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

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

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GOH MEI FONG

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
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28 APRIL 2008

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.

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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

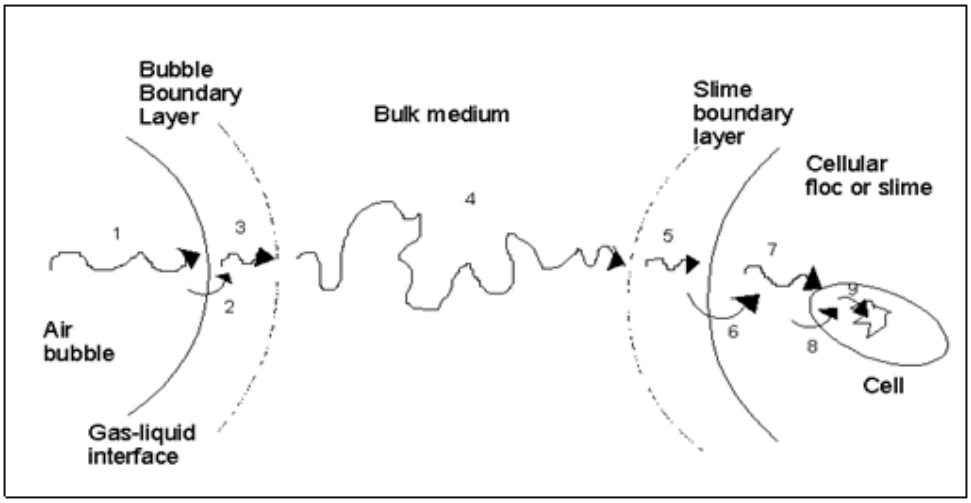

Figure 2.1 Oxygen moves from a bubble to an immobilized cell system

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.

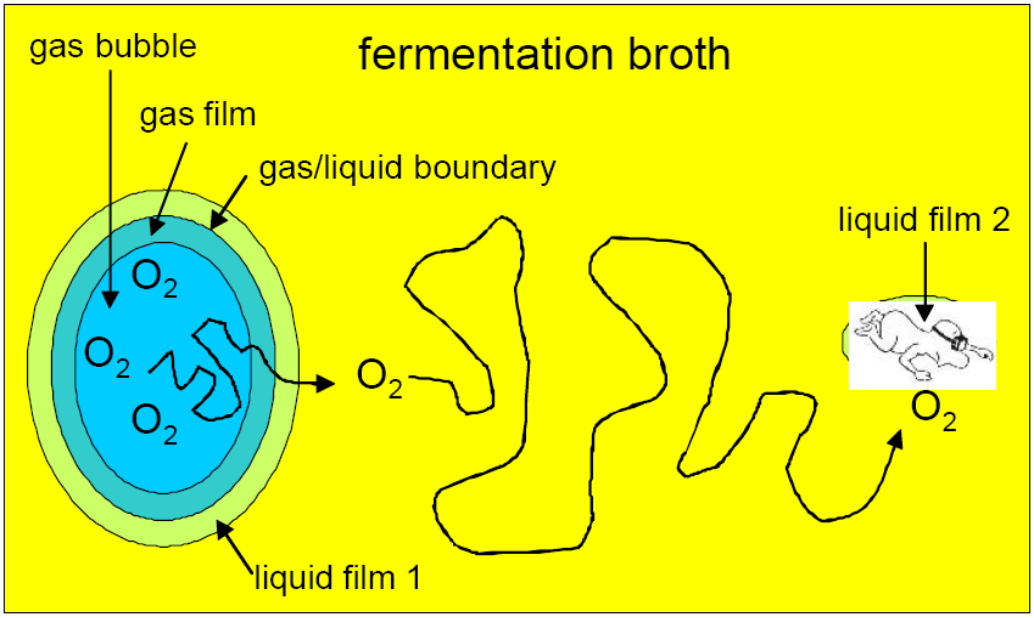

Figure 2.2 Two film model

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.

3.1.2 Regeneration of the bacteria

The culture was maintained at slant medium. Regeneration was conducted every two weeks. Slant was prepared as the following procedure. Prepare NGY (Nutrient Glucose Yeast) agar medium 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	15
Aqueduct	Added until total volume= 1L

The solution was heated in a beaker glass with continuous stirring on laboratory hot plate until the solution comes into boiling. About 10 ml of the hot agar solution was poured into each sterilized test tube. The tube was closed with sterile cotton and wrap in aluminum foil. The tubes were sterilized in autoclave for 30 minutes at 121ºC. The tubes were put in incline position so that the agar will set with inclined surface in the tubes. Let it set for one night in sterile incubator. The bacteria were transferred from the old slant to the new slant in sterile laminar air flow hood with the following procedure:

Firstly, the metal loop was heated until burning red. Then, the old slant containing bacteria to be regenerated was opened. Next, the loop was cooled down by touching it on the agar surface. After that, one loop full of bacteria was scraped and quickly transfer it to the new slant by slightly scratch the agar surface.

The slant was incubated in the sterile incubator at room temperature for about 24 hours until the bacteria seem to grow. Then, keep in the refrigerator at 4ºC for long time maintenance.

3.1.3 Fermentation for Starter 1

Table 3.2 : Medium for Starter 1 NGY without agar

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

20 ml of NGY medium was put in 100 ml Erlenmeyer, the flask was closed with sterile cotton then was sterilized in autoclave for 30 minutes at 121ºC. Let it stand in sterile incubator for 24 hours at room temperature. One loop of the bacteria from the slant was taken and is put it into the incubated medium. Transfer was conducted in sterile laminar air flow hood. Then, it was incubated for 24 hours before move the content into Starter 2.

3.1.4 Fermentation for Starter 2

Table 3.3 : Ramsay Medium for Starter 2 with the composition

chemicals	Amount (g/L)
Glucose	10
$Na_2HPO_4.7H_2O$	6.7
KH_2PO_4	1.5
$(NH_4)_2SO_4$	1.0
$MgSO_4$	0.2
$CaCl_2.2H_2O$	0.01
Ferri ammonium sulfate	0.06
Trace element	1ml
Aqueduct	To make total volume 1L

Table 3.4 : Trace elements composition

Chemicals	Amount (g/L)
H_3BO_4/H_3BO_3	0.3
$CoCl_2.6H_2O$	0.2
$ZnSo_4.7H_2O$	0.1
$MnCl_2.4H_2O$	0.03
$NaMoO_4.2H_2O$	0.03
Aqueduct	To make total volume 1L

To grow bacteria in Starter 2, 180 ml of Ramsay medium was put in 500 ml Erlenmeyer (plug the inlet with sterile cotton) and sterilized in autoclave for 30 minutes at 121ºC.After sterilization, the flask was let to stand for 24 hours in sterilize incubator. Then, starter 1 was poured in to the flask and was shaking on shaker for 24 hours.

3.1.5 Fermentation in 10L bioreactor

The bioreactor was cleaned thoroughly before using. Then, 1800 ml of Ramsay medium was poured into the bioreactor and wais sterilized for 30 minutes at 121˚C. 0.5 M NaOH and 0.5 M HCl is prepared for pH control. Palm oil was prepared for antifoaming. The lines were connected to sterilized bioreactor containing medium, the bioreactor was turned on and the cooling tower and air supply were opened. Starter 2 was flowed into the bioreactor through the automatic input. The bioreactor was run. Samples were withdrawn through the sampling line for every 6 hours intervals.

3.2 Analysis Methods

3.2.1 Glucose analysis

Glucose analysis was determined by the DNS (dinitrosalicylic acid) method.10ml of sample was withdrawn and was centrifuged at 5000rpm and 4˚C for 12 minutes. The supernatant was used for the glucose analysis. 1ml of supernatant was reacted with 1ml of 1% DNS reagent and then two drops of 0.1M NaOH was added. The mixture was placed in boiling water for 5 minutes. Then, the mixture was cooled under water flow. Then, 10ml of distilled water was added and the mixture was mixed evenly. The optical density of the mixture was read under the absorbance at 540nm using UV-VIS spectrophotometer.

3.2.2 Dry Cell Weight analysis

Biomass content was evaluated by gravimetric method. The pellet that obtained from steps as above was washed with 10ml of deionized water. Then, it was recovered by centrifuge at 5000rpm and 4˚C for 12 minutes. After that, it was dried at 90ºC for 24 hours. Then wait it to cool and weight it. The biomass yield coefficient on glucose ($Y_{X/S}$) as the cell dry weight produced per unit mass of glucose consumed was calculated.

3.2.3 PHB analysis

10ml of 0.625% commercial sodium hypochlorite solution was added to the biomass pellet obtained as described for biomass measurements. The mixture was recovered by centrifugation at 5000rpm and 4˚C for 12 minutes. Then, the supernatant was removed and 10ml of 100µm hydrogen peroxide was added to the pellet and the mixture was shaking in the water bath shaker at 30˚C for 4 hours. Then, the mixture was centrifuged at 5000rpm and 4˚C for 12 minutes. After that, 10ml of chloroform

was added to the pellet and mixed it by vortex. The mixture was poured into the Petri dishes and let it dried. Then, 5ml of concentrated acid sulfuric was added and well mixed. The mixture was poured into the test tubes and was boiled in water bath for 10 minutes. The optical density of the mixture was read under the absorbance at 238nm using UV-VIS spectrophotometer. The PHB yield coefficient relative to biomass ($Y_{P/X}$) as the mass of PHB obtained per unit cell dry weight was calculated.

3.3 Solving Techniques

3.3.1 Effect of aeration on OTR and kLa values

$$YR(t) = C^*\{[(kap.\exp(-kLa.t))/(kap-kLa)] - [(kLa.\exp(-kap.t))/(kLa-kap)]\}$$

This equation was taken from the article by Ahmad Jaril Asis, Zulaikha Paidi, Michael A. Winkler and Jailani Salihon in Jurnal Kejuruteraan 2 (1990) p179-195. The equation above was solved by using MATLAB software with Fibonacci error minimisation search method. There are two unknown, namely k_La and ka_p. The k_La and ka_p values were calculated by solving the equation with the DOT data. The aim was to get the best fit curves for the 500ml shake flask and 10L fermentor and to get the k_La value.

CHAPTER 4

RESULTS AND DISCUSSIONS

In this project, the volumetric mass transfer coefficient K_La was selected to be the scale up criterion, so the the K_La in both the 500ml Erlenmeyer flask and 10L bioreactor has to be the same, that is (K_La) shake flask = (K_La) 10L bioreactor. This criterion is usually applied to aerobic systems where oxygen concentration is most important and affects metabolism of the microbial cell. Since the role of oxygen on microorganism growth and metabolism is important, the DO transfer due to oxygen transfer rate (OTR) into a system during fermentation needs to be investigated. For aerobic fermentations, it is equally important to satisfy both agitation rate and the oxygen supply requirements.

4.1 DOT versus Time Curves

The percent DOT versus time curve for 500ml shake flask was obtained at the optimum conditions of the PHB, which is 200rpm, at room temperature and compressed air flow rate is 1 L/min. The 500ml shake flask was filled with 200ml of distilled water. There are some assumptions: firstly, the optimum temperature of PHB was assumed equal to room temperature even though the exact optimum temperature of PHB is 30˚C. Next, the rotational diameter of the orbital shaker that used to shake the shake flask is 25mm. The rotational diameter of the orbital shaker will affect the shaking geometry of the distilled water. 25mm to 50mm is best range of diameter that suitable for shaking the *Cupriavidus necator*.

The percent DOT versus time curve for 10L fermentor was obtained by trial and error on the agitation rate and the compressed air flow rate. The 10L fermentor was filled with 8L of distilled water. After trying 4 times, finally, at the agitation rate of 310rpm and 1 L/min, the percent DOT curve in 10L fermentor was produced nearly the same pattern as in the 500ml shake flask above. In the trials, the agitation rate cannot be set too much higher because the shear rate can cause damage to the cells growing inside the 10L fermentor. So, the compressed air flow rate needs to adjust to achieve the same DOT as in the 500ml shake flask. During the experiment, the calibration of DOT must be very accurate. The wrong step of calibration will strongly affect the DOT values.

The DOT values will increase dramatically to a maximum level and then keep constant afterwards. The k_La value will increase with increase of aeration rates. The positive correlation of k_La values on biopolymer productivity is expected with aeration effect. DO concentrations directly correlate with the OTR and k_La values. The mixing intensification may lead to a reducing of k_La and this effect is more pronounced at lower aeration rate and lower biomass concentrations. The magnitude of blocking effect on k_La values also depends on microorganism type. Therefore, the k_La around the impellers is high since the mixing is appropriate while k_La low where mixing is heterogeneous (Kouda, 1997).

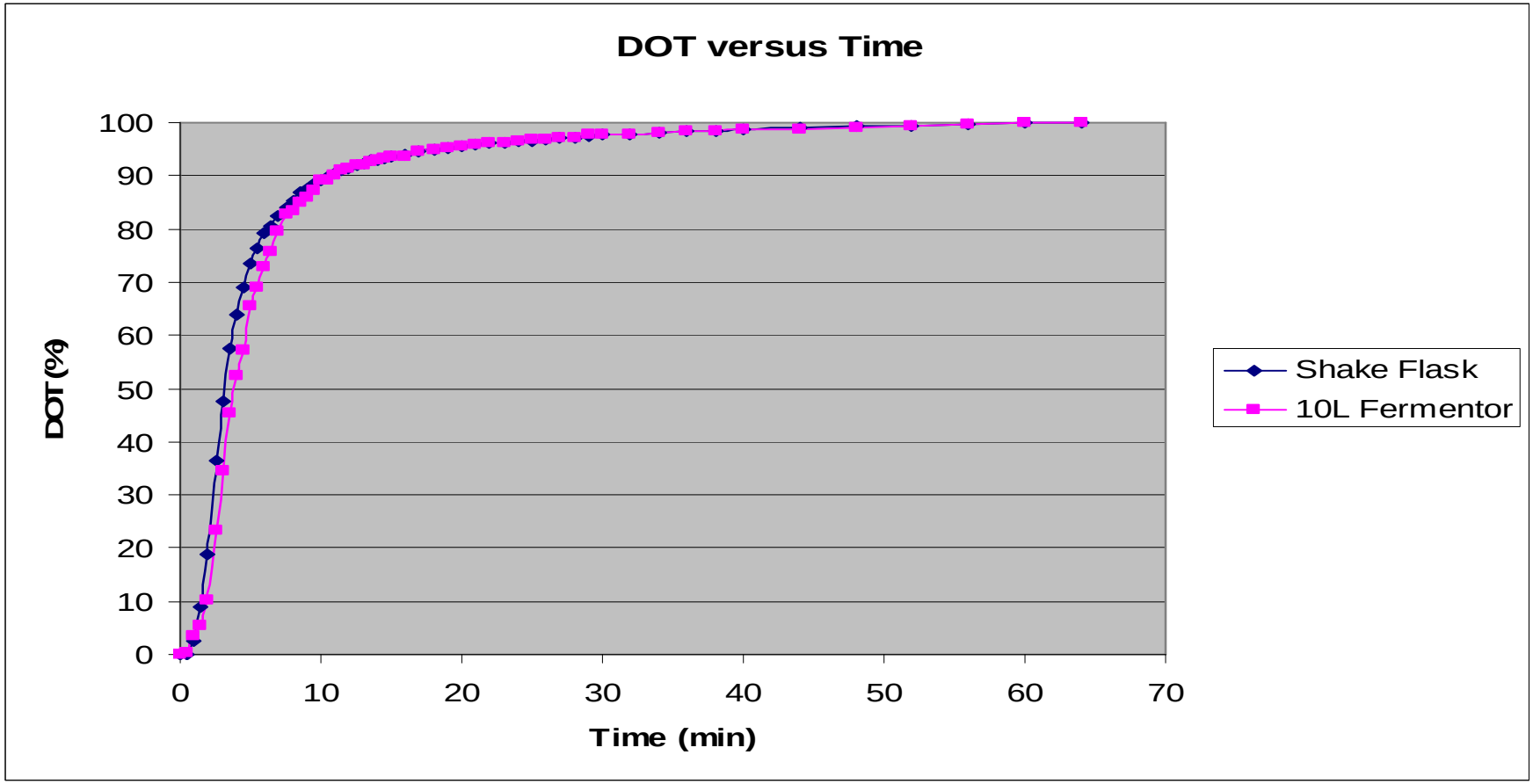

Figure 4.1 DOT (%) versus time curves for 500ml shakes flask and 10L fermentor

4.2 MATLAB Fitted Curves for 500ml shake flask and 10L fermentor

MATLAB software was used to get the k_La and ka_p values. The Fibonacci Min Search method was selected to calculate both the values. With the mass transfer equation

$$YR(t) = C*\{[(kap.exp(-kLa.t))/(kap-kLa)] – [(kLa.exp(-kap.t))/(kLa-kap)]\}$$

This equation was taken from Ahmad Jaril Asis, Zulaikha Paidi, Michael A. Winkler and Jailani Salihon from Jurnal Kejuruteraan 2 (1990) 179-195.) From the DOT and time data, the k_La and ka_p values for 500ml shake flask were 0.2809 min^{-1} and 0.0010 min^{-1}. However, the k_La and ka_p values for 10L fermentor were 0.2533 min^{-1} and 0.0009 min^{-1}.

The 500ml shake flask fitted curves and the 10L fermentor fitted curves shown the comparisons between the experimental curve and the MATLAB fixed curve. For the k_La, the error is 9.83%, however for the ka_p, the error is 10.00%

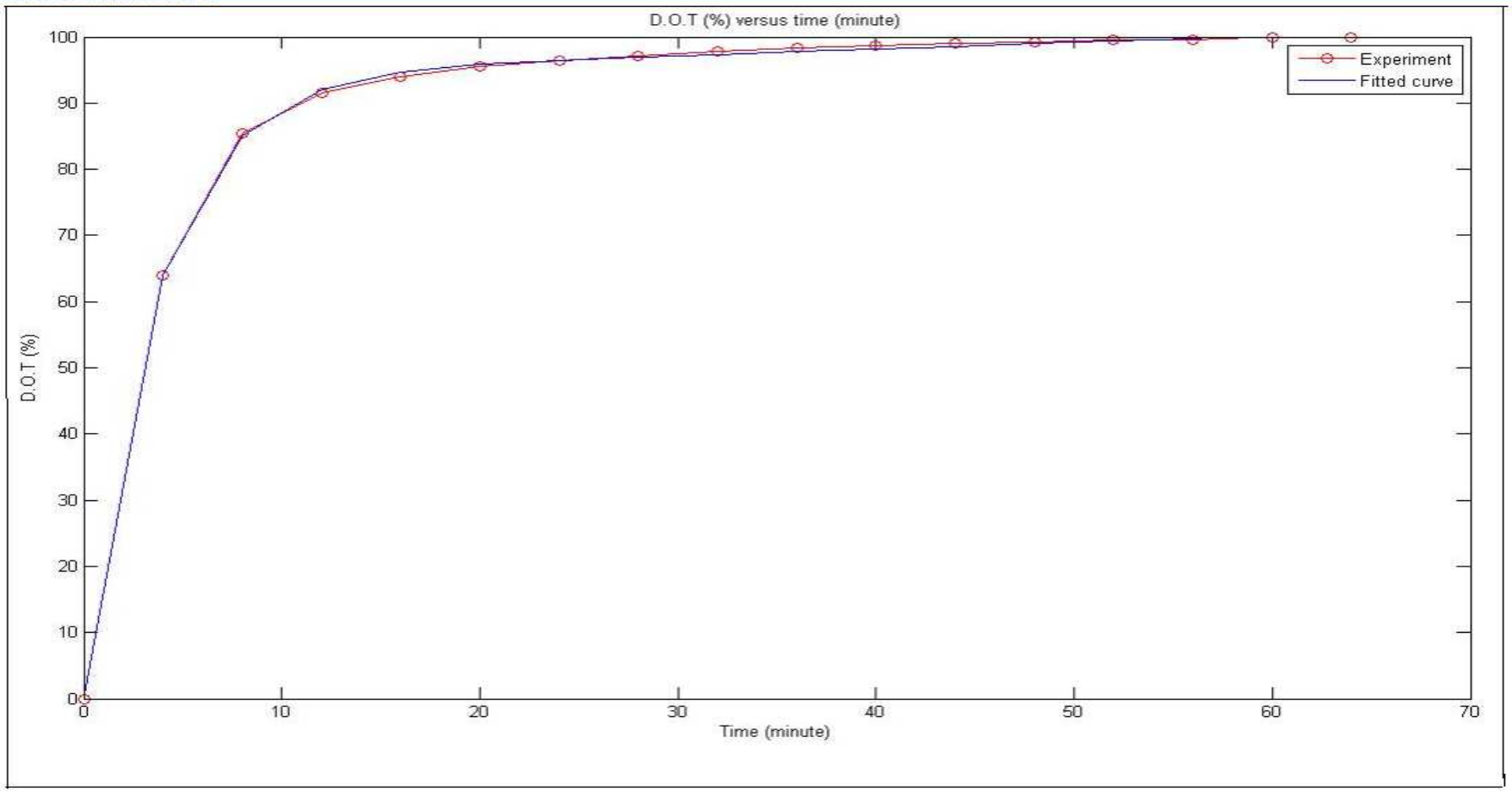

Figure 4.2 500ml shake flask fitted curve

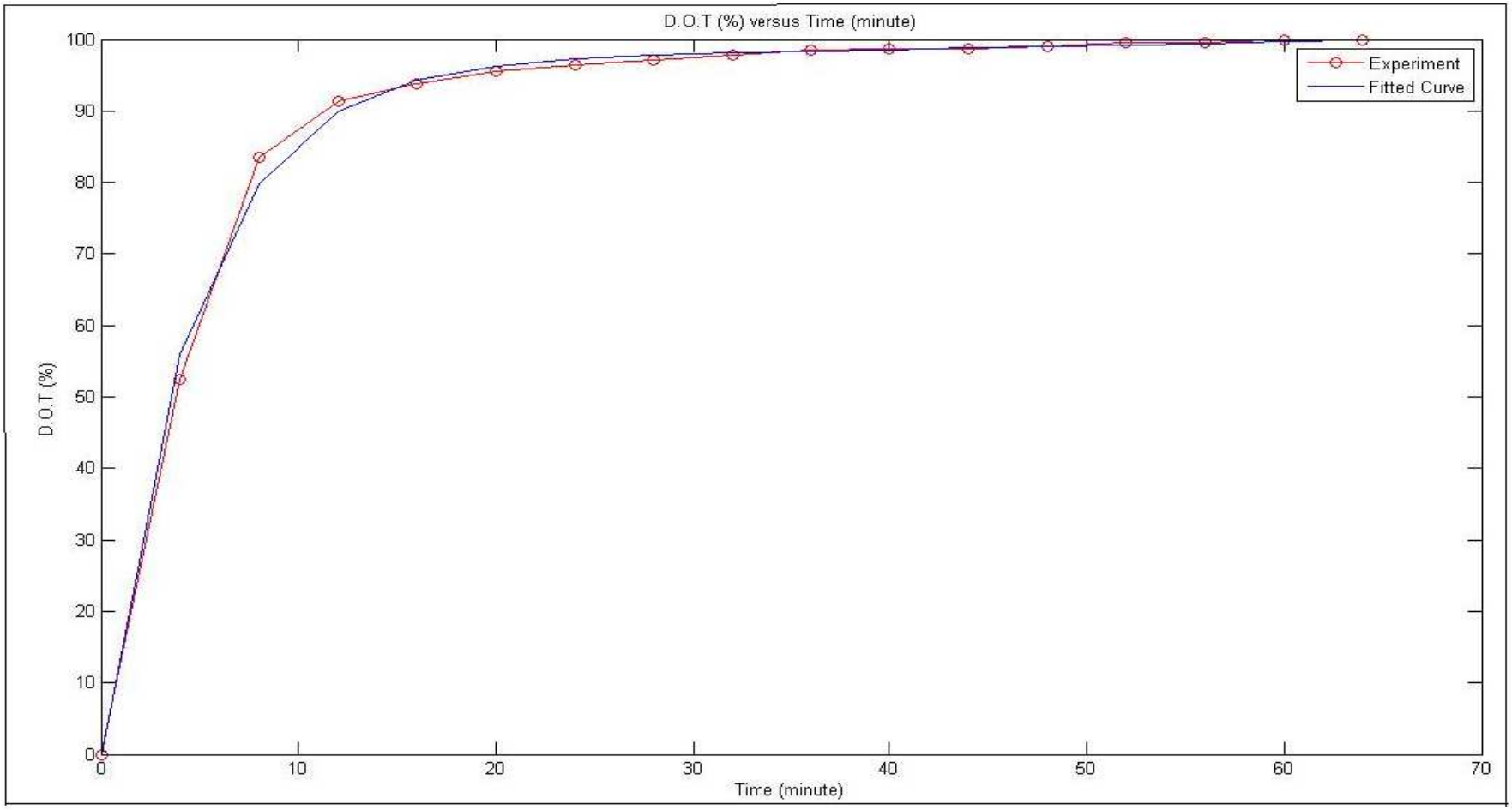

Figure 4.3 10L fermentor fitted curve

4.3 Glucose Analysis

Glucose analysis of PHB was evaluated by DNS method. From the glucose concentration standard curve, the straight line with equation of y = 0.9109x was obtained. Y represented the optical density of the glucose samples for every six hours; however x represented the remaining glucose concentration in the medium. The glucose concentration was then multiplied by 10 because of 10 times dilution.

From the graphs, the glucose concentrations were declined from the beginning sixth hours until the 72nd hours. For the 500ml shake flask fermentation, the glucose concentration declined slightly from sixth to 30th hours, but then declined greatly from 30th to 48th hours. After that, it was slightly declined until 72nd hours. However for the 10L bioreactor fermentation, the glucose concentration declined slightly from sixth to 24th hours, but then declined greatly from 24th to 48th hours. After that, it was slightly declined until 72nd hours. From the trend of the curves, for both the fermentation in 500ml shake flask and 10L bioreactor, the bacteria consumed glucose largely in the exponential phase, which is from 24th to 48th hours. During the exponential phase, the bacteria already adapted with the situation and growth healthy and the reproduction also goes fast.

Glucose analysis is important in the PHB production from bacteria of *Cupriavidus necator* because insufficient of glucose for the growth of the bacteria will cause the bacteria to consume PHB as the source of Carbon. Then, the desired PHB production will reduce.

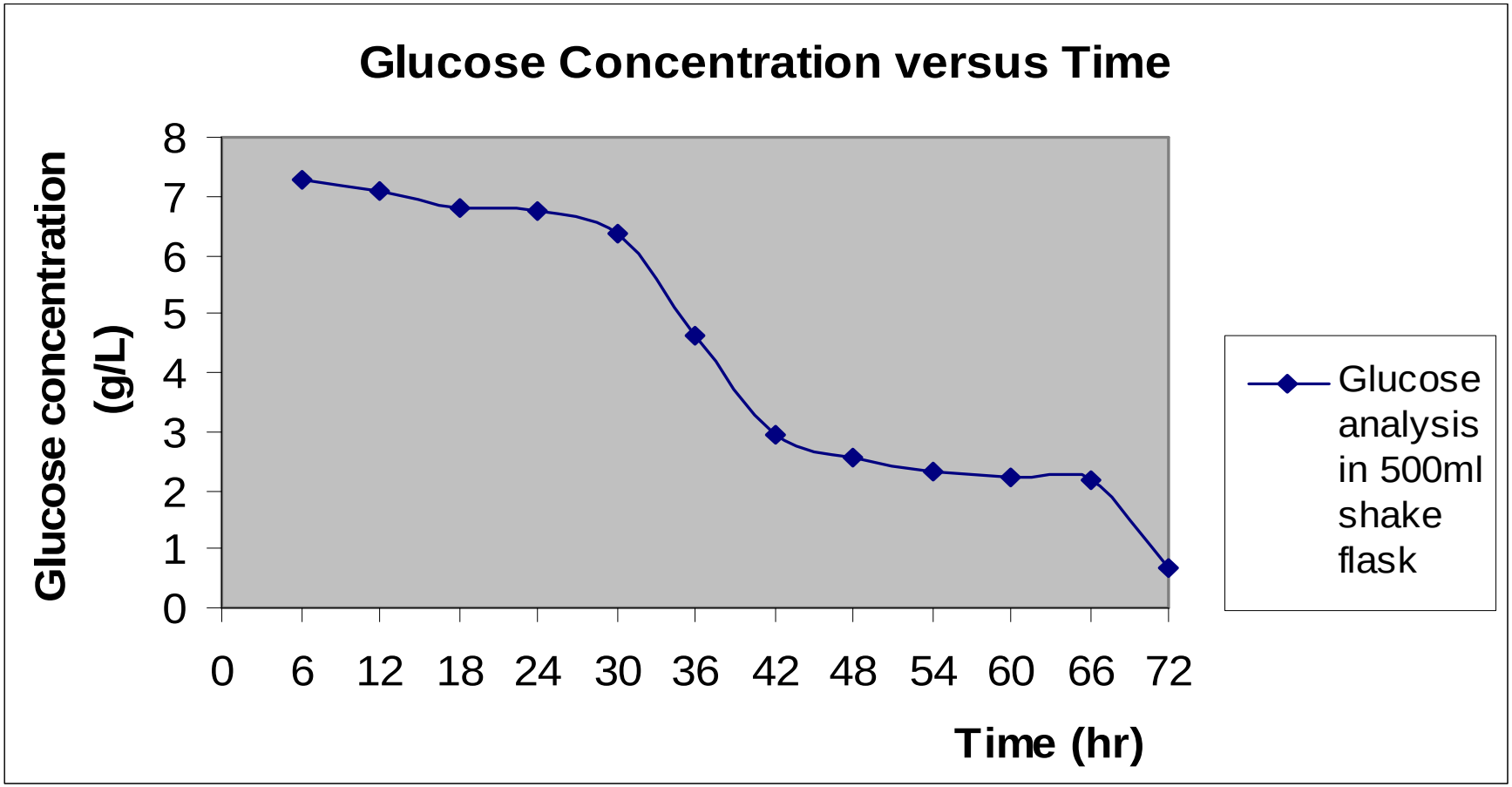

Figure 4.4 Glucose concentration versus time for 500ml shake flask

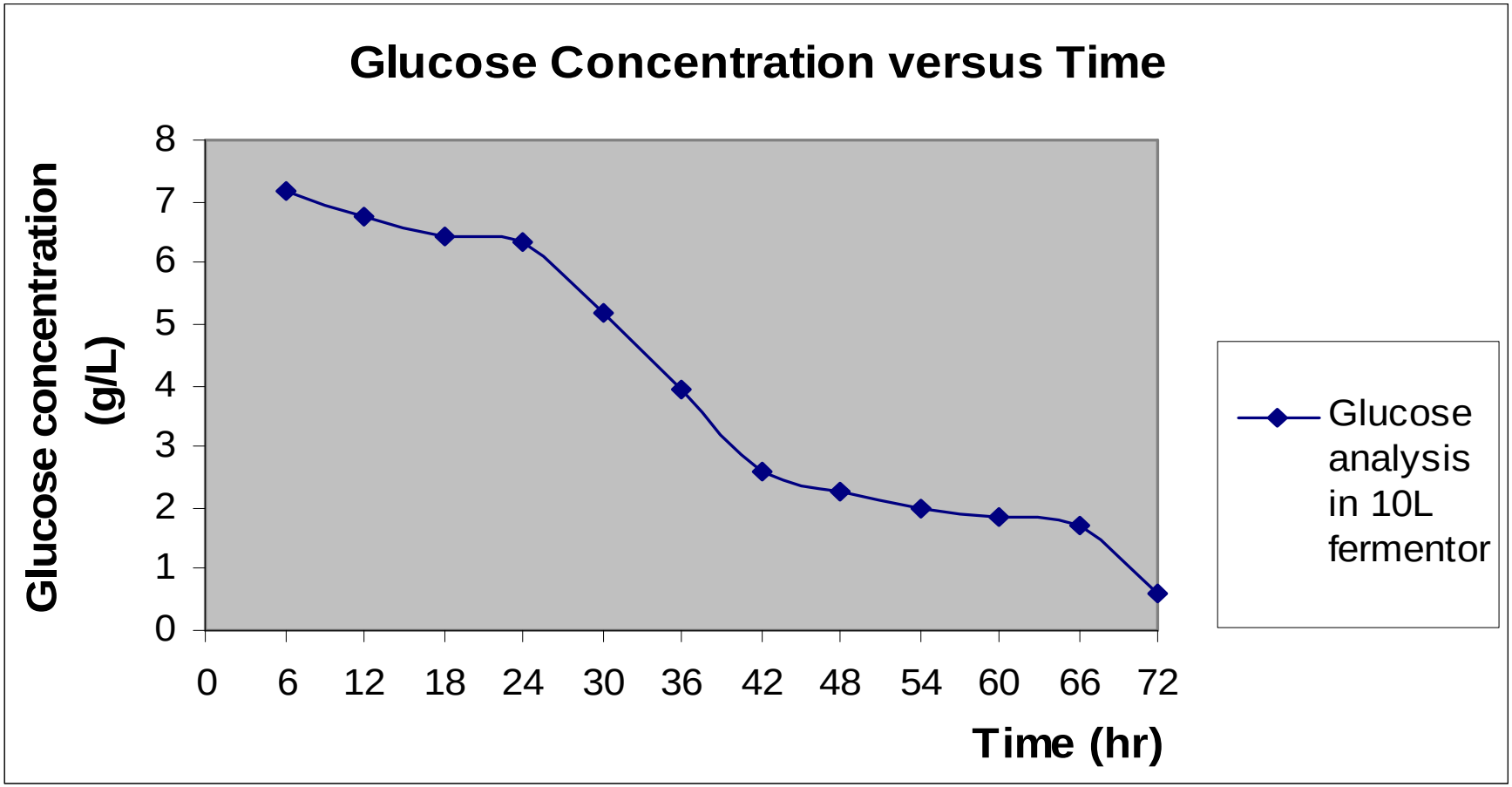

Figure 4.5 Glucose concentration versus time for 10L fementor

4.4 Dry Cell Weight Analysis

Dry cell weight or biomass content analysis was evaluated by the gravimetric method. Gravimetric analysis, by definition, includes all methods of analysis in which the final stage of the analysis involves weighing. The net weight of dry cells produced was in a sample of 10ml. The net weights were simply multiple by 100 to get the dry cell weight in gram per 1L.

For both the 500ml shake flasks and 10L bioreactor fermentations, the dry cells weights were increased from the beginning until the maximum of 60^{th} hours. Then, it was slightly decreased and constant after that until the end of 72^{nd} hours fermentation. The dry cells weight increased with increasing the duration of fermentation. From the curves, the cell mass increased rapidly at the 24^{th} to 30^{th} hours.

The maximum dry cell weight for the 500ml shake flask fermentation is 7.75g/L at 60^{th} hours. However, for the 10L bioreactor is 7.60 g/L at 60^{th} hours. There is 0.15 g/L decrease in dry cell weight when scaled up to the 10L fermentation. This is because of the factors such as aeration and agitation of the bioreactor that affect the oxygen transfer rate to the bacteria.

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

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

= (7.75 g cells / 1L medium) / (10-2.2408 g glucose / 1 L medium)

= 0.9988 g cells / g glucose

The highest biomass yield coefficient on glucose for 10L fermentor

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

= (7.60 g cells / 1L medium) / (10-1.8400 g glucose / 1L medium)

= 0.9314 g cells / g glucose

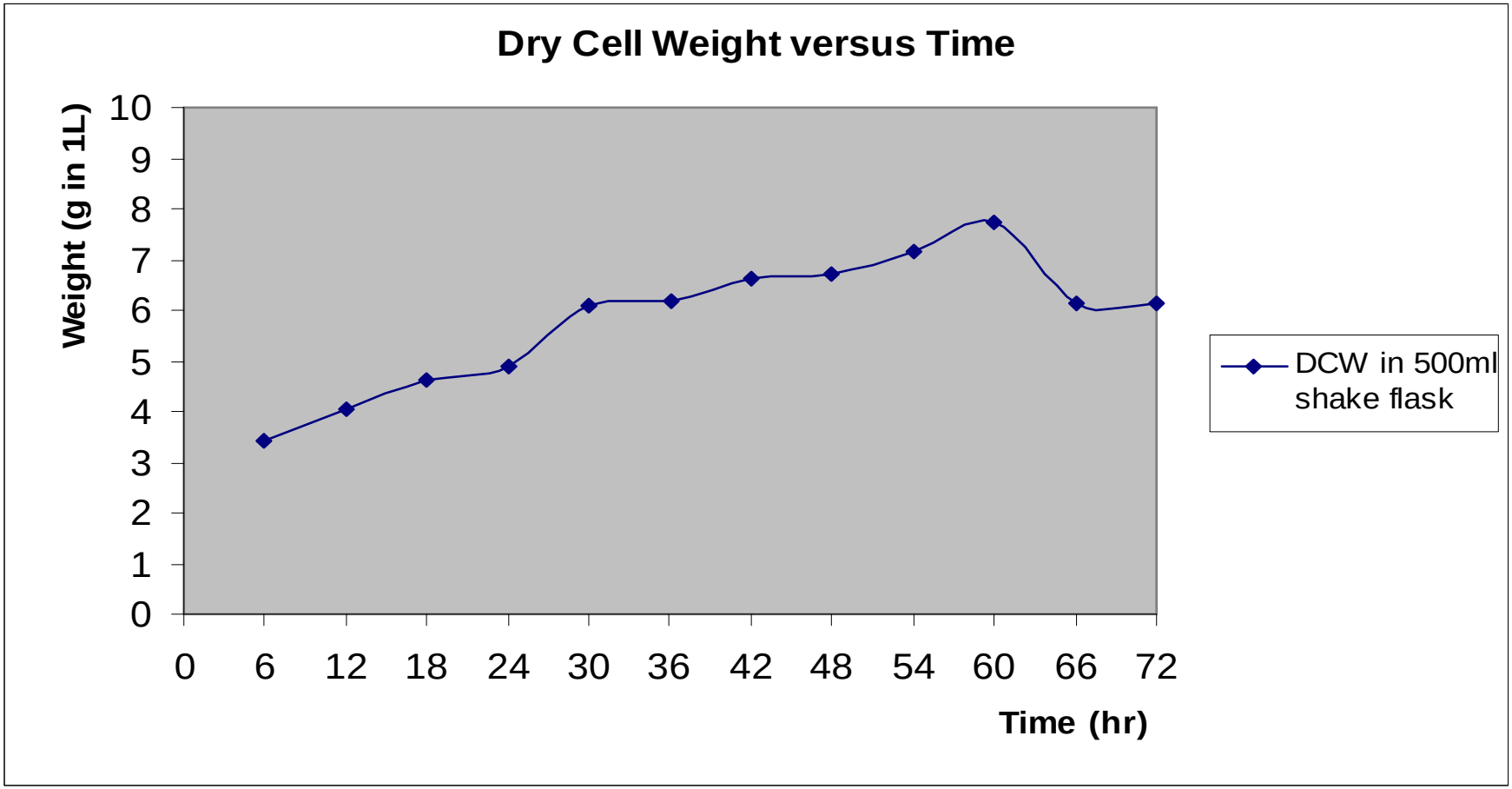

Figure 4.6 Dry cell weights versus time for 500ml shake flask

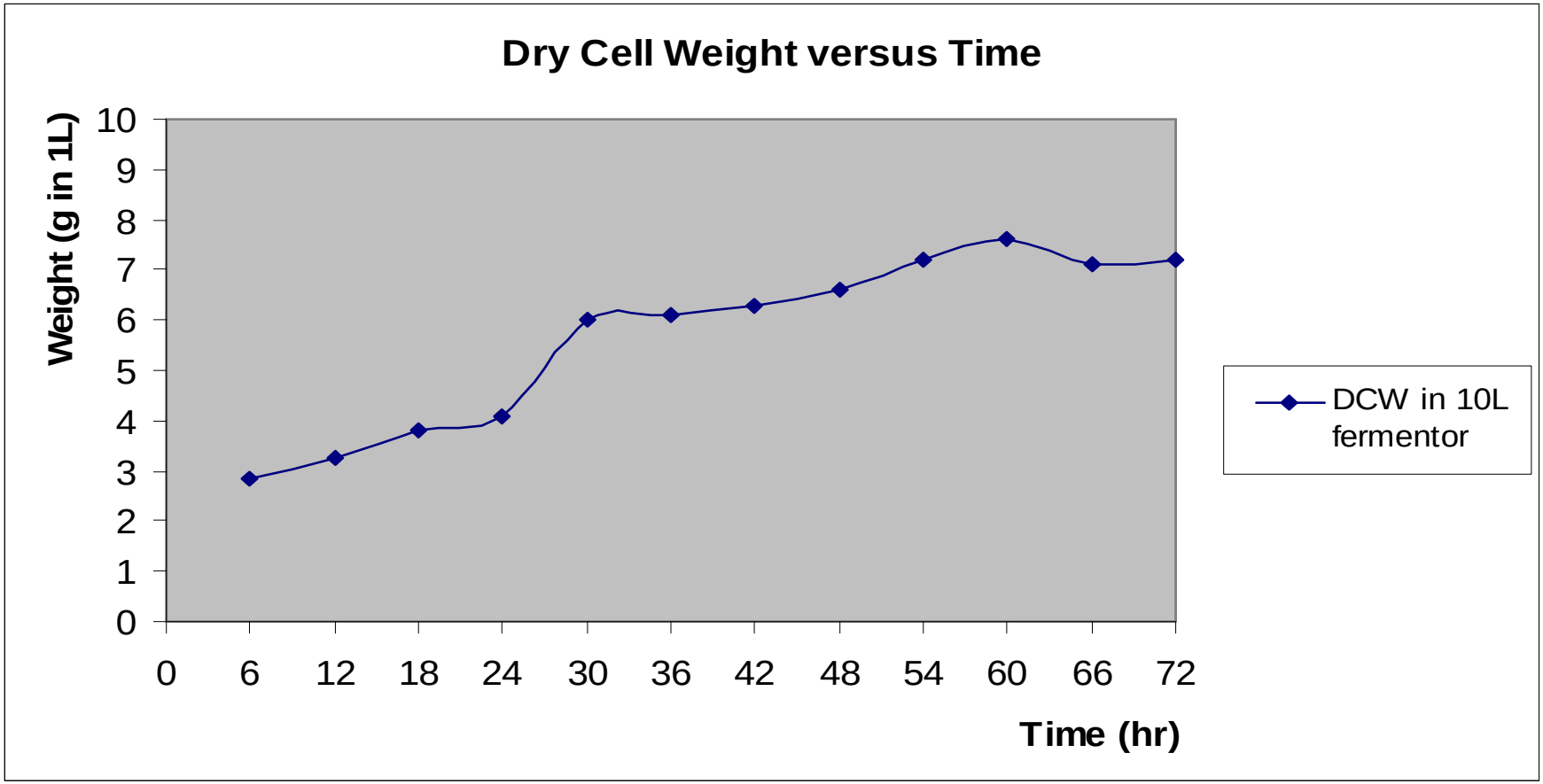

Figure 4.7 Dry cell weights versus time for 10L fermentor

4.5 PHB Analysis

This PHB analysis method was recommended by Dr Wiratni from Universiti Gadjah Mada (UGM), Indonesia. From the PHB concentration standard curve, the straight line with equation of y = 0.1492x was obtained. Y represented the optical density of the PHB samples for every six hours; however x represented the PHB concentration obtained. The PHB concentration was then multiplied by 10 because of 10 times dilution. During the analysis, natrium chloride was used to wash the cells of bacteria; hydrogen peroxide was used to lysis the bacteria cells wall and chloroform was used to extract the PHB from the bacteria.

From the graphs of PHB concentrations for 500ml shake flask and 10L bioreactor fermentation, clearly shown that the PHB concentrations were increased from beginning until the maximum at 54^{th} hours, then slightly decreased after that until 72^{nd} hours. The maximum PHB concentration for the 500ml shake flask fermentation is 1.0190g/L at 54^{th} hours, however for the 10L bioreactor fermentation is 1.0071g/L at 54^{th} hours. Compare with journal, the maximum PHB will achieve at 55^{th} to 60^{th} hours of fermentation. There are 0.0119 g/L of PHB concentration decreased when scaled up to 10 L fermentor.

The sources of glucose and nitrogen are the important factors that affect the yield of PHB. However, the oxygen transfer rate are the major factor to ensure the scale up meet the same yield in both shake flask scale and in 10L bioreactor scale. The compressed air flow rate and agitation rate of the bioreactor cause the PHB yield slightly lower than in the 500ml shake flask scale.

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

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

= (1.0190 g PHB / 1L medium) / (7.15 g dry cell / 1L medium)

= 0.1425 g PHB / g dry cell

The highest PHB yield coefficient on dry cell weight for 10L fermentor

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

= (1.0071 g PHB / 1L medium) / (7.20 g dry cell / 1L medium)

= 0.1399 g PHB / g dry cell

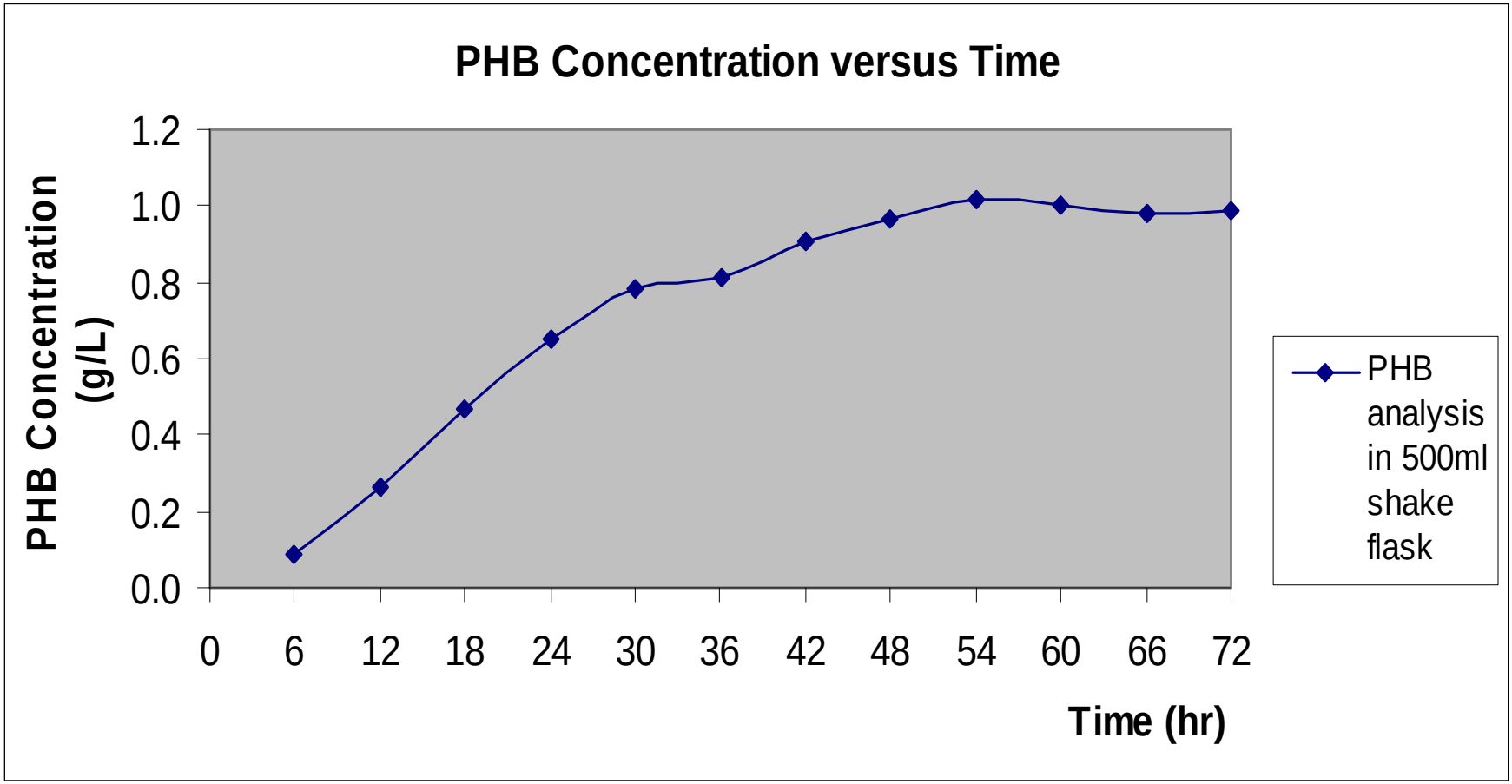

Figure 4.8 PHB concentration versus time for 500ml shake flask

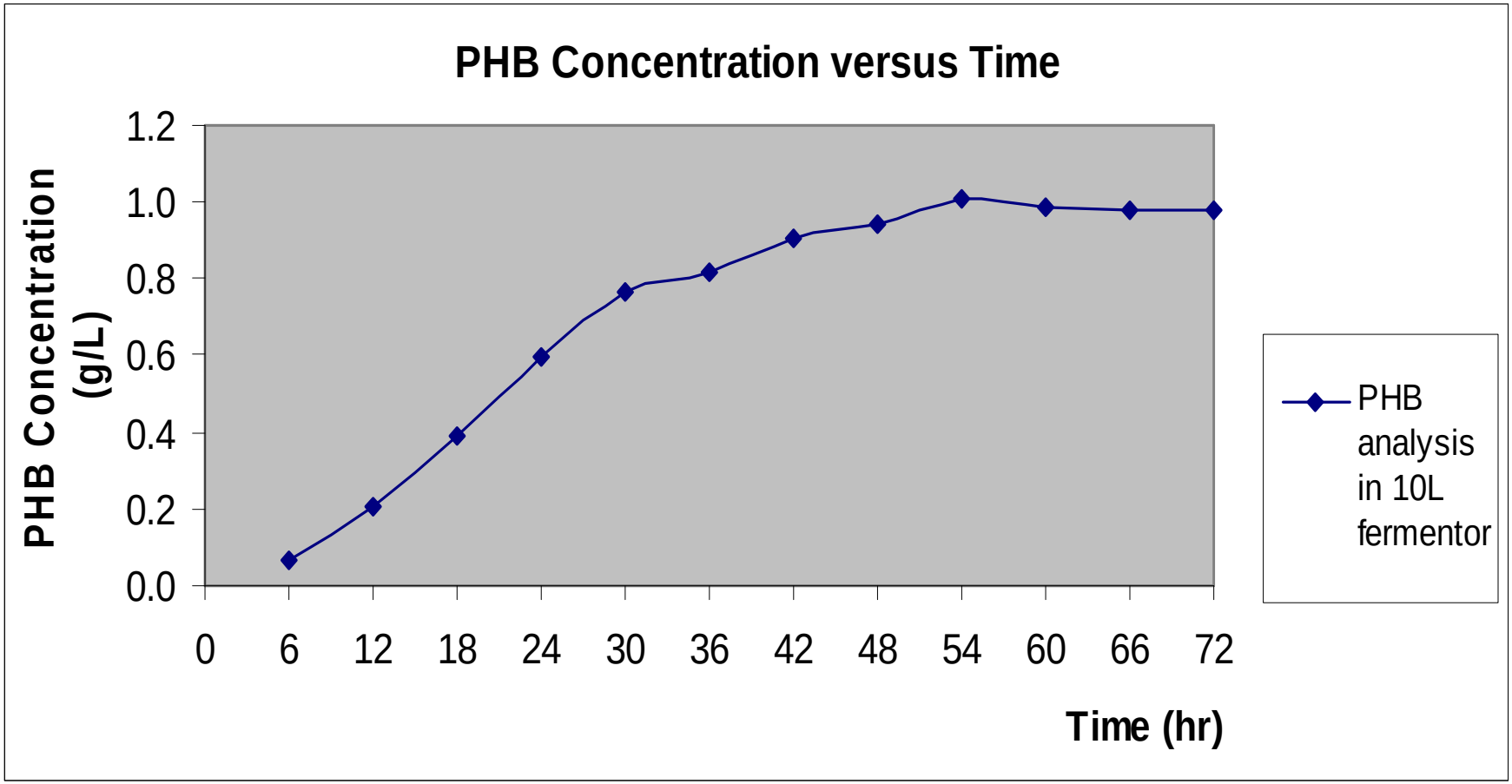

Figure 4.9 PHB concentration versus time for 10L fermentor

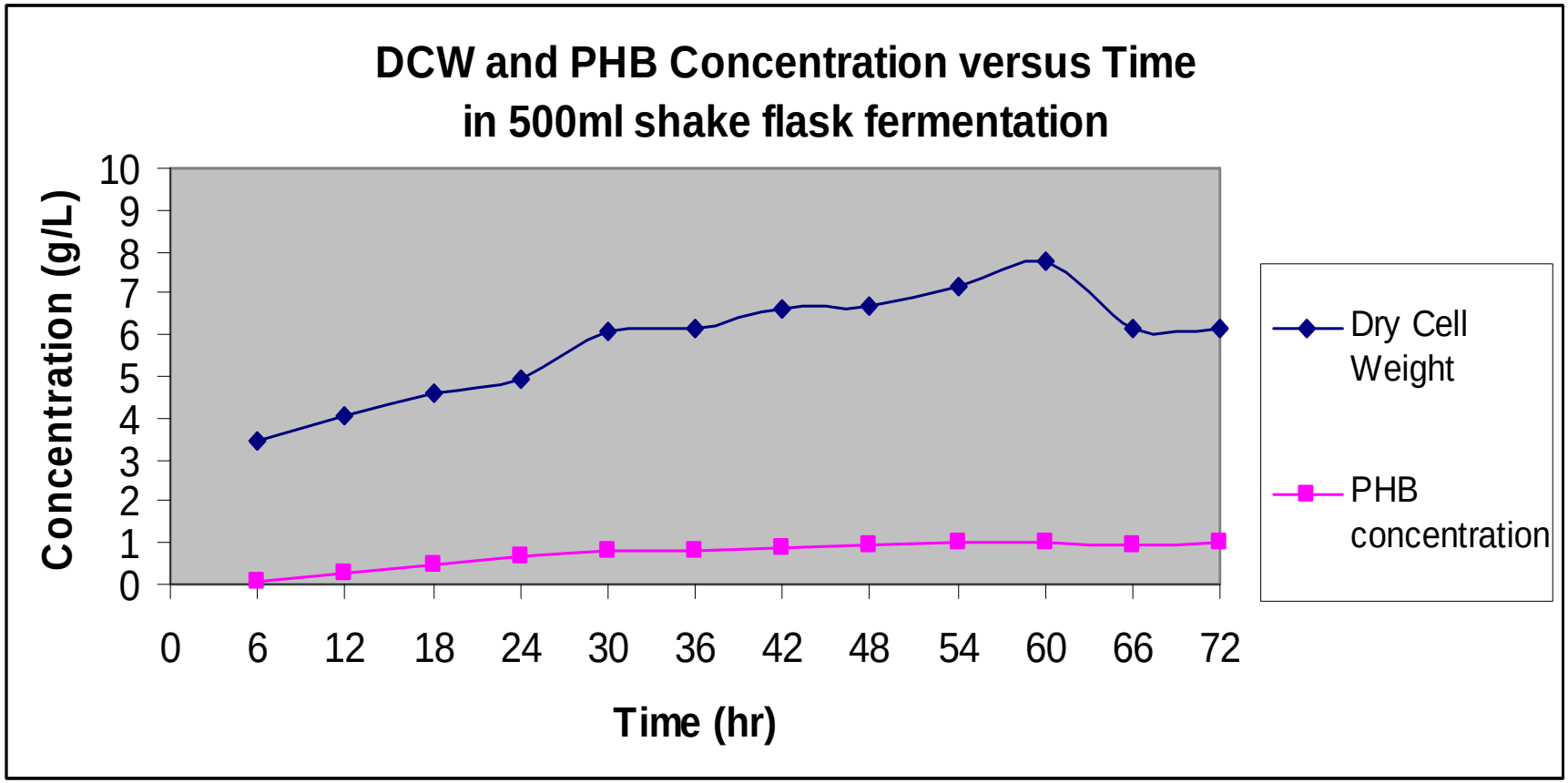

Figure 4.10 DCW and PHB concentration versus time for 500ml shake flask

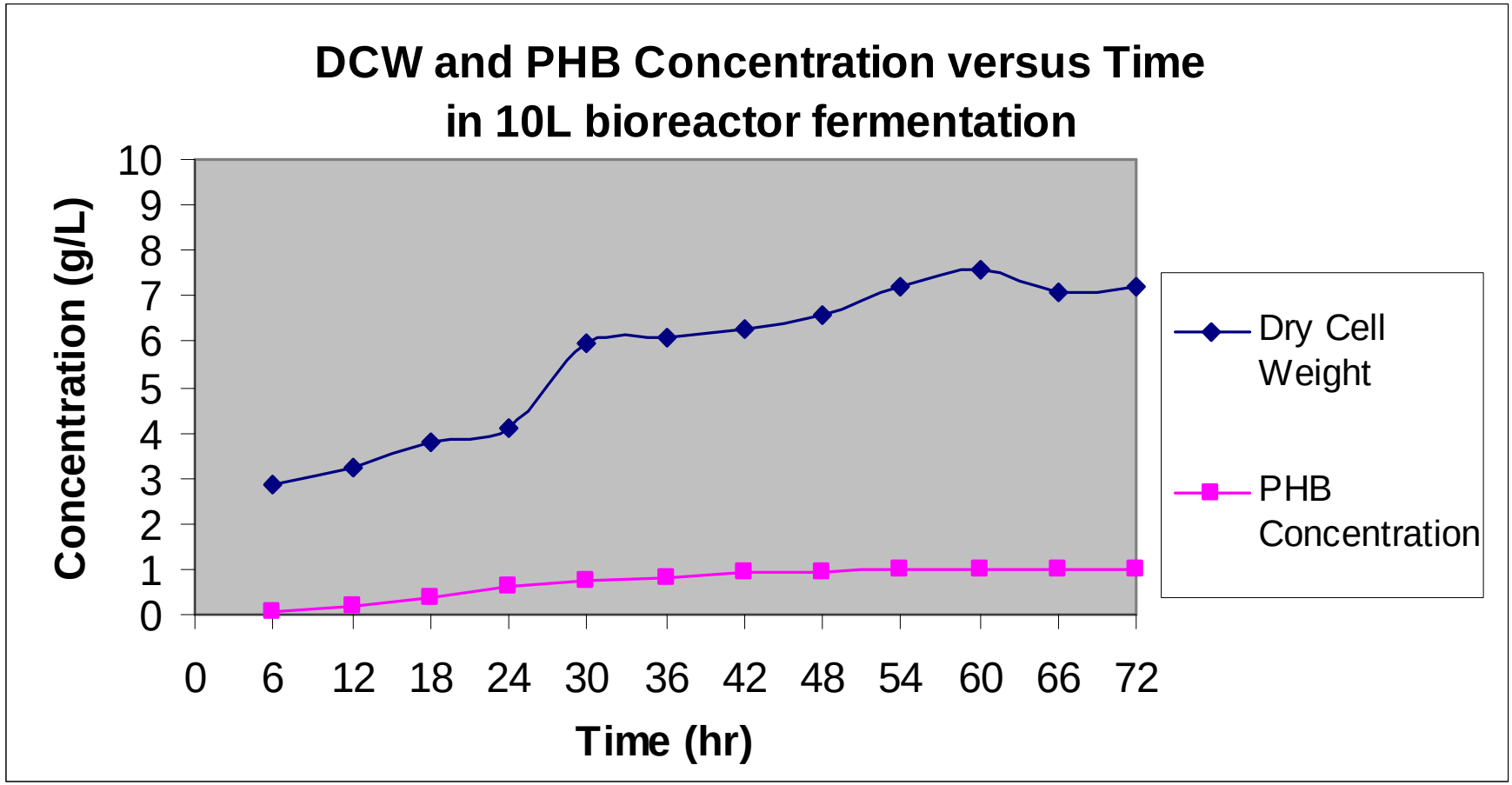

Figure 4.11 DCW and PHB concentration versus time for 10L

4.6 Comparison between DCW and PHB yield

The dry cell weight and PHB concentration yields had shown a big difference in both the shake flask and bioreactor scales. This is because the scale up had carried on none optimize condition of *Cupriavidus necator*. The optimize conditions of *Cupriavidus necator* can only be obtained once after completing the factors analysis and then optimization of the bacteria.

The result that obtained from the journal of *Fermentation optimization for the production of poly (β-hyroxybutyric acid) microbial thermoplastic* by Yusuf Chisti dated 10 February 1999, shows that when the conditions of *Cupriavidus necator* was optimized, that is the carbon to nitrogen ratio is 28.3, the maximum yield of PHB concentration was 4.5 g/L when the dry cell weight was 8 g/L. This means that 56.25% of PHB of its dry cell weight will produce if the carbon to nitrogen ratio is optimized.

Overall, the scale up was successful but with the PHB yield is not comparable if compare the result with the journal's result.

CHAPTER 5

CONCLUSION

Cell growth and biopolymer yield of *Cupriavidus necator* under aerobic fermentation is affected by oxygen transfer rate from air bubbles into the fermentation medium. The highest biopolymer yield at shake flask scale will obtain at optimum condition. The k_La values at both scales will increase with increasing of aeration and agitation rates. Scale up using the method of constant volumetric transfer coefficient (k_La) of oxygen will produce PHB yield comparable in both the shake flask and 10L stirred tank fermentor. From this research, the PHB concentration for shake flask fermentation is 1.0190 g/L and for the 10L fermentation is 1.0071 g/L. The objective of this research is achieved because there are only small differences in both scales, which is 0.0119 g/L or 1.1678%.

RECOMMENDATIONS

For the future research, it is proposed that the scale up may be carried out in the optimized conditions of production of PHB by *Cupriavidus necator*. A detailed research must be done, such as the significance of the sources of Carbon, Nitrogen, and Phosphate on the bacteria growth and yield. However, the agitation rate and aeration rate also are the important factors because the higher the agitation rate the higher the shear stress to the bacteria.

For the analysis method of PHB yield, it is suggested that for future researcher, they may try to get the PHB concentration by using the gravimetric method instead of reading the optical density by using UV-VIS spectrophotometer. This can compare which methods are more suitable to get the result.

REFERENCES

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.

Byrd, J. J., Zeph, L. R. and Casida, L. E. (1985). Bacterial control of Agromyces ramosus in soil. *Can J Microbiol* 31, 1157–1163.

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.

Casas, J. A., Santos, V. E., and Garcia-Ochoa, F. (2000). Xanthan gum production under several operational conditions: molecular structure and rheological properties. *Enzyme and Microbial Technology*, 26, 282–291.

Choi, J., Lee, S.Y., Han, K., 1998. Cloning of Alcaligenes latus polyhydroxyalkanoate bio-synthesis genes and use of these genes for enhanced production of poly(3-hydroxy butyrate) in *Escherichia coli. Appl. Environ. Microbiol*. 64, 4897–4903.

Diaz, A., and Acevedo, F. (1999). Scale-up strategy for bioreactors with Newtonian and non-Newtonian broths. *Bioprocess Engineering*, 21, 21–23.

Eickenbusch, H., Brunn, P.O., and Schumpe, A. (1995). Mass transfer into viscous pseudoplastic liquid in large-diameter bubble columns. *Chemical Engineering Process*, 34, 479–485.

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

Flores, E. R., Peres, F., and De La Torre, M. (1997). Scale-up of *Bacillus thuringensis* fermentation based on oxygen transfer. *Journal of Fermentation and Bioengineering*, 83(6), 561–564.

Galaction, A.I., Cascaval, D., Oniscu, C., and Turnea, M. (2004). Prediction of oxygen mass transfer coefficients in stirred bioreactors for bacteria, yeasts and fungus broths. *Biochemical Engineering Journal*, 20, 85–94.

Garcia Ochoa, F., Gomez Castro, E., and Santos, V. E. (2000). Oxygen transfer and uptake rates during xanthan gum production. *Enzyme and Microbial Technology*, 27, 680–690.

Gibbs, P. A., and Seviour, R. J. (1996). Does the agitation rate and/or oxygen saturation influence exopolysaccharide production by *Aureobasidium pullulans* in batch culture. *Applied Microbial and Biotechnology*, 46, 503–510.

Kobayashi, Y., Okamoto, A., and Nishinari, K. (1994). Viscoelasticity of hyaluronic acid with different molecular weights. *Biorheology*, 31(3), 235–244.

Kouda, T., Yano, H., and Yoshinaga, F. (1997). Effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture. *Journal of Fermentation and Bioengineering*, 38(4), 371–376.

Kurane, R., Hatamochi, K., Kakuno, T., Kiyoshara, M., Hirano, M., and Taniguchi, Y. (1994). Production of a bioflocculant by *Rhodococcus erythropolis* S-1 grown on alcohols. *Bioscience, Biotechnology, and Biochemistry*, 58(2), 428–429.

Kwon, G. S., Moon, S. H., Hong, S. D., Lee, H. M., Mheen, T. I., Oh, H. M., et al. (1996). Rheological properties of extracellular polysaccharide, Pestan, produced by *Pestalotiopsis sp*. *Biotechnology Letters*, 18(12), 1465–1470.

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

Lo, Y. M., Hsu, C. H., Yang, S. T., and Min, D. B. (2001). Oxygen transfer characteristics of a centrifugal, packed-bed reactor during viscous xanthan fermentation. Bioprocess and Biosystems Engineering. *Springer Verlag, pp* 1–16.

Makkar, N. S. & Casida, L. E., Jr (1987). *Cupriavidus necator* gen. nov., sp. nov a nonobligate bacterial predator of bacteria in soil. *Int J System Bacteriol* 37, 323–326.

Miura, S., Arimura, T., Hoshino, M., Kojima, M., Dwiarti, L., and Okabe, M. (2003). 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. Nakayama, K. (1981). Microbial aspects of scale-up. In H.-J. Rehm & G. Reed (Eds.), Biotechnology: Volume 1 Microbial fundamentals (pp. 401–408). Weinheim: Verlag Chemie. Sutherland, I W. (1998). *Novel and established applications of microbial polysaccharides. Trade in Biotechnology*, 16, 41–46.

Nakayama, K. (1981). Microbial aspects of scale-up. In H.-J. Rehm and G. Reed (Eds.), *Biotechnology: Volume 1 Microbial fundamentals* (pp. 401–408). Weinheim: Verlag Chemie.

Pfeffer, J.T., 1992. *Recycling and Solid Waste Management Engineering*, 72–84.

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

Serafim, L.S., Lemos, P.C., Oliveira, R., Reis, M.A.M., 2004. Optimization of polyhydroxy butyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. *Biotechnology Egineering* 45–160.

Sillman, C. E.Casida, L. E. (1986). Isolation of non-obligate bacterial predators from soil. *Can J Microbiology*, 760–762.

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

Thiry, M., and Cingolani, D. (2002). Optimizing scale-up fermentation rocesses. *Trends in Biotechnology*, 20(3), 103–105. Tuffile, C. M., & Pinho, F. (1970). Determination of oxygen transfer coefficients in viscous *Streptomycetes* fermentations. *Biotechnology and Bioengineering*, 12, 849–871.

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

Vaneechoutte, M., Kampfer, P., De Baere, T., Falsen, E. and Verschraegen, G. (2004). *Wautersia gen. nov*., a novel genus accommodating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of Ralstonia *[Pseudomonas] syzygii* (Roberts et al. 1990)

Wang, F., Lee, S.Y., 1997. Production of poly(3-hydroxybutyrate) by fedbatch culture of filamentation suppressed recombinant *Escherichia coli. Appl. Environ. Microbiol*. 63, 4765–4769.

Wernersson, E. S., and Tragardh, C. (1998). Scaling of turbulence characteristics in a turbine-agitated tank in relation to agitation rate. *Journal of Chemical Engineering*, 70, 37–45.

Weuster-Botz, D., Hnnekes, E., and Hartbrich, A. (1998). Scale-up and application of a cyclone reactor for fermentation processes. *Bioprocess Engineering*, 18(6), 433–436.

Winkler, M. A. (1983). Scale-up in biotechnology. In A. Winkler (Ed.), *Principles in Biotechnology* (pp. 134–136). New York: Surrey University Press.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. & Nishiuchi, Y. (1995). Transfer of two Burkholderia and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. *Novel microbiol Immunol* 39, 897–904.

Yabuuchi, E., Kawamura, Y., Kosako, Y. and Ezaki, T. (1998). Emendation of the genus *Achromobacter* and *Achromobacter xylosoxidans* (Yabuuchi and Yano) and proposal of *Achromobacter ruhlandii* (Packer and Vishniac) comb. nov., *Achromobacter piechaudii* (Kiredjian et al.) comb. nov., and *Achromobacter xylosoxidans* subsp. denitrificans (Ru¨ger and Tan) comb. nov. Microbiol Immunol 42, 429–438.

Yuh-Lih, H., and Wen-Teng (2002). A novel approach for scaling-up a fermentation system. *Biochemistry Engineering Journal*, 11, 123–130.

Zeph, L. R. and Casida, L. E. (1986). Gram-negative versus Gram positive (*actinomycete*) bacterial predators of bacteria in soil. *Applied Environmental Microbiol* 52, 819–823.

APPENDIX A

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

500ml shake flask with 200ml distilled water
Compressed air flow rate = 1L/min
Agitation = 200 rpm
Temperature = 23.9 ˚C

Table : DOT (%) and concentration data for 500ml shake flask

Time (minutes)	DOT (%)	Concentration (ppm)
0	0.00	0.00
0.5	0.09	0.01
1	2.51	0.20
1.5	9.08	0.74
2	18.76	1.52
2.5	36.30	2.95
3	47.71	3.88
3.5	57.48	4.67
4	64.05	5.21
4.5	68.89	5.60
5	73.47	5.97
5.5	76.49	6.22
6	79.08	6.43
6.5	80.55	6.55
7	82.46	6.70
7.5	84.10	6.84
8	85.39	6.94
8.5	86.78	7.05
9	87.55	7.12
9.5	88.42	7.19
10	89.28	7.26
10.5	89.72	7.29
11	90.49	7.36
11.5	91.01	7.40
12	91.53	7.44
12.5	92.05	7.48
13	92.31	7.50
13.5	92.83	7.55
14	93.09	7.57
14.5	93.35	7.59
15	93.60	7.61
16	93.95	7.64
17	94.56	7.69
18	94.81	7.71
19	95.16	7.74
20	95.51	7.76

21	95.77	7.79
22	96.02	7.81
23	96.28	7.83
24	96.46	7.84
25	96.63	7.86
26	96.72	7.86
27	96.98	7.88
28	97.06	7.89
29	97.58	7.93
30	97.75	7.95
32	97.84	7.95
34	98.01	7.97
36	98.27	7.99
38	98.53	8.01
40	98.62	8.02
44	98.96	8.05
48	99.22	8.07
52	99.48	8.09
56	99.65	8.10
60	100.00	8.13
64	100.00	8.13

APPENDIX B

DOT (%) and Concentration Data for 10L Fermentor

10L fermentor with 8L distilled water
Compressed air flow rate = 1L/min
Agitation = 310 rpm
Temperature = 24 ˚C

Table : DOT (%) and concentration data for 10L fermentor

Time (minutes)	DOT (%)	Concentration (ppm)
0	0.00	0.00
0.5	0.34	0.03
1	3.67	0.30
1.5	5.45	0.44
2	10.35	0.84
2.5	23.46	1.91
3	34.51	2.81
3.5	45.52	3.70
4	55.45	4.51
4.5	62.34	5.07
5	65.50	5.33
5.5	68.90	5.60
6	72.87	5.92
6.5	75.67	6.15
7	79.62	6.47
7.5	82.67	6.72
8	83.48	6.79
8.5	85.11	6.92
9	85.90	6.98
9.5	87.10	7.08
10	89.09	7.24
10.5	89.14	7.25
11	90.11	7.33
11.5	91.00	7.40
12	91.34	7.43
12.5	91.98	7.48
13	92.10	7.49
13.5	92.63	7.53
14	92.99	7.56
14.5	93.21	7.58
15	93.52	7.60
16	93.76	7.62
17	94.45	7.68
18	94.76	7.70
19	95.26	7.74
20	95.52	7.77

21	95.76	7.79
22	96.05	7.81
23	96.30	7.83
24	96.46	7.84
25	96.67	7.86
26	96.72	7.86
27	97.00	7.89
28	97.08	7.89
29	97.63	7.94
30	97.75	7.95
32	97.84	7.95
34	98.03	7.97
36	98.45	8.00
38	98.53	8.01
40	98.62	8.02
44	98.65	8.02
48	99.01	8.05
52	99.48	8.09
56	99.54	8.09
60	100.00	8.13
64	100.00	8.13

APPENDIX C

MATLAB Window Command for 500ml Shake Flask

```
>> t = (0:4:64)';
>> y = [0 64.04 85.39 91.53 93.95 95.51 96.46 97.06 97.84 98.27 98.62 98.96 99.22
99.48 99.65 100 100]';
>> 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 =

    0.2809
    0.0010
```

APPENDIX D

MATLAB Window Command for 10L Fermentor

```
>> t = (0:4:64)';
>> y = [0 52.45 83.48 91.34 93.76 95.52 96.46 97.08 97.84 98.45 98.62 98.65 99.01
99.48 99.54 100.00 100.00]';
>> 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 =

    0.2171
    0.0005
```

APPENDIX E

Glucose Analysis Data

1. Glucose concentration for *cupriavidus necator* in 500ml shakes flask fermentation

   y = 0.9109x

Table : Glucose analysis for 500ml shakes flask fermentation

Time (hr)	Optical density, x	Concentration, y (in 1ml)	Concentration, y (10X dilution)
6	0.800	0.7287	7.2872
12	0.778	0.7087	7.0868
18	0.747	0.6804	6.8044
24	0.742	0.6759	6.7589
30	0.700	0.6376	6.3763
36	0.507	0.4618	4.6183
42	0.325	0.2960	2.9604
48	0.281	0.2560	2.5596
54	0.254	0.2314	2.3137
60	0.246	0.2241	2.2408
66	0.240	0.2186	2.1862
72	0.074	0.0674	0.0641

2. Glucose analysis for *cupriavidus necator* in 10L bioreactor fermentation

Table : Glucose analysis for 10L bioreactor fermentation

Time (hr)	Optical density, x	Concentration, y (in 1ml)	Concentration, y (10X dilution)
6	0.785	0.7151	7.1506
12	0.742	0.6759	6.7589
18	0.704	0.6413	6.4127
24	0.696	0.6340	6.3399
30	0.567	0.5165	5.1648
36	0.430	0.3917	3.9169
42	0.286	0.2605	2.6052
48	0.247	0.2250	2.2499
54	0.218	0.1986	1.9858
60	0.202	0.1840	1.8400
66	0.186	0.1694	1.6943
72	0.068	0.0619	0.6194

APPENDIX F

Dry Cell Weight Data

1. Dry cell weights for *Cupriavidus necator* in 500ml shake flask fermentation

Table : Dry cell weight for 500ml shakes flask fementation

Time (hr)	Filter paper + dry cells	Filter paper	Net DCW (g in 10 ml)	Dry cell weight (g in 1L)
6	2.1325	2.0982	0.0343	3.43
12	2.0988	2.0585	0.0403	4.03
18	2.1032	2.0570	0.0462	4.62
24	2.0977	2.0486	0.0491	4.91
30	2.1225	2.0617	0.0608	6.08
36	2.0954	2.0338	0.0616	6.16
42	2.0541	1.9881	0.0660	6.60
48	2.1034	2.0363	0.0671	6.71
54	2.0990	2.0275	0.0715	7.15
60	2.0930	2.0155	0.0775	7.15
66	2.1241	2.0629	0.0612	6.12
72	2.0744	2.0132	0.0612	6.12

2. Dry cell weights for *Cupriavidus necator* in 10L bioreactor fermentation

Table : Dry cell weight for 10L bioreactor fermentation

Time (hr)	Filter paper + dry cells	Filter paper	Net DCW (g in 10 ml)	Dry cell weight (g in 1L)
6	2.1345	2.1061	0.0284	2.84
12	2.1563	2.1239	0.0324	3.24
18	2.1373	2.0993	0.0380	3.80
24	2.1498	2.1088	0.0410	4.10
30	2.1459	2.0860	0.0599	5.99
36	2.1508	2.0898	0.0610	6.10
42	2.0978	2.0348	0.0630	6.30
48	2.1299	2.0639	0.0660	6.60
54	2.1345	2.0625	0.0720	7.20
60	2.1422	2.0662	0.0760	7.60
66	2.1478	2.0768	0.0710	7.10
72	2.1388	2.0668	0.0720	7.20

APPENDIX G

PHB Analysis Data

1. PHB concentration for *Cupriavidus necator* in 500ml shake flask fermentation

   $y = 0.1492x$

Table : PHB analysis for 500ml shakes flask fermentation

Time (hr)	Optical density, x	Concentration, y (g in 10ml)	Concentration, y (10X dilution)
6	0.060	0.0090	0.0895
12	0.175	0.0261	0.2611
18	0.312	0.0466	0.4655
24	0.437	0.0652	0.6520
30	0.523	0.0780	0.7803
36	0.543	0.0810	0.8102
42	0.610	0.0910	0.9101
48	0.649	0.0968	0.9683
54	0.683	0.1019	1.0190
60	0.670	0.1000	0.9996
66	0.656	0.0979	0.9788
72	0.664	0.0991	0.9907

2. PHB concentration for *Cupriavidus necator* in 10L bioreactor fermentation

Table : PHB analysis for 10L bioreactor fermentation

Time (hr)	Optical density, x	Concentration, y (mg in 10ml)	Concentration, y (10X dilution)
6	0.045	0.0067	0.0671
12	0.136	0.0203	0.2029
18	0.260	0.0388	0.3879
24	0.402	0.0600	0.5998
30	0.511	0.0762	0.7624
36	0.550	0.0821	0.8206
42	0.608	0.0907	0.9071
48	0.633	0.0944	0.9444
54	0.675	0.1007	1.0071
60	0.660	0.0985	0.9847
66	0.654	0.0976	0.9758
72	0.657	0.0980	0.9802

APPENDIX H

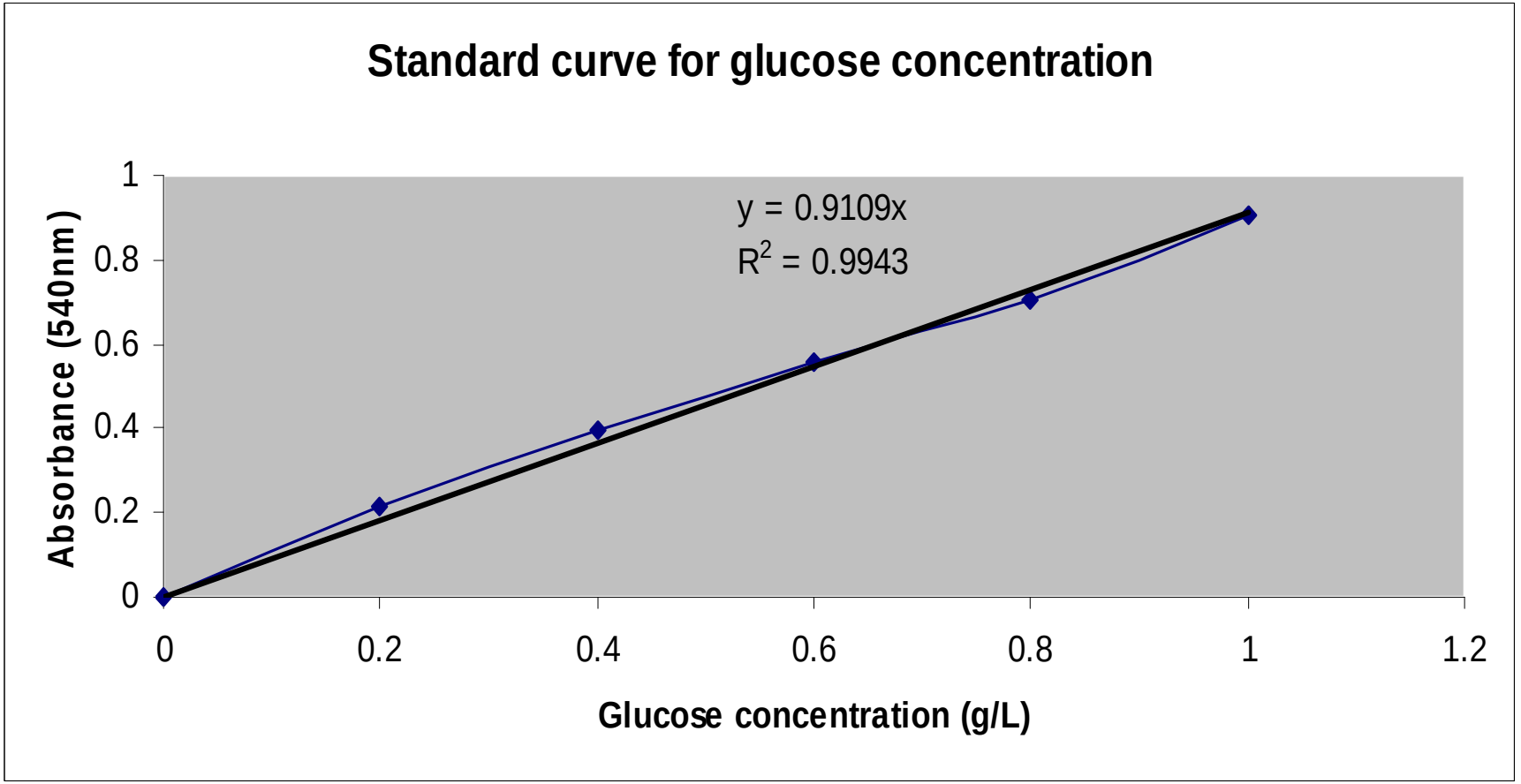

Figure Standard curve for glucose concentration

APPENDIX I

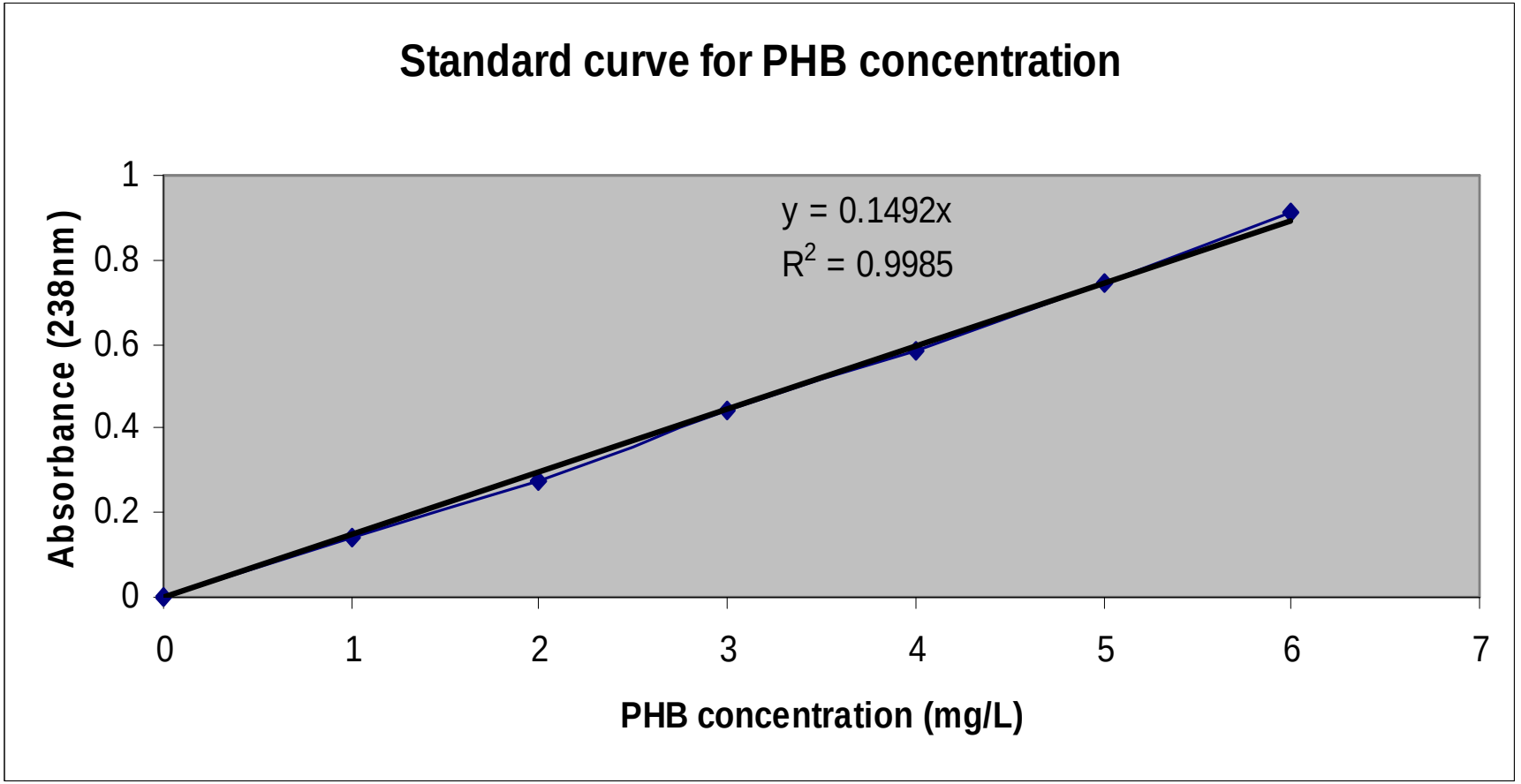

Figure Standard curve for PHB concentration