SCALE UP OF BIOPOLYMER FROM SHAKE FLASK TO 20L STIRRED TANK FERMENTER

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I declare that this thesis entitled "*Scale up of biopolymer from shake flask to 20 l stirred tank fermenter*" is the result of my own research except as cited in the references. This thesis has not been accepted for any degree and is not currently submitted in candidature of any other degree.

Signature : ……...

Name : ……....

Date : ……

DEDICATION

To my beloved mother, father, sisters and brother……

ACKNOWLEDGEMENT

In preparing this thesis, I was in contact with so many people, researchers, academicians as well as new friends. They have contributed towards my understanding and thoughts. In particular, I wish to express my sincere appreciation to my PSM supervisor, Prof. ir Jailani Bin Salihon for encouragements, guidance and critics.

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ABSTRACT

This study is about the scaling up for production of poly-β-hydroxybutyrate (PHB) from shake flask to 20 liter stirred tank fermenter. This scale-up operation used the method of constant volumetric transfer coefficient (K_La) of oxygen. K_La values were derived by fitting the dissolved oxygen tension (DOT) versus time on computer with two unknowns, namely K_La and the electrode mass transfer coefficient (Kap) of oxygen. The stirrer speed (RPM) and the air flow rate (A) in the 20 liter fermenter that produce the value of K_La found in the shake flask was approximated using trial and error method which is 200 rpm for both scale. A fermentation run in 20 liter fermenter using these values of rpm and A gave a PHB yield comparable to that achieved in shake flask. From the research work, the K_La for 500 ml shake flask and 20 l stirred tank fermenter were 0.2809 and 0.2564 respectively. Meanwhile the Kap for 500 ml shake flask and 20 l stirred tank fermenter were 0.0010 and 0.0008 respectively. The holding time for sterilization cycle is also calculated at 7.75 minutes which is recommended to make it longer for safety precautions. The results for cell dry weight of shake flask and 20 l fermentor are 7.15g/L and 6.90g/L respectively. Meanwhile, the PHB yield for shake flask and 20 l are 1.0190 g/L and 0.926 g/L respectively. From these results, it can be concluded that this research work had achieved its objectives.

ABSTRAK

Kajian ini adalah mengenai produksi poly-β-hydroxybutyrate (PHB) dalam fermenter tangki teraduk yang diskala naik daripada kelalang goncang ke 20 liter tangki teraduk. Operasi menskala naik ini menggunakan kaedah pekali pemindahan jisim kepada data tekanan oksigen terlarut (DOT) menentang masa dengan komputer dengan 2 pembolehubah iaitu K_La dan pekali pemindahan jisim (Kap) bagi oksigen. Kadar pengadukan (RPM) dan kadar alir udara (A) dalam fermenter 20 liter yang menghasilkan nilai K_La yang didapati daripada kelalang goncang dengan menggunakan kaedah cuba-cuba di mana telah didapati sebagai 200 rpm pada kedua-dua skala. Fermentasi di dalam fermenter 20 l menggunakan nilai rpm dan A tersebut telah menghasilkan paras enzim yang hampir sama dengan yang didapati dalam kelalang goncang. Daripada kajian, didapata bahawa nilai K_La untuk kelalang goncang dan 20 l fermenter tangki aduk adalah masing-masing 0.2809 dab 0.2564. Nilai Kap untuk kelalang goncang dan 20 l fermenter tangki aduk masing-masing ialah 0.0010 dan 0.0008. Tempoh masa diperlukan untuk pengekalan suhu pada 121^oC ialah sebanyak 7.75 minit. Bagaimana pun, adalah digalakkan untuk memanjangkan tempoh masa itu sebagai langkah berjaga-jaga. Nilai untuk berat berat kering pada kelalang goncang dan 20 l fermentor tangki aduk ialah masing-masing sebanyak 7.15g/L dan 6.90g/L. Manakala, nilai PHB adalah masing-masing sebanyak 1.0190 g/L and 0.926 g/L. Daripada keputusan ini, dapatlah dirumuskan bahawa kajian ini berjaya mencapai objecktif.

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LIST OF SYMBOLS

%	-	percentage
wt	-	weight
PHB	-	poly-β-hydroxybutyrate
PHA	-	polyhydroxyalkanoate
PHV	-	polyhydroxyvalerate
PHH	-	polyhydroxyhexanoate
PHO	-	polyhydroxyoctanoate
l	-	liter
g	-	gram
rpm	-	rotation per minute
pH	-	potential hydrogen
M	-	molar
K_La	-	volumetric transfer coefficient of oxygen
Kap	-	mass transfer coefficient of oxygen
^0C	-	degree celcius
A	-	air flow rate

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CHAPTER 1

INTRODUCTION

1.1 Background of study

The research on the production and application possibilities of such polymers was commenced at the beginning of the 1970's when the economically developed western countries encountered very serious problems caused by the amount of polymeric materials of everyday applications building up in waste. The biotechnology produced (PHB) turned out to be the most promising biodegradable to counter this problem.

Poly-β-hydroxybutyrate (PHB) is an intracellular storage compound that provides carbon and energy reserves in several microorganisms. PHB is produced by microorganisms like *Alcaligenes eutrophus or Bacillus megaterium* 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 microorganisms as a form of energy reserve when other common energy resources are not available. *Alcaligenes eutrophus* accumulate PHB during the stationary phase of growth when the cells become limited for an essential nutrient but have excess of carbon source.

Definitions of scale up is a process used to go from a small scale production (several culture dishes) to a large scale (a reactor of several liters) or to perform an experiment in bulk, after the optimal conditions have been determined by a screening experiment. Both definitions referred to a process in which the data from

an experimental scale operation is used in a larger scale (scaled up) unit for larger production.

The basis of constant volumetric transfer coefficient (K_La) of oxygen is used in order to scale up. The purpose of scaling up is to obtain the same product per volume in both small scale and big scale at the same time. During scale up, there are three factors should be stressed which are inoculum development, medium sterilization and aeration.

1.2 Problem statement

Development of fermentation process is usually carried out in three steps. The to steps are firstly, the flask scale to screen strains and evaluate medium composition and second are pilot scale to establish optimal fermentation condition and finally are the industrial scale process to produce desired product economically.

Usually, the productivity of the desired product is high in flask scale, and will be gradually reduced as the scale is enlarged because of the complexity of fermentation process. This will affect the efficiency of industrial fermentation process.

During fermentation, another factor that is very important is the sterilization process. When in small scale, the effect of sterilization is minimized because of the shorter time exposure towards the high temperature and pressure. However, when in larger scale the exposure is longer so the productivity is reduced significantly.

So, it is important to study scale up in fermentation process and adopt suitable strategy of scaling up in order to increase the productivity of the desired product on the industrial level.

1.3 Objective of research project

The objective of the research project is to scale up biopolymer (PHB) from shake flask to 20 l stirred tank fermentor.

1.4 Scope of research project

The scope of research project is to determine these following aspects. The aspects are:

- Determine the volumetric transfer coefficient (KLa) of oxygen of biopolymer in shake flask.
- Obtain the similar value of KLa in 20 L stirred tank fermentor.
- Determine the maintenance time of sterilization temperature in 20 L stirred tank fermentor.
- Fermentation in 500 ml shake flask
- Fermentation in 20 l fermentor

CHAPTER 2

LITERATURE REVIEW

2.1 History

PHAs (polyhydroxylalkanoates) is the chemical term for a naturally occurring form of polyester ultimately derived from sunlight, carbon dioxide and water through the process photosynthesis. They were first identified by the French microbiologist Maurice Lemoignr in 1925. Lemoigne discovered polyhydroxybutyrate (PHB), one of the most abundant PHAs in nature. PHB is a highly crystalline thermoplastic and melts closely to its decomposition temperature making it very difficult to process commercially.

Over the past two decades, over a hundred more PHA polymers have been described in the scientific literature. The properties of these polymers range from stiff, highly crystalline materials like PHB to soft, low melting thermoplastics like polyhydroxyoctanoate (PHO) to other PHAs which are completely amorphous and tacky substances.

2.2 Microorganisms

There are many organisms that produce PHB under nutrient stress while having an excess of carbon sources. A wide variety of prokaryotic organisms have

been shown to accumulate this polymer, including numerous heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria, gliding bacteria, cyanobacteria and recently in anaerobic, fatty acid-oxidizing, gram negative bacterium*(Anderson and Dawes, 1990)*.

The microorganism that is used to produce PHB in this research is *Cupriavidus necator* (also known as *Ralstonia eutropha* or *Alcaligenes eutrophus*). The reason for choosing this microorganism is because it had been found out that *Alcaligenes eutrophus* is the prime PHB producer that accumulates PHB up to 80% of its dry weight *(Doi et al, 1987)*

Alcaligenes eutrophus can use inexpensive carbon sources, which is important in industrial scale production. The organisms show differences in their growth and polymer production conditions but they were chosen because of their high polymer production capacity. The other criterion for the selection is the ease of separation of the polymer from the cells.

2.3 Poly-β-hydroxybutyrate (PHB)

2.3.1 Characteristics of Poly-β-hydroxybutyrate (PHB)

Poly-β-hydroxybutyrate (PHB) is the storage polymer separated from cytoplasm. β-hydroxyl butyrate is connected by ester linkage and form PHB. PHB is a biodegradable thermoplastic which can be extracted from a wide range of bacteria. The polymer which provides a reserve of carbon and energy accumulates as intracellular granules. Reusch and Sadoff have shown that PHB is an important molecule on cytoplasm and cell wall in 1983.

PHB is accumulated when culture medium is unbalanced due to limited sources of oxygen, nitrogen, phosphorus, sulphur or magnesium and excess of carbon source *(Kim et al, 1994; Lee, 1996)*

Biopolymers which are synthesized by microorganisms from agricultural substrates (PHB) are able to become degraded to carbon dioxide and water in aerobic conditions or to methanol in anaerobic conditions in such diverse habitats as soil, sea, stagnant water or sewage *(Lee, 1996; Reusch, 2002)*

2.3.2 **Poly-β-hydroxybutyrate (PHB) formation**

In their metabolism, bacteria produce acetyl-coenzyme-A (acetyl0CoA), which is converted into PHB by three biosynthetic enzymes which are 3-kethiolase (PhaA), acetoacetyl-CoA reductase (PhaB) and PHB synthase (PhaC). In the first step, -kethiolase (PhaA) combines 2 molecules of acetyl-CoA to form acetoacetyl-CoA. acetoacetyl-CoA reductase (PhaB) allows the reduction of acetoacetyl-CoA by NADH to 3-hdroxybutyryl-CoA. Finally PHB syntase (PhaC) polymerizes 3-hdroxybutyryl-CoA to PHB, coenzyme-A being liberate. Only (R) – isomers are accepted as substrates for the polymerizing enzyme *(Tsuge et al, 2005)*.

During normal bacterial growth, the 3-ketothiolase will be inhibited by free coenzyme-A coming out of the Krebs cycle. But when entry of acetyl-coA into the Krebs cycle is restricted (during non carbon nutrient limitation), the surplus acetyl-CoA is channeled into PHB biosynthesis *(Ratledge and Kristiansen, 2001)*.

PHB is a homopolymer whose monomer units have a D (-) configuration due to the stereospecificity of the enzymes involved in the synthesis *(Lee, 1996)*.

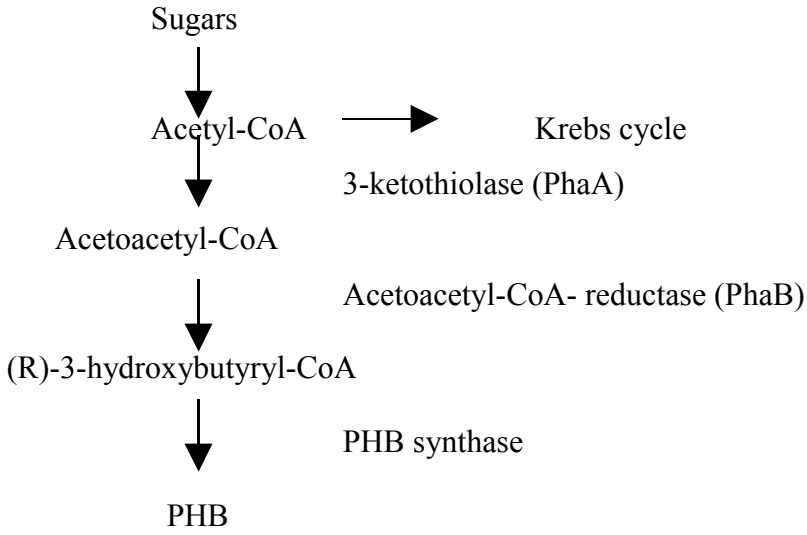


Figure 2.1: Metabolic pathway to PHB

The poly-3-hydroxybutyrate form of PHB is probably the most common type of polyhydroxyalkanoate, but many other polymers of this class are produced by a variety of organisms, these include poly-4-hydroxybutyrate, polyhydroxyvalerate, polyhydroxyhexanoate, polyhydroxyoctanoate and their copolymers. Some of the structures are shown as in Figure 2.2.

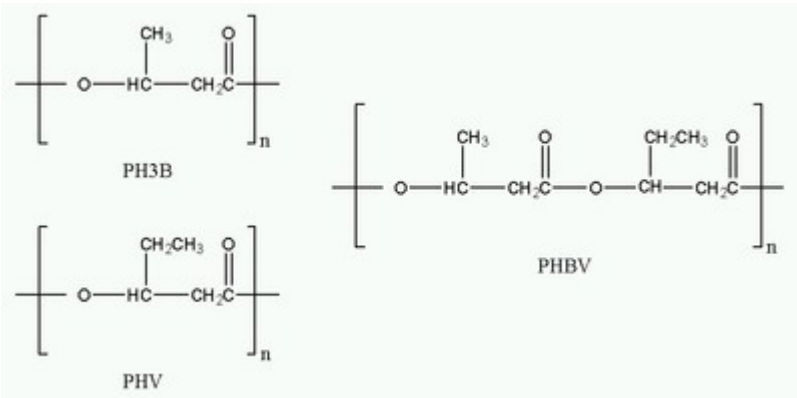


Figure 2.2: Chemical structures of copolymers of PHA

As PHA is insoluble in water, the copolymers 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.

The surface of a PHA granule is coated with a layer of phospholipids and proteins. Phasins, a class of proteins are the predominant compounds in the interface of a granule. The phasins influence the number and size of PHA granules *(Potter et al, 2002 and Steinbuchel, 2005).*

2.3.3 **Advantages of Poly-β-hydroxybutyrate (PHB)**

The biopolymer is a biodegradable and biocompatible thermoplastic with an isotactic structure (Isotatactic refers to those polymers formed by branched monomers that have the characteristics of having the entire branch group on the same side of the polymeric chain), a high degree of crystallinity (approximately 80%), a high number average molecular weight (approximately 105±106) and a high melting temperature (about 175^0C) *(Dawes, 1990; Scandola, 1995; Madison and Huisman, 1999.*

Production of organic polymeric materials is currently one of the principal areas of PHB is a thermoplastic material that has attracted much attention due to such properties as biocompatibility and biodegradability.

Microorganisms in nature are able to degrade PHa using their enzymes such as PHA hydrolase and PHA depolymerases (*Jendrossek and Handrick, 2002; Choi et al, 2004).* 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) *(Madison and Huisman, 1999).* Ultraviolet light can accelerate the degradation of PHAs (*Shangguan et al, 2006).*

PHAs have been proved biocompatible, which means they have no toxic effects in living organisms (*Volova et al, 2003).* Within mammals, the polymer is

hydrolysed only slowly. After a 6 months period of implantation in mice, the mass loss was less than 1.6% (w/w) (*Pouton and Akhtar, 1996).*

2.3.4 **Disadvantages of Poly-β-hydroxybutyrate (PHB)**

There are drawbacks of using PHB as a plastic material such as its tendency to be brittle. When it was spun into fibres it behaves as a hard-elastic material *(Antipov et al, 2006).* This problem could be solved by using by synthesis of copolymers of 3-hydroxybutyrate and other hydroalkanoates with a relatively low molecular weight and melting point *(De Koning, 1995; Scandola, 1995; Fukui and Doi, 1997).*

2.4 **Comparisons of Poly-β-hydroxybutyrate (PHB) to propylene**

Within the last 50 years petrochemical plastics have become one of the most applied materials. Their versatility, outstanding technical properties and relatively low price (1 kg of propylene costs about US$ 0.70) caused their success.

Today's applications are nearly universal; components in automobiles, home appliances, computer equipments, constructions, sport and leisure equipments, packages and even medical applications are areas where plastics clearly have become indispensable. However, we all know that these plastics are environmentally unfriendly; they are not biologically degraded *(Manfred Zinn et al, 2001).*

The current cost of the PHB production is considerably more than that of the synthetic plastics (Byroms, 1987). The current costs are approximated about US$ 15-30 per 1 kg of PHB.

2.5 **Material applications**

The majority of expected applications of PHB are as replacements for petrochemical polymers.

The plastics currently used for packaging and coating applications can be replaced partially or entirely by PHB. The extensive range of physical properties of the PHA family and the extended performance obtainable by chemical modification *(Zinn and Hany, 2005)* or blending *(Zhang et al, 1997; Avella et al, 2000; Lee and Park, 2002; Wang et al, 2005; Gao et al, 2006; Kunze et al, 2006)* provide a broad range of potential end-use applications.

Applications focus in particular on packaging such as containers and films *(Bucci and Tavares, 2005)*. In addition, their use as biodegradable personal hygiene articles such as diapers and their packaging have already been described *(Noda, 2001)*. It is also processes into toners for printing applications and adhesives for coating applications *(Madison and Huisman, 1999.*

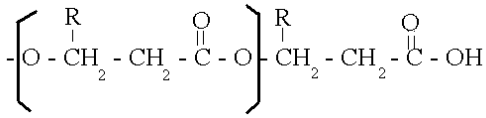
Composites of bioplastics are already used in electronic products, like mobile phones (NEC Corporation and UNITIKA Ltd.). Potential agricultural applications include encapsulation of seeds, encapsulation of fertilizers for slow release, biodegradable plastic films for crop protection and biodegradable containers for hothouse facilities.

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

In pure form or as composites, PHB can be used as sutures, repair patches, orthopedic pins, adhesion barriers, stents, nerve guides and bone marrow scaffolds.

It was concluded hat PHB and its copolymers may be a promising alternatives to the materials of petrochemical origin in the treatment of osteomyelitis,

due to their being biodegradable and eliminating the need for a second operation. The copolymer used in osteomyelitis operation is PHV. Figure 3 shows the difference between PHB and PHV in their chemical structures.



Poly (3-hydroxyalkanoate) (PHA)

R = CH3, Poly(3 hydroxybutyrate)
R = CH2-CH3, Poly(3-hydroxyvalerate)

Figure 3: structures of PHB and PHV

Research shows that PHA materials can be useful in bone healing processes. PHA together with hydroxyapatite (HA) can find applications as bioactive and biodegradable composite for applications in hard tissue replacement and regeneration *(Chen and Wu, 2005)*.

Polymer implants for targeted drug delivery, an emerging medical application can be made out of PHAs *(Chen and Wu, 2005; Park et al, 2005)*. However, because of the high level of specifications for plastics used in the human body not every PHA can be used in medical applications *(Vert, 2005)*. PHA can be used in contact with blood which 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)*.

2.6 Factors affecting K_La and OTR

The value of the volumetric mass transfer coefficient K_La depends among other factors. First factor is medium viscosity which when viscosity increased, the K_La decrease. Second factor is degree of mixing. Increased mixing caused K_La to increase. Third factor is velocity increase which reduces the K_La and vice versa. Finally, antifoaming agents decrease K_La substantially.

The following methods are frequently used to increase the oxygen transfer rate (OTR). First is to increase the stirrer speed and second is to increase the aeration rate.

2.6 In-situ sterilization

Steam sterilization is commonly used in sterilization for in-situ procedure. The sterilization system design must ensure that the steam comes in contact with all sites exposed to process materials and gasses, culture materials or product. All inlet ports, outlet ports, supply line, harvest line, sensors, regulators, the reactor vessel itself and any stretch of piping that carried materials critical to the process must be sterilized.

Steam should reach each of these sites at a temperature and pressure adequate to destroy contaminating organisms and must remain at these levels for a specified period of time. The design of bioreactors and their associated components are usually designed to compatible with steam sterilization. For examples, the pitch of the piping and the positioning of ports, feed lines and regulatory devices can affect their accessibility to the steam). Sterilization is important to be designed to eliminate probability of microorganisms evading the sterilization and stopping them from flourish.

Medium sterilization within the fermentor vessel is performed in the batch mode by direct steam sparging. Sterilization cycle is composed of heating, holding and cooling stages. This cycle can be calculated using the total Del Factor (measure the size of a task to be completed) required for a complete sterilization should be equal to the sum of the Del Factor for heating, holding and cooling. (Deindoerfer and Humphrey, 1960)

CHAPTER 3

METHODOLOGY

3.1 Theory

3.1.1 DOT curve

Gassing out Technique was used to obtain the value of K_La for distilled water. One oxygen probe which had been connected to the monitor that can show the value of dissolved oxygen tension was immersed into a 500 mL shake flask which had been filled with 200 mL distilled water.

Nitrogen gas was supplied to the distilled water until the reading of the DOT reached zero. After that, the shake flask was shook using orbital shaker at 200 RPM at the room temperature. Simultaneously, the reading of DOT is taken to develop the DOT curve versus time.

3.1.2 K_La and Kap determination

$$YR(t) = C^*[((Kap.\exp(-K_La.t)/(Kap- K_La))- (K_La.\exp(-Kap.t)/(K_La-Kap))]$$

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

By applying this equation in matlab software (Fminsearch) it can be solved which will give results of two variables involved which are K_La and Kap.

3.2 Experimental methodology

There are 4 stages in the production processes:

Regeneration of the bacteria (for routine maintenance of the culture)

Fermentation for Starter 1

Fermentation for Starter 2

Fermentation in 2L bioreactor (batch)

3.2.1 Regeneration of the bacteria

1. The culture was maintained at slant medium. Regeneration is conducted every two weeks. Slant was prepared as the following procedure:
2. NGY agar medium was prepared with the following composition:

Table 3.1 NGY agar medium composition

No.	Chemicals	Amount (g/L)
1	Peptone	5
2	Glucose	10
3	Yeast extract	3
4	Beef extract	0.3
5	Agar	15
6	Aqueduct	Added until total volume= 1L

3. The solution was heated in a beaker glass with continuous stirring on laboratory hot plate until the solution comes into boiling.
4. 10 ml of the hot agar solution was poured into each sterilized test tube.
5. The tube was closed with sterile cotton and wrap in aluminum foil.

6. The tubes were sterilized in autoclave for 20 minutes at 121ºC.
7. The tubes were put in incline position so that the agar will set with inclined surface in the tubes. The tubes were left to set for one night in sterile incubator.
8. The bacteria were transferred from the old slant to the new slant in sterile laminar air flow hood with the following procedure:

   The metal loop was heated until burning red.

   The old slant containing bacteria to be regenerated was opened.

   The loop was cooled down by touching it on the agar surface.

   One loop full of bacteria was scrapped.

   The loop was transferred to the new slant by slightly scratching the agar surface quickly.
9. The slant was incubated in the sterile incubator at room temperature for about 24 hours until the bacteria seem to grow.
10. It was kept in the refrigerator at 4ºC for long time maintenance.

3.2.2 Fermentation for Starter 1

Table 3.2 Medium for Starter 1 NGY without agar

No.	Chemicals	Amount (g/L)
1	Peptone	5
2	Glucose	10
3	Yeast extract	3
4	Beef extract	0.3
5	Aqueduct	Added until total volume= 1L

1. The medium for starter 1 NGY without agar was prepared by following the medium compositions.
2. 20 ml of NGY medium was put into 100 ml Erlenmeyer flask.
3. The flask was closed with sterile cotton.
4. The flask was sterilized in autoclave for 20 minutes at 121ºC.
5. It was left to stand in sterile incubator for 24 hours at room temperature.

6. One loop of the bacteria was taken from the slant and was put into the incubated medium. The transfer was conducted in sterile laminar air flow hood.
7. It was incubated for 24 hours before moving the content into Starter 2.

3.2.3 Fermentation for Starter 2

Table 3.3 Medium for Starter 2 is Ramsay medium

No.	Chemicals	Amount (g/L)
1	Glucose	10
2	$Na_2HPO_4.7H_2O$	6.7
3	KH_2PO_4	1.5
4	$(NH_4)_2SO_4$	1.0
5	$MgSO_4$	0.2
6	$CaCl_2.2H_2O$	0.01
7	Ferri ammonium sulfate	0.06
8	*Trace element	1ml
9	Aqueduct	To make total volume 1L

Table 3.4 Trace elements composition

No.	Chemicals	Amount (g/L)
1	H_3BO_3	0.3
2	$CoCl_2.6H_2O$	0.2
3	$ZnSo_4.7H_2O$	0.1
4	$MnCl_2.4H_2O$	0.03
5	$(NH_4)_6MO_7O_4.4H_2O$	0.03
6	CuSO4.5H2O	0.01
7	NiSO4.6H2O	0.02
8	Aqueduct	To make total volume 1L

To grow bacteria in Starter 2, the following procedure is conducted:

1. 180 ml of Ramsay medium was put into 500 ml Erlenmeyer (plug the inlet with sterile cotton) and was sterilized in autoclave for 20 minutes at 121ºC.
2. After sterilization, let the flask to stand for 24 hours in sterile incubator.
3. Starter 1 was poured into the flask.
4. It was shook on shaker for 24 hours.

3.2.4 Fermentation in batch bioreactor (20 l)

1. The bioreactor was cleaned thoroughly before using.
2. 1800 ml of Ramsay medium was poured into the bioreactor, sterilized for 20 minutes at 121ºC.
3. 0.5 M NaOH and 0.5 M HCl were prepared for pH control.
4. Si solution was prepared for antifoaming (dilute 100x from the original solution)
5. The lines were connected to sterilize bioreactor (containing medium), turned on the bioreactor and the cooling tower and air supply were .opened
6. Starter 2 was flowed into the bioreactor through the automatic input.
7. The bioreactor was let to run. If samplings were needed, samples were withdrawn through the sampling line periodically.

3.3 Solving techniques

The solving technique used is to perform dissolved oxygen tension (DOT) curve by plotting Dissolved Oxygen Tension, DOT (%).versus time (minute) in MATLAB 7.1 to solve for values of $K_L a$ and Kap.

Firstly, the DOT curve in shake flask will be obtained by setting the RPM and air flow rate to the optimum condition which is 200 RPM and 30^0C. From the DOT curve, the values of $K_L a$ and Kap will be obtained by using Fmin search in MATLAB. By trial and error, the values of RPM and air flow rate that produce the similar value of $K_L a$ and Kap will be chosen for scaling up in 20 l fermenter .

3.4 Method of analysis

The methods of analysis that is applied in this research are:

1. Cell dry mass. This analysis is done periodically by taking 10ml of fermentation sample every 6 hours.
2. PHB harvesting. This analysis is done by taking 10 ml of fermentation sample every 6 hours.
3. Dinitrosalycilic method (DNS method) for glucose analysis

3.4.1 Cell dry mass

The method for cell dry mass analysis is by doing the following steps:

1. 10 ml sample was taken at designated time intervals (every 6 hours).
2. The sample was centrifuged using the refrigerant centrifuge at 5000rpm, 4^{o}C for 12 minutes.
3. The supernatant of the centrifuged samples will be used for glucose analysis meanwhile the pellet is used for cell dry mass analysis.
4. The pellet was added with 10ml deionized water for washing.
5. Then, it will be vortex to mix the pellet with deionized water.
6. Steps 2, 4 and 5 were repeated.
7. The sample was filtered with filter paper and left to dry at 90^{o}C for 15-20 hours.
8. The dried filter paper will be cooled by using desiccator for 30 minutes.
9. The filter paper was weighed.

Cell dry weight = (weight of filter paper+ dried sample) – (weight of filter paper)

3.4.2 Glucose analysis

The method for glucose analysis is as follows:

1. 1 ml of supernatant was added with 1 ml DNS reagent and mixed evenly.
2. 2 drops of Natrium Hydroxide (NaOH) was added to the mixture.
3. A blank was also prepared which consist of 1ml deionized water, 1ml DNS reagent and 2 drops of NaOH.
4. The mixture from step 3 and 4 were soaked in water bath for 5 minutes at 100^{o}C.
5. The samples were cooled under running water.
6. The samples including the blank were added with 10 ml of deionized water and mixed evenly.
7. The absorbance was read using uv-vis at 540nm.

3.4.3 PHB analysis

The steps for PHB analysis is as follows:

1. 10 ml of sample was centrifuge at 4^{o}C, 5000 rpm for 12 minutes.
2. The pellet was taken for PHB analysis.
3. The pellet was added with 10 ml NaCl (0.625%).
4. The mixture was centrifuged at the same parameters as in step 1.
5. The pellet was added with 10 ml, 100 µm hydrogen peroxide (H_2O_2).
6. The mixture was soaked in water bath for 4 hours at 30^{o}C.
7. Step 4 was repeated.
8. The pellet was added with 10 ml chloroform.
9. The mixture was mixed using vortex and was poured to a petri dish to dry.
10. 5 ml of sulphuric acid (H_2SO_4) was added into the petri dish.
11. The mixture was then collected into a centrifuge bottle.
12. It was left soaked in boiling water at 100oC for 10 minutes.
13. It was cooled under running water before reading the absorbance at 238nm.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

4.1.1 K_La and Kap determination

The results from this research project are the K_La from 20 l fermenter is similar to the K_La from 500 ml shake flask. After running the data in MATLAB 7.1 using the Fminsearch with Nelder-Mead implementation, the K_La for 500 ml shake flask and 20 l stirred tank fermenter were 0.2809 and 0.2564 respectively. Meanwhile the Kap for 500 ml shake flask and 20 l stirred tank fermenter were 0.0010 and 0.0008 respectively.

Using trial and error method, the values of agitation speed and air flow rate were manipulated to achieve the similar value of K_La and Kap.in 500 mL shake flask. After many trials and error, the agitation speed of 20 l fermenter that had achieved the similar result in shake flask was 200 RPM. Meanwhile, the value of air flow rate is 5 l/min. These values of K_La and Kap will be used in scaling up process from shake flask to 20 l fermenter.

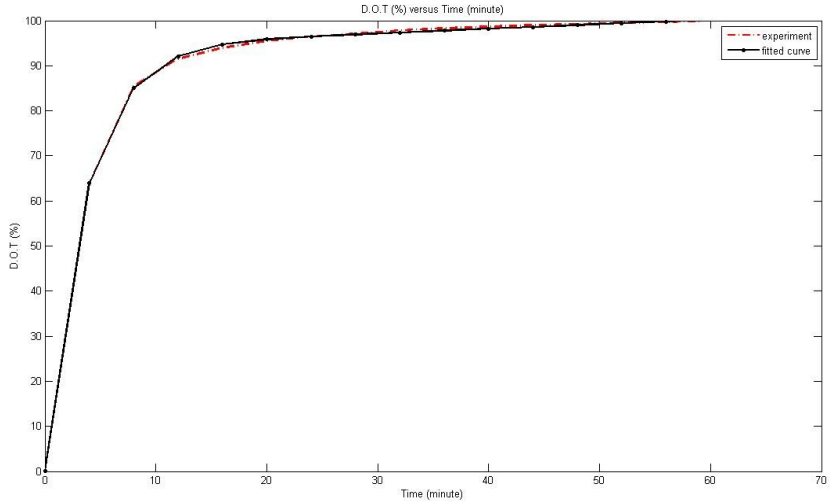


Figure 4.1 Comparison between DOT by experiment and calculation for 500mL shake flask.

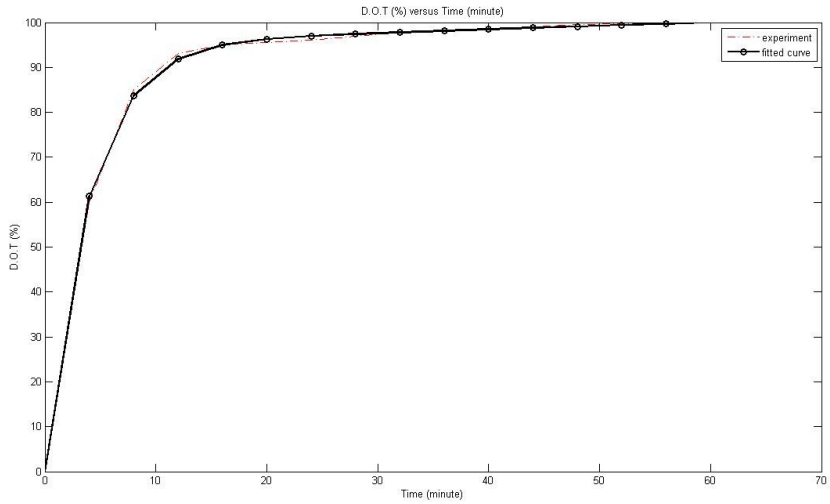


Figure 4.2 Comparisons between DOT by experiment and calculation for 20 l stirred tank fermenter.

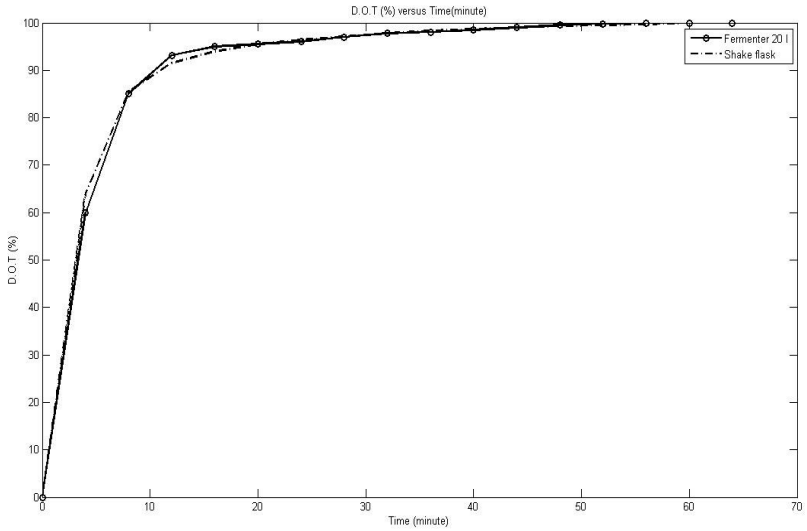


Figure 4.3 Dissolved Oxygen Tension (DOT) for shake flask and 20 l stirred tank fermenter.

4.1.2 **Sterilization cycle**

Deindoerfer and Humprey (1960) have investigated the thermal destruction of *B. stearothermophilus* spores by moist heat and have calculated the values of the activation energy and Arrhenius constant as follows:

Activation energy = 67.7 Kcal/mol

Arrhenius constant = 1 x $10^{36.2}$ sec^{-1}

N_o = 5 x 10^6 cells/mL x 10^3 x 20L

N = 10^{-3} (1:1000 probability of contamination)

$$\nabla_t = \log_e\left(\frac{N_o}{N}\right)$$

= 32.236 min

$$\nabla_t = \nabla_h + \nabla_m + \nabla_c$$

So $\nabla_m = \nabla_t - \nabla_h - \nabla_c$

$\nabla_m = 32.236 - 12.55$

$\nabla_m = 19.686$

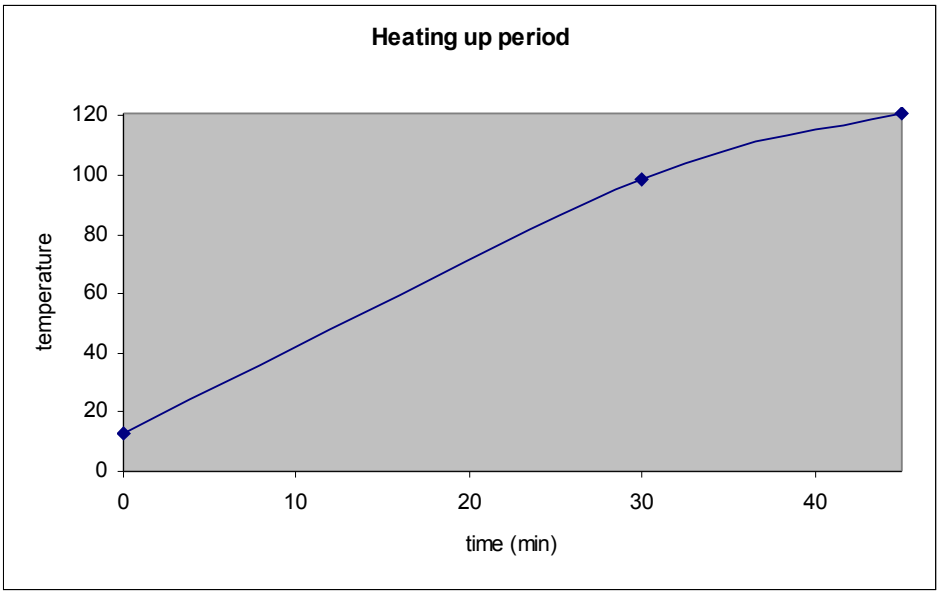


Figure 4.4 The heating up period for in-situ steam sterilization

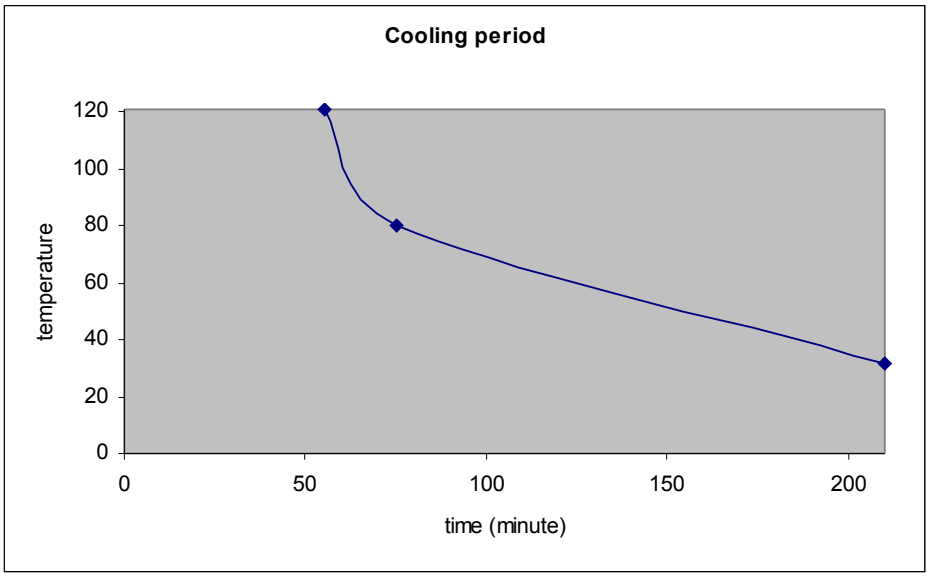


Figure 4.5 the cooling period for in-situ steam sterilization

$\nabla_m = k_m t_m$

$\therefore t_m = \left(\frac{\nabla_m}{k_m} \right)$

k_m = thermal death rate constant at sterilization temperature (121°C)

t_m = holding time at sterilization temperature

$$k = A.e^{\left(\frac{-E}{RT}\right)}$$

Temperature at the sterilization temperature:

T = 121+273 = 394 K

$$k_m = \left(1x10^{36.2}\right)e^{\left(\frac{-67.7\,Kcal\,/\,mol}{1.987\,cal\,/\,molKx394\,K}\right)}$$

$$k_m = 2.54\,\mathrm{min}^{-1}$$

$$t_m = \frac{19.686}{2.54}$$

$$t_m = 7.75\,\mathrm{min}$$

So, the calculated holding time at sterilization temperature is for 7.75minutes

4.1.3 Shake flask fermentation

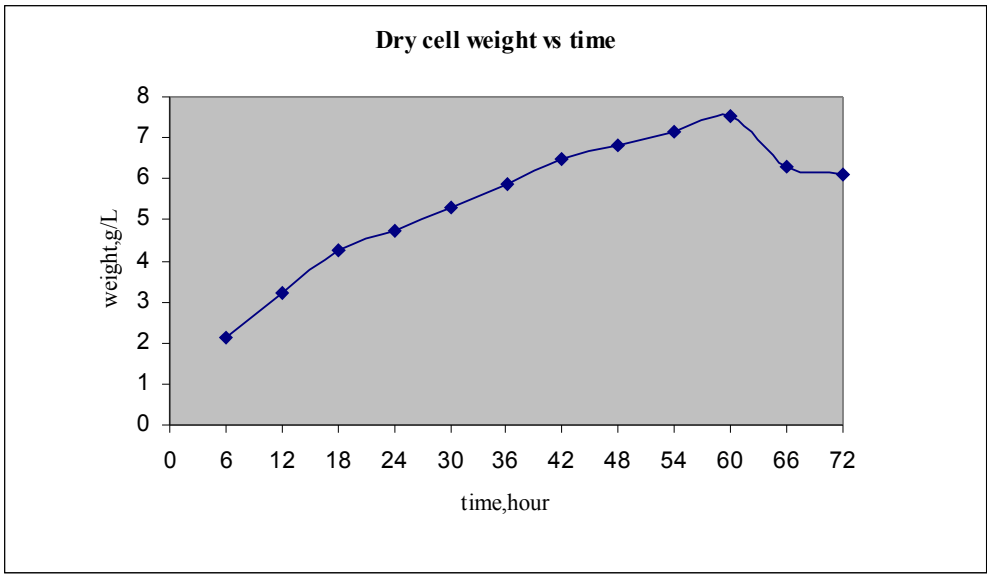


Figure 4.6 Dry cell weight versus time in shake flask fermentation

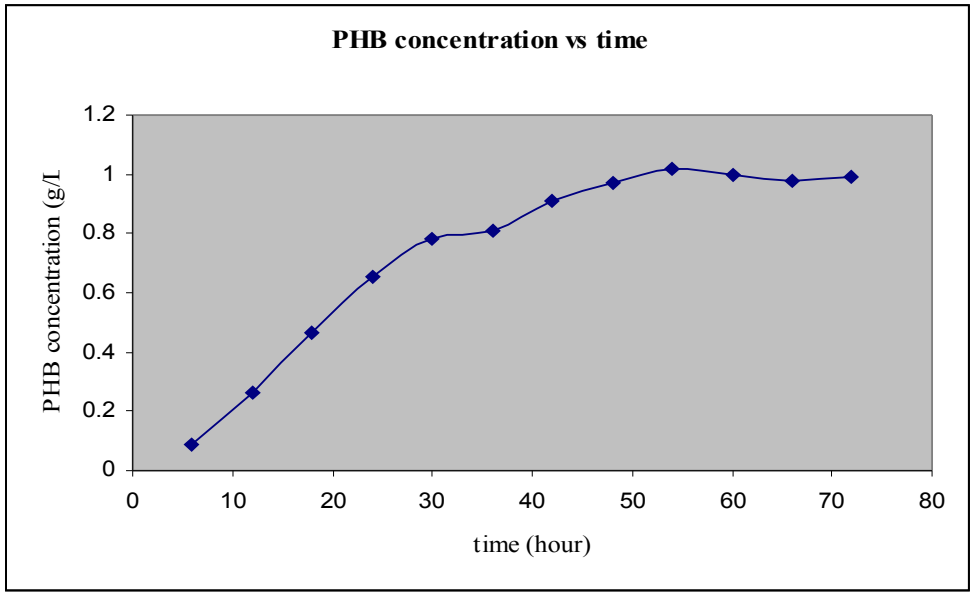


Figure 4.7 PHB concentrations versus time for shake flask fermentation

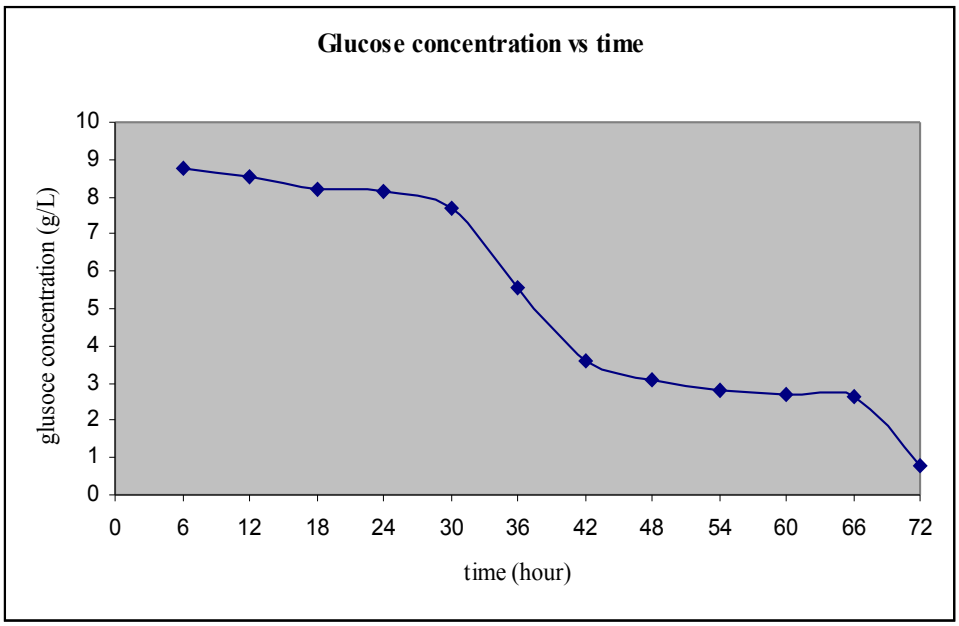


Figure 4.8 Glucose concentration versus time for shake flask fermentation