BIOSYNTHESIS OF POLY(3-HYDROXYBUTYRATE) (PHB) BY *Cupriavidus necator* H16 FROM JATROPHA OIL AS CARBON SOURCE

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Thesis submitted in fulfillment of the requirements
for the award of the degree of
Master of Engineering (Chemical)

Faculty of Chemical and Natural Resources Engineering
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Dedicated to those who stumbled, fumbled and found their way out

If we knew what we were doing it would not be called research, would it?

-Albert Einstein

ACKNOWLEDGEMENTS

Syukur Alhamdulillah, I get to finally submit this paper. This dissertation would not have seen the light of the day without the guidance and assistance of several individuals who have contributed and extended their invaluable assistance in the preparation and completion of this study.

First and foremost, I am deeply indebted to my supervisor, Assoc. Prof. Dr. Maksudur Rahman Khan. Without his patience and persistent help this research would not have been possible. I started off with little knowledge in research works. His guidance and perseverance has helped me all the way to the end of this study. I would also like to express my gratitude to Dr. Reddy Prasad for all his help during this study.

None of this research works would have been possible without the financial assistance provided throughout this study. Thus, I would like to thank the Malaysian Ministry of Higher Education under the Fundamental Research Grant Scheme (RDU100107) for funding this research. I would also like to express my gratitude to Universiti Malaysia Pahang (UMP) for the always-on-time monthly allowance through its Graduate Research Scheme (GRS).

My special thanks are extended to all the laboratory staffs of Faculty of Chemical and Natural Resources Engineering (FKKSA) for their consistent guidance and support during my experimental works. My deepest gratitude goes to Dr. Mior Ahmad Khusairi for his valuable assistance in this study. A gazillion thanks goes to Kamal Sukardi and Baranitharan for relentlessly teaching this newbie the basics of bacterial fermentation. Not to mention Sifu Roy for all his help from the very first day I stepped into UMP till the final days of my studies. Without these people I would have been totally helpless.

Friends and roommates, thanks for making this journey a memorable one. Mama and Papa, thanks for letting go of your only child to further her studies. I know it was hard for both of you. Your endless prayers have helped me to overcome all hurdles. Last but not least, husband, thanks for being my pillar of strength throughout my long-drawn-out studies. Your patience and moral support got me through this roller-coaster ride.

ABSTRACT

The increasing non-biodegradable plastic waste materials have created critical need in finding a better replacement to the currently available conventional plastic. Researches have shown great interest in the production of polyhydroxyalkanoate (PHA) biopolymer from bacterial fermentation. Although successful attempts have been made in producing these short to medium-chain length biopolymers, there are still problems in terms of yield and cost effectiveness that needs to be resolved. Poly(3-hydroxybutyrate) (PHB), a homopolymer of PHA is one particular example of bioplastic that are naturally produced by bacteria like *Cupriavidus necator* sp. By using plant oils such as jatropha oil as an alternative, a higher yield of PHB can be obtained and thus reducing the overall production cost of the biopolymers. In this study, *Cupriavidus necator* H16 was used to synthesize PHB by using jatropha oil as its sole carbon source. Different variables mainly jatropha oil and urea concentrations, and agitation speed were investigated to determine the optimum condition for microbial fermentation in batch culture. Based on the results, the highest cell dry weight and PHB concentration of 20.1 g/L and 15.5 g/L respectively was obtained when 20 g/L of jatropha oil was used along with 1 g/L of urea at 200 rpm of agitation speed. Ethanol was used as external stress factor and the addition of 1.5% (v/v) ethanol at 38 h had a positive effect with a high PHB yield of 0.987 g PHB/g jatropha oil. The kinetic studies for cell growth rate and PHB production were conducted and the data were fitted with Logistic and Leudeking-Piret models. The rate constants were evaluated and the theoretical values were in accordance with the experimental data obtained. Optimization through Response Surface Methodology (RSM) at the condition of 0.9 g/L urea, 23.6 g/L jatropha oil and 251 rpm agitation speed resulted in 5% increase in PHB concentration to 17.92 g/L compared to the previously obtained PHB concentration of 17.05 g/L. The present work has succeeded in obtaining a high yield of PHB from an inexpensive raw material.

ABSTRAK

Peningkatan bahan buangan plastik telah mewujudkan keperluan drastik untuk mencari pengganti kepada plastik konvensional yang sedia ada. Bioplastik yang dihasilkan daripada fermentasi bakteria telah mendapat sambutan yang hangat di kalangan para pengkaji. Beberapa kajian telah berjaya menghasilkan biopolimer rantaian singkat ke rantaian sederhana polihidroksialkanoat (PHA). Walau bagaimanapun, masalah dari segi keberkesanan dan kos penghasilan masih perlu diatasi. Poli(3-hidroksibutirat) (PHB), sejenis homopolimer PHA adalah salah satu contoh bioplastik yang dihasilkan secara semulajadi oleh bakteria seperti *Cupriavidus necator* sp. Dengan penggunaan minyak tumbuhan seperti minyak jatropa sebagai satu alternatif, penghasilan PHB yang lebih tinggi boleh diperolehi dan secara tidak langsung dapat mengurangkan kos penghasilan biopolimer. Dalam kajian ini, *Cupriavidus necator* H16 digunakan untuk mensintesis PHB dengan menggunakan minyak jatropa sebagai sumber karbon utama. Beberapa pemboleh ubah seperti kepekatan minyak jatropa dan urea serta kelajuan kelalang goncang telah dikaji untuk menetukan keadaan optimum fermentasi bakteria di dalam kultur berkelompok. Berdasarkan keputusan yang diperolehi, berat sel kering dan kepekatan PHB paling tinggi diperolehi pada kepekatan minyak jatropa 20 g/L, urea 1 g/L dan kelajuan kelalang goncang 200 rpm; iaitu sebanyak 20.1 g/L berat sel kering dan 15.5 g/L kepekatan PHB. Penggunaan etanol sebagai faktor tekanan luaran memberi kesan positif dengan penambahan 1.5% etanol pada masa inkubasi yang ke-38 dengan penghasilan sebanyak 0.987 g PHB/g jatropa. Kajian kinetik bagi kadar pertumbuhan sel dan penghasilan PHB dijalankan dan data yang diperolehi diselaraskan dengan model *Logistic* dan *Leudeking-Piret*. Kadar tetap yang diperolehi dikaji dan didapati nilai-nilai teori adalah setara dengan nilai-nilai ujikaji. Kaedah Respon Permukaan (RSM) digunakan untuk mengenal pasti keadaan yg optimum untuk penghasilan PHB. Pada kepekatan urea 0.9 g/L, jatropa 23.6 g/L dan kelajuan kelalang goncang 251 rpm, penghasilan PHB didapati meningkat daripada keadaan asal, iaitu sebanyak 17.05 g/L kepada 17.92 g/L. Keadaan optimum ini memperlihatkan peningkata penghasilan PHB sebanyak 5%. Kajian ini telah berjaya menghasilkan jumlah PHB yang tinggi dengan menggunakan bahan mentah kos rendah.

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LIST OF SYMBOLS

M_N	Average molecular weight
M_w	Molecular weight
P	Product concentration (g/L)
P_0	Initial product concentration (g/L)
T_d	Thermodegradation temperature
T_g	Glass transition temperature
T_m	Melting temperature
t	Time (h)
t_m	Time when maximum cell concentration is reached (h)
x	Cell concentration (g/L)
x_m	Maximum cell concentration (g/L)
x_0	Initial cell concentration (g/L)
α	Growth associated constant (g/g)
β	Non-growth associated constant (g g^{-1} h^{-1})
μ_m	Maximum specific cell growth rate (h^{-1})

LIST OF ABBREVIATIONS

BOD	Biological oxygen demand
CDW	Cell dry weight
COD	Chemical oxygen demand
CPKO	Crude palm kernel oil
CPO	Crude palm oil
FDA	Food and Drug Administration
GC	Gas chromatography
HBME	Hydroxybutyric methyl esters
HHx	Hydroxyhexanoate
HPLC	High Performance Liquid Chromatography
HV	Hydroxyvalearate
mcl-PHA	Medium chain length PHA
MMC	Mixed microbial concortia
NADPH	Nicotinamide adenine dinucleotide phosphate
PHA	Polyhydroxyalkanoate
PHB, P(3HB)	Poly-3-hydroxybutyrate
PLA	Polylactic acid
phaA	β-ketothiolase
phaB	Acetoacetyl–CoA reductase
phaC	PHA synthase
PO	Palm oil
POME	Palm oil mill effluent
P&G	Procter & Gamble
PTFE	Polytetrafluoroethylene

RSM	Response Surface Methodology
scl-PHA	Short chain length PHA
SPKO	Saponified palm kernel oil
TCA	Tri-carboxylic acid cycle
SBR	Sequencing batch reactors
USD	US Dollars
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Undeniably, petroleum-based synthetic plastics offer a wide range of industrial and domestic applications due to their convenience and durability. The short-term convenience of using and throwing these conventional plastics have created major problem since they cannot be degraded naturally in the environment. These plastic wastes pile up in landfills and take hundreds of years to degrade (Wurpel et al., 2011). The situation is made worse when these plastic wastes are thrown carelessly into the ocean, endangering marine life. Apart from that, the diminishing worldwide petroleum resources compels for a better alternative for petroleum-based plastics. Based on the report by US Energy Information Administration, about 191 million barrels of liquid petroleum gases and natural gas liquids were used in the United States to make plastic products in the plastic materials and resins industry which is equivalent to about 2.7% of total U.S. petroleum consumption (US Enery Information Administration, 2010).

The current concerns over the increasing usage of non-biodegradable plastics and its impact to the nature have pushed researchers to develop bioplastics that are biodegradable and environmental-friendly. Biodegradable plastics have the potential to replace conventional plastics as they are environmentally-friendly. These biopolymers can be synthesized from renewable raw materials and thus reducing the greenhouse gas effect. Polyhydroxyalkanoates (PHA) and polylactic acid (PLA) are an example of biodegradable plastic that are produced by fermentation using agricultural products and microorganisms (Tokiwa et al., 2009).

This research emphasis is on PHA biopolymers which are produced by various microbes under nutrient-limiting conditions (e.g.: limitation of sodium and phosphorus) but with an excess of carbon source (Luengo et al., 2003). The bacterial strains used for PHA biosynthesis are categorized based on the culture medium used during fermentation. The first group, consisting of microbes such as *Cupriavidus necator*, *Protomonas extorquens* and *Protomonas oleovorans*, requires the presence of excess carbon source and limitation of nutrients like nitrogen or phosphorus. Meanwhile, the second group can accumulate PHA during growth phase itself at a nutrient-sufficient condition. Bacterial strain in this group include *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii* and recombinant *E. coli* harboring the PHA biosynthetic operon of *C. necator* (Khanna and Srivastava, 2005; Lee, 1996).

The specific biopolymer synthesized in this research was poly-3-hydroxybutyrate (PHB). PHB, the most common type of PHA, can be accumulated by various microorganisms. These short-chain length biopolymers have been studied and characterized comprehensively (Madison and Huisman, 1999). This research focusses on elucidating the influence of various process parameters to determine the optimum condition for the bacterial fermentation of *Cupriavidus necator* H16 to produce PHB by using jatropha oil as its main carbon source.

1.2 PROBLEM STATEMENT

Although researches on PHB synthesis from microorganisms are abundant, the large-scale manufacture of PHB-based bioplastics is still limited due to its high production cost. Since the raw material cost is one of the major elements in the production of PHB, a good choice of the feed substrate may reduce significantly the overall PHB production cost. At present, large-scale production of PHB uses sugars like fructose and glucose as their carbon source. These sugars at USD 0.50/kg are expensive (Choi and Lee, 1997) and on top of that, they also give a low yield of PHB (Kahar et al, 2004). Thus, in order to make the biopolymer production a more practical approach, an alternate raw material that is cost-effective and at the same time does not affect the yield of PHB should be used.

One better way of achieving this goal is to substitute the carbon source into a more cost efficient ones that are derived from plant oils. Plant oils such as jatropha oil are known to give a theoretical yield of PHA of over 1.0 g-PHA per g-plant oils used compared to glucose which only gives a yield of 0.32–0.48 g-PHA/g-glucose (Kahar et al, 2004). Thus, with the usage of jatropha oil as its carbon source, the production cost of PHB-based biopolymers can be radically reduced without compromising the PHB yield. Ng et al. (2010) have reported convincing results of 13.1 g/L cell dry weight and 11.4 g/L of PHB from the bacterial fermentation of *Cupriavidus necator* H16 using 12.5 g/L jatropha oil as their sole carbon source. Various nitrogen sources were studied by Ng et al. (2010) and urea at 0.54 g/L was concluded as the most suitable nitrogen source that gives high PHB accumulation.

The outstanding result had encouraged us to explore the possibility of further enhancing the PHB yield by studying the influence of various process parameters on PHB accumulation. Mainly, we examined the effect of external stress factor on the bacterial growth and PHB accumulation. Research on the optimization and kinetic studies on production of PHB from *Cupriavidus necator* sp. by using jatropha oil were also limited. Therefore, additional research concerning these aspects was done to improve the overall understanding on PHB production from jatropha oil as carbon source.

1.3 RESEARCH OBJECTIVES

The main objective of this research is to study the reaction kinetics of the production of PHB from *Cupriavidus necator* sp. by using jatropha oil as its sole carbon source. Different aspects such as the agitation speed, oil and urea concentration and stress factor effect were analyzed to determine the best condition for PHB production. These conditions were optimized further to increase the yield of PHB and mathematical models were be developed for cell growth and PHB accumulation. The specific objectives of this study include:-

1. To study the effect of various process parameters (agitation speed, oil and urea concentration etc.) in the production of PHB from *Cupriavidus necator* sp. by using jatropha oil as its main carbon source.

2. To study the kinetics and develop the corresponding mathematical model of PHB production and conduct optimization of PHB by using Response Surface Methodology (RSM).

1.4 RESEARCH SCOPE

The study plan focuses on the production of PHB by using jatropha oil as carbon source. In order to achieve the above stated objectives, the following scope of research has been identified:

1. The bacteria *Cupriavidus necator* sp. were fermented in shake flask and the study of different variables (agitation speed, oil and urea concentration, stress factor effect) were conducted.

2. Quantitative analysis were done on the biopolymer produced by using Gas-Chromatography (GC) analysis to determine the PHB concentration in cells.

3. Subsequently, the research were expanded to study the optimization of the biopolymer synthesis using Response Surface Methodology (RSM) to gain the optimum conditions for the highest PHB concentration in cells.

4. The kinetic studies for cell growth rate and PHB production were conducted and the data were fitted with Logistic and Leudeking-Piret models. The rate constants were evaluated and the data obtained were compared with the calculated theoretical values.

1.5 SIGNIFICANCE OF RESEARCH

Through this research, it is believed that a higher yield of PHB can be obtained by optimizing the variable conditions and by doing kinetic studies on the biopolymer synthesis. Moreover, using jatropha oil as the carbon source for PHA production may lessen the overall production cost considerably and thus making it a more feasible approach for large-scale production. In addition, jatropha oil has an added advantage of being non-edible oil. Therefore, utilizing it for bioplastic production would not interfere with the existing global food shortage issue.

1.6 THESIS OVERVIEW

This thesis comprises of five main chapters. Chapter 1 discloses the introduction and Chapter 2 has a detailed review on literatures related to polyhydroxyalkanoate (PHA). Meanwhile Chapter 3 discusses the methodology, apparatus and experimental equipment used throughout this research. Chapter 4 holds comprehensive discussions on the experimental results obtained and Chapter 5 discusses the overall summary and recommendations for future work. References and appendices are also included for better understanding of the research.

CHAPTER 2

LITERATURE REVIEW

This chapter mainly consists of findings from previous researches with regards to production of PHB. Detailed discussions on the properties, synthesis mechanism, and the comparison of various bacterial strains and carbon sources used in the production of PHB and its homopolymers were presented in the following sections.

2.1 INTRODUCTION

Presently, the diminishing global petroleum resources have created urgent need towards finding sustainable alternate sources for value-added chemicals. In addition, petroleum-based plastics are known to be hazardous given that they cannot be degraded naturally in the environment. Thus, a more enhanced approach would be to implement the usage of biodegradable plastics that are cheap and have similar properties to the commercial plastics.

In general, bioplastics are defined as a kind of biomaterial that are cultivated under specific nutrient and environmental conditions by using a variety of microorganism and carbon substrate as their raw material. These polymers are used as storage materials by microbes to survive under nutrient-deficient condition (Madison and Huissman, 1999). Although numerous researches have been done with regards of bioplastics, the large-scale production is still limited due to the low productivity and also high manufacturing cost. As for the past few years, there are a few types of bioplastics available such as starch and cellulose based plastics, polylactic acid (PLA) plastics and also polyhydroxyalkanoate (PHA) based polyester plastics.

PHAs are completely biodegradable and biocompatible polymers with properties such as thermoplastic, elastomer, insoluble in water and also non-toxic in nature (Ng et. al, 2010). These polyesters have characteristics similar to those of polyethylene and polypropylene, and can therefore be used as a substitute to conventional plastics. Apart from that, they are also degraded completely under aerobic and anaerobic conditions by microorganisms (Luengo et al., 2003), and thus putting an end to the increasing non-biodegradable municipal solid waste problems. Figure 2.1 shows the general structure of PHAs.

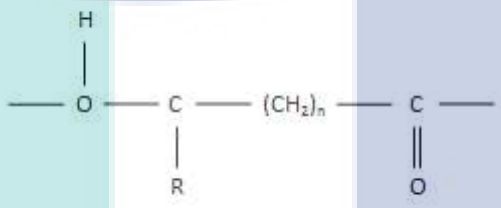

Figure 2.1: General structure of polyhydroxyalkanoates

Source: Volova (2004)

PHAs have a variety of usage in the industries due to the similarities of physical and thermal properties between commercial plastics and bioplastics produced from PHA polymers. PHA bioplastics have great potential to be used as packaging films in bags, containers and paper coatings. These polymers can also be used as a replacement of the regular commercial plastic to manufacture disposable items such as razors, utensils, cosmetic containers and so on. The comparison between physical and mechanical properties of PHA and polypropylene, a common synthetic plastic, is shown in Table 2.1.

Table 2.1: Physical and Mechanical Properties of PHA

Properties	PHA	Polypropylene
Molecular Weight, M_w x 10^4	10 – 1000	-
Melting Temperature, T_m (^{0}C)	60 – 177	176
Glass Transition Temperature, T_g (^{0}C)	-50 – 4	-10
Thermodegradation Temperature, $T_{d(5\%)}$ (^{0}C)	227 – 256	-
Young's Modulus (GPa)	0.7 – 3.5	1.7
Elongation at Break (%)	2 – 1000	400
Tensile Strength (MPa)	17 – 104	34.5

Source: Chen (2009) and Ojumu et al. (2004)

Generally, PHAs can be divided into two main groups according to the number of carbon atoms in the monomeric units. These include short chain length PHAs (scl-PHA) which consist of 3-5 carbon atoms in the constituting monomeric unit of the polymer and also medium chain length PHAs (mcl-PHA) consisting of 6-14 carbon monomers (Ojumu et al., 2004). One particular example of PHA is poly(3-hydroxybutyrate) (PHB) which is a homopolymer that contains monomers of 3-hydroxybutyrate. The molecular structure of PHB is displayed in Figure 2.2. PHB has crystalline properties with a melting point of around 170 ^{0}C (Kulkarni et al., 2010). With degradation temperature (185^{0}C) recorded just slightly above its melting temperature, PHB has an unstable nature during its melting stage (Ojumu et al., 2004). Furthermore, its crystallinity, hardness and brittleness forces it to be used only as specialty plastics for certain types of industries. Thus, to overcome these problems, several attempts have been made by incorporating comonomers such as 3-hydroxyvalearate (HV) and 4-hydroxybutyrate (HB) into PHB to reduce its brittleness.

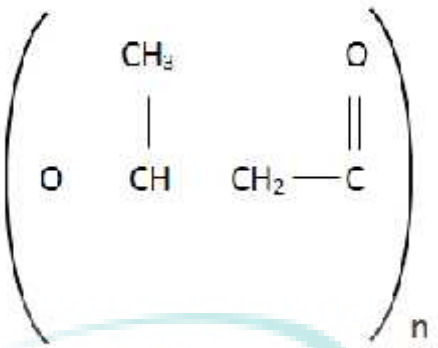

Figure 2.2: Molecular structure of PHB, n can range from 100 to several thousands

Source: Salakkam (2012)

2.2 PHA APPLICATION

PHA based biopolymers have garnered immense interest due to their biodegradability and biocompatibility. The following subtopics discuss the current and future applications of PHA.

2.2.1 As Packaging Material and Disposable Items

The similarity of PHA and other synthetic plastics renders it beneficial to be used as packaging films mainly in bags, containers and paper coatings. Likewise, its biodegradability makes it a suitable choice as a substitute for regular plastic disposable items such as razors, utensils, diapers, feminine hygiene products and cosmetic containers like shampoo bottles and cups (Reddy et al., 2003). Procter & Gamble (P&G, USA) had developed, Nodax ™, a bioplastic based on polyhydroxybutyrate-hexanoate (PHB-HHX). Nodax ™ can be used to manufacture a variety of plastic materials including packaging, laminates and coatings, and nonwoven fibers (Noda et al., 2010). Figure 2.3 portrays the various products based on Nodax ™ bioplastic.

Figure 2.3: Various products from Nodax ™ bioplastic

Source: Paliakoff and Noda (2004)

2.2.2 In Medical and Pharmaceutical Industries

The non-toxicity and biocompatibility of PHB based biopolymers offers great potential to be used in medical and pharmaceutical industries. Upon degradation, these bioplastics will be degraded into D-3-hydroxybutyrate is a common intermediate metabolic compound in all higher organisms (Lee, 1996). PHA biopolymers can be used as surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements in healthcare industries (Reddy et al., 2003). The main advantage of using PHA bioplastic in surgical implantation is its biodegradability which enables it to degrade naturally without the need for surgical removal of the implant. In the pharmaceutical industry, PHB is used in drug delivery system as a matrix material for slow release drugs and in vitro cell cultures (Suriyamongkol et al., 2007). Nevertheless, their applications in the pharmaceutical and medical fields are still restricted due to their slow biodegradation and high hydraulic stability in sterile tissues (Wang and Bakken, 1998).

2.2.3 In Agricultural Industries

The agricultural industry may offer a vast array of application which includes seed encapsulation, encapsulation of fertilizers and protective material for crops in the form of biodegradable plastic films. The biopolymer P(3HB-3HV) could be used in the controlled release of insecticides for crops. The commercially available Nodax ™

bioplastic could also be used as coating for urea fertilizers in rice fields. The biopolymer, which can be degraded anaerobically, can also be used as herbicides and insecticides (Yogesh et al., 2012).

2.3 COMMERCIALISATION OF PHA

Although PHA has great potential to be used as a substitute for conventional plastics, its large scale production is still restricted due to its cost effectiveness. As mentioned earlier, the cost of raw material and also the recovery process play a major role in the overall production cost of PHA biopolymer. Numerous researches were conducted to address this crucial problem so that PHA based biopolymer can be made commercially viable. Through constant research, the price for Biopol^{TM} plastics was reduced from 16 USD/kg to 4 USD/kg. Nevertheless, the price is still expensive when compared to plastics made from polypropylene and polyethylene (0.25 – 0.5 USD/kg) (Chandrasekharaiah, 2005). Biopol is produced industrially by bacterial fermentation of *Cupriavidus necator* with glucose as its carbon source. The annual production of Biopol was about 10,000 tonnes (Lee, 1996). In 1990, the product was successfully used for the marketing of German's hair care company, Wella's Sanara shampoo bottle (Chen, 2010). Table 2.2 presents a list of PHA producing companies around the world.

Table 2.2: Worldwide PHA producers

Type of PHAs	Company and origin	Trade name	Price (USD/kg)	Microorganism
PHB	Biocycles, Brazil	Biocycle	3.12-3.75 (2010)	*Alcaligenes* sp.
PHB	Biomer, Germany	Biomer™	3.75-6.25 (2010)	*A. latus*
PHB	Chemie Linz, Austria	-	-	-
PHB	Jiangsu Nan Tian, China	-	-	-
PHB	Mitsubishi Gas Chemical, Japan	Biogreen™	2.75 (2010)	Methanol utilising bacteria
PHB/PHV	Metabolix, USA	Biopol™	4 (2005)	Glucose utilizing mutant of *C. necator*
PHA/PHB/ PHO*	Metabolix, USA	Metabolix PHA	-	Recombinant *E. coli* K12
PHA copolymer	Meredian, USA	Nodax™	-	*Aeromonas caviae* and *C. necator*

*PHO – polyhydroxyoxanoate

Source: Salakkam (2012)

Currently, there are several brands of PHA that are available in the market. These PHAs are produced at a large scale by using sugar as their carbon source. Some examples of commercially produced PHAs include Biopol™ (copolymer of hydroxybutyrate (HB) and hydroxyvalerate (HV)), Biomer™ (homopolymer of HB), Nodax™ (copolymer of HB and hydroxyhexanoate (HHx)) and Biocycle™ (homopolymer of HB, copolymer of HB and HV) (Mumtaz et al., 2010). Tepha, a PHA bioplastic producing company, have succeeded in commercializing medical devices such as the Food and Drug Administration (FDA) approved PHA-based sutures. Nevertheless, usages of bioplastics from PHA are still limited mainly because their production cost are still very high when compared to petroleum-based polyesters. One of the major problems faced in reducing its production cost include selecting a relatively cheap but equally viable carbon source.

2.4 PHB SYNTHESIS

PHA can be synthesized either by chemical or biological methods. The biological approach gives a higher molecular weight of PHA compared to the ones obtained from chemical methods. Nonetheless, biosynthesis of PHA poses some difficulties when it comes to controlling the monomer structure of PHA polymers since the polymers produced are strictly dependant on the microorganism and carbon source used during the fermentation process. Thus, a good choice of bacterial strain and carbon substrate is necessary in order to acquire the desired PHA monomer structure.

Generally, PHAs are produced in a two-stage production method. In the first stage, cells are cultivated in the culture medium while in the second stage, the microorganisms are exposed to nutrient-deficit conditions to induce the production of PHA (Kulkarni et al., 2010). So, in order to obtain a high product yield, a high cell density needs to be inoculated in the production medium (Madison and Huisman, 1999). In another research done by López-Cuellar et al. (2010), three-stage fermentation process was used to obtain PHA with an enhanced thermal and mechanical property. In the first stage, cells are cultivated in the growth medium followed by the second stage where the feeding substrates are controlled to achieve a high cell density. In the final stage, a carbon source is added to produce the required medium-chain length PHA.

As for PHB production, previous researches have reported the usage of shake flask and bioreactors to produce PHB. The yield of PHB from shake flask fermentation is lower since PHB production in this batch process is mainly inhibited by carbon source limitation. Fed-batch process using bioreactors gives a higher yield of PHB since the carbon source is intermittently added to ensure a constant supply of feed substrate throughout the process. For instance, Park and Kim (2011) had reported the production of a homopolymer of PHB with a dry cell weight of 32 g/L and a PHB content of 78 wt% with fed-batch fermentation of *C. necator* KCTC2662 using soybean oil as its sole carbon source. Meanwhile, Ng et al. (2010) produced a total biomass of 65.2 g/L and PHB accumulation of 76 wt% from the fed-batch fermentation of *C. necator* H16 with jatropha oil as its sole carbon source. The fermentation was conducted in a 10 L fermentor for 48 h with 2 g/L of urea and initial jatropha oil concentration of 20 g/L.

The jatropha oil concentration was maintained at 10 g/L with 40% dissolved oxygen during the course of the fermentation.

2.4.1 PHB Metabolic Pathway

In an effort to understand the intracellular PHB production, there had been immense interest in deducing its metabolic pathway in bacterial cells. The biosynthetic mechanism of PHB production in *C. necator* H16 is known to consist of three reactions catalyzed by three different enzymes when cultivated in carbohydrates, pyruvate or acetate. The first reaction consists of the condensation of two acetylcoenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA (*phb*A). This reaction is followed by the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by a nicotinamide adenine dinucleotide phosphate (NADPH) dependent acetoacetyl–CoA reductase (*phb*B). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by PHB synthase (*phb*C) (Madison and Huisman, 1999). Figure 2.4 shows the outline of metabolic pathway in *C. necator*.

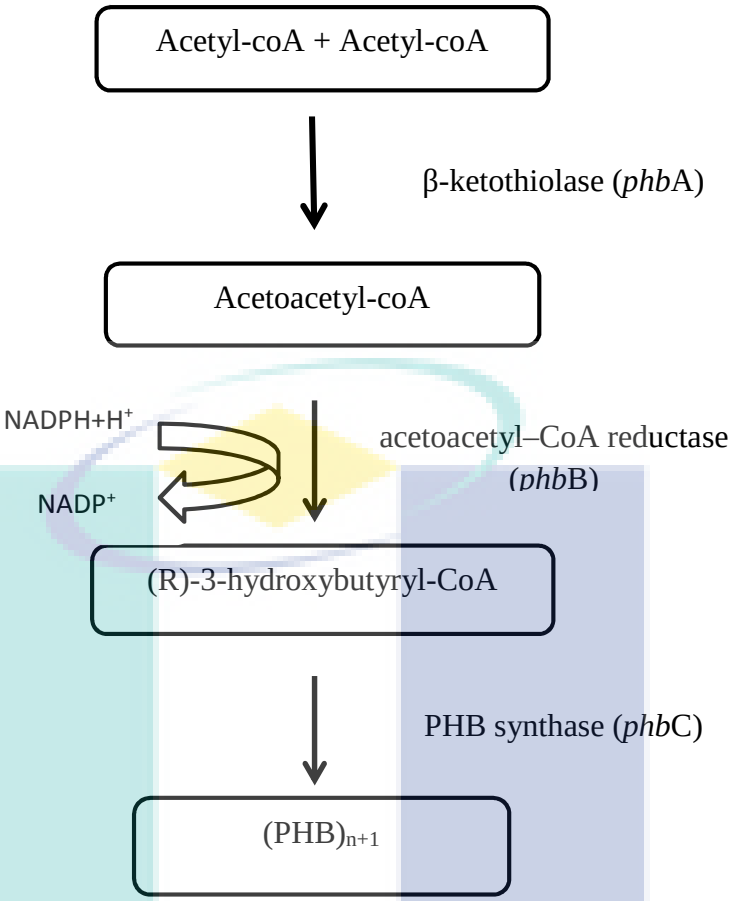

Figure 2.4: Metabolic pathway of PHB biosynthesis in *C. necator*

Source: Lee et al. (1996)

2.5 PHB PRODUCERS

Previous researches have reported several types of hosts mainly microorganisms and transgenic plants that can be utilized to produce PHA. These include natural PHA producing microorganisms, recombinant bacterial strains and also transgenic plants which will be discussed comprehensively in the following sections.

2.5.1 Natural Producers

PHAs are mainly accumulated in many microorganisms as intracellular energy storage compound due to the deficiency of one or more environmental or nutritional factors such as nitrogen, phosphorus, potassium, or oxygen in the presence of excess

carbon (Anderson and Dawes, 1990). One example of such bacteria is *Cupriavidus necator* sp. (formerly known as *Ralstonia eutropha*). It is a gram-negative bacteria that is capable of accumulating between 8 to 13 PHB granules in the cell with a diameter ranging from 0.2 to 0.5µm (López-Cuellar et al., 2010). By far, this natural PHB producing bacteria is the most comprehensively studied microorganism owing to the fact that it can accumulate up to 80% (w/w) of PHBs (Anderson and Dawes, 1990) by using various carbon source such as carbohydrates, vegetable oils and so on. Apart from exposure to limiting nutrients, *C. necator* is also known to tolerate adverse stress conditions such as heat, osmotic pressure, UV radiation and toxins like ethanol and hydrogen peroxide (Kadouri et al., 2005). Previous study by Obruca et al. (2010) had reported positive results with almost 40% increase in their PHB yield with the addition of 1% ethanol in their culture medium at the beginning of stationary phase.

Similarly, other bacterial strain like *Alcaligenes latus* sp. also has high potential for PHB production. Since *A. latus* utilizes sucrose as its carbon source, a range of sugar-based industrial by-products like molasses and cane sugar can be used as its carbon feedstock (Volova, 2004). Interestingly, this particular strain has distinctive characteristics wherein the PHB is produced during exponential growth (Hanggi, 1990) instead of other natural PHA producers which need nutrient deficit condition in order to do so. Hence, the synthesis of PHB from *A. latus* can be done in one stage (Hrabak, 1992) or in continuous mode. Nonetheless, the PHB content of this strain is somewhat low with a PHB content of about 50% from its cell dry weight (Yamane et al., 1996). Apart from PHB, *A. latus* can also synthesize PHB-co-3HV by using sucrose and 3HV precursors like valerate or propionate (Volova, 2004).

2.5.2 Recombinant Strain

Most natural producers take a long time to grow during fermentation stage and cell lysis is difficult (Suriyamongkol et al., 2007), causing considerable polymer lose during the extraction process. Besides that, the existence of degradation pathway in natural PHA producers (Reddy et al., 2003) may have also contributed to the somewhat low yield of PHA. Through genetic engineering, these drawbacks can be solved by using non-PHA producing bacteria like *Escherichia coli*, which is considered to give a

better yield of PHA due to its fast growth and easy cell lysis (Suriyamongkol et al., 2007). Recombinant *E. coli* for example, is created by introducing specific PHA biosynthesis harbouring genes from *C. necator* to induce the production of PHA (Zhang et al., 1994). However, there were a few obstacles faced in using these recombinant microorganisms because the instability of the introduced *pha*A genes reduces the yield of biopolymer (Madison and Huisman, 1999). Table 2.3 shows the comparison of PHA content produced from several types of microorganisms using different carbon substrates. From the table, it can be seen that *C. necator* produces high PHB content when vegetable oils are used as its substrate.

Table 2.3: Comparison of PHA produced by different bacterial strains and substrate

Microorganism	Carbon Source	Polymer	PHA Content (%w/v)
C. necator	Gluconate	PHB	46-85
	Propionate	PHB	26-36
	Octanoate	PHB	38-45
	Crude palm kernel oil	PHB	67
	Olive oil	PHB	80
A. latus	Sucrose	PHB	50
P. putida	Palm kernel oil	PHA	37
	Lauric acid	PHA	25
	Myristic acid	PHA	28
	Oleic acid	PHA	19
P. oleovorans	Glucanoate	PHB	1.1-5.0
	Octanoate	PHB	50-68
S. natans	Glucose	PHB	40

Source: Reddy et al. (2003)

2.5.3 Transgenic Plants

In addition to PHA biosynthesis from bacterial fermentation, studies have also been conducted on transgenic plants as hosts for PHA production. The concept of PHA production from genetically modified crops seems intriguing since only carbon dioxide and sunlight will be required as raw material for the plant growth. This approach might be more feasible because the large-scale plantation of these transgenic crops are less problematic compared to conducting large-scale bacterial fermentation in a sterile environment. Research on PHB production in plants was conducted by Poirier et al.

(1992) on *Arabidopsis thaliana* harbouring the PHA genes (*phaB* and *phaC*) of *C. necator*. It resulted in low PHB accumulation with severely reduced plant growth. Further attempts were made by expressing all the genes (*phaA*, *phaB* and *phaC*) in the chloroplast of *A. thaliana* to give PHB accumulation of up to 14% of dry weight with minimal damage to the plant growth (Nawrath et al., 1994). According to Bohlmann (2004), genetically modified oilseed as carbon source also gave promising results with 85% yield of PHB. Nevertheless, the low PHA content achieved from these transgenic plants may prove to be an obstacle in employing an efficient PHA extraction method (Budde, 2010).

2.6 CARBON SOURCE

One of the main concerns when it comes to commercializing PHA bioplastics is the high production cost when compared to other petroleum-based synthetic plastics. The non-economical price of PHA has caused consumers to still be dependent upon the much cheaper conventional plasticwares. Since the carbon source is one of the main raw material in PHA production, the right choice of carbon feedstock may significantly reduce the PHA production cost. At present, pure fructose and glucose are the main carbon substrate used by companies for large-scale PHA production. Apart from the high raw material cost, the low carbon content in the sugars gives a low yield of PHA. Thus, a potential carbon substrate would be one that is cheap and also at the same time has a high carbon content per weight. Plant oils might be a good answer to this problem since they are relatively cheap (about 0.3 USD/kg plant oil) (Kahar et al., 2004) and they also have higher carbon content per weight when compared to glucose or fructose.

In addition, previous researches by Akiyama et al. (2003) have established that the PHB yield from plant oils is almost twice higher than that from glucose. Their theoretical yield coefficients is known to be as much as over 1 g PHB per g of plant oil used compared to glucose, which only has a theoretical yield of around 0.32–0.48 g-PHB/g-glucose. However, an issue of concern would be the use of food-grade oils in bioplastic production as it may affect the global food supply and thus increasing the overall food price. The food shortage crisis that occurred in recent years was mainly caused by the depletion of agricultural lands and also the utilization of edible oils for the

production of biofuel. Thus, it is unethical to further aggravate the situation by using vegetable oils for bioplastic production. Therefore, a better alternative is by using non-edible oils like jatropha oil which is relatively cheap and at the same time does not disrupt the global food supply chain. Some options of carbon sources will be discussed further in the following subtopics.

2.6.1 Waste Materials

Alternatively, researchers have also focussed on the idea of exploiting industrial and municipal wastes for the synthesis of PHA. Wastes from the industries contain high levels of BOD and COD which can be utilized as carbon source for PHA production. Plus, this approach has an added advantage of reducing the sludge handling cost. Nevertheless, these organic wastes need to be digested under anaerobic condition to form organic acids before it can be consumed by the PHA producing microorganism and this may add up the overall cost of polymer production. Thus, proper optimization is necessary to make it into a more feasible process.

Previous researches have reported the use of various industrial wastes such as activated sludge, dairy waste, cheese whey, palm oil mill effluent (POME), molasses and so forth. In a research done by Chua et al. (2003), it was shown that PHA accumulation was higher with slight alteration of the sludge by adding acetate into the municipal wastewater to give a PHA yield of up to 30% of the sludge's dry weight. Sludge that was adapted to municipal wastewater alone produced only 20% PHA content. Studies done by Rogers and Wu (2010) suggested the use of enhanced biological phosphorus removal in activated sludge to give a yield of 50% PHB content under aerobic and anaerobic condition. In another research done by Kasemsap and Wantawin (2007), PHA content of up to 51% was obtained from an 8% polyphosphate content of sludge by using acetic acid as their only substrate. Satoh et al. (1998) proposed the use of microaerophilic condition with an end result of up to 61% of PHA content using sodium acetate as their main substrate. Cheese whey is another type of solid waste that can be used for PHA synthesis. As a by-product of dairy industry, it is considered as a pollutant due to its high content of BOD (Orhon et al., 1993). In a study

done by Pandian et al. (2009), a yield of up to 11.32 g/L of PHB dry weight was obtained by using this dairy waste as their main substrate.

Aside from the organic wastes discussed above, POME is another promising substrate that can be used in PHA biosynthesis. According to Hassan et al. (1997), PHA synthesized from POME could be produced at an approximate unit cost of 2 USD/kg with a PHA content of 50% in the dried cell and 2% dissolved in chloroform. By increasing the PHA content from 50% to 80% and PHA dissolved in chloroform from 2% to 5%, a further decline in the production cost to less than 1 USD/kg can be achieved. In a research done by Mumtaz et al. (2010), it was reported that POME as a substrate for PHA biosynthesis could give PHA yield of up to 90% (w/w) of the cell content. This crucial finding could further bring down the overall production cost of PHA from the previously mentioned 1 USD/kg. Biodiesel waste that consists mainly of crude glycerol has also shown to successfully synthesis the biopolymer. Dobroth et al. (2011) have reported the use of mixed microbial concortia (MMC) to produce PHB by using crude glycerol as their carbon source. In this study, it was observed that the MMC synthesized PHB by utilizing methanol in the crude glycerol. The highest PHB dry cell weight of 62% was obtained through this method.

On top of that, Rusendi and Sheppard (1995) have also proposed the use of potato processing wastes as substrate for bacterial fermentation in PHB production. In this study, potato starch waste was first converted to concentrated glucose solution before being used as the carbon substrate for the synthesis of PHB. A conversion efficiency of almost 96% was achieved with a final yield of 5 g/L PHB consisting of 77% of the biomass dry weight. A summary of PHA synthesis from different types of waste material is shown in Table 2.4.

Table 2.4: PHA synthesis yield from different types of waste material

Microorganism	Type of waste	Fermentation conditions	Polymer	PHA content (%)	Productivity (g PHA /L/h)	PHA yield (g/g substrate)
C. necator DSM545	Waste glycerol, Ammonium sulphate	2 L STR, fed batch	PHB	50	1.1	-
C. necator NCIMB 11599	Saccharified waste potato starch	Phosphate limitation	PHB	46	1.47	0.22
Activated sludge	Malt waste, Soya waste	SBR	PHBV	70	-	-
C. necator TF93	Fermented organic waste	Batch, pH 8 and airflow of 0.24 mol O_2/h.kg biomass	PHBV	40	-	0.16
Recombinant *E. coli*	Whey	pH-stat, fed-batch, 2.5L fermentor, 49 h	PHB	80	1.4	0.22
Activated sludge	Malt waste	Fed-batch, 70.1 h	PHB	69	0.33	
Bacillus megaterium	Date syrup/beet molasses	48 h fermentation	PHB	52	-	-
Recombinant *E. coli*	Xylose	Flask culture	PHB	35.8	-	-

Source: Mumtaz et al. (2010)

2.6.2 Plant Oil

There were several researches done in the past with regards to plant oil as feed substrate in the synthesis of PHA. Park and Kim (2011) reported the use of soybean oil and g-butyrolactone as their carbon source for PHA synthesis by *Ralstonia eutropha* KCTC 2662. A 2.5 L fermentor was used for the production of the homopolymer of PHB by using soybean oil as their carbon source to give a dry cell weight and PHA content of 15-32 g/L and 78-83% respectively. A yield of 0.80-0.82 g PHA/g soybean oil was obtained through both batch and fed-batch fermentation. The same group had used both soybean oil and g-butyrolactone for the production of the copolymer poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] with dry cell weight of 10-21 g/L and yield 0.45-0.56 g PHA/g soybean oil used. By using soybean oil as their carbon source, Kahar et al. (2004) also have established that a high dry cell weight (118-126 g/L) with a high yield of PHA (0.72-0.76 g PHA/ g soybean oil) can be obtained from the wild-type strain H16.

Aside from soybean oil, outstanding results were also achieved from palm oil as the carbon source for PHB production. In a research made by Fukui and Doi (1998), nearly 80 wt% of PHB was produced by *C. necator* H16 using palm oil as its sole carbon substrate. Studies using different strains of bacteria other than *C. necator* have also been done with promising results. Chee et al. (2010) reported utilizing *Burkholderia* sp. with crude palm kernel oil (CPKO) as its carbon substrate with a yield of almost 70 wt% PHB. *Chromobacterium* sp. USM2 was also employed to produce PHB with CPKO substrate with a 23 wt% of PHB accumulation (Bhubalan et al., 2010). PHB-co-3HV was also synthesized successfully from *C. necator* H16 using CPKO and sodium propionate as carbon substrate and precursor respectively, with 90 wt% accumulation from 7.5 g/L cell biomass (Lee et al., 2008).

Additionally, canola oil also showed a high potential as carbon source for PHA production. In a research done by López-Cuellar et al. (2010), three-stage fermentation process was applied using *C. necator* sp. After fermentation for 40 hours, a PHA content of 90 wt% of cell dry mass was obtained with PHA concentration of 18.27 g/L. As for jatropha oil, research by Ng et al. (2010) using *C. necator* H16 strain have

proven PHB accumulation of up to 87 wt% with 13.1 g/L of cell dry mass. Further studies using a 10 L lab-scale fermentor for 48 h managed to give a yield of 0.78 g PHB/ g jatropha oil. Previously, it was believed that the toxicity of jatropha oil would affect bacteria fermentation (Viswanathan et al., 2004) but the findings by Ng et al. (2010) proved otherwise because even at high amount of jatropha oil (12.5 g/L), the yield of PHB by *C. necator* H16 was not disrupted. A summary of PHA production from different carbon sources are shown in Table 2.5.

Table 2.5: Summary of PHA production from plant oil

Carbon Source	Microorganism	Polymer	Dry cell weight (g/L)	PHA content (wt%)	PHA yield (g PHA/ g carbon source)	Reference
Soybean oil	*C. necator* KCTC2662	PHA	15-32	78-83	0.80-0.82	Park and Kim (2001)
Soybean oil	*C. necator* H16	PHB	118-126	72-76	0.72-0.76	Kahar et al. (2004)
soybean oil	PHB^{-}4 / pJRDEE32d13	P(3HB-co-5 mol% 3HHx)	128-138	71-74	0.72-0.74	Kahar et al. (2004)
CPKO	*Chromobacterium* sp. USM2	PHB	3	23	-	Bhubalan et al. (2010)
SPKO	*Pseudomonas putida* PGA1	mcl-PHA	3-8.8 g/L	19-37	-	Annuar et al. (2007)
Spent cooking oil	*C. necator* H16	PHB	3.8-6.3	49-73	-	Sudesh et al. (2011)
Canola oil	*C. necator*	mcl-PHA	20.3	90	-	Lopez-Cuellar (2011)
Jatropha oil	*C. necator* H16	PHB	13.1	87	0.91	Ng et al. (2010)
PO	*C. necator* PHB^{-}4	P(3HB-co-4mol%3HHx)	3.6	81	-	Fukui and Doi (1998)
Olive oil with sodium propionate	*C. necator* H16	P(3HB-co-8mol%3HV)	6	78	-	Lee et al. (2008)
CPKO	*Burkholderia* sp.	PHB	2.2	70	-	Chee et al. (2010)

During fermentation process, *C. necator* cells make use of oleic acids (C18:1), palmitic acids (C16) and linoleic acids (C18:2) for cell growth but linolenic acids (C18:3) are poorly utilized throughout this stage (Kahar et al., 2004). The minimal content of linolenic acid and high content of oleic, linoleic and palmitic acid in jatropha oil (Salimon and Abdullah, 2008) justifies the high PHB accumulation that can be obtained from this substrate. Similarly, the low composition of linolenic acid in palm kernel oil also makes it a potential carbon source for polymer synthesis. Table 2.6 shows a comparison of fatty acid composition in jatropha oil and palm kernel oil.

Table 2.6: Comparison of fatty acid content in palm kernel oil and jatropha oil

Fatty acid composition (%)	Palm Kernel Oil[a]	Jatropha oil[b]
Saturated		
Lauric C12:0	44.2	Trace
Myristic C14:0	14.4	0.1
Palmitic C16:0	8.2	17.1
Stearic C18:0	2.5	4.3
Unsaturated		
Palmitoleic C16:1	-	1.2
Oleic C18:1	16.9	42.0
Linoleic C18:2	2.9	34.8
Linolenic C18:3	-	0.1

Source: Zazali and Irene (2005)[a] and Ng et al. (2010)[b]

2.7 PHB EXTRACTION AND PURIFICATION

Apart from the raw material cost, the cost of downstream process of PHB production also contributes to a major part in the overall PHB production cost. In order to extract PHB from the bacterial cell, the cell membrane needs to be lysed and the PHB in the cytoplasm is dissolved and separated from the residual biomass. The cell will first undergo pretreatment step where the bacterial cell will be destroyed using either alkaline or salt pretreatment process. Later on, the PHB is isolated from the cell residue through extraction process (Jacquel et al., 2008). This step is critical in the large-scale manufacturing of PHB because the cost efficiency and purity of the biopolymer

produced depends a lot on the extraction method chosen for this process. There are several methods generally used for PHA extraction mainly solvent extraction, digestion and also mechanical cell disruption.

In the solvent extraction method, the basic principle of the solubility of PHB in chloroform and its insolubility in methanol is used. Once the cells are harvested, they will be subjected to hot methanol reflux treatment where the components in the bacterial cells are washed out. Later on, soxhlet apparatus will be used with warm chloroform as its solvent to solubilise the PHB. The solubilised PHB is retrieved by precipitation through the addition of methanol (Valappil et al., 2007). This method provides a high purity PHB without degradation of PHB molecules (Ramsay et al., 1994). Nevertheless, this method is not environmentally friendly due to the large amount of hazardous solvents used and thus it would be inappropriate for large-scale production of these biopolymers.

Hypochlorite extraction is another method normally used for PHB extraction process. Although it may cause extensive degradation of polymer chains, the level of degradation depends a lot on the type of microorganism used. According to Valappil et al. (2007), the polymer degradation is more apparent in *C. necator* (75% reduction in number of average molecular weight, M_N) compared to recombinant *E. coli* with just 15% reduction in its M_N. In a research done by Ramsay et al. (1990), a more enhanced approach was proposed by using a combination of hypochlorite and surfactant method to lessen the effect of polymer degradation. Although surfactant treatment alone is efficient, it gives out a lower purity of PHA when compared to the ones produced by hypochlorite-surfactant method.

Aside from that, PHA extraction can also be done using chemical or enzymatic digestion. According to Posada et al. (2011), chemical digestion requires different chemicals to break up the carbohydrates, lipids, enzymes and protein components in the bacterial cells. These include digestion by surfactants (e.g: anionic sodium dodecyl sulphate (SDS) and synthetic palmitoyl carnitine), sodium hypochlorite and chloroform digestion, surfactant-chelate digestion and so on. Meanwhile in enzymatic digestion method, various enzymes are used to disintegrate the bacterial cell wall. Enzymes like

protease, nuclease and lysozyme are used together with detergents to destroy bacterial organelles without disrupting the PHA inside the cell (Steinbuchel, 1996). In addition to the methods discussed above, a few more PHA extraction techniques are presented in Table 2.7.

Table 2.7: Advantages and disadvantages of different PHA extraction method

Extraction Method	Advantages	Disadvantages	Results (%)
Solvent extraction	Elimination of endotoxin/high purity. No polymer degradation	Break PHA granules morphology. Hazards connected with halogenated solvents. High price/low recovery	Purity 99.5%. Recovery > 90%
Digestion by surfactants	Treatment of high cell densities.	Low purity. Waste treatment needed. Polymer degradation	Purity 95%. Release rate >90%
Digestion by sodium hypochlorite, NaOCl and chloroform	High purity. Low polymer degradation	High quantity of solvent needed	Purity >97%. Recovery 91%
Enzymatic digestion	Good recovery	High cost of enzymes	Purity 92.6 wt%. Recovery 90%
Bead mill disruption	No chemicals used	Require several passes	-
High pressure homogenization	No chemicals used	Poor disruption rate for low biomass level. Low micronization	Yield 98% Purity 95%
Supercritical carbon dioxide, CO_2	Low cost. Low toxicity	Low recovery	Recovery 89%

Source: Posada et al. (2011)

2.8 PHB ANALYSIS

In the past, gravimetric method was the most common analytical method used for PHB estimation (Lemoigne, 1926). This method was employed by extracting PHB from lyophilized cells with chloroform and later on precipitating the PHB with diethyl ether or acetone. Soon, Law and Slepecky (1961) introduced spectrophotometric method for PHB estimation by heat treatment with sulphuric acid which converts the PHB into crotonic acid. A more convenient approach was suggested by Braunegg et al.

(1978) which involves the quantification of PHB with gas chromatographic method. Lyophilized cells were treated with in mild acid or alkaline conditions and undergo methanolysis to form hydroxyalkanoate methylyesters. These methylesters were then collected and analysed by gas chromatography (GC) to obtain the corresponding peaks that quantifies the amount of PHB in samples. This method offers a high accuracy and excellent reproducibility within a short period of time of sample analysis (4 h). In another report, and increased PHB recovery with less polymer degradation was achieved by Riis and Mai (1988) by conducting PHB propanolysis in hydrochloric acid compared to acidic methanolysis in sulphuric acid as suggested by Braunegg et al. (1978).

Apart from GC, High Performance Liquid Chromatography (HPLC) is also used for quantitative determination of PHA. At first, HPLC was only used for PHB analysis by converting it into crotonic acid followed by insertion of the acid into ion exchange column to generate the required chromatograph. Continued research on this method has yielded modified approaches that could detect a combination of R-3-hydroxybutyric acid and R-3-hydroxyvaleric acid (Parry et al., 1980). Meanwhile, Karr et al. (1983) and Hesselmann et al. (1999) managed to fractionate 3-hydroxybutyric acid, 3-hydroxyvaleric acid and 3-hydroxyhexanoic acid through HPLC method. This method proves to be convenient and less time consuming since centrifuged samples could be used directly without need for lyophilization of cells.

2.9 KINETIC STUDY OF CELL GROWTH AND PHB ACCUMULATION

The kinetics of cell growth during PHB production can be determined using logistic equation. Mulchandani et al. (1989) had suggested a simplified form of the logistic equation that depicts the batch kinetics of cell growth in biopolymer synthesis. The cell growth is categorized into two phases; exponential growth ($\frac{d}{d} = \mu_m x$ with μ being constant) and stationary growth phase ($\frac{d}{d} = 0$).The logistic equation, originally proposed by Verhulst (1838) is presented in Eq. (2.1)

$$\frac{dP}{dt} = rP\left(1 - \frac{P}{K}\right) \tag{2.1}$$

where P represents the population size and K denotes the carrying capacity. The constant r defines the growth rate.

As for the kinetics of PHB accumulation, PHB synthesis in *C. necator* is known to be non-growth associated in which the production of PHB is influenced by the cell concentration and not the growth rate. Nevertheless, experimental results have proved otherwise with PHB accumulation beginning at the growth phase itself (Mulchandani et al., 1989). Hence, the PHB accumulation rate can be described using the product formation model originally proposed by Luedeking and Piret (1959) which incorporates both growth associated and non-growth associated terms (Mulchandani et al. 1989).

These kinetic models were employed by Divyashree et al. (2009) to represent the kinetics of PHA production in *Bacillus flexus*. Their model indicated that the product formation rate was linearly proportional to the biomass growth rate and the instantaneous biomass concentration. Similarly, Pirouz et al. (2011) and Qaderi et al. (2012) also employed logistic and Leudeking-Piret model for cell growth and PHB accumulation, respectively and both researches obtained good agreement between their theoretical and experimental values.

2.10 OPTIMIZATION OF PHB PRODUCTION

PHB production depends a lot on the process parameters that may influence PHB accumulation in cells. The appropriate concentration of carbon and nitrogen source along with the correct agitation speed, pH and temperature is necessary to get an optimum PHB concentration (Tripathi et al., 2013). Optimization proves to be a crucial phase in PHB synthesis because a comprehensive PHB optimization analysis would further facilitate the large-scale PHB production by increasing its productivity without affecting its overall cost. There have been several attempts in optimizing PHB synthesis by conducting one factor optimization strategy and also by using statistical methods. Although PHB optimization can be done manually by using the one-variable-at-a-time

approach, this process is known to be painstaking and time consuming (Khanna and Srivastava, 2005). Hence, experimental designs based on statistical methods such as Response Surface Methodology (RSM) comes as a handy tool that assists in finding a suitable combination of variables which gives the most optimum PHB concentration. RSM offers an adept and well-organized research strategy to study the interactions between these variables to better understand their effect on PHB production.

Previous researches have successfully attempted multivariable PHA optimization using RSM. For instance, Khanna and Shrivastava (2005) optimized the medium for the growth of *Ralstonia eutropha* NRRL B14690 and obtained a maximum of 6.65 g/L cell dry weight and 6.75 g/L PHB under optimized medium concentrations. In another research, Sharma et al. (2007) conducted nutrient optimization for PHB yield in cyanobacterium, *Nostoc muscorum* using RSM and achieved better PHB yield at reduced level of nutrients. Meanwhile, Grothe et al. (1999) had also successfully achieved higher PHB yield upon media and process parameter optimization through Response Surface Methodology (RSM).

2.11 PHB BIODEGRADATION

One of the main reasons behind the efforts in reducing petroleum based synthetic plastic is the environmental impact caused by these conventional plastics due to their inability to disintegrate naturally in the environment. PHA based biopolymers have garnered immense interest because these bioplastics can be biodegraded in both aerobic and anaerobic condition. The biodegradation of PHA under aerobic conditions produces harmless CO_2 and H_2O, whereas in anaerobic conditions, the degradation by-products are CO_2 and CH_4 (Santhanam and Sasidharan, 2010). There are a number of factors affecting PHA biodegradation such as the microbial activity of the environment, the exposed surface area, moisture, temperature, pH and molecular weight of the PHA (Boopathy, 2000). PHA based bioplastic are known to last under normal conditions of storage, and is stable indefinitely in air (Lee, 1996; Mergaert et al., 1993). The biodegradation mechanism of PHA begins with microbial enzymes such as PHA hydrolases and PHA depolymerases that are secreted to break down the PHAs into its monomeric hydroxyacids, which are utilized as a carbon source for microbial growth.

The enzyme activity differs with the variation in PHA composition and the environmental conditions (Choi et al., 2004). The degradation rate of a piece of PHB bioplastic takes only a few months in anaerobic sewage compared to several years if kept in seawater (Madison and Huisman, 1999). Meanwhile, Lee (1996) reported that P(HB-HV) were completely degraded after 6, 75 and 350 weeks in anaerobic sewage, soil and sea water, respectively. A list of PHA degrading microorganism is presented in Table 2.8.

Table 2.8: PHA degrading microorganism

Strain	Source	Class of polymer
Alcaligenes faecalis	Activated sludge	PHB
Acidovorax faecalis	Soil	P(HB-HV)
Commamonas sp.	Fresh water	PHB
Ralstonia pickettii	Soil	P(HB-HV)
Pseudomonas stutzeri	Lake water	mcl-PHA
Pseudomonas lemoignei	Activated sludge	P(HB-HV)
Pseudomonas fluorescens	Activated sludge	PHB and mcl-PHA
Aspergillus fumigatus	Soil	PHB

Source: Davis (2008)

2.12 SUMMARY

A survey of the literature suggests that there are some issues that have not been thoroughly explored in regards of PHB production. Although there are findings on PHB production from jatropha oil, the influence of different fermentation parameters and the kinetics involved during the bacterial fermentation still remain unclear. Likewise, the effect of stress factor on PHB fermentation from jatropha oil are also unknown. Therefore, the present study aims at investigating these aspects for a clearer perspective of the biosynthesis of PHB from jatropha oil.

CHAPTER 3

MATERIALS AND METHOD

3.1 INTRODUCTION

In this chapter the methodology of the research as well as the materials used will be explained in detail. These include the medium required for bacterial fermentation, cell lyophilization and also analytical procedures.

3.2 INOCULATION AND CULTIVATION METHOD

C. necator H16 was pre-cultivated in nutrient-rich medium consisting of 2 g/L yeast extract, 10 g/L meat extract and 10 g/L peptone (Doi et al., 1995). Ten 100 mL shake flask containing 10 mL mineral medium and 0.01 mL trace elements were inoculated with 0.4 mL of the pre-culture and incubated at 30 ^{0}C for 100 hours. The composition of mineral medium and trace element are presented in tables 3.1 and 3.2 respectively. The shake flasks, culture mediums and jatropha oil were autoclaved at 121^{0}C for 15 minutes before being used. All inoculations were done in sterile condition by using laminar flow hood.

The variable studies were conducted by manipulating the variables through one-variable-at-a-time approach. The effect of nitrogen source on cell dry weight (CDW) and PHB concentration was determined by varying urea concentration in the culture medium with jatropha oil concentration and agitation speed fixed at 12.5g/L and 200 rpm, respectively. Similarly, the influence of carbon source on CDW and PHB concentration was examined by conducting bacterial fermentation at jatropha oil concentration of 5 g/L, 12.5 g/L and 20 g/L at a fixed urea concentration and agitation

speed of 1 g/L and 200 rpm, respectively. Likewise, the agitation speed effect on CDW and PHB concentration was studied by conducting experiment at various agitation speed, mainly 100 rpm, 200 rpm and 250 rpm, at a fixed urea concentration and oil concentration of 1 g/L and 12.5 g/L, respectively. The influence of ethanol as external stress factor on CDW and PHB concentration was examined with the addition of ethanol to the culture medium at various time intervals. The cultivation was conducted for 87 h at an agitation speed of 200 rpm with oil and urea concentration fixed at 12.5 g/L and 1 g/L respectively. All the experiments were conducted in duplicates to minimize the chances of errors.

At the end of the incubation period, the cells were harvested by centrifugation (8000 rpm, 10 min, 4 ^{0}C) of the medium in a pre-weighed centrifuge tube. The physical appearance of culture medium at 0 h, 30 h and 65 h is shown in Figure 3.1. Cell pellets were centrifuged twice after washing with hexane and distilled water respectively. The washed cell pellets were frozen at -20 ^{0}C for 24 h (Ng et al., 2010). These procedures were repeated for all the shake flasks and the frozen cell pellets were freeze dried and the CDW were obtained. Figure 3.2 shows the dried cells obtained after freeze drying.

Table 3.1: Culture medium composition

Mineral	Per litre
Potassium dihydrogen phosphate, KH_2PO_4	1.5 g
Disodium phosphate dodecahydrate $Na_2HPO_4.12H_2O$	9 g
Magnesium sulfate heptahydrate $MgSO.7H_2O$	0.2 g
Urea, $CO(NH_2)_2$	1 g
Trace element	1 mL
Jatropha oil	12.5 g

Source: Park and Kim (2011)

Table 3.2: Trace element composition

Mineral	Per litre
Boric acid, H_3BO_3	0.3 g
Cobalt(II) chloride hexahydrate, $CoCl_2 \cdot 6H_2O$	0.2 g
Zinc sulfate heptahydrate, $ZnSO_4 \cdot 7H_2O$	0.1 g
Manganese(II) cloride tetrahydrate $MnCl_2 \cdot 4H_2O$	30 mg
Sodium molybdate dihydrate, $Na_2MoO_4 \cdot 2H_2O$	30 mg
Nickel(II) chloride hexahydrate, $NiCl_2 \cdot 6H_2O$	20 mg
Copper(II) sulfate pentahydrate, $CuSO_4 \cdot 5H_2O$	10 mg

Source: López-Cuellar et al. (2010)

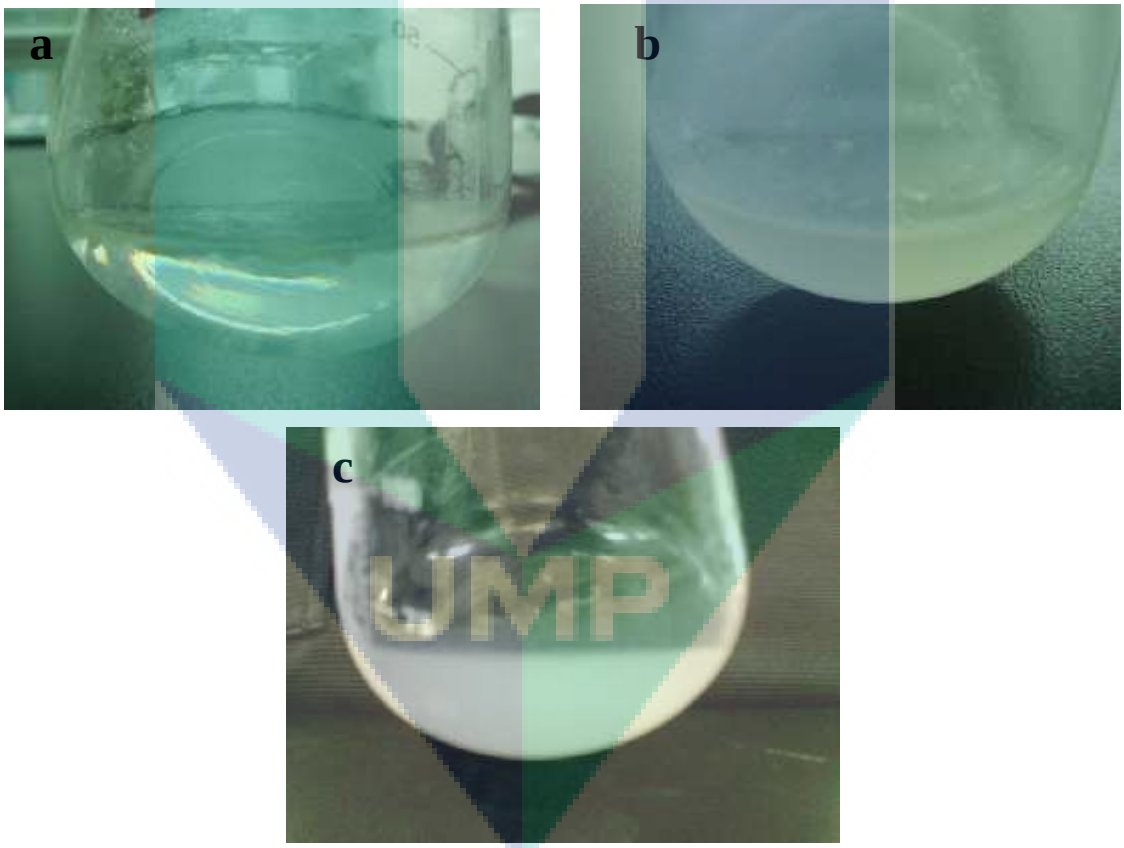

Figure 3.1: The physical appearance of culture medium at (a) 0 h, (b) 30 h, and (c) 65 h respectively

Figure 3.2: Dried cells obtained after freeze drying

3.3 Analytical Procedures

The PHB concentration was determined with slight modifications from the method suggested by Braunegg et al. (1978). Braunegg et al. (1978) performed 3.5 hours of methanolysis reaction with 2 mL of acidified methanol and 2 mL of chloroform. Preliminary studies using this method resulted in the presence of other peaks in the GC chromatographs which may be due to the incomplete methanolysis of PHB in the dry cells. Hence, the method proposed by Braunegg et al. (1978) was altered by adding the volume of acidified methanol to 4 mL and increasing the reaction time to 4 hours. Therefore, in this research, approximately 10 mg to 20 mg of lyophilized cells were subjected to methanolysis with 4 mL of acidified methanol (10% (v/v) sulphuric acid) and 2 mL of chloroform along with 20 mg of benzoic acid as internal standard. Reactions were conducted in a digester for 4 hours at 100 °C as shown in Figure 3.3. As soon as the reaction ends, the sample vials were immersed in cold water for 1 min to stop the reaction and anhydrous sodium sulphate were added to remove excess water. 2 mL of distilled water were added for separation of layers. Figure 3.4 shows the sample at initial stage and after the separation of layers due to the addition of distilled water. The bottom layer (chloroform) were taken and dried on anhydrous sodium sulphate. Samples were filtered using PTFE membrane filter and collected in HPLC vial. 1 µL of these 3-hydroxybutyric methyl esters (HBME) sample were injected into Gas Chromatography (GC) (6890N Series, Agilent Inc.) for analysis.

HP-Innowax column (30 m x 0.25 mm x 0.15 µm) was used for the GC analysis and the initial column temperature was set to 80 °C with a temperature ramp of 5 °C/min until a final temperature of 240 °C was reached. Helium was used as carrier gas at a flow rate of 20 mL/min. Known amounts of pure PHB was treated similarly to obtain a calibration curve and the resulting PHB concentration was calculated based on the HBME peak areas obtained from the chromatograms. The method for constructing calibration curve is discussed in detail in the following subtopic. An example of GC chromatograph obtained for PHB standard and sample are shown in Appendix A.

Figure 3.3: Sample methanolysis conducted in a digester at 100 °C for 4 h

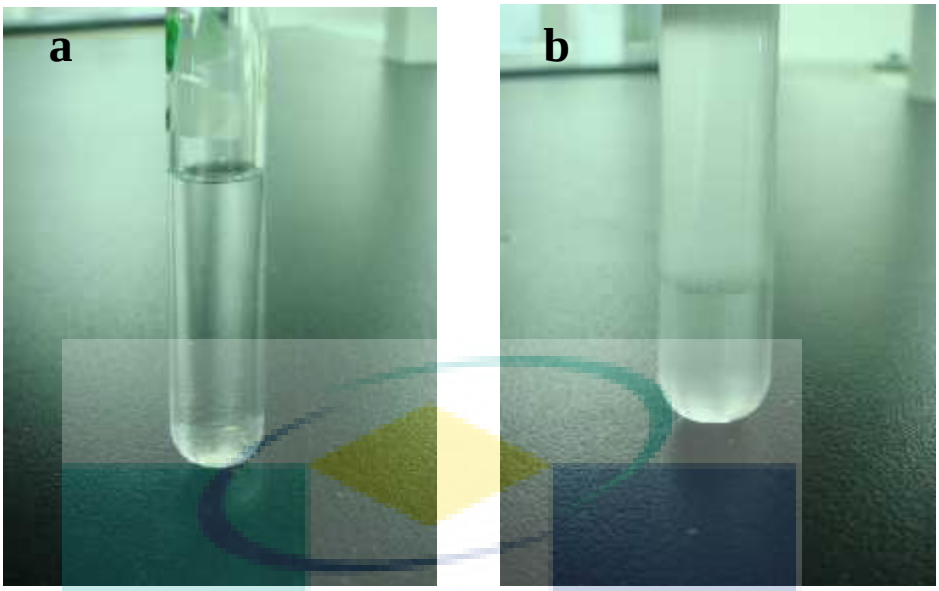

Figure 3.4: (a) Appearance of sample before methanolysis and, (b) after separation of layers

3.3.1 Calibration Curve Construction

The GC results for PHB have to be compared with calibration curve to obtain the required PHB concentration in samples. To construct the calibration curve, known amount of standard PHB samples were methanolysed using the same method as mentioned above and the resulting HBME were analysed in a GC. The area obtained for each sample was tabulated and presented in Appendix A. A plot of area versus pure PHB concentration was constructed using Microsoft Excel 2007 software and a trendline was generated to fit all the points as presented in Figure 3.5. The equation obtained was used to calculate the PHB concentration in samples. The calibration curve was constructed regularly to ensure a high accuracy of the GC results.

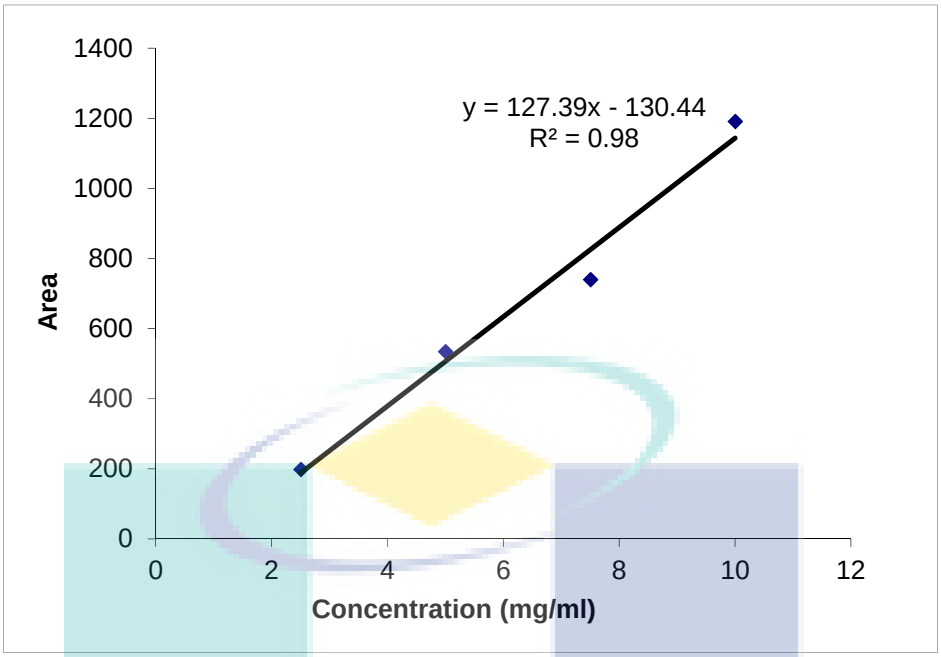

Figure 3.5: Calibration curve for PHB concentration

3.4 KINETIC STUDY

3.4.1 Cell Growth Rate

Logistic equation, a substrate-independent model was used to represent the rate of cell growth by determining the inhibition effect on cell growth. This model depicts the microbial growth in a nutrient-limited environment instead of the inhibitory effects of product accumulation (Wachenheim et al., 2003). The originally proposed logistic equation (Eq. 2.1) can be applied for bacterial growth as described in Eq. (3.2)

$$\frac{d}{d} = \mu_m x(1 - \frac{x}{x_m}) \qquad (3.2)$$

where μ_m is the maximum specific growth rate (h^{-1}) and x_m is the maximum cell concentration (g/L). The integrated form of Eq. (3.2) gives the value of x as a function of t. Integration from x_o to x and t_0 to t yields Eq. (3.3)

$$x = \frac{x_0 e^{\mu_m t}}{(1-(\frac{x_0}{x_m})(1-e^{\mu_m t})} \qquad t \leq t_m \qquad (3.3)$$

Rearrangement of Eq. (3.3) gives

$$ln\left(\frac{x}{x_m - x}\right) = \mu_m t - \ln(\frac{x_m}{x_0} - 1) \qquad (3.4)$$

3.4.2 PHB Production Rate

Since PHB synthesis in *C. necator* occurs during both exponential and stationary phase, Mulchandani et. al, (1989) had suggested the use of Leudeking-Piret model to represent the PHB production rate associated with both the growth and non-growth stage. The model, originally proposed by Luedeking and Piret (1959) can be described as follows:

$$\frac{d}{d} = \alpha \frac{d}{d} + \beta \qquad (3.5)$$

Whereby α and β are the growth and non-growth associated constant, respectively. Substituting Eq. (3.2) and (3.3) into (3.5) and integrating will yield Eq. (3.6)

$$P = P_0 + \alpha x_0 \left[\frac{e^{\mu_m t}}{1-(x_0/x_m)(1-e^{\mu_m t})} - 1\right] + \beta \frac{x_m}{\mu_m} \ln\left[1 - \frac{x_0}{x_m}(1 - e^{\mu_m t})\right] \qquad (3.6)$$

Since PHB production starts mainly at exponential phase, the initial PHB concentration, P_0 was assumed to be negligible. Thus, Eq. (3.6) can be simplified further as

$$P = \alpha A(t) + \beta B(t) \qquad (3.7)$$

where

$$A(t) = x_0 \left[\frac{e^{\mu_m t}}{1-(x_0/x_m)(1-e^{\mu_m t})} - 1\right] \qquad (3.8)$$

$$B(t) = \frac{x_m}{\mu_m} \ln\left[1 - \frac{x_0}{x_m}(1 - e^{\mu_m t})\right] \quad (3.9)$$

At stationary phase, $\frac{d}{d}$ = 0 and $x = x_m$. Therefore the value of β can be obtained from Eq. (3.5). The value of α can be obtained from the linear plot of [P - $\beta B(t)$] against $A(t)$.

3.5 OPTIMIZATION OF PHB PRODUCTION BY RESPONSE SURFACE METHODOLOGY (RSM)

A second order Central Composite Design (CCD) with three variables (jatropha oil concentration, urea concentration, agitation speed) was used to demonstrate the interaction between variables to give optimum CDW and PHB concentration. Experiments were conducted in 20 runs and RSM was employed on the experimental data using Design Expert 7.0.0 software (Stat-Ease Inc., Minneapolis, USA). Table 3.3 presents the range of actual and coded value for each process variable. The alpha value, as determined by the software, signifies the outlier for each process variable. Model validation was done by using the point prediction feature from the same software.

Table 3.3: Range of experimental values for each process variable

Factor	Name	Units	Low Actual	High Actual	Low Coded	High Coded	-alpha	+alpha
A	Urea concentration	g/L	0.50	1.50	-1.00	1.00	0.16	1.84
B	Oil Concentration	g/L	12.50	30.00	-1.00	1.00	6.53	35.97
C	Agitation speed	rpm	200	300	-1.00	1.00	165.91	334.09

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this chapter, a comprehensive review of the results obtained from the experiments was provided. This chapter was divided into several parts discussing the different variables involved that could influence cell growth and PHB accumulation. The optimization of PHB was conducted using Response Surface Methodology (RSM). The kinetic model were also be evaluated to determine the fitness of the experimental data with the theoretical values.

4.2 INFLUENCE OF DIFFERENT VARIABLES ON CELL GROWTH AND PHB ACCUMULATION

4.2.1 Effect of Urea Concentration

As mentioned in Chapter 2, the production of PHB is triggered by the limiting nitrogen concentration in the mineral medium. However, nitrogen is also one of the essential nutrients that is required to achieve high cell growth during bacterial fermentation. Thus, adequate amount of nitrogen has to be present to achieve rapid cell growth in the early stage and also simultaneously induce high rate of PHB production at later stage. Previous research done by Ng et al. (2010) reported the highest PHB concentration when 0.54 g/L of urea was used. Subsequent increase in the urea concentration had caused decline in the PHB concentration.

The effect of urea concentration on CDW and PHB concentration are presented in figures 4.1 to 4.3. In-depth experimental results are given in Appendix B. At urea concentration of 0.5 g/L (Figure 4.1), highest PHB concentration of 8.4 g/L was

achieved at 75.5 h. A maximum PHB yield of 0.673 g PHB/g oil was achieved at this stage (Table 4.1). Meanwhile, by increasing the urea concentration to 1 g/L, the highest PHB concentration of 8.6 g/L was achieved earlier at 61.5 h (Figure 4.2). The highest PHB yield achieved was 0.686 g PHB/g oil (Table 4.2). The cell growth showed a typical pattern with exponential phase of up to 55 h followed by stationary phase. Prolonged incubation time showed decrease in both CDW and PHB concentration. This is probably due to the insufficient nutrient in the culture that might have prompted the cells to degrade PHB to produce more energy.

Additional increase in urea concentration to 1.5 g/L (Figure 4.3) shows a slight decrease in the highest CDW achieved (10.5 g/L at 52.5 h) compared to the ones obtained from urea concentration of 0.5 g/L (12.1 g/L at 70.5 h) and 1 g/L (11.6 at 55 h). Nevertheless, the PHB concentration decreased significantly to a maximum of 6.5 g/L at 70.5 h with PHB content of 64.6% (Figure 4.3) and yield of 0.521 g PHB/g oil (Table 4.3). The reduced PHB concentration indicates that higher urea concentration inhibits PHB production. Higher nitrogen concentration may have facilitated the cells to undergo tri-carboxylic acid cycle (TCA) to generate more energy and thus reducing the availability of acetyl-coA, which is the main precursor in generating the mechanism for PHB synthesis (Doi, 1990; Patnaik, 2006).

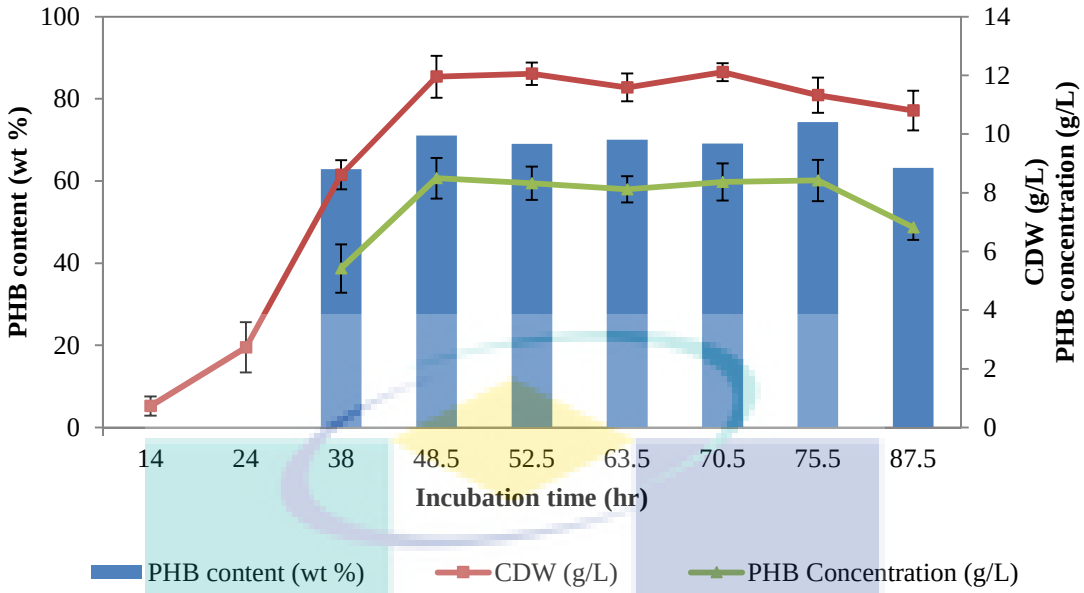

Figure 4.1: Time course of CDW, PHB concentration and PHB content at 0.5 g/L urea

Table 4.1: Summary of data for effect of 0.5g/L urea with time. All results were means of duplicate

Incubation time (hr)	Average CDW (g/L)	Average PHB concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)
14	0.730			
24	2.731			
38	8.610	5.417	62.912	0.433
48.5	11.950	8.494	71.076	0.679
52.5	12.052	8.321	69.046	0.666
63.5	11.587	8.113	70.018	0.649
70.5	12.109	8.369	69.118	0.670
75.5	11.322	8.418	74.356	0.673
87.5	10.800	6.823	63.172	0.546

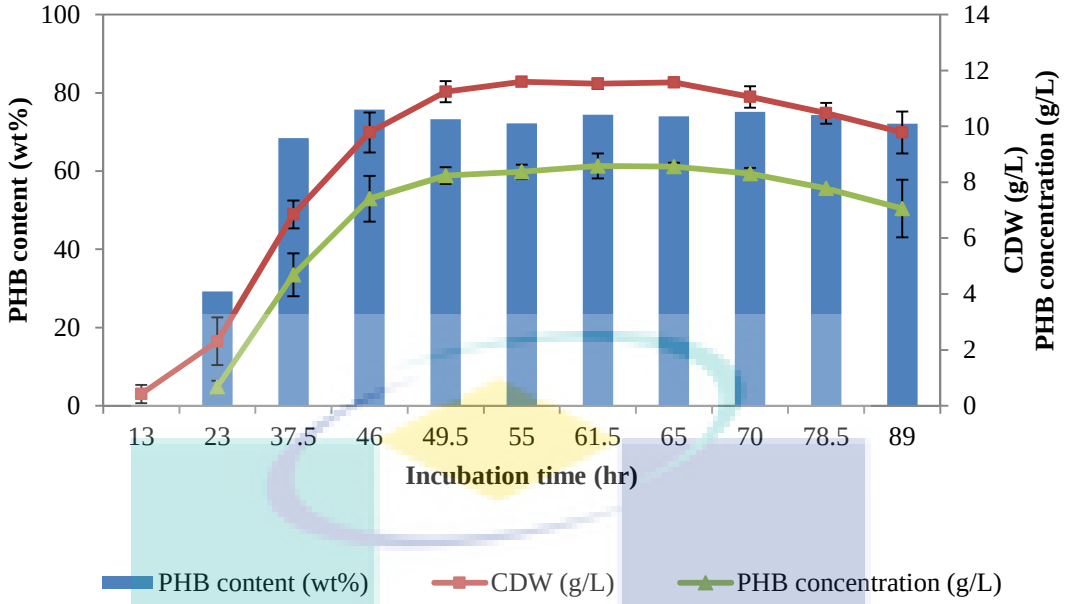

Figure 4.2: Time course of CDW, PHB concentration and PHB content at 1 g/L urea

Table 4.2: Summary of data for effect of 1 g/L urea with time. All results were means of duplicate

Incubation time (hr)	Average CDW (g/L)	Average PHB concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)
13	0.422			
23	2.309	0.675	29.234	0.054
37.5	6.851	4.686	68.393	0.375
46	9.781	7.409	75.753	0.593
49.5	11.246	8.238	73.252	0.659
55	11.595	8.373	72.210	0.670
61.5	11.530	8.580	74.411	0.686
65	11.576	8.561	73.956	0.685
70	11.054	8.306	75.135	0.664
78.5	10.468	7.776	74.283	0.622
89	9.781	7.056	72.138	0.564

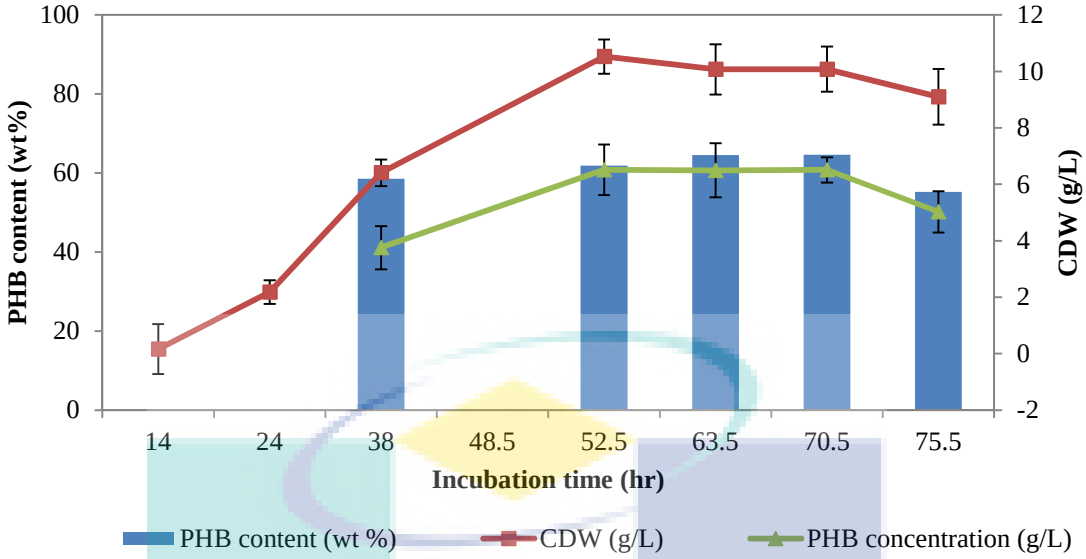

Figure 4.3: Time course of CDW, PHB concentration and PHB content at 1.5 g/L urea

Table 4.3: Summary of data for effect of 1.5 g/L urea with time. All results were means of duplicate

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
14	0.161			
24	2.181			
38	6.410	3.753	58.555	0.300
52.5	10.525	6.510	61.852	0.521
63.5	10.070	6.495	64.499	0.520
70.5	10.080	6.511	64.599	0.521
75.5	9.103	5.027	55.225	0.402

4.2.2 Effect of Jatropha Oil Concentration

The influence of jatropha oil on CDW and PHB production were investigated by varying jatropha oil concentration in the culture medium as presented in figures 4.4 and 4.5. The detailed experimental results are given in Appendix C. At jatropha oil concentration of 5 g/L, the highest CDW obtained was 3.5 g/L at 69.5 h (Figure 4.4) with a corresponding PHB concentration of 2.7 g/L and PHB content of 69.6%. The PHB yield obtained at this stage was only 0.488 g PHB/g oil as shown in Table 4.4. The low CDW and PHB concentration obtained were may be due to insufficient carbon

source which hindered the cell growth significantly. An increase in jatropha oil concentration to 12.5 g/L (refer Figure 4.2) shows considerable increase in both the maximum CDW (11.6 g/L at 55 h) and PHB concentration (8.6 g/L at 65 h). Nevertheless, Ng et al. (2010) had obtained better results with similar jatropha oil concentration. They reported the highest CDW of 13.1 g/L and PHB concentration of 11.4 g/L when 12.5 g/L jatropha oil and 0.54 g/L urea was used at an agitation speed of 200 rpm. Meanwhile, when the jatropha oil concentration was increased to 20 g/L, higher CDW and PHB concentration of 20.1 g/L and 15.5 g/L respectively was observed (Figure 4.5). A significant increase in its PHB content and yield was observed with 83.6% PHB content achieved at 61 h with a corresponding PHB yield of 0.776 g PHB/g oil (Table 4.5). Increasing the jatropha oil concentration to 30 g/L caused decrease in both the CDW and PHB concentration (data not shown).

The results obtained contradict with the ones reported by Ng et al. (2010) who observed a decrease in their CDW and PHB concentration when the oil concentration was increased beyond 12.5 g/L. The conflicting results were probably attributed by the different mineral medium used in both cases. To the best of our knowledge, there are no findings relating the choice and composition of mineral medium to the cell growth and PHB accumulation in *C. necator*. Nonetheless, there are several researches that have proved the importance of regulating and optimizing the mineral medium composition in achieving high cell density in bacterial fermentation of various methylotrophic bacterial strains (Suzuki et al., 1986; Daniel et al., 1992; Bourque et al., 1995). Thus, it might be possible that the choice and composition of mineral medium in our study may have resulted in a higher cell and PHB yield.

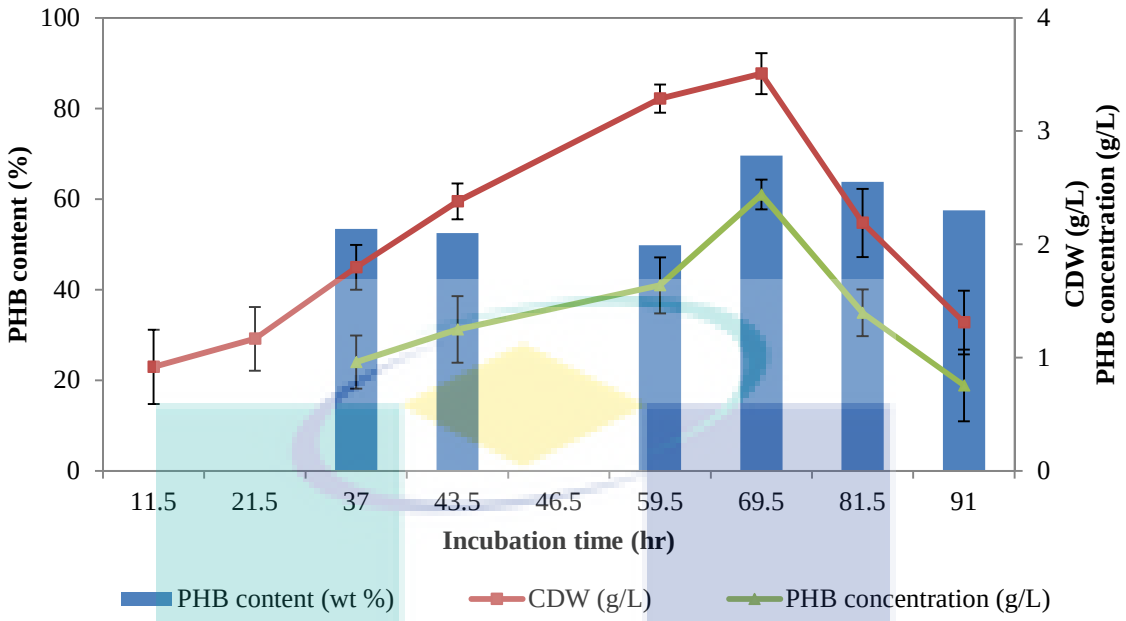

Figure 4.4: Time course of CDW, PHB concentration and PHB content at 5 g/L jatropha oil

Table 4.4: Summary of data for effect of 5g/L jatropha oil with time. All results were means of duplicate

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
11.5	0.917			
21.5	1.166			
37	1.797	0.960	53.412	0.192
43.5	2.380	1.249	52.490	0.250
59.5	3.287	1.638	49.811	0.326
69.5	3.507	2.440	69.556	0.488
81.5	2.188	1.396	63.776	0.279
91	1.309	0.753	57.502	0.151

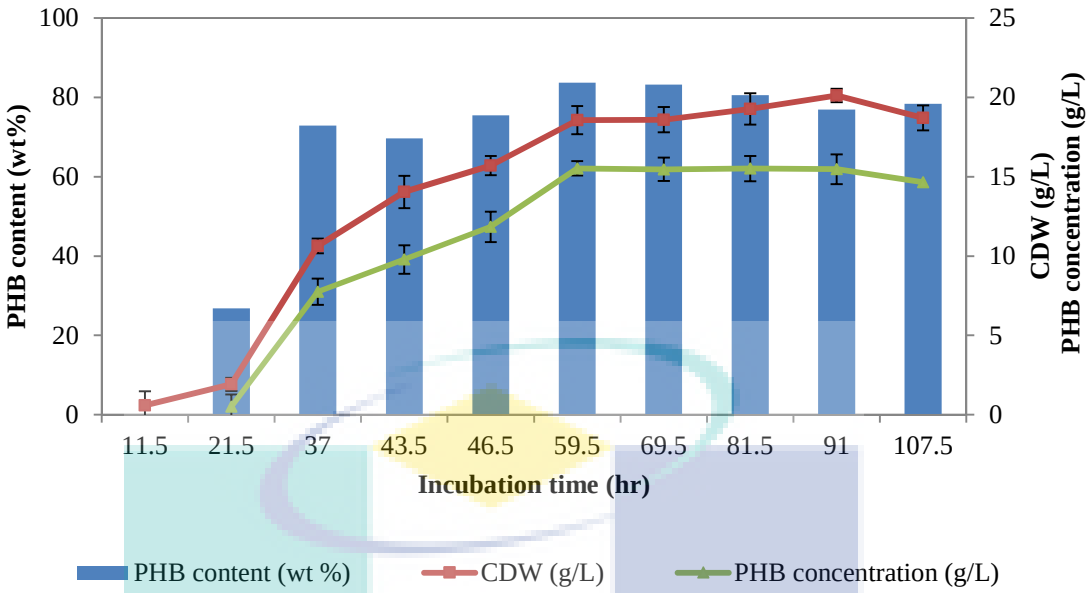

Figure 4.5: Time course of CDW, PHB concentration and PHB content at 20 g/L jatropha oil

Table 4.5: Summary of data for effect of 20g/L jatropha oil with time. All results were means of duplicate

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
13	0.589			
23	1.915	0.513	26.815	0.026
37.5	10.638	7.750	72.856	0.388
43	14.040	9.783	69.682	0.489
47.5	15.693	11.837	75.429	0.592
61	18.561	15.526	83.647	0.776
69	18.585	15.464	83.207	0.773
84	19.258	15.506	80.521	0.775
91	20.114	15.469	76.909	0.773
101.5	18.702	14.653	78.346	0.733

4.2.3 Effect of Agitation Speed

Agitation speed plays vital role in ensuring that the bacteria receives sufficient aeration to facilitate rapid cell growth. Figures 4.6 and 4.7 shows the effect of agitation speed on CDW and PHB concentration and the detailed experimental data were presented in Appendix D. Higher CDW and PHB concentration were observed in both 200 rpm (refer Figure 4.2) and 250 rpm (Figure 4.7) flasks when compared to 100 rpm (Figure 4.6) flasks. In the meantime, culture medium agitated at 250 rpm showed increase in PHB content and yield when compared to 100 rpm flasks with the highest PHB content of 73.4% (Figure 4.7) and PHB yield of 0.714 g PHB/g oil (Table 4.7) at 83.5 h. At 100 rpm of agitation speed, the PHB yield obtained was very low at 0.182 g PHB/g oil (Table 4.6).

Slower agitation may have caused the cells not to be dispersed well into the medium and thus causing the medium to be more heterogeneous (Zahari et al., 2012). The increase in aeration might have enabled more oxygen to be consumed by the bacterial cells and thus enabling rapid cell growth. Nevertheless, agitation speed that is too high may decrease the PHB accumulation in cells since the increase in shear stress during fermentation may reduce the bacterial growth (Raghul, 2012).

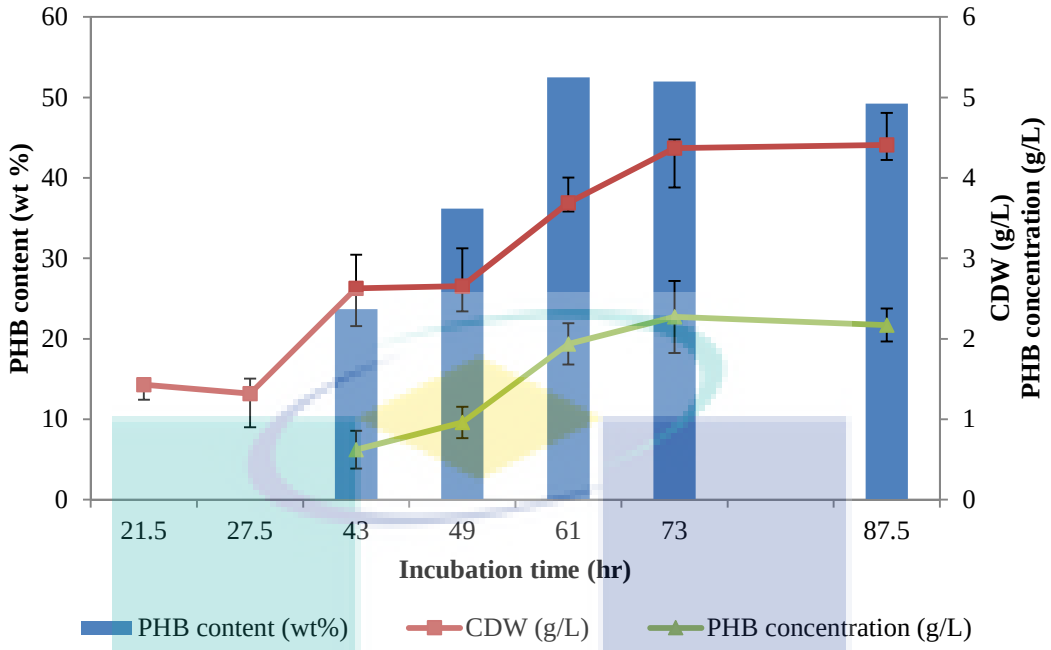

Figure 4.6: Time course of CDW, PHB concentration and PHB content at 100 rpm agitation speed

Table 4.6: Summary of data for effect of 100 rpm agitation speed with time. All results were means of duplicate

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
21.5	1.432			
27.5	1.318			
43	2.627	0.622	23.685	0.050
49	2.655	0.960	36.161	0.077
61	3.689	1.935	52.471	0.155
73	4.371	2.271	51.958	0.182
87.5	4.409	2.171	49.240	0.174

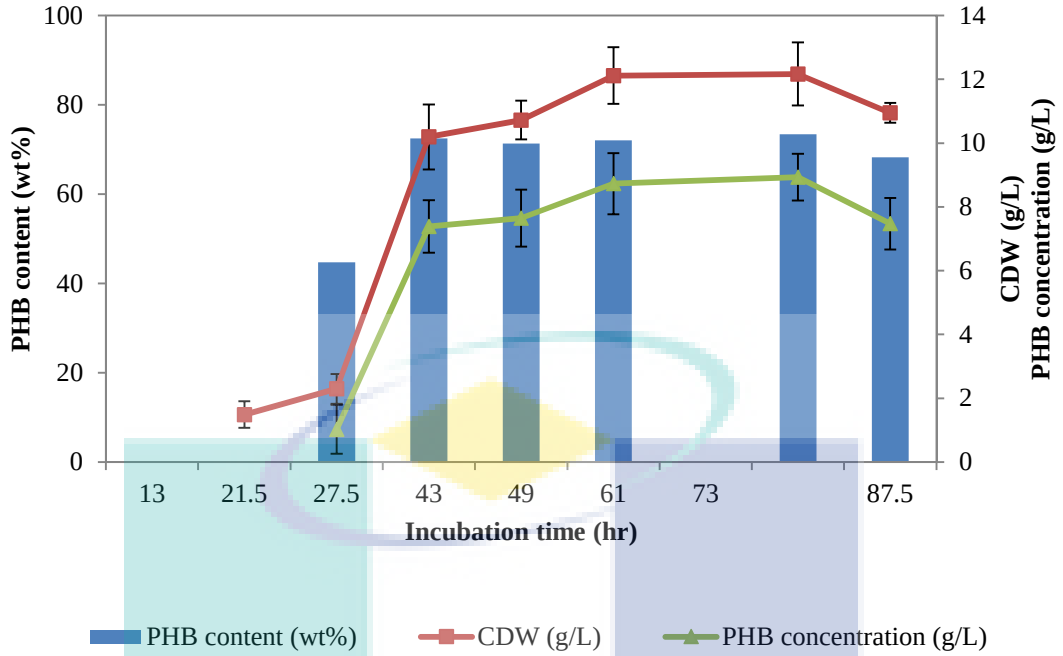

Figure 4.7: Time course of CDW, PHB concentration and PHB content at 250 rpm agitation speed

Table 4.7: Summary of data for effect of 250 rpm agitation speed with time. All results were means of duplicate

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)
21.5	1.489			
27.5	2.295	1.027	44.757	0.082
43	10.193	7.391	72.504	0.591
49	10.724	7.649	71.320	0.612
61	12.118	8.730	72.037	0.698
83.5	12.166	8.931	73.412	0.714
87.5	10.952	7.474	68.246	0.598

4.2.4 Effect of Ethanol Addition

In the present study, ethanol was used instead of methanol since Obruca et al.(2010) had reported a lower PHB concentration when methanol was used as external stress factor. Therefore, the effect of ethanol addition on the bacterial growth was investigated by adding different concentration of ethanol at specified time intervals and

the results were presented in Figure 4.8. Detailed experimental results were given in Appendix E. From Figure 4.8, it was observed that the PHB yield increased to 0.75 g PHB/g jatropha oil when 1.5 % (v/v) of ethanol was added at 38 h compared to the control experimental data which only produced a yield of 0.48 g PHB/g jatropha oil. Further increase in ethanol addition time reduced the PHB content (data not shown).

Since our aforementioned results showed high PHB accumulation from using 20 g/L jatropha oil and agitation speed of 250 rpm, the experiment was carried on to observe the influence of ethanol addition on bacterial fermentation with 20 g/L jatropha oil at 250 rpm. The results, as portrayed in Figure 4.8, showed a substantial increase in PHB production when 1.5% (v/v) of ethanol was added at 38 h with a yield of up to 0.987 g PHB/g jatropha oil. The fermentation was ended at 68 h in this case because continuation of incubation time lead to degradation of PHB. The summary of data for this experiment is tabulated in Table 4.8. The PHB yield achieved in this study was higher than the ones reported by Ng et al. (2010) who obtained a yield of 0.911 g PHB/g jatropha oil. The high yield of PHB obtained in this research might prove to be beneficial for large-scale PHB production since this approach can be easily applied and most importantly, the inexpensive ethanol would only contribute a small part in the overall cost of PHB production.

Although the relation between ethanol and PHB accumulation in cells remain unknown, Obruca et al. (2010) deduced that the addition of ethanol activated alcohol dehydrogenase and the ethanol metabolism produces a final product of acetyl-coA along with reduced coenzymes NADPH. The NADPH inhibited the tri-carboxylic acid (TCA) cycle which in turn prompted more acetyl-coA into the PHB biosynthetic pathway. The inhibition of TCA cycle also reduces the formation of free coA. This in turn facilitated the free coA inhibited enzyme β-ketothiolase in PHB biosynthetic pathway to synthesize more PHB.

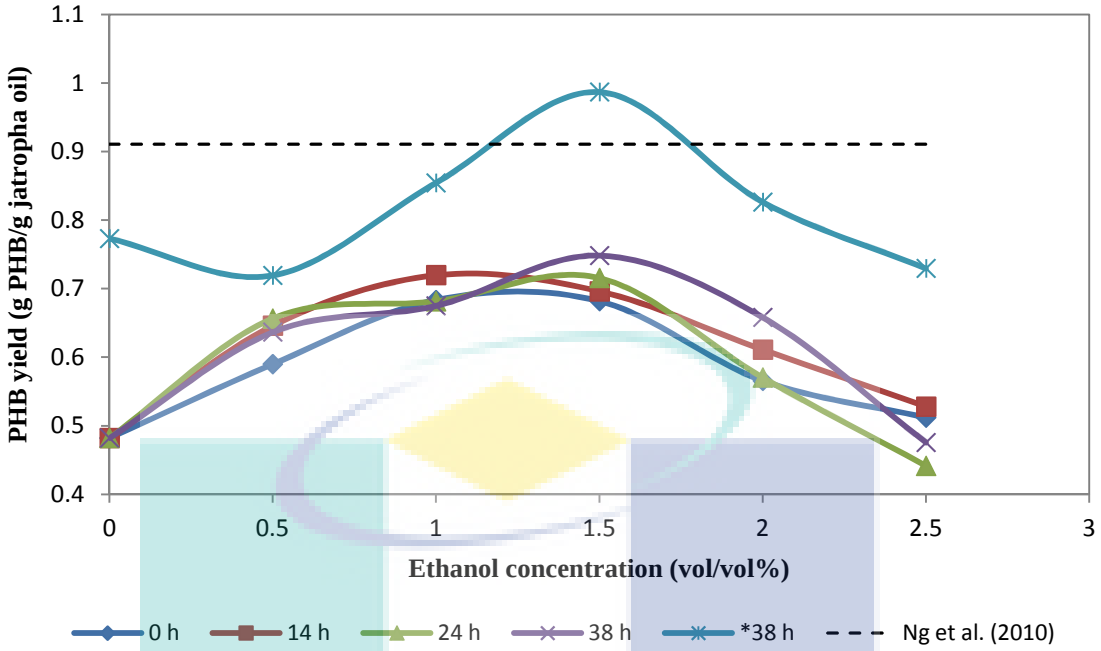

Figure 4.8: Effect of ethanol addition at various time intervals. Oil and urea concentration was fixed at 12.5 g/L and 1 g/L respectively. The cultivation was conducted in 100 mL shake flasks, and incubated at 30 ^{0}C at 200 rpm for 87 h. All results were means of duplicate.

*38 h – Effect of ethanol addition at 38hr with oil and urea concentration fixed at 20g/L and 1g/L respectively. The cultivation was conducted in 100 mL shake flasks, and incubated at 30 ^{0}C at 250 rpm for 68 h. Results were means of duplicate.

Ng et al. (2010) – PHB yield from fermentation of *C. necator* with 12.5 g/L of jatropha oil and 0.54 g/L of urea.

Table 4.8: Summary of data for effect of ethanol addition at 38 h with oil and urea concentration fixed at 20 g/L and 1 g/L respectively. The cultivation was conducted in 100 mL shake flasks, and incubated at 30 ^{0}C at 250 rpm for 68 h. Results were means of duplicate

Ethanol addition time (hr)	***38**			
Ethanol concentration (v/v%)	**Total CDW (g/L)**	**PHB concentration (g/L)**	**PHB Content (%)**	**Yield (g PHB/g oil)**
0	18.921	15.513	81.988	0.776
0.5	16.834	14.387	85.461	0.719
1	19.873	17.089	85.987	0.854
1.5	21.543	19.743	91.647	0.987
2	19.013	16.528	86.932	0.826
2.5	16.293	14.590	89.547	0.730

4.3 KINETIC STUDY

Based on the results obtained, the kinetic model for microbial fermentation using 12.5 g/L of jatropha oil and 1 g/L of urea was determined by using Eq. (3.2) to (3.7). The linear plot of Eq. (3.4) as shown in Figure 4.9, gives the values of μ_m and x_0. Substituting these values along with x_m obtained from the experimental data into Eq. (3.3), yielded the theoretical CDW as depicted in Fig. 4.11. From the calculated constants, the values for A(t) and B(t) in Eq. (3.7) can be calculated to give the theoretical PHB concentration values (Appendix F). The slope of the linear plot of P – βB(t) vs A(t) (Figure 4.10) will then give the α value in Eq. (3.7). As shown in Figure 4.11, the experimental data matches well with the kinetic model at exponential phase and early stationary phase. However, the experimental values deviate slightly towards the end of stationary phase because the logistic equation used does not portray the decrease in cell density that normally occurs at the end of stationary phase (Wachenheim et al., 2003). Similarly, the kinetic model for PHB concentration agrees well with the experimental data. Nevertheless, at stationary phase, the model tends to underestimate the PHB concentration obtained. The α value (0.6814 g/g) obtained was considerably higher than β (0.001768 g g^{-1} h^{-1}) which indicates that the PHB production mostly confirms to the growth associated kinetic pattern with low rate of PHB production during non-growth stage. The summary of the kinetic data and its parameters

for microbial cell growth and PHB formation is given in Table 4.9 and Table 4.10 respectively.

The kinetic model appears to overestimate the PHB production at the end of stationary phase. This is probably due to PHB degradation at death phase that might have caused the PHB concentration to decline in our experimental data. It has to be noted that the kinetic parameters may vary with change in fermentation condition such as agitation, pH and substrate concentration. Thus, the kinetic model for different experimental condition should be calculated individually.

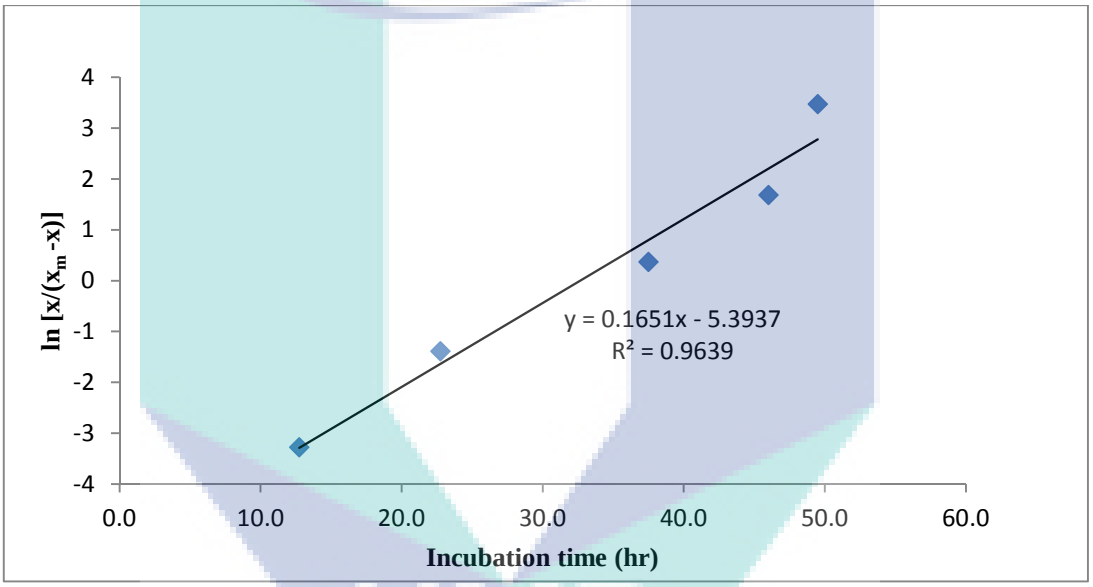

Figure 4.9: Linear plot of Eq. (3.4)

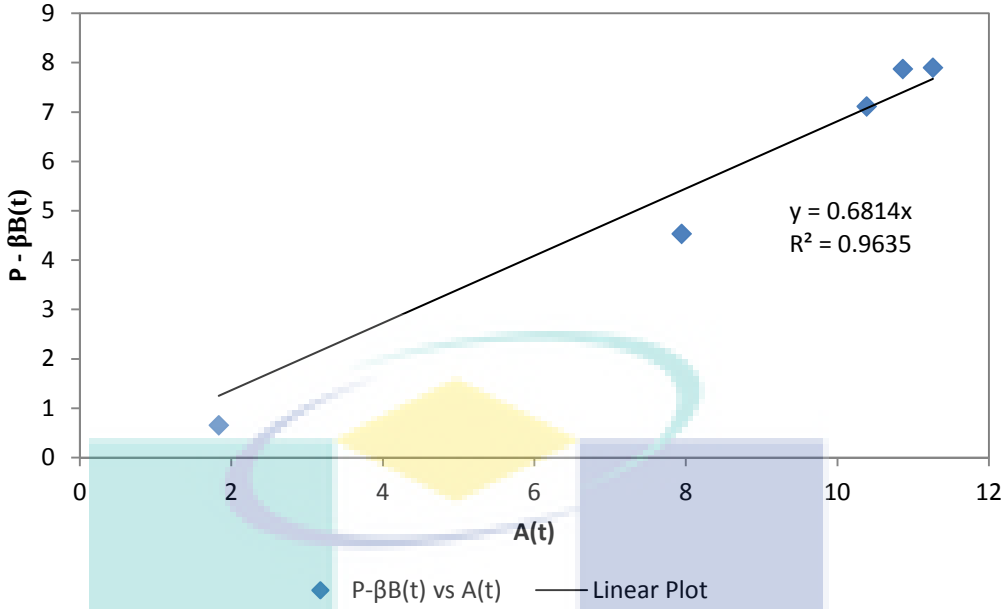

Figure 4.10: Linear plot of P – βB(t) against A(t)

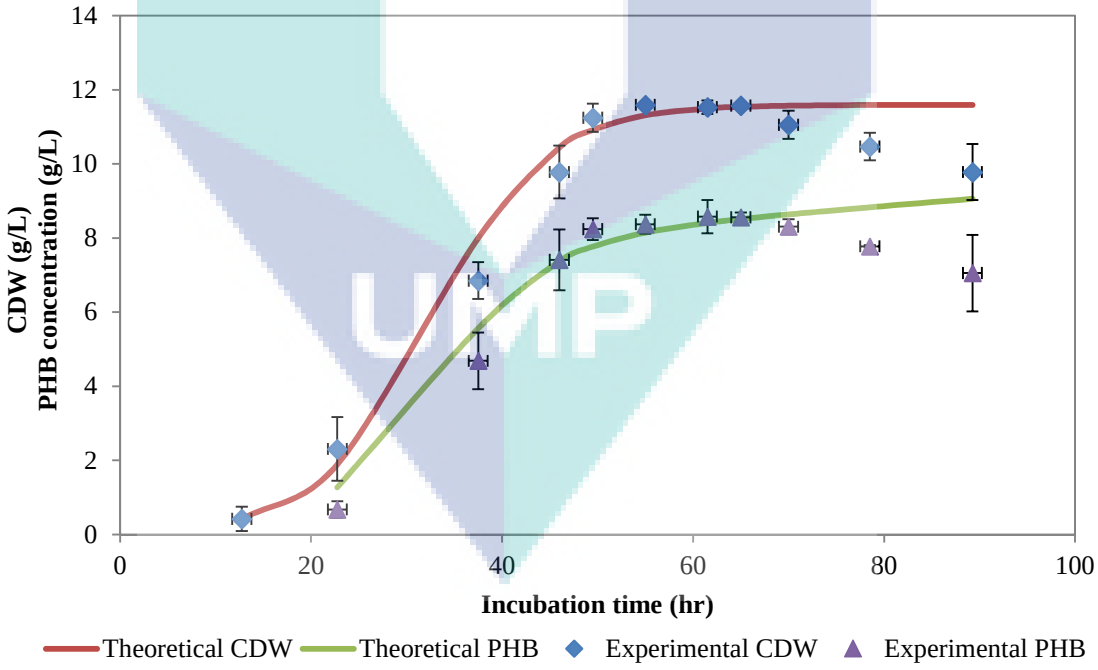

Figure 4.11: Kinetic data for microbial cell dry weight and PHB concentration. Oil and urea concentration was fixed at 12.5 g/L and 1 g/L respectively. The cultivation was conducted in 100 mL shake flasks, and incubated at 30 ^{0}C at 200 rpm for 90 h. All results were means of duplicate.

Table 4.9: Summary of kinetic data for microbial cell dry weight and PHB concentration

Incubation time (hr)	Experimental CDW (g/L)	Experimental PHB concentration (g/L)	Theoretical CDW (g/L)	Theoretical PHB concentration (g/L)
0			0.053	
13	0.422		0.417	
23	2.309	0.675	1.889	1.573
37.5	6.851	4.686	7.996	4.669
46	9.781	7.409	10.440	6.666
49.5	11.246	8.238	10.917	7.664
55	11.595	8.373	11.312	7.902
61.5	11.530	8.580	11.496	7.858
65	11.576	8.561	11.539	7.889
70	11.054	8.306	11.571	7.534
78.5	10.468	7.776	11.589	7.134
89	9.781	7.056	11.594	6.666

Table 4.10: Summary of kinetic model parameters for microbial cell growth and PHB formation.

Constants	Values
Cell Growth Rate	
x_m (g/L)	11.59486
μ_m (1/h)	0.1651
x_0 (g/L)	0.0525
PHB Formation Rate	
α (g/g)	0.6814
β (g/g h)	0.001768

4.4 OPTIMIZATION OF PHB PRODUCTION BY RESPONSE SURFACE METHODOLOGY (RSM)

The studies on the combined effect of these variables, mainly oil concentration, urea concentration and agitation speed were conducted to obtain the optimum condition for CDW and PHB concentration. The range of oil and urea concentration selected was between 5 g/L to 30 g/L and 0.5 g/L to 1.5 g/L respectively. Meanwhile, agitation speed were in the range of 100 rpm to 300 rpm. The values for predicted and actual responses for CDW and PHB are tabulated in Table 4.11.

Table 4.11: Experimental designs with consequent actual and predicted values for CDW and PHB concentration

Standard order	A: urea concentration (g/L)	B: oil concentration (g/L)	C: agitation speed (rpm)	Actual CDW (g/L)	Predicted CDW (g/L)	Actual PHB concentration (g/L)	Predicted PHB concentration (g/L)
1	0.5	12.5	200	10.59	9.21	8.11	7.68
2	1.5	12.5	200	9.07	8.43	6.09	5.35
3	0.5	30	200	14.65	14.13	10.19	9.52
4	1.5	30	200	15.95	15.30	10.60	9.96
5	0.5	12.5	300	11.02	10.17	7.13	7.14
6	1.5	12.5	300	10.69	9.71	3.26	3.29
7	0.5	30	300	14.83	13.97	10.98	11.08
8	1.5	30	300	15.59	15.46	10.19	9.99
9	0.16	21.25	250	12.19	13.62	9.22	9.50
10	1.84	21.25	250	13.52	14.22	6.01	6.63
11	1	6.53	250	4.52	6.08	2.72	3.09
12	1	35.97	250	14.50	15.06	9.74	10.27

Table 4.11: Continued

Standard order	A: urea concentration (g/L)	B: oil concentration (g/L)	C: agitation speed (rpm)	Actual CDW (g/L)	Predicted CDW (g/L)	Actual PHB concentration (g/L)	Predicted PHB concentration (g/L)
13	1	21.25	165.91	11.43	12.60	9.89	11.06
14	1	21.25	334.09	12.59	13.55	10.90	10.64
15	1	21.25	250	21.09	20.35	17.03	17.32
16	1	21.25	250	19.94	20.35	17.20	17.32
17	1	21.25	250	18.84	20.35	16.95	17.32
18	1	21.25	250	21.25	20.35	18.03	17.32
19	1	21.25	250	19.50	20.35	17.84	17.32
20	1	21.25	250	21.86	20.35	17.05	17.32

4.4.1 Statistical Analysis

The analysis of variance (ANOVA) for both CDW and PHB concentration were presented in Table 4.12 and 4.13 respectively. The mean squares were obtained by dividing the sum of squares of each of the variable terms, the model and the error variance, by their respective degrees of freedom. P-values indicate the significance of each model terms. Model terms with P-value less than 0.05 are considered as significant (Guo, et al., 2009). The influence of each variable on the CDW was identified by considering their respective P-values. The linear model term B and quadratic model terms (A^2, B^2, and C^2) were significant with $P < 0.05$ with the other model terms (A, C, AB, AC, BC) being insignificant ($P > 0.05$). As for PHB concentration, model terms A, B, AB, A^2, B^2, and C^2 proved to be significant with $P < 0.05$ with the rest being insignificant.

The high F-value of 21.84 for CDW model and 98.06 for PHB model proves that both models were adequate in describing the response. In addition, the lack of fit value of 1.93 and 3.71 for CDW and PHB respectively, implies that the lack of fit is insignificant relative to pure error. The coefficient of determination, R^2 for CDW was 0.9516 which indicated that 95.16% of the variability in the response can be expressed by the model. The same goes to PHB concentration model which showed an R^2 value of 0.9888 suggesting a good agreement between predicted values and experimental data in this model. A more reliable predicted value will be achieved when the R^2 value is closer to unity. Both models also had reasonable agreement between its Predicted R squared value and Adjusted R squared value.

Table 4.12: Analysis of variance (ANOVA) results for CDW response surface quadratic model.

Source	Sum of Squares	Df	Mean Square	F value	p-value Prob > F	Singnificance
Model	389.81	9	43.31	21.84	< 0.0001	Significant
A	0.44	1	0.44	0.22	0.6487	
B	97.22	1	97.22	49.02	< 0.0001	
C	1.07	1	1.07	0.54	0.4787	
AB	1.89	1	1.89	0.95	0.3515	
AC	0.052	1	0.052	0.026	0.8740	
BC	0.62	1	0.62	0.31	0.5872	
A^2	74.48	1	74.48	37.56	< 0.0001	
B^2	172.31	1	172.31	86.89	< 0.0001	
C^2	95.40	1	95.40	48.11	< 0.0001	
Residual	10.83	10	1.98			
Lack of Fit	13.06	5	2.61	1.93	0.2441	Not significant
Pure Error	6.77	5	1.35			
R squared	0.9516					
Adj-R squared	0.9080					

Table 4.13: Analysis of variance (ANOVA) results for PHB response surface quadratic model.

Source	Sum of Squares	Df	Mean Square	F value	p-value Prob > F	Significance
Model	445.93	9	49.55	98.06	< 0.0001	Significant
A	9.94	1	9.94	19.67	0.0013	
B	62.25	1	62.25	123.20	< 0.0001	
C	0.22	1	0.22	0.43	0.5279	
AB	3.81	1	3.81	7.54	0.0206	
AC	1.17	1	1.17	2.31	0.1597	
BC	2.20	1	2.20	4.35	0.0637	
A^2	154.32	1	154.32	305.40	< 0.0001	
B^2	203.97	1	203.97	403.67	< 0.0001	
C^2	75.44	1	75.44	149.30	< 0.0001	
Residual	5.05	10	0.51			
Lack of Fit	3.98	5	0.80	3.71	0.0883	Not significant
Pure error	1.07	5	0.21			
R squared	0.9888					
Adj-R squared	0.9787					

4.4.2 Development Of Regression Model Equation

The regression model equations were developed in terms of coded factors and the results for both CDW (Y_1) and PHB (Y_2) concentration model were fitted into second order polynomial equation as presented in Eq. (4.1) and (4.2) respectively. Coded terms A, B and C represent urea concentration, oil concentration and agitation speed respectively.

$$Y_1 = 20.35 + 0.18A + 2.67B + 0.28C + 0.49A \quad + 0.081A \quad - 0.28B \quad - 2.27A^2 - 3.46B^2 - 2.57C^2 \tag{4.1}$$

$$Y_2 = 17.32 - 0.85A + 2.14B - 0.13C + 0.69A \quad - 0.38A \quad + 0.52B \quad - 3.27A^2 - 3.76B^2 - 2.29C^2 \tag{4.2}$$

The fitted polynomial equations were displayed as 3D surface plot to distinguish the interaction between each variable that results in a particular response and also to determine the optimum level of the variables that gives maximum response. The elliptical curve obtained from the 3D surface plots demonstrates mutual interactions between all variables. These are obtained when there is perfect interaction between the independent variables (Muralidhar et al., 2001). Figures 4.12 – 4.17 portrays the response surface of CDW by retaining one variable at zero level and varying the other two variables within the specified experimental ranges. Figure 4.12 depicts positive interaction between oil and urea concentration with an increase in CDW up to a certain level and later on declines at higher oil and urea concentration. At an optimum oil concentration, CDW increases with an increase in urea and agitation speed (Figure 4.13). The increase in agitation and urea concentration provides sufficient aeration and nitrogen source ensuring sufficient cell growth. Continued increase in both variables shows slight decline in CDW. Meanwhile, Figure 4.14 displays the interaction between agitation speed and oil concentration and its effect on CDW. Increase in oil concentration shows profound increase in CDW. This proves that adequate amount of jatropha oil as the sole carbon source is essential for optimal cell growth.

Similar interaction can be seen for PHB when oil and urea concentration were varied at an optimum agitation speed. Since PHB production is triggered by limiting nitrogen concentration, adequate amount of nitrogen has to be present in mineral medium to achieve rapid cell growth in the early stage of fermentation. Figure 4.15 indicates increase in PHB concentration with increase in urea concentration. Soon after, the PHB concentration declines when the oil and urea concentration continues to rise. Higher urea concentration may have facilitated the cells to undergo tri-carboxylic acid cycle (TCA) metabolic pathway to generate more energy and thus reducing the availability of acetyl-coA, which is the main substrate in generating the mechanism for PHB synthesis (Du et al., 2001). This in turn reduces the PHB synthesis and causes PHB concentration to decline at a higher urea concentration. Meanwhile, high oil concentration also has negative impact on PHB accumulation in cells as shown in Figure 4.15. Excessive oil might have caused insufficient oxygen in mineral medium which inhibits cell growth and thus lowers PHB accumulation. The interaction between agitation speed and urea concentration (Figure 4.16) and agitation speed and oil concentration (Figure 4.17), exhibit similar trend with PHB concentration increasing up to a certain level when the variable values are increased. Further increase in these variables resulted in a declined PHB accumulation.

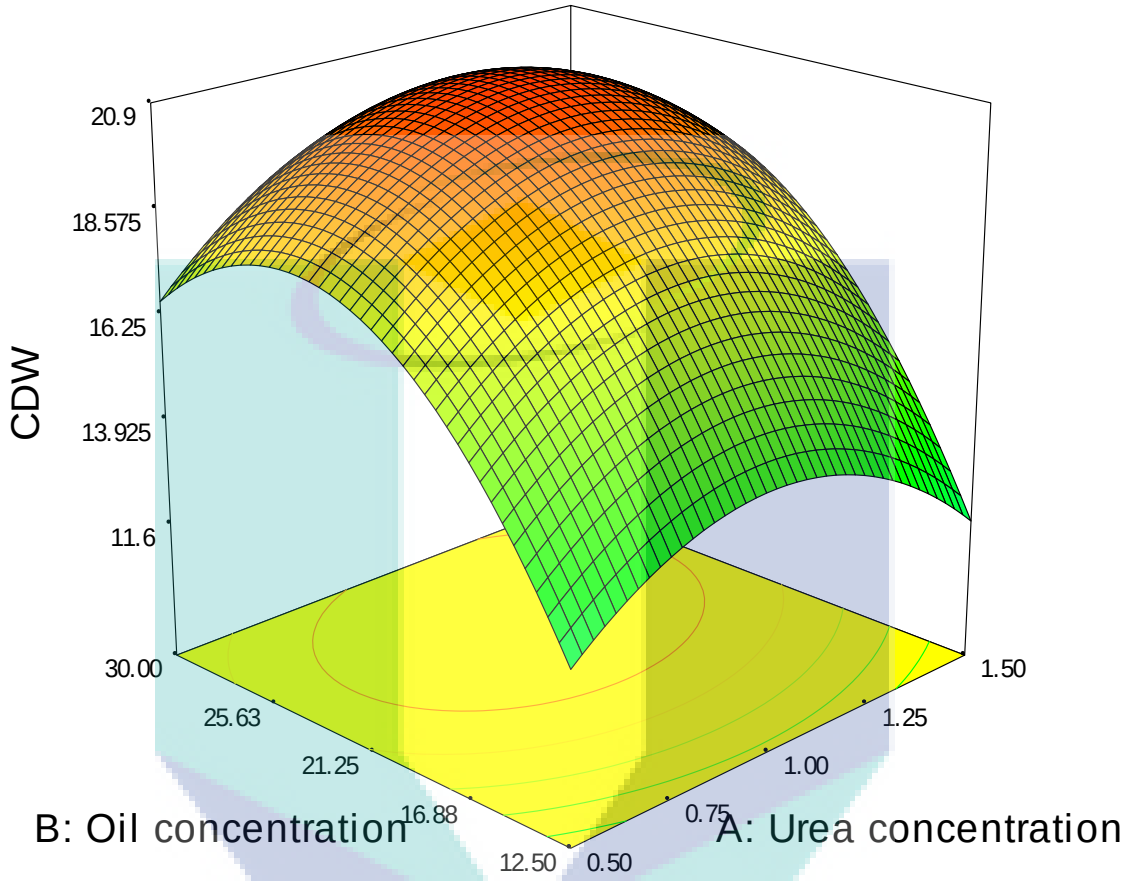

Figure 4.12: 3D response surface for CDW model. Interactive effect of varied urea (A) and oil concentration (B)

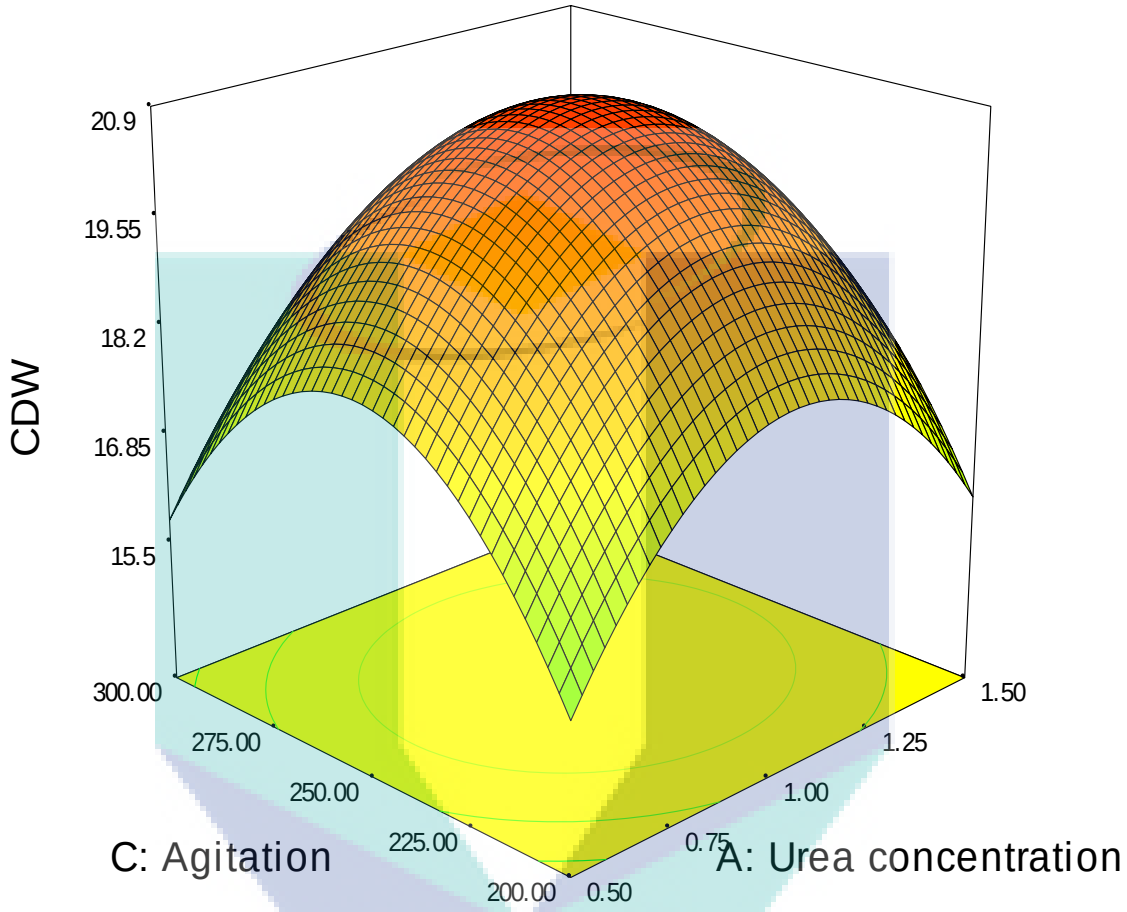

Figure 4.13: 3D response surface for CDW model. Interactive effect of varied urea (A) and agitation speed (C)

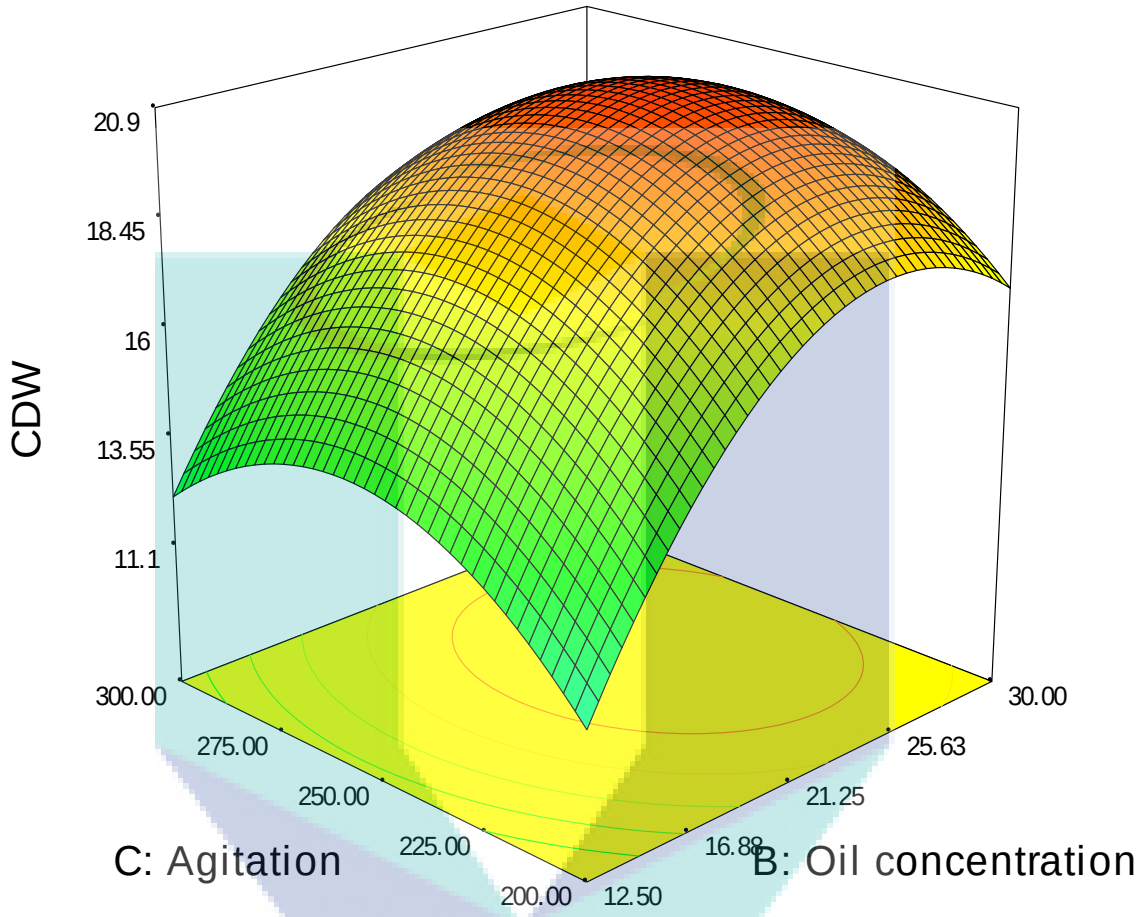

Figure 4.14: 3D response surface for CDW model. Interactive effect of varied oil (B) and agitation speed (C)

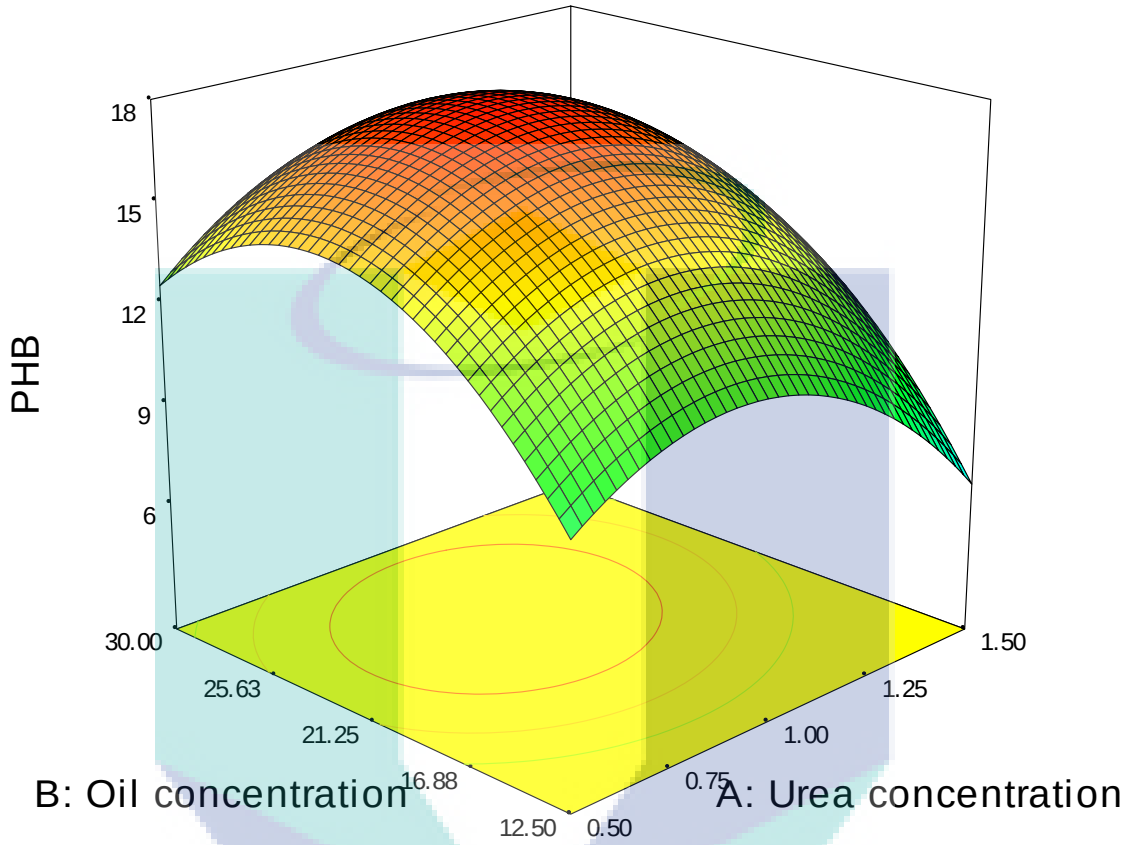

Figure 4.15: 3D response surface for PHB model. Interactive effect of varied urea (A) and oil concentration (B)

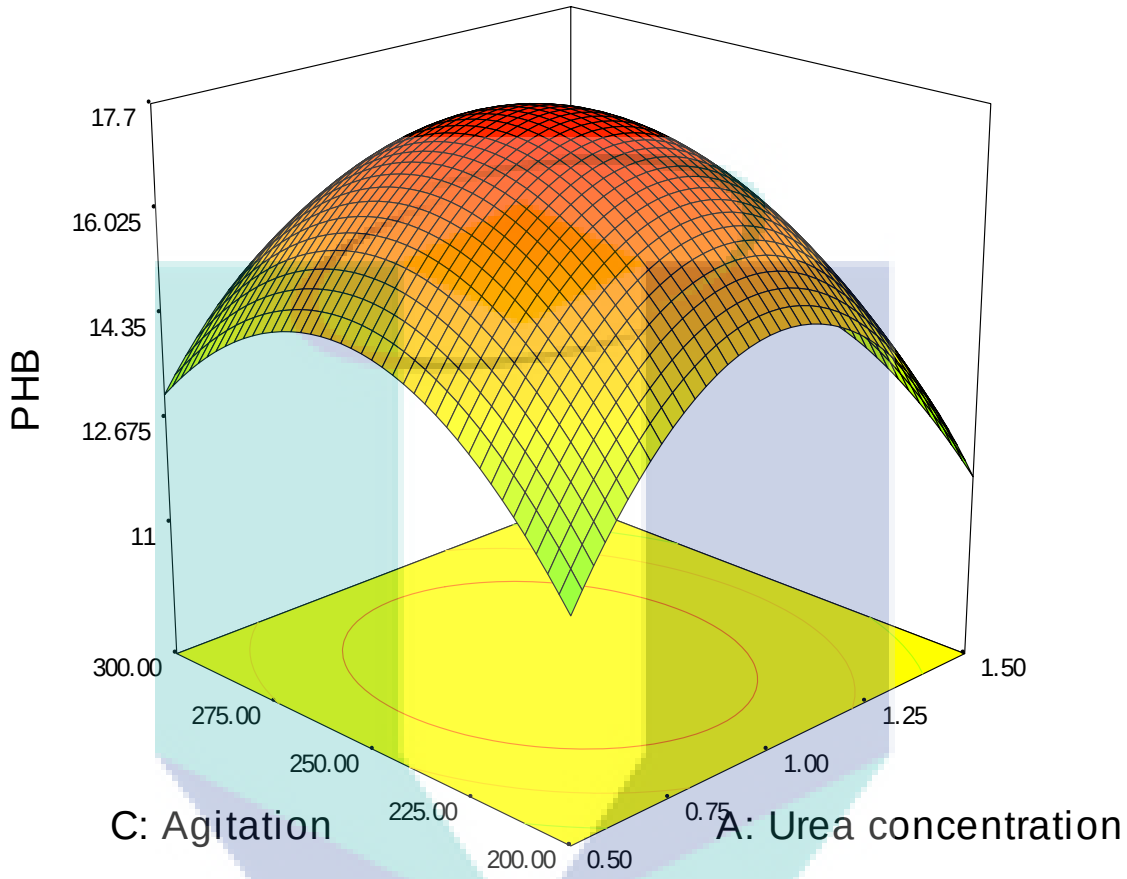

Figure 4.16: 3D response surface for PHB model. Interactive effect of varied urea concentration (A) and agitation speed (C)

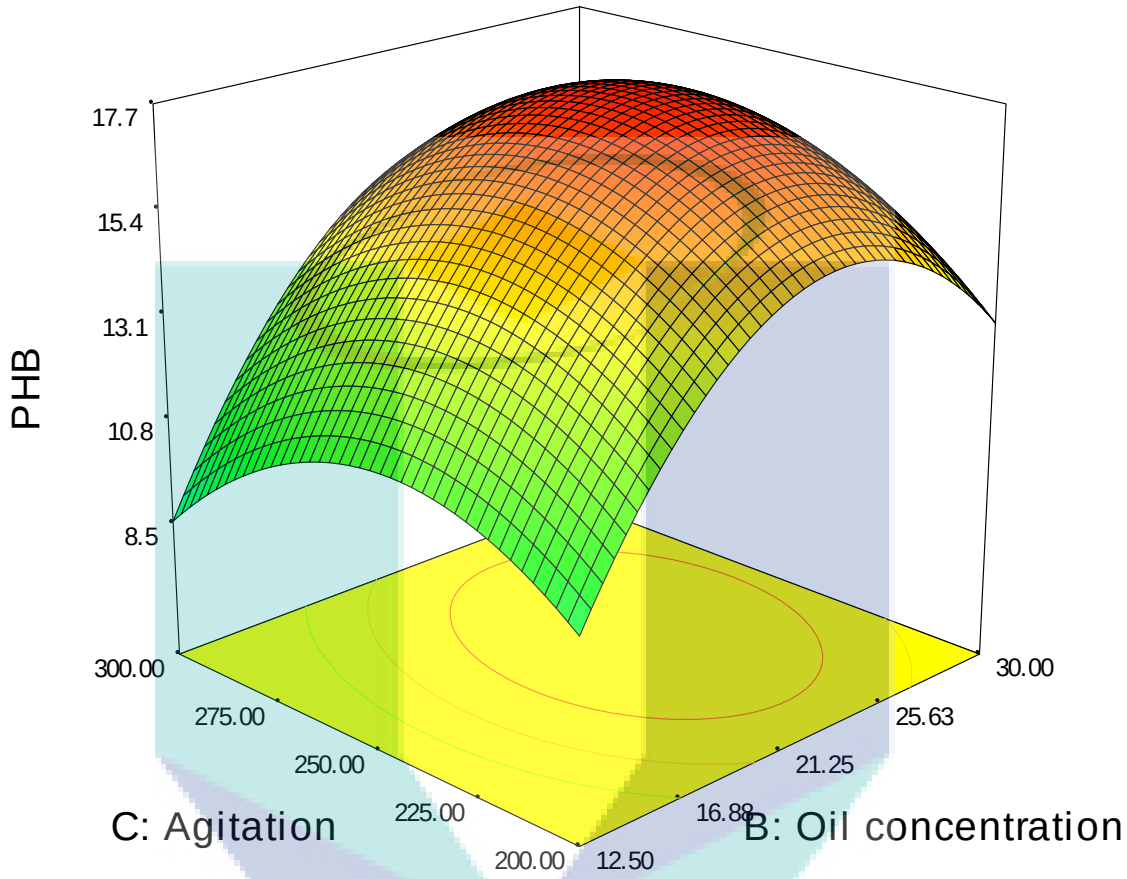

Figure 4.17: 3D response surface for PHB model. Interactive effect of varied oil (B) and agitation speed (C)

The predicted CDW and PHB concentration values obtained from the Design Expert software were plotted against the experimental data as presented in Figure 4.18 and 4.19 respectively. The plots for CDW and PHB showed close proximity between the predicted values and the experimental values. This indicate that the models developed were satisfactory in capturing the correlation between process parameter and the response.

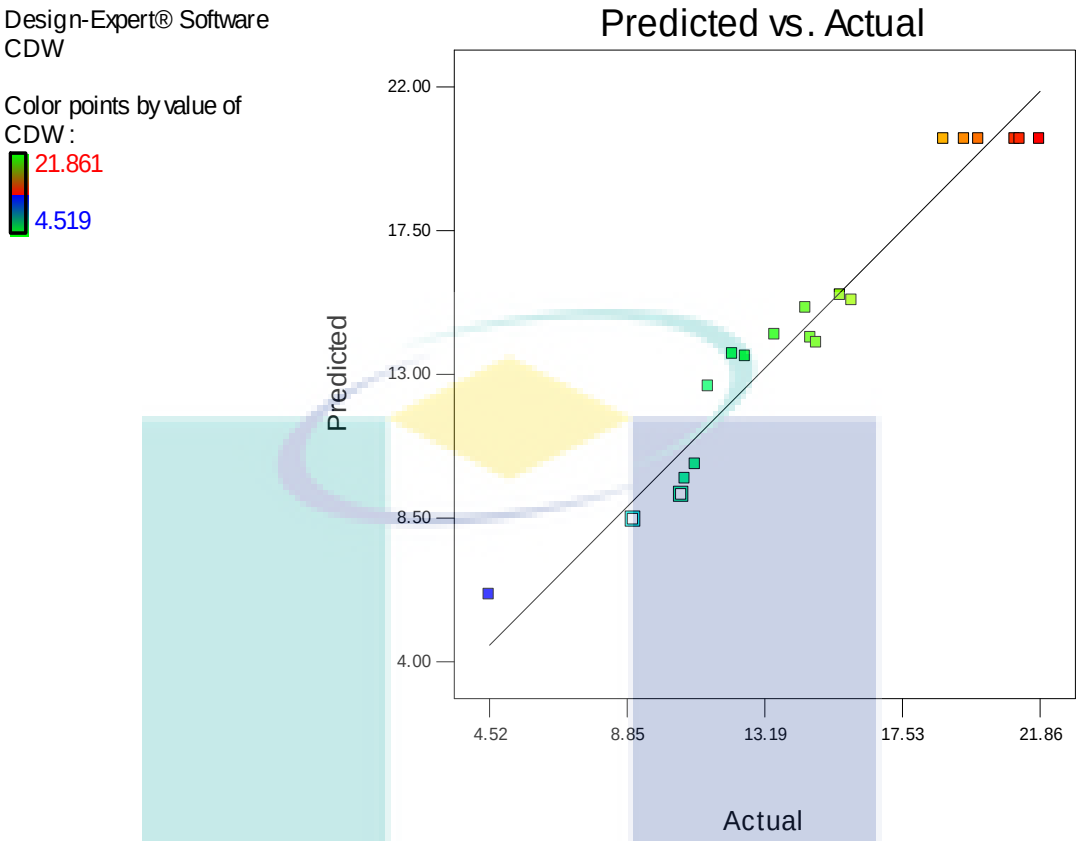

Figure 4.18: Graph of predicted values against actual values for CDW model

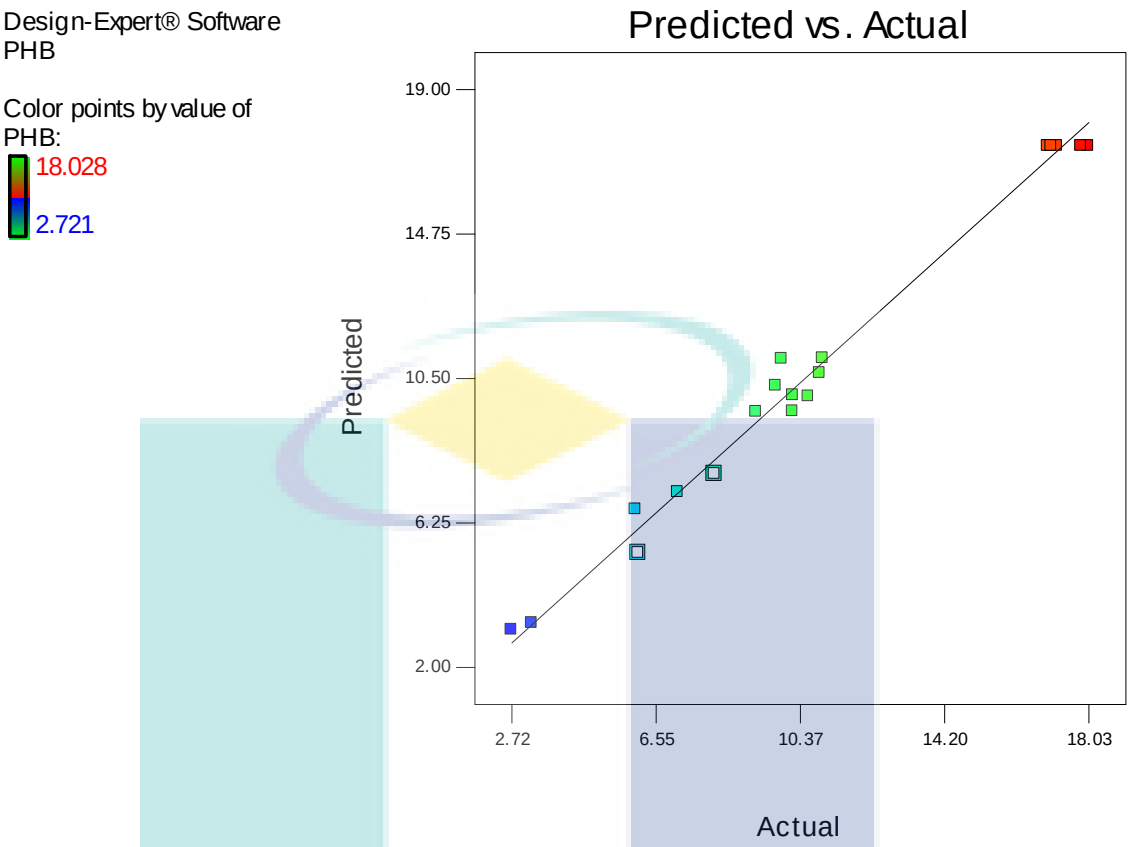

Figure 4.19: Graph of predicted values against actual values for PHB model

4.4.3 Model Validation

The 3D surface plot provided a clear picture of the different combination of variables that need to be incorporated to achieve the desired CDW and PHB. Subsequently, point optimatization technique was used to obtain the specific values of these variables to get an optimized CDW and PHB concentration. Experiments were done in triplicates and the optimum urea concentration, oil concetration and agitation speed obtained were as displayed in Table 4.14. Actual PHB concentration increased by 5% after optimization. The results confirmed that the predicted and actual values were in close proximity which suggests that the models were indeed functional.

Table 4.14: CDW and PHB concentration before and after optimization of process parameters

Variable	Before	After	Before		After			
					Predicted		Actual	
			CDW (g/L)	PHB (g/L)	CDW (g/L)	PHB (g/L)	CDW (g/L)	PHB (g/L)
Urea (g/L)	1	0.95	21.86	17.05	20.77	17.66	21.30	17.92
Oil (g/L)	21.25	23.63						
Agitation (rpm)	250	250.4						

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

In this research, the effect of various process parameters mainly jatropha oil and urea concentrations, and agitation speed were investigated to determine the optimum condition for microbial fermentation in batch culture. The present work has succeeded in obtaining the highest PHB accumulation with the following process parameters of 20 g/L jatropha oil, 1 g/L urea and agitation speed of 250 rpm respectively. At jatropha oil concentration of 20 g/L, a high CDW and PHB concentration of 20.1 g/L and 15.5 g/L respectively was observed. The results proved to be better than the ones obtained by Ng et al. (2010).

The results encouraged us to further investigate the effect of stress factor on PHB production from jatropha oil. The addition of ethanol as stress factor resulted in an increased PHB yield. An optimized fermentation condition using 1.5 % (v/v) ethanol addition at 38 h produced convincing result with a PHB yield of 0.987 g PHB/g jatropha oil. The high yield of PHB obtained by adding ethanol as stress factor might prove to be beneficial for large-scale PHB production since this approach is convenient and most importantly, the inexpensive ethanol would only contribute a small fraction in the overall cost of PHB production. The low cost of jatropha oil as raw material coupled with the high yield of PHB obtained would certainly reduce the overall PHB production cost.

The kinetic model for CDW was constructed using Logistic equation, which depicts the microbial growth in a nutrient-limited environment without taking into account the inhibitory effects of product accumulation. As for PHB accumulation,

Leudeking-Piret model was used to construct the PHB production rate which occurred at both the growth and non-growth stage of *C. necator*. The theoretical model for both CDW and PHB production rate matches well with the experimental data obtained from the research. The PHB accumulation mostly adhered to growth-associated kinetic pattern with insignificant amount of PHB produced during the non-growth stage. A well-defined kinetic model could facilitate in problem-solving during large-scale fermentation process. In addition, it could also increase the production efficiency resulting in a better PHB yield.

RSM was implemented to study the influence of key variables which include jatropha oil concentration, urea concentration and agitation speed on CDW and PHB concentration in *C. necator* H16. Based on the statistical analysis, it was found that the interaction between the variables (jatropha oil concentration and urea concentration; urea concentration and agitation speed; jatropha oil concentration and agitation speed) had mutual effect on both CDW and PHB. Cultivation at optimized condition resulted in 5% increase in PHB concentration to 17.92 g/L compared to the previously obtained PHB concentration of 17.05 g/L. The predicted and actual experimental values were in close range which demonstrates the practicality of the model.

5.2 RECOMMENDATION

To better understand the microbial synthesis of PHB, the study needs to be expanded to include mineral medium optimization for the fermentation of *C. necator* H16 with jatropha oil as its carbon source. Additionally, the characteristics of the polymer produced from the said method of PHB production needs to be examined to establish its chemical and mechanical properties. Further research on PHB degradation is also necessary to ensure the efficacy of PHB based bioplastics. Most importantly, it is highly recommended for the study to be conducted in larger scale to evaluate the feasibility of PHB production. The most reliable method for PHB recovery also needs to be determined to ensure its efficiency and cost-effectiveness.

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APPENDIX A

GC RESULT OF PHB STANDARD AND PHB SAMPLE

Table A1: Pure PHB weight and its corresponding GC results

Pure PHB Weight (mg)	Concentration (mg PHB/ml chloroform)	Area
5	2.5	198.2219
10	5	533.8388
15	7.5	739.8976
20	10	1191.16

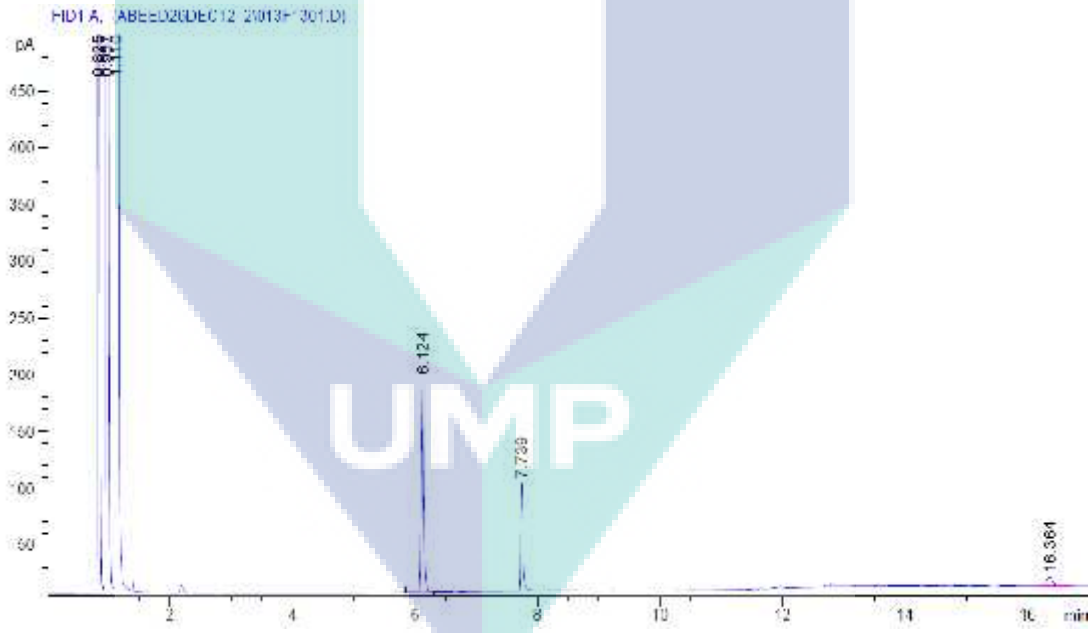

Figure A1: An example of GC result obtained for 10 mg of pure PHB. Peak for PHB was observed at 6.124 min

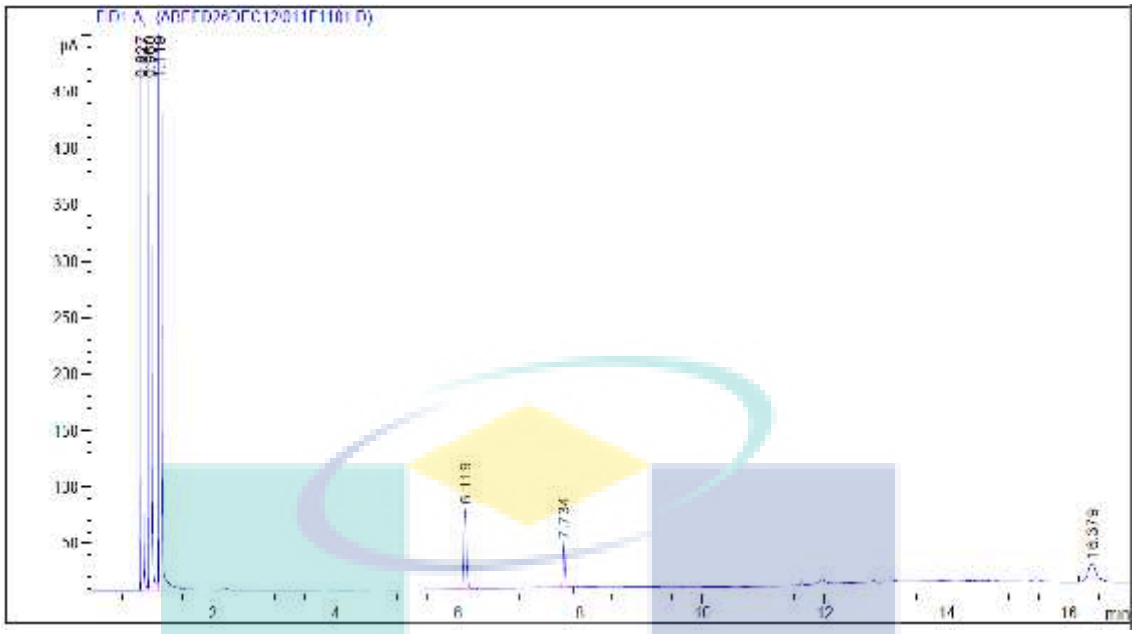

Figure A2: An example of GC result obtained for 0.5 g/L urea at 87.5 h (Set 1). Peak for PHB was observed at 6.119 min

APPENDIX B

EFFECT OF UREA CONCENTRATION

Table B1: Experimental data for urea concentration of 0.5 g/L (set 1)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
14	0.510			
24	1.921			
38	8.299	5.975	71.369	0.478
48.5	11.208	7.795	69.723	0.624
52.5	11.621	8.899	76.057	0.712
63.5	11.258	8.733	77.158	0.699
70.5	11.732	7.997	68.223	0.640
75.5	10.926	7.787	71.484	0.623
87.5	11.366	7.340	64.728	0.587

Table B2: Experimental data for urea concentration of 0.5 g/L (set 2)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
14	0.950			
24	3.540			
38	8.921	4.858	54.454	0.389
48.5	12.692	9.193	72.429	0.735
52.5	12.483	7.744	62.036	0.620
63.5	11.917	7.493	62.878	0.599
70.5	12.486	8.742	70.013	0.699
75.5	11.718	9.050	77.228	0.724
87.5	10.234	6.306	61.615	0.504

Table B3: Experimental data for urea concentration of 0.5 g/L (average of set 1 and set 2)

Incubation time (hr)	Average CDW (g/L)	Average PHB concentration (g/L)	Average PHB content (%)	Yield (g PHB/g oil)	CDW Standard Deviation	PHB Standard Deviation
14	0.730				0.220	
24	2.731				0.809	
38	8.610	5.417	62.912	0.433	0.311	0.559
48.5	11.950	8.494	71.076	0.679	0.742	0.699
52.5	12.052	8.321	69.046	0.666	0.431	0.578
63.5	11.587	8.113	70.018	0.649	0.330	0.620
70.5	12.109	8.369	69.118	0.670	0.377	0.372
75.5	11.322	8.418	74.356	0.673	0.396	0.631
87.5	10.800	6.823	63.172	0.546	0.566	0.517

Table B4: Experimental data for urea concentration of 1 g/L (set 1)

Incubation time (hr)	Total CDW (g/L)	PHB concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)
13	0.095			
23	1.451	0.450	31.018	0.036
37.5	6.353	3.920	61.706	0.314
46	10.497	8.233	78.431	0.659
49.5	10.867	8.532	78.515	0.683
55	11.620	8.629	74.266	0.690
61.5	11.711	9.028	77.093	0.722
65	11.529	8.693	75.400	0.695
70	10.673	8.100	75.893	0.648
78.5	10.838	7.745	71.457	0.620
89	10.535	8.088	76.776	0.647

Table B5: Experimental data for urea concentration of 1 g/L (set 2)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)
13	0.749			
23	3.167	0.900	28.417	0.072
37.5	7.349	5.451	74.174	0.436
46	9.065	6.586	72.651	0.527
49.5	11.625	7.944	68.332	0.636
55	11.570	8.116	70.146	0.649
61.5	11.350	8.132	71.644	0.651
65	11.430	8.430	73.753	0.674
70	11.436	8.511	74.428	0.681
78.5	10.099	7.808	77.317	0.625
89	9.027	6.023	66.726	0.482

Table B6: Experimental data for urea concentration of 1 g/L (average of set 1 and set 2)

Incubation time (hr)	Average CDW (g/L)	Average PHB concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)	CDW Standard deviation	PHB Standard deviation
13	0.422				0.327	
23	2.309	0.675	29.234	0.054	0.858	0.225
37.5	6.851	4.686	68.393	0.375	0.498	0.765
46	9.781	7.409	75.753	0.593	0.716	0.823
49.5	11.246	8.238	73.252	0.659	0.379	0.294
55	11.595	8.373	72.210	0.670	0.025	0.257
61.5	11.530	8.580	74.411	0.686	0.180	0.448
65	11.576	8.561	73.956	0.685	0.050	0.132
70	11.054	8.306	75.135	0.664	0.381	0.206
78.5	10.468	7.776	74.283	0.622	0.370	0.032
89	9.781	7.056	72.138	0.564	0.754	1.032

Table B7: Experimental data for urea concentration of 1.5 g/L (set 1)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
14	0.285			
24	1.784			
38	5.924	2.984	50.378	0.239
52.5	10.995	5.738	52.190	0.459
63.5	10.796	7.294	67.563	0.584
70.5	10.948	6.932	63.316	0.555
75.5	9.750	5.742	58.896	0.459

Table B8: Experimental data for urea concentration of 1.5 g/L (set 2)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
14	0.037			
24	2.577			
38	6.896	4.522	65.579	0.362
52.5	10.056	7.282	72.415	0.583
63.5	9.345	5.696	60.959	0.456
70.5	9.211	6.091	66.125	0.487
75.5	8.456	4.312	50.993	0.345

Table B9: Experimental data for urea concentration of 1.5 g/L (average of set 1 and set 2)

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/ g oil)	CDW standard deviation	PHB standard deviation
14	0.161			0.000	0.124	
24	2.181			0.000	0.397	
38	6.410	3.753	58.555	0.300	0.486	0.769
52.5	10.525	6.510	61.852	0.521	0.469	0.772
63.5	10.070	6.495	64.499	0.520	0.726	0.799
70.5	10.080	6.511	64.599	0.521	0.869	0.421
75.5	9.103	5.027	55.225	0.402	0.647	0.715

APPENDIX C

EFFECT OF JATROPHA OIL CONCENTRATION

Table C1: Experimental data for oil concentration of 5 g/L (set 1)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
11.5	1.183			
21.5	1.345			
37	2.007	1.176	58.585	0.235
43.5	2.206	0.971	44.021	0.194
59.5	3.466	1.849	53.349	0.370
69.5	3.738	2.615	69.946	0.523
81.5	1.813	1.148	63.306	0.230
91	1.077	0.519	48.211	0.104

Table C2: Experimental data for oil concentration of 5 g/L (set 2)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
11.5	0.652			
21.5	0.986			
37	1.586	0.744	46.867	0.149
43.5	2.553	1.527	59.807	0.305
59.5	3.109	1.426	45.866	0.285
69.5	3.277	2.264	69.111	0.453
81.5	2.564	1.644	64.109	0.329
91	1.542	0.987	63.988	0.197

Table C3: Experimental data for oil concentration of 5 g/L (average of set 1 and set 2)

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/ g oil)	CDW Standard deviation	PHB Standard deviation
11.5	0.917				0.266	
21.5	1.166				0.180	
37	1.797	0.960	53.412	0.192	0.210	0.216
43.5	2.380	1.249	52.490	0.250	0.174	0.278
59.5	3.287	1.638	49.811	0.326	0.179	0.212
69.5	3.507	2.440	69.556	0.488	0.231	0.175
81.5	2.188	1.396	63.776	0.279	0.376	0.248
91	1.309	0.753	57.502	0.151	0.233	0.234

Table C4: Experimental data for oil concentration of 20 g/L (set 1)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
13	0.678			
23	1.496	0.487	32.553	0.024
37.5	10.643	7.195	67.603	0.360
43	13.023	9.495	72.909	0.475
47.5	15.385	10.949	71.167	0.547
61	19.345	15.916	82.274	0.796
69	19.385	15.314	78.999	0.766
84	19.356	15.023	77.614	0.751
91	19.695	14.983	76.075	0.749
101.5	19.281	15.012	77.859	0.751

Table C5: Experimental data for oil concentration of 20 g/L (set 2)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
13	0.500			
23	2.334	0.539	23.093	0.027
37.5	10.633	8.305	78.106	0.415
43	15.057	10.071	66.886	0.504
47.5	16.001	12.725	79.526	0.636
61	17.777	15.136	85.144	0.757
69	17.785	15.614	87.793	0.781
84	19.160	15.989	83.450	0.799
91	20.533	15.955	77.704	0.798
101.5	18.123	14.294	78.872	0.715

Table C6: Experimental data for oil concentration of 20 g/L (average of set 1 and set 2)

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/ g oil)	CDW standard deviation	PHB standard deviation
13	0.589				0.089	
23	1.915	0.513	26.815	0.026	0.419	0.026
37.5	10.638	7.750	72.856	0.388	0.005	0.555
43	14.040	9.783	69.682	0.489	1.017	0.288
47.5	15.693	11.837	75.429	0.592	0.308	0.888
61	18.561	15.526	83.647	0.776	0.784	0.390
69	18.585	15.464	83.207	0.773	0.800	0.150
84	19.258	15.506	80.521	0.775	0.098	0.483
91	20.114	15.469	76.909	0.773	0.419	0.486
101.5	18.702	14.653	78.346	0.733	0.579	0.359

APPENDIX D

EFFECT OF AGITATION RATE

Table D1: Experimental data for agitation speed of 100 rpm (set 1)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
21.5	1.034			
27.5	1.754			
43	2.935	0.455	15.494	0.036
49	2.756	0.866	31.411	0.069
61	3.232	1.635	50.567	0.131
73	3.896	1.957	50.225	0.157
87.5	4.735	1.945	41.085	0.156

Table D2: Experimental data for agitation speed of 100 rpm (set 2)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/g oil)
21.5	1.829			
27.5	0.883			
43	2.319	0.790	34.052	0.063
49	2.554	1.054	41.288	0.084
61	4.145	2.236	53.955	0.179
73	4.847	2.586	53.351	0.207
87.5	4.083	2.397	58.697	0.192

Table D3: Experimental data for agitation speed of 100 rpm (average of set 1 and set 2)

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB Content (%)	Yield (g PHB/ g oil)	CDW standard deviation	PHB standard deviation
21.5	1.432				0.397	
27.5	1.318				0.435	
43	2.627	0.622	23.685	0.050	0.308	0.167
49	2.655	0.960	36.161	0.077	0.101	0.094
61	3.689	1.935	52.471	0.155	0.456	0.301
73	4.371	2.271	51.958	0.182	0.476	0.315
87.5	4.409	2.171	49.240	0.174	0.326	0.226

Table D4: Experimental data for agitation speed of 250 rpm (set 1)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)
21.5	1.079			
27.5	1.888	1.045	55.335	0.084
43	10.579	6.877	62.546	0.550
49	10.134	6.860	67.690	0.549
61	12.675	7.908	60.981	0.633
83.5	12.985	9.569	73.695	0.766
87.5	10.634	6.789	63.836	0.543

Table D5: Experimental data for agitation speed of 250 rpm (set 2)

Incubation time (hr)	Total CDW (g/L)	PHB Concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)
21.5	1.899			
27.5	2.702	1.010	37.367	0.081
43	9.807	7.905	80.600	0.632
49	11.314	8.437	74.571	0.675
61	11.562	9.552	82.614	0.764
83.5	11.347	8.293	73.088	0.663
87.5	11.270	8.160	72.407	0.653

Table D6: Experimental data for agitation speed of 250 rpm (average of set 1 and set 2)

Incubation time (hr)	Average CDW (g/L)	Average PHB Concentration (g/L)	PHB content (%)	Yield (g PHB/g oil)	CDW standard deviation	PHB standard deviation
21.5	1.489				0.410	
27.5	2.295	1.027	44.757	0.082	0.407	0.713
43	10.193	7.391	72.504	0.591	0.801	0.845
49	10.724	7.649	71.320	0.612	0.590	0.789
61	12.118	8.730	72.037	0.698	0.557	0.822
83.5	12.166	8.931	73.412	0.714	0.819	0.638
87.5	10.952	7.474	68.246	0.598	0.318	0.686

APPENDIX E

EFFECT OF ETHANOL ADDITION

Table E1: Experimental data for the effect of ethanol addition with increase in incubation time

Ethanol addition time (hr)			**0**				**14**	
Ethanol concentration (v/v %)	**Total CDW (g/L)**	**PHB concentration (g/L)**	**PHB Content (%)**	**Yield (g PHB/g oil)**	**Total CDW (g/L)**	**PHB concentration (g/L)**	**PHB Content (%)**	**Yield (g PHB/g oil)**
0	9.045	6.024	66.600	0.482	9.045	6.024	66.600	0.482
0.5	9.257	7.373	79.644	0.590	12.772	8.074	63.216	0.646
1	10.439	8.545	81.857	0.684	12.820	8.995	70.163	0.720
1.5	9.896	8.524	86.133	0.682	10.831	8.702	80.345	0.696
2	9.628	7.072	73.453	0.566	10.264	7.638	74.415	0.611
2.5	10.228	6.399	62.564	0.512	10.392	6.600	63.508	0.528

Table E1: Continued

Ethanol addition time (hr)	**24**				**38**			
Ethanol concentration (v/v %)	**Total CDW (g/L)**	**PHB concentration (g/L)**	**PHB Content (%)**	**Yield (g PHB/g oil)**	**Total CDW (g/L)**	**PHB concentration (g/L)**	**PHB Content (%)**	**Yield (g PHB/g oil)**
0	9.045	6.024	66.600	0.482	9.045	6.024	66.600	0.482
0.5	10.738	8.206	76.423	0.656	10.354	7.956	76.841	0.636
1	10.848	8.519	78.534	0.682	10.259	8.436	82.234	0.675
1.5	10.925	8.944	81.871	0.716	10.175	9.354	91.931	0.748
2	9.278	7.130	76.847	0.570	10.241	8.228	80.346	0.658
2.5	8.922	5.519	61.855	0.442	9.123	5.943	65.146	0.475

APPENDIX F

DETERMINATION OF α FOR THEORETICAL PHB CONCENTRATION

Table F1: Calculated values to obtain α

Incubation time (h)	PHB concentration (g/L)	B(t)	A(t)	P-βB(t)
23	0.675	12.539	1.836	0.653
37.5	4.686	84.343	7.944	4.536
46	7.409	166.605	10.388	7.115
49.5	8.238	205.190	10.865	7.875
55	8.373	268.339	11.259	7.898
61.5	8.580	344.833	11.444	7.970
65	8.561	386.383	11.487	7.878
70	8.306	445.932	11.518	7.517
78.5	7.776	547.381	11.536	6.809
89	7.056	675.798	11.541	5.861

APPENDIX G

PUBLICATIONS

1. Mohidin Batcha, A.F, Prasad, D., Khan, M. and Abdullah, H. 2013. Biosynthesis of poly(3-hydroxybutyrate) (PHB) by *Cupriavidus necator* H16 from jatropha oil as carbon source. *Bioprocess and Biosystems Engineering*.

2. Khan, M. R., Prasad, D.M., Abdullah, H. and Mohidin Batcha, A.F. 2013. Kinetic analysis on cell growth and biosynthesis of poly (3-hydroxybutyrate) (PHB) in *Cupriavidus necator* H16. *International Journal of Bioscience, Biochemistry and Bioinformatics.* **3**(5), 516-519.