OPTIMISATION AND SCALE-UP OF PRODUCTION OF BIODEGRADABLE POLYMER IN STIRRED TANK FERMENTATION

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ABSTRACT

Polyhydroxybutyrates (PHB) are natural, biodegradable polymers which are accumulated as an energy reserve material by a large number of bacteria when nutrient such as nitrogen source is available in limiting concentrations in the present of excess carbon source. The major problem associated with the industrial production of PHB is its high production cost. In the present study, effort was made to screen out the variables that influence the production of biomass and PHB by the bacteria *Cupriavidus necator* in shake-flask fermentations using the method of Factor Analysis. The variables studied are the concentrations of glucose, $CaCl_2.2H_2O$, trace element, $(NH_4)_2SO_4$ and $Na_2HPO_4.7H_2O$, and agitation and temperature. The levels of variables that were found to have significant influence on the production of biomass and PHB were then optimized in shake-flask fermentations using the methods of factorial experiments and composite design. The optimized shake-flask fermentation was then scaled-up to 10L stirred tank fermentation using the method of constant volumetric mass transfer coefficient of oxygen (k_La) at both scales. The k_La value was derived by fitting the mass transfer equation to the data of dissolved oxygen concentration [which was derived from the data of dissolved oxygen tension (DOT)] versus time on computer using the Simplex Method on Matlab with two unknowns, namely k_La and the electrode mass transfer coefficient (ka_p) of oxygen. The stirrer speed (rpm) and the air flow rate (A) in the 10L fermentor that produced the same value of k_La found in the optimized conditions in shake-flask was approximated by trial and error.

ABSTRAK

Polyhydroxybutyrates (PHB) adalah polimer yang mempunyai sifat mudah terurai dengan adanya aktiviti mikroorganisma (*Cupriavidus necator*). PHB berperanan sebagai pembekal tenaga kepada mikrooganisma apabila nutrin seperti nitrogen berada dalam keadaan terhad manakala kehadiran karbon dalam keadaan berlebihan. Faktor utama yang menghadkan pengeluaran PHB dalam industri ialah kos untuk menghasilkan PHB terlalu tinggi berbanding dengan polimer berasakan petrokimia. Oleh sebab itu, banyak kajian yang telah dibuat untuk mengurangkan kos penghasilan biopolimer ini. Dalam kajian ini, kaedah analisis faktor telah digunakan untuk menapis pembolehubah-pembolehubah yang memberi kesan kepada penghasilan biojisim dan PHB dalam fermentasi kelalang goncang. Pembolehubah-pembolehubah yang di kaji kesannya ialah adalah gula, $CaCl_2.2H_2O$, unsur penyurih, $(NH_4)_2SO_4$ dan $Na_2HPO_4.7H_2O$ serta suhu dan kadar goncang. Seterusnya, aras pembolehubah-pembolehubah yang mempunyai kesan yang signifikan terhadap penghasilan biojisim dan PHB telah dioptimumkan menggunakan kaedah ujikaji faktorial dan rekabentuk komposit. Kemudian fermentasi kelalang goncang yang telah dioptimumkan itu telah diskalanaik kepada fermenter tangki teraduk isipadu 10L menggunakan kaedah pekali perpindahan isipadu oksigen (k_La) tetap pada kedua-dua skala. Nilai-nilai k_La diterbitkan dengan memadankan persamaan permindahan jisim kapada data kepekatan oksigen larut [(yang diterbitkan daripada data tekanan oksigen terlarut (DOT)] menentang masa menggunakan Kaedah Simplex daripada Matlab dengan dua pembolehubah iaitu k_La dan pekali permindahan jisim elektrod (ka_p) bagi oksigen. 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.

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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 Research

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. Global Environmental Pollution has become a serious issue nowadays. The relationships among all the living and non-living 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 part 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.

Polyhydroxybutyrates (PHB) is thermoplastic that widely produced by many bacteria such as *Protomonas extroquens*, *Candida utilis* ATCC 8205, *Azetobacter vinelandii* and *Cupriavidus necator* which is accumulated in the form of intracellular granules. This granules act as energy reserve materials when nutrient such as

nitrogen and phosphorus sources are available in limiting concentrations in the present of excess carbon source.

PHB is biodegradable, biocompatible and has similar physical properties to polypropylene. The use of PHB as biodegradable plastic is desirable since the disposal of non-biodegradable plastics, after they are used, causes significant ecological problems. The availability of landfills is limited and the incineration of plastic increases greenhouse gases and releases toxic compounds (Howells, 1972).

This research is associated with *Cupriavidus necator,* since it accumulate PHB more than any other wild type microbes (Kim et al. 1994).

1.2 Problem Statement

Currently the main problem, which limits the widespread use of PHB and its copolymers, is its relatively high cost compared with plastics based on petrochemicals. One of the major factors adding to the cost of PHB is the cost in product recovery in fermentation process and substrates used for production. Several studies have tackled this problem using different approaches. Some researcher have focus on reducing cost by optimizing fermentation processes of *Cupriavidus necator,* expressing the operon responsible for PHB production in other organism such as *Escherichia coli* (Howells, 1972). Another approach is to minimize the consumption of glucose in order to optimize the usage. Therefore, less expensive substrates, improved cultivation strategies and easier downstream processing methods are required for reducing the cost. Thus, utilization of media containing cheaper carbon and nitrogen sources should be used to reduce the production cost of PHB (Brom, 1987).

1.3 Objective of Research

The objective of this study is to optimize the fermentation for the production of PHB and to scale-up the optimized fermentation.

1.4 Scopes of Research

The scopes of this research are (1) to screen and select the variables relevant to the PHB yield in fermentation from among the variables concentrations of glucose, $CaCl_2.2H_2O$, trace element, $(NH_4)_2SO_4$ and $Na_2HPO_4.7H_2O$) and agitation and temperature, (2) to optimize the levels of the relevant variables, and (3) to scale-up the optimized fermentation.

CHAPTER 2

LITERATURE REVIEW

2.1 Types of Biodegradable Plastics

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, 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).

PHAs are produced by a wide range of bacteria when they find themselves in an environment with an available carbon source but limited in additional nutrient(s) required for growth. The short-chain-length PHAs, where R is a methyl or ethyl, have properties of thermoplastics and are biodegradable (Figure 2.1).

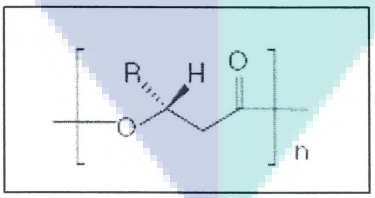

Figure 2.1: Poly (3-hydroxyalkanoates)

2.2 What is PHB?

The simplest and most commonly occurring form of PHA is the fermentation produced polyhydroxybutyrates (PHB). 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 the 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).

As PHBs are insoluble in water, the polymers are accumulated in intracellular granules inside the cells. It is advantageous for bacteria to store excess nutrients inside their cells, especially as their general physiological fitness is not affected. By polymerizing soluble intermediates into insoluble molecules, the cell does not undergo alterations of its osmotic state. Thus, leakage of these valuable compounds out of the cell is prevented and the nutrient stores will remain securely available at a low maintenance cost (Peters and Rehm, 2005).

2.3 Chemical structure of PHA's

Besides PHB, there are many other PHAs composed of 3- hydroxy fatty acids. The pendant group (R) varies from methyl (C_1) to tridecyl (C_{13}). Fatty acids with the hydroxy group at position 4, 5 or 6 and pendant groups containing substituents or instaurations are also known. Within bacterial metabolism, carbon substrates are converted into hydroxyacyl-CoA thioesters. As shown in Figure 2.2, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer. This polymerization reaction is catalysed by the host's PHA synthase.

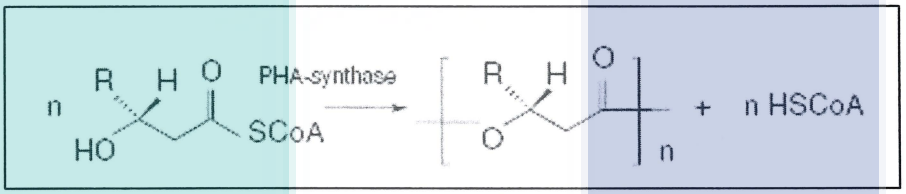

Figure 2.2: Synthesis of PHAs in bacteria hydroxyacyl-CoA thioesters as precursor.

In all PHAs that have been characterized so far, the hydroxyl-substituted carbon atom is of the stereochemica (R)-configuration. There is an enormous variation possible in the length and composition of the side chains. This variation makes the PHA polymer family suitable for an array of potential applications. The most common are shown in Table 2.1.

Table 2.1: PHAs and corresponding R-groups

R-Group	Full name	Short
CH3	Poly (3-hydroxybutyrate)	PHB
CH2CH3	Poly (3-hydroxyvalerate)	PHV
CH2CH2CH3	Poly (3-hydroxyhexanoate)	PHHx

2.4 Physical properties of PHAs

The material characteristics of these biopolymers are similar to conventional plastics such as polypropylene (Marchesault and Yu, 2004). The properties of PHB (homopolymer), PHBV, PHB4B (scl-copolymers) and PHBHx (mcl-copolymer) are compared with polypropylene (PP) in Table 2.2. PHB homopolymer is a highly crystalline (Padermshoke etal., 2005), stiff but brittle material. When spun into fibres it behaves as a hard-elastic material (Antipov et al.,2006). Copolymers like PHBV or mcl-PHAs are less stiff and brittle than PHB, while retaining most of the other mechanical properties of PHB. Homopolymer PHB has a helical crystalline structure, this structure seems to be similar in various copolymers. Melting behaviour and crystallization of PHAs have recently been studied by Gunaratne and Shanks (2005). In this study, PHAs show multiple melting peak behaviour and melting–recrystallization–remelting. When processing biopolymers, it is important to know the point of thermal degradation. Carrasco et al. (2006) recently determined that PHB (Biopol) decomposition starts at 246.3°C, while the value for PHBV (Biopol) is 260.4°C. This indicates that the presence of valerate in the chain increases the thermal stability of the polymer

Table 2.2: Properties of PHAs and polypropylene (PP). PHBV contains 20% 3HV monomers, PHB4B) contains 16% 4HB-monomers, PHBHx contains 10% 3HHx monomers (Tsuge, 2002)

Parameter	PHB	PHBV	PHB4B	PHBHx	PP
Melting temperature (°C)	177	145	150	127	176
Glass transition temperature (°C)	2	-1	-7	-1	-10
Crystallinity (%)	60	56	45	34	50-7-
Tensile strength (MPa)	43	20	26	21	38
Extension to break (%)	5	50	444	400	400

2.5 Biodegradability of PHAs

Besides the typical polymeric properties described above, an important characteristic of PHAs is their biodegradability. Micro-organisms in nature are able to degrade PHAs by using PHA hydrolyses and PHA depolymerases (Jendrossek and Handrick, 2002). The activities of these enzymes may vary and depend on the composition of the polymer and the environmental conditions. The degradation rate of a piece of PHB is typically in the order of a few months in anaerobic sewage to years in seawater. UV light can accelerate the degradation of PHAs. PHAs have been proved biocompatible, which means they have no toxic effects in living organisms. Within mammals, the polymer is hydrolysed only slowly. After a 6-month period of implantation in mice, the mass loss was less than 1.6% w/w (Pouton and Akhtar, 1996). Figure 2.3 shows the biodegradability of PHB.

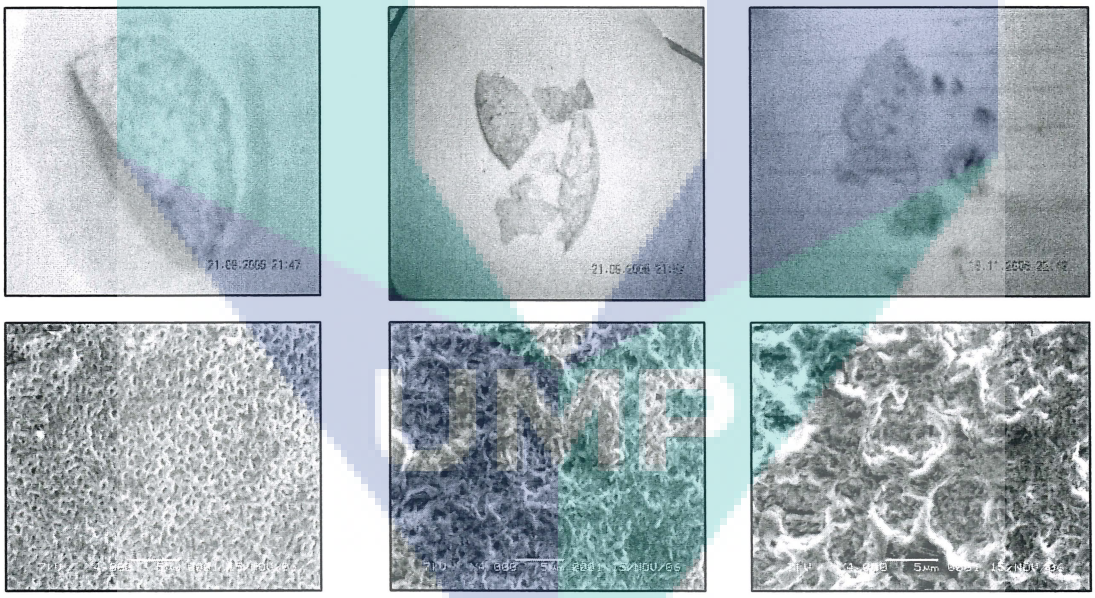

Figure 2.3: The structure of the PHB during (a) One week (b) Two weeks (c) Four weeks

2.6 Applications of PHAs

The majority of expected applications of PHAs are as replacements for petrochemical polymers. The plastics currently used for packaging and coating applications can be replaced partially or entirely by PHAs. The extensive range of physical properties of the PHA family and the extended performance obtainable by chemical modification or blending (Zhang et al., 1997) provide a broad range of potential end-use applications. Applications focus in particular on packaging such as containers and films. In addition, their use as biodegradable personal hygiene articles such as diapers and their packaging have already been described (Noda, 2001).

PHAs have also been processed into toners for printing applications and adhesives for coating applications (Madison and Huisman). Composites of bioplastics are already used in electronic products, like mobile phones (NEC Corp. and Unitika Ltd, 2006). Potential agricultural applications include encapsulation of seeds, encapsulation of fertilizers for slow release, biodegradable plastic films for crop protection and biodegradable containers for hothouse facilities.

PHAs also have numerous medical applications. The main advantage in the medical field is that a biodegradable plastic can be inserted into the human body and does not need to be removed again. PHA has an ideal biocompatibility as it is a product of cell metabolism and also 3-hydroxy butyric acid (the product of degradation) is normally present in blood at concentrations between 0.3 and 1.3 $mmol^{-1}$) . In pure form or as composites with other materials, PHAs are used as sutures, repair patches, orthopedic pins, adhesion barriers, stents, nerve guides and bone marrow scaffolds. An interesting aspect of PHA scaffolds is the fact that the tissue engineered cells can be implanted with the supporting scaffolds. Research shows that PHA materials can be useful in bone healing processes. PHA together with hydroxyapatite (HA) can find an applications as a bioactive and biodegradable composite for applications in hard tissue replacement and regeneration (Chen and Wu, 2005a).

Polymer implants for targeted drug delivery, an emerging medical application, can be made out of PHAs (Chen and Wu, 2005b) However, because of the high level of

specifications for plastics used in the human body, not every PHA can be used in medical applications. PHA used in contact with blood has to be free of bacterial endotoxins and consequently there are high requirements for the extraction and purification methods for medical PHAs (Sevastianov et al., 2003).

2.7 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)).

2.8 Liquid-side Mass Rate Transfer of Oxygen

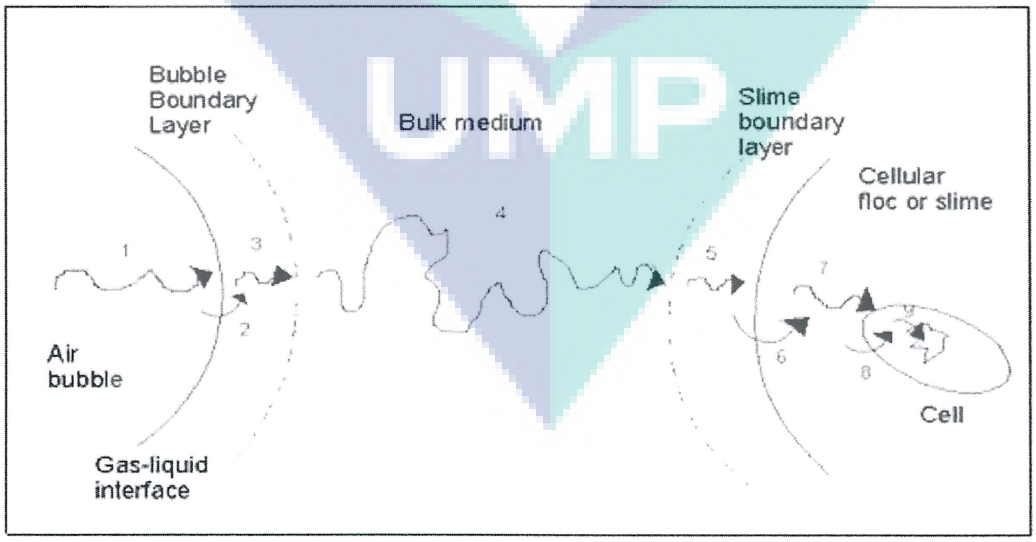

Figure 2.4: Oxygen moves from a bubble into medium and into a cell

Figure 2.4 shows 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 is shown I figure 2.5 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.

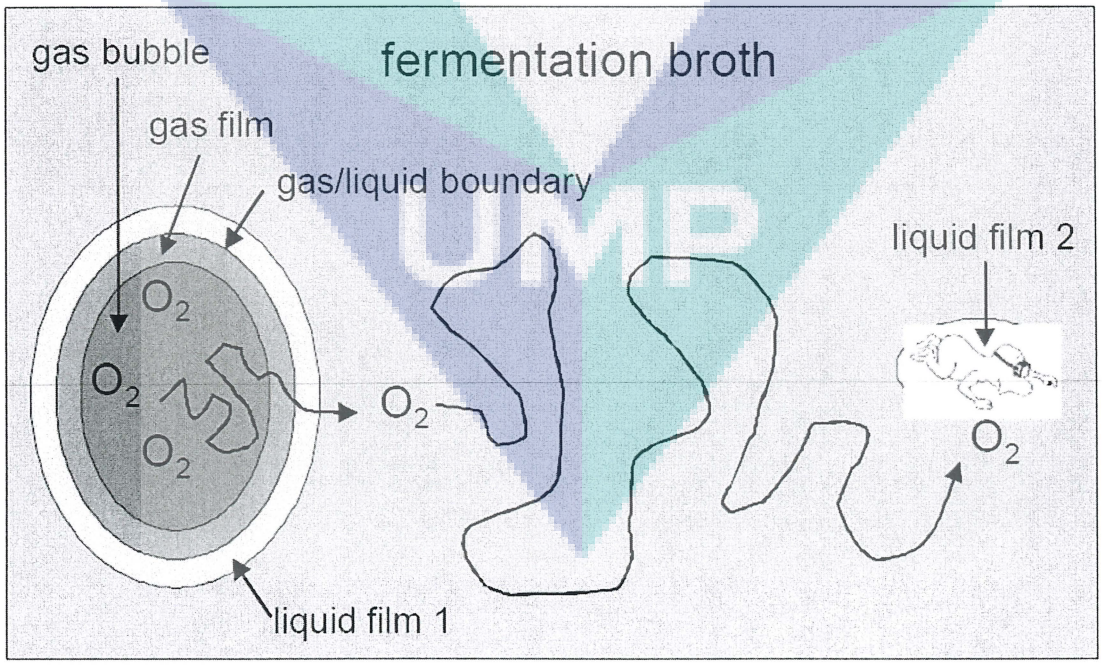

Figure 2.5: Two-film model

2.9 Effect of Aeration and Agitation Rates on Volumetric Oxygen Transfer Coefficient (k_La) and Its Importance in PHB Fermentation

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 the 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 oxygen transfer rate (OTR) can be the controlling step in industrial bioprocesses, and in the 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 by 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, etcetra. 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.

To reduce the complication of various variables, 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 k_La values has been a commonly used criterion for scale-up of aerobic fermentations (Garcia-Ochoa, Gomez-Castro, & Santos, 2000; Gibbs & Seviour, 1996; Miura, 2003). The rationale of using constant k_La value 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 parameter.

CHAPTER 3

MATERIALS AND METHODS

3.2 Microorganism

Cupriavidus necator CUG 52238 is used in all experiments.

3.2 Regeneration of the bacteria

The culture is maintained on medium slant. Regeneration is conducted every two weeks. Slant is prepared by the following procedure: Firstly NGY agar medium is prepared with composition as in Table 3.1.

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 is heated in a glass beaker with continuous stirring on laboratory hot plate until the solution comes into boiling. About 10 ml of the hot agar solution is poured into each sterilized test tube. The tube is closed with sterile cotton and wrap in aluminum foil. The tubes are sterilized in autoclave for 30 minutes at 121°C. The tubes are 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 are transferred from the old slant to the new slant in sterile laminar air flow hood with the following procedure: Firstly, a metal loop is heated until burning red. Then, the old slant containing bacteria to be regenerated is opened. Next, the loop is cooled down by touching it on the agar surface. After that, one loop full of bacteria is scraped and quickly transferred to the new slant by lightly scratching the agar surface.

The slant is incubated in the sterile incubator at room temperature for about 24 hours. After that it is kept in the refrigerator at 4°C for two weeks or until used, whichever is earlier.

3.3 Starter 1 Fermentation

20 ml of NGY medium with composition as in Table 3.2 is put in 100 ml Erlenmeyer flask, the flask is closed with sterile cotton then it is sterilized in autoclave for 30 minutes at 121°C. Let it stand in sterile incubator for 24 hours at room temperature.

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	Added until total volume= 1L

One loop of the bacteria from the slant is taken and is put it into the incubated medium as Starter 1 Fermentation. Transfer is conducted in sterile laminar air flow hood. Then, it is incubated for 24 hours before the content is transferred into Starter 2.

3.5 Starter 2 Fermentation

To grow bacteria in Starter 2, 180 ml of Ramsay medium (Ramsay et al., 1983) with composition as in Table 3.3 (and with trace elements composition as in Table 3.4)

was put in 500 ml erlenmeyer flask and sterilized in autoclave for 30 minutes at 121°C. After sterilization, the flask was let to stand for 24 hours in a sterile incubator. Then, 10% of the content of starter 1 fermentation was poured into the flask and the flask was put on the shaker for 24 hours.

Table 3.3 : Ramsay Medium for Starter 2

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
Ferric 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

3.5 The Shake-Flask Fermentation for Screening of Relevant variables and The Shake Flask Fermentation for Optimisation of Levels of Relevant Variables

Glucose is weighed and dissolved in distilled water and sterilized in erlenmeyer flasks separately from the rest of the components of the medium to prevent any reaction which may occur at high temperatures. The rest of the components are put in a separate erlenmeyer flask and dissolved in distilled water, with the volume adjusted so that the combined volume gives the intended total volume in the fermentation. After sterilization, both flasks are left to cool down.

Once cooled down, the two parts of the medium are mixed in a single flask in a laminar flow chamber, inoculated using starter 2, and put in an incubator shaker at the designated temperature and agitation speed.

3.6 Fermentation in 10L bioreactor

The bioreactor was cleaned thoroughly before using. Then, 7000 ml of the optimized medium minus glucose was poured into the bioreactor and was sterilized for 30 minutes at 121°C. Glucose was dissolved in 2000 ml distilled water, sterilized separately and was pumped in after cooling, using a peristaltic pump. 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 system and air supply were opened. Innoculum comprising of five (5) 200 ml starter 2 cultures in 500 ml Erlenmeyer flasks was pumped into the bioreactor aseptically using a peristaltic pump. The bioreactor was run. Samples were withdrawn through the sampling line at 6 hours intervals.

3.7 Cell Dry Mass Determination

Biomass content was evaluated by gravimetry. Culture sample at 10ml was centrifuged for 4 minutes at 5000rpm and 4°C. The supernatant is refrigerated for further analysis and the cell pellet was washed in DI water, recovered in the same condition. The pallet was dried to constant weight at 90°C for 24 hrs and cooled in the desiccators and weighed. The biomass yield coefficient on glucose ($Y_{x/g}$) was calculated as the cell dry weight produced per unit mass of glucose consumed. All measurements were duplicated.

3.8 PHB harvesting

Gravimetric method similar to those employed previously by Marchessault and Yu (2004) and Ramsay et al. is used. Sample in 10 ml was centrifuged for 12 minutes at 500 rpm and 4°C. Sodium chloride was added to the pallet in 10 ml and centrifuge again in the same condition. After that, pallet was mixed with the hydrogen peroxide

in 10 ml and soaked in water bath for 4 hrs at 30°C. After 4 hrs, the solution was centrifuged at the same conditions. Pallet was mixed with 10 ml chloroform and the solution was pour into glass petri dish and dried in a fume hood for 1 day. Then, the mixture was added with 5 ml acid sulfuric 98% and the mixture was observed by spectrophotometer at 238 nm.

3.9 Screening Method

3.9.1 A Brief Theory of Factor Analysis

The method of Factor Analysis [3, 4, 5] have been used to screen the experimental variables which are most relevant to the fermentation. This method has been shown to allow an efficient screening of the experimental variables which are most relevant to the biomass yield in a shake flask.

The method of Factor Analysis (Harman, 1967a; Harman, 1967b) enables us to describe the various experimental variables in terms of mutually orthogonal factors which are uncorrelated to each other but which have the same mean and the same variance as the standardized from the experimental variables. Mutually orthogonal factors are important in that only such factors may be use to construct linear models, where the interactions between factors are not taken into account. Empirical models are constructed to describe the yields in terms of mutually orthogonal factors. The significance of each factor in its effect on the yield is the determined by removing the particular factor from the model involving all the factors and comparing the mean square difference between the actual data and the prediction of the resulting model with the mean square difference between the actual data and the predictions of the model involving all the factors using the F-test.

These factors can then be classified into categories according to how significantly each of them affect the yield. If an experimental variable contribute only to factors that do not affect the yield significantly then it can be concluded that it is not relevant to the yield and can be dropped from subsequent experiments. If an experimental variable contributes to one or more factors which have significant effects on the yield

then the experimental variables is relevant to the yield and should be retained for future investigation an optimization.

3.9.2 The Application of Factor Analysis in Screening Variables Relevant to Biomass and PHB Yields in Shake-Flask Fermentations

A table consisting of seven variables shown in table appendix A that have been used for screening was constructed using dices. Each variable was assigned a dice. There were six levels for each variable and each level was marked on a face of the dice. To get combination of levels of variables, the dices were throwing all at once.

Experiments were run using the combinations of levels of variables thus achieved and results of biomass yield and PHB yield were taken.

The combinations of levels of variables used in the experiments were then standardized to give mean of zero and variance 1.0.

The 2-variables correlation between standardized variables were then calculated. The eigen values and associated eigen vectors of the correlation matrix of standardized variables were then obtained using Matlab.

Orthogonal factors were then calculated using successive coefficients of each eigen vector as coefficients of successive experimental variables, thus transforming the table of levels of standardized experimental variables into its corresponding table of orthogonal factors.

A linear regression between these factors and the PHB yield achieved by the corresponding experiment was then constructed. Then a linear regression between these factors and the biomass yield achieved by the corresponding experiment was constructed. The evaluation of the significance of each factor to the PHB yield, and to the biomass yield, is the done by the statistical F-test.

In this work, the estimation of the regression coefficients off all models were done by using Matlab which operates by minimizing the sum of squared differences between the actual and predicted yields.

The results of the regression analysis were tested for significant at level of the confidence 95%. The breakdown of the significance of the results into this confidence level were considered sufficient for this preliminary work.

3.10 Otimisation Method

3.10.1 The Method of Factorial Experiments

The experimental variables which were found to have significant effects on PHB and biomass yields in the screening exercise were considered for this experiments while the rest were set at constant values.

The level of each selected variable in the combination which gives the highest biomass yield were taken as the centre point of the first factorial experiments, where each variable were given two levels ; the low level and the high level (to the left and right of the centre point respectively) , the distance between the levels being adjusted so that the effect of experimenting at the two different levels should be distinguishable from the experimental error.

A 2^n factorial design was constructed, where n is the number of experimental variables. The resulting biomass and PHB yields were analysed using Yates' Method for Main Effects and Interactive Effects.

3.10.2 The Method of Steepest Ascent

A linear regression was constructed between the biomass yield as the dependent variable, and the experimental variables as the independent variables. This process was repeated for the PHB yield.

The resulting linear equation was tested for the presence of the point of maximum yield in the response surface. If the result of the test is positive, then the factorial experiments are complemented with the necessary additional experimental points to make it a composite design. If the result of the test is negative, the method of steepest ascent is used to reach the area containing the point of maximum yield, where a new 2^n factorial experiment is designed and the process repeated.

3.10.3 The Method of Composite Design

Once the area of the response surface containing the point of maximum yield has been identified as above, the factorial experiment for the area is complemented with the necessary additional experimental points to make it a composite design.

The resulting combined experimental results are then fitted with a quadratic equation and the values of the coefficients of the quadratic equation which gives the best fit is achieved by regression.

The values of the coefficients of the quadratic equation are then used to calculate the levels of the experimental variables at the point of maximum yield.

3.11 DOT curves

The gassing out technique had been used to get the values of k_La and ka_p for distilled water. The oxygen probe from the 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 becomes zero. Then, the shake flask was shaken on the orbital shaker at 200rpm at room temperature, which are the optimum conditions for

PHB production. At the same time, a stop-watch was started and the values of DOT are taken at specified time intervals until it reached 100%.

The steps above were repeated a few times using the 10L fermentor with 8L of distilled water in it to get the DOT curve that is almost the same as in the shake-flask above. These were done by trial and error on the air flow rate and rpm. The air flow rate and rpm that produced the DOT curve which is almost the same as in the shake flask above will be used in the fermentation in 10L fermentor later.

3.12 Analysis Methods

3.12.1 Glucose analysis

Glucose concentration 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 by running tap water over the test tube containing it. 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.

3.12.2 Dry Cell Weight analysis

Biomass content was evaluated by gravimetric method. The pellet that was 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 90°C for 24 hours. Then it was cooled and weighed. The biomass yield coefficient on glucose ($Y_{X/S}$) as the cell dry weight produced per unit mass of glucose consumed was calculated.

3.12.3 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, followed by mixing by vortex. The mixture was then poured into petri dishes and left to dry. Then, 5ml of concentrated sulphuric acid was added and well-mixed. The mixture was poured into 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 then calculated.

3.13 Approximation of the Values of k_La and ka_p

3.13.1 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 et al., (1990). 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 DISCUSSION

4.1 Experimental Results in the Screening of Experimental variables Relevant to Biomass and PHB Yields in Fermentations

Table for experimental results was shown in Table 1 of appendix A. The correlation matrix of the Standardized Experimental Variables are shown in Table 4.1. The coefficients of the eigen vectors are shown in Table 4.2. The values of the orthogonal factor was shown in Table 4.3. The coefficients and constant of the linear models for the biomass was shown in Table 4.4. Table 4.5 shows the evaluation of linear model for the biomass. Table 4.6 shows the coefficients and constant of the linear models for the PHB and Table 4.7 shows the evaluation of linear model for the PHB.

Table 4.1: The Correlation Matrix of the Standardized Variables

	RPM	T	G	P	A	C	TE
RPM	1.0000	0.0241	-0.3713	-0.0290	-0.3726	0.1964	-0.1659
T	0.0241	1.0000	0.1528	0.0268	-0.0970	-0.1537	0.0250
G	-0.3713	0.1528	1.0000	-0.0548	0.2668	-0.5245	0.0546
P	-0.0290	0.0268	-0.0548	1.0000	0.0578	-0.0850	-0.1804
A	-0.3726	-0.0970	0.2668	0.0578	1.0000	0.1042	-0.3060
C	0.1964	-0.1537	-0.5245	-0.0850	0.1042	1.0000	0.0151
TE	-0.1659	0.0250	0.0546	-0.1804	-0.3060	0.0151	1.0000

Table 4.2 : The Coefficient of the Eigen Vectors

	RPM	T	G	P	A	C	TE
F1	0.5076	0.2339	-0.5993	-0.1156	-0.3448	-0.2950	0.2811
F2	-0.1423	0.0533	0.0858	-0.3301	-0.3782	0.7074	0.5253
F3	-0.6207	-0.5131	-0.5548	-0.1201	-0.0082	-0.1679	0.1208
F4	-0.0489	-0.0572	-0.2264	0.2776	-0.5675	0.2397	-0.6805
F5	-0.3305	0.5418	-0.2723	0.6134	0.1518	0.2022	0.2362
F6	0.4746	-0.5271	-0.2710	0.3028	0.3292	0.469	0.1326
F7	-0.0052	0.3234	-0.3561	-0.5642	0.5334	0.2571	-0.3066

Table 4.3: The Values of the Orthogonal Factors

no of exp.	F1	F2	F3	F4	F5	F6	F7
1	-1.2372	-0.3854	1.4761	-1.7886	0.2127	0.7998	-0.6003
2	-2.2663	-0.2764	1.1237	0.6782	0.1958	1.6423	0.9925
3	-0.6615	0.7183	0.5155	-1.7348	-1.0096	-1.6057	-0.7074
4	0.4391	0.1622	1.4676	0.2705	0.6674	-0.4118	-0.8924
5	-0.4367	0.5579	0.2231	-0.3503	0.1149	-1.4141	-0.9899
6	-1.6993	-1.8797	-0.8084	0.5203	1.0410	-1.4411	-0.9455
7	-0.8920	1.7643	-0.1254	-0.6994	1.7815	0.1456	0.0882
8	-0.2537	0.2603	0.4021	-0.2440	0.6863	-0.9905	2.4359
9	0.1805	2.3178	1.5346	0.0337	-0.3810	1.2825	-0.2175
10	-1.1093	-2.0070	0.6898	0.8861	-0.3922	0.3209	0.4939
11	0.4751	0.3436	0.3852	-1.3534	-0.1879	-1.0874	-0.4376
12	-1.4263	-1.7943	-1.0794	-0.2120	0.1423	-1.2993	0.0895
13	0.2881	1.4737	0.0032	-0.3297	-1.0407	-0.1588	0.0142
14	0.3784	-1.2710	0.5571	1.0226	1.1449	1.2590	-0.1855
15	0.2921	-0.1680	-0.2826	0.2887	-1.0184	-1.0063	-1.4604
16	1.0810	0.0436	0.4645	-0.2528	-0.6669	-0.5486	0.8925
17	-0.5725	-1.1674	-1.4065	-0.6214	0.5248	-0.8182	-1.0044
18	-0.3256	0.2874	-1.0293	-1.3858	0.1625	-0.3904	2.1236

Table 4.3 continued

19	1.3700	0.0293	-0.1011	2.0667	-0.2318	0.2470	0.4252
20	1.6100	0.2141	-0.5714	1.0359	0.5185	-0.1280	-0.4966
21	0.7221	0.4939	0.2999	1.2485	-0.8720	2.5330	0.5726
22	-0.4705	-0.5940	-0.5351	-0.1280	-1.4017	1.9322	0.3020
23	1.0831	-0.5782	-0.7140	0.1116	-2.1304	-0.4693	-0.0099
24	2.5415	0.3478	-0.6427	0.5874	1.5452	0.9111	-1.3992
25	0.8898	1.1074	-1.8465	0.3499	0.5949	0.6961	0.9169

Table 4.4: The Coefficients and Constant of the Linear Models for the Biomass

Model	**a**	**a_1**	**a_2**	**a_3**	**a_4**	**a_5**	**a_6**	**a_7**
1234567	4.699	-1.269	0.645	-1.052	-0.194	-0.826	-0.205	-1.383
123456	4.699	-0.918	0.388	-0.771	-0.278	-0.683	-0.479	
12345	4.699	-0.866	0.254	-0.885	-0.577	-0.654		
1234	4.699	-0.777	0.192	-0.804	-0.673			
123	4.699	-1.054	0.428	-0.880				
12	4.699	-0.852	0.208					
1	4.699	-0.783						

Table 4.5: The Evaluation of Linear Model for the Biomass

No	**Model**	**MSE**	**d.f**	**MSE_{new}/MSE_7**	**$F_{0.95}$**
1	1234567	1.6266	16	1	
2	123456	3.1829	17	1.957	
3	12345	3.3884	18	2.083	
4	1234	3.7387	19	2.299	/
5	123	4.0283	20	2.477	/
6	12	4.5675	21	2.808	/
7	1	4.6102	22	2.834	/

Note: / indicates significance at 95% confidence level

Table 4.6: The Coefficient and Constant of the Linear Models for the PHB

Model	a	A_1	a_2	a_3	a_4	a_5	a_6	a_7
1234567	0.502	-0.037	-0.018	-0.143	-0.057	-0.120	0.006	-0.07
123456	0.502	-0.020	-0.031	-0.128	-0.061	-0.113	-0.007	
12345	0.502	-0.019	-0.033	-0.130	-0.066	-0.112		
1234	0.502	-0.002	-0.044	-0.116	-0.082			
123	0.502	-0.037	-0.015	-0.126				
12	0.502	-0.008	-0.047					
1	0.502	-0.024						

Table 4.7: The Evaluation of Linear Model for the PHB

No	Model	MSE	d.f	MSE_{new}/MSE_7	$F_{0.95}$
1	1234567	0.005518	16	1	
2	123456	0.009510	17	1.72	
3	12345	0.009559	18	1.73	
4	1234	0.019906	19	3.61	/
5	123	0.024220	20	4.39	/
6	12	0.035190	21	6.38	/
7	1	0.037330	22	6.7	/

Note: / indicates significance at 95% confidence level

4.2 Discussion on the Experimental Results in the Screening of Experimental Variables Relevant to Biomass and PHB Yields in Fermentations

From the results of the experiments, 17 experiments produced biomass and PHB in large amounts relative to the other experiments. Yield of biomass at 9.68 g/l was achieved while production of PHB was 0.855 g/l. It is because bacteria will grow rapidly in excess glucose at 200 rpm ie aerobic conditions.

Table 4.6 for the evaluation of the linear model for the biomass production shows that removing F_6 and F_7 do not cause the model to incur significant errors at 95%

confidence level. However, removing F_5, F_6 and F_7 causes the model for the biomass yield to incur significant errors at 95% confidence level.

Since the Factors are arranged in order of decending eigen values, it follows that removing any of F_4, F_3, F_2 or F_1 (each of which is associated with progressively bigger eigen values) will cause even bigger errors in the models. Hence the best (the simplest model which does not cause significant errors) for the biomass yield contains the factors F_1, F_2, F_3, F_4, and F_5.

By similar arguments, the best model for the PHB yield also contains the factors F_1, F_2, F_3, F_4, and F_5.

A check with Table 4.2 shows that the factors F_1, F_2, F_3, F_4, and F_5 between them actually contains large (more than ± 0.3) from all the experimental variables. Hence this screening exercise has shown that all the experimental variables tested actually have significant effects on biomass yield and PHB yield.

4.3 Experimental Results and Discussion on the Optimization of Shake-Flask Fermentations.

4.3.1 The Results of the Factorial Experiments

Even though the screening exercise has shown that all the experimental variables tested actually have significant effects on biomass yield and PHB yield, because of shortage of time, it was decided that only the levels of temperature (T), glucose concentration (G) and agitation rate (RPM) are to be optimized while the levels of the rest of the variables are kept constant.

The levels of these variables in the first factorial experiments are as shown in Table 4.8, while the 2^3 Factorial Design and the experimental results are shown in Table 4.9. The results of the replication at centre point are shown in Figure 4.10.

Table 4.8: Levels of Experimental Variables in the First 2^3 Factorial Experiments

Variables	α = -1	α = 0	α = 1	Units
Temperature	28	31.5	35	°C
Glucose concentration	10	20	30	g/L
Agitation	150	225	300	rpm

Table 4.9: The Plan for the 2^3 Factorial Experiments and the Untreated Result

No	X_1	X_2	X_3	Biomass (g/L)	PHB (g/L)
1	-1	-1	-1	2.745	0.18755
2	1	-1	-1	3.08	0.5096
3	-1	1	-1	3.55	0.16243
4	1	1	-1	2.01	0.37208
5	-1	-1	1	3.13	0.28426
6	1	-1	1	2.11	0.52378
7	-1	1	1	3.76	0.27899
8	1	1	1	2.98	0.52378

Table 4.10 the Plan of the Replication at the Center Point and the Untreated Result of the Experiments

No	T X_1	Glucose conc. X_2	rpm X_3	PHB (g/L)	Biomass g/L
9	0	0	0	0.54092	3.80
10	0	0	0	0.54289	4.20
11	0	0	0	0.55344	3.10
12	0	0	0	0.56729	2.30
13	0	0	0	0.57916	4.10
14	0	0	0	0.56729	2.40
			Average	0.55850	3.3167
			Standard Deviation	0.000232	0.8424
			Mean Square Error	0.01523	0.7097

From the experiment results, the coefficients of the linear equation 4.1 were found for the biomass yield and PHB yield to be as in Table 4.11.

$$y = a_0 + a_1 x_1 + a_2 x_2 + a_3 x_3 \qquad [4.1]$$

Table 4.11: The Regression Coefficients of the Linear Regression Equation of the Response Surface of the 2^3 Factorial Experiments

Coefficients.	Biomass	PHB
a_0	2.9206	0.3553
a_1	-0.3756	0.1270
a_2	0.1544	-0.0210
a_3	0.0744	0.0474

The performance of the linear regression equations of the response surface of the 2^3 factorial experiments for biomass and PHB are then evaluated by calculating the errors in the prediction by each equation as in Tables 4.12 and 4.13.

Table 4.12 The Evaluation of the Linear Regression Equation of the Response Surface of the 2^3 Factorial Experiments for PHB

No	True Yield (g/L)	Predicted Yield (g/L)	Squared Error
1	0.18755	0.2019	0.000206
2	0.50960	0.4559	0.002884
3	0.16243	0.1599	0.000006
4	0.37208	0.4139	0.001749
5	0.28426	0.2967	0.000155
6	0.52378	0.5507	0.000725
7	0.27899	0.2547	0.000590
8	0.52378	0.5087	0.000227
Mean Squared Error			0.000818
Mean Squared Error / Mean Experimental Error			0.053693

Table 4.13 The Evaluation of the Linear Regression Equation of the Response Surface of the 2^3 Factorial Experiments for Biomass

No	True Yield (g/L)	Predicted Yield (g/L)	Squared Error
1	2.745	3.0674	0.10394
2	3.08	2.3162	0.58339
3	3.55	3.3762	0.03021
4	2.01	2.625	0.37823
5	3.13	3.2162	0.00743
6	2.11	2.465	0.12603
7	3.76	3.525	0.05523
8	2.98	2.7738	0.04252
Mean Squared Error			0.165873
Mean Squared Error / Mean Experimental Error			0.233722

4.3.2 Discussion on the Experimental Results of the Factorial Experiments

For the PHB yield, Table 4.11 shows that the coefficients of two of the experimental variables are small compared to the constant, while the coefficient of the experimental variable temperature (T) is about a third of the value of the constant, indicating that the surface is still quite flat. Table 4.12 shows that the fit of the equation to the data is good, indicating that the surface is a plateau which may contain the point of maximum yield.

For the biomass yield, Table 4.11 shows that the coefficients of all the experimental variables are small compared to the constant, indicating that the response surface is flat. Table 4.13 shows that the fit of the equation to the data is good, indicating that the surface is a plateau which may contain the point of maximum yield.

Hence the factorial experiments were complemented with the necessary extra points to make the composite design

4.3.3 The Results of the Experiments in The Composite Design

The levels of experimental variables of the additional points making the rotatable composite design are as given in Table 4.14.

Table 4.14 Levels of Experimental Variables of the Additional Points Making the Rotatable Composite Design

Variables	$\alpha = -1.68$	$\alpha = 1.68$	Units
Temperature	25.61	37.39	°C
Glucose concentration	3.18	36.82	g/L
Agitation	98.87	351.13	rpm

The results of the experiments at the additional points are as given in Table 4.15.

Table 4.15 the Plan of the Additional Experiments Making the 2^3 Rotatable Composite Design and the Result of the Experiments

No	T X_1	Glucose conc. X_2	RPM X_3	PHB (g/L)	Biomass g/L
15	-1.68	0	0	0.15762	2.760
16	1.68	0	0	0.23067	2.810
17	0	-1.68	0	0.55608	2.400
18	0	1.68	0	0.58575	1.700
19	0	0	-1.68	0.03394	0.300
20	0	0	1.68	0.05701	0.830

The coefficients of the quadratic equation representing the response surface of the composite design as found by regression are given in Table 4.16.

Table 4.16 The Coefficients of the Quadratic Equation of the Response Surface of the Rotatable Composite Design

Coefficients	Value	
	Biomass	PHB
b_0	3.16	0.55
b_1	-0.21	0.083
b_2	0.004228	-0.008641
b_3	0.11	0.031
b_{11}	0.082	-0.11
b_{22}	-0.18	0.027
b_{33}	-0.70	-0.16
b_{12}	-0.20	-0.013
b_{13}	-0.74	-0.005924
b_{23}	0.22	0.020

The evaluation of the performance of the quadratic equation for biomass yield give results as in Table 4.17.

Table 4.17 The Evaluation of the Quadratic Equation of the Response Surface of the 2^3 Rotatable Composite Design for Biomass

No	True Yield (g/L)	Predicted Yield (g/L)	Squared Error
1	2.745	2.403772	0.11074
2	3.080	2.531772	0.29135
3	3.550	2.372228	1.40714
4	2.010	1.700228	0.10127
5	3.130	2.331772	0.62374
6	2.110	2.163772	0.00387
7	3.760	3.180228	0.34601
8	2.980	2.212228	0.60253
9	3.80	3.16	0.40960
10	4.20	3.16	1.08160
11	3.10	3.16	0.00360
12	2.30	3.16	0.73960
13	4.10	3.16	0.88360
14	2.40	3.16	0.57760
15	2.760	3.744237	0.96872
16	2.810	3.038637	0.05227
17	2.400	2.644865	0.06712
18	1.700	2.659071	0.89277
19	0.300	0.99952	0.48933
20	0.830	1.36912	0.29065

The evaluation of the performance of the quadratic equation for PHB yield give results as in Table 4.18.

Table 4.18 The Evaluation of the Quadratic Equation of the Response Surface of the 2^3 Rotatable Composite Design for PHB

No	True Yield (g/L)	Predicted Yield (g/L)	Squared Error
1	0.18755	0.20364	0.00026
2	0.50960	0.40564	0.01081
3	0.16243	0.17236	0.00010
4	0.37208	0.32236	0.00247
5	0.28426	0.23564	0.00236
6	0.52378	0.41764	0.01127
7	0.27899	0.28436	0.00003
8	0.52378	0.41436	0.01197
9	0.54092	0.55000	0.00008
10	0.54289	0.55000	0.00005
11	0.55344	0.55000	0.00001
12	0.56729	0.55000	0.00030
13	0.57916	0.55000	0.00085
14	0.56729	0.55000	0.00030
15	0.15762	0.10010	0.00331
16	0.23067	0.37898	0.02199
17	0.55608	0.64072	0.00716
18	0.58575	0.61169	0.00067
19	0.03394	0.04634	0.00015
20	0.05701	0.15050	0.00874

The coefficients of the quadratic equation (4.2) for PHB (ie last column of Table 4.16) were then used for the calculation of the levels of the variables at the point of maximum PHB yield.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}^2x + b_{33}^2x + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \text{ --[4.2]}$$

Using matrix algebra, the levels of the experimental variables at the theoretical maximum yield of PHB were found as follows:

$$X_{\max} = -\left(\frac{1}{2}\right) b_{ii}^{-1} b_i^T \qquad \text{[eq.4.3]}$$

$$y_{\max} = b_0 + b_i X_{\max} + X_{\max}^T b_{ii} X_{\max} \qquad \text{[eq.4.4]}$$

From these equations, levels of the experimental variables at the theoretical maximum yield of PHB were found to be as in Table 4.19.

Table 4.19: Levels of the Experimental Variables at the Point of Theoretical Maximum Yield of PHB

X_i	Variable	Level	Value	Unit
X_1	Temperature	0.7341	34.07	°C
X_2	Glucose concentration	-0.4373	15.63	g/L
X_3	Agitation	-0.0290	222.83	rpm

Equation (4.2) was then used to calculate the theoretical yields of PHB and biomass at this point of maximum PHB yield and the results are as in Table 4.20.

Table 4.20: Predicted maximum yield

	Value (g/L)
Biomass	3.07855
PHB	0.564093

Figure 4.3 gives the 3-dimensional model of the response surface for biomass yield involving the variables glucose concentration (G) and temperature (T), with agitation rate held constant.

Figure 4.4 gives the 3-dimensional model of the response surface for PHB yield involving the variables glucose concentration (G) and temperature (T), with agitation rate held constant.

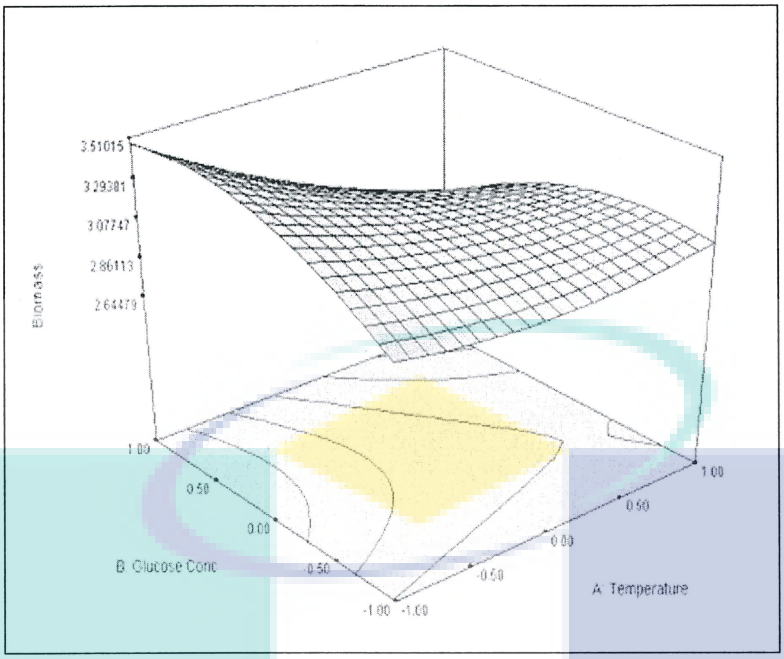

Figure 4.1: 3-D Model of Biomass optimization graph

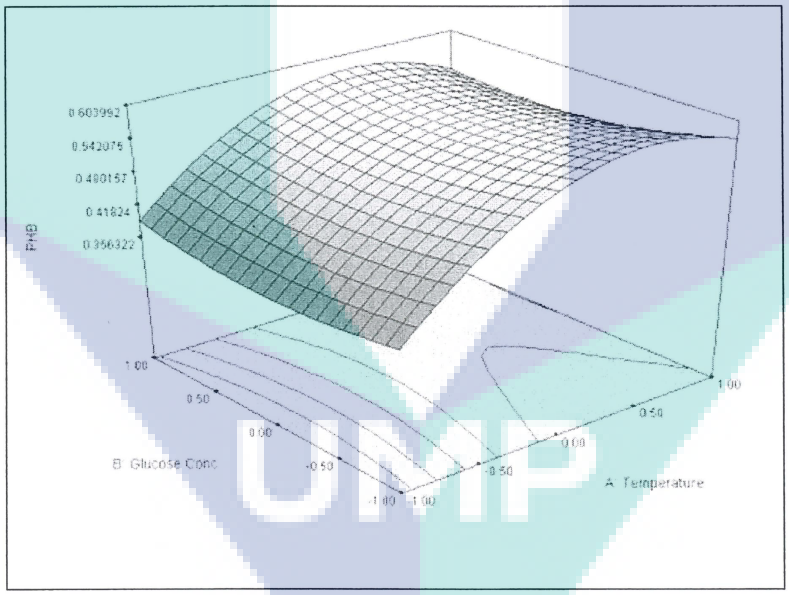

Figure 4.2: 3-D model of PHB optimization graph

4.3.4 Discussion on the Experimental Results in the Composite Design

The optimization of the levels of the experimental variables to give the maximum yield of PHB in shake-flask fermentation has succeeded in determining the optimized levels of the variables.

4.4 Experimental Results and Discussions in the Scale-Up to 10L Stirred-Tank Fermentation

4.4.1 Experimental Results for DOT versus Time Curves

Figure 4.5 shows the DOT versus Time curve for the optimum fermentation conditions in shake-flask, and the DOT versus Time curve of the 10L fermenter which is closest to it.

The percent DOT versus time curve for 500ml shake flask was obtained at the optimum conditions of the PHB production, which is 200rpm, at room temperature.

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 air flowrate 1 L/min, the percent DOT curve in 10L fermentor was nearly the same as in the 500ml shake flask above.

4.4.2 Discussion on the DOT versus Time Curves

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 was used to shake the shake-flask is 25mm. The rotational diameter of the orbital shaker will affect the shaking of the distilled water.

In the trial and error with the 10L stirred tank fermenter , the agitation rate cannot be set too high because the shear rate can cause damage to the cells growing inside the 10L fermentor. So, the compressed air flow rate needs to be adjusted 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.

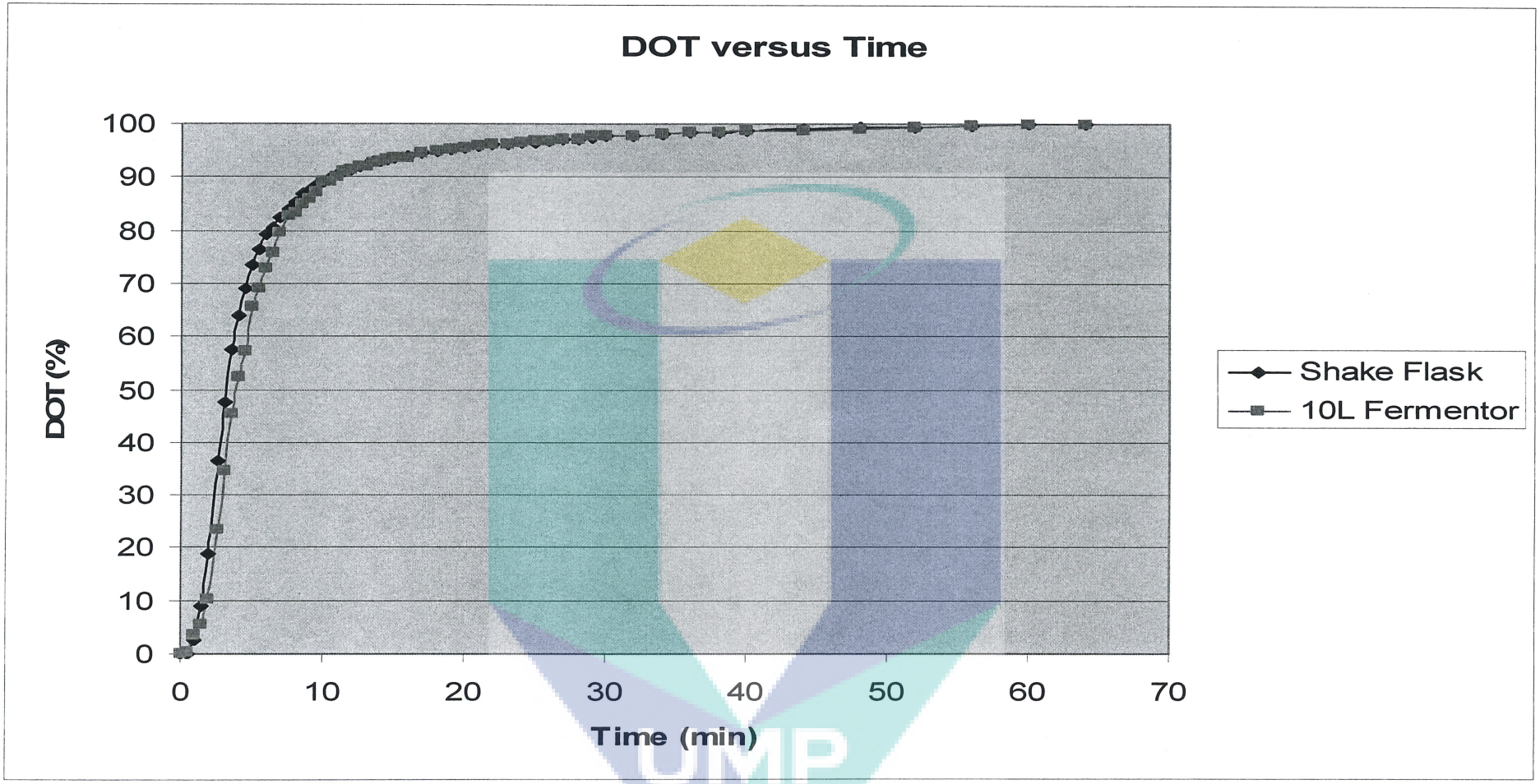

Figure 4.3: DOT (%) versus time curves for 500ml shakes flask and 10L fermentor

4.4.3 Experimental Results of Fitting the Oxygen Mass Transfer Equation to DOT versus Time Curves of 500ml Shake-Flask and 10L fermentor

Figure 4.6 shows the 500ml shake-flask Matlab fitted curve while Figure 4.7 shows the 10L fermentor Matlab fitted curve, each in comparison with the corresponding experimental curve.

4.4.4 Discussion on the Fitting of the Oxygen Mass Transfer Equation to DOT versus Time Curves of 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 et al., (1990). 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}. For the k_La, the error is 9.83%. However for the ka_p, the error is 10.00%.

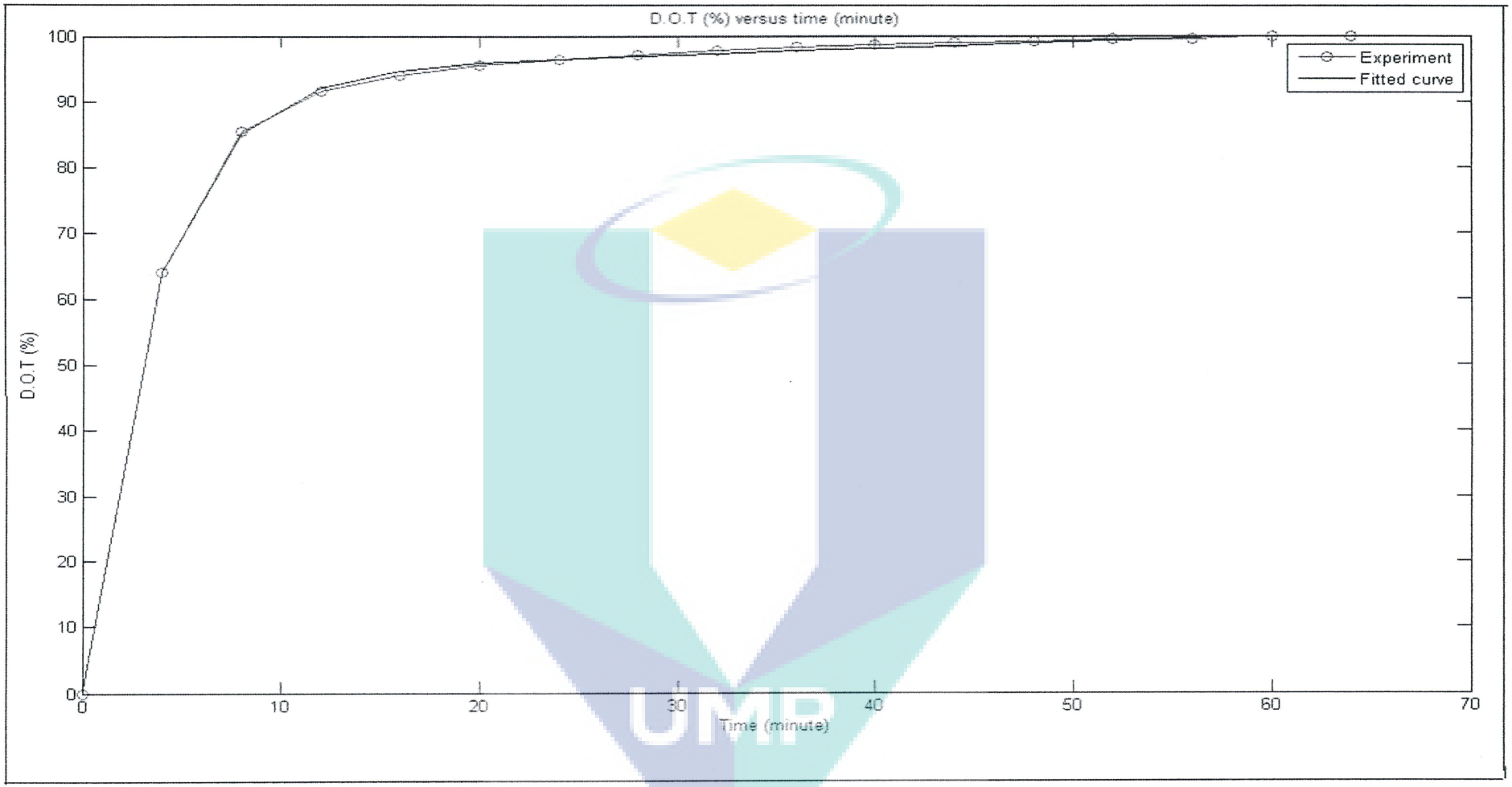

Figure 4.4: 500ml shake flask fitted curve

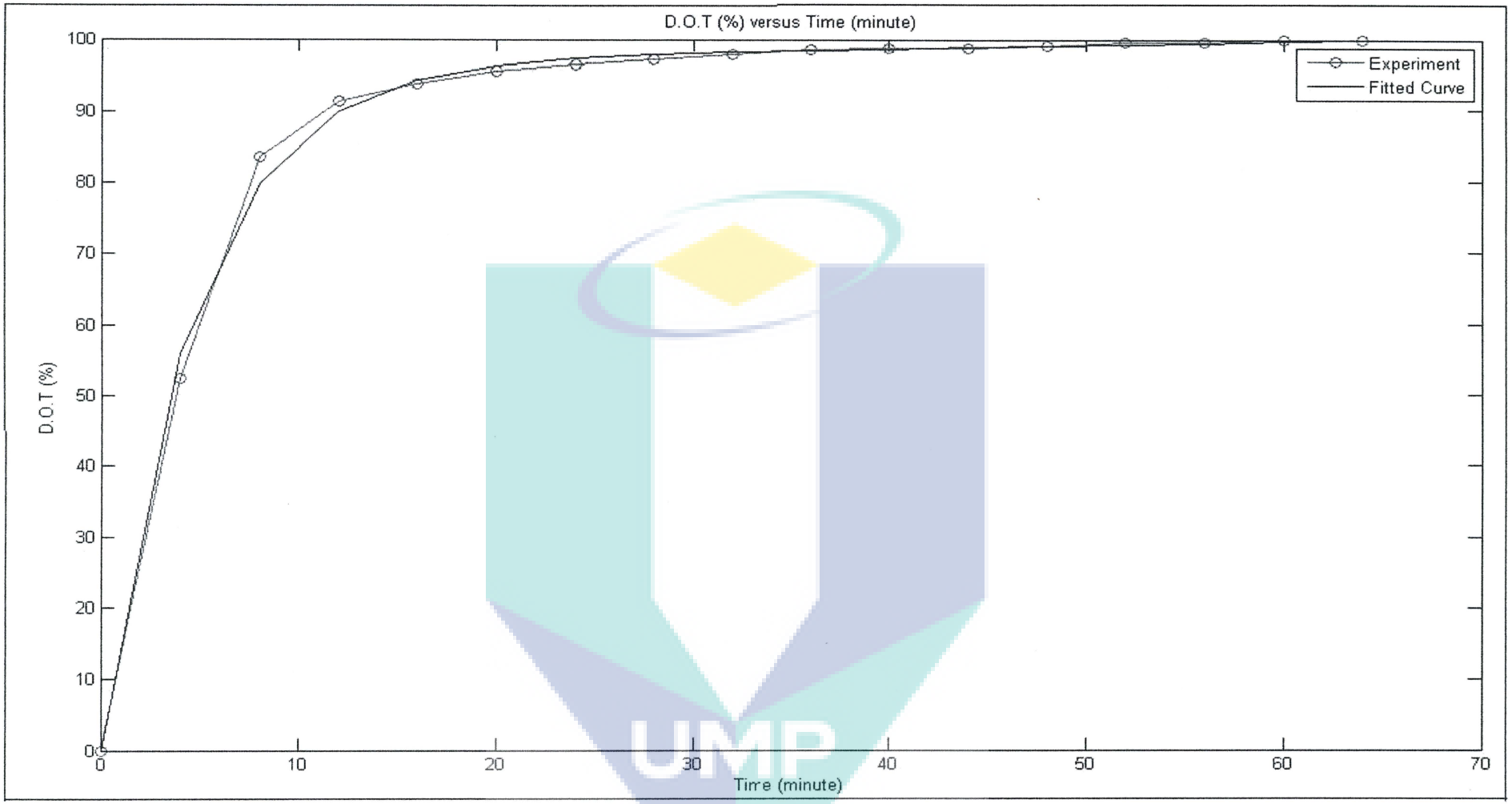

Figure 4.5: 10L fermentor fitted curve

4.5 Experimental Results and Discussions in the 10L Stirred-Tank Fermentation

4.5.1 Glucose Analysis

Glucose analysis of PHB was evaluated by DNS method. From the glucose concentration standard curve in Apendix I, 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 in Figures 4.8 and 4.9, the glucose concentrations declined from the sixth hour until the 72nd hours. For the 500ml shake flask fermentation (Figure 4.8), 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 (Figure 4.9), 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 fementation 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.

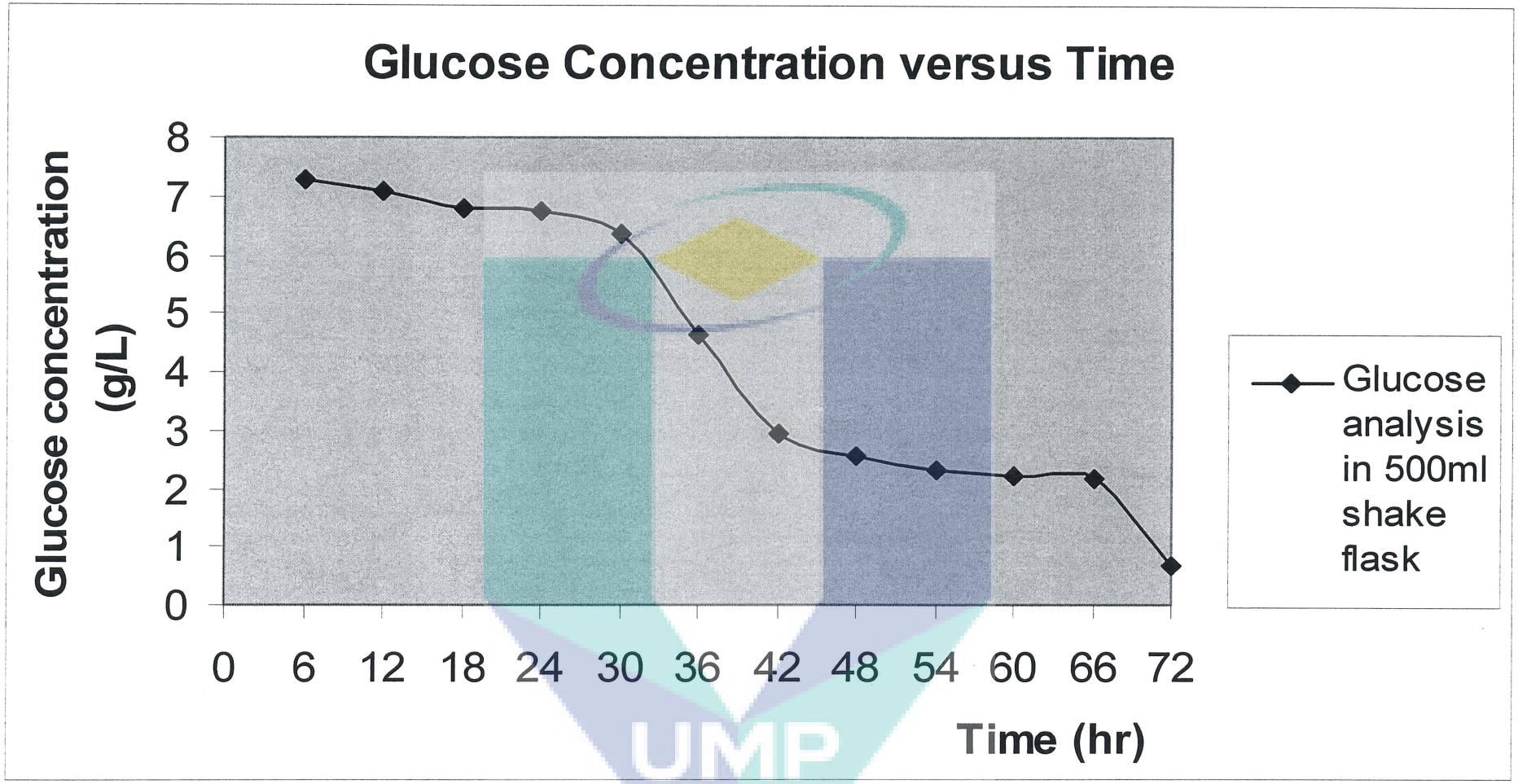

Figure 4.6: Glucose concentration versus time for 500ml shake flask

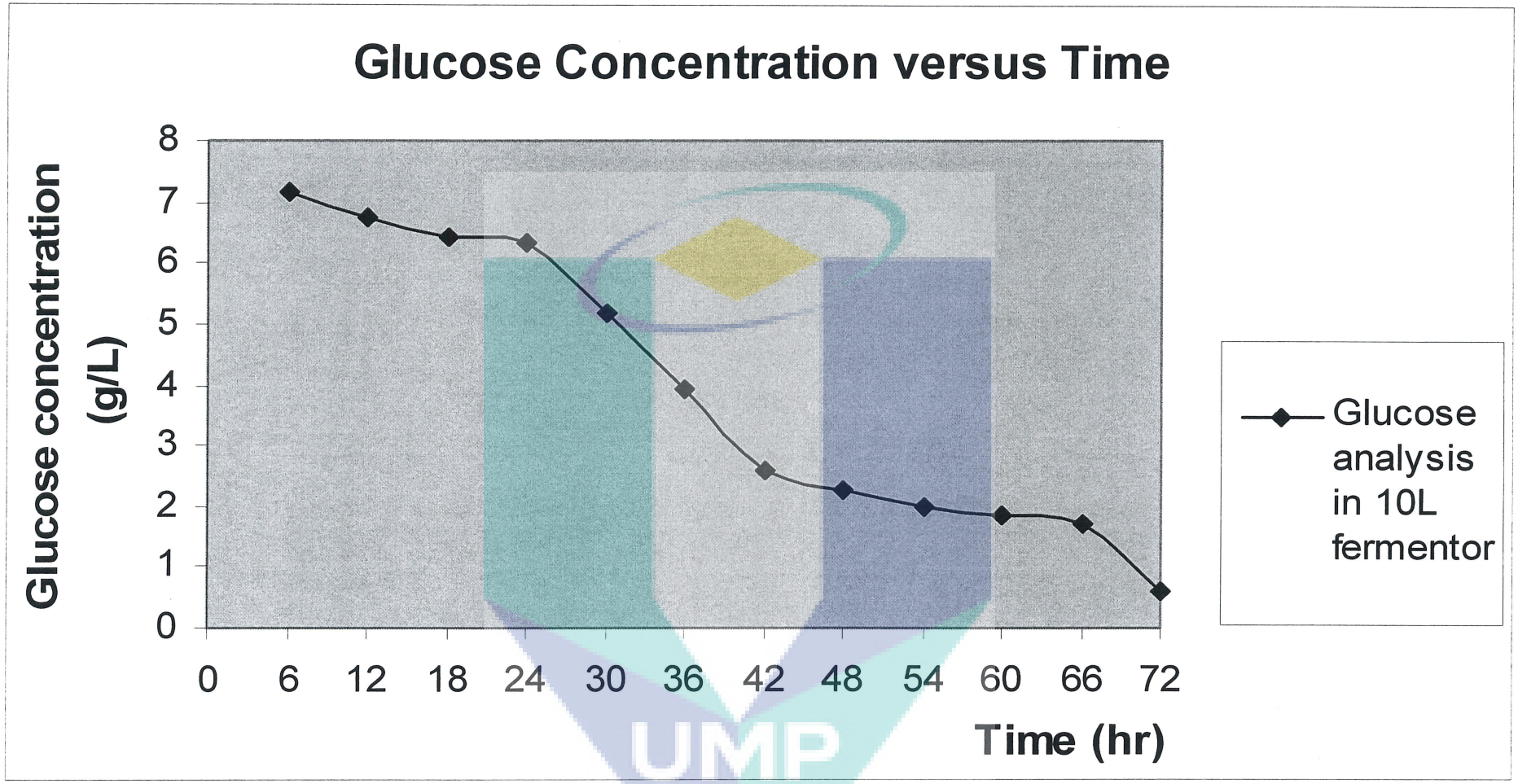

Figure 4.7: Glucose concentration versus time for 10L fementor

4.5.2 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 (Figure 4.10) and 10L bioreactor (Figure 4.11) 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 lucose

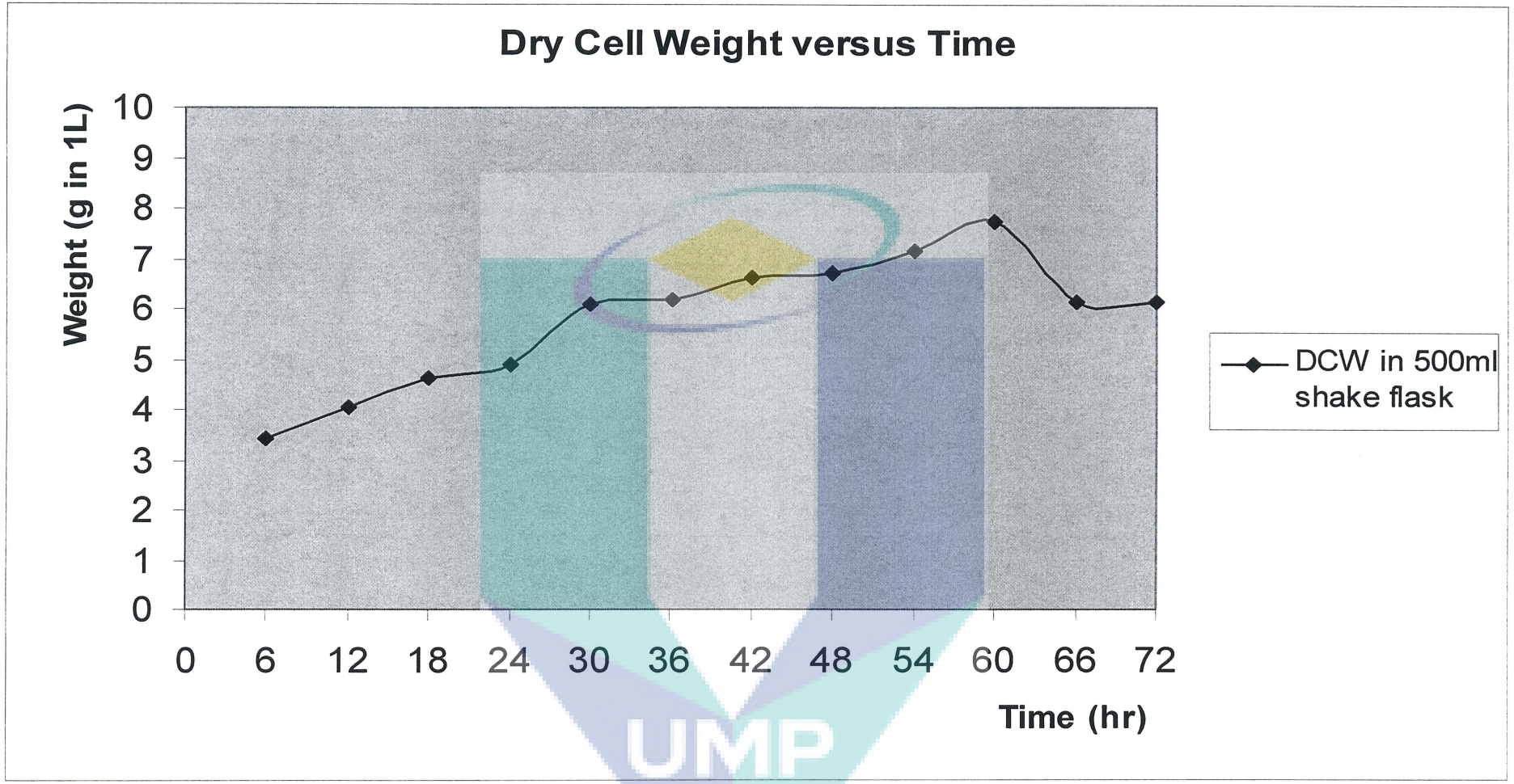

Figure 4.8: Dry cell weights versus time for 500ml shake flask

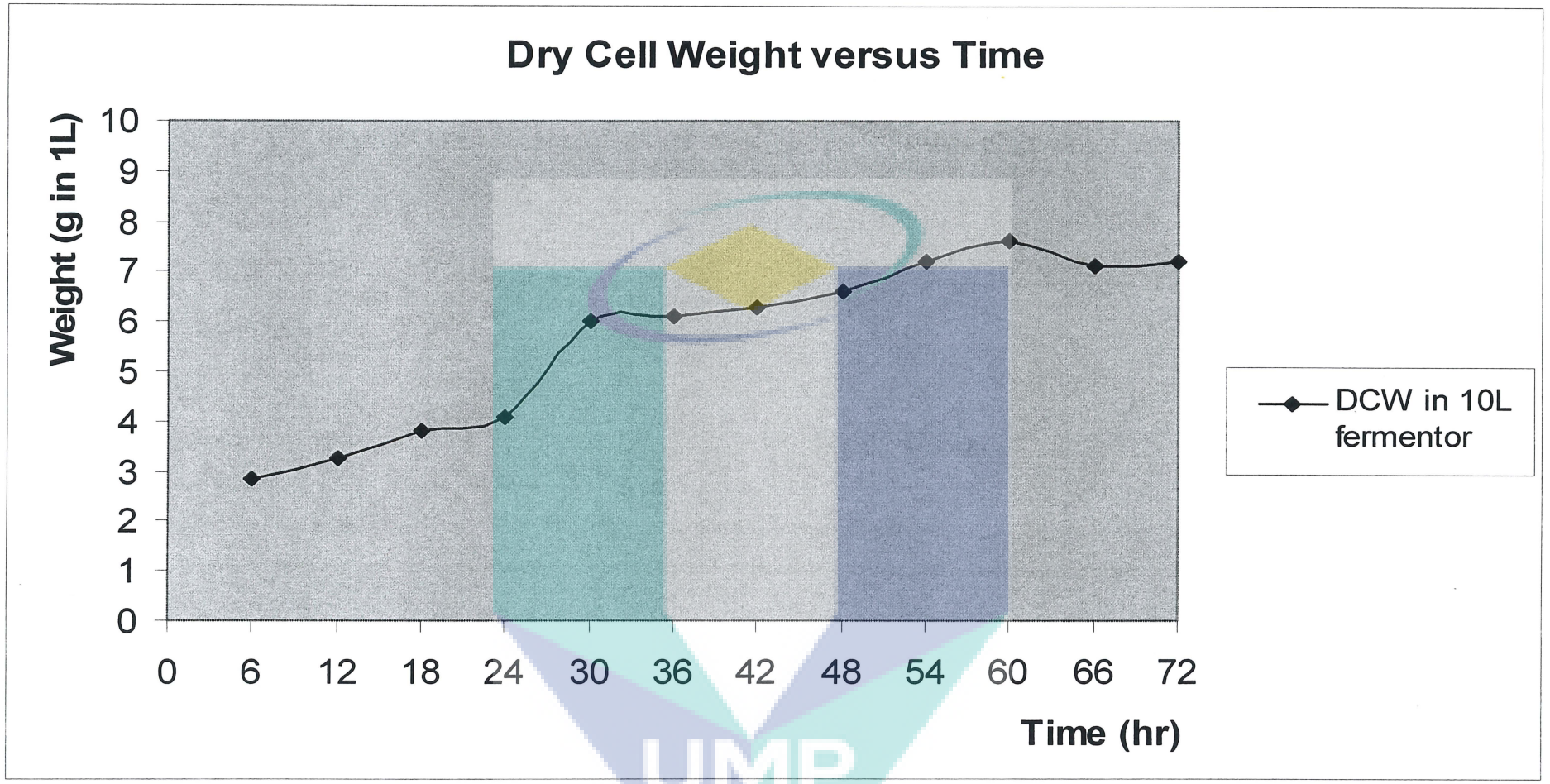

Figure 4.9: Dry cell weights versus time for 10L fermentor

4.5.3 PHB Analysis

This PHB analysis method was recommended by Dr Wiratni from Universiti Gadjah Mada (UGM), Indonesia. From the PHB concentration standard curve (Appendix J), 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 fementation. 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

4.5.4 Comparisons in Dry Cell Weight (DCW) Yield and PHB Yield Between 500ml Shake-Flask Fermentation and 10L Stirred Tank Fermentation

The highest biomass yield coefficient on glucose for 500ml shake flask at 0.9988gcells / g glucose is compared with the highest biomass yield coefficient on glucose for 10L fermentor at 0.9314 g cells / g glucose

The highest PHB yield coefficient on dry cell weight for 500ml shake flask at 0.1425g PHB / g dry cell is compared with the highest PHB yield coefficient on dry cell weight for 10L fermentor at 0.1399 g PHB / g dry cell.

The dry cell weight and PHB concentration yields in 10L fermenter are both lower than at 500 ml shake-flask fermenter. Since the medium is identical, and the innoculum preparation is identical at both scales, the problems would be in aeration and agitation, and sterilization.

The sterilization cycle at 10L fermenter has been optimized so that the sterility probability of 1:1000 contamination has been achieved while incurring the least damage to nutrient quality. Hence the only improvement that can be done is in the re-optimisation of air flowrate and impeller speed RPM at 10L fermenter.

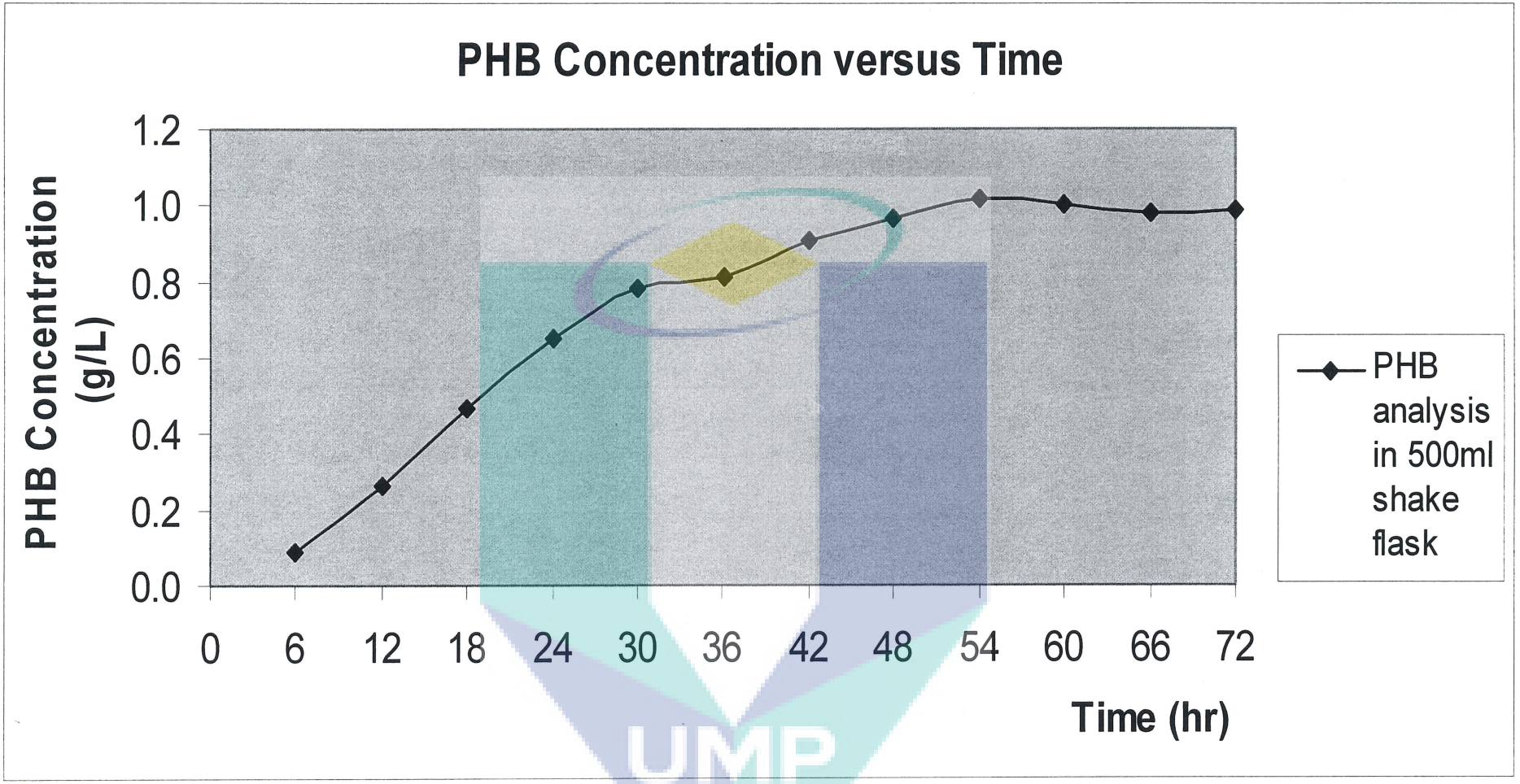

Figure 4.10: PHB concentration versus time for 500ml shake flask

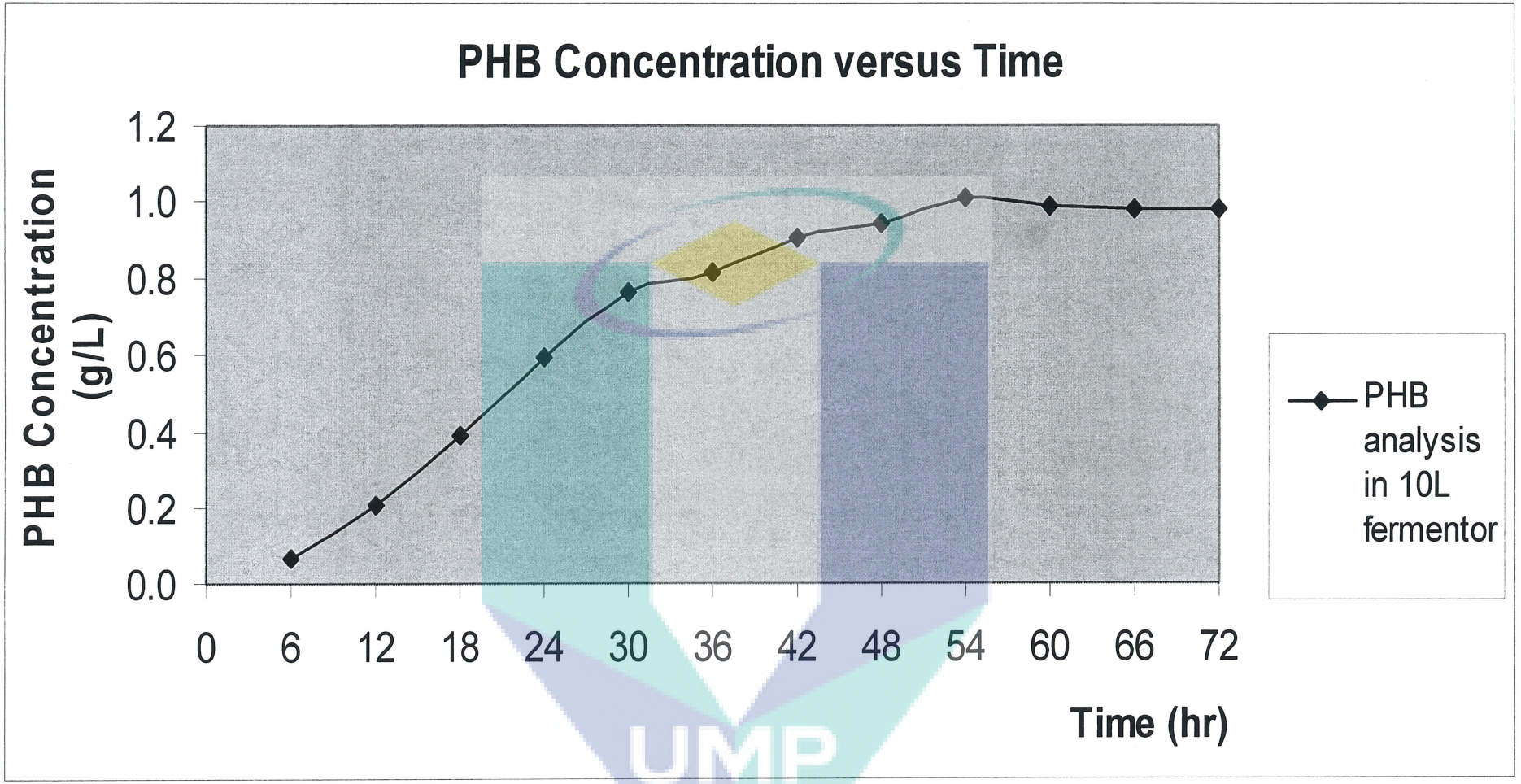

Figure 4.11: PHB concentration versus time for 10L fermentor

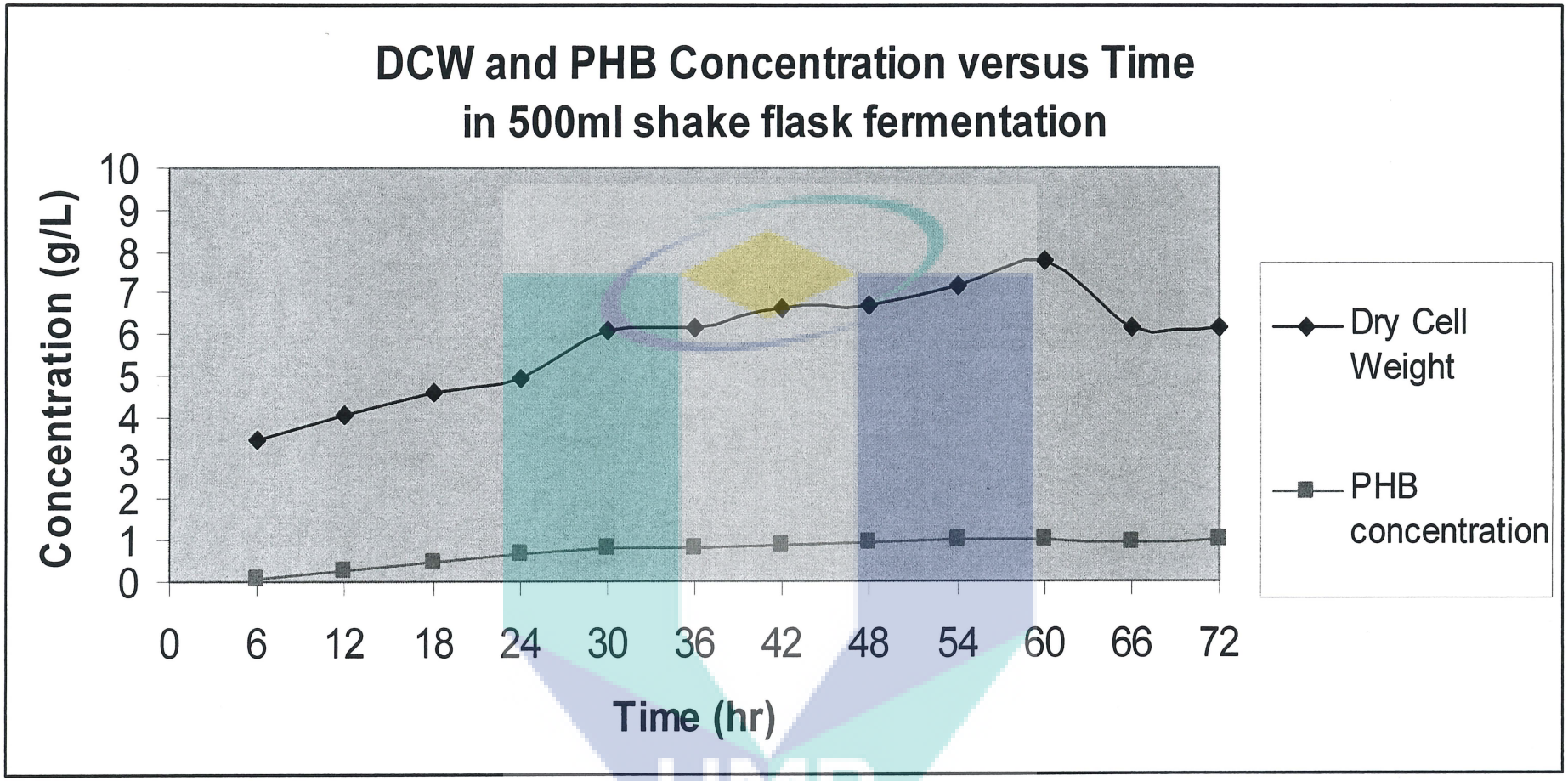

Figure 4.12: DCW and PHB concentration versus time for 500ml shake flask

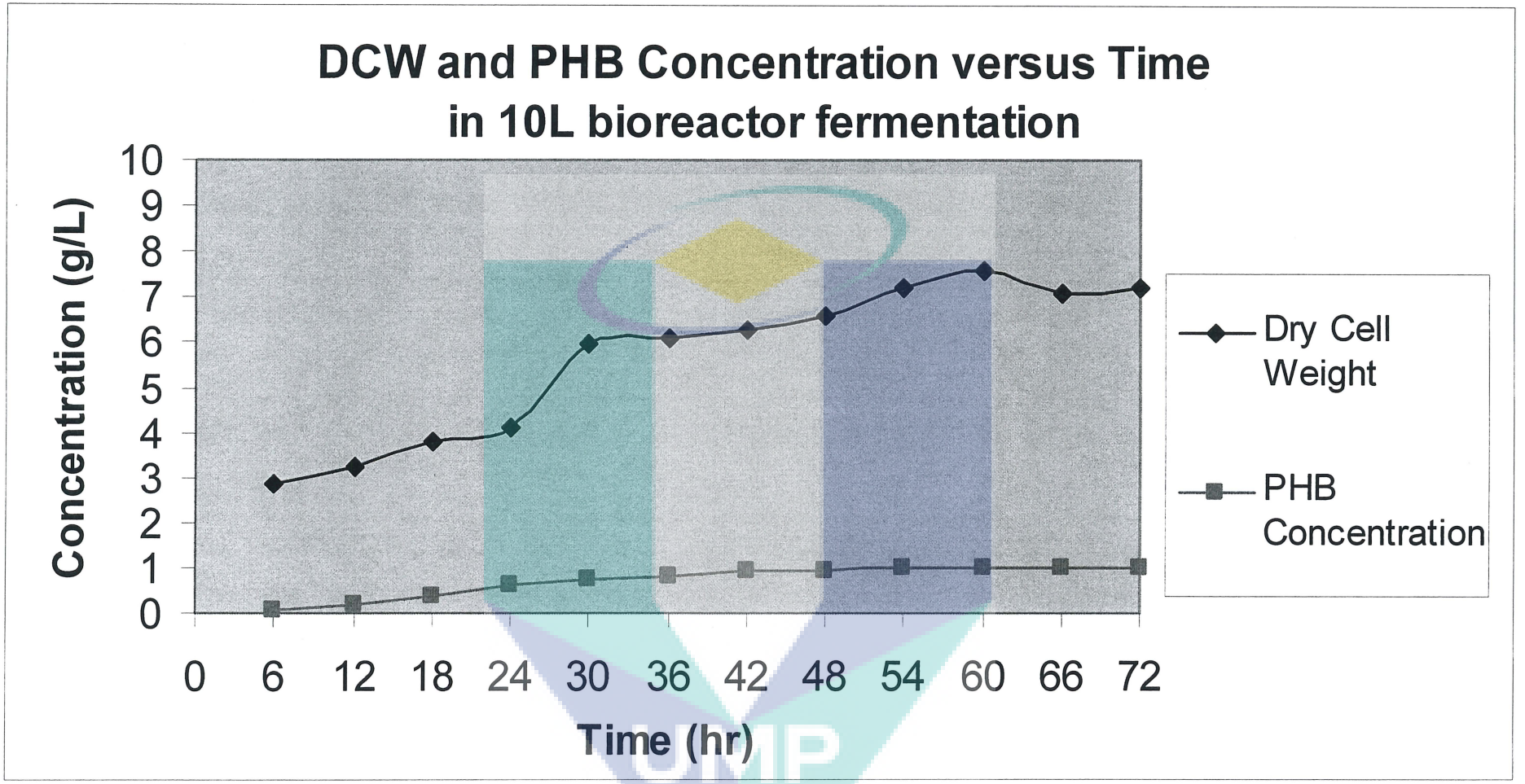

Figure 4.13: DCW and PHB concentration versus time for 10L

CHAPTER 5

CONCLUSION

This research showed that the scale up of the fermentation process for the production of PHB was successful. However, improvements might be possible in the 10L fermenter by optimizing the air flowrate and the impeller speed.

CHAPTER 6

RECOMMENDATION

For further research, it is recommended that the air flowrate and the impeller speed of the 10L fermenter be optimized to achieve maximum PHB production.

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APPENDIX A : Experimental Results of the Screening Experiment

Table 1 : Levels of Variables and Experimental Results of the Screening Experiment

no	RPM	T (°C)	G (g/l)	P (g/l)	A (g/l)	C (g/l)	TE (ml)	Biomass (g/l)	PHB (g/l)
1	140	26	22	4	6	0.02	10.00	4.74	0.396
2	140	26	18	4	6	0.06	2.80	2.94	0.255
3	140	30	30	1	3	0.01	10.00	5.93	0.574
4	140	30	14	4	2	0.02	8.20	2.13	0.231
5	140	32	26	3	2	0.02	8.20	6.30	0.526
6	140	34	30	6	4	0.01	2.80	6.21	0.527
7	140	34	22	4	4	0.06	10.00	5.26	0.341
8	140	36	14	1	5	0.04	4.60	1.79	0.224
9	170	26	14	2	2	0.06	10.00	8.96	0.380
10	170	28	18	4	5	0.02	1.00	3.68	0.440
11	170	32	22	2	3	0.01	10.00	5.39	0.611
12	170	34	30	4	5	0.01	2.80	8.42	0.836
13	200	30	22	1	2	0.04	8.20	5.61	0.712
14	200	30	10	6	4	0.03	4.60	2.48	0.357
15	200	30	26	3	1	0.01	6.40	8.34	0.830
16	200	32	14	1	3	0.02	6.40	2.56	0.322
17	200	34	30	5	4	0.01	6.40	9.68	0.855
18	200	36	22	1	6	0.04	6.40	3.57	0.652
19	230	32	10	3	1	0.04	2.80	2.03	0.321
20	230	34	14	4	1	0.03	6.40	2.81	0.374
21	260	26	10	3	3	0.06	4.60	2.13	0.489
22	260	26	22	3	5	0.04	4.60	5.33	0.721
23	260	30	22	1	2	0.01	4.60	5.48	0.783
24	260	34	10	6	1	0.03	10.00	2.28	0.364
25	260	36	18	3	3	0.06	6.40	3.42	0.431

APPENDIX B: 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 1: 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

Table 1: Continued

Time (minutes)	DOT (%)	Concentration (ppm)
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 C: 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 1 : 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

Table 1 : Continued

Time (minutes)	DOT (%)	Concentration (ppm)
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 D: MATLAB Window Command for 500ml Shake Flask

Document 1

```
>> 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 E: MATLAB Window Command for 10L Fermentor

Document 1

```
>> 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 F: Glucose Analysis Data

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

   y = 0.9109x

Table 1: 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 2: 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 G: Dry Cell Weight Data

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

Table 1: 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 2: 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 H: PHB Analysis Data

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

   $y = 0.1492x$

Table 1: 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 I: Standard Curve for Glucose Concentration

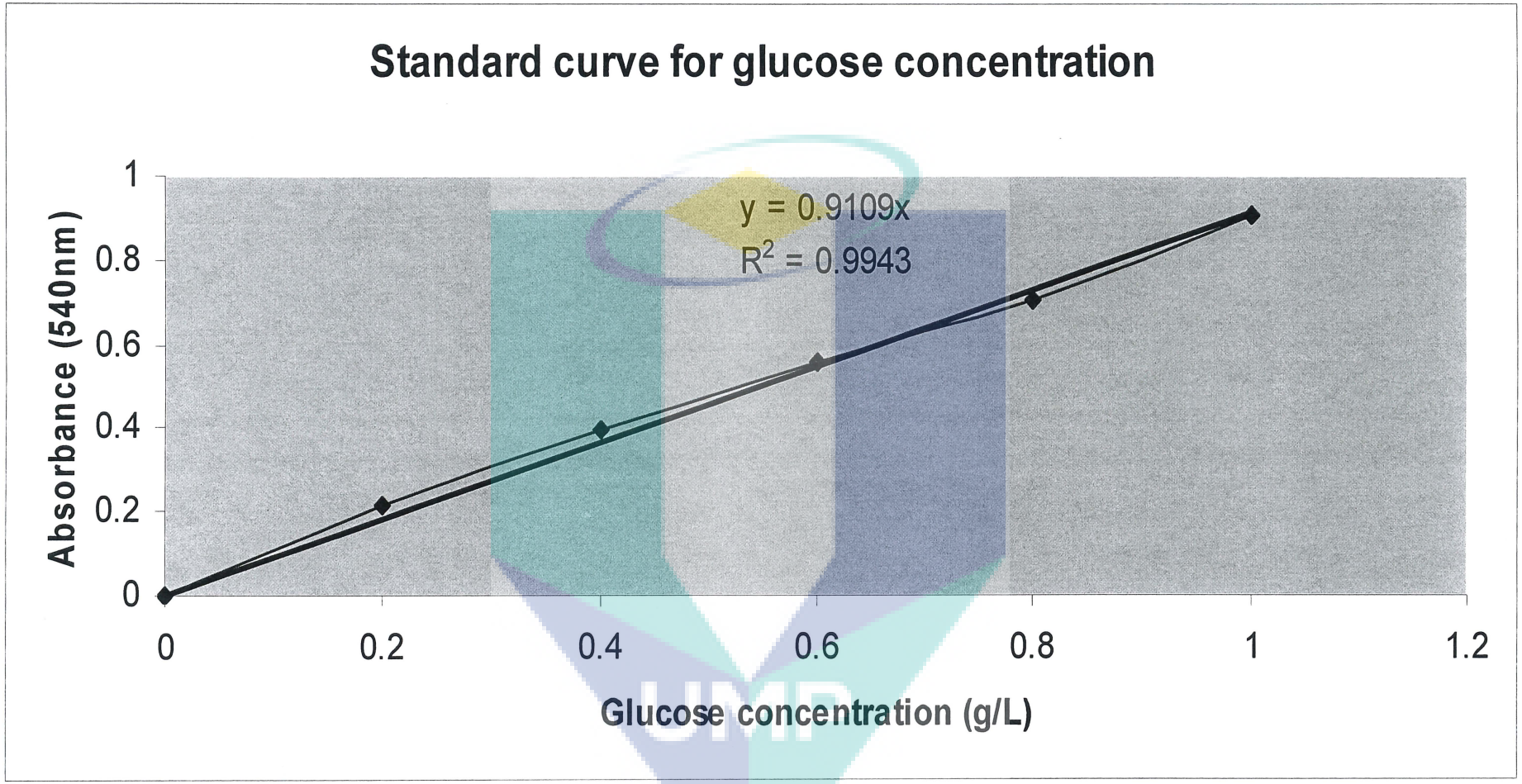

Figure 1: Standard curve for glucose concentratio

APPENDIX J: Standard Curve for PHB Concentration

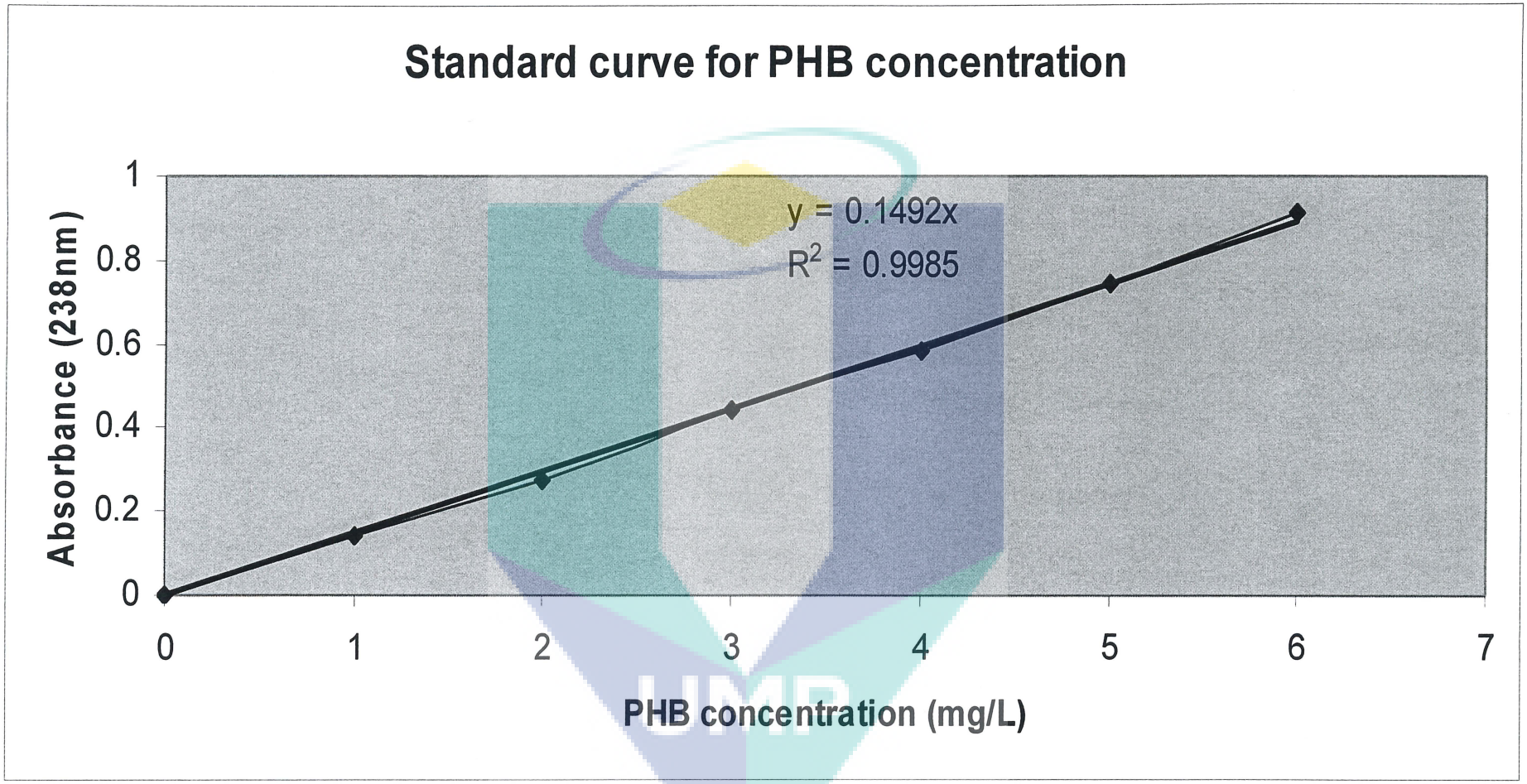

Figure 1: Standard curve for PHB concentration