EFFECTS OF FERMENTATION TIME AND TEMPERATURE ON BUTANOL PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY USING *CLOSTRIDIUM ACETOBUTYLICUM*

SITI NADIAH BT MUSTAFA KAMAL

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

BORANG PENGESAHAN STATUS TESIS

JUDUL : **EFFECTS OF FERMENTATION TIME AND TEMPERATURE ON BUTANOL PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY USING *CLOSTRIDIUM ACETOBUTYLICUM***

SESI PENGAJIAN: **2010**

Saya **SITI NADIAH BT MUSTAFA KAMAL**
(HURUF BESAR)

mengaku membenarkan tesis (PSM/~~Sarjana~~/~~Doktor Falsafah~~)* ini disimpan di Perpustakaan Universiti Malaysia Pahang dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Pahang.
2. Perpustakaan Universiti Malaysia Pahang dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. **Sila tandakan (✓)

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)
☐	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
✓	TIDAK TERHAD	

Disahkan oleh

(TANDATANGAN PENULIS) (TANDATANGAN PENYELIA)

Alamat Tetap: **NO 20, JALAN BIDARA 1**
TAMAN BIDARA,
09000 KULIM, KEDAH.

Nama Penyelia
SHARIZA JAMEK

Tarikh: 6th Disember 2010

Tarikh: 6th Disember 2010

CATATAN:
* Potong yang tidak berkenaan.
** Jika tesis ini **SULIT** atau **TERHAD**, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai **SULIT** atau **TERHAD.**
◆ Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau disertai bagi pengajian secara kerja kursus dan penyelidikan, atau Laporan Projek Sarjana Muda (PSM).

"I hereby declare that I have read this thesis and in

my opinion this thesis is sufficient in terms of scope and

quality for the award of the degree of

Bachelor of Chemical Engineering (Biotechnology)"

Signature : .....

Supervisor : MISS SHARIZA JAMEK

Date : .....

EFFECTS OF FERMENTATION TIME AND TEMPERATURE ON BUTANOL PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY USING *CLOSTRIDIUM ACETOBUTYLICUM*

SITI NADIAH BINTI MUSTAFA KAMAL

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
University Malaysia Pahang

DECEMBER 2010

DECLARATION

I declare that this thesis entitled "Effects of Fermentation Time and Temperature on Butanol Production by Using *Clostridium acetobutylicum*" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature :…….

Name of Candidate : Siti Nadiah binti Mustafa Kamal

Date : 6th December 2010

DEDICATION

Special dedication to my beloved mother (Fatimah bt Yusoff) and father (Mustafa Kamal bin Ahmad), for their love and encouragement.

and,

Special thanks to my friends, my fellow course mates and all faculty members for all your care, support and best wishes.

Sincerely,

Siti Nadiah binti Mustafa Kamal

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere appreciation to my supervisor, Ms. Shariza Bt Jamek, for constantly guided and encouraged me throughout this study. Thanks a lot for giving me guidance, continuous advice and suggestions to bring this project to its final form.

I am very thankful to the staffs of Chemical & Natural Resources Engineering Faculty, Universiti Malaysia Pahang (UMP) especially Mr. Mohamad Zaki B. Sahad and Dr. Chua @ Yeo Gek Kee for their guidance and facilitate throughout this project. In addition, I am appreciative for all staffs of UMP for guided and helped me during my experimental work.

In addition, my sincere thankful is also extends to all my team members, Nur Hidayah Bt. Maslan, Siti Hazriah Bt. Hamzah and Ellie Fazlin Bt. Mohd Sarian, who have provided support at various occasions. Their views and tips were useful indeed. My personal thanks also to everybody who were directly and indirectly involved in helping me to complete this thesis.

Lastly, I also want to thank my family for their encouragement and understanding in completing this project. Last but not least, I appreciate the support and encouragement from Muhammad B. Nor Shakiman.

ABSTRACT

Malaysia is the world's largest producer of palm oil. Waste from palm oil production factories has been increasing rapidly and one of the serious problems in the palm oil fruit processing is the managing of the waste generated by the processes. Recently, fermentative energy production from several sources of biomass has been carried out which is Acetone–butanol–ethanol (ABE) fermentation (Shinto *et al.*, 2008). Due to this, this study is focusing on the utilization of palm oil mill effluent (POME) for ABE fermentation by *Clostridium acetobutylicum* (NCIMB 13357). The main objective of this study is to develop conversion of POME into useful material which is butanol. Investigations were carried out on the effects of fermentation temperature within 35°C to 45°C and fermentation time within 48 to 72 hours to the butanol production. The experiment were conducted in Schott bottle with 90% concentration of culture medium and 10% v/v of inoculums concentration by using POME and reinforced clostridia medium (RCM) as growth medium. This anaerobic fermentation was done in batch culture. It was observed that the optimum condition for butanol fermentation by *C.acetobutylicum* is at 40°C through 48 hours which produced the highest yield of butanol (3.1344 g/L). From this study, it showed that the butanol production was decreased as the fermentation temperature and time were increased. The results from this study hence showed that the POME is a viable media for butanol fermentation. For improvement, research on other parameter such as pH of substrate and concentration of substrate that effect on butanol production should be carried out to optimize the production of butanol.

ABSTRAK

Malaysia merupakan pengeluar minyak sawit yang terbesar di dunia. Sisa buangan dari kilang pengeluaran minyak sawit telah meningkat dengan cepat. Salah satu masalah serius dalam pemprosesan minyak sawit adalah pengurusan sisa yang dihasilkan oleh proses itu sendiri. Kebelakangan ini, penghasilan pelarut asiton, butanol dan etanol (ABE) daripada sisa buangan kilang sawit telah dilakukan. Kajian telah dijalankan adalah untuk memanfaatkan sisa kilang kelapa sawit (POME) untuk fermentasi ABE oleh *Clostridium acetobutylicum* (NCIMB 13357). Tujuan utama kajian ini dilakukan adalah untuk menukarkan POME kepada bahan yang bermanfaat iaitu butanol. Eksperimen dilakukan pada pengaruh lingkungan suhu 35°C hingga 45°C dan lingkungan masa 48 hingga 72 jam untuk penghasilan butanol yang tertinggi. Kajian dilakukan di dalam botol Schott dengan kepekatan medium 90% dan 10% kepekatan inokulum dengan menggunakan media fermentasi efluen loji minyak sawit (POME) dan dikawal oleh medium kawalan (RCM) sebagai media pertumbuhan *Clostridium acetobutylicum* dalam ruangan anaerobik untuk mengekalkan keadaan anaerobik. Diamati bahawa keadaan optimum untuk fermentasi butanol oleh *C.acetobutylicum* ialah pada suhu 40°C selama 48 jam untuk menghasilkan butanol yang tertinggi iaitu sebanyak 3,1344g/L. Dari ujikaji ini menunjukkan bahawa pengeluaran butanol menurun apabila suhu fermentasi dan masa meningkat. Hasil dari kajian menunjukkan bahawa POME adalah media yang sesuai untuk penghasilan butanol.

TABLE OF CONTENT

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENT	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	xii
	LIST OF FIGURES	xiii
	LIST OF SYMBOLS / ABBRECIATIONS	xvi
	LIST OF APPENDICES	xvii
1	**INTRODUCTION**	

1.1	Background of Research	1
1.2	Problem Statement	3
1.3	Objective	5
1.4	Scopes of Research	5
1.5	Rational and Significance	6

2 LITERATURE REVIEW

2.1	Butanol	7
2.2	Anaerobic Fermentation	9
2.3	Acetone-butanol-ethanol (ABE) fermentation	10
2.4	*Clostridium acetobutylicum*	11
2.5	Palm Oil Mill Effluent (POME)	12

3 METHODOLOGY

3.1	Materials	15

3.1.1 Chemicals and Biological Materials 15

3.1.2 Media 16

3.2 Equipments 16

3.3 Experimental Procedures 17

3.3.1 Bacteria Culturing 17

3.3.1.1 Agar Preparation 17

3.3.1.2 *Clostridium acetobutylicum* Cultivation 18

3.3.1.3 Inoculums Preparation 18

3.3.2 Media Preparation 20

3.3.2.1 Pretreatment POME 20

3.3.2.1 Reinforced Clostridial Medium 20

3.3.3 Determination of Growth Profile 20

3.3.4 Fermentation 21

3.3.5 Centrifugation 22

3.3.6 Product Analysis 23

3.3.6.1 Determination of Butanol Production 23

3.3.6.2 Determination of Glucose Consumption 23

4 RESULT AND DISCUSSION

4.1 Growth Profile of *C.acetobutylicum* 25

4.2 Butanol Production 28

4.2.1 Effect of Fermentation Temperature 28

4.2.1.1 Butanol Production in 48 hours 29

4.2.1.2 Butanol Production in 72 hours 30

4.2.2 Effect of Fermentation Time 31

4.2.2.1 Butanol Production at 35°C 31

4.2.2.2 Butanol Production at 40°C 32

4.2.2.3 Butanol Production at 45°C 32

4.2.3 Overall Study on Butanol Production in POME 33

4.2.4 Overall Study on Butanol Production in RCM 33

4.3 Glucose Consumption 36

4.3.1 Glucose Consumption at 35°C 36

4.3.2 Glucose Consumption at 40°C 37

4.3.3 Glucose Consumption at 45°C 38

4.3.4 Glucose Consumption in POME 39

4.3.5 Glucose Consumption in RCM 40

5 CONCLUSION AND RECOMMENTDATIONS

5.1 Conclusion 41

5.2 Recommendation 41

REFERENCES 44

APPENDIX A-D 49

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Typical characteristic of POME	14
4.1	Growth profile of *C. acetobutylicum* in POME and RCM	26

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	Structure of butanol	7
2.2	Metabolic pathway in *Clostridium acetobutylicum* ATCC 824 with glucose	22
3.2	Preparation of reinforced clostridial agar in laminar flow.	17
3.3	Bactron Anaerobic Chamber	19
3.4	Centrifuge 5810R Eppendorf	22
4.1	Growth profile of *C.acetobutylicum* in POME and RCM as growth medium.	26
4.2	The concentration in g/L of butanol produced in POME and RCM throughout 48 hours at 35, 40 and 45°C.	29
4.3	The concentration in g/L of butanol produced in POME and RCM throughout 72 hours at 35, 40 and 45°C.	30
4.4	The concentration in g/L of butanol produced in POME and RCM for 48 and 72 hours at 35°C	31
4.5	The concentration in g/L of butanol produced in POME and	32

	RCM for 48 and 72 hours at 40°C	
4.6	The concentration in g/L of butanol produced in POME and RCM for 48 and 72 hours at 45°C	32
4.7	The concentration in g/L of butanol produced in POME for 48 and 72 hours for control different fermentation temperature (35°C, 40°C and 45°C)	33
4.8	The concentration in g/L of butanol produced in RCM for 48 and 72 hours for control different fermentation temperature (35°C, 40°C and 45°C)	33
4.9	Glucose consumption at 35°C	36
4.10	Glucose consumption at 40°C	37
4.11	Glucose consumption at 45°C	38
4.12	The concentration of glucose consumption in g/L in POME throughout 72 hours	39
4.13	The concentration of glucose consumption in g/L in RCM throughout 72 hours	39

LIST OF SYMBOLS / ABBREVIATIONS

ABE	-	Acetone-Butanol-Ethanol
ATP	-	Adenosine triphosphate
adc		Acetoacetic acid decarboxylase
C.acetobutylicum	-	*Clostridium acetobutylicum*
CO_2		Carbon dioxide
DNS	-	Dinitrosalicylic Colorimetric Method
GC		Gas Chromatography
GC-FID	-	Gas Chromatography equipped with Flame Ionization Detector
NaOH	-	Sodium Hydroxide
N_2		Nitrogen gas
OD	-	Optical Density
POME	-	Palm Oil Mill Effluent
RCM	-	Reinforced Clostridium Medium
RNA	-	Ribonucleic acid
USA	-	United States of America
UV-VIS	-	Ultraviolet-Visible Spectroscopy

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A-1	Optical Density value of *C.acetobutylicum* in POME and RCM	49
B-1	Standard of Butanol	50
B-2	Effect of fermentation temperature	51
B-2-1	Effect of fermentation temperature on butanol production in 48 hours	51
B-2-2	Effect of fermentation temperature on butanol production in 72 hours	51
B-3	Effect of fermentation time	52
B-3-1	Effect of fermentation time in butanol production at 35°C	52
B-3-2	Effect of fermentation time in butanol production at 40°C	52
B-3-3	Effect of fermentation time in butanol production at 45°C	52
B-4	Overall study on butanol production	53
B-4-1	Overall study on butanol production in POME	53

B-4-2	Overall study on butanol production in RCM	53
C-1	Standard Curve of glucose	54
C-2	Concentration of glucose	52
C-2-1	Concentration of glucose (35°C, 48 hours)	56
C-2-2	Concentration of glucose (35°C, 72 hours)	56
C-2-3	Concentration of glucose (40°C, 48 hours)	57
C-2-4	Concentration of glucose (40°C, 72 hours)	57
C-2-5	Concentration of glucose (45°C, 48 hours)	58
C-2-6	Concentration of glucose (45°C, 72 hours)	58

CHAPTER 1

INTRODUCTION

1.1 Background of Research

The Malaysian palm oil industry has grown rapidly over the years. Malaysia has become the world's largest producer and exporter of palm oil and its products (Wu *et al.*, 2009). Malaysia is producing more than 80% of the world's crude palm oil. Palm oil is produced from palm fruit mill through steaming and squeezing process. One of the serious problems in the palm fruit processing is the managing of the wastes generated during the processes. The waste consists of a significant amount of solid waste and a wastewater called palm oil mill effluent (POME) (Miura *et al.*, 2001). If the effluent discharged untreated, it can certainly cause considerable environmental problems. POME has great potential as a substrate for acetone-butanol-ethanol (ABE) fermentation because it contains a mixture of carbohydrates including starch, hemicelluloses, sucrose and other carbohydrates that can be utilized by saccharolytic clostridia.

The production of acetone-butanol-ethanol (ABE) by solvents producing strains of Clostridium was one of the first large-scale industrial fermentation process developed (Kalil *et al.*, 2003). The fermentation of ABE was widely carried out using renewable sources. ABE producing clostridia posses two distinct characteristic phases in energy acquiring pathway, especially acidogenesis and solventogenesis. During acidogenesis,

cell growth is exponential and products are acetic acid and butyric acid with ATP formation. During solventogenesis, cell growth enters the stationary phase and the above organic acids are reutilized and acetone, butanol and ethanol are produced (Tashiro *et al.,* 2004). These metabolism pathways of clostridia offer an opportunity to establish the ABE fermentation as an economically viable process (Sillers *et al.,* 2009).

Acetone, butanol and ethanol are dominant products of the acetone-butanol-ethanol (ABE) process which butanol constitutes 60-70% (w/w) of the total solvents, acetone 20-30%, and ethanol about 10%. Butanol is the most valuable of these solvent products (Jiang *et al.,* 2009). Since butanol has remarkable features such as hydrophobicity, high energy content, and ease of storage and transportation, it has been proposed as a substitute and supplement of gasoline as a transportation fuel (Shinto *et al.,* 2008). Current utilization strategies for biomass have focused on ethanol production; producing butanol instead of ethanol offers several advantages for biofuel-gasoline blending. Butanol has lower vapor pressure but higher energy content than ethanol, which makes the former safer for blending with gasoline as well as offering better fuel economy than ethanol-gasoline blends (Hipolito *et al.,* 2008).

Butanol has a higher tolerance to water contamination in gasoline blends and therefore butanol-gasoline blends are less susceptible to separation and that facilities its use in existing gasoline supply and distribution channels. Furthermore, butanol can be blended with gasoline at higher concentrations than ethanol without the need to retrofit vehicles. Therefore, optimizing acetone-butanol-ethanol (ABE) fermentation to enhance butanol production over ethanol appears to be more commercially and technologically attractive opinion. Besides that, palm oil mill effluent (POME) utilization would further increase profitability of palm oil mill industry and solve an environmental problem. The availability of cheap and readily available sources of substrate such as POME should enhance the economic viability of fermentation process for ABE production (Takriff *et al.,* 2009).

1.2 Problem Statement

This research is proposed to convert waste into wealth. Malaysia is producing more than 80% of the world's crude palm oil. One of the serious problems in the palm fruit processing is the managing of the wastes generated during the processes. By-products from palm oil mill constitute the most abundant renewable resources available in Malaysia. The abundance of oil palm empty fruit bunches has created a vital environmental issue. In the year 2004, more than 40 million tons of palm oil mill effluent (POME) was generated from 372 mills in Malaysia. If the effluent is discharged untreated, it can certainly cause considerable environmental problems due to its high biochemical oxygen demand (25,000 mg/l), chemical oxygen demand (53,630 mg/l), oil and grease (8370 mg/l), total solids (43,635 mg/l) as well as suspended solids (19,020 mg/l).

The palm oil mill industry in Malaysia is identified as the one that produces the largest pollution load into the rivers throughout the country. The discharge of untreated palm oil mill effluent (POME) though creates adverse impact to the environment, the notion of nurturing POME and its derivatives as valuable resources should not be dismissed (Wu *et al.*, 2007). Besides that, it is also an efficient solution when using POME as an alternative to produce a butanol because it is a renewable fuel instead of using fossil fuel. Depleting non-renewable natural resources such as mineral petroleum has raised the concern for the need of renewable sources to overcome the shortage of transportation fuel in the coming era and at the same time to protect the environment from pollutants (Fei *et al.*, 2008).

Butanol is an important industrial solvent and potentially a better fuel extender than ethanol. In the 1970s, the primary focus for alternative fuels was on ethanol. People were familiar with its production and did not realize that dehydration was necessary in order to blend it with fossil fuels. Nor did people realize the difficulty of distribution, since ethanol cannot be transferred through the existing pipeline infrastructure. The

selection of ethanol, a lower-grade and corrosive, hard-to-purify, dangerously explosive, and very evaporative alcohol is the result. Ethanol is still subsidized by the government, since it is not profitable enough to compete with gasoline.

On the other hand, butanol which is a new alternative is the good biofuel which when it consumed in an internal combustion engine yields no carbon monoxide all environmentally harmful byproducts of combustion. Carbon dioxide (CO_2) is the combustion byproduct of butanol, and is considered environmentally 'green' (Fei *et al*., 2008). Butanol is far less corrosive than ethanol and can be shipped and distributed through existing pipelines and filling stations. Reformed butanol has four more hydrogen atoms than ethanol, resulting in a higher energy output and is used as a fuel cell fuel. Current butanol prices as a chemical are at $3.75 per gallon, with a worldwide market of 370 million gallons per year. The market demand is expected to increase dramatically if green butanol can be produced economically from low cost biomass.

Widespread adoption of butanol as an alternative fuel to replace gasoline would stimulate an increased demand for corn and other organics as well as waste biomass. In this research, palm oil mill effluent (POME) is used as an alternative to produce a butanol instead of using corn because corn will contribute a food crisis. Numerous carbon sources such as glucose, molasses, starch and corn has been utilized in the acetone-butanol-ethanol (ABE) fermentation. However the cost of substrate has a dramatic influence on the economic viability of ABE production via fermentation (Ezeji *et al.,* 2007 and Takriff *et al.,* 2009).

Many developed countries heavily subsidies the growing of food crops to produce ethanol before. However, the subsidy for corn has reduced the land used for the production of other food crops such as wheat and soya. Therefore, world prices of all these crops have gone up sharply. This, in turn, has affected millions of poor people all over the world in terms of their ability to have access to affordable food. The public too must play a role by reducing their consumption of resource-intensive food. Thus, the

availability of cheap and readily available sources of substrates such as palm oil mill effluent (POME) should enhance the economic viability of fermentation processes for acetone-butanol-ethanol (ABE) production (Takriff *et al.,* 2009).

Therefore, solution should be found and developed. Various technically feasible means of transforming the palm oil mill effluent (POME) into different benefit products through cleaner production and environmentally sound biotechnologies for enabling the balance between environmental protection and a sustainable reuse of biosources found in POME (Wu *et al.,* 2009).

1.3 Objective

The main objective for this research is to study the effects of fermentation temperature and fermentation time in producing higher butanol from the anaerobic fermentation of Palm Oil Mill Effluent (POME) by using *Clostridium acetobutylicum*.

1.4 Scopes of research

The main scopes of this research are:

(i) To study the growth profile of *Clostridium acetobutylicum.*

(ii) To study the effect of fermentation time within the range 48 to 72 hours to the butanol production.

(iii) To study the effect of fermentation temperature within 35°C to 45°C to the butanol production.

(iv) To study the glucose consumption in fermentation broth by using dinitrosalicylic acid (DNS) method.

1.5 Rationale and Significance

(i) This research applies concept of ‘waste to wealth’ due to abundant supply of palm oil mill effluent (POME) as a substrate to yield butanol in huge amount.

(ii) Butanol is an alternative to replace the use of ethanol which butanol has a lot of advantages compare to ethanol.

(iii) The increased demand of corn for bioethanol fuel production will contribute a food crisis. The price of corn will rapidly increase and people will suffer for food.

CHAPTER 2

LITERATURE REVIEW

2.1 Butanol

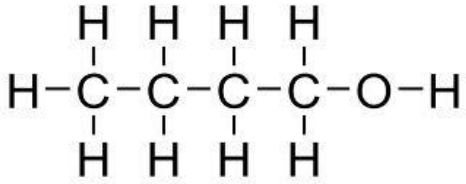

Figure 2.1 Structure of butanol

Butanol is a 4-carbon alcohol that has more energy than ethanol; 25% more energy per unit volume (Shapovalov and Ashkinazi, 2008). Butanol is a cleaner and superior extender than ethanol with octane numbers 113 and 94 as compared with that of 111 and 94 for ethanol (Qureshi *et al.*, 2008). Butanol can be produced from renewable resources such as corn, sugar beets, sorghum, cassava, sugarcane, corn stalks, and other biomass. Unlike conventional biofuels, such as ethanol and biodiesel that are mainly derive from food-based feedstocks, biobutanol is an advanced biofuel that can be derived from non-food sources.

Butanol is produced by solventogenic clostridia via the acetone-butanol-ethanol (ABE) fermentation. Historically, butanol (or ABE in fermentation broth, the typical ratio of ABE is 3:6:1, where butanol is a major product) fermentation is second to ethanol and there were plants that operated during World War I (Qureshi *et al.,* 2008). Butanol is now recognizing as an important transport fuel with superior characteristics to ethanol. Its inherent chemical properties make it superior to ethanol for use in combustion engines:

i. Butanol has a lower vapor pressure and higher flashpoint than ethanol, making it easier to store and safer to handle.
ii. Butanol is safer to handle with a Reid Value of 0.33 psi, which is a measure of a fluid's rate of evaporation when compared to gasoline at 4.5 and ethanol at 2.0 psi.
iii. Butanol is less corrosive than ethanol and can be transported using existing infrastructures.
iv. Butanol when consumed in an internal combustion engine yields no carbon monoxide, all environmentally harmful byproducts of combustion. CO_2 is the combustion byproduct of butanol, and is considered environmentally 'green' (Shapovalov and Ashkinazi, 2008).
v. Butanol is an industrial commodity, with 370 million gallons per year market with a selling price of $3.75 per gallon.
vi. Hydrogen generated during the butanol fermentation process is easily recovered, increasing the energy yield of a bushel of corn by an additional 18 percent over the energy yield of ethanol produced from the same quantity of raw material.
vii. Butanol is a pure alcohol with energy content similar to that of gasoline. It does not have to be stored in high-pressure vessels like natural gas (Hipolito *et al.*, 2008).
viii. Butanol is more “hydrophobic” than ethanol, meaning it has a higher tendency to repel water. This quality allows it to blend well with gasoline, could be used to

improve ethanol / gasoline blending, and might mean it is potentially suitable for transport in pipelines.

On the other hand, butanol is used as an ingredient in perfumes and as a solvent for the extraction of essential oils. Butanol is also used as an extractant in the manufacture of antibiotics, hormones and vitamins and as solvent for paints, coatings, natural resins, gums, synthetic resins, dyes, alkaloids, and camphor. Other miscellaneous applications of butanol are as a swelling agent in textiles, as a component of brake fluids, cleaning formulations, degreasers, and repellents and as a component of ore floatation agents, and of wood-treating systems.

2.2 Anaerobic fermentation

The anaerobic process has been developed for the efficient treatment of waste and high organic wastewater. One of the advantages of the anaerobic process is the recovery of the useful matters such as solvent and methane (Hwang *et al.*, 2004). Given the need to reduce carbon dioxide (CO_2) accumulation in the Earth's atmosphere, anaerobic processes should attract more attention than aerobic ones because much less the former generates CO_2 (Somrutai *et al.*, 1996). Anaerobic fermentation is a promising method of sustainable hydrogen production since organic matter, including waste products, can be used as a feedstock for the process. The highest yields of hydrogen have been reported with strains of clostridia in pure cultures or mixed cultures where clostridia are predominant (Alalayah *et al.*, 2009).

Acetone, butanol and ethanol production is a strictly anaerobic process due to the strictly anaerobic Clostridium. Actually the process was industrialized for production of acetone. Then, it was used to produce butanol which has varied applications. At present, both acetone and butanol are obtained by chemical synthesis, and this fermentation is rarely used *Clostridium acetobutylicum* which has an ability to utilize

various substrates. The fermentation yields number of products of which acetone, butanol and ethanol are the major ones. The fermentation process uses conversion of starch to acetone by *C. acetobutylicum*. According to Somrutai *et al.* (1996), this strain is able to convert the carbohydrates in molasses to acetone and butanol.

Normally in anaerobic fermentation, the cultivations were conducted anaerobically at 30ºC without pH control and the inoculum formed 10% (v/v) of the culture volume. For a batch fermentation which exceeding a working volume of 100 ml, the headspace of the culture was purged with oxygen-free nitrogen gas for 30 minutes after inoculation. Much anaerobic fermentation does, however, require mild aeration for the initial growth phase, and sufficient agitation for mixing and maintenance of temperature.

2.3 Acetone-butanol-ethanol (ABE) fermentation

Acetone–butanol–ethanol (ABE) fermentation was industrially carried out during the first half of last century, but was subsequently unable to compete economically with the petrochemical industry (Jones and Wood, 1986). However, there has been a revival of interest in ABE fermentation, since renewable resources as such domestic and agro-industrial wastes have become possible alternative substrates for the production of chemicals and liquid fuels (Kobayashi *et al.,* 2005).

Acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fermentations. It is one of the largest biotechnological processes ever known. The metabolic pathways of ABE-producing clostridia consist of two distinct characteristic phases namely acidogenesis and solventogenesis (Shinto *et al.,* 2008). During acidogenesis, cell growth is exponential and products are acetic acid and butyric acid with ATP formation. During solvetogenesis, cell growth enters the stationary phase and the above organic acids are reutilized and acetone, butanol and

ethanol are produced (Tashiro *et al.,* 2004). These solvents produced in a ratio of 3:6:1, or 3 parts acetone, 6 parts butanol and 1 part ethanol.

2.4 *Clostridium acetobutylicum*

Solvent-producing clostridia were used extensively from the beginning of the 20th century for the industrial production of acetone and butanol (Jones and Wood, 1986). This research has remained because of their potential application in biotechnology. Strains classified as *Clostridium acetobutylicum* were the first industrial cultures to be successfully isolated, patented and used for a large scale production of solvents from starched-based substrates (Keis *et al.,* 2001). *C.acetobutylicum* is a gram-positive, spore-forming, rod shaped, forming terminal or subterminal spores and strictly anaerobic. Therefore, it will not grow in the presence of oxygen. It is able to ferment various sugars to form the highest yields of acetone and butanol from a variety starchy substrate by using the ABE process (Zhao *et al.,* 2003). It normally can be found in soils, lake sediments, well water, and bovine, canine, and human feces.

The metabolic pathways of acetone-butanol-ethanol ABE-producing clostridia consist of two distinct characteristic phase; acidogenesis and solventogenesis. A figure below shows the metabolic pathways of *C.acetobutylicum* ATCC 824 by Jones and Wood (1986). In general, during acidogenesis, ABE-producing clostridia grow exponentially, and acetic and butyric acids are produced with ATP formation. Further, in the subsequent solventogenesis, cell growth attains a stationary phase; organic acids are reassimilated and acetone, butanol, and ethanol are produced. ABE fermentation includes substrate inhibition by glucose and xylose and product inhibition by butanol and these lead to low productivity and yield of solvents (Shinto *et al.,* 2008).

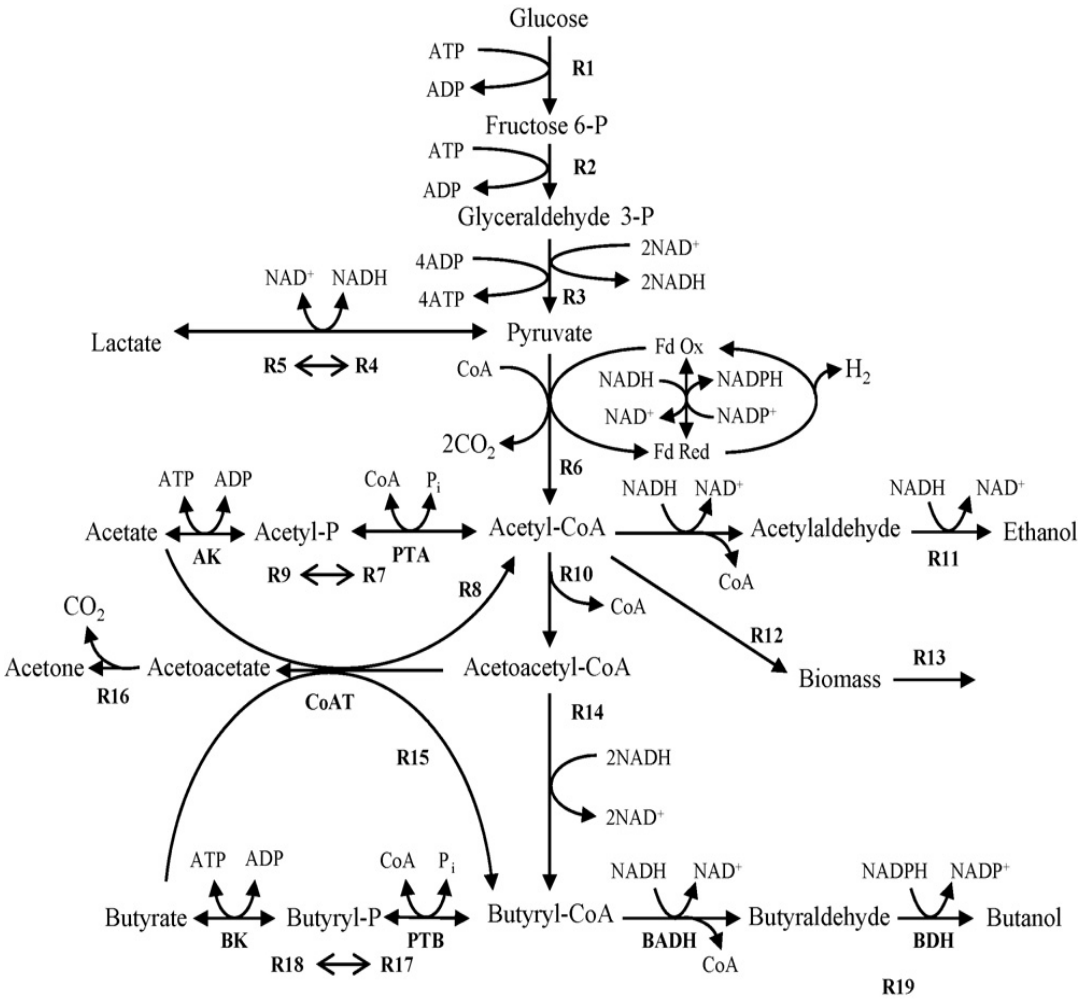

Figure 2.2 Metabolic pathway in *Clostridium acetobutylicum* ATCC 824 with glucose.

2.5 Palm Oil Mill Effluent (POME)

The production of palm oil is one of the major edible oils consumed in the world. It has increased tremendously in the last decade and is led by Malaysia, Indonesia and Thailand. However, the milling process produces a huge volume of palm oil mill effluent (POME). Disposal of these wastes is already an economic burden on communities and industries, so creating a marketable product from these wastes would reduce the treatment cost. Recovery of energy from waste might reduce the cost of

wastewater treatment, and contribute to reducing our dependence on fossil fuel (O-Thong *et al.,* 2008).

In general, the palm oil milling process can be categorized into a dry and a wet process. The wet process of palm oil milling is the most common and typical way of extracting palm oil, especially in Malaysia. It is estimated that for each ton of crude palm oil that is produced, 5–7.5 t of water are required, and more than 50% of this water ends up as palm oil mill effluent (POME). If the untreated effluent is discharged into watercourses, it is certain to cause considerable environmental problems. The palm oil mill industry in Malaysia has thus been identified as the one discharging the largest pollution load into the rivers throughout the country (Hwang *et al.,* 1978).

Palm oil mill effluent (POME) is thick, brownish liquid with a discharge temperature in range of 80 to 90°C and pH 4.2-4.5. Palm oil waste consists mainly of mixture of carbohydrates that include starch, hemicelluloses, sucrose and other carbohydrate that can be utilized in acetone-butanol-ethanol (ABE) fermentation (Takriff *et al.,* 2009). Wu (2009) reported that POME also contains certain powerful water soluble, anti oxidants, phenolic acids and flavonoids that may inhibit the growth development of microorganism (Lin *et al.,* 2005; Uzel *et al.,* 2005). The characteristic of a typical POME is shown in Table 2.1.

Table 2.1 Typical characteristic of POME (Ma, 2000; Lorestani, 2006)

Parameter	*Average	Metal	*Average
pH	4.7	Phosphorous	180
Oil and Grease	4000	Potasium	2270
Biochemical Oxygen Demand (BOD5)	25000	Magnesium	615
Chemical Oxygen Demand (COD)	50000	Calcium	439
Total Solids	40500	Boron	7.6
Suspended Solids	18000	Iron	46.5
Total Volatile Solids	34000	Manganese	2.0
Ammonical Nitrogen	35	Copper	0.89
Total Nitrogen	750	Zinc	2.3

*All in mg/l except pH.

The characteristics of palm oil mill effluent (POME) have made POME as a potential substrate for acetone-butanol-ethanol (ABE) fermentation. However, the cost of substrate has a dramatic influence on the economic viability of ABE production via fermentation. Thus this availability of cheap and readily available sources of substrates such as POME enhances the economic viability of fermentation process for ABE production (Takriff *et al.*, 2009).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemicals and Biological materials

In this research, all chemicals were supplied by Faculty of Chemical and Natural Resources Engineering. The chemicals were bought from Sigma and Merck companies. Chemicals that have been used in this research were reinforced clostridia agar, reinforced clostridia medium, sodium hydroxide (NaOH), potassium sodium tatrate, phenol, sodium sulfite and 3,5 DNS acid, isobutanol, 1-butanol, acetone and ethanol.

The bacteria strain, *Clostridium acetobutylicum* NCIMB 13357 was obtained from Universiti Kebangsaan Malaysia (UKM).

3.1.2 Media.

The fresh sample of palm oil mill effluent (POME) was collected from Kilang Sawit Felda Lepar Hilir, Pahang. The sample was kept in heat resistant bottle and sediment for one day

Reinforced clostridial agar was used for cultivating and enumerating clostridia, anaerobes and other species of bacteria. This medium was superior to others in supporting growth and producing high cell counts of *clostridia*. It contains beef extract dextrose, yeast extract, soluble starch peptone, sodium chloride, sodium acetate, l-cysteine hydrochloride and bacteriological agar. The peptone and beef extract are sources of nitrogen, vitamins and aminoacids. The yeast extract provides b-complex vitamins. Dextrose is a complex carbohydrate and sodium chloride maintains the osmotic balance. The starch detoxifies metabolic by-products. l-cysteine hydrochloride is the reducing agent and sodium acetate is the buffer. Bacteriological agar is the solidifying agent. The final pH was 6.8 at 25°C.

Reinforced Clostridia Medium (RCM) is a semi-solid medium for the enumeration and cultivation of anaerobes. The medium is more fertile and enabled for bacteria growth.

3.2 Equipments

The equipments used in this study were autoclave Hirayama, Bactron Anaerobic Chamber (Sheldon Manufacturing Inc., USA), Hybridization Incubator Shaker model SI-100D, Centrifuge 5810R Eppendorf, Ultravilot-Visible Spectrophotometer (UV-Vis) Hitachi U-1800 and Gas Chromatography Agilent 6890 equipped with flame ionization (GC-FID) detector (Agilent Technology, USA).

3.3 Experimental procedures

3.3.1 Bacteria culturing

3.3.1.1 Agar preparation

52.5g of reinforced clostridial agar powder was suspended in 1 L of distilled water and thoroughly mixed. The mixture was heated with frequent agitation and boiled for 5 minutes to completely dissolve the powder. Then the mixture was sterilized in an autoclave for 20 minutes at 121 ºC. After autoclaving, the agar was poured into the plate under UV light and left it around 30 minutes.

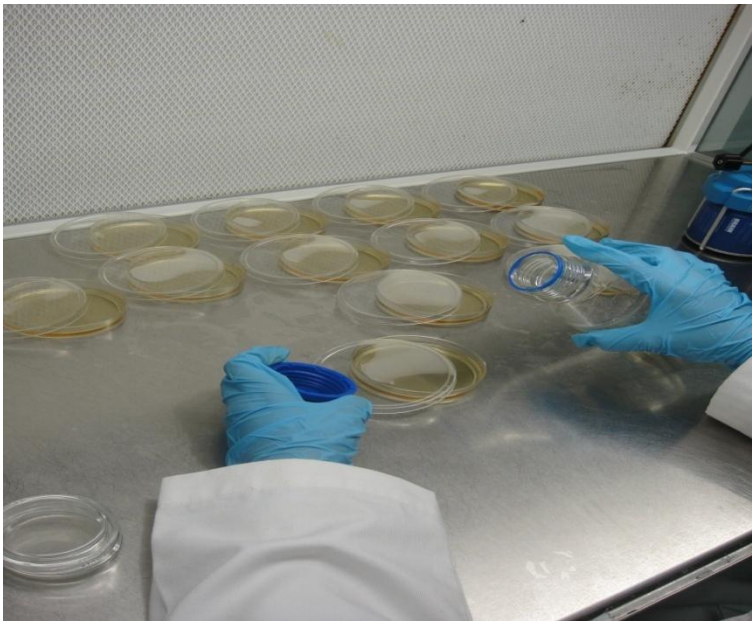

Figure 3.2 Preparation of reinforced clostridial agar in laminar flow.

3.3.1.2 *Clostridium acetobutylicum* cultivation

Strain *Clostridium acetobutylicum* (NCIMB 13357) was used in this study. It was obtained from Universiti Kebangsaan Malaysia (UKM). The strain from the glycerol stock vial was taken by using the toothpicks. A portion of the stock was transferred into the 150mL broth medium. Then, the broth was incubated for 2-3 days in anaerobic chamber at 37°C. After 2 days, the broth was examined. Only the broth with growth of bacteria strain can be inoculated on the agar.

The active strain was inoculated on agar by applying streaking method. All the plates were labeled and dated at the bottom of the plate. The inoculated plates were always sealed and incubated them upside down. This will help prevent contamination and aid in the formation of isolated colonies. Then the plates were moved into the incubator and incubated at 37°C for 24 hours.

After 24 hours inoculating, the plate was examined and only one colony type on the plate was chosen for inoculums preparation. The other plates with good colonies were kept in the chiller 4°C for the next use. They can be maintained for a month.

3.3.1.3 Inoculums preparation

After incubate period, the active growth from the agar was transferred into 250mL conical flask contained 150mL broth medium and was inoculated at 37°C for 2-3 days in anaerobic chamber. Figure 3.2 below shows the Bactron Anaerobic Chamber which helps to maintain the anaerobic condition. After 2 days, the inoculums was transferred in sterile centrifuge tubes and centrifuged at 10 000 rpm at 4°C in 10 minutes.

The supernatant was decanted and replaced with 0.85% saline solution. After that, the solution was centrifuged for one more time. The supernatant was decanted again and replaced with saline solution. The solution was shaken until the pellet totally dissolved. Approximately 5ml of solution was taken out for optical density reading at 660 nm by using UV-Vis Spectrophotometer. Only the inoculums with optical density value that greater than 0.7 can be used for fermentation.

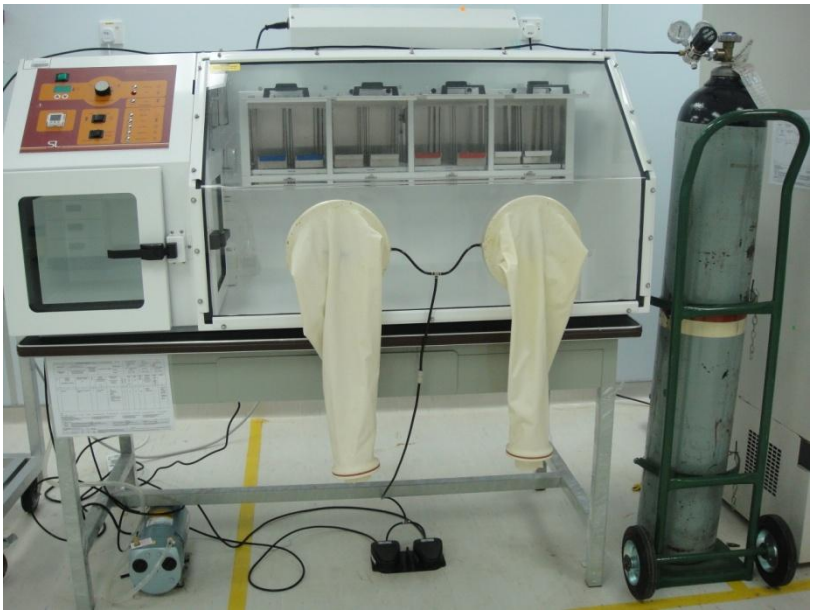

Figure 3.3 Bactron Anaerobic Chamber

3.3.2 Media preparation

3.3.2.1 Pretreatment Palm oil mill effluent (POME)

Samples of palm oil mill effluent (POME) were collected from Kilang Sawit Felda Lepar Hilir, Pahang. Fresh POME was sediment in a chiller at 4°C for 24 hours before use. The supernatant (upper layer) was decanted and sediment POME sludge (lower part) was used as the medium of the fermentation. Initial pH of the POME was adjusted to pH 5.8 by added in 5M sodium hydroxide (NaOH). The pH 5.8 was maintained before the fermentation start. The substrate medium was autoclaved at 121°C for 20 minutes. The concentration of POME used in fermentation was 90%.

3.3.2.2 Reinforced Clostridial Medium (RCM)

38g of the powder was suspended in 1 L of distilled water and thoroughly mixed. The mixture was heated with frequent agitation and boiled for 5 minutes to completely dissolve the powder. Then the mixture was sterilized in an autoclave for 20 minutes at 121ºC. The concentration of RCM used in fermentation was 90%.

3.3.3 Determination of Growth Profile

For the growth profile of the bacteria strain *Clostridium acetobutylicum*, the optimum condition was used to check the pattern of the bacteria growth. The fermentation was run at temperature 37 ºC with the initial pH of palm oil mill effluent (POME) 5.8 and 6.8 for clostridial medium (RCM). The concentration of the substrate

was 90% and inoculums concentration was 10% v/v. An agitation rate was 200rpm. The growth profile of *C.acetobutylicum* in POME and RCM were taken for each 6 hours until the graph shows a constant pattern. UV-VIS Spectrophotometer was used to determine the reading of optical density value of each sample at 660 nm.

3.3.4 Fermentation

In this research, fermentation process was carried out in the 500mL of Schott bottles with working volume of 300 ml under anaerobic condition. 10% v/v of inoculums was transferred into Schott bottle containing reinforced clostridial medium which was our control medium. The same step was applied for Schott bottle containing 90% palm oil mill effluent (POME). After transferring inoculums into medium, the first value of OD was read. Then, capped the Schott bottle and the medium were purged with Nitrogen gas (N_2) at the headspace of the medium.

A clip at a tube of the bottle was opened a little bit for N_2 to pass through. The Nitrogen valve was opened until the bubble appeared and the medium was purged for 30 minutes. The N_2 must be purged every time after the medium of fermentation was exposed to air especially after put the microbes or taking the samples.

For first run, the two bottles of RCM and two bottles of POME are fermented in Hybrid Incubator Shaker at 35°C at constant speed 200 rpm. One of the RCM bottle and one of the POME bottle were run for 48 hours while the others were run for 72 hours. For second and third run, the temperatures that have been run were at 40°C and 45°C for 48 hours and 72 hours for each run. The sample for OD reading was taken every 6 hours while for glucose determination was done for every 18 hours.

3.3.5 Centrifugation

After fermentation, the samples were centrifuged at 5000 rpm for 5 minutes to get the supernatant by using Centrifuge 5810R Eppendorf as shown in Figure 3.3. Centrifugation separates on the basis of the particle size and density difference between the liquid and solid phases. Then the pellet was decanted and the supernatant was used in product analysis.

Figure 3.4 Centrifuge 5810R Eppendorf

3.3.6 Product Analysis

3.3.6.1 Determination of Butanol Production

The concentration of butanol was assayed by a gas chromatography (GC) equipped with high pressure inowax column. The carrier gas was Nitrogen gas (1.2kg/cm^2), with detection using flame ionization detector. The temperatures of injection and detector were 250°C respectively. The column temperature was programmed to change from 50°C to 170°C at a rate 10°C/min. Isobutanol was used as an internal standard.

For sample preparation, each sample was mixed with 4% isobutanol at ratio 1:10. Then the mixture was filtered approximately 1.5mL into GC vial by using 0.2µm syringe filter. The standard that is used to detect concentration of butanol and ethanol in the sample of solution are pure butanol respectively.

3.3.6.2 Determination of Glucose Consumption

Glucose assays were important to detect of reducing sugars in medium. Miller method (1959) was applied to determine the glucose. The 3,5 - Dinitrosalicylic acid (DNS) reagent ingredients were prepared (10g NaOH, 182g Potassium Sodium Tatrate, 2g Phenol, 0.5 g Sodium Sulfite and 10g 3,5 DNS acid). Then the DNS reagent was wrapped with aluminum foil and kept in refrigerator at 4°C.

By following method for 10^2 dilutions, 0.1mL of sample (RCM and POME) was added into 9.9mL of distilled water in test tube and shake vigorously. Then, 1mL from dilution sample was taken and put into another test tube with 1mL DNS reagent and 0.1mL 0.1M NaOH. The solution was boiled at 90°C for 10 minutes. The sample was cooled in the cabinet for few minutes. After cooled, 10mL of distilled water was added and test the solution by using UV- Vis Spectrometer at 540 nm.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter discuss on the results from the experiment that was done in order to produce butanol from *C. acetobutylicum.* Butanol has been produced at different temperature of 35, 40 and 45°C for 48 and 72 hours. The concentration of fermentation medium was set at 90%, agitation rate was at 200 rpm and pH of the culture medium was set constant. For analysis, UV-Visible Spectrometer was used to check the concentration of cell and Gas Chromatography Flame Ion Detector (GCFID) was used to determine the butanol production. Summaries of results are presented into graphs and figures in this chapter. The raw data tables related to the experiments conducted were shown in Appendix section.

4.1 Growth profile of *C.acetobutylicum*

Table 4.1 Growth profile of *C. acetobutylicum* in POME and RCM

Optical density value (abs)		
Time (hour)	POME	RCM
0	0.887	0.553
12	2.175	1.478
24	2.172	1.961
36	2.563	1.704
48	2.543	2.078
60	2.085	1.965
72	2.268	1.688

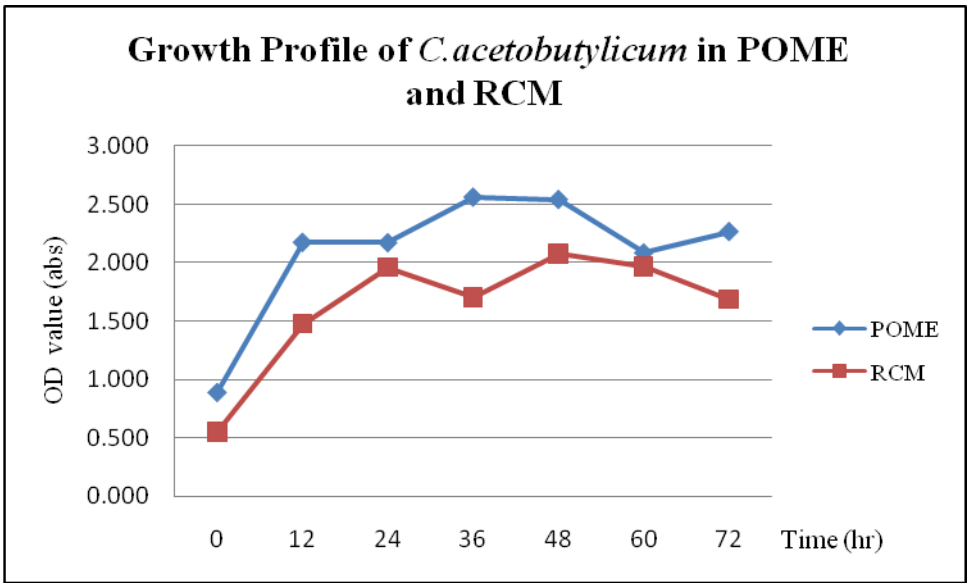

Figure 4.1 Growth profile of *C.acetobutylicum* in POME and RCM as growth medium.

Figure 4.1 showed the growth profile pattern of *C.acetobutylicum* in POME and RCM. The pattern of bacteria growth in both of the medium is slightly the same. Optical density values of growth profile were identified by using UV-Vis Spectrophotometer for every 12 hours for 72 hours. In a spectrophotometer, light was transmitted through a dilution of the culture (usually a 1:10 dilution). As microbe numbers increase, light passing through the culture decreases. The output was often absorbance or optical density. Absorbance was a logarithmic expression of the amount of light that gets through the culture.

From the Figure 4.1, the lag phase was at 0 to 12 hours. At this stage, the cell started to adapt to medium and synthesis new components such as refilled spent materials. After the cells have adapted to the new environment, the cell division occurred at increasing frequency until the maximum growth rate reached.

The log phase occurred when the strains' population was uniform in terms of chemical and physical properties. This phase took place at 12 to 24 hours of the microbial growth. At this stage supposedly the cells were dividing and doubling in number at regular intervals. In this research, for reinforced clostridial medium (RCM), the growth of microbe was slowly increase which it started divide at time 24 hours compared to palm oil mill effluent (POME) medium which was the growth faster. The unbalanced growth happened under certain condition such as change in nutrient level and environmental condition. When level of substrate decreases, it became limiting and no longer sustain maximum growth rate.

During 24 to 48 hours, the total number of viable cells remains constant because of metabolically active cell stop dividing or reproductive. This phase called stationary phase. Possible reasons microbes turned into stationary phase were because of the nutrient limitation or limited oxygen availability. The other possible reasons were because of the decrease in size of microbes and production of starvation proteins. For POME medium, the bacteria were active divided at this time until 48 hours. Death phase exist at time after 48 hours. This phase was which cell deaths exceed cell maturity. Michael and Morgan (2001) assumed that a variety of unusual shapes make them difficult to identify.

The *C.acetobutylicum* only active approximately 48 hours because the bacteria used was not fresh, inactive or too old. After that period, the growth of bacteria slowly decreased. Secondly, improper handling during streaking method, preparing medium and standard inoculums or lack of lab skills also could affect the growth rate of bacteria.

4.2 Butanol production

Butanol has been produced from *C.acetobutylicum* using POME and RCM as growth medium. The other condition or parameters have been controlled and set constant except fermentation time and temperature that have been investigated in this experiment.

4.2.1 Effect of fermentation temperature

Other parameters were set at the optimum condition to study the effect of fermentation temperature. The concentration of the substrate (POME and RCM) was 90% and the inoculum of 10% v/v was used. The agitation rate was set at 200 rpm and the fermentation time was taken about 48 and 72 hours for each run.

The results of butanol production in each of the run were shown in Figure 4.2 and 4.3.

4.2.1.1. Effect of fermentation temperature on butanol production in 48 hours

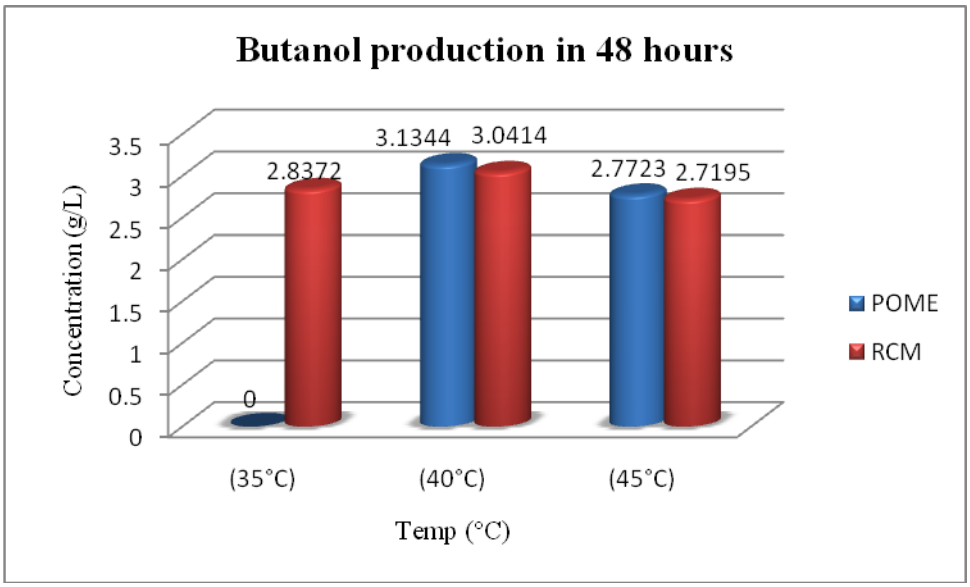

Figure 4.2 The concentration in g/L of butanol produced in POME and RCM throughout 48 hours at 35, 40 and 45°C.

The graph (Figure 4.2) showed the concentration of butanol produced in POME and RCM in 48 hours. It can be observed that the highest concentration of butanol in POME was 3.1344 g/L while the highest production of butanol in RCM was 3.0414 g/L. In POME, there was no production of butanol at 35°C while at 45°C, the butanol produced was 2.7723 g/L. On the other hand, butanol in RCM at 35°C and 45°C were 2.8372 g/L and 2.7195 g/L.

4.2.1.2 Effect of fermentation temperature on butanol production in 72 hours

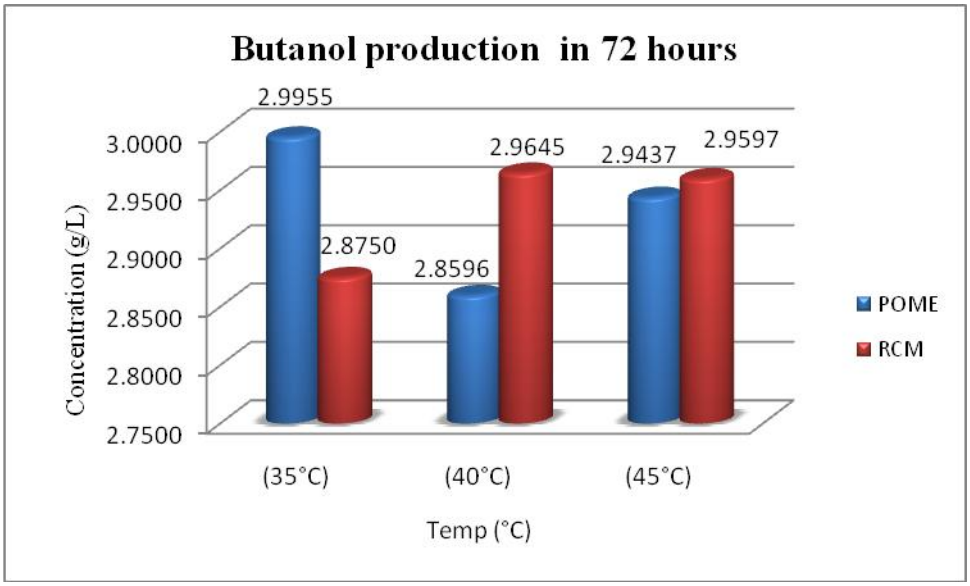

Figure 4.3 The concentration in g/L of butanol produced in POME and RCM throughout 72 hours at 35, 40 and 45°C.

The graph (Figure 4.3) showed the concentration of butanol produced in POME and RCM in 72 hours. It can be observed that the highest concentration of butanol in POME was 2.9955 g/L while the highest production of butanol in RCM was 2.9645 g/L. In POME, the butanol produced was low at 40°C (2.8596g/L). At 45°C, the production was 2.9437 g/L. On the other hand, butanol in RCM at 35°C and 45°C were 2.8750 g/L and 2.9597 g/L.

4.2.2 Effect of fermentation time

Other parameters were set at the optimum condition to study the effect of fermentation time. The concentration of the substrate (POME and RCM) was 90% and the inoculum of 10% v/v was used. The agitation rate was set at 200 rpm and the fermentation temperature was at 35, 40 and 45°C for each run.

The results of butanol production in each of the run were shown in Figure 4.4, 4.5 and 4.6.

4.2.2.1 Effect of fermentation time in butanol production at 35°C

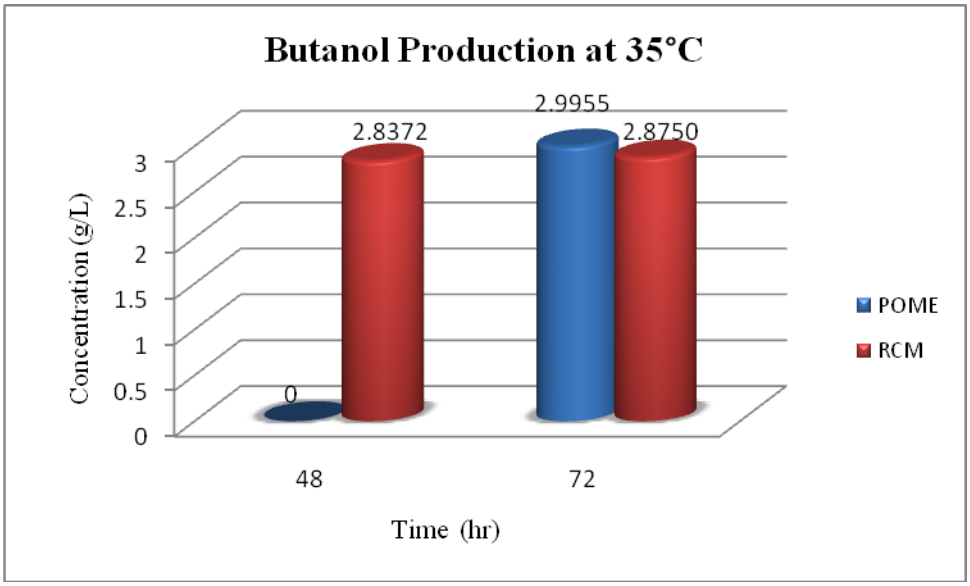

Figure 4.4 The concentration in g/L of butanol produced in POME and RCM for 48 and 72 hours at 35°C

4.2.2.2 Effect of fermentation time in butanol production at 40°C

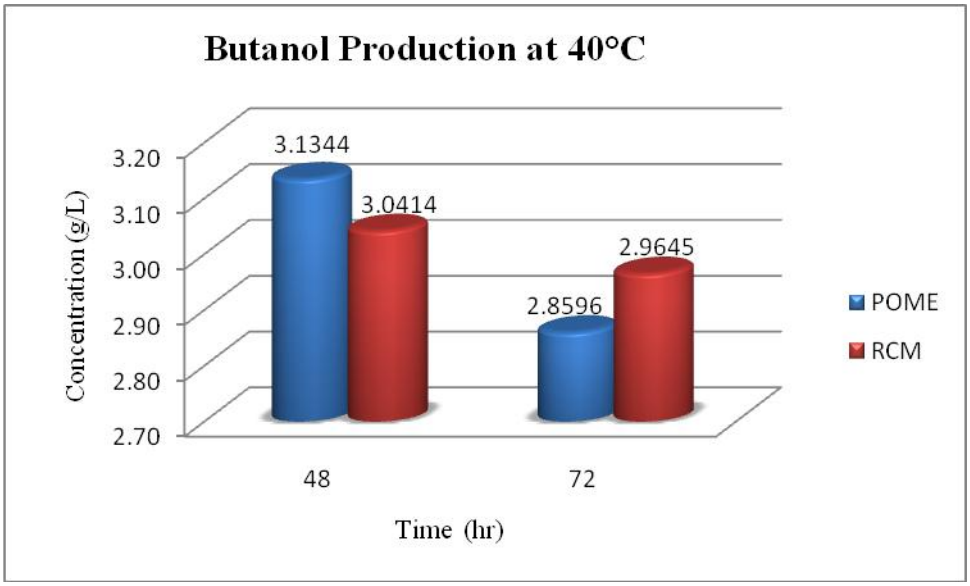

Figure 4.5 The concentration in g/L of butanol produced in POME and RCM for 48 and 72 hours at 40°C

4.2.2.3 Effect of fermentation time in butanol production at 45°C

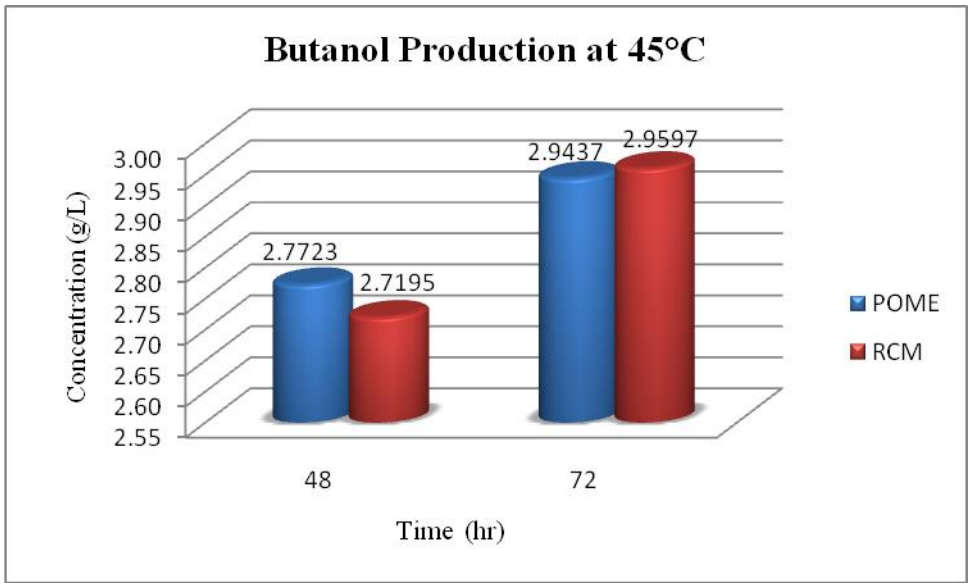

Figure 4.6 The concentration in g/L of butanol produced in POME and RCM for 48 and 72 hours at 45°C

4.2.3 Overall study on butanol production in POME

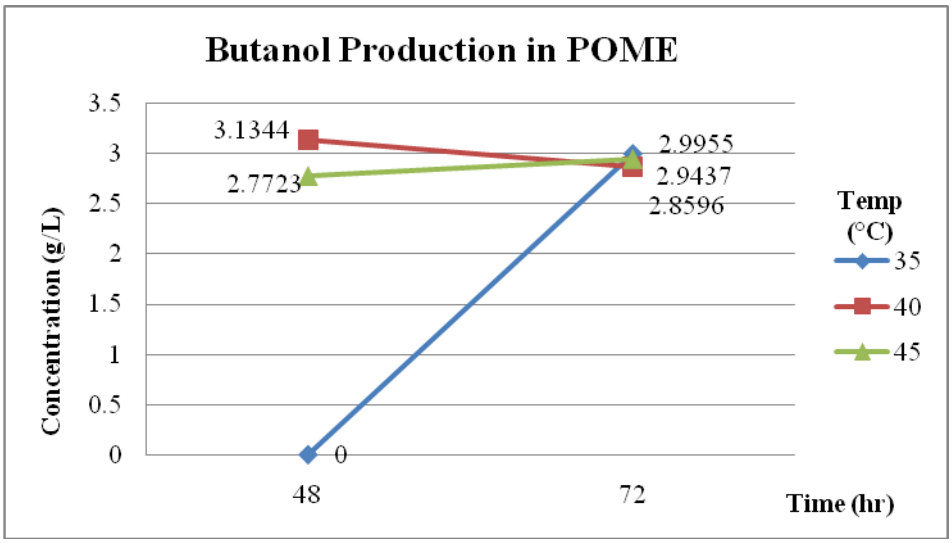

Figure 4.7 The concentration in g/L of butanol produced in POME for 48 and 72 hours for control different fermentation temperature (35°C, 40°C and 45°C)

4.2.4 Overall study on butanol production in RCM

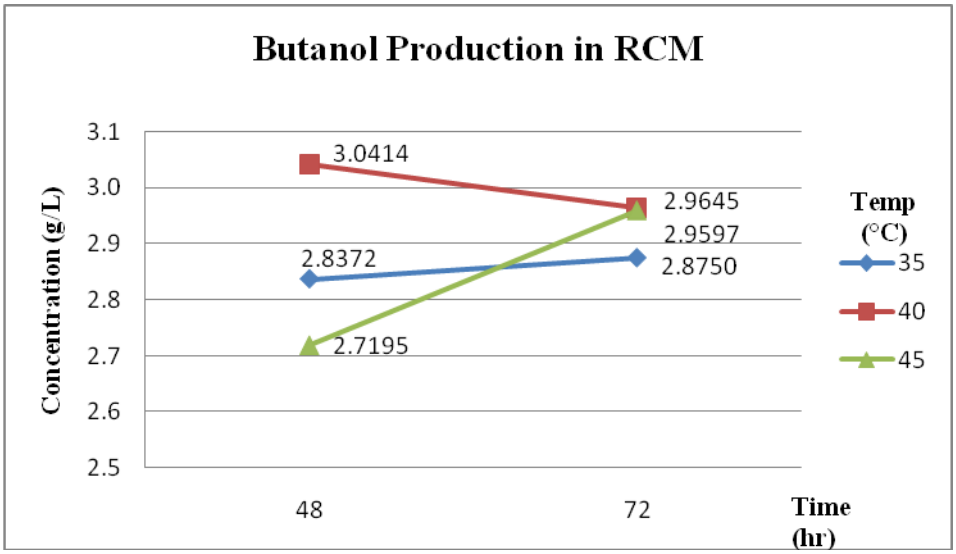

Figure 4.8 The concentration in g/L of butanol produced in RCM for 48 and 72 hours for control different fermentation temperature (35°C, 40°C and 45°C)

Figure 4.7 and 4.8 showed the overall production of butanol in POME and RCM at temperature 35, 40 and 45°C for 48 and 72 hours. Product butanol was determined by Gas Chromatography equipped with flame- ionization detector (GCFID). In the medium of palm oil mill effluent (POME), the production of butanol was detected to be highest during 40°C and 48 hours with value 3.1344g/L. This production of butanol was greater than previous study which Jones and Wood stated in the journal 1986, the ABE obtained was 1.5-2.0 g/L and this value was greater than reported by Khaw *et al.* (1999) which was only 0.94 g/L ABE produced by *C.acetobutylicum.*

From the result, a greater efficiency of conversion to butanol has been produced. According to Lin and Blaschek (1982), based on the metabolic pathways in *C.acetobutylicum*, the metabolism is such that acids are produced first followed by a shift in the fermentation to acetone and butanol. Although the mechanism is still under debate, the timing and magnitude of this shift appear depend on the pH and composition of acetate and butyrate. The possibility was the faster growth rate and higher final stationary-phase cell population together with more rapid attainment of the pH breakpoint, resulted in greater amount of butanol being produced much earlier in the fermentation.

Microorganisms encounter dynamic environmental challenges during butanol fermentation including high concentration of substrates, limitation of nutrients, sensitivity to acetic acid, rapid shifts of pH and temperatures. The butanol toxicity is one of the major barriers for commercial scale production of this fuel and chemical. The main limitation of ABE fermentation relates to the butanol toxicity on test microorganism, leading to low solvents synthesis. According to Qureshi and Maddox (1991), higher initial sugar concentration cannot be used because of butanol toxicity.

The bacterial responses or adaptations to butanol stress were initially examined at a physiological level, looking at the changes of cell membrane compositions. Butanol, as an organic solvent, tends to partition into the cytoplasmic membranes and changes the

membrane structures interfering with normal functions. The *C. acetobutylicum* cells synthesized increased levels of saturated acyl chains accompanied by decreased unsaturated chains in the presence of butanol (Liu and Qureshi, 2009)

Result from Figure 4.7 showed that the highest production of butanol in palm oil mill effluent (POME) was at 40°C and 48 hours. Lin and Blaschek (1982), have stated that the optimum fermentation temperature was at 37°C and optimum fermentation time was 96 hours. At temperature 35°C and 45°C, the strain unsuccessfully produced high yield of butanol because condition of the strain was not suitable for growth and produce ABE product. Only at temperature 40°C, *C.acetobutylicum was* able to produce high butanol product. This strain can survive for only 72 hours because it was not fresh and too old.

4.3 Glucose Consumption

Figure 4.9, 4.10, 4.11, 4.12 and 4.13 below showed the glucose consumption in POME and RCM at temperature 35, 40 and 45°C. The samples were taken for every 18 hours for glucose concentration.

4.3.1. Glucose consumption in POME and RCM at 35°C

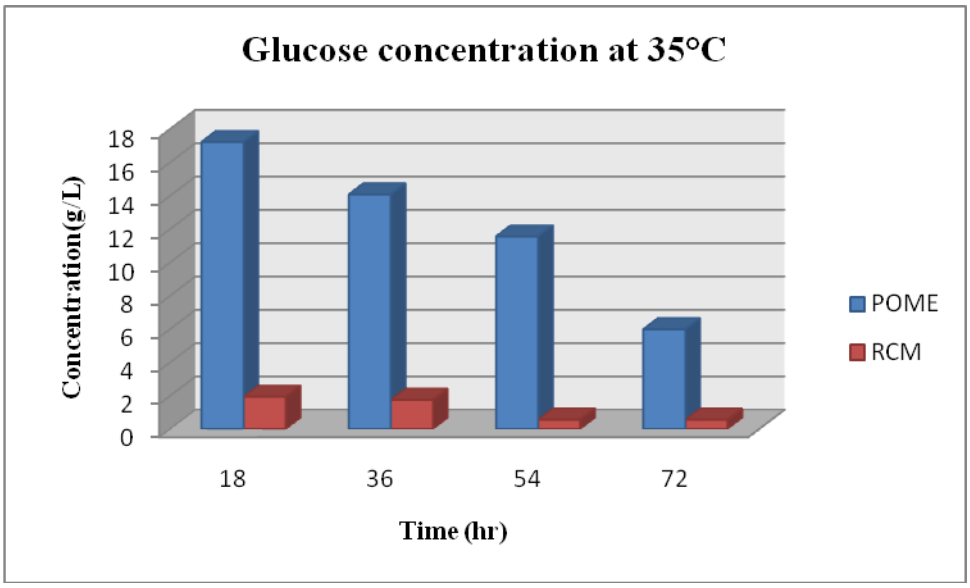

Figure 4.9 The concentration of glucose consumption in g/L at 35°C

Figure 4.9 showed the concentration of glucose in POME and RCM at 35°C throughout 72 hours. The concentration of glucose in POME and RCM was decreased as the increased of fermentation time. The value of glucose concentration in POME and RCM was decreased rapidly from initial value of 17.227g/L to 5.987g/L and 1.939g/L to 0.561g/L.

4.3.2 Glucose consumption in POME and RCM at 40°C

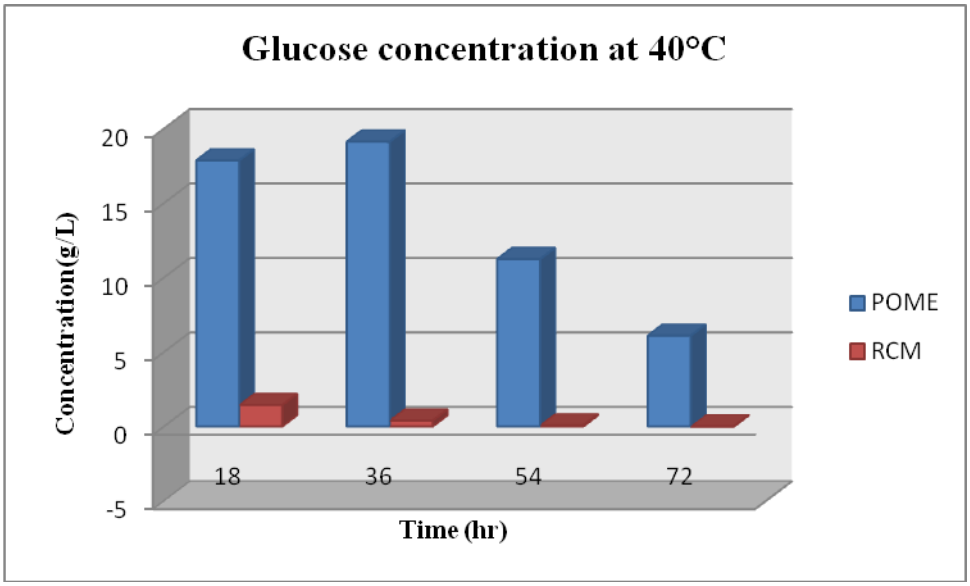

Figure 4.10 The concentration of glucose consumption in g/L at 40°C

Figure 4.10 showed the concentration of glucose in POME and RCM at 40°C throughout 72 hours. The concentration of glucose in POME and RCM was decreased as increased of fermentation time. The value of glucose concentration in POME and RCM was decreased rapidly from initial value of 17.86g/L to 6.092g/L and 1.432g/L to zero.

4.3.3 Glucose consumption in POME and RCM at 45°C

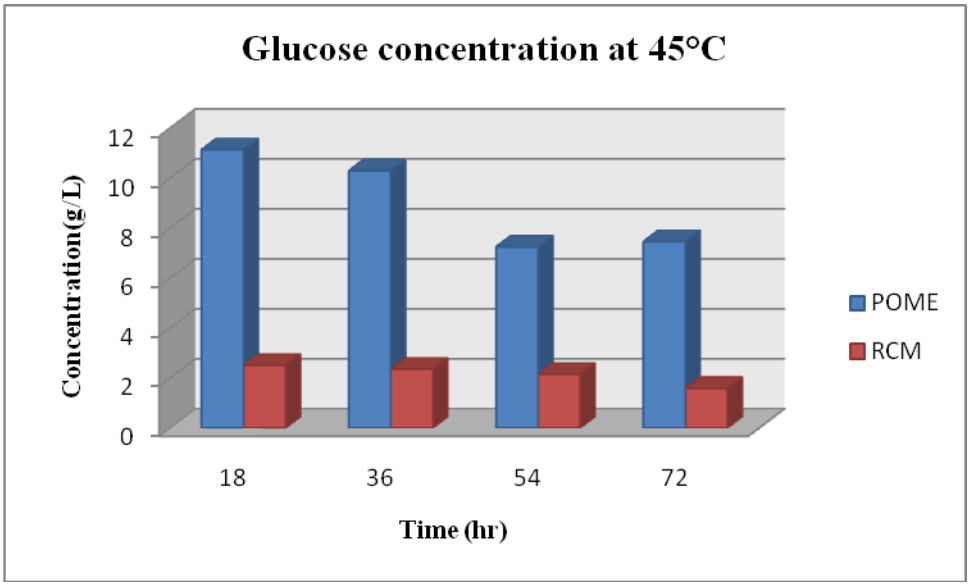

Figure 4.11 The concentration of glucose consumption in g/L at 45°C

Figure 4.11 showed the concentration of glucose in POME and RCM at 45°C during 72 hours. The concentration of glucose in POME and RCM was decreased as increased of fermentation time. The value of glucose concentration in POME and RCM was decreased rapidly from initial value of 11.134g/L to 7.458g/L and 2.512g/L to 1.591g/L.

4.3.4 Overall study of glucose consumption in POME

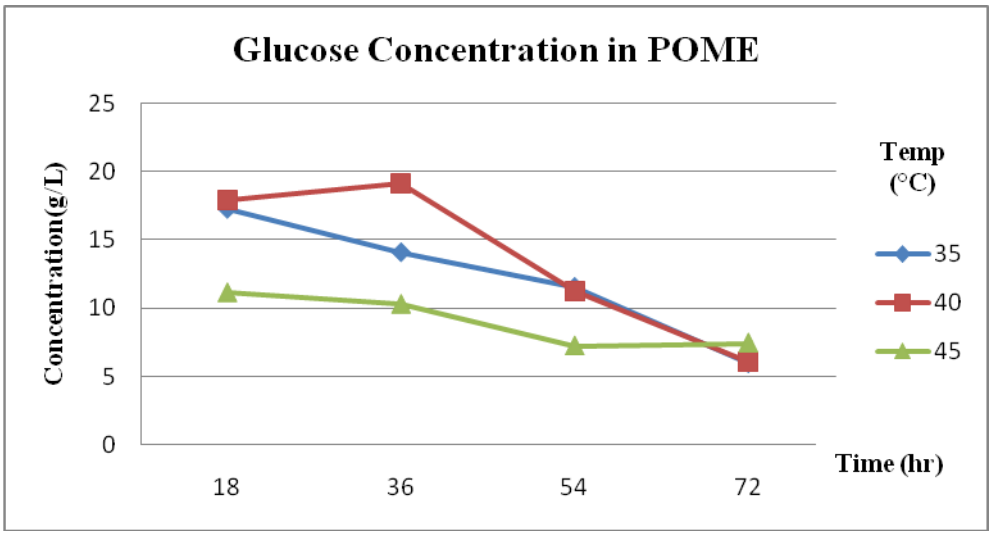

Figure 4.12 The concentration of glucose consumption in g/L in POME throughout 72 hours

4.3.5 Overall study of glucose consumption in RCM

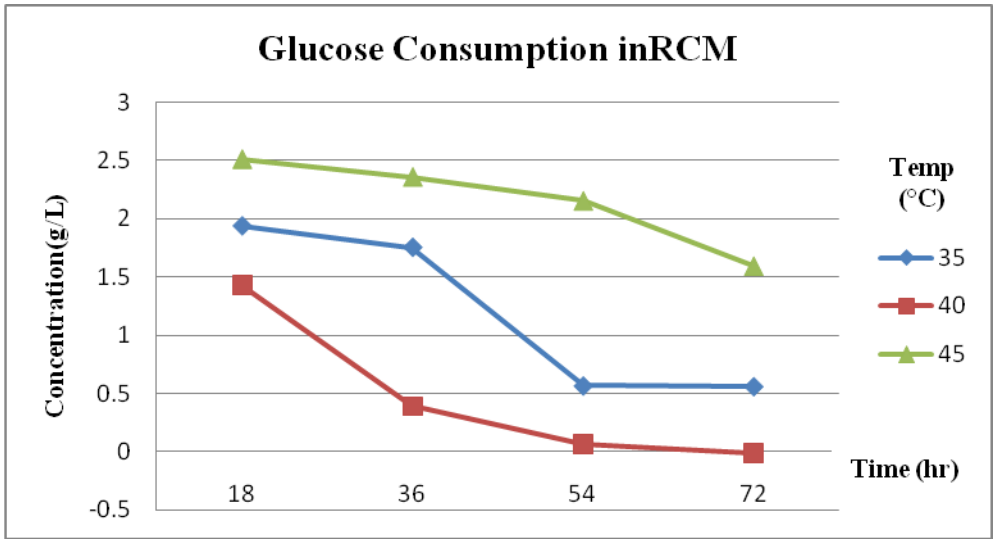

Figure 4.13 The concentration of glucose consumption in g/L in RCM throughout 72 hours

From the Figure 4.12 and 4.13, the concentration of the glucose in POME was decreased as time increased. The concentration of glucose declined from 17.86 g/L to 6.092 g/L at 40°C. At temperature 35°C and 45°C, the graph pattern was same. This pattern of graph is supported by research on bioconversion of industrial wastewater from palm oil processing to butanol by using clostridia (Hipolito *et al.,* 2008). The research reported that as the cells growth, the concentration of sugar will be decreased.

The concentration of sugar was decreased because the glucose was used during fermentation process as a nutrient growth. As a growth medium, the total sugar and the nutrients in sludge efficiently supported for microbe development and could be used to raise any amount of inoculums needed for fermentation process. The sludge offered additional benefits as a source of nutrients in high concentrated glucose medium used for butanol production and would reduced the amount of nutrients in the medium.

According to Kalil *et al.* (2003), in batch fermentation, there will be no addition of glucose or nutrient because *C.acetobutylicum* was able to grow in POME without addition of nutrients and ABE produced nearly 1g/L after 48 hours incubation. Tashiro *et al.,* (2004) have stated that when only butyric acid was fed, butanol production was similar to that without feeding butyric acid after glucose depletion.

When a solution of butyric acid containing glucose was fed, butyric acid utilization and butanol production were observed. Butyric acid was reutilized and converted to butanol via three metabolic enzymes such as acetoacetyl- CoA: acetate/butyrate: CoA transferase, NADH-dependent butyraldehyde dehydrogenase and NADH-dependent butanol dehydrogenase. Since these dehydrogenases require reducing power, such as NADH obtained by glycolysis from glucose, no butanol production by feeding of only butyric acid may result from insufficient NADH due to the absence of glucose. Consequently, energy sources like glucose are necessary for butyric acid utilization and butanol production.

CHAPTER 5

CONSLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the experiment, it can be concluded that the growth of the bacteria for fermentation has produced the butanol. The importance of the growth profile is to indicate the most suitable time in the growth of bacteria for highest butanol production. For the butanol production, the highest butanol produced was 3.1344 g/L. End product inhibition might occur if the fermentation proceeds for a long time and production of butanol might be decreased. Glucose has high influence towards the production of butanol in fermentation. The concentration of glucose was decreased as time increased because the used of glucose by *C.acetobutylicum* as a nutrient growth during the fermentation. The determination of glucose consumption also has been successfully done.

As a conclusion, butanol is suitable to be produced from palm oil mill effluent (POME) by using *C.acetobutylicum* in anaerobic condition at temperature 40°C through 48 hours. POME is a viable media for acetone-butanol-ethanol (ABE) fermentation.

5.2 Recommendations

There are several recommendations to this study in order to increase the production of butanol and improve the experiments methods.

- This experiment used the sediment POME 90% concentration. According to Kalil *et al.* (2003), higher concentration of POME gave better results because sedimentation helped to remove the traces of oil and soluble toxic substances leaving less inhibitory POME which is more suitable for growth of Clostridia. To get higher product, the concentration can be increased into 100%.

- It is suggested that research on other parameters that effect on butanol production should be carried out. Parameters such as inoculums concentration and pH of substrate can be investigated to optimize the production of butanol. The concentration of ABE will increase with the increase of initial pH up to pH 5.8. Jones and Wood (1986) reported that the production of ABE was optimum at the initial values of 5.0-6.5 by using other clostridia strains.

- Use of the fed batch culture with pH-stat continuous butyric acid and glucose feeding method can improve the production of butanol. Tashiro *et al.* (2004) reported that the addition of acetic acid and butyric acid to cultures of *C.acetobutylicum* and *C.beijerinkii* will increase the yield and production of solvents. It was observed that butyric acid elevated the specific butanol production rate, acetone-butanol production and yield of solvent with the increasing the added butyric acid concentration, although acetic acid only could enhance acetone production. When only butyric acid was fed, butanol production was similar to that without feeding butyric acid after glucose depletion. However, when a solution of butyric acid containing glucose was fed, butyric acid utilization and butanol production was observed.

- Another possible way to improve the economic efficacy of biobutanol production is to increase the butanol ratio by eliminating the production of other solvent by-products such as acetone. In this way, the cost of product separation can also be reduced in the acetone-butanol-ethanol (ABE) process. Genetic modification of *C.acetobutylicum* to further increase the butanol ratio by eliminating acetone production. Shao *et al.* (2007) used TargeTron technology to completely disrupt the *adc* (acetoacetic acid decarboxylase) gene in *C.acetobutylicum*, to vercome the problem leaky expression during antisense RNA downregulation (Jiang *et al.*, 2009).

REFERENCES

Alalayah, W. M., Kalil, M. S., Kadhum, A. A., Jahim, J. & Alauj, N. (2009). Effect of Environmental Parameters on hydrogen Production using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). *American Journal of Environmental Sciences,* 5(1): 80 – 86.

Ezeji, T., Qureshi, N. & Blascheck, H. P. (2007). Butanol Production from Agriculture Residues: Impact of Degradation Products on *Clostridium beijerinckii* Growth and Butanol Fermentation. *Biotechnology Bioengineering*. 97: 1460-1469.

Fei, Y. K. & Teong, L. K. (2008). Palm Oil As Feedstocks For Biodiesel Production Via Heterogeneous Transesterification: Optimization Study. *International Conference on Environment* (Icenv 2008).

Hipolito, C. N., Crabbe, E., Badillo, C. M., Zarrabal, O. C., Mora, M. M., Flores, G.P.,Cortazar,M.H. & Ishizaki,A. (2008). Bioconversion Of Industrial Wastewater From Palm Oil Processing to Butanol by *Clostridium Saccharoperbutylacetonicum N1-4* (ATCC 13564). *Journal of Cleaner Production,* 16: 632-638.

Hwang, M. H., Jang, N. J., Hyun, S. H. & Kim, I. S. (2004). Anaerobic Bio hydrogen Production from Ethanol fermentation. *Journal of Biotechnology*, 111: 297-309.

Jiang, Y., Xu, C., Dong, F., Yang, Y., Jiang, W. & Yang, S. (2009) Disruption of The Acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* Increase the Butanol Ratio. *Metabolic Engineering*, 11: 284 291.

Jones, D. T. & Woods, D. R. (1986). Acetone-butanol fermentation revisited. *Microbiol*. *Rev*. 50(94): 484–524.

Kalil, M. S., Kit, P. W., Yusoff, W. M. W., Sadazo,Y. & Rahman, R. A. (2003). Direct Fermentation of Palm Oil Mill Effluent to Acetone-butanol-ethanol by Solvent Producing Clostridia. *Pakistan Journal of Biological Sciences.* 6(14): 1273-1275.

Keis, S., Shaheen, R., & Jones, D. T. (2001). Emended Descriptions of *Clostridium acetobutylicum* and *Clostridium beijirinckii*, and Descriptions of *Clostridium saccharoperbutylacetonicum* sp. Nov. and *Clostridium saccharobutylicum* sp. Nov. *International Journal of Systematic and Evolutionary Microbiology*, 51: 2095-2103.

Khaw, T. S., Arbakariya, A., H. Mohd. Ali & Ismail, A. K. (1999). Utilization of Fermentable Sugars from Oil Palm Empty Fruit Bunch (OPEFB) Fibers for Acetone-Butanol-Ethanol (ABE) Fermentation. *Seminar of Efficient Use of Palm Oil Waste as Renewable Resource for Energy and Chemical, Bali, Indonesia.*

Kobayashi, G., Eto, K., Tashiro, Y., Okuba, K., Sonomoto, K., & Ishizaki, I. (2005). Utilization of Excess Sludge by Acetone-Butanol-Ethanol Fermentation Employing *Clostridium Saccharoperbutylacetonicum* N1-4 (ATCC 13564). *Journal of Bioscience and Bioengineering*, 99(5): 517-519.

Lin, Y. L. & Blaschek, H.P. (1983). Butanol Production by a Butanol-Tolerant Strain of *Clostridium acetobutylicum* in Extruded Corn Broth. *Applied and Environmental Microbialogy*. 45(3): 966-973.

Lin, Y.T., Labbe, R.G. & Shetty, K. (2005). Inhibition of Vibrio Parahaemolyticus in Seafood Systems Using Oregano and Cranberry Phytochemical Synergies and Lactic Acid. *Innovative Food Sci Emerging Technology,* 6:453–8.

Lorestani, A. A. Z. (2006). Biological Treatment of Palm Oil Mill Effluent (POME) Using An Up-Flow Anaerobic Sludge Fixed Film (Uasff) Bioreactor.

Ma, A. N. (2000). Environmental Management for the Palm Oil Industry. *Palm Oil Dev.*, 30:19.

Miura, K., Masuda, T., Funazukuri, T., Sugawara, K., Shirai, K., Hayashi, J., Karim, M. I., Ani, F. N. & Susanto, H. (2001). Efficient Use of Oil Palm Wastes as Renewable Resource for Energy & Chemicals.

O-Thong, S., Prasertsan, P., Intrasungkha, N., Dhamwichukorn, S., & Birkeland, N. (2008). Optimization of Simultaneous Thermophilic Fermentative Hydrogen Production and COD Reduction from Palm Oil Mill Effluent by Thermoanaerobacterium-Rich Sludge. *Journal of Hydrogen Energy,* 33: 1221-1231.

Qureshi, N., Saha, B. C. & Cotta, M. A. (2008). Butanol Production from Wheat Straw by Simultaneous Saccrification and Fermentation Using *Clostridium Beijerinckii*: Part II- Fed batch Fermentation. *Biomass and Bioenergy,* 32: 1/6-183.

Shao, L. (2009). Targeted Gene Disruption by Use of a Group II Intron (Targetron) Vector in *Clostridium acetobutylicum*. *Cell Res.*, 17: 963-965.

Shapovalov, O. I. & Ashkinazi L. A. (2008). Biobutanol: Biofuel of Second Generation. *Russian Journal of Applied Chemistry*. 81(12): 2232 – 2236.

Shinto, H., Tashiro, Y., Kobayashi, G., Sekiguchi, T., Hanai, T., Kuriy, Y., Okamoto, M. & Sonomoto, K. (2008). Kinetic study of substrate dependency for higher butanol production in acetone–butanol–ethanol fermentation. *Process Biochemistry*, 43: 1452–1461.

Sillers, R., Al-Hinai, M. A. & Papoutsakis, E. T. (2009). Aldehyde-alcohol Dehydrogenase and/or thiolase Overexpression Coupled with CoA Transferase Downregulation Fermentations. *Biotecnology Bioengineering*, 102: 38-49.

Somrutai, W., Takagi, M. & Yoshida, T. (1996). Acetone-Butanol Fermentation by *Clostridium aurantibutyricum* ATCC1777 from a Model Medium for Palm Oil Mill Effluent. *International Center for Biotechnology*. 81(6): 543-547.

Takriff, M. S, Masngut, N., Khadum, A. A. H., Khalil. M. S. & Mohamad, A. W. (2009). Solvent Fermentation from Palm Oil Mill Efluent using *Clostridium acetobutylicum* in Oscillatory Flow Bioreactor. *Sains Malaysiana*, 38(2): 191-196.

Tashiro, Y., Takeda, K., Kobayashi, G., Sonomoto, K., Ishizaki, A., & Yoshino, S. (2004). High Butanol Production by *Clostridium Saccaharoperbutylacetonicum* N1-4 in Fed Batch Culture with pH Stat Continuous Butyric Acid and Glucose Feeding Method. *Journal of Bioscience and Bioengineering*, 98(4): 263-268.

Uzel, A., Sorkun, K., Önçağ, Ö., Çoğulu, D., Gençay, Ö., Sali ̇h B. (2005). Chemical Compositions and Antimicrobial Activities of Four Different Anatolian Propolis Samples. *Microbiol Res*, 160:189–95.

Wu, T. Y., Mohammad, Q. W., Jahim, J. M. & Anuar, N. (2007). Palm oil mill effluent (POME) treatment and bioresources recovery using ultrafiltration membrane: Effect of pressure on membrane fouling. *Biochemical Engineering Journal*, 35: 309–317.

Wu, T. Y., Mohammad, Q. W., Jahim, J. M. & Anuar, N. (2009). A Holistic Approach to Managing Palm Oil Mill Effluent (POME): Biotechnological Advances in The Sustainable Reuse of POME. *Biotechnology Advances*, 27: 40–52.

Zhao, Y., Hindorff, L. A., Chuang, A., Monroe-Augustus, M., Lyristis, M., Harrison, M. L., Rudolph, F. B., & Bennett, G. N. (2003). Expression of a Cloned Cyclopropane Fatty Acid Synthase Gene Reduces Solvent Formation in *Clostridium acetobutylicum* ATCC 824. *Applied and Environmental Microbiology*. 2831–2841.

APPENDIX A

GROWTH PROFILE

APPENDIX A-1: Optical Density value of *C.acetobutylicum* in POME and RCM

Table A-1-1: Optical Density value of *C.acetobutylicum* growth

SAMPLE	POME (abs)				RCM (abs)			
READING / TIME (hr)	1	2	3	Ave	1	2	3	Ave
0	0.89	0.89	0.89	**0.89**	0.55	0.55	0.55	**0.55**
6	1.46	1.46	1.46	**1.46**	0.59	0.59	0.59	**0.59**
12	2.18	2.18	2.18	**2.18**	1.48	1.48	1.48	**1.48**
18	2.18	2.18	2.18	**2.18**	1.66	1.66	1.66	**1.66**
24	2.17	2.17	2.17	**2.17**	1.96	1.96	1.96	**1.96**
30	2.38	2.38	2.38	**2.38**	1.69	1.69	1.69	**1.69**
36	2.56	2.56	2.56	**2.56**	1.70	1.70	1.70	**1.70**
42	2.75	2.75	2.75	**2.75**	1.81	1.81	1.81	**1.81**

48	2.54	2.54	2.54	**2.54**	2.08	2.08	2.08	**2.08**
54	2.16	2.16	2.16	**2.16**	1.99	1.99	1.99	**1.99**
60	2.08	2.08	2.08	**2.08**	1.97	1.97	1.97	**1.97**
66	2.36	2.36	2.36	**2.36**	1.64	1.64	1.64	**1.64**
72	2.27	2.27	2.27	**2.27**	1.69	1.69	1.69	**1.69**

APPENDIX B

BUTANOL PRODUCTION

APPENDIX B-1: Standard of Butanol

Table B-1-1: Standard of Butanol

CONCENTRATION (%)	PEAK AREA (Pa*s)
1.0	197.7136
2.0	397.1444
3.0	614.6014
4.0	839.5170
5.0	990.3093

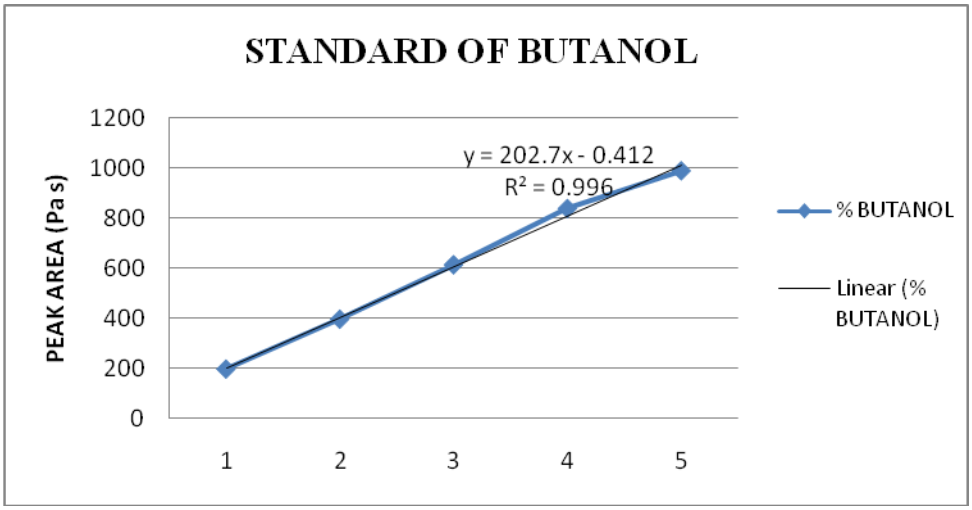

Figure A-1-1 Standard Curve for Butanol Production

APPENDIX B-2: Effect of fermentation temperature

APPENDIX B-2-1: Effect of fermentation temperature on butanol production in 48 hours

Table B-2-1: Butanol Production in g/L by 48 hours

TEMPERATURE (°C) / SAMPLE	35	40	45
POME	0	3.1344	2.7723
RCM	2.8372	3.0414	2.7195

APPENDIX B-2-2: Effect of fermentation temperature on butanol production in 72 hours

Table B-2-2: Butanol Production in g/L by 72 hours

TEMPERATURE (°C) / SAMPLE	35	40	45
POME	2.9955	2.8596	2.9437
RCM	2.8750	2.9645	2.9597

APPENDIX B-3: Effect of fermentation time

APPENDIX B-3-1: Effect of fermentation time in butanol production at 35°C

Table B-3-1: Butanol Production in g/L at 35°C

TIME (hours) / SAMPLE	48	72
POME	0	2.9955
RCM	2.8372	2.8750

APPENDIX B-3-2: Effect of fermentation time in butanol production at 40°C

Table B-3-2: Butanol Production in g/L at 40°C

TIME (hours) / SAMPLE	48	72
POME	3.1344	2.8596
RCM	3.0414	2.9645

APPENDIX B-3-3: Effect of fermentation time in butanol production at 45°C

Table B-3-3: Butanol Production in g/L at 45°C

TIME (hours)	48	72
SAMPLE		
POME	2.7723	2.9437
RCM	2.7195	2.9597

APPENDIX B-4: Overall study on butanol production

APPENDIX B-4-1: Overall study on butanol production in POME

Table B-4-1: Butanol production in POME

TIME (hours) / TEMPERATURE (°C)	48	72
35	0	2.9955
40	3.1344	2.8596
45	2.7723	2.9437

APPENDIX B-4-2: Overall study on butanol production in RCM

Table B-4-2: Butanol production in RCM

TIME (hours) / TEMPERATURE (°C)	48	72
35	2.8372	2.8750
40	3.0414	2.9645
45	2.7195	2.9597

APPENDIX C

GLUCOSE CONSUMPTION

APPENDIX C-1: Standard curve of glucose

Table C-1-1: Concentration of Glucose

CONCENTRATION (g/L)	Optical Density
0.0	0
0.2	0.092
0.4	0.368
0.6	0.524
0.8	0.710
1.0	0.931

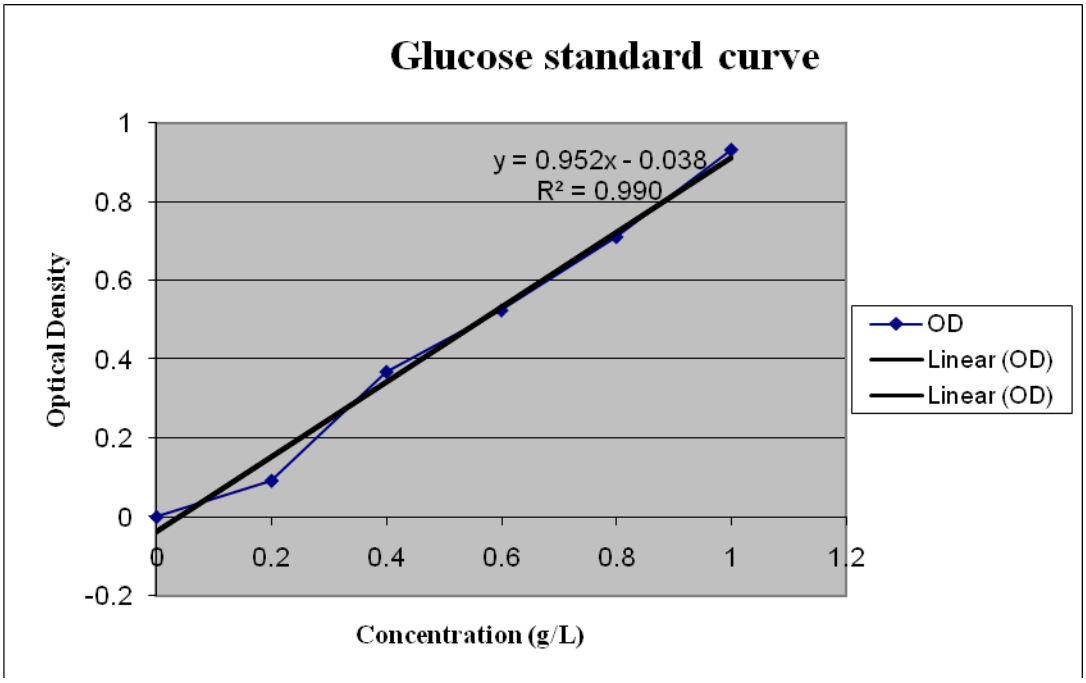

Figure C-1-1: Concentration of Glucose

The standard curve equation:

$$y = 0.952x - 0.038$$

$$R^2 = 0.990$$

APPENDIX C-2: Concentration of glucose

APPENDIX C-2-1: Concentration of glucose (35°C, 48 hours)

Table C-2-1: Optical density value of glucose consumption

SAMPLE	POME (abs)				RCM (abs)			
READING / TIME (hr)	1	2	3	Ave	1	2	3	Ave
18	0.088	0.090	0.092	0.090	1.209	1.208	1.216	1.211
36	0.063	0.060	0.069	0.064	0.440	0.452	0.447	0.446
48	0.043	0.043	0.043	0.043	0.202	0.202	0.202	0.202

APPENDIX C-2-2: Concentration of glucose (35°C, 72 hours)

Table C-2-2: Optical density value of glucose consumption

SAMPLE	POME (abs)				RCM (abs)			
READING / TIME (hr)	1	2	3	Ave	1	2	3	Ave
18	0.126	0.128	0.123	0.126	1.805	1.821	1.797	1.808
36	0.071	0.073	0.071	0.072	1.638	1.631	1.629	1.633
54	0.017	0.019	0.021	0.019	0.502	0.501	0.504	0.502
72	0.085	0.099	0.105	0.096	0.559	0.570	0.358	0.496

APPENDIX C-2-3: Concentration of glucose (40°C, 48 hours)

Table C-2-3: Optical density value of glucose consumption

SAMPLE	POME (abs)				RCM (abs)			
READING TIME (hr)	1	2	3	Ave	1	2	3	Ave
18	0.052	0.045	0.000	0.032	0.375	0.375	0.375	0.375
36	0.017	0.017	0.017	0.017	0.793	0.793	0.793	0.793
48	0.015	0.015	0.015	0.015	0.200	0.200	0.200	0.200

APPENDIX C-2-4: Concentration of glucose (40°C, 72 hours)

Table C-2-4: Optical density value of glucose consumption

SAMPLE	POME (abs)				RCM (abs)			
READING TIME (hr)	1	2	3	Ave	1	2	3	Ave
18	-0.007	-0.050	-0.007	-0.021	1.325	1.325	1.325	1.325
36	0.077	0.177	0.177	0.144	0.333	0.333	0.333	0.333
54	0.069	0.069	0.069	0.069	0.026	0.026	0.026	0.026
72	0.020	0.020	0.020	0.020	-0.013	-0.144	0.000	-0.052

APPENDIX C-2-5: Concentration of glucose (45°C, 48 hours)

Table C-2-5: Optical density value of glucose consumption

SAMPLE	POME (abs)				RCM (abs)			
READING / TIME (hr)	1	2	3	Ave	1	2	3	Ave
18	0.040	0.184	0.029	0.084	1.943	1.760	1.943	1.882
36	0.036	0.026	0.036	0.033	1.555	1.025	1.025	1.202
48	0.047	0.015	0.047	0.036	1.670	1.670	1.670	1.670

APPENDIX C-2-6: Concentration of glucose (45°C, 72 hours)

Table C-2-6: Optical density value of glucose consumption

SAMPLE	POME (abs)				RCM (abs)			
READING / TIME (hr)	1	2	3	Ave	1	2	3	Ave
18	0.070	0.065	0.070	0.068	2.366	2.347	2.347	2.353
36	0.041	0.010	0.041	0.031	2.208	2.208	2.208	2.208
54	0.043	0.028	0.028	0.033	2.000	2.018	2.018	2.012
72	0.036	0.109	0.036	0.060	1.509	1.414	1.509	1.477

APPENDIX D

Figure D-1: Active culture of *C.acetobutylicum* in agar slant

Figure D-2: Incubator 36.9°C

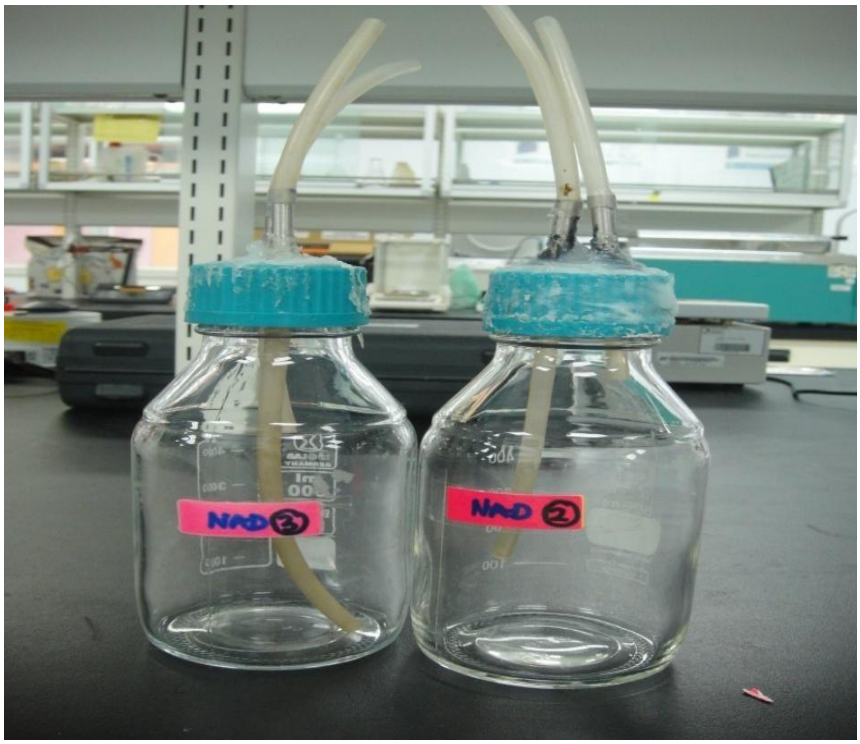

Figure D-3: Schott bottle 500mL

Figure D-4: Autoclave Hirayama

Figure D-5: Hybridization Incubator Shaker model SI-100D,

Figure D-6: Chiller at 4°C

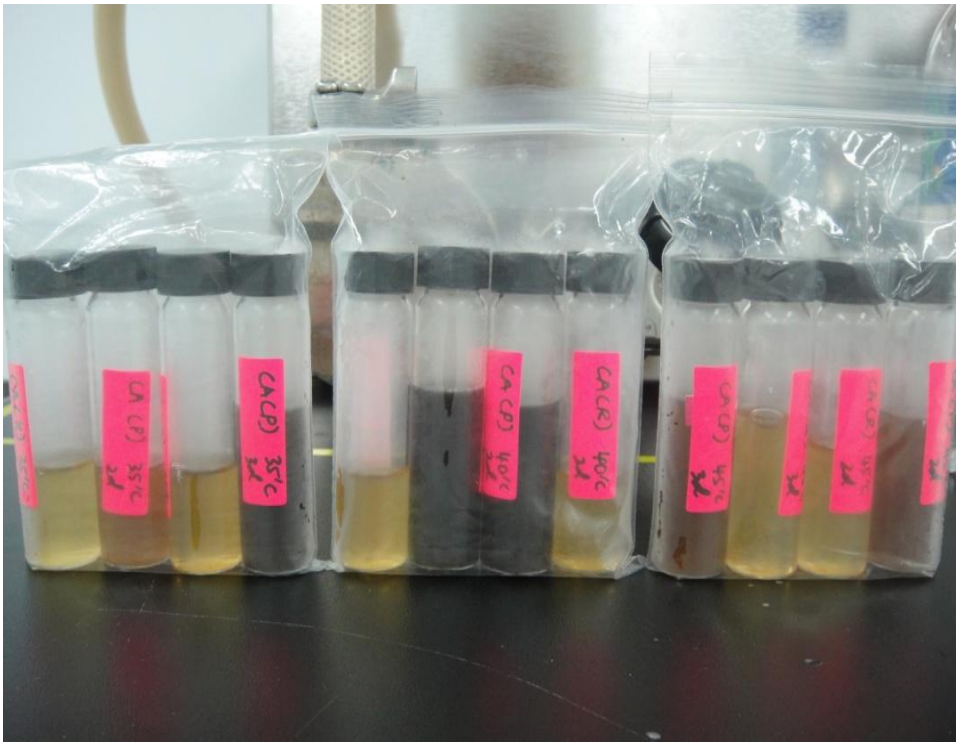

Figure D-7: Samples of POME and RCM at each run of the experiments