EFFECTS OF VARIABLES ON BIPOLYMER PRODUCTION IN 20L BATCH STIRRED TANK FERMENTATIONS

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EFFECTS OF VARIABLES ON BIOPOLYMER PRODUCTION IN 20L BATCH STIRRED TANK FERMENTATIONS

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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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APRIL 2010

"I declare that this thesis entitled "Effect of Variables On Biopolymer Production in 20L Batch Stirred Tank Fermentations" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

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To my beloved mother

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ABSTRACT

This research is conducted to study the effects of variables on polyhydroxybutyrate(PHB) production in 20L batch stirred tank fermentations by using *Cupriavidus necator*. It consist of three major phases starting with scale up process, followed by fermentation process and mathematical analysis. The fermentation process is scaled up from the optimum condition in 500mL shake flask to 20L stirred tank bioreactor by approximating the set point values of air flowrate and agitation speed which gives the same dissolve oxygen tension (DOT) curve versus time. The closest DOT curve pattern to that of the optimum condition in 500mL shake flask is achieved by the combination 230 RPM agitation speed and 10L/min compressed air flowrate. This experiment is followed with fermentatin process involving the manipulation of the parameters agitation speed, temperature and initial glucose concentration. The 2^3 factorial experiment method is used to design the experiment around the set points of agitation speed and air flowrate which gave the optimum DOT curve. Among the experimental points, it is found that the highest yield of PHB is produced in condition of 210RPM agitation speed, 32°C temperature and 23g/L initial glucose concentration. This experiment was followed by mathematical analysis using Yates' Method to study the main effect and interactive effect of changing the level of experimental variables. The calculation proved that temperature has the biggest main effect on PHB production while the biggest interactive effect is from the combination of agitation speed and initial glucose concentration.

ABSTRAK

Penyelidikan ini dijalankan adalah untuk mengkaji kesan pembolehubah ke atas penghasilan polyhydroxybutyrate(PHB) di dalam tangki penampaian kelompok teraduk dengan menggunakan *Cupriavidus necator*. Ia mengandungi tiga fasa yang dimulai dengan proses menskala naik, diikuti oleh proses penampaian dan analisis matematik. Proses penampaian diskala naik dari persekitaran optimum di dalam kelalang goncang berisipadu 500mL kepada tanki reaktor teraduk berisipadu 20L dengan mendapatkan bentuk lengkuk tekanan oksigen terlarut (DOT) menentang masa yang paling sama. Lengkuk DOT yang paling hampir sama dengan persekitaran optimum di dalam 500mL kelalang goncang diperoleh daripada kombinasi 230 RPM kelajuan pengadukan dan 10L/min kadar aliran udara mampat. Eksperimen ini dikuti oleh proses penampaian yang melibatkan manipulasi pembolehubah kelajuan pengadukan, suhu dan kepekatan awal glukosa. Teknik eksperimen 2^3 faktorial digunakan untuk merekabentuk ekspeiment di sekitar titik kawalan bagi kelajuan pengadukan dan kadar kelajuan udara yang memberikan lengkuk DOT optimum. Daripada kesemua proses eksperiment, didapati bahawa PHB paling banyak dihasilkan dalam persekitaran 210 RPM kelajuan pengadukan, 32°C suhu dan 23g/L kepekatan awal glukosa. Eksperimen ini seterusnya diikuti oleh analisis matematik menggunakan taknik Yates untuk mengkaji kesan utama dan kesan saling tindak akibat perubahan aras pembolehubah eksperiment. Pengiraan menunjukkan bahawa suhu mempunyai kesan utama terbesar terhadap penghasilan PHB manakala kesan saling tindak terbesar pula adalah daripada kombinasi kelajuan pengadukan dan kepekatan awal glukosa

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LIST OF SYMBOLS

PHB	-	Poly-β-hydroxybutyrate
PHA	-	Poly-β-hydroxyalkonoate
DOT	-	Dissolve Oxygen Tension
RPM	-	Rotation Per Minutes
g	-	Gram
L	-	Liter
W%	-	Weight Percent
m	-	Molar
OD	-	Optical Density
mg	-	Miligram
Min	-	Minutes

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CHAPTER 1

INTRODUCTION

1.1 Background Study

In the world that full of fascinating technology and the concern about the facing of the demand, biotechnology is couraged to offer an increasing potential for the production of goods to meet various human needs. The goal of these approach is to meet the criteria sustainability in concept that is introduced by the World Commission on Environment and Development (WCED, 1987). The concept of sustainability aim to promote "..development that meets the needs of the present without compromising the ability of future generation to meet their own needs". To determine the application of the concept, criteria that concern is its economic, environmental and social impact perspective.

In the objective to fulfill the human needs, the plastic is invented from oil and petroleum about in 1933 by German chemist. The plastic that we daily used is the polyethylene plastic which is inexpensive material and be very durable. However, the common property of this polyethylene plastic is the high resistance to natural

degradation process and cause a crucial problem to environment in space for landfill (Huisman *et al*.,) So, it is not environmental friendly and contribute to the pollution as the increasing of the solid waste, whose main part is plastic. Furthermore, it is opposite to the concept of sustainable development.

In conjunction to settle this problem, there is the obligation for us to find another alternative material to replace the polyethylene plastic based material that can be easily degrade or biodegradable material in force to save the environment for the next generation. The awareness of this situation lead to the research and invention of biodegradable plastic and environmental friendly. One of them is production of polyhydroxyalkanoates (PHAs) and polyhydroxybutyrate which is microbially-formed. The presence of the polyhydroxybutyrate was confirmed as an energy and carbon source and storage in various bacteria apparently in response to conditions of physiological stress (Kim *et al.,* 1994*)*. The most advantage of PHB is given by its properties that is biodegradable in reasonable time period.

Polyhydroxybutyrate (PHB) is a high molecular weight polyester. It is a biodegradable intracellular microbial thermoplastic that provide carbon and energy reserves in several microorganism. PHB polymer is produced by microorganisms such as *Alcaligenes eutrophus* or *Basillus megaterium* and *Cupriavidus necator* under unfavourable conditions (Lee, 1996). Polyhydroxybutyrate has attracted much commercial interest as a plastic. These polymers have similar properties to some of petrochemical-derived thermoplastics such as polyethylene in term of molecular weight, melting point, stiffness, brittleness and glass transition temperature (Steinbuchel, 2002). PHB polymer is resistant to water and ultravioletradiation and also impermeable to oxygen. Therefore, it is suitable to replace the polyethylene in application for food and drink packaging. Besides, it can help to reduce land pollution due to its characteristic that is biodegradable in soil.

1.2 Problem Statement

Commonly, the production of biopolymer is known as a relatively expensive process especially in large scale or industrial scale compare to the production of polyethylene based material plastic. This is happen due to biological process used that required the usage of growth medium in large scale such as glucose and other mineral which quite expensive, in the other hands, the preparation of the inoculum in the culture media needs 3 days and the extraction process to obtain the biolpolymer or PHB need the usage of specific and expensive solution also contribute to the high cost production process. The cost that required is seems high as it is compared to the concentration of biopolymer that may produce from the fermentation process after several days. Therefore, it is a very strong obligation to optimize the production of bioplastic by study the effect of changing the variables of production and at the same time minimize the cost. Hence it would strengthen the promotion on public to use PHB based material for daily usage.

1.3 Scope of Research Work

The study for effect of variables on biopolymer production in 20L batch stirred tank fermentations by using *Cupriavidus necator* involve the following steps:

- Obtaining from the previous jurnal for optimum condition for the production of PHB of fermentation in 500 mL shake flask.
- Scale up process from 500mL shake flask to 20L fermenter
- The fermentation process in 20L tank fermenter by manipulating the condition of the fermentation.

- Experimental analysis to obtain the production profiles.
- Mathematical analysis to study the effect of variables on production biopolymer in 20L batch stirred tank fermentations.

1.4 Objectives

- To produce polyhydroxybutyrate (PHB) which is biodegradable plastic as a replacement to polyethylene plastic that non-biodegradable.
- To scale-up biopolymer (PHB) fermentation process from scale of 500mL shake flask to scale of 20L stirred tank fermenter.
- To study the effect of variables on production of PHB in scale of 20L stirred tank fermenter by manipulating the parameters which are the agitation speed, initial glucose concentration and temperature of the fermentation process.

CHAPTER 2

LITERATURE REVIEW

2.1 Development of Bioplastic

As the technology skyrocket with new fascinating inventions to fulfill the human demands, a group of materials never seen before on our planet is produced. They were synthetic polymers, also known as plastics. The application of this type of material is widely accepted in daily usage to improve the lifestyle around the world. Contrarily, the wide application of plastic give negative impact on earth due to high resistance of the material to natural degradation process. Statistically, in Malaysia, the per capita generation of solid waste varies from 0.45 to 1.44kg/day depending on the economic status of an area and most of the waste are consist of plastic based material. In general, the per capita generation rate is about 1kg/day. Disposal of solid waste is done solely through landfill method with capacity about 177 disposal sites in Peninsular Malaysia (Malaysia Country Report, 2001).

The usage of the conventional type of plastic, which is polyethylene based material contribute to the huge amount of the solid waste. Hence cause the limitation problem in landfill space due to its non-biodegradable property. Biodegradation process refer to the chemical breakdown of materials by a physiologically environment where the materials are degraded aerobically with oxygen or anaerobically without oxygen. Sometimes, this process is related to the environmental remediation or bioremediation (Diaz E., 2008)

Basically, biodegradable matter is organic material such as plant and animal or other substances originated from living organisms or artificial that similar enough to plant and animal matter to be put to use by microorganisms (Diaz E., 2008). In conjunction to overcome the problem of limitation in landfill space, the production of bioplastic is looked as a precious invention to meet the human demand and at the same time does not give negative effect on the environment. This is due to its biodegradable property compared to the conventional type. In fact, one of the the bioplastic that invented is polyhydroxybutyrate (PHB)

2.2 Polyhpolyhydroxybutyrate (PHB)

2.2.1 Introduction of Polyhpolyhydroxybutyrate (PHB)

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate (PHA), a polymer belonging to the polyesters class that was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne in the bacterium *Bacillus megaterium*. In subsequent years, it was also found in other species of bacteria as at

the end of 1950s, the presence of PHB is confirmed as an a carbon source and energy (Poirier *et al*, 1995). The structure of PHB is shown in the Figure 2.1

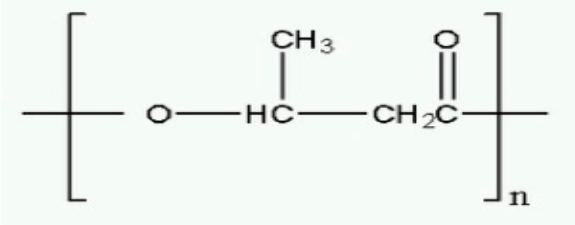


Figure 2.1 Structure of PHB

Bacteria that produce PHB can be divide into two groups. The first one include the *Ralsotnia eutropha (Alcaligenes eutropha),* and consist of bacteria that produce short-chain PHA with C6-C5 monomers, while the second, which contain for example *Pseudomonas oleovorans,* involves microorganisms that produce medium-chain PHA with C6-C14 monomers (Poirier et al., 1995)

PHB is produced by micro-organisms apparently in response to conditions of physiological stress. The polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by micro-organisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA. This latter compound is then used as a monomer to polymerize PHB.

2.2.2 Properties Of Polyhydroxybutrate

Historically, PHB has been studied most extensively and has triggered the commercial interest in this type of polymer. PHB is the common type of PHA , and the ability of bacteria to accumulate PHB is often used as a taxonomic characteristic. Copolymers of the PHB can be formed by cofeeding of substrates and may result int the formation of polymer containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers (Brandl *et a*l, 1988).

In general, PHB have the similar properties as the polyethylene plastic with further advantage of PHB for biological degradable and nontoxic material. As addition, PHB have the ability of water insoluble and relatively resistant to hydrolytic degradation which differentiate it with the another biopolymer (Peijs, 2002). The biodegradable ability is the most important characteristic of PHB. In nature, the degradation process depend on the activities of enzyme in the microorganisms. Therefore, the process may vary and depend on the composition of the polymers, its physical form (amorphous or crystalline), the dimensions of the sample and the most important is the environmental conditions (Jendrossek et al., 1998). Typically, the degradation rate of a piece of PHB is around a few month in anaerobic sewage to years in seawater (Mergaert, *et al*, 1995)

Within the cell, PHB exist in a fluid and amorphous state. However, after the extraction process from the cell with organic solvent, PHB becomes highly crystalline and in this state, it is stiff but brittle (Doi, Y., 1995). Besides, the PHB is good oxygen permeability and good ultra-violet resistance but poor resistance to acids and bases. PHB also soluble in chloroform and other chlorinated hydrocarbon and have the ability of biocompatible. It have the melting point of 175°C and glass transition temperature 15°C (Lara L., 1999). PHB has the tensile strength 40 MPa,

which close to polypropylene strength. Other physical property of PHB is sinking in water (while polypropylene floats), and cause facilitating its anaerobic biodegradation in sediments.

In the other hands, the production of PHB which is from the fermentation process attract the world attention since the fermentation process is based on the renewable resources and agriculture products such as sugars and fatty acid as the carbon source. Thus, it is looked as the answer to the problem of diminishing fossil fuel stockpiles that used in production of conventional plastic. However, in using the PHB as the plastic material have some disadvantage due to its tendency to be brittle. When it was spun into fibers it behaves as a hard-elastic material (Antipov *et al*, 2006). Besides, the synthesis process of PHB is economically challenging compare to the production of polyethylene plastic.

2.2.3 Application of Polyhydroxybutrate

The main issues that become the driving force the production of PHB is as biodegradable plastic and as replacement to the usege of the non-biodegradable plastics disposal such as polyethylene based material. Recently, the usage of the non-biodegradable plastics may causes significant ecological problems. That is, the availability of landfills is limited and the incineration of plastics increases greenhouse gases and releases toxic compounds. In contrarily, PHB is a biodegradable product, biocompatible thermoplastic and has similar physical properties to polypropylene. It has similar piezoelectric properties to natural bone and is optically active (all of its monomers are the d-isomer).

For further application of PHB, there are numerous research done by extending the physical properties of PHB and the PHA family of polymer by compounding and blending to provide a corresponding broad range of end-use applications. For example is the molding applications in consumer packaging and coating items. Besides, PHB can be used in toner and developer compositions or as the ion-conducting polymers. Instead, PHB also applicable in medicine, veterinary practice and agriculture due to its biodegradability. Its biocompatibility is the reason of medical applications such as surgical pins and sutures.

2.2.4 Metabolic Pathways of PHB synthesis

The biosynthetic pathway of PHB consists of three enzymatic reactions catalyzed by three distinct enzymes. The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl- CoA by b-ketoacylCoA thiolase (encoded by phbA). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH- dependent acetoacetyl-CoA dehydrogenase (encoded by phbB). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by PHB polymerase, encoded by phbC. PHB is synthesized by the successive action of b-ketoacyl-CoA thiolase (phbA), acetoacetyl-CoA reductase (phbB) and PHB polymerase (phbC) in a three-step pathway (Steinbuchel and Fuchtenbusch, 1998). The genes of the phbCAB operon encode the three enzymes. The promoter (P) upstream of phbC transcribes the complete operon (phbCAB) (Daae *et al*, 2008).

2.3 *Cupriavidus necator*

Cupriavidus necator was described as accommodatation of non-obligate bacterial predator of various Gram-negative and Gram-positive soil bacteria and fungi. 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; and *Alcaligenes eutrophus* (first reclassified in the genus *Ralstonia* and recently transferred again, to the novel genus *Wautersia.*

2.4 Study for Effects of variables

2.4.1 The First Area Of Response Surface Investigation

Once the experimental variables or variables relevant to the fermentation have been screened by using the method of Factor Analysis, the combination of levels of experimental variables that gives the highest yield among other combinations is obtained. Besides, the levels of combination variables can be obtained from scale up process. The combination of levels of variables that gave the highest yield is used as the centre point of first area of response surface investigated for presence of the point of maximum yield. The area around this centre point can be investigated for presence of the point for maximum yield using the method of factorial experiment.

2.4.1.1 The Method of Factorial Experiments

There are many types of factorial experiments, however the common type used is the one that involving two levels or known as the 2^n factorial experiments (Cochran et al., 1957). This method has been designed to allow the effects of a number of experimental variables on the yield to be investigated simultaneously.

This method gives the 'main effects' and the 'interactive effects' of changing the levels of the experimental variables from the lower level to the upper level. The main effect of an experimental variable is defined as the average of the effect of changing its value from the lower level to the upper level among all experiments. It is derived by assuming that the experimental variable is an independent parameter and all variations in its effects are due to experimental only.The interactive effects between two or more experimental variables are calculated on the assumption that the experimental parameters are not independent but are in fact interacting between them.

2.4.1.2 Yates' Method

This mathematical method is used to analyze the main effects and interactive effects. At the same time, the result that obtained from the calculation of this method also indicate whether the yield response surface in the area of examined is curved or incurved, whether it is flat or not, whether increasing or decreasing with relation to one or more experimental variables and in which direction.

CHAPTER 3

METHODOLOGY

3.1 Research Background

The study effects of variables on production in 20L batch stirred tank fermentations is carried out with 15L working volume by using microorganism *Cupriavidus necator*, formerly known as *Ralstonia eutropha* to produce PHB which is the biopolymer.

Previously, the optimization process of production PHB already carried out in small scale fermentation process which is conducted in 500mL shake flask with 200mL of working volume. Project is extended by optimizing the production of PHB in fermentation process that carried out in big scale of 20L stirred tank fermenter with 15L working volume. The research consists 6 phase of experimental and mathematical methodology which are:

i. Obtaining the information from previous research, (Aisyah Azmi, 2009)
ii. Scale up process.

iii. Mathematical method of Factorial experiment.

iv. Fermentation.

v. Sample analysis.

vi. Mathematical method for optimization process.

3.2 Materials

In this experiment, the material that used are microorganism *Cupriavidus necator,* mineral agar medium for cultivation of microbe, growth medium, glucose as the carbon source, nitrogen gas and compress air.

3.3 Previous Research

The highest concentration of PHB that produce at the optimum parameter is 1.0802 g/L with 5.8350 g/L biomass. The optimum values of parameters are 28.46 °C, initial glucose concentration of 28.66 g/L, agitation speed of 251.93 RPM and initial peptone concentration of 10.05 g/L. Besides, the journal also show that the main effect of the initial concentration of peptone give low effect on the production of PHB and can be negligible compare to another parameters. Therefore, in this extending research, the parameters that manipulated are agitation speed of impeller, temperature of fermentation and initial concentration of glucose(Azmi, A.,2009)

3.4 Scale Up Process

Scale up process is carried out to obtain similar amount of product per volume for small scale and large scale in the same time duration. The main part in scale up process is by maintaining the rate of oxygen transfer in the large scale like already achieve in the small scale. The method for scale up process that used is applied in this is known as Gassing Out Technique is used to obtain the dissolve oxygen transfer (DOT) curve in small scale of shake flask and in 20L fermenter (Salihon, J., 1995)

An oxygen probe is connected to the oxygen meter and immersed in a 500mL shake flask that filled with 200mL of distilled water. Nitrogen gas is sparged into the flask until the reading of oxygen probe show zero. Calibration method is required as the reading of oxygen probe unable to achieve value of zero. After that, the flask is shaked in the orbital shaker with the speed of 250 RPM and air flowrate of 1L/min. The values that used are the optimum value of parameters for production PHB in 500mL shake flask which already obtained by the previous research. At the same time, the reading of the oxygen probe is taken for each minutes. Graphically, the oxygen probe reading versus time is plotted.

The steps above is repeated by using the scale of 20L stirred tank fermenter with 15L of working volume. This equipment is filled with distilled water to obtain the same pattern of DOT curve as already obtained in 500mL shake flask. The values of the agitation speed and the air flowrate are determined by using trial-and-error technique. The values of parameter that obtain is used in the next step of research.

3.5 Mathematical Method of Factorial Experiment

This method is used to determine the number of experiments that should carried out in order to investigate the effect of three parameters simultaneously. The mathematical equation that used is shown as below:

Number of experiment = 2^n n= 3 parameters used.

Therefore,

number of experiment= 2^3 = 8

From the calculation, the table that show the combination of the parameter are constructed as shown in table below:

Table 3.1 Experimental Table Constructed

Number	Agitation Speed (RPM)	Temperature (°C)	Glucose Concentration (g/L)
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1

3.6 Fermentation Process

Fermentation process consist of 3 stage of experimental process which are cultivation of microbe, inoculum development 1, inoculum development 2, inoculum development 3 and fermentation production stage.

3.6.1 Cultivation of Microbe

The cultivation of microbe is conducted every two weeks. For regeneration of the bacteria, the culture is maintained at slant medium. Regeneration is conducted every two weeks. The agar slant medium was prepared with composition of 5g fructose, 2.5g peptone, 1.5g yeast extract, 0.15g beef extract, 15g agar and distilled water that added until total volume of 500mL.

The solution is heated in a Scott bottle with continuous stirring on laboratory hot plate until the solution comes into boiling. After that, the agar medium in the Scott bottle is sterilize in the autoclave for 20 minutes at temperature of 121 °C. After the sterilization process ended, the agar is is poured into each sterilized agar plate. The agar is allowed to cold down and solidify about in 15 minutes. The bacteria are transferred from the old slant to the new slant in sterile laminar air flow hood. The slant was incubated in the sterile incubator at room temperature for about 24 hours until the bacteria seem to grow. Then, the new slant agar that contain the microbe is kept in the refrigerator at 4ºC for long time maintenance.

3.6.2 Inoculum Development 1

Growth medium is prepared at the initial process which the composition of the materials inside are 20g fructose, 10g peptone 6g yeast extract and additional distilled water until the total volume is 2L. The solution is stirred until well mixed. Then, the growth medium solution is sterilized in autoclave for 20 minutes at 121 °C. The solution is allowed to cold down to room temperature.

20 ml of growth medium solution is put in 100 ml Erlenmeyer flask that already sterilize(20 minutes at 121 °C). The One loop of the bacteria from the slant was taken and is put into the growth medium and closed with the flask was closed with sterile cotton that already sterilized in autoclave for 30 minutes at 121ºC. In attention, all the step involve in transferring microbe is conducted in sterile laminar air flow hood. The flask that contain the growth medium and microbe is incubated in incubator shaker for 24 hours with condition of 30 °C and 200 RPM for agitation speed. After 24 hours, the content is ready to move into inoculum development 2.

3.6.3 Inoculum Development 2

After 24 hours, the inoculum development 1 is ready for the next step of experiment. 20mL of inoculum development 1 is put into a 500mL Erlenmeyer with 180mL of growth medium and plug the inlet with sterile cotton. In additional, all glassware and apparatus that used in this transferring process which that have direct contact with microbe including cylindrical measurement are sterilize in the autoclave first.Besides, all the transferring process is conducted in the laminar flow hood.

The flask is incubated in the incubator shaker for 24 hours with condition of 28 °C and 250 RPM for agitation speed which the optimum values of parameters that obtained from the previous research. After 24 hours, the content is ready to move into inoculum development 3.

3.6.4 Inoculum Development 3

After 24 hours, 200mL of inoculum is obtained from the inoculum development 2 and ready for the next step. 20mL of inoculum development 2 is put into a 500mL Erlenmeyer with 180mL of growth medium. Thus, making the working volume for this stage is 200mL and the inlet is plugged with sterile cotton. These technique is applied for 8 Erlenmeyer flask with same volume and composition of growth medium and inoculum. This step is carried out in order to obtain 1500mL of inoculum and at the same time, the optimum values of parameter can be used in this condition.

The flask is incubated in the incubator shaker for 24 hours with condition of 28 °C and 250 RPM for agitation speed which the optimum values of parameters from the previous research. After 24 hours, the content is ready to move into fermentation production stage.

3.6.5 Fermentation in 20L Stirred Tank Fermenter

Before bioreactor is used, some solution need to prepared. For pH control, 2M of sodium hydroxide solution is made by adding 53mL of concentrated NaOH with 50% purity into distilled water until the total volume of solution is 500mL. For acidic agent, 54mL of concentrated sulphuric acid with 98% purity is added into distilled water until the total volume of solution is 500mL. Besides, mineral salt medium is prepared for the fermentation process. The composition of the substances is as in table 3.2 and the medium is diluted in 2L distilled water.

Table 3.2 The Composition of Substances for Mineral Salt Medium.

Chemicals	Amount (g/L)
Peptone	75.0
$Na_2HPO_4.7H_2O$	100.5
KH_2PO_4	22.5
$(NH_4)_2SO_4$	30.0
$MgSO_4$	3.0
Distilled Water	Added until total volume 2L

The bioreactor was cleaned throughly before every usage by using deionized water. After the deionized water is removed from the fermenter, Mineral Salt Medium without glucose that prepared was poured into the bioreactor. Then, 9.5L distilled water and 15mL trace element is added into the bioreactor and making the current working volume is 11.5L. The trace element is prepared by diluting 0.713g $NiCl_26H_2O$, 0.682g $CuCl_22H_2O$, 1.33g $CrCl_36H_2O$, 0.396g $MnCl_24H_2O$, 0.2g $CuSO_45H_2O$, 0.863g $ZnSO_47H_2O$, 0.484g $Na_2MoO_42H_2O$ and 0.019g $CoCl_26H_2O$ in

1L of distilled water (Hassan *et al,* 1997) The bioreactor is switched on and air, steam and water supply are opened and connected to the bioreactor. The medium inside the bioreactor is sterilized by using in-situ sterilization unit of the bioreactor.

At the same time, glucose is diluted in 2L distilled water. The mass of glucose are vary because the initial glucose concentration is one the parameter that manipulated. The solution is sterilize separately from the mineral salt medium in autoclave for 20 minutes with 121°C. 1500mL inoculum that obtain from step inoculum developmetn 3 is collected and gather in a sterilize inoculum bottle and ready to flow into the bioreactor after the sterilization process end.

After sterilization process in 20L stirred tank bioreactor finish, glucose is flowed into it first. The conditions of the fermentation process is set up. Then, the inoculum is flowed and the bioreactor is run. Samples were withdrawn through the sampling line for every 6 hours intervals.

3.7 Analysis Methods.

3.7.1 Biomass Analysis

The samples were taken at every 6 hours time intervals is tested for biomass content. Then, 10mL of sample is recovered by centrifuge at 6000 RPM and 4°C for 10 minutes. Pallet and supernatant that obtained after centrifuge is proceeded to next where the pellet is used in biomass analysis while the supernatant is used for glucose

detection. The pellet that obtained was added with 2mL of deionized water and transfer into test tube for further dilution. After the dilutions were done, the optical density of the mixture was read under the absorbance at 600nm using UV-VIS spectrophotometer. The concentration of the biomass was determined by using standard curve

3.7.2 Glucose Analysis

Glucose analysis was determined by using the DNS (dinitrosalicylic acid) method. The DNS reagent is prepared by dissolve 1g NaOH, 18.2g Potassium sodium tartarate, 1g 3, 5-dinitrosalicylic acid, 0.2g Phenol and 0.05g Sodium sulphite in 100mL of distilled water. Wrap the bottle with aluminium foil to prevent phenol oxidation to phenolic compound. Keep in fridge with 4°C. Other solution that prepare are 0.1 M NaOH and glucose standard (0.2 to 1.0 mg of glucose per mL). To produce 0.1M NaOH, 4.08g of NaOH with purity of 98% is dissolve in 1L of distilled water. Standard glucose is prepared by diluting 0.2g, 0.4g, 0.6g, 0.8g and 1.0g glucose respectively with distilled water.

To analyze the glucose, 1ml supernatant is mixed with 1.0 ml DNS reagent and 2 drops of 0.1M NaOH. The same method is done to the standard glucose and distilled water (blank solution).Place in boiling water for 5 minutes and cool under water flow. Then, add 10 ml of distilled water and mix evenly. Read absorbance at 540 nm using spectrophotometer. Quantify glucose in culture broth according to glucose standard curve between 0- 1.0g/ml.

3.7.3 PHB Analysis

10mL of culture sample is centrifuge at 5000rpm, 12minutes, and 4°C.the cell pellet are re-suspended with 10mL of 0.625% sodium chloride, then centrifuge again (5000rpm, 12min, 4ºC), the cell pellet is re-suspended again using 10mL of 100µM hydrogen peroxide. The mixture is then put into shaker water bath for 4 hour, at 30°C. The mixture is centrifuge again (5000rpm, 12min, 4ºC). The cell pellet is then re-suspended again by adding 10mL of chloroform and the solution is dried in a glass petri dish. After the chloroform is totally evaporated, 5mL of hydrochloric acid is added into the petri dish. The solution is then transferred into glass test tube and put into boiling water for 10 minutes. After the solution is cooled down, the absorbance of the solution is readed at 238nm using spectrophotometer. Noting that, for PHB absorbance reading, quartz cuvette is used. Then, the PHB concentration in time interval sample is quantified according to PHB standard curve with concentration between 0- 7.0mg/ml.

3.8 Mathematical Method

The values that optimized in production of PHB in shake flask is calculated by using Design Expert software which is factorial design. Besides, Yates' Method is used to determined the main effect and interactive effect.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

As shown in previous chapter, this research is an extended project to determine the optimum value of parameter in 20L stirred tank fermenter. Starting with scale up process based on the optimum values in shake flask, the process continue with mathematical method of calculation to construct sequence of experiment for fermentation process. Then, the parameter that may result the highest concentration of PHB is discussed.

4.2 Scale Up Process

Dissolve oxygen transfer curve versus time is determined from both scale which is in 500mL shake flask and 20L bioreactor. In scale of 500mL with 200mL working volume, the parameters value is obtained from previous research which are 250 RPM for agitation speed and 1L/min for compress air flowrate. In the bioreactor scale with 15L working volume, the values of parameter is determined by using trial-and-error method where the agitation speed of impeller and compress air flowrate are manipulated. The result that colected is shown in Appendix A. Based on the result obtained, the combination of 230 RPM agitation speed and 10L/min compress air flowrate in bioreactor of 20L show the most similar pattern of DOT curve versus time. The comparison for DOT curve versus time for both scale is shown graphically in Figure 4.1.

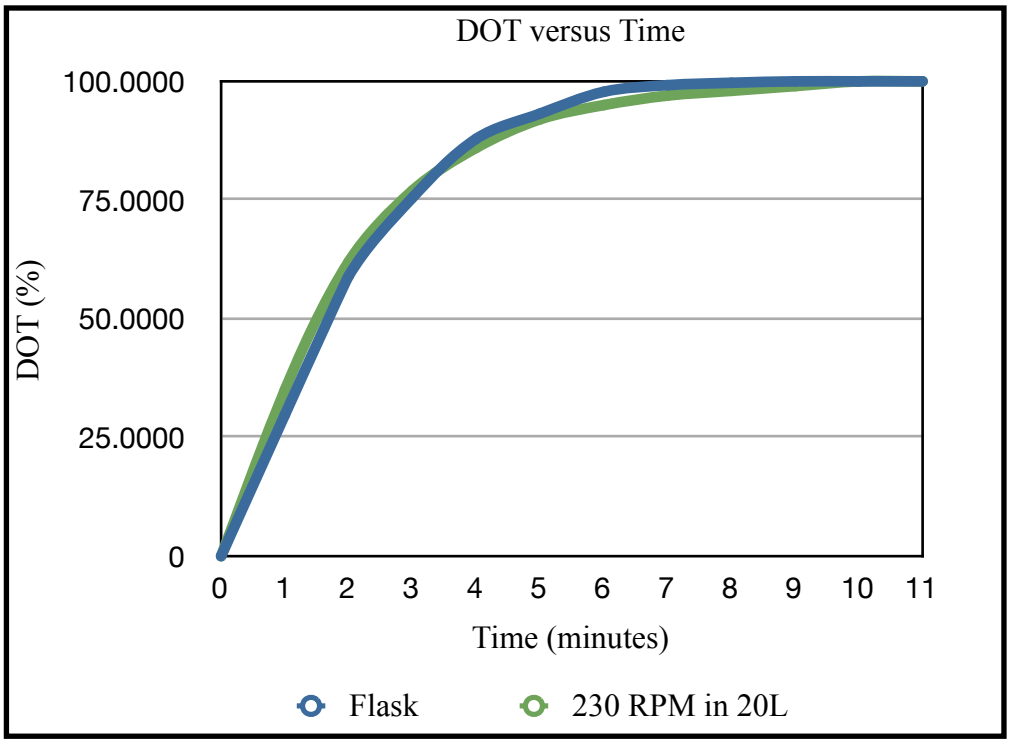


Figure 4.1 DOT (%) versus Time Curves for 500mL Shakes Flask and 20L Fermenter

Scale up method by comparing the pattern of DOT curve is a common method in fermentation process especially involving aerobic fermentation like production of PHB by using *Cupriavidus necator*. The main idea is to maintain the oxygen transfer rate to the microbe is from the small scale to the large scale. If the oxygen transfer rate is different between both scale, it may cause a shocking condition of aeration rate on the microbe and cause the microbe died or lack of productivity. However, this method have some weakness such as the usage of oxygen probe is only at a point in the medium. Therefore, if the oxygen is not well mixed or dissolve in the medium can cause the error in the probe reading. Besides, this method required large volume of nitrogen gas in order to remove oxygen until the reading of the oxygen probe show zero. Furthermore, the trial and error activities is done in 20L bioreactor and involve a huge amount of nitrogen gas.

4.3 Fermentation Result

From the result that obtained in the scale up process, the values that use as the centre point to manipulate the parameters values is determined and the level of parameters that used is shown in the table 4.1 below:

Table 4.1 Level of Experimental Parameters in 2^3 Factorial Experiment

	Agitation Speed (RPM)	Temperature (°C)	Glucose Concentration
α= +1	250	32	33
α= 0	230	30	28
α= -1	210	28	23

The plan for experimental process was constructed by the Expert Design software according to the 2^3 factorial plan. Thus, the Table 4.2 below is the combination of the parameters that constructed with the highest concentration of biomass and PHB produced in every set of data.

Table 4.2 The Plan for The 2^3 Factorial Experiments and The Highest Production Concentration of PHB and Biomass.

No. Run	Agitation Speed (RPM)	Temperature (°C)	Glucose Concentration (g/L)	PHB (g/L)	Biomass (g/L)
1	210	28	23	2.362	8.4634
2	250	28	23	1.029	4.728
3	210	32	23	2.937	9.720
4	250	32	23	0.969	5.789
5	210	28	33	0.3068	11.280
6	250	28	33	0.3457	11.280
7	210	32	33	0.2294	18.854
8	250	32	33	0.3801	26.459

Production profile of PHB for all eight run is plotted graphically for further comparison and determine the highest production of PHB, highest concentration of biomass and profile of glucose concentration. The comparison for biomass concentration is shown in Figure 4.2 while the profile analysis of glucose concentration for all set of data are shown in Figure 4.3 and the PHB concentration profile are shown in Figure 4.4.

4.3.1 Biomass Analysis

Profile analysis of biomass concentration show that the concentration of the biomass increase the with time. The concentration of biomass is used to investigate the microbes' growth in the fermenter. Since PHB is an intracellular product, the quantity of biomass is important in predicted the concentration of biomass. Besides, biomass concentration is used to determine the percentage of conversion for PHB production in the microbe cell.

The highest concentration of biomass occur at during eighth run at 60 hours reading. Some of data in figure show that the concentration of biomass decrease after the highest concentration is achieved. This is due to growth of the microbe that starting with initial phase, which the concentration of biomass is low and start to increase with time and move into the exponential phase. Most of the exponential phase occur at time 18 hours to 42 hours. Then, the microbe are in the stationary phase where the optimum concentration is obtained. Final phase is death phase which indicate the decreasing in biomass concentration. However, some of combination parameter able to extend the stationary phase more than 60 hours such as in fifth, seventh and eighth run.

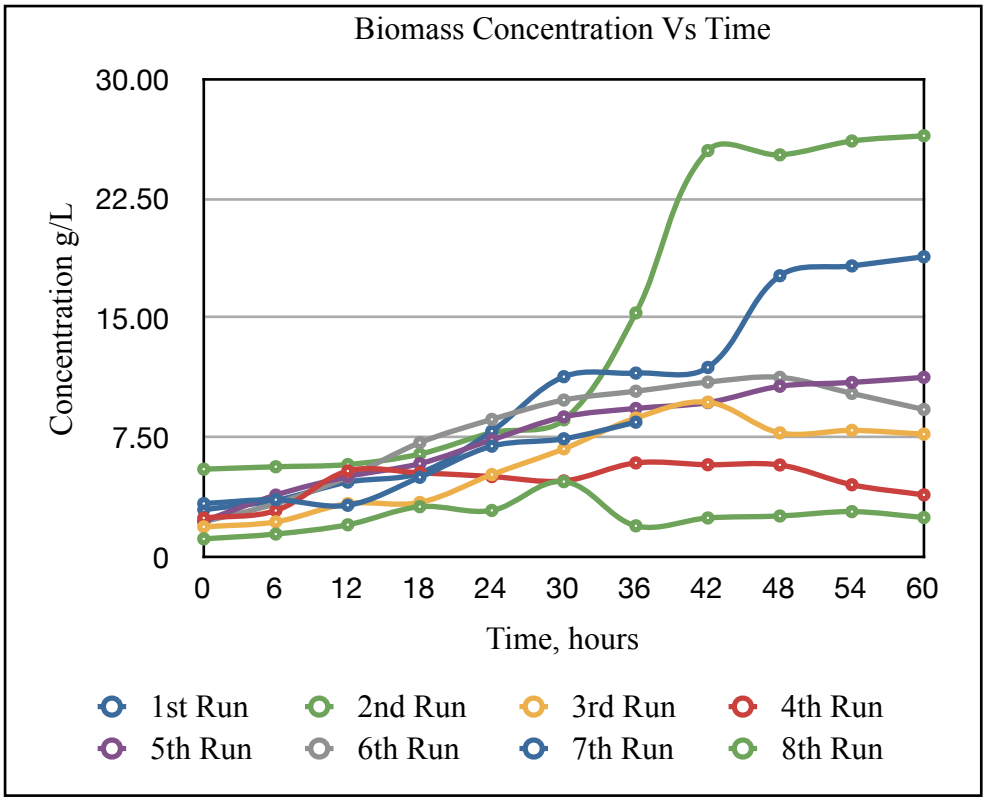


Figure 4.2 Profile Analysis of Biomass Concentration for Eight Set of Data.

4.3.2 Glucose Analysis

The profile of glucose is important to determine the activities of microbe. Theoretically, *Cupriavidus necator* will take glucose as the food in growing. Therefore, the decreasing in glucose concentration proportional to time indicate that the microbes take the glucose for living. In the other hands, the concentration of glucose is inversely proportional to the concentration of biomass. Thus, the decreasing in concentration of glucose show that the numbers of microbe in the medium increase by replication of cells and may influence the production of PHB as the number of cell increase.

Based on the result that plotted in the Figure 4.3 below, the lowest concentration of glucose occur in sixth run at time 36 hours. The profile start with high concentration which indicate the initial glucose values inside the medium, then the concentration of the glucose decrease rapidly as the time moving until it achieve constant value at the end of the experiment. This is due to the usage of the glucose by the microbes as the carbon source. The decreasing of concentration rapidly is as result of exponential phase of microbes' growth on that time. As the experiment nearly end,the reading show some fluctuation and some show the constant values. Besides the experimental error, this situation happened because the microbe enter the death phase which is the number of microbe decrease and no replication in microbe. Therefore, glucose in the medium is not used and remain constant in concentration.

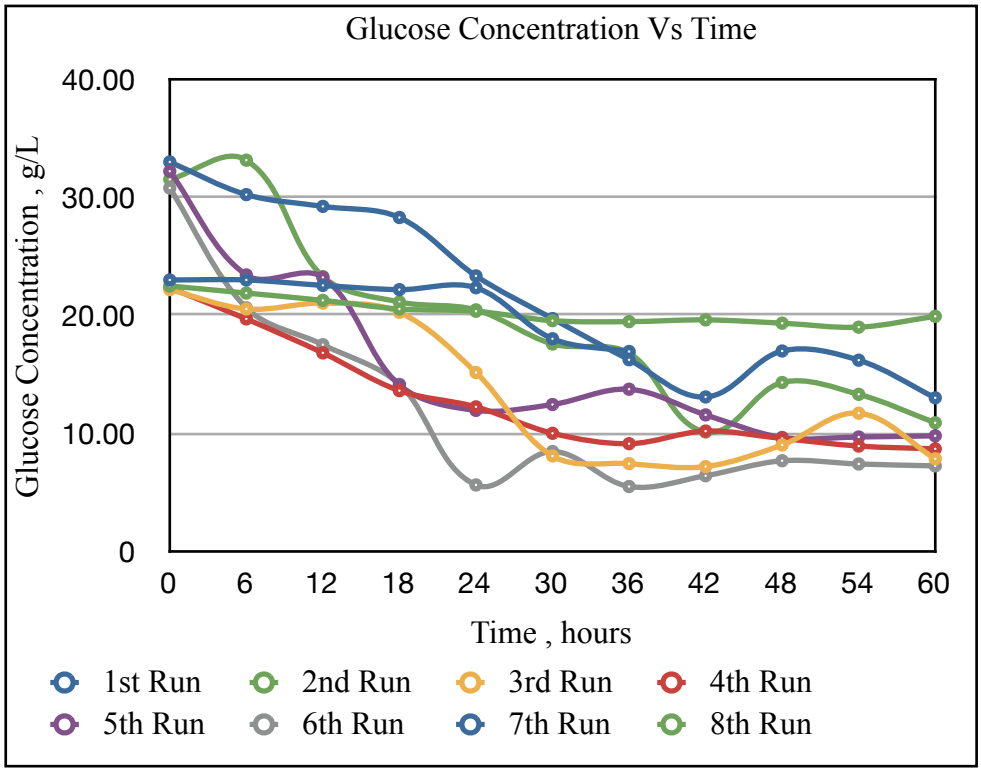


Figure 4.3 Profile Analysis of Glucose Concentration for All Set of Data

4.3.3 PHB Analysis

PHB is an intercellular cell product that produce in response to conditions of physiological stress conditions. The result that obtained from the experiment is plotted in the graph as shown in Figure 4.4. This figure contain the information about the production profile of the PHB that start to increase by time. At the initial of fermentation process, the concentration of PHB is low which the quantity of cell in the medium is still low. The cell that obtained at the initial process is the cell or microbe that obtained from the inoculum development that prepared in the previous step of fermentation. Basically, the volume of inoculum that used in the fermentation process is about 10% of total working volume. Therefore, the inoculum that used is 1500mL and this volume is low compare to the total working volume of fermentation production phase. This is the reason to low concentration of PHB at the initial process.

From the observation on the graph, the highest production of PHB occur in the third run of experiment at the time 42 hours. Most of highest concentration of PHB is produce at the middle part of fermentation duration which around time of 24 hours to 48 hours. The production of PHB is proportional to the concentration of biomass since PHB is the intracellular cell product. The middle part of the experiment is the part where the microbe in the exponential phase of growth and achieving the stationary phase. Thus, the highest production of PHB is occur at the area time.

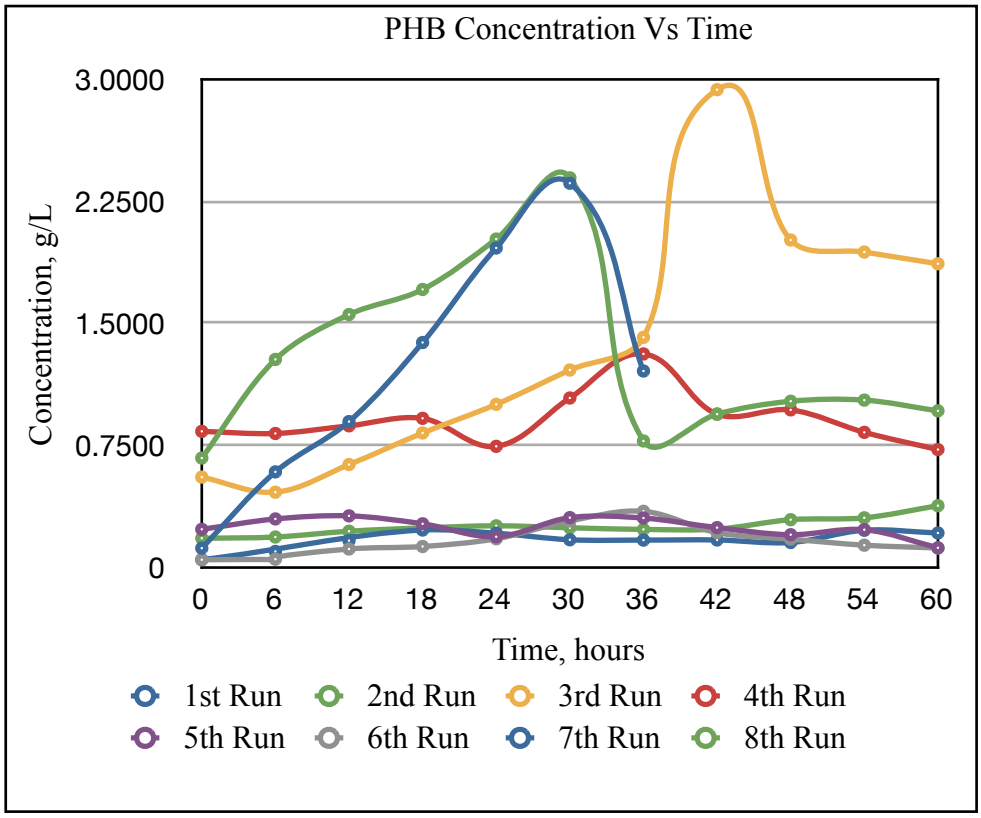


Figure 4.4 Profile Analysis of PHB Production for All Set of Data

After comparing all set of data that obtained and plotted in graphs above, the third run of fermentation with combination parameters of 210 RPM agitation speed, 32 °C temperature and 23 g/L glucose initial glucose concentration is chose as the best since the highest value of PHB produced. The profile of the third run is shown in Figure 4.5 which consist the glucose analysis profile, biomass analysis profile and PHB production profile. The figure also show that the concentration of the biomass increase proportional to the increasing the concentration of PHB. This support the understanding of PHB as the intracellular product and its production influence by the concentration of biomass and the replication activities of microbe *Cupriavidus necator*. The concentration of glucose show the deduction from the initial values as the time moving since the usage of glucose as the carbon source for microbe.

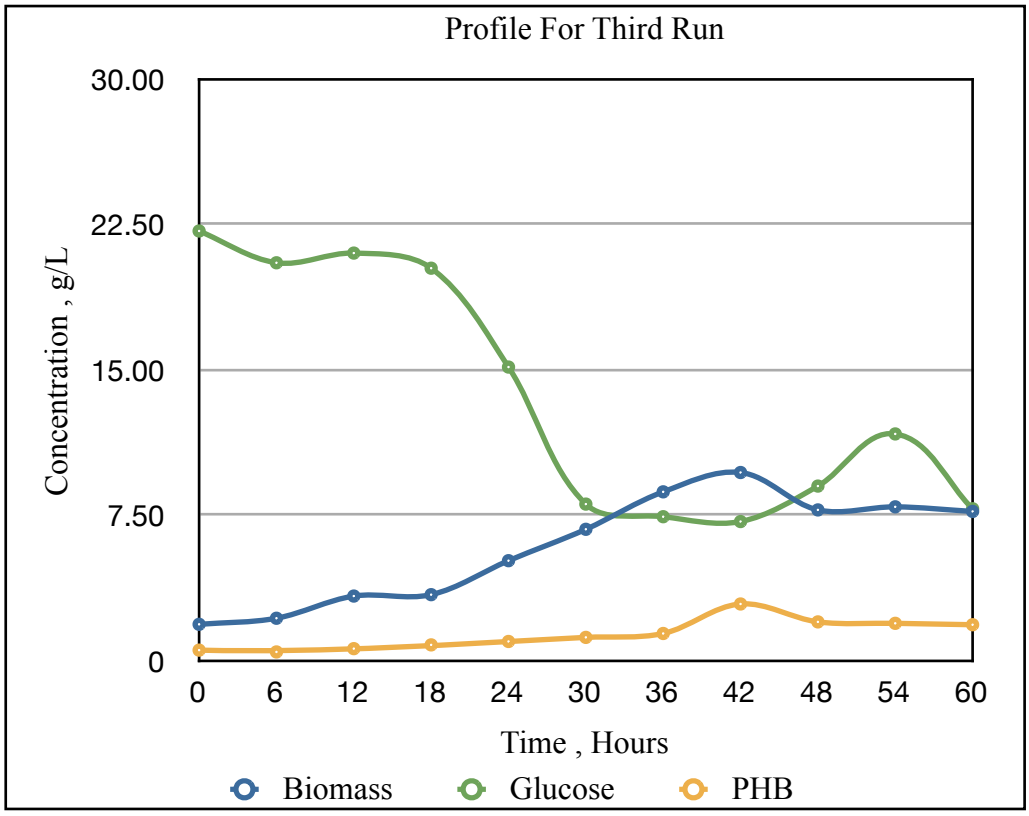


Figure 4.5 Production Profile for Third Run With The Highest Concentration of PHB

Highest concentration of biomass is obtained in the eighth run at time 60 hours. Contrarily the concentration of PHB is low at the same point even PHB is intracellular product and the concentration of PHB is directly proportional to the concentration of biomass. The reason for this situation in due to the combination of parameter that result the condition during the fermentation process. PHB is produce as response to the phycological stress condition of microbe. Thus, the condition that exist as the result of variables combination is good for microbe replication but do not cause stress condition for PHB production. Another seven production profile is shown in appendix.

4.4 Mathematical Analysis

4.4.1 Yates' Method

Yates' method is used in calculation process to study the main effects and interactive effects and at the same time examined the pattern of the response curve. The calculation is carried out by using the highest yield produced for PHB and biomass concentration for each set of data. The result that obtained is shown in the Table 4.3 below. Further calculation step is shown in appendix B. Based on the Yates' Method Analysis, the main effect that most influence the production of PHB by changing from lower value to higher value is temperature. While the interactive effect is combination of initial glucose concentration and agitation speed. Besides, there are main effect that give negative effect on the production which is agitation speed and initial glucose concentration.

Table 4.3 The Calculation of Yates' Method on PHB Production

No. Of Run	PHB (g/L)	Effect	Identity
1	2.362	1.1069	Grand Total Effect
2	1.029	-0.3889	RPM
3	2.937	0.0590	T
4	0.969	-0.0654	RPM - T
5	0.3068	-0.7544	[G]
6	0.3457	0.4363	RPM - [G]
7	0.2294	-0.06975	T-[G]
8	0.3801	0.09335	RPM - T - [G]

Yates' method also applied for the concentration of biomass. The highest values of biomass is taken for calculation and the result that obtained is shown in Table 4.4 below. The most effluent main effect in biomass yield is glucose concentration followed by temperature. The agitation speed give negative effect on biomass the value is increase to the higher level. This is due to cell disruption that happened as result of shear stress on the cell wall. The interactive effect that effluent the most is combination of temperature and glucose concentration. Initial glucose concentration give highest effluent on biomass concentration because glucose is used as the carbon source or food for microbe. Therefore, the high quantity of food will encourage the replication of cell.

Table 4.4 Yates' Method Calculation On Concentration of Biomass

No. Of Run	Biomass (g/L)	Effect	Identity
1	8.4634	12.0716	Grand Total Effect
2	4.728	-0.007675	RPM
3	9.720	3.1338	T
4	5.789	0.9262	RPM - T
5	11.280	4.8966	[G]
6	11.280	1.9089	RPM - [G]
7	18.854	2.5544	T-[G]
8	26.459	0.9751	RPM - T - [G]

4.4.2 Linear Regression Coefficient

From the result that obtained and shown in Table 4.2, Linear Regression Coefficient can be calculated by using Design Expert software. The values of the coefficient that calculated are shown in Table 4.5 as below:

Table 4.5 The Linear Regression Coefficient of the 2^3 Factorial Experiment

Coefficients	Value	
	PHB	Biomass
α_0	8.88201	-62.27331
α_1	-0.019446	-0.000409
α_2	0.0295	1.56716
α_3	-0.15088	0.97942

From the linear regression coefficient, the area that satisfied the criterion for the area that may contain maximum yield able to determine by inserting the values into the Equation 4.1 below:

$$Y= \alpha_0 + \alpha_1 X + \alpha_2 X_2 + \alpha_3 X_3 \qquad \text{[eq.4.1]}$$

Based on the equation, the evaluation of the linear regression equation of the 2^3 Factorial Experiment for biomass and PHB concentration are as follow in Table 4.6 and table 4.7:

Table 4.6 The Evaluation Of Linear Regression Equation of The Factorial Experiment for PHB

No.	True Yield (g/L)	Predicted Yield (g/L)	Square Error
1	1.03	1.38	0.1225
2	0.97	1.49	0.2704
3	2.94	2.27	0.4489
4	2.36	2.15	0.0441
5	0.31	0.65	0.1156
6	0.23	0.76	0.2809
7	0.35	-0.13	0.2304
8	0.38	-0.014	0.1552
Mean Square Error			0.1043
Percentage Error of Average Yield (%)			1.0869

Table 4.7 The Evaluation Of Linear Regression Equation of The Factorial Experiment for Biomass

No.	True Yield (g/L)	Predicted Yield (g/L)	Squared Error
1	4.72	4.03	0.4761
2	5.79	10.30	20.3401
3	9.72	10.32	0.36
4	8.46	4.05	19.4481
5	11.28	13.84	6.5536
6	18.85	20.11	1.5876
7	11.28	13.83	6.5025
8	26.46	20.09	40.5769
Mean Square Error			11.9806
Percentage Error of Average Yield (%)			34.613%

Based on the result that obtained from the calculation, the parameter that used give effect on the production of PHB and biomass concentration. The Linear Regression Equation show 1.0869% error for PHB production and 34.613% for biomass production by comparing the true yield and predicted yield. Thus, the experimental process can be continue in second order polynomial to complete the optimization methodology and obtain the exact values of optimum parameter that produce highest yield of PHB.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

As conclusion for project entitle optimization of biopolymer batch fermentation in 20L stirred tank fermenter able to carry out the PHB is produced. Scale up process is successfully done by comparing the most similar pattern of DOT curve for both scale. The most similar pattern of DOT curve in 20L bioreactor is achieve in combination parameters of 230 RPM agitation speed and 10 L/min compress air flow rate and it is suitable to used as the centre point for Factorial Experiment Method. Based on yield analysis, the highest production of PHB is occur at third run with parameters of 230 RPM, 32 °C temperature and 23g/L initial glucose concentration. For optimization process, parameters that investigated are agitation speed, temperature and initial glucose concentration. Yates' Method show that the production of PHB have huge influent from main effect of temperature and interaction effect of Agitation speed-Initial glucose concentration.

5.2 Recommendation

From the research that performed, there are some recommendations that should be concern and understand to improve the research in the future. The recommendation are listed as below:

2. The exact values of parameter that yield PHB at the optimum concentration should be carried out by extend the research with second order polynomial of optimization.
3. The usage of glucose in tis research is relatively expensive, thus, some alternative should be done to obtained improve the production of PHB and encourage the application.
4. In the seventh run, observation find that foam is produced with huge amount and high rate. The foam existence may effect dissolve oxygen transfer through the medium since the foam are float at the medium surface. Some research should carried out to understand the process that would produce foam and the process to avoid it.

LIST OF REFERENECES

Asis, A. J., Paldi, Z. Winkler, M. A. and Salihon, J. (1990). Menskala Naik Satu Process Fermentasi Berdasarkan Pekali Pemindahan Isipadu (k_La) Oksigen Tetap. *Jurnal Kejuruteraan*. 2, 179-195.

Asis, A. J., and Salihon, J. (1995). Cocunut-Water as Fermentation medium for Enazyme Production (3): Optimization of Batch 5L Stirred Tank Fermentation. *Jurnal Kejuruteraan.* 7, 67-76.

Azmi. S. A (2009). *Optimization of Levels of Relevant Variables for Maximum Biopolymer Yield in Shake Flask Fermentations*. Undergraduate Thesis. Universiti Malaysia Pahang, Gambang.

Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller. (1988). *Pseudomonas oleovorans* as a Source of Polyhydroxyalkanoates for Potential Applications as Biodegradable Polyesters. *Appl. Environ. Microbiol*. 54:1977–1982.43.

Box, G. E. P., Hunter, W. G. (1978). *HuntExperimenters*. New York, Wiley.

Choi J. , Lee S. Y. (2000). Economic considerations in the production of poly(3-hydroxybutyrate- co-3-hydroxyvalerate) by bacterial fermentation. Applied Microbiology and Biotechnology. 53, 646-649. ISSN 0175-7598.

Cochran, W.G. and Cox, G.M., (1957). *Experimental Design*. John Wiley and Sons, New York.

Consumer Association of Penang (2001). Waste Not Asia, *Malaysia Country Report.* Taipei.

Daae, E.B., Dunnill, P., Mitsky, T.A., Padgette, S.R., Taylor, N.B., Valentin, H.E., Gruys, K.J., (1999). Metabolic modeling as a tool for evaluating polyhydroxyalkanoate copolymer production in plants. *Metabolic. Engineering*. 1, 243–254.

Diaz E. (Ed.). (2008). *Microbial Biodegradation: Genomics and Molecular Biology* (1st ed.). Caister Academic Press. ISBN 978-1-904455-17-2

Doi, Y. (1995). *Microbial Synthesis, Physical Properties, and Biodegradability of Polyhydroxyalkanoates*. Macromol. Symp. 98:585–599.

Hassan, M.A, Shirai, Y., Umeki, H., Yamazumi, H., Jin, S., Yamamoto, S., Abdul Karim, M.I., Nakanishi, K., Hashimoto, K. (1997). Acetic Acid Separation From Anaerobically Treated Palm Oil Mill Effluent by Ion Exchange Resins for the Production Of Polyhydroxyalkanoate by Alcaligenes eutrophus. *Bioscience Biotechnology Biochemical*, 61 (9), 1465-1468

Huisman, G.W., Leeuwde, O., Eggink, G., Witholt, B., (1989). Synthesis of Poly-3-hydroxyalkanoates is a Common Feature of Fluorescent *Pseudomonads*. *Application Environmental Microbiol*. 55, 1949–1954

Husin. M. H. (2009). *Scale Up Of Biopolymer (PHB) Fermentation From Shake Flask to 10L Stirred Tank Fermenter and Oprimization of Level of Variables at 10L.* Undergraduate Thesis. Universiti Malaysia Pahang, Gambang.

Jendrossek, D., A. Schirmer, and H. G. Schlegel. (1996). Biodegradation of Polyhydroxyalkanoic Acids. *Application Microbiol. Biotechnol*ogy. 46:451–463.

John, M. E. and Keller, G. (1996). Metabolic Pathway Engineering in Cotton: Biosynthesis of Polyhydroxybutrate in Fiber Cells. *Applied Organic Sciences*. 93. 12768-12773. Proc. Natl. Acad. Sci. USA.

Kim B.S, Lee, S.C., Lee, S.Y., Chang, H.N. (1994) Production of Poly(3-hydroxybutyrate) by Fed-batch Culture of *Alcaligenes eutrophus* with Glucose Concentration Control. *Biotechnology and Bioengineering*. ;43:892–8

Madison. L. And Huisman. G. W. (1999). Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. *Microbiology and Molecular Biology Review*. 63(1), 21-53.

Mergaert, J., A. Wouters, C. Anderson, and J. Swings. (1995). *In-situ Biodegradation of Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in Natural Waters*. Can. J. Microbiol. 41(Suppl. 1):154–159.

Mehmet Inan, Vijay Chiruvolu, Kent M. Eskridge, George P. Vlasuk, Kenneth Dickerson, Scott Brown and Michael M. Meager. (1999). Optimization of The Temperature-Glycerol-pH Condition for a Fed Batch Fermentation Process for Recombinant Hookworm (*Ancylostoma caninum*) Anticoagulant Peptide (AcAP-5) Production by *Pichia Pastoris*. University of Nebraska-Lincoln.

Husin. M. H. (2009). *Scale Up Of Biopolymer (PHB) Fermentation From Shake Flask to 10L Stirred Tank Fermenter and Oprimization of Level of Variables at 10L.* Undergraduate Thesis. Universiti Malaysia Pahang, Gambang.

Peijis.T., (2002). Composites turn green! *E-Polymers*. 2:1-2:13,

Poirier, Y., Nawrath, C., Somerville, C. (1995). Production of Polyhydroxyalkanoates, Family of Biodegradable Plastics and Elastromers, in Bacteria and Plants. *BIO/Technology.* 13, 142-150.

Saruul, P., Srienc, F., Somers, D. A., and Samac. D. A. (2002). Production of a Biodegradable Plastic Polymer, Poly-hydroxybutyrate in Transgenic Alfalfa. *Crop Science.* 42, 919-927.

Steinbuchel, A. (1991). Polyhydroxyalkanoic acids. Byrom, D. (Ed.), *Novel Biomaterials from Biological Sources*. MacMillan, New York.

Steinbuchel. A. and Doi. Y., (2002). *Biopolymers*. Weinheim, Germany: Wiley-VCH.

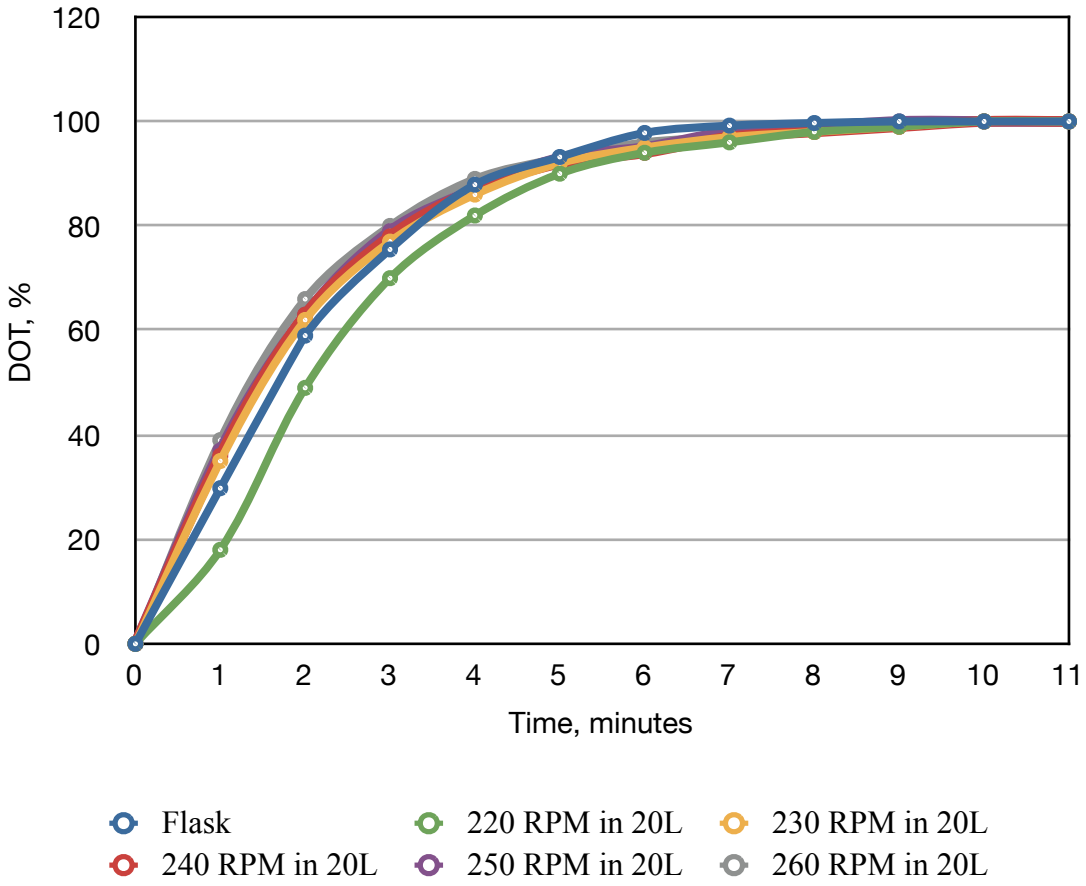
United Nations General Assembly (1987). *Report of the World Commission on Environment and Development: Our Common Future*

Wróbel, M., Zebrowski,J., Szopa, J. (2003). Polyhydroxybutyrate Synthesis in Transgenic Flax. *Journal of Biotechnology.* 107, 41–54.

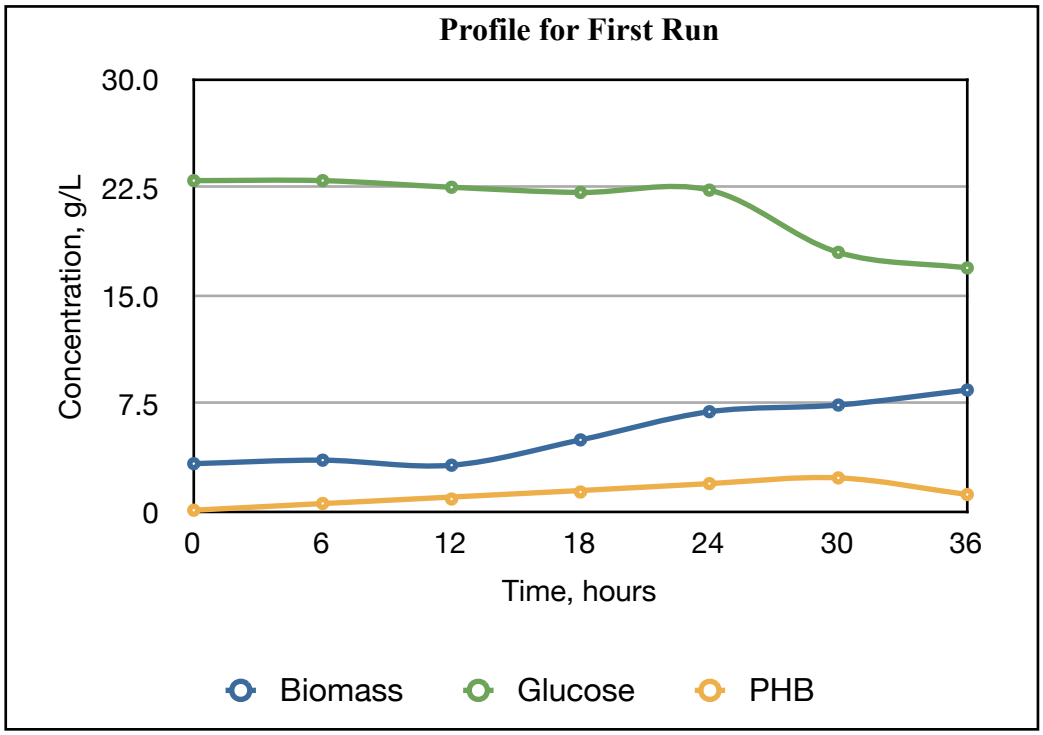
APPENDIX A

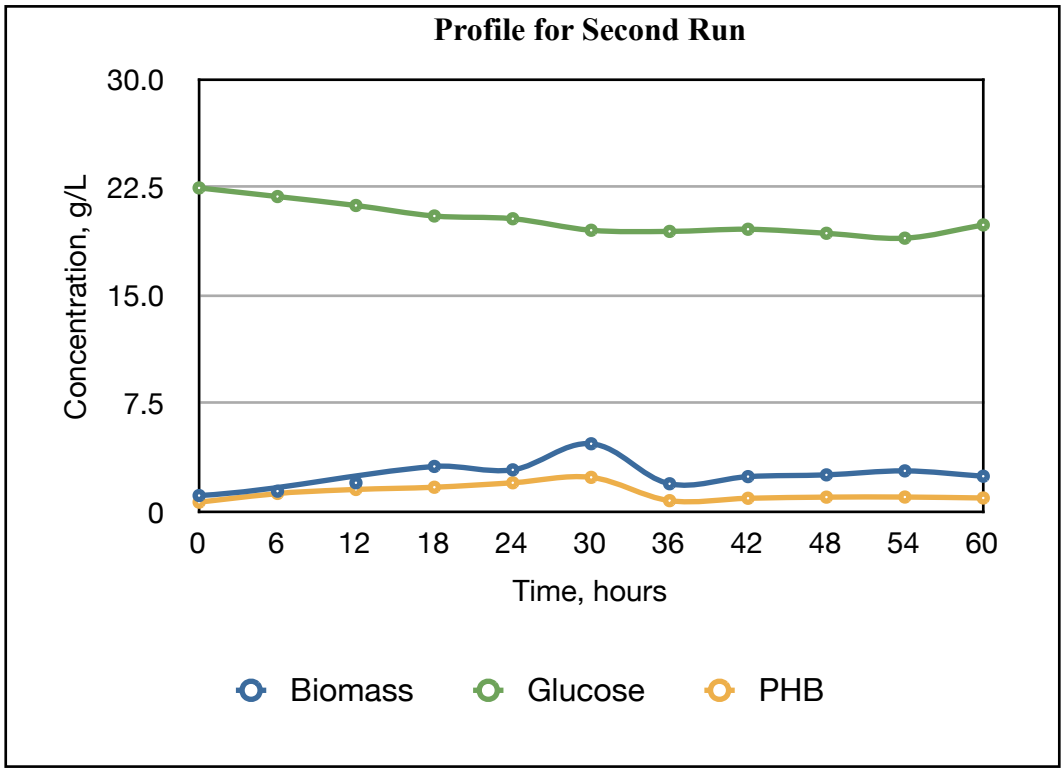
Time	Flask	220 RPM in 20L	230 RPM in 20L	240 RPM in 20L	250 RPM in 20L	260 RPM in 20L
0	0	0	0	0	0	0
1	29.8	18	35	36	37	39
2	59.0	49	62	63	63	66
3	75.5	70	77	78	79	80
4	87.9	82	86	87	87	89
5	93.2	90	92	92	93	93
6	97.8	94	95	94	95	96
7	99.2	96	97	97	98	97
8	99.7	98	98	98	99	98
9	100.0	99	99	99	100	99
10	100.0	100	100	100	100	100
11	100.0	100	100	100	100	100

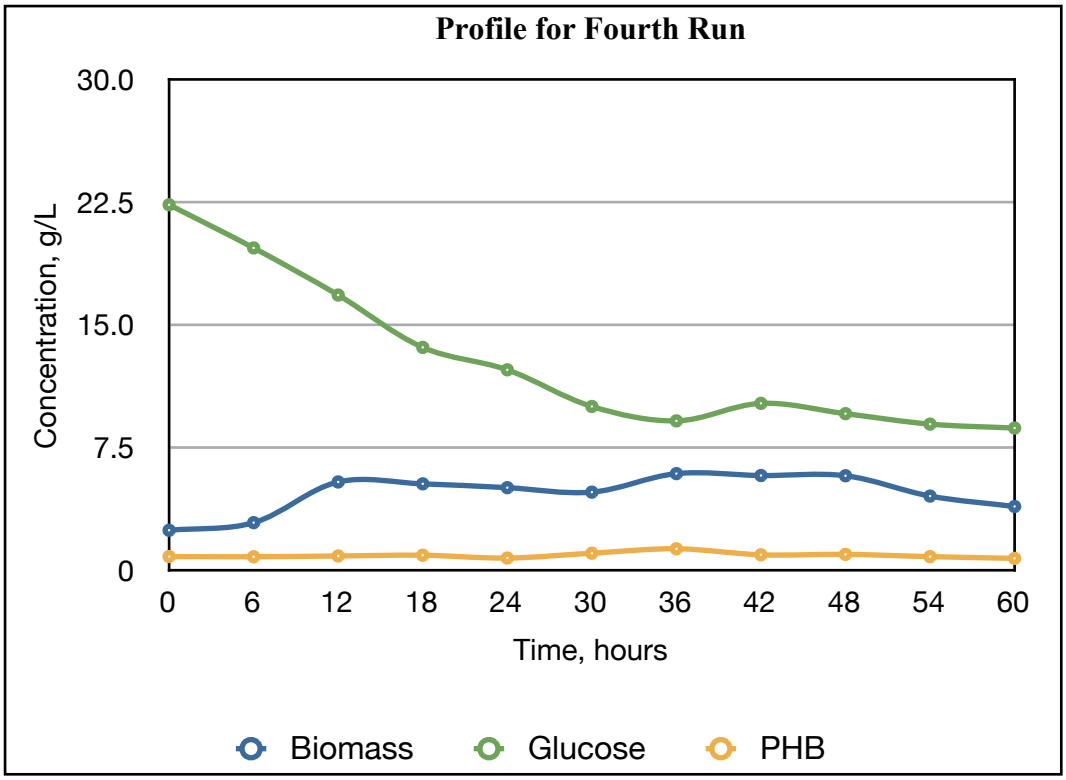
Comparison of DOT curves

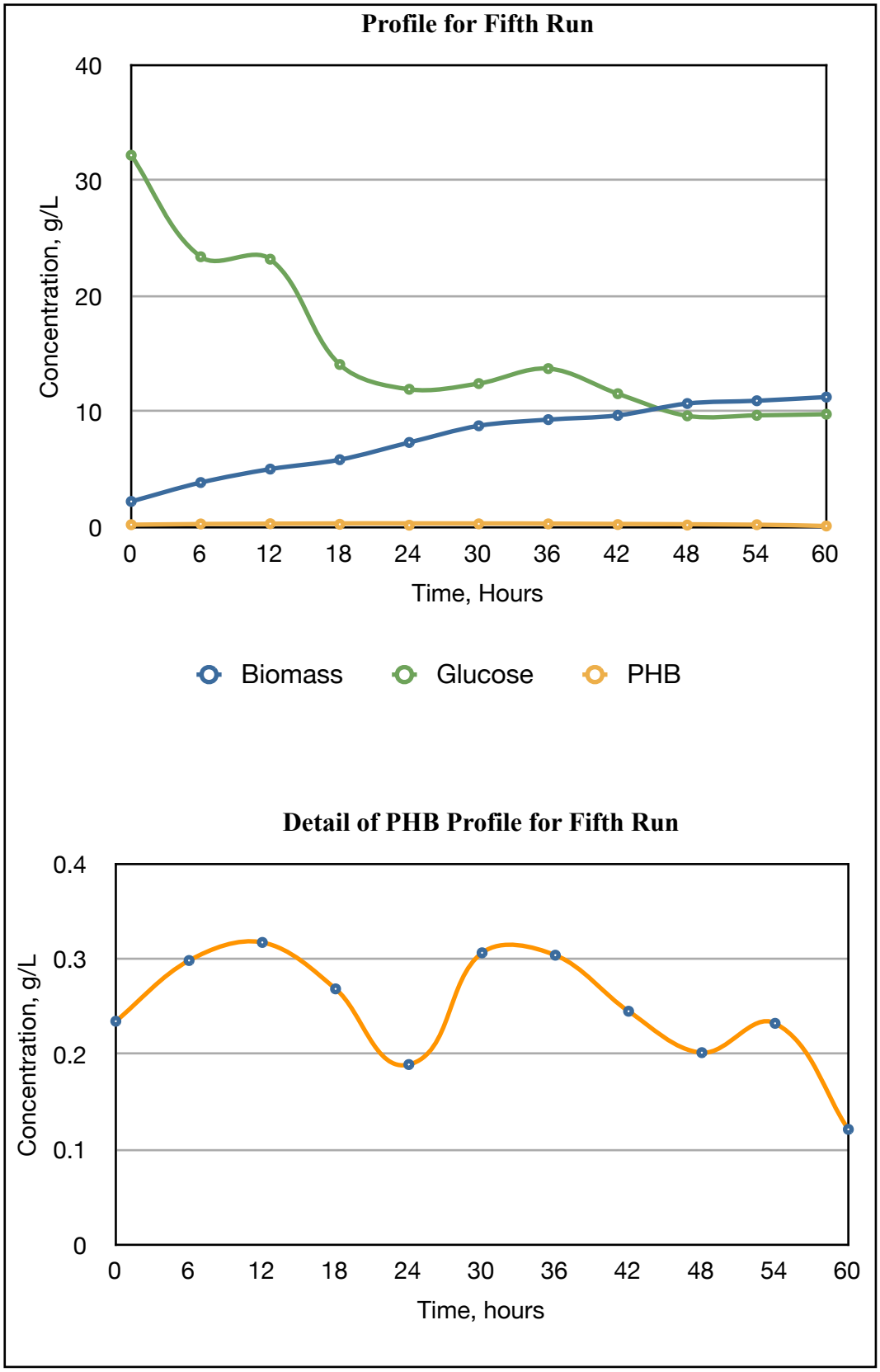


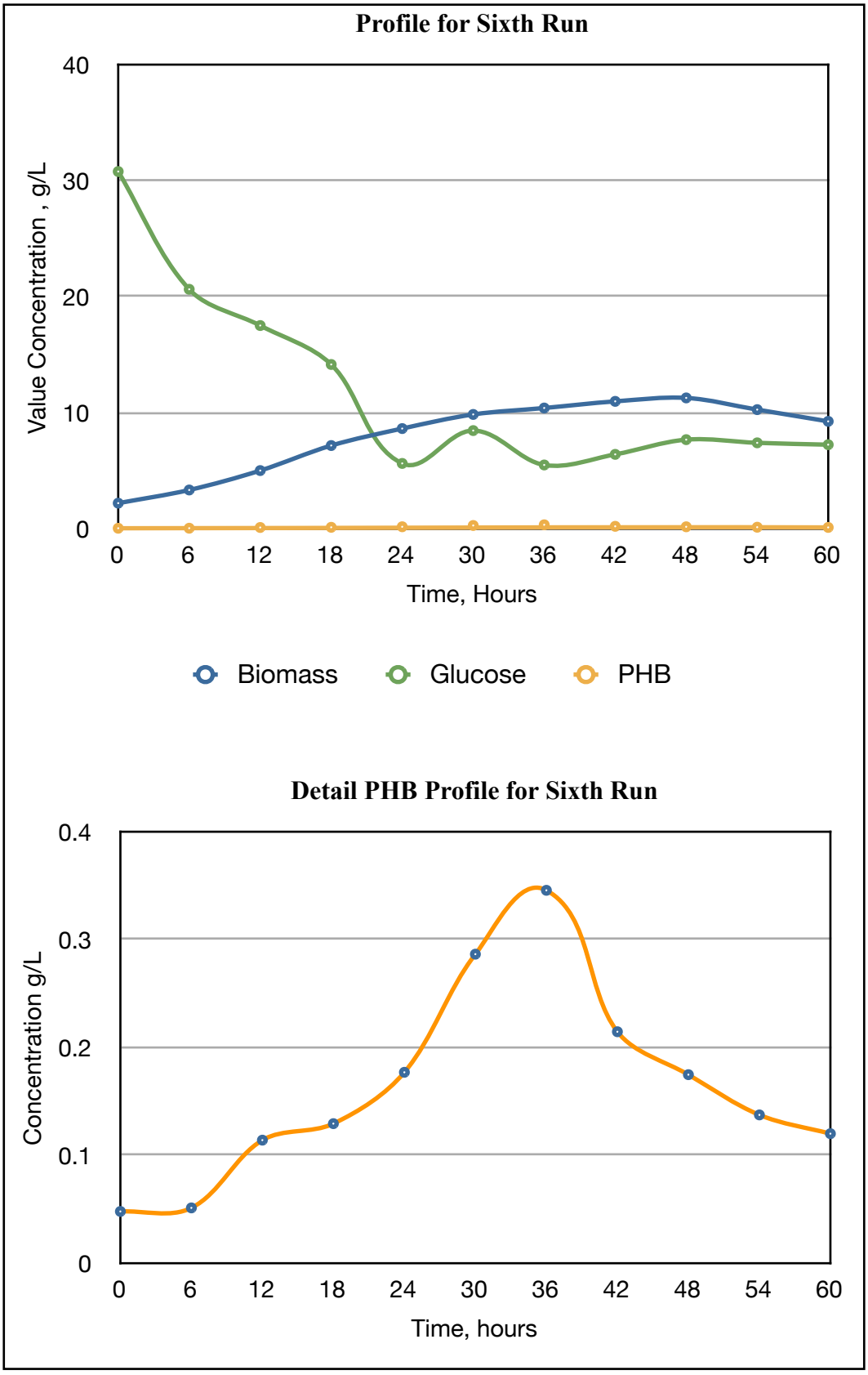
APPENDIX B

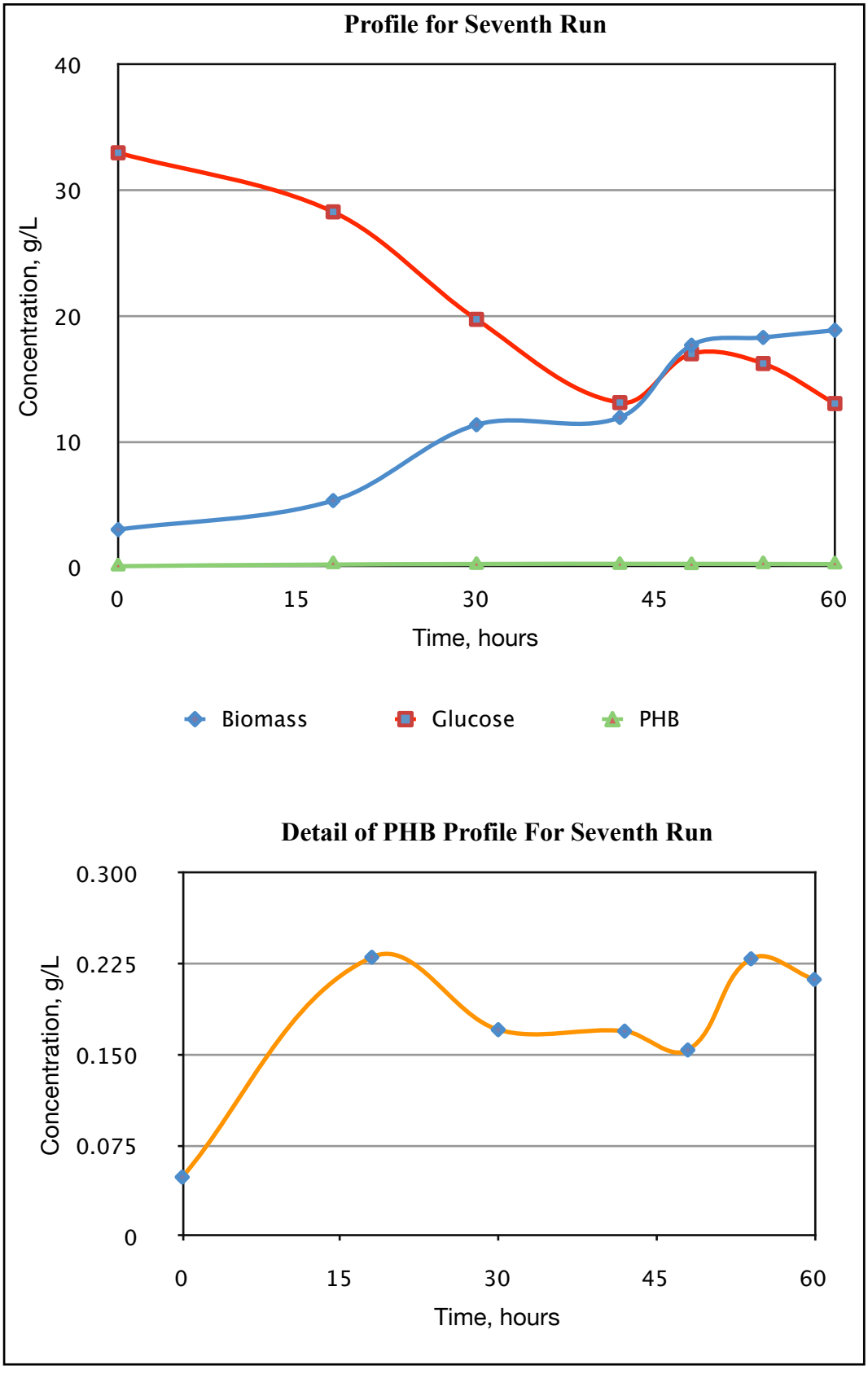

Profile for First Run
30.0
22.5
15.0
7.5
0
Concentration, g/L
0
6
12
18
24
30
36
Time, hours
Biomass
Glucose
PHB

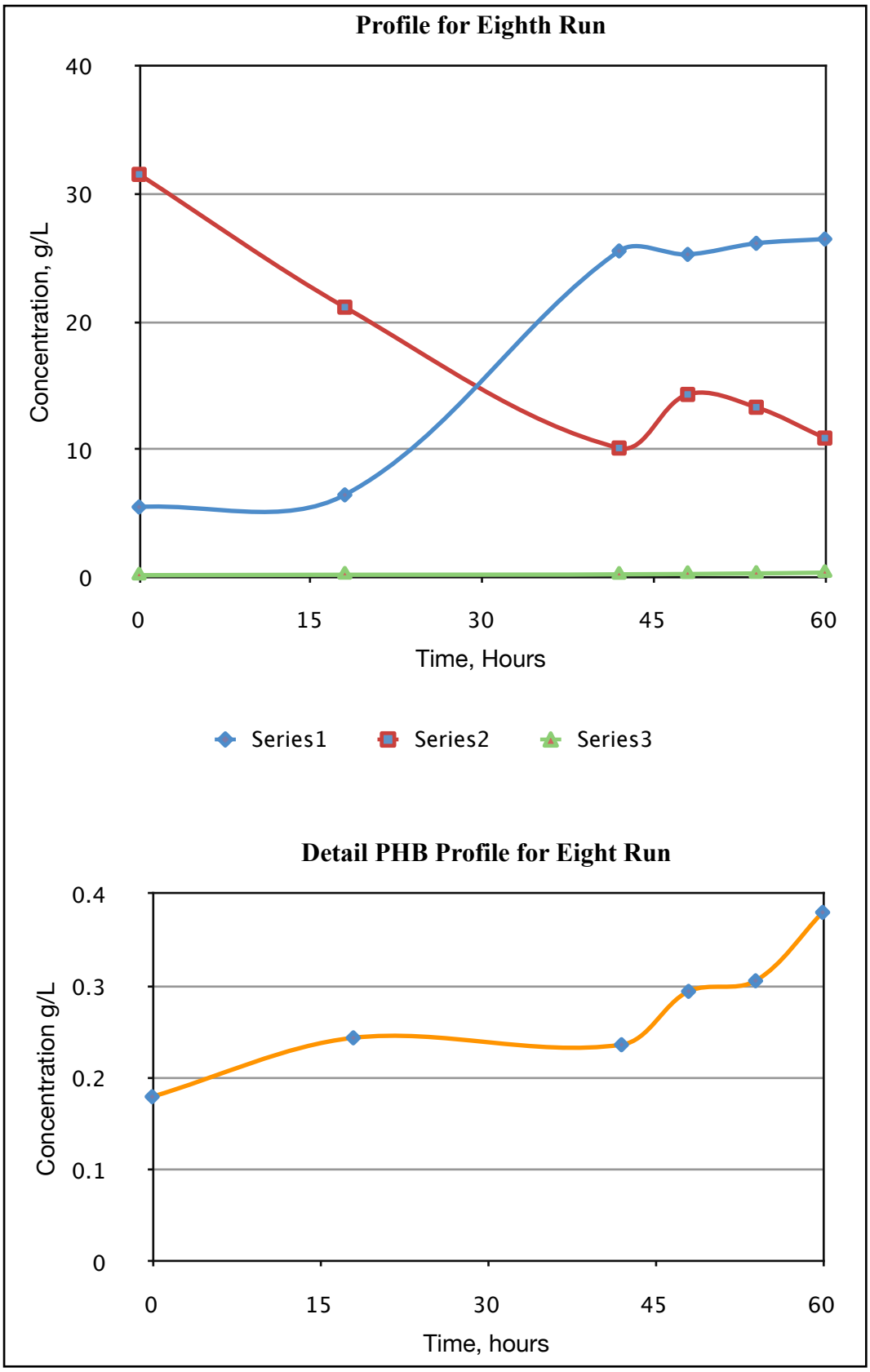

Profile for Second Run
30.0
22.5
15.0
7.5
0
Concentration, g/L
0
6
12
18
24
30
36
42
48
54
60
Time, hours
Biomass
Glucose
PHB


Profile for Fourth Run
30.0
22.5
15.0
7.5
0
Concentration, g/L
0
6
12
18
24
30
36
42
48
54
60
Time, hours
Biomass
Glucose
PHB


Profile for Fifth Run
Concentration, g/L
40
30
20
10
0
0
6
12
18
24
30
36
42
48
54
60
Time, Hours
Biomass
Glucose
PHB
Detail of PHB Profile for Fifth Run
Concentration, g/L
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
36
42
48
54
60
Time, hours


Profile for Sixth Run
Value Concentration , g/L
40
30
20
10
0
0
6
12
18
24
30
36
42
48
54
60
Time, Hours
Biomass
Glucose
PHB
Detail PHB Profile for Sixth Run
Concentration g/L
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
36
42
48
54
60
Time, hours


Profile for Seventh Run
Concentration, g/L
40
30
20
10
0
0
15
30
45
60
Time, hours
Biomass
Glucose
PHB
Detail of PHB Profile For Seventh Run
Concentration, g/L
0.300
0.225
0.150
0.075
0
0
15
30
45
60
Time, hours


Profile for Eighth Run
Concentration, g/L
40
30
20
10
0
0
15
30
45
60
Time, Hours
Series1
Series2
Series3
Detail PHB Profile for Eight Run
Concentration g/L
0.4
0.3
0.2
0.1
0
0
15
30
45
60
Time, hours

APPENDIX C

No. Of Run	PHB (g/L)	Column 1	Column 2	Column 3	Effect	Identity
1	2.362	3.391	7.297	8.8559	1.1069	Grand Total
2	1.029	3.906	1.262	-3.1114	-0.3889	RPM
3	2.937	0.6525	-3.301	0.472	0.0590	T
4	0.969	0.6095	0.1896	-0.5232	-0.0654	RPM - T
5	0.3068	-1.333	0.515	-6.035	-0.7544	[G]
6	0.3457	-1.968	-0.043	3.4906	0.4363	RPM - [G]
7	0.2294	0.0389	-0.635	-0.558	-0.06975	T-[G]
8	0.3801	0.1507	0.1118	0.7468	0.09335	RPM - T - [G]

No. Of Run	Biomass (g/L)	Column 1	Column 2	Column 3	Effect	Identity
1	8.4634	13.1914	28.7004	96.5734	12.0716	Grand Total
2	4.728	15.509	67.873	-0.0614	-0.007675	RPM
3	9.720	22.560	-7.6664	25.0706	3.1338	T
4	5.789	45.313	7.605	7.4094	0.9262	RPM - T
5	11.280	-3.7354	2.3176	39.1726	4.8966	[G]
6	11.280	-3.931	22.753	15.2714	1.9089	RPM - [G]
7	18.854	0.0000	-0.1956	20.4354	2.5544	T-[G]
8	26.459	7.605	7.605	7.8006	0.9751	RPM - T - [G]