn,'TolX',0.1);
>> estimated_lambda=fminsearch(@(x)fitfun(x,t,y),start,options)

estimated_lambda =

    0.2969
    0.0220
```

APPENDIX C

DOT (%) and Concentration Data for 2L stirred tank fermentor

Trial 1

3. Rpm 200
4. Air Flow rate = 1L/min

Time(min)	1st	Replicate	Average
0	0	0	0
1	9.3	11.3	10.3
2	30.3	30.5	30.4
3	48	48.6	48.3
4	62.1	57.8	59.95
5	72.8	67.6	70.2
6	80.5	74.7	77.6
7	86.3	85.1	85.7
8	90.4	88.5	89.45
9	93.5	91.8	92.65
10	95.7	94.3	95
11	97.4	96	96.7
12	98.6	97.4	98
13	99.4	98.5	98.95
14	100	99.7	99.85
15	100	100	100

Trial 2(average)

- Rpm = 200
- Air flow rate =1.5L/min

Time(min)	1st	Replicate	Average
0	0	0	0
1	19.3	19.9	19.6
2	44.9	45.5	45.2
3	61.4	61.8	61.6
4	72.1	73.5	72.8
5	81.0	81.6	81.3
6	88.5	88.9	88.7
7	93.0	93.8	93.4
8	95.8	96.2	96
9	97.5	99.9	97.7
10	99.0	99.2	99.1
11	99.8	100	99.9
12	100	100	100

Trial 3(average)

- Rpm = 200
- Air Flow rate= 2L/min

Time(min)	1st	Replicate	Average
0	0	0	0
1	26.3	26.9	26.6
2	55.3	55.5	55.4
3	74.4	74.6	74.5
4	84.9	87.7	86.3
5	89.0	90.8	89.9
6	95.3	96.1	95.7
7	97.5	97.7	97.8
8	98.3	99.1	98.7
9	98.5	99.9	99.2
10	99.5	99.9	99.7
11	99.8	100	99.9
12	100	100	100

Trial 4

- Rpm=200
- Air Flow rate= 1.75 L/min

Time(min)	1st	Replicate	Average
0	0	0	0
1	23.2	21.5	22.35
2	51.1	50	50.55
3	71.9	68.5	70.2
4	83.2	81.9	82.55
5	89.8	88.9	89.35
6	95.1	94.8	94.95
7	97.1	98	97.55
8	98.4	98.9	98.65
9	99	99.3	99.15
10	99.6	99.8	99.7
11	99.9	100	99.95
12	100	100	100
13	100	100	100

APPENDIX D

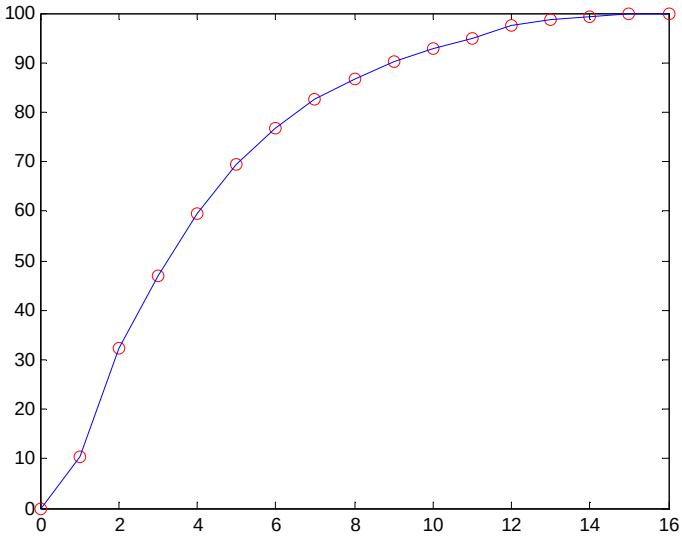
MATLAB Command Window for 2L stirred tank fermentor

Trial 1

- Rpm 200
- Air Flow rate = 1L/min

```
>> t=(0:1:16);
>> y=[0 10.3 32.2 46.9 59.5 69.4 76.7 82.5 86.7 90.1 92.9 95 97.4 98.6 99.4 100
100]';
>> plot(t,y,'ro');hold on;h=plot(t,y,'b');hold off;
>> title('DOT(%)vs time(min)');ylim([0 158])
>> type fitfun

function err = fitfun(lambda,t,y)
%FITFUN Used by FITDEMO.
%   FITFUN(lambda,t,y) returns the error between the data and the values
%   computed by the current function of lambda.
%
%   FITFUN assumes a function of the form
%
%     y =  c(1)*exp(-lambda(1)*t) + ... + c(n)*exp(-lambda(n)*t)
%
%   with n linear parameters and n nonlinear parameters.

%   Copyright 1984-2004 The MathWorks, Inc.
%   $Revision: 5.8.4.1 $  $Date: 2004/11/29 23:30:50 $

A = zeros(length(t),length(lambda));
for j = 1:length(lambda)
   A(:,j) = exp(-lambda(j)*t);
end
c = A\y;
z = A*c;
err = norm(z-y);


>> start=[1;0];
>> outputFcn=@(x,optimvalues,state)fitoutputfun(x,optimvalues,state,t,y,h);
>> options=optimset('OutputFcn',outputFcn,'TolX',0.1);
>> estimated_lambda=fminsearch(@(x)fitfun(x,t,y),start,options)



estimated_lambda =
```

0.1514
0.0225



*Percentage of error (as compared with shake flask value)

For kla,

$$\frac{0.2969 - 0.1514}{0.2969} \times 100\% = 49.01\%$$

For kap,

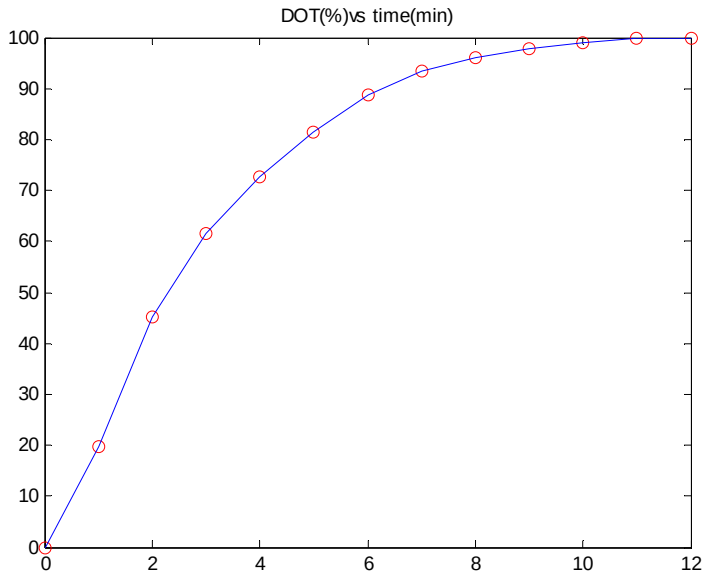
$$\frac{0.0225 - 0.0220}{0.0220} \times 100\% = 2.27\%$$

Trial 2(average)

- Rpm = 200
- Air flow rate =1.5L/min

```
>> t=(0:1:12);
>> y=[0 19.6 45.2 61.6 72.8 81.3 88.7 93.4 96 97.7 99.1 99.9 100]';
>> plot(t,y,'ro');hold on;h=plot(t,y,'b');hold off;
>> title('DOT(%)vs time(min)');ylim([0 100])
>> type fitfun

function err = fitfun(lambda,t,y)
%FITFUN Used by FITDEMO.
%   FITFUN(lambda,t,y) returns the error between the data and the values
%   computed by the current function of lambda.
```

```
%
%   FITFUN assumes a function of the form
%
%     y =  c(1)*exp(-lambda(1)*t) + ... + c(n)*exp(-lambda(n)*t)
%
%   with n linear parameters and n nonlinear parameters.

%   Copyright 1984-2004 The MathWorks, Inc.
%   $Revision: 5.8.4.1 $  $Date: 2004/11/29 23:30:50 $

A = zeros(length(t),length(lambda));
for j = 1:length(lambda)
   A(:,j) = exp(-lambda(j)*t);
end
c = A\y;
z = A*c;
err = norm(z-y);


>> start=[1;0];
>> outputFcn=@(x,optimvalues,state)fitoutputfun(x,optimvalues,state,t,y,h);
>> options=optimset('OutputFcn',outputFcn,'TolX',0.1);
>> estimated_lambda=fminsearch(@(x)fitfun(x,t,y),start,options)

estimated_lambda =

    0.1970
    0.0348
```



*Percentage of error (as compared with shake flask value)

For kla,

$$\frac{0.2969 - 0.1970}{0.2969} \times 100\% = 33.65\%$$

For kap,

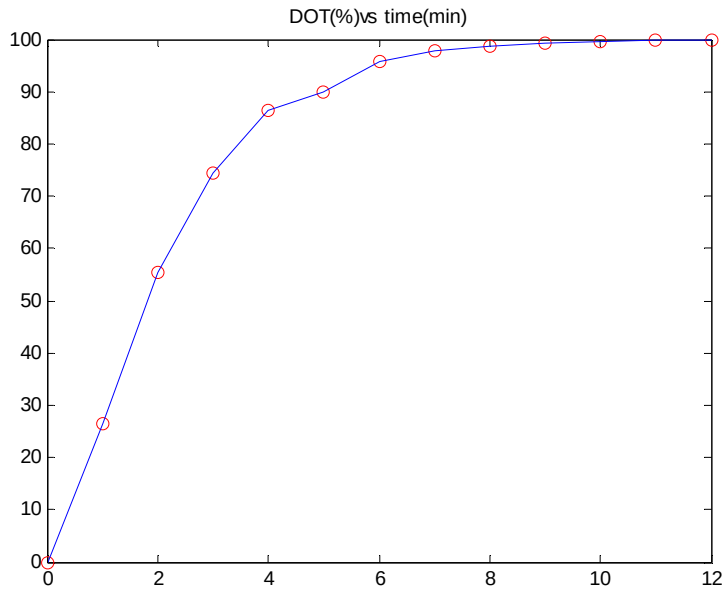
$$\frac{0.0348 - 0.0220}{0.0220} \times 100\% = 58.18\%$$

Trial 3(average)

- Rpm = 200
- Air Flow rate= 2L/min

```
>> t=(0:1:12);
>> y=[0 26.6 55.4 74.5 86.3 89.9 95.7 97.8 98.7 99.2 99.7 99.9 100]';
>> plot(t,y,'ro');hold on;h=plot(t,y,'b');hold off;
>> title('DOT(%)vs time(min)');ylim([0 100])
>> type fitfun

function err = fitfun(lambda,t,y)
%FITFUN Used by FITDEMO.
%   FITFUN(lambda,t,y) returns the error between the data and the values
%   computed by the current function of lambda.
%
%   FITFUN assumes a function of the form
%
%     y =  c(1)*exp(-lambda(1)*t) + ... + c(n)*exp(-lambda(n)*t)
%
%   with n linear parameters and n nonlinear parameters.

%   Copyright 1984-2004 The MathWorks, Inc.
%   $Revision: 5.8.4.1 $  $Date: 2004/11/29 23:30:50 $

A = zeros(length(t),length(lambda));
for j = 1:length(lambda)
   A(:,j) = exp(-lambda(j)*t);
end
c = A\y;
z = A*c;
err = norm(z-y);


>> start=[1;0];
>> outputFcn=@(x,optimvalues,state)fitoutputfun(x,optimvalues,state,t,y,h);
```

```
>> options=optimset('OutputFcn',outputFcn,'TolX',0.1);
>> estimated_lambda=fminsearch(@(x)fitfun(x,t,y),start,options)

estimated_lambda =

    0.3300
    0.0193
```



*Percentage of error (as compared with shake flask value)

For kla,

$$\frac{0.3300-0.2969}{0.2969}\times 100\% = 11.15\%$$

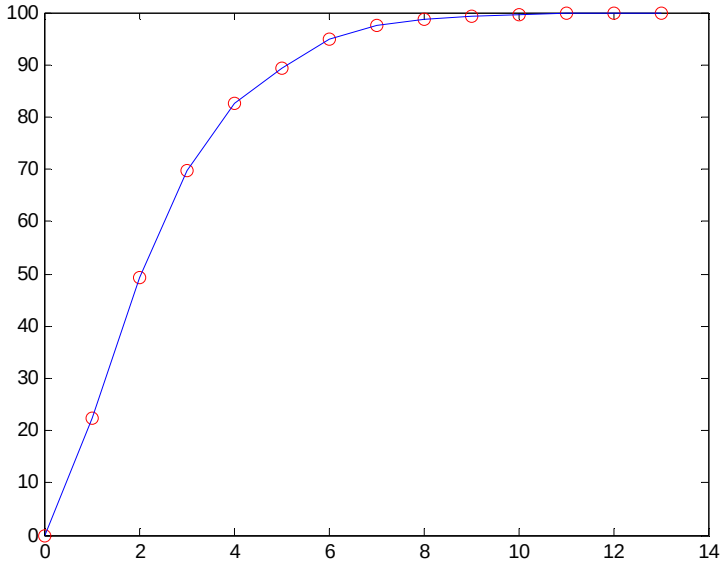
For kap,

$$\frac{0.0220-0.0193}{0.0220}\times 100\% = 12.27\%$$

Trial 4

- Rpm=200
- Air Flow rate= 1.75 L/min

```
>> t=(0:1:13);
>> y=[0 22.35 50.55 70.2 82.55 89.35 94.95 97.55 98.65 99.15 99.7 99.95 100 100]';
>> plot(t,y,'ro');hold on;h=plot(t,y,'b');hold off;
>> title('DOT(%)vs time(min)');ylim([0 100])
>> type fitfun

function err = fitfun(lambda,t,y)
%FITFUN Used by FITDEMO.
%   FITFUN(lambda,t,y) returns the error between the data and the values
%   computed by the current function of lambda.
%
%   FITFUN assumes a function of the form
%
%     y =  c(1)*exp(-lambda(1)*t) + ... + c(n)*exp(-lambda(n)*t)
%
%   with n linear parameters and n nonlinear parameters.

%   Copyright 1984-2004 The MathWorks, Inc.
%   $Revision: 5.8.4.1 $  $Date: 2004/11/29 23:30:50 $

A = zeros(length(t),length(lambda));
for j = 1:length(lambda)
   A(:,j) = exp(-lambda(j)*t);
end
c = A\y;
z = A*c;
err = norm(z-y);


>> start=[1;0];
>> outputFcn=@(x,optimvalues,state)fitoutputfun(x,optimvalues,state,t,y,h);
>> options=optimset('OutputFcn',outputFcn,'TolX',0.1);
>> estimated_lambda=fminsearch(@(x)fitfun(x,t,y),start,options)

estimated_lambda =

    0.2763
    0.0248
```



*Percentage of error (as compared with shake flask value)

For kla,

$$\frac{0.2969 - 0.2763}{0.2969} \times 100\% = 6.94\%$$

For kap,

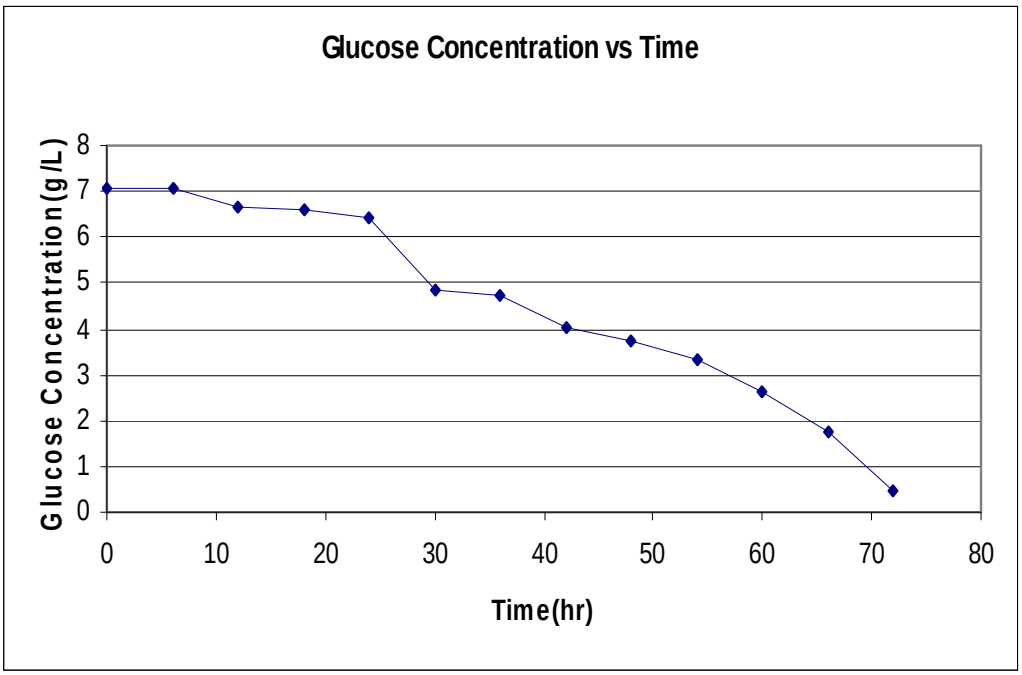
$$\frac{0.0248 - 0.0220}{0.0220} \times 100\% = 12.72\%$$

APPENDIX E

Glucose Analysis Data

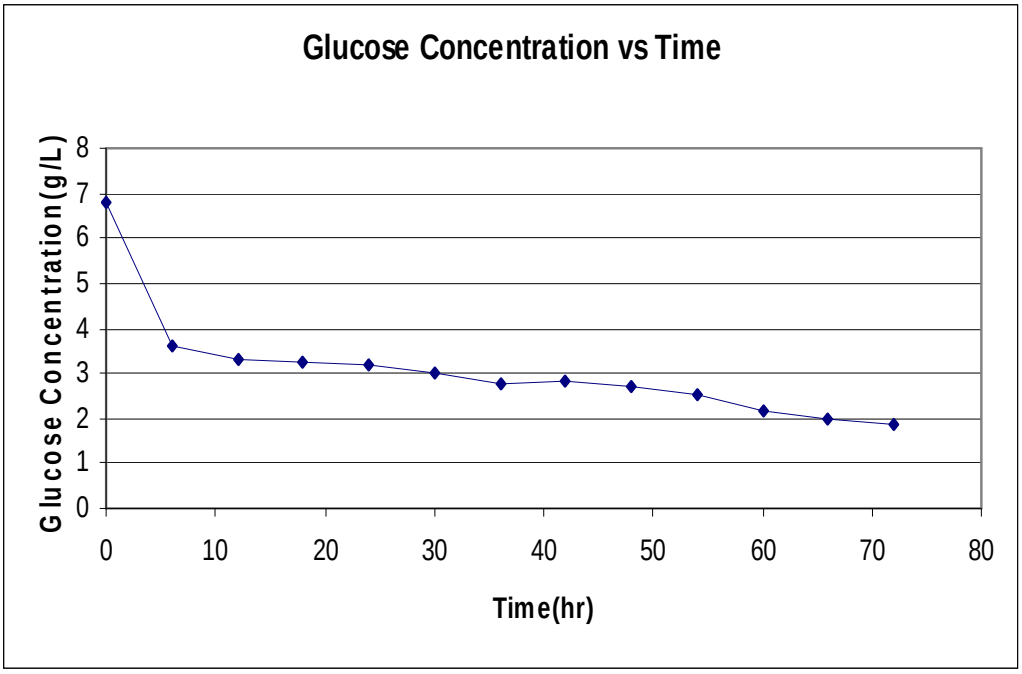
1. Average data for 500 mL shake flasks

Time	OD Reading (y)	Raw Glucose I (g/L)	Dilution factor	Actual Glucose (g/L)
0	0.549	0.50647	14	7.090573
6	0.546	0.503829	14	7.053604
12	0.515	0.476543	14	6.671596
18	0.51	0.472142	14	6.609982
24	0.494	0.458058	14	6.412816
30	0.367	0.346272	14	4.847813
36	0.356	0.33659	14	4.712261
42	0.3	0.287299	14	4.022181
48	0.278	0.267934	14	3.751078
54	0.242	0.236247	14	3.307455
60	0.189	0.189596	14	2.654344
66	0.118	0.127101	14	1.779421
72	0.013	0.03468	14	0.485521



2. Average data for 2L fermentor

Time	OD Reading (y)	Raw Biomass I (g/L)	Dilution factor	Actual Biomass (g/L)
0	0.284	0.226741	30	6.802218
6	0.163	0.120236	30	3.607077
12	0.151	0.109673	30	3.290203
18	0.149	0.107913	30	3.237391
24	0.147	0.106153	30	3.184579
30	0.141	0.100871	30	3.026142
36	0.132	0.09295	30	2.788487
42	0.133	0.09383	30	2.814893
48	0.129	0.090309	30	2.709269
54	0.121	0.083267	30	2.49802
60	0.108	0.071825	30	2.15474
66	0.101	0.065663	30	1.969897
72	0.098	0.063023	30	1.890679

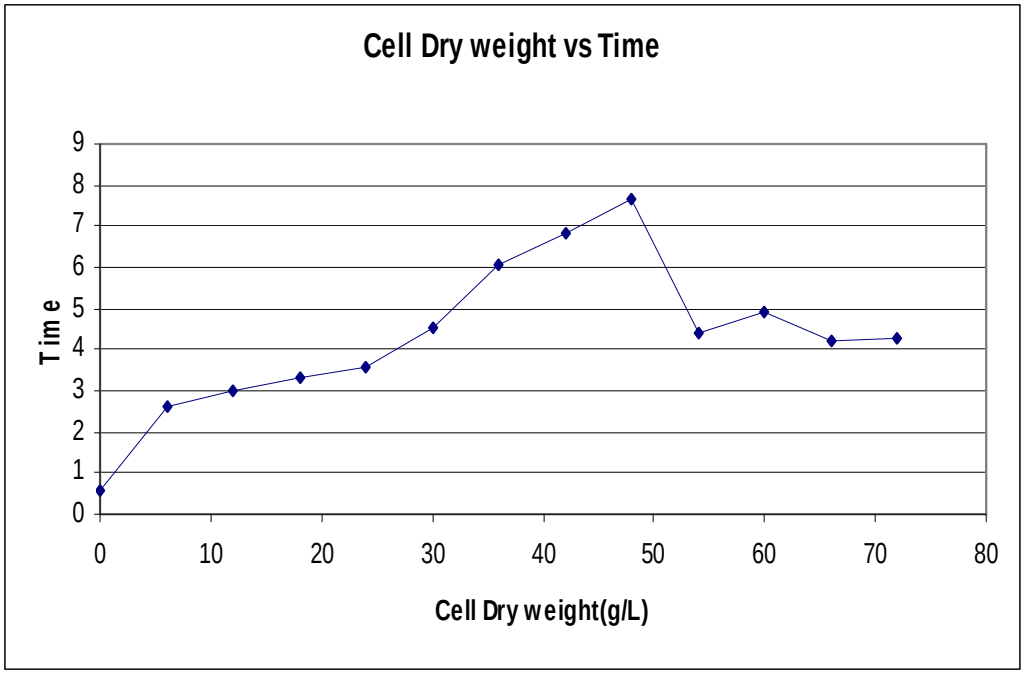


APPENDIX F

Dry Cell Weight Data Analysis data

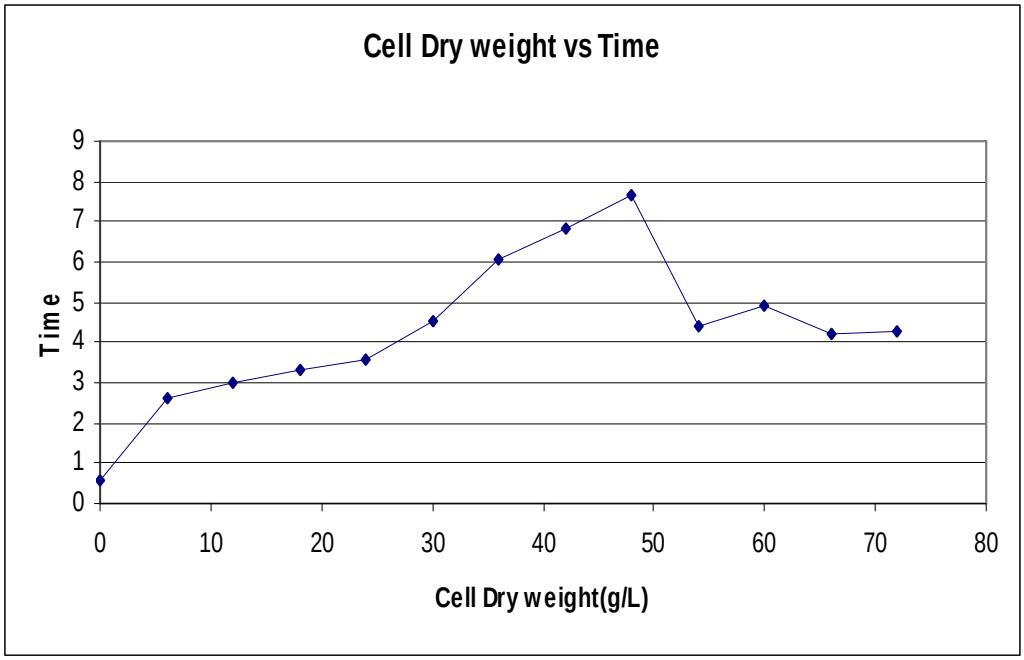
1. Average data for 500 mL shake flasks

Time	OD Reading (y)	Raw Biomass I (g/L)	Dilution factor	Actual Biomass (g/L)
0	0.263	0.113527	5	0.567633
6	1.178	0.522813	5	2.614063
12	1.359	0.603775	5	3.018876
18	1.493	0.663714	5	3.318572
24	1.602	0.712471	5	3.562355
30	2.032	0.904813	5	4.524065
36	2.721	1.213008	5	6.065038
42	1.539	0.684291	10	6.842906
48	1.724	0.767042	10	7.670424
54	1.987	0.884684	5	4.423421
60	2.215	0.98667	5	4.933351
66	1.883	0.838164	5	4.190821
72	1.917	0.853373	5	4.266863



2. Average data for 2L fermentor

Time	OD Reading (y)	Raw Biomass I (g/L)	Dilution factor	Actual Biomass (g/L)
0	0.436	0.190911	5	0.954554
6	2.149	0.957148	5	4.78574
12	2.377	1.059134	5	5.29567
18	2.523	1.124441	5	5.622204
24	2.537	1.130703	5	5.653516
30	2.678	1.193773	5	5.968867
36	2.721	1.213008	5	6.065038
42	2.745	1.223743	5	6.118715
48	2.958	1.31902	5	6.595098
54	2.745	1.223743	5	6.118715
60	2.602	1.159778	5	5.798891
66	2.745	1.223743	5	6.118715

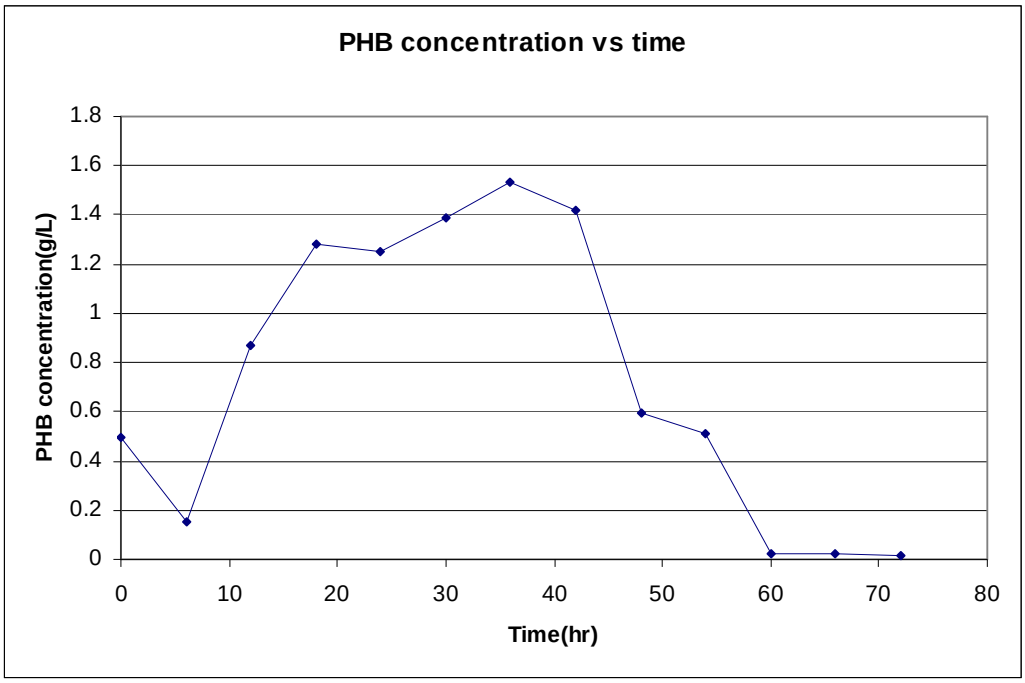


APPENDIX G

PHB Analysis Data

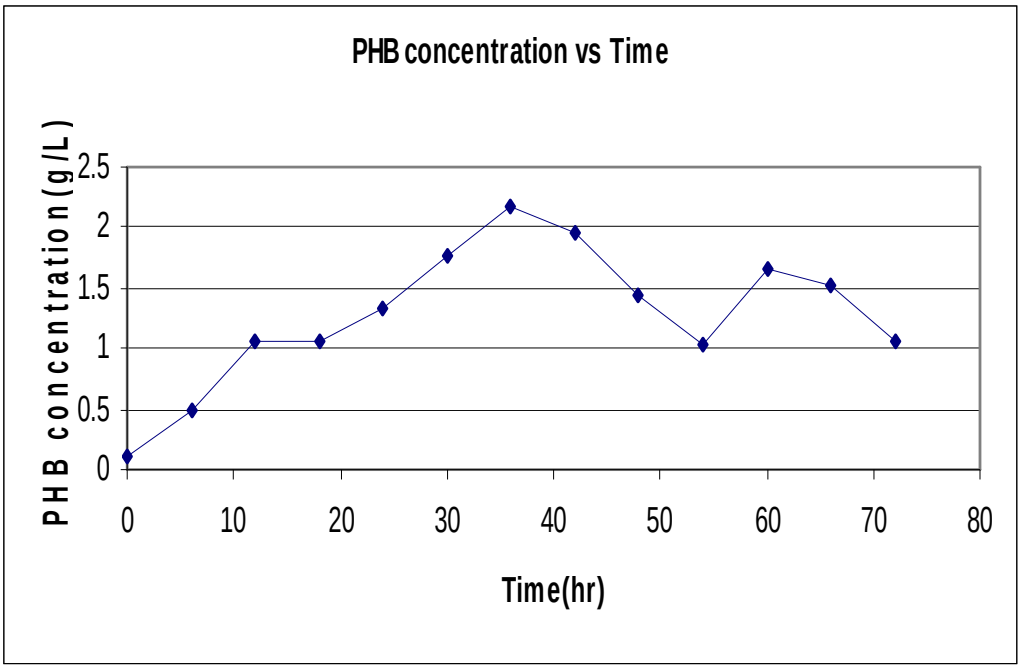
1. Average data for 500 ml shake flasks

Time	Wt of boat (g)	Wt of boat + PHB (g)	Wt of PHB (g)	Conversion factor	Actual wt of PHB (g/L)
0	0.9337	0.9436	0.0099	50	0.495
6	0.9457	0.9488	0.0031	50	0.155
12	0.9422	0.9596	0.0174	50	0.87
18	0.9375	0.9631	0.0256	50	1.28
24	0.9514	0.9764	0.025	50	1.25
30	0.9451	0.9729	0.0278	50	1.39
36	0.9422	0.9728	0.0306	50	1.53
42	1.25	1.2783	0.0283	50	1.415
48	1.268	1.2799	0.0119	50	0.595
54	1.2703	1.2805	0.0102	50	0.51
60	1.266	1.2665	0.0005	50	0.025
66	1.278	1.2785	0.0005	50	0.025
72	1.2744	1.2747	0.0003	50	0.015



2. Average data for 2L fermentor

Time	Wt of boat (g)	Wt of boat + PHB (g)	Wt of PHB (g)	Conversion factor	Actual wt of PHB (g/L)
0	0.9185	0.9195	0.001	100	0.1
6	0.9252	0.9301	0.0049	100	0.49
12	0.9161	0.9266	0.0105	100	1.05
18	0.9251	0.9356	0.0105	100	1.05
24	0.9224	0.9358	0.0134	100	1.34
30	0.9183	0.936	0.0177	100	1.77
36	0.9263	0.948	0.0217	100	2.17
42	0.9353	0.955	0.0197	100	1.97
48	0.9346	0.9491	0.0145	100	1.45
54	0.9238	0.9342	0.0104	100	1.04
60	0.9275	0.944	0.0165	100	1.65
66	0.9276	0.9428	0.0152	100	1.52
72	0.9161	0.9267	0.0106	100	1.06



APPENDIX H

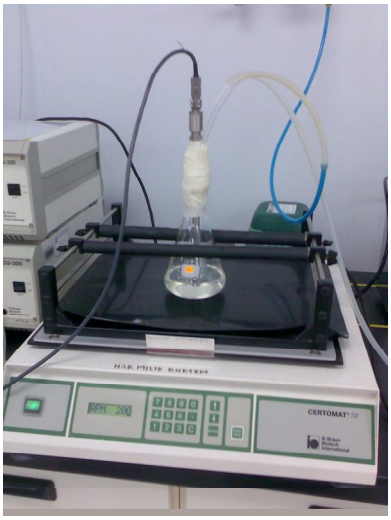


Figure H.1: k_La determination at 500 mL shake flask

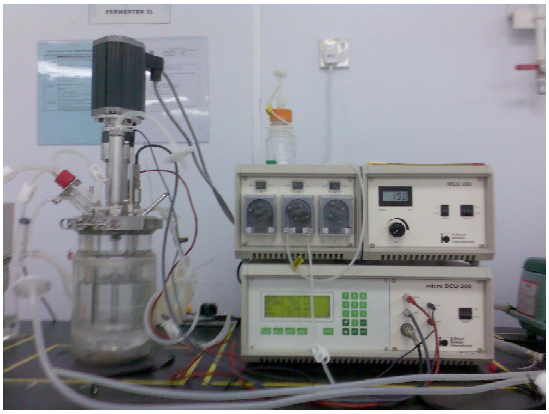


Figure H.2: k_La determination at 2L fermentor

Figure H.3: PHB sample at selected harvest time