EFFECT OF SUBSTRATE CONCENTRATION IN PRODUCING HIGHER BUTANOL COMPARED TO ETHANOL BY USING *CLOSTRIDIUM ACETOBUTYLICUM*

PEARLJEET KAUR A/P KARTAR SINGH

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

BORANG PENGESAHAN STATUS TESIS♦

JUDUL: EFFECT OF SUBSTRATE CONCENTRATION IN PRODUCING HIGHER BUTANOL COMPARED TO ETHANOL BY USING *CLOSTRIDIUM ACETOBUTYLICUM*

SESI PENGAJIAN : 2009/2010

Saya **PEARLJEET KAUR A/P KARTAR SINGH**

(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.55, KG. BARU BANIR** **SHARIZA JAMEK**

JALAN TEMOH Nama Penyelia

35000 TAPAH, PERAK

Tarikh : Tarikh:

CATATAN : * Potong yang tidak berkenaan.

** Jika tesis ini **SULIT** atau **TERHAD**, sila lampirkan surat daripada pihak berkuasa/organisasiberkenaan 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 disertasi bagi pengajian secara kerja kursus dan penyelidikan, atau Lapuran 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 Degree of Bachelor of Chemical Engineering (Biotechnology)”

Signature :
Name of Supervisor : MISS SHARIZA JAMEK
Date :

EFFECT OF SUBSTRATE CONCENTRATION IN PRODUCING HIGHER BUTANOL COMPARED TO ETHANOL BY USING *CLOSTRIDIUM ACETOBUTYLICUM*

PEARLJEET KAUR A/P KARTAR SINGH

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

Faculty of Chemical & Natural Resources Engineering
University Malaysia Pahang

MAY 2010

I declare that this thesis entitled “Effect of Substrate Concentration in Producing Higher Butanol Compared to Ethanol by Using *Clostridia 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	: Pearljeet Kaur A/P Kartar Singh
Date	:

Dedicated especially to my beloved Father, Mother, Siblings, Friends, Lecturers and the ones who gave me inspiration and support that made this work possible.

ACKNOWLEDGEMENT

My greatest prayers and thank to the Great Almighty Guru Granth Sahib Ji whom made my degree thesis a realism. My sincere love and gratitude to my adored family because of whom I sternly moved forward towards my passion in pursuing my degree and gave me moral support to successfully accomplish my studies.

To would like to express deepest appreciation and sincere gratitude of the patience guidance of my dearest supervisor Miss Shariza Jamek. I owe my debts to her for her selfless advice and support in both related and non-related matters to my project.

Customarily, I would also like to thank Madam Chua@ Yeo Gek Kee and Encik Meor, for giving me a helping hand in various fields through which I had managed to complete my skills and competencies. I owe debt to all the technical staffs at the lab of Chemical Engineering and Natural Resources in attaining all the needed resources for my project.

I am also very grateful to my group members, Einayah Kamaruzaman, Zuriana bt Sidi Ahmad and Mohd Zaidi who helped me throughout the project. My personal thanks also to everybody who were directly and indirectly involved in helping me to complete this thesis.

Last but not least, there will be errors, inconsistencies and over-simplifications in this thesis and I bear absolute responsibility for the erratic judgments I made. None of the above mentioned people should be held responsible for any errors in this thesis.

ABSTRACT

In this present experiment, the main objective is to study the effect of substrate concentration in producing higher butanol compared to ethanol by using *Clostridium acetobutylicum.* Palm oil mill effluent (POME) was used as the main substrate in this fermentation because it imposes negative effect towards the environment which in vast amount besides the availability as a low cost substrate and reinforced clostridium medium (RCM) was used as a control substrate. Study was also done to investigate the growth profile of *C. acetobutylicum,* type of sugars in POME, and glucose consumption of *C. acetobutylicum* during fermentation. The HPLC analysis result for sugar component showed that the reducing sugars; fructose, glucose, galactose, sucrose and lactose exist in POME and are utilized as substrate for solvents fermentation by *C. acetobutylicum.* Main study on the effect of four substrate concentrations 70%, 80%, 90% and 100% in POME and RCM were tested using Schott bottle as fermentor in anaerobic chamber to maintain the anaerobic condition for *C. acetobutylicum* growth condition for 72 hours at temperature of 35^{o}C, pH 5.8 and speed of 200 rpm. The results showed that butanol and ethanol were produced at the end of the fermentation hence proving POME is a viable substrate for the fermentation. After 20 hours fermentation it was observed that at 90% substrate concentration butanol produced was higher compared to ethanol. However, in overall the result showed higher ethanol production compared to butanol production for all the four different substrate concentration throughout the experiment. The core factor contributing in this result is the substrate inhibition by butanol besides the phenolic component in POME which also acts as inhibitor, strain degeneration, and also extraction of butanol from fermentation broth. In conclusion, many efforts need to be taken to ensure higher butanol can be produced especially in decreasing the inhibition factor towards butanol.

ABSTRAK

Objektif dalam menjalankan kajian terbaru ini adalah mengkaji kesan kepekatan substrat dalam menghasilkan butanol yang lebih tinggi berbanding ethanol dengan menggunakan *Clostridium acetobutylicum.* Sisa buangan dari kilang memproses kelapa sawit (POME) digunakan sebagai substrat utama kerana sisa dalam jumlah sangat banyak ini mengakibatkan kesan negatif ke atas persekitaran selain kebolehsediaannya sebagai substrat berkos rendah dan 'Reinforced Clostridium Media' (RCM) digunakan sebagai substrat kontrol. Kajian juga dilakukan untuk mengkaji kadar profil pertumbuhan *C. acetobutylicum*, jenis gula dalam POME dan penggunaan glukosa oleh *C. acetobutylicum* semasa fermentasi. Keputusan analisa HPLC menunjukkan bahawa gula menurun; fruktosa, slukosa, galaktosa, sucrosa dan laktosa hadir dalam POME dan digunakan sebagai substrat untuk fermentasi. Kajian utama ke atas kesan empat kepekatan substrat yang berbeza 70%, 80%, 90% dan 100% dalam POME dan RCM dikaji menggunakan botol Schott dalam ruang anaerobik untuk mengekalkan keadaan anaerobik bagi pertumbuhan *C. acetobutylicum* selama 72 jam pada suhu 35^{o}C, pH 5.8 dan kelajuan 200rpm. Butanol dan ethanol terhasil dari fermentasi membuktikan POME adalah substrat yang sesuai untuk fermentasi ini. Selepas 20 jam fermentasi dapat diperhatikan bahawa pada kepekatan substrat 90%, butanol yang terhasil adalah lebih tingi berbanding ethanol. Walaubagaimanpun, secara keseluruhannya, ethanol dihasilkan lebih tinggi berbanding butanol di sepanjang eksperimen ini. Sebab utama yang menyumbang kepada keadaan ini adalah perencatan oleh butanol itu sendiri selain daripada komponen fenol dalam POME yang bertindak sebagai perencat. Selain itu, degenerasi strain dan juga penyaringan butanol dari pati fermentasi juga dilihat sebagai penyebab kepada keadaan di atas. Secara kesimpulannya, pelbagai usaha perlu dilaksanakan untuk memastikan penghasilan butanol yang lebih tinggi.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS / ABBREVIATIONS

ABE	-	Acetone-Butanol-Ethanol
BOD	-	Biological Oxygen Demand
C.acetobutylicum	-	*Clostridium acetobutylicum*
DNS reagent	-	Dinitrosalicylic Colorimetric Method
EFB	-	Empty Fruit Bunch
GC-FID	-	Gas Chromatography equipped with Flame Ionization Detector
HPLC	-	High Performance Liquid Chromatography
ME	-	Metabolic Engineering
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
OD	-	Optical Density
POME	-	Palm Oil Mill Effluent
RCM	-	Reinforced Clostridium Medium
RVP	-	Reid Vapor Pressure
SI	-	Spark Ignition
SS	-	Separator Sludge
USA	-	United States of America
UV-VIS	-	Ultraviolet-Visible Spectroscopy

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Under strain of human demand, oil prices have been fluctuating and resulted in the research on production of liquid fuels, such as butanol and ethanol, by fermentation. Deliberate actions have been taken towards production of alcohol fuels from easily and extensively-produced renewable resources prior to the constant conflict in oil-supply region of the world, and also the cascading decline of the fossil fuels.

The acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fementations (second to ethanol) and is one of the largest biotechnological processes ever known. However, since the 1950's industrial ABE fermentation has declined continuously, and almost all butanol is now produced via petrochemical routes (Ramey & Yang, 2004). Butanol is an important industrial solvent and potentially a better fuel extender than ethanol. The market demand is expected to increase dramatically if butanol can be produced economically from low-cost biomass (Durre, 1998).

Butanol is an alcohol that can be used as a transport fuel. It is a higher member of the series of straight chain alcohols with each molecule of butanol ($C_4H_{10}O$) containing four carbon atoms rather than two as in ethanol (Brekke, 2007). Because it is longer hydrocarbon chain causes it to be fairly non-polar, it is more similar to gasoline than it is to ethanol. Butanol which is produced from biomass such as molasses, corn, corn fiber, and other agricultural byproducts or processing wastes which require proper disposal to avoid pollution problems for example the palm oil mill effluents (POME) is known as "biobutanol". Both biobutanol and petrobutanol (from fossil fuels) have the same chemical properties.

Petroleum-derived butanol is currently used in food and cosmetic industries as an extractant. Bio-butanol is preferred, because there are concerns of its carcinogenic aspects associated with the residual petroleum components (Ramey, 2004). Butanol has the propensity to solve hydrogen infrastructure problems associated with fuel cell use of the future. Dispersed through existing pipelines and filling stations and then butanol can be reformed onboard the fuel cell vehicle, butanol offers a safer fuel with more hydrogen than methanol (very dangerous) or ethanol.

Palm oil mill effluent (POME) is a kind of byproduct of palm oil, but huge amount of it has been discarded in the vicinity of the palm oil mill plant. POME is a negative byproduct that might cause huge pollution of environment if not treated well before disposing it to the environment. Though, due to its high biological oxygen demands (BOD), it could be a kind of sustainable resource. Development of effective fermentation method for POME that contains fermentative sugars and fatty acid will make it real sustainable resource (Ngan *et al.*, 2003).

Acetone-butanol-ethanol (ABE) fermenting clostridia can catabolize various sustainable bio-resources including bio-wastes, due to its wide substrate specificities to various sugar substrate including cellulosic and hemicellulosic materials (Hayasida & Ahn, 1990). Glucose and fatty acid in POME could be expected as possible substrates for ABE fermenting clostridia. Moreover, ABE-clostridia, as anaerobes, do not require any aeration process or other facilities needed for aerobic fermentation system.

1.2 Problem Statement

Over the past decades, there has been heavy reliance on the fuels to be used in the cars. This is because cars make up the largest portion of the road and the percentage is increasing day by day. Oil prices have been fluctuating and resulted in the research on production of liquid fuels, such as butanol and ethanol, by fermentation. Deliberate actions have been taken towards production of alcohol fuels from easily and extensively-produced renewable resources prior to the constant cascading decline of the fossil fuels.

The need to produce fuel from raw material which is significantly less cost compared to petrochemical raw materials is very important. Therefore, these biomass based production will maintain or significantly increase in the demand in the market as the raw material used will be cheap and easy to be gained. Hence here the operating cost is directly reduced as POME is used because of the availability as a low cost raw material.

Besides that, industrialization is important to spur economic growth in a country. Malaysia is the largest producer and exporter of palm oil in the world. As a result of industrialization, palm oil manufacturing companies releases a big amount of effluent to the environment. The raw POME has an extremely high content of degradable organic matter, which is due in part to the presence of unrecovered palm oil, thus, POME should be treated before discharge to avoid serious environmental pollution. Raw POME has Biological Oxygen Demand (BOD) values averaging around 25,000 mg/litre, making it about 100 times more polluting than domestic sewage

Not only that, the main problem addressed here is the costly treatment of the waste. Treatment done by the Kualiti Alam Company at the moment is very costly. Hence the treatment to produce butanol is the best solution in reducing the cost of treatment. Where the focus is on changing the negative money, the money spent to treat something to positive money.

1.3 Objectives of study

To study the effect of substrate concentration in producing higher butanol compared to ethanol by using *Clostridium acetobutylicum.*

1.4 Scope of Study

To accomplish these objectives, the scope of work has been identified;

i. To study the growth profile of *Clostridium acetobutylicum*
ii. To complete the composition analysis of selected batch fresh POME by using HPLC
iii. To study the effect of substrate concentration on the higher butanol production compared to ethanol.
iv. To study glucose consumption in the fermentation broth.

CHAPTER 2

LITERATURE REVIEW

2.1 Fermentation

Fermentation is the conversion of a carbohydrate such as sugar into an acid or an alcohol. More specifically, fermentation can refer to the use of yeast to change sugar into alcohol or the use of bacteria to create lactic acid in certain foods. Fermentation occurs naturally in many different foods given the right conditions, and humans have intentionally made use of it for many thousands of years (McGuigan, 2009). The earliest uses of fermentation were most likely to create alcoholic beverages such as mead, wine, and beer.

Acetone-Butanol-Ethanol (ABE) fermentation is a process that uses bacterial fermentation to produce acetone, butanol and ethanol from starch. The process is anaerobic (it does not require oxygen), similar to how yeast ferments sugars to produce ethanol for wine, beer, or fuel. The process produces these solvents in a ratio of 3-6-1, or 3 parts acetone, 6 parts butanol and 1 part ethanol. It usually uses a strain of bacteria from the Clostridia Class (Clostridium Family) where *C. acetobutylicum* is the most well known strain. ABE fermentation for butanol production is gaining interest over the petrochemical route (Jones and Woods, 1986).

2.1.1 Anaerobic Fermentation

Anaerobic fermentation of carbohydrates by yeasts and bacteria leads to the production of a range of alcohols, acids and esters. Three alcohols, ethanol, isopropanol and butanol, are currently made industrially by fermentation, though, in most places, production from petroleum is cheaper than the biological conversion. The conversion of glucose to ethanol can be achieved at approaching the theoretical maximum efficiency of 51% (by mass) based on the biochemical route, retaining 93% of the energy content of the carbohydrate (Righelato, 1980).

2.2 Butanol over Ethanol

With the recent rise in oil prices to record levels alternative fuel sources are increasingly in demand. One option would be to switch to butanol produced by biological sources like bacteria using biomass as a fuel. This would mean using alternative fuels to power our cars, homes, appliances, computers and other oil dependant machines. The main sources of our current fuels (crude oil) are from fossils which are extracted from decayed bodies of ancient creatures.

The use of alcohol in spark ignition (SI) engines began in 1954 in countries like United States, Germany, and France. During World War I and II, gasoline shortages occurred in France and Germany, and alcohol was used in all types of vehicles, including military planes. Nowadays it is used with gasoline (a mixture) in the United States and has become a major fuel in Brazil (Nag, 2008). Any new fuel which is going to be introduced should be evaluated from the aspect of availability, renewability, safety, and cost adaptability to existing engines performance, economy and finally emission.

Butanol can generate energy when used in internal combustion engines similar to gasoline. However, for a variety of reasons it may actually be better than gas. Talking about its compatibility with existing vehicles, the air to fuel mixture ratio is 11.2 compared to gasoline which is 14.7. The energy content of Butanol is 105,000 Btu per gallon compared to gasoline's 114,000 Btu per gallon. This similarity between air to fuel mixture and energy content means conversion of existing vehicles would be very simple.

Butanol is a chemical with excellent fuel characteristics; butanol can solve many problems associated with the use of ethanol. Butanol has the following advantages over ethanol:

a) butanol has 25% more Btu per gallon
b) butanol is less evaporative/explosive with a Reid vapor pressure (RVP) 7.5 times
c) lower than ethanol
d) butanol is safer than ethanol because of its higher flash point and lower vapor
e) pressure
f) butanol has a higher octane rating
g) butanol is more miscible with gasoline and diesel fuel but less miscible with water.
h) butanol has energy density is only 10 to 20% lower than gasoline's.
i) It can be produced using existing ethanol production facilities with relatively minor modifications.
j) It is compatible with the current gasoline distribution infrastructure and would not require new or modified pipelines, blending facilities, storage tanks, or retail station pumps.

At the moment the main key challenge is the cost of the substrates used, the ability to use low cost substrates in producing cost effective butanol. Much work is being done to develop microbes that can be used with a variety of substrates. Palm **o**il **m**ill **e**ffluent is a potential exchange to the substrates used at the moment in the production of butanol as the main target of our world which is ‘Waste to Wealth’.

2.3 Palm Oil Mill Effluent

One of the components in the suspended solids of POME is separator sludge. Separator sludge acts as substrate to support production of solvents by *C. saccharoperbutylacetonicum* N1-4 without any need for mineral supplements. Besides that, enzymatic hydrolysis by cellulose prior to fermentation found to increase the yield of butanol by 75% (from 2.47g/l to 4.37g/l) (Mun *et al.*, 1995).

The current treatment technology of POME typically consists of biological aerobic and anaerobic digestion. Biologically treated effluent is disposed of via land application system, thus providing essential nutrients for growing plants (Wong *et al.*, 2002).

2.4 Solventogenic Clostridia

Clostridium is one of the largest bacterial genera with an enormous potential for biotechnical and medical applications. Despite growing scientific, medical, and industrial interest, information on basic methods, biochemical fundamentals, clinical practice, industrial applications, and novel developments remains scattered.

Solventogenic clostridia are strictly anaerobic, endospore forming bacteria that produce a large array of primary metabolites, like butanol, by anaerobically degrading simple and complex carbohydrates, including cellulose and hemicellulose (Papoutsakis, 2008). Solventogenic, butyric acid clostridia can produce a large array of metabolites, and metabolic engineering (ME) driven strain development could enhance these native capabilities and lead to production of chemicals including butyric and acetic acids, butanediol, propanol, and acetone (Jones & Woods, 1986).

2.4.1 *Clostridium acetobutylicum* in Butanol production.

In butanol production method, two types of microbes were used in two separate process steps. The first pass optimizes the production of hydrogen and butyric acid, while the second pass converts this acid into butanol. Each step utilizes a different Clostridium strain (Ramey, 2005).

Study by Monot *et al.* (1984) in the influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *C. acetobutylicum* showed that at lower pH, growth occurs in two consecutive phases and solvents are the main excreted metabolites. At the higher pH, there is a single growth phase with only acid formation. The influence of the pH can be correlated with a critical role of the concentration of undissociated butyric acid in the medium. Reducing the intracellular acid dissociation by lowering the intracellular pH also favours the production of acetone and butanol

POME fermentation using *C. acetobutylicum* NCIMB 13357 in an oscillatory flow bioreactor showed that POME is a viable media for ABE fermentation. Oscillatory flow bioreactor has an excellent potential as an alternative fermentation device. Fermentation was carried out for 72 hours at 35^{o}C using POME and reinforced clostridia medium as a growth medium in batch culture (Takriff *et al.*, 2009)

CHAPTER 3

METHODOLOGY

3.1 Introduction

Fermentation is is the main process to convert Palm Oil Mill Effluent (POME) to Butanol using Solventogenic Clostridia. There are few phases in this experiment before a product can be produced;

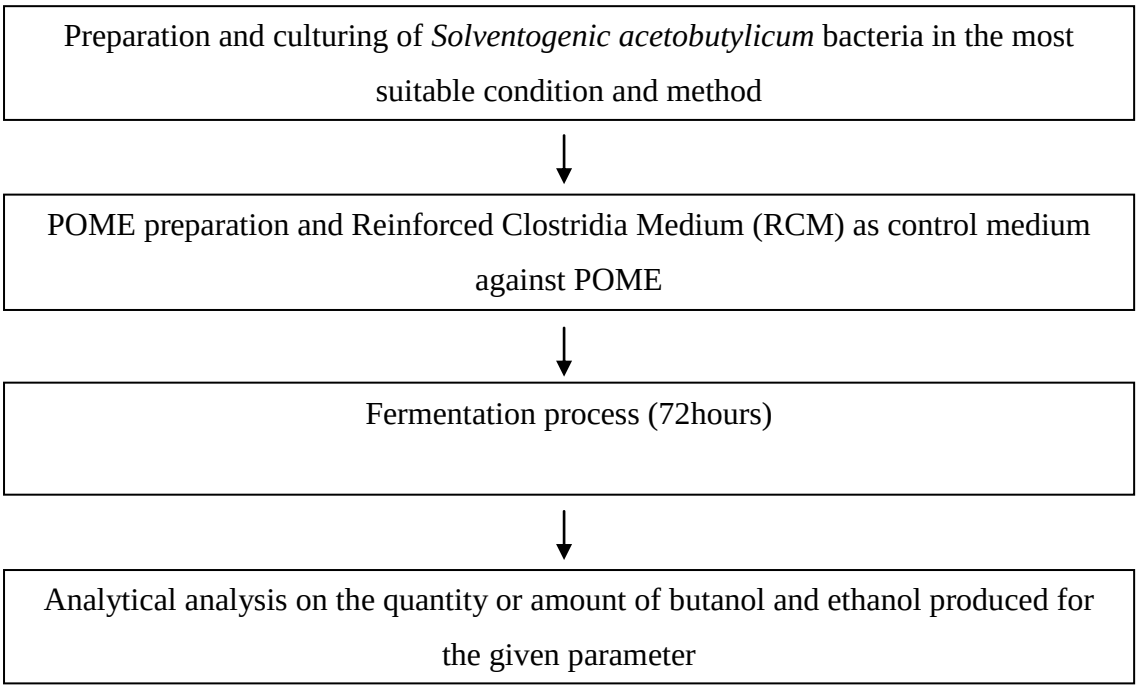

Figure 3.1: Flow Chart of experimental procedures

3.2 Material

3.2.1 Bacterial Strain

The bacterial strain, *C.acetobutylicum* NCIMB 13357 was obtained from University Kebangsaan Malaysia and used throughout the experiment. *C.acetobutylicum* is an anaerobic, saccharolytic and proteolytic bacterium that has been isolated from a number of environments.

3.2.2 Substrate

The fresh sample of palm oil mill effluent (POME) was obtained from Felda Palm Industries Sdn. Bhd., Lepar Hilir, Gambang, Kuantan. This sample consist of separator sludge which is the medium used for the experiment throughout. Separator **s**ludge contains fermentative sugars and fatty acids making it as a real sustainable resource.

3.2.3 Media

3.2.3.1 Reinforced Clostridium Medium

Reinforced Clostridium Medium that contained 5.0 g pancreatic digest of casein, 5.0 g proteose peptone, 10.0 g beef extract, 3.0 g yeast extract, 5.0 g

dextrose, 5.0 g NaCl, 1.0 g soluble starch, 0.5 g cysteine hydrochloride, 3.0 g sodium acetate and 0.5 g agar per liter was used as the germination and inoculums media.

3.2.3.2 Reinforced Clostridium Agar

Reinforced Clostridium Agar that contained 10.0g casein enzymatic hylosate, 10.0g beef extract, 3.0g yeast extract, 5.0g dextrose, 5.0g NaCl, 1.0g soluble starch, 3.0g sodium acetate, 0.5g L-Cysteine hydrochloride and 13.5g agar per liter was also used as the agar medium for the growth of the bacteria culture.

3.3 Equipments

3.3.1 Anaerobic Chamber

The laboratory scale anaerobic chamber (Sheldon Manufacturing Inc., USA) was used for the anaerobic fermentation of *C.acetobutylicum* in POME.

3.3.2 Gas Chromatography – flame Ionization Detector

Gas Chromatography Agilent 6890 equipped with flame ionization (GC-FID) detector (Agilent Technology, USA) was used to detect the presence of butanol in

the fermentation product. The FID works by directing the gas phase output from the column into a hydrogen flame. A voltage of 100-200V is applied between the flame and an electrode located away from the flame. The increased current due to electrons emitted by burning carbon particles is then measured. The Specification of GC-FID that is used for product analysis is shown in Table 3.1.

Table 3.1: Specification of GC-FID for fermentation analysis

Specification of GC-FID	
Column	Hp-Innowax
Oven Temperature	$50^{o}C – 180^{o}C$
Carrier Gas	Hydrogen

3.3.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography Agilent 1200 (HPLC) (Agilent Technology, USA) was used for the analytical measurement of the sugar composition of selected batch fresh POME before the fermentation occurs. The Specification of HPLC that is used for product analysis is shown in Table 3.2

Table 3.2: Specification of HPLC for sugar analysis

Specification of HPLC	
Column	Supelcosil LC-NH_3
Injection Range	1ml/minute
Retention time	15minutes
Mobile Phase	75% Acetonitrile 25% Water
Standard Preparation	20 g/l ,40 g/l, 60 g/l, 80 g/l & 100 g/l for each sample of the standard

3.3.3 Ultraviolet-visible Spectrophotometer (UV-VIS)

Ultraviolet-visible Spectroscopy (UV-Vis) (HITACHI, Japan) refers to absorption spectroscopy in the UV-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum,molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state.

UV-VIS was used for the determination of glucose before, during each 20 hours of fermentation and after fermentation reading. The wavelength used for the glucose reading was 540 nm.

3.4 Experimental Procedure

3.4.1 Bacteria Culturing

3.4.1.1 Preparation of Agar Medium

Reinforce clostridia agar was prepared by dissolving 52.5 g of the powder in 1 liter of distilled water. After that, it was brought to the boil to dissolve the agar completely. Later agar medium was sterilized by autoclaving at 121^{o}C for 20 minutes. After autoclaving the medium, the medium was cooled at room temperature. Finally a tube of melted agar was poured into a sterile Petri dish and kept until the agar hardens.

3.4.1.2 Bacterial Strain and Cultivation Condition

C.acetobutylicum was used throughout the experiments. Spore from the bacteria culture was transferred in a small vial containing RCM with cultivation at 37^{o}C for 24 hours as an enrichment step. This process is used as a subculture for further experiment.

3.4.1.3 Subculture Striking

Anaerobic chamber was used to maintain anaerobic condition during the culturing of the bacteria on the petri dish. Three parallel lines about 5 mm distance from each others was inoculated on the agar plate. The bacteria on petri dish were then sealed with parafilm and incubated in the incubator of anaerobic chamber at 37^{o}C for 2 days until a single colony growth is observed on the agar plate.

3.4.1.4 Inoculum Preparation

The single colony of bacteria from the agar plate was used to cultivate the bacteria in 167 ml liquid medium of RCM in 250ml **s**chott bottle and kept for incubation for 18 hours at 37^{o}C in anaerobic chamber. The growth of bacteria was monitored by measuring an optical density at 680 nm using spectrophotometer. Cells in RCM were harvested at late-log phase (OD 680 nm = 0.7).

3.4.2 Media Preparation

3.4.2.1 Pretreatment POME

Fresh POME from Felda Palm Industries Sdn Bhd Gambang, Kuantan was passively sediment at 4^{o}C over a period of 24 hours.

3.4.2.2 Preparation of Substrate & Media

After 24 hours, the supernatant of the POME was decanted and 300 ml of the sedimented POME was taken as the working volume. The initial pH of the POME adjusted to pH 5.8 by the addition of 5M NaOH (Kalil et al, 2003) because pH 5.8 was found to be the optimal pH for solvent production. Since the parameter for this experiment is substrate concentration, each time preparation, the working volume has to be calculated separately for 70%, 80%, 90% and 100% of substrate concentration.

Reinforce Clostridia Media (RCM) medium was prepared separately by dissolving 38 g of the powder into 1 L distilled water. Both mediums were autoclaved at 121^{o}C for 20 minute and then later the RCM was transferred into the schott bottle of 500 ml with working volume of 300 ml. After autoclaving, the medium of POME and RCM was deoxidized by gassing with the nitrogen gas for 10 minutes. For each substrate concentration (70%, 80%, 90% and 100%) of RCM, calculation needs to be done separately.

POME and RCM were used directly as fermentation medium without additional nutrient.

3.4.3 Fermentation Process

Anaerobic chamber was used for all the transfer of products. 10% v/v (30 ml) of inoculum was transferred into the POME and RCM medium, both the mediums were incubated at temperature, 35^{o}C and optimum speed of 200 rpm.

3.4.4 Analysis

Before the fermentation process was done, analysis on growth profile of *C. acetobutylicum* using UV-VIS and analysis on the composition of selected batch of POME using HPLC was done. During the fermentation process, samples were taken every 20 hours until 72 hours to determine the production of butanol and ethanol using GC-FID in POME and RCM besides checking the glucose consumption using UV-VIS spectrophotometer by dinitrosalicylic colorimetric method (DNS Method).

Readings taken for the analysis process was first centrifuged at 10000 x g for 30 min at 25^{o}C before proceeding with the respecting method.

3.4.4.1 Determination of Growth Profile

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 was used to determine the reading of OD value of each of the samples at 680 nm.

3.4.4.2 Determination of Composition in Selected Batch POME

The supernatant from the sample of POME was taken to check the component inside the POME. The components of POME were analyzed by using high performance liquid chromatography (HPLC) with capillary column Supelcosil LC-NH_2. The flow rate that used was 1 ml/minute and the retention time around 15 minutes.

The mobile phase for this HPLC is acetonitrile and water. The ratio of mobile phase is 75% of acetonitrile and 25% of water. The standard for the detection of glucose, galactose, fructose, sucrose, lactose and xylose in the sample of POME was prepared using the pure HPLC grade solid. The calibration curve for each sugar (glucose, galactose, fructose, xylose, sucrose and lactose) was prepared for 20 g/L, 40 g/L, 60 g/L, 80 g/L and 100 g/L. The samples and standard solutions were filtered using the filter 0.45 micro meters into the HPLC vial before using it.

3.4.4.3 Determination of Butanol and Ethanol Production

After 20 hours, 40 hours. 60 hours and 72 hours, the sample of POME medium and RCM medium were taken. The supernatant from the centrifuged medium was used in this process. Each supernatant of RCM and POME was entered in the different of separating funnel respectively. Then each the sample was mixed with universal solvent (toluene) with ratio of 1:1. The toluene function is to absorb the butanol and ethanol produced in the sample of POME and RCM.

The samples were mixed nicely and left for separation for 24 hours. The toluene separated sample with lower density will be above, and hence it is taken and transferred into a biker. The sample in the biker was added with Na_2SO_4 to absorb the molecule of water in the sample, closed with aluminum foil and stored in the chiller

at 4^{o}C for another 24 hours. After 24 hours the sample was filtered by using syringe filter of 0.2 µm.

The prepared samples were used to analyze the composition of butanol and ethanol using GC-FID. 10 µl of sample POME and RCM was mixed with 990 µl of hexane. After process mixture between the sample and hexane the sample was transfer into vial by using filter of 0.25µm. The standard that is used to detect concentration of butanol and ethanol in the sample of solution are pure butanol and ethanol respectively.

3.4.4.4 Determination of Glucose Consumption

The glucose consumption of POME was determined throughout the fermentation process by using DNS Method. The readings before and after the fermentation were taken. 3ml of supernatant from POME and RCM was added with 3 ml of DNS Reagent respectively. Then it was heated to 90^{o}C until the color of both the solution changes. UV-VIS was used to determine the reading of OD value of each of the samples using 540nm.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth Profile

The results are shown in figure 4.1 for POME and RCM. POME is suitable for fermentation as it contains major carbon sources, lipids and glucose (Masngut *et al.*, 2007) whereas this semisolid medium of RCM supports the growth of clostridia from small inocula and produces higher viable counts (http://www.bd.com/ds/technicalCenter/inserts/Reinforced_Clostridial_Medium.pdf).

RCM acts as control in this experiment to show the difference in the growth of the *C. acetobutylicum*. This is important to know the viability of the bacteria for the usage in fermentation process to ensure the best medium that can be used.

4.1.1 Growth Profile of *Clostridium acetobutylicum* in POME and RCM

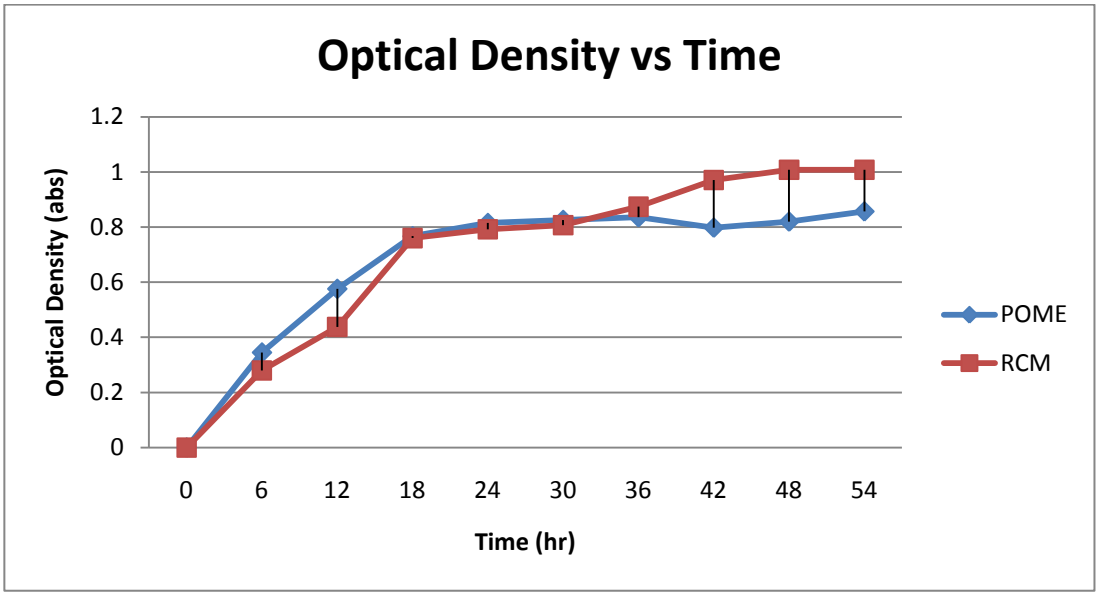

Figure 4.1: Growth profile of *C. acetobutylicum* in Schott bottle utilizing POME and RCM as growth medium.

From Figure 4.1, *C.acetobutylicum* displays a characteristic three-phase pattern of growth in both medium. The initial lag phase is a period of slow growth during which the bacteria are adapting to the conditions in the fresh medium occurs from 0-6 hours for both POME and RCM. This is followed by a log phase during which growth is exponential, doubling every replication cycle, where for RCM, replication occurs from the 6 hours to 42 hours. On the other hand, for POME replication occurs from the 6 hours to 18 hours.

Stationary phase occurs when the nutrients become limiting and the rate of multiplication equals the rate of death. Here the phase for RCM occurs from 42 hours to 54 hours and will undergo logarithmic decline phase after that when cells die faster than they are replaced (This latter occurs over a much longer period of time that the previous three). For POME, the stationary phase starts from the 18th hour to 54th hour.

In the production of solvents in fermentation process, *C.acetobutylicum* utilizes the carbon sources in each POME and RCM in two phases, acidogenic phase and solventogenic phase (Masngut *et al*., 2007). That is the time where POME and

RCM actively produce acid. Solventogenic phase on the other hand utilizes the produced acids into solvents. Study by Masngut *et al.* (2007) also stated in that during the phase of solventogenic, the metabolism of cells undergoes a shift to produce solvents by reassimilation of the organic acids.

4.2 Composition Analysis of selected fresh POME

Separator sludge (SS) is a better substrate to support the production of solvents without the need of any mineral supplementation (Mun *et al.*, 1995). Hence, for this particular experiment we have used separator sludge accumulated from Palm Oil Mill, Lepar Hilir.

For this particular analysis, High Performance Liquid Chromatography (HPLC) was used in regards to analyze the nutrition's in SS of palm oil mill effluent (POME). Three replicates of supernatant from the sedimented POME samples were taken for analysis.

The result for this experiment is shown in Table 4.1.

Table 4.1: Sample Analysis of POME

Sugar compound	Concentration (g/l)
Glucose	3.6702
Galactose	8.8594
Sucrose	2.2442
Lactose	1.3120

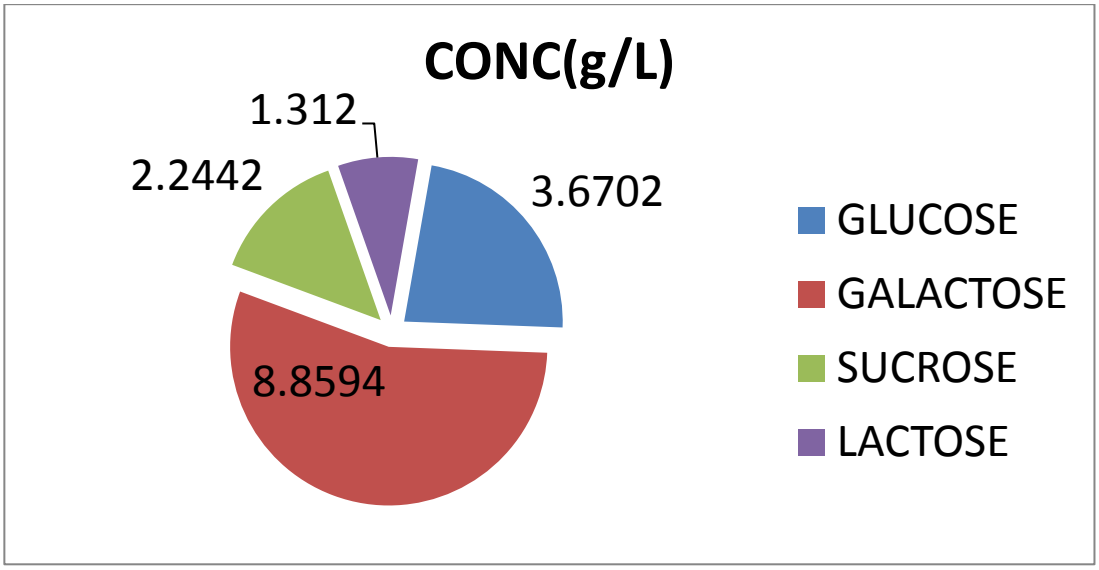

Figure 4.2: Average concentration in g/l of reducing sugars in SS of POME for three different replicates of samples.

From Figure 4.2, it can be observed that the basic components of reducing sugars that consist in SS are glucose, galactose, sucrose and lactose. These sugars are the component to be used as substrate and support material for the growth of *C. acetobutylicum*. In average, concentration of galactose is the highest among all the sugars with value of 8.8594 g/l. It follows by the concentration of sucrose and glucose with 3.6702 g/l and 2.2442 g/l respectively. Lactose is the lowest component as the concentration is 1.312 g/l.

In overall, the total amount of reducing sugars in average is very low in this sample of SS which is below 15g/l. This may be caused from the POME sample itself which was taken from Lepar Hilir. During the cleaning process of each effluent, excess water could have been used to clean and dilute the effluent before being discharged from the company. The excess water content in the sample caused the sample to dilute the organic components in the SS.

Even though the sugar components are low, SS from POME is seen as an essential and very useful substrate for the conversion of sugars to solvents as the variety of sugar components in the SS will increase the chances of the solvent production. Study by Bahl *et al.* (1986), has shown that reducing sugar plays an important role in the higher production of solvents. Co-fermentation of two different

sugars increases the production of butanol and decreases the production of other solvents.

As a whole, it can be conclude that separator sludge from POME contains enough reducing sugar that can act as carbon source to enhance the solvent production by *C. acetobutylicum* if a fresh POME is used and not adulterated by dilution of water.

4.3 Butanol and Ethanol Production

Lab scale fermentation using schott bottle was used to produce the butanol and ethanol from *C. acetobutylicum*. All the condition or parameter have been controlled and set constant. Butanol and ethanol have been produced at different substrate concentration of 70%, 80%, 90% and 100% by adding distilled water to dilute the substrate concentration according to its percentage.

The results of the butanol and ethanol production in each of the fermentation run are shown in Figure 4.3, 4.4, 4.5 and 4.6.

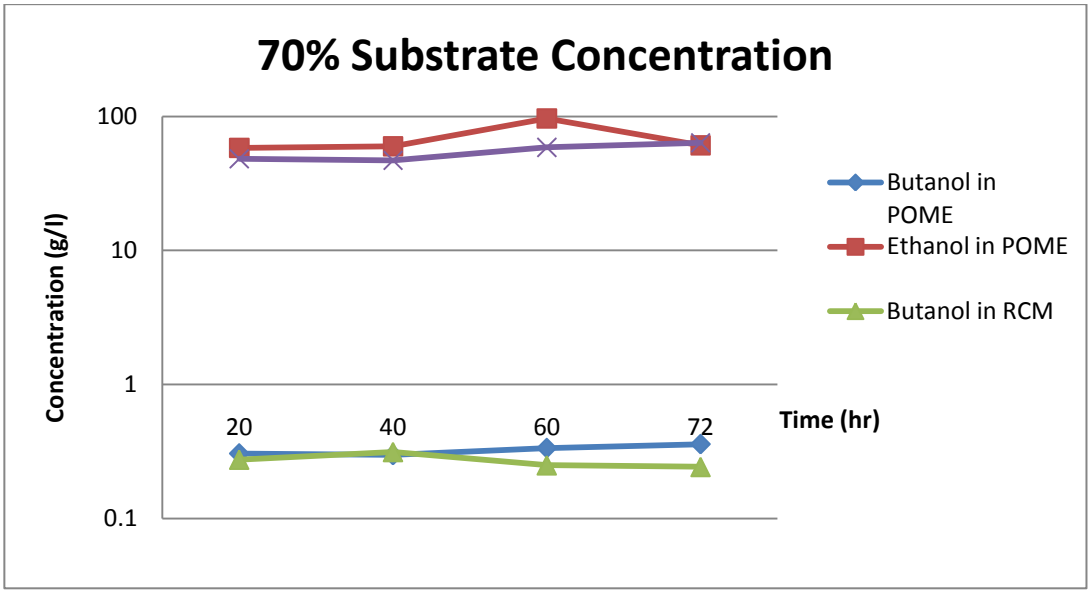

Figure 4.3: The concentration in g/l of butanol and ethanol produced for POME and RCM in 70% substrate concentration

From the graph (Figure 4.3), it can be observed that the concentration of ethanol is much higher compared to the butanol production in both RCM and POME. Value of butanol in POME increased from 0.3054 g/l to 0.3584 g/l while in RCM the value decreased from the initial value of 0.2751 to 0.2457 g/l. On the other hand, ethanol in RCM and POME increased from 48.2524 g/l to 63.6762 and 58.1711g/l to 60.7609 g/l respectively.

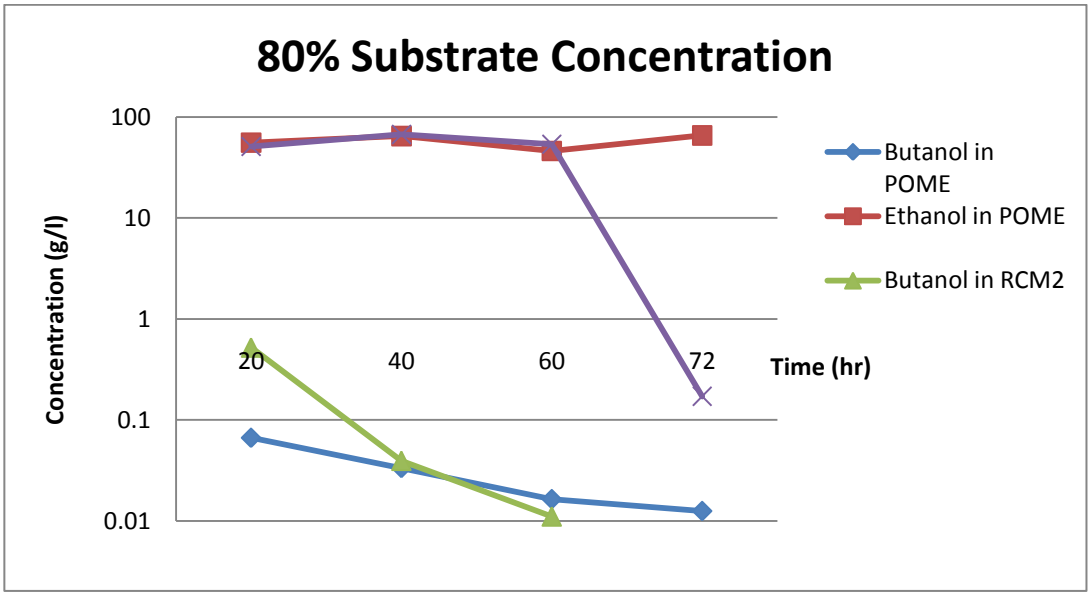

Figure 4.4: The concentration in g/l of butanol and ethanol produced for POME and RCM in 80% substrate concentration

From the graph (Figure 4.4), it can be observed that the concentration of ethanol is much higher compared to the butanol production in both RCM and POME. The production in overall decreases as the fermentation reaches the 72 hours. The value of butanol in each RCM and POME decreases rapidly from initial value of 0.5222 to 0.0111 and 0.0666 g/l to 0.0125 g/l respectively. Ethanol value in RCM also decreased from 50.8754 g/l to 0.1713 g/l. On the other hand, the value of ethanol in POME increases from 55.6526 g/l to 65.6153 g/l.

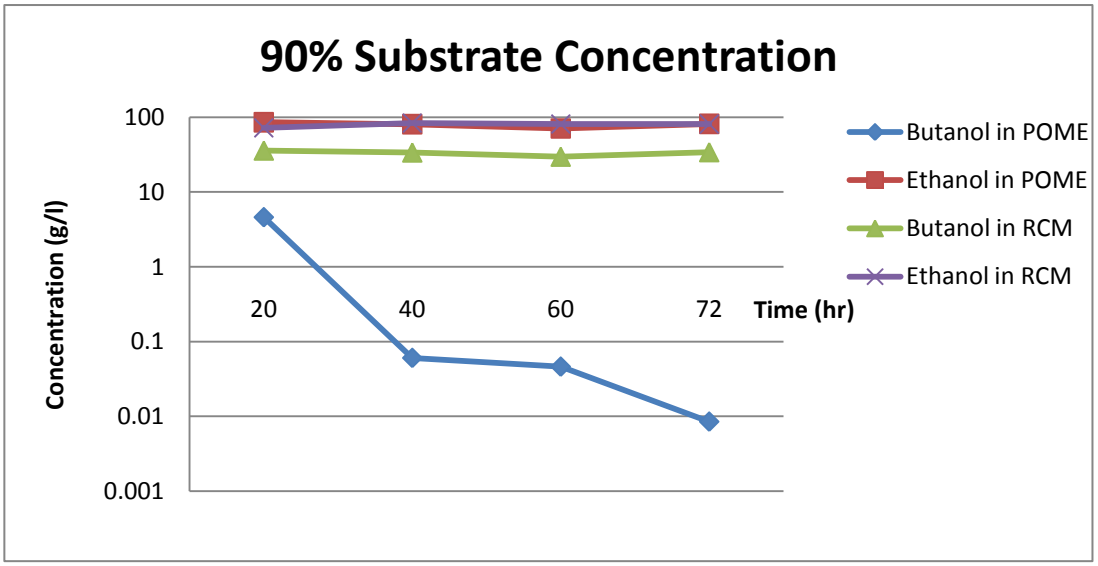

Figure 4.5: The concentration in g/l of butanol and ethanol produced for POME and RCM in 90% substrate concentration

From the graph (Figure 4.5), it can be observed that the concentration of ethanol in POME, ethanol in RCM and butanol in RCM is quite high throughout the fermentation process but butanol in RCM decreases as it reaches the 72 hours of fermentation. The concentration of butanol in POME decreases rapidly 4.6045 g/l to 0.0085 g/l. Eventhough the value of butanol in RCM is high but it decreases in small value from 35.8125 g/l to 34.0325 g/l. The ethanol value in POME also decreases from 85.5993 g/l to 81.3450 g/l and ethanol in RCM on the other hand, increases from 71.9671 g/l to 80.9993 g/l.

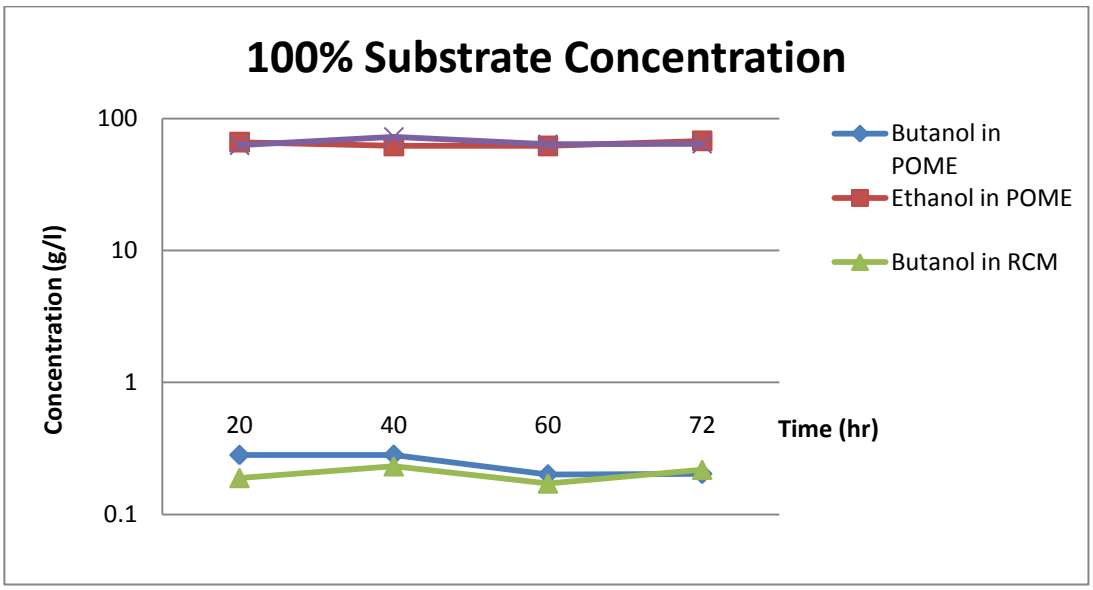

Figure 4.6: The concentration in g/l of butanol and ethanol produced for POME and RCM in 90% substrate concentration

As observed in this graph (Figure 4.6), the value of ethanol in RCM and POME is much higher above 61 g/l compared to butanol production which is lower than 0.3 g/l. The value of ethanol in POME and RCM increases from 66.1905 g/l to 67.7019 g/l and 62.6304 g/l to 64.1466 g/l respectively. Butanol production in POME anyhow decreases from 0.2819 g/l to 0.2030 g/l and the value of butanol in RCM on the other hand increases from 62.6304 g/l to 64.1466 g/l.

4.3.1 Overall Study on Butanol and Ethanol Production

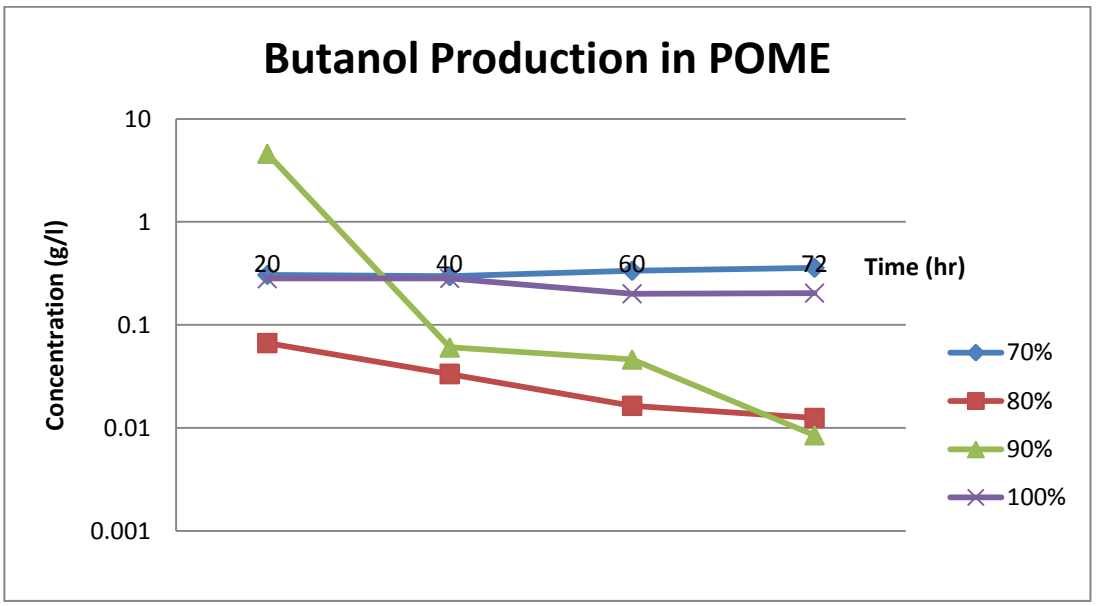

Figure 4.7: The concentration in g/l of butanol produced in POME throughout 72 hours fermentation of different concentration of POME (70%, 80%, 90% and 100%)

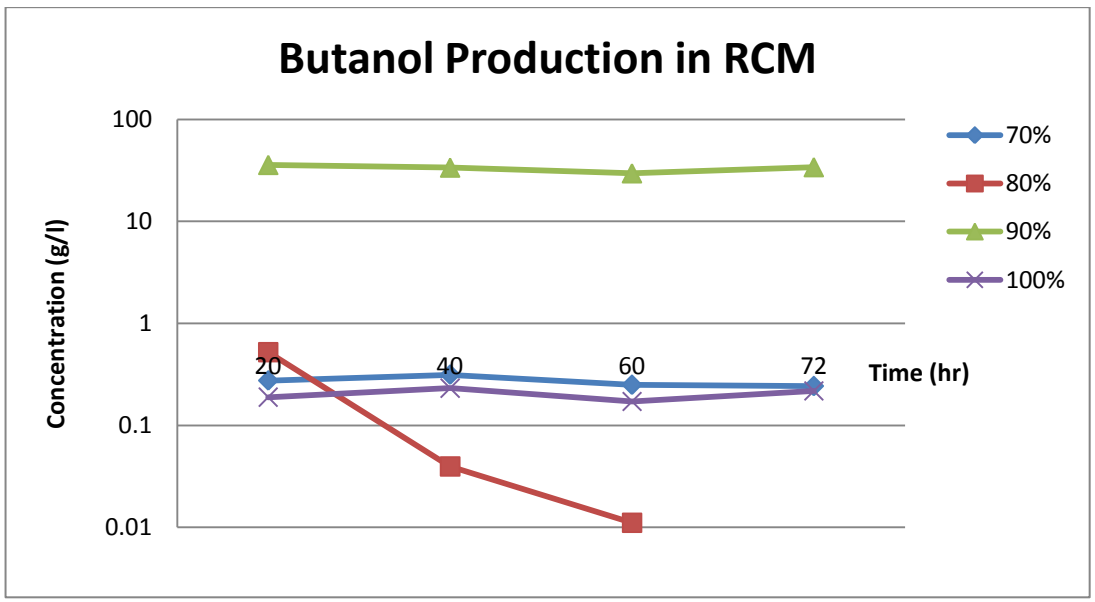

Figure 4.8: The concentration in g/l of butanol produced in RCM throughout 72 hours fermentation for control of different concentration of RCM (70%, 80%, 90% and 100%)

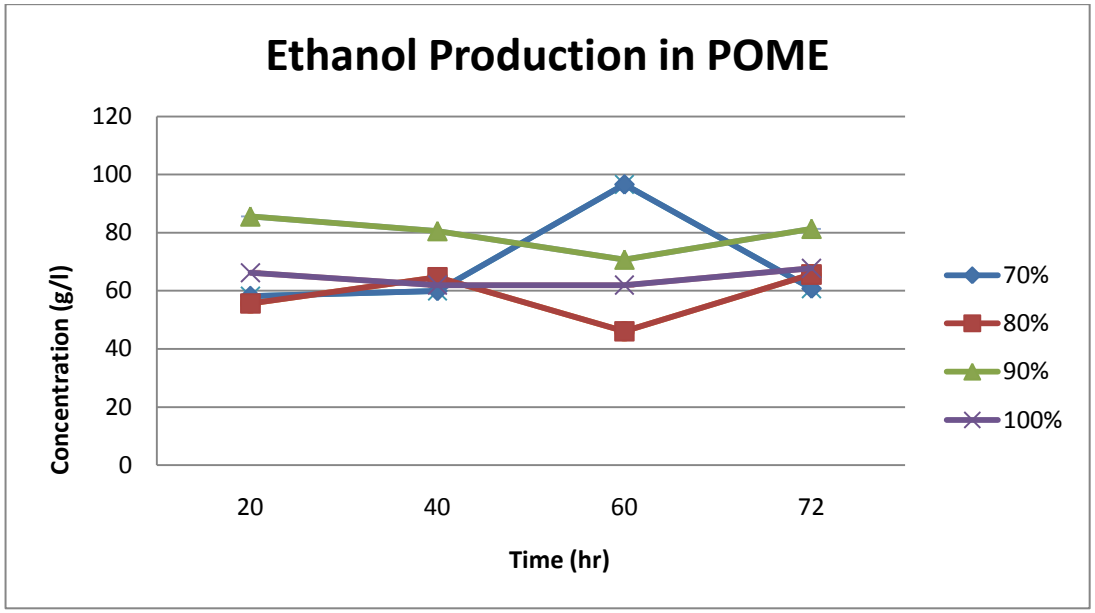

Figure 4.9: The concentration in g/l of ethanol produced in POME throughout 72 hours fermentation of different concentration of POME (70%, 80%, 90% and 100%)

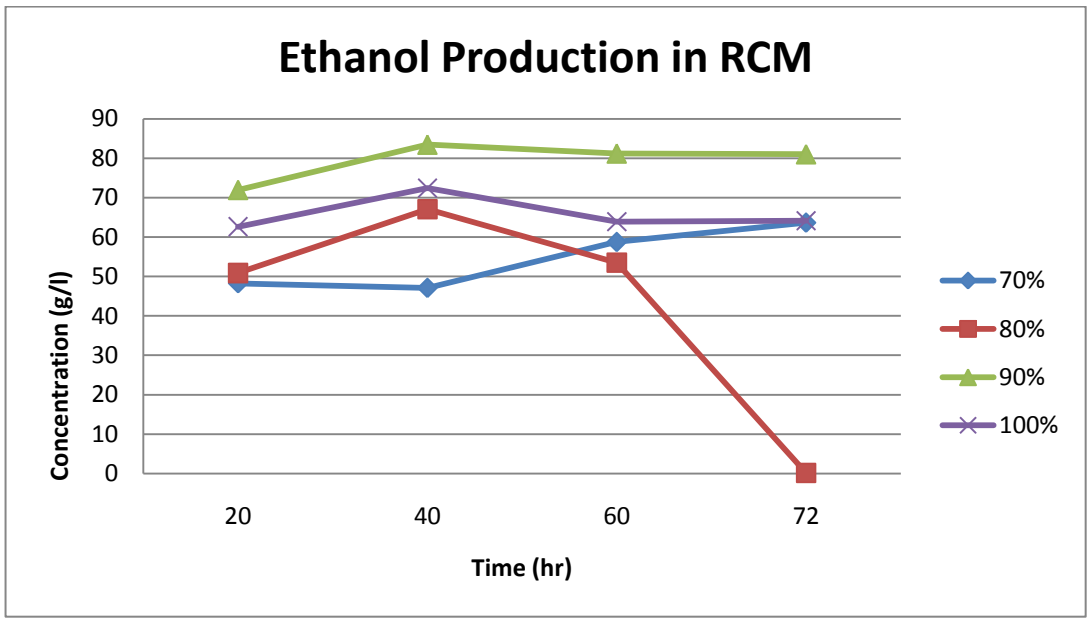

Figure 4.10: The concentration in g/l of ethanol produced in RCM throughout 72 hours fermentation for control of different concentration of RCM (POME 70%, 80%, 90% and 100%)

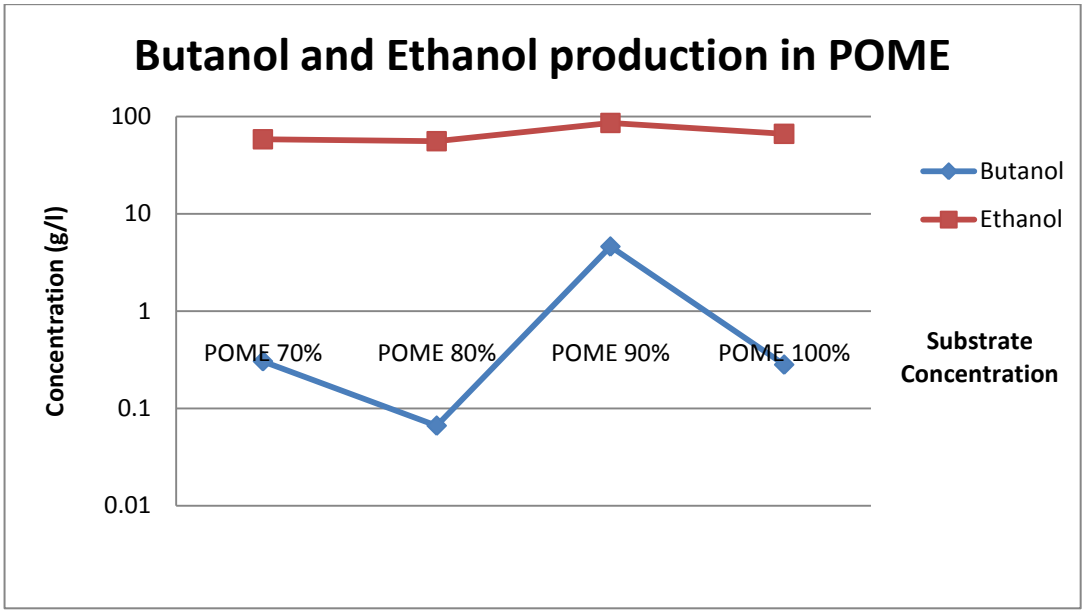

Figure 4.11: The concentration in g/l of butanol and ethanol produced after 20 hours cultivation using different concentration of POME (70%, 80%, 90% and 100%)

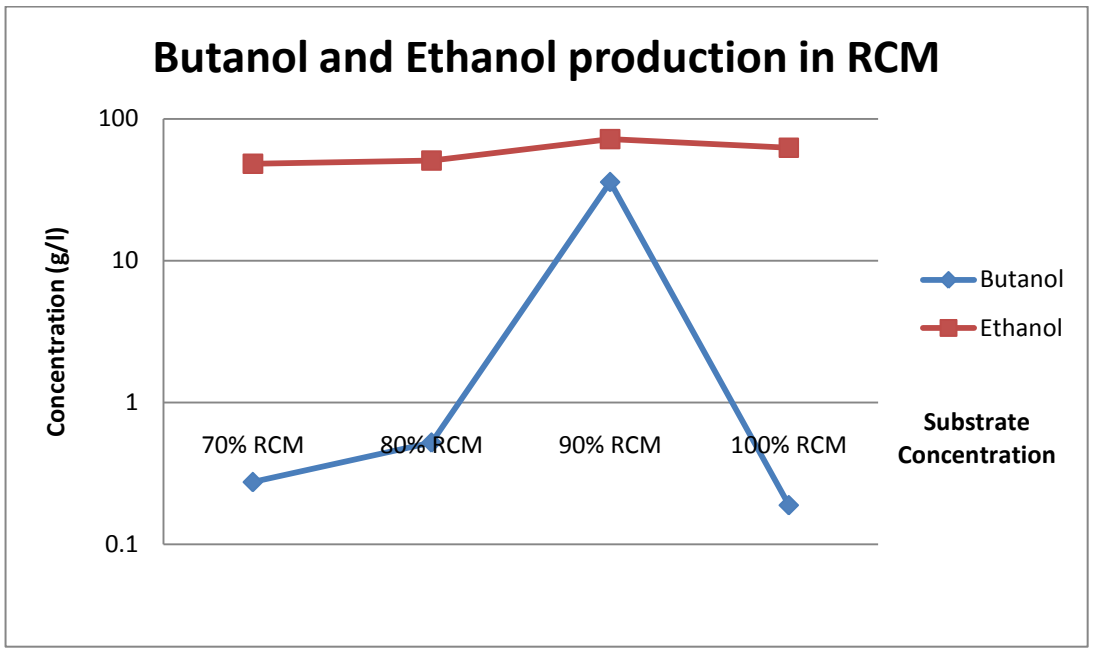

Figure 4.12: The concentration in g/l of butanol and ethanol produced after 20 hours cultivation using RCM for different concentration of RCM (70%, 80%, 90% and 100%)

For this particular experiment, two main figures, Figure 4.11 and Figure 4.12 shows the overall butanol production in g/l after 20 hours cultivation for POME and

RCM respectively. Figure 4.11 shows the (g/l) of butanol and ethanol produced after 20 hours cultivation in different concentration of POME (70%, 80%, 90% and 100%).

In the medium of POME, the production of butanol was detected to be highest during the 90% concentration of POME with value of 5.1377 g/l and lowest during the 70% & 100% concentration POME with 0.3429 g/l and 0.3167 g/l respectively. The concentration of butanol production is very low because of few important reasons. The main cause for the limited solvent production may be because of severe product inhibition. Butanol at a concentration of 10 g/L can significantly inhibit cell growth and the fermentation. Consequently, butanol titers in conventional ABE fermentations are usually lower than 13 g/L (1.3%) (Ramey & Yang, 2004).

However in overall, it can be observed that the pattern of the butanol production is very low compared to the ethanol production in each sample. The lower production of butanol compared to the ethanol production can be caused by many possibilities. The main problem detected in this fermentation could be the substrate inhibition. Main effects of each factor (acetate, butyrate, butanol, acetone, and ethanol), as well as the mutual interactions of these factors, on the maximum specific cell growth rate were examined in a fractional experiment was found that acetate, butyrate and butanol exhibit synergistic inhibitory effects on the cell growth of *C. acetobutylicum* while acetone and ethanol was non inhibitory solvents (Yang and Tsao, 1994).

Strain degeneration can also be a prior problem in the fermentation process for culturing maintenance and also for inoculums preparation. Initial sub-culture of the *Clostridium butylicum* bacteria showed improvement in the sugar utilization, solvent concentration and cell numbers. However, after third sub-culture, degeneration was rapid and by the sixth no solvent was detected (Gapes *et al.*, 1983).

Another factor that may affect the butanol production is the pH. pH values shows different growth pattern as lower pH, favors the undissociated form of the acid inhibiting cellular growth with depletion of nitrogen gas while higher pH shows single growth phase as it does not reach a sufficiently high level to stop growth

before depletion of nitrogen (Monot *et al.*, 1984; Yang and Tsao, 1994; Bowles and Ellefson, 1985).

Not only that, lignocellulosic materials are abundant and contains cellulose and hemicelluloses which liberate sugars by hydrolytic methods. However the lignocellulosic hyrolysates contains not only fermentable sugars but also non-fermentable compounds such as furan, weak acids, and various phenolic compounds that inhibit the microbial fermentation to the desired products. (Cho *et al.*, 2009). The toxicity of the phenolic compound inhibits the cell growth of the *C.acetobutylicum* in this fermentation and then inhibits the butanol production throughout the experiment. The values of butanol shown in the figures above are very low except for the butanol concentration in the 90% substrate concentration fermentation.

Apart from all, another reason on the low production of the butanol could be from the extraction during the recovery of butanol from the fermentation broth. In this experiment toluene was used as an extractant to extract the solvents using liquid-liquid extraction. Liquid-liquid extraction was used for this fermentation because it was the most compatible method to be used in order to extract the solvent. As solvents, alcohols, esters, and phenols have higher distribution coefficients for ethanol, butanol, and acetone from aqueous solutions (Kim *et al.*, 1999; Dadger and Foutch, 1985).

Masahito *et al*, (1980) stated that oleyl alcohol (cis-9-octadecen-1-ol) was an excellent extracting solvent for butanol contrary from the research done by Chuichulcherm and Chutmanop, (2004), where they stated that 2-ethyl-1-hexanol was the best extractant compared to oleyl alcohol crude palm oil ester. However, both extractant could not be used for this fermentation because of their very high selling price besides the times constrain to get the extractant from other resources.

Sedimentation of POME on the other hand, helped to remove toxics and also traces of oil leaving less inhibitory POME for a better and suitable growth of *C. acetobutylicum*. The concentration (g/l) of **b**utanol and **e**thanol produced after 60 hours cultivation using RCM for different concentration of POME (70%, 80%, 90%

and 100%) showed almost similar pattern as the butanol production with the highest production during the 90% POME substrate concentration. RCM was basically used as an indicator and act as the control process for the production of butanol and ethanol.

Another main reason was the POME which was collected from Lepar Hilir. There are 2 reasons on how POME could have been affecting the results in solvents production:

1) The sample supplied from lepar hilir was taken at different times of production. When the sample was taken during the morning, the Lepar Hilir Company would have just started their production and hence the sample was very dilute since not much production has been done. Comparatively, when the POME was taken during the evening, the production would have reached its peak production and the waste produced was much thicker. This gives a big effect on the solvent production as this experiment is based on the concentration of the POME.
2) In Malaysia there are two seasons, from October to February is the rainy season and from June to September is comparatively dry season while oil palm is able to harvest all the year around. The pH's from October to January were higher than pHs of the other months (Ramey & Yang, 2004). Hence not only the acidity but also the nutrients were diluted with water.

Furthermore, this fermentation process was quite complex and extra safety precaution needed to be taken in order run the fermentation under very sterile conditions. Contamination, particularly due to phage infections can cause problems making it impossible for the growth of wanted bacteria (Jones and Ramey, 1986; Chauvatcharin *et al.*, 1998). This is because, during the fermentation, even though the most safety precautions were taken, mishaps do occur during the fermentation.

Lower production in butanol compared to ethanol in overall may be also contributed by the contamination during the preparation of this experiment. This may be caused by many factors such as contaminated vials during the preparation of the bacteria enrichment and also may be due to the contamination in the Schott bottle after the taking of the first reading for the GC-FID test on butanol and ethanol.

4.4 Glucose Consumption Using DNS Method

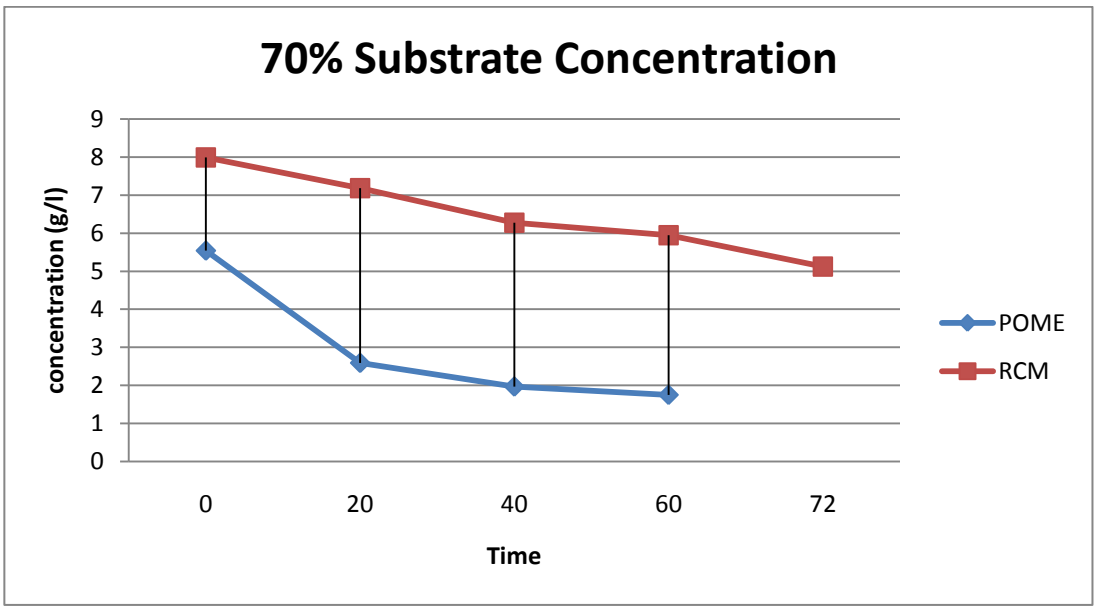

Figure 4.13: The concentration of glucose consumption in g/l versus time during 70% POME substrate concentration.

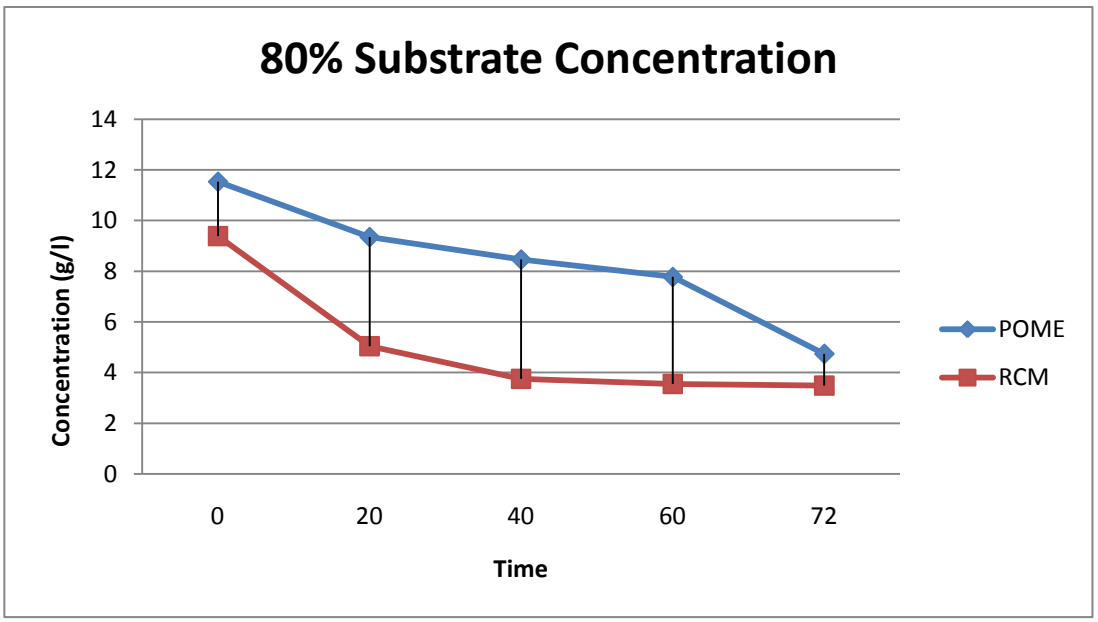

Figure 4.14: The concentration of glucose consumption in g/l versus time during 80% POME substrate concentration.

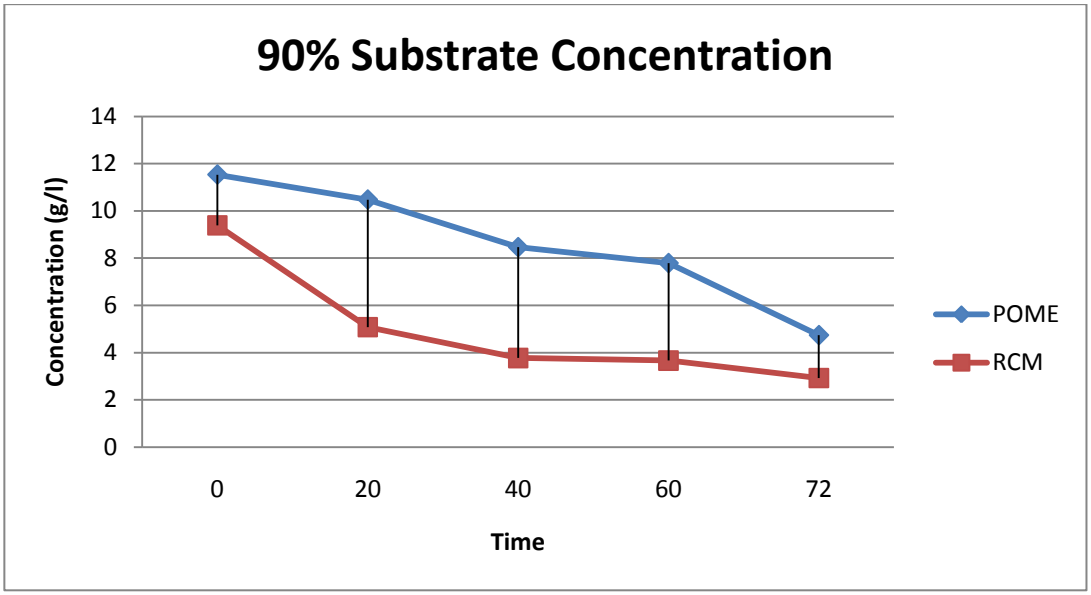

Figure 4.15: The concentration of glucose consumption in g/l versus time during 90% POME substrate concentration.

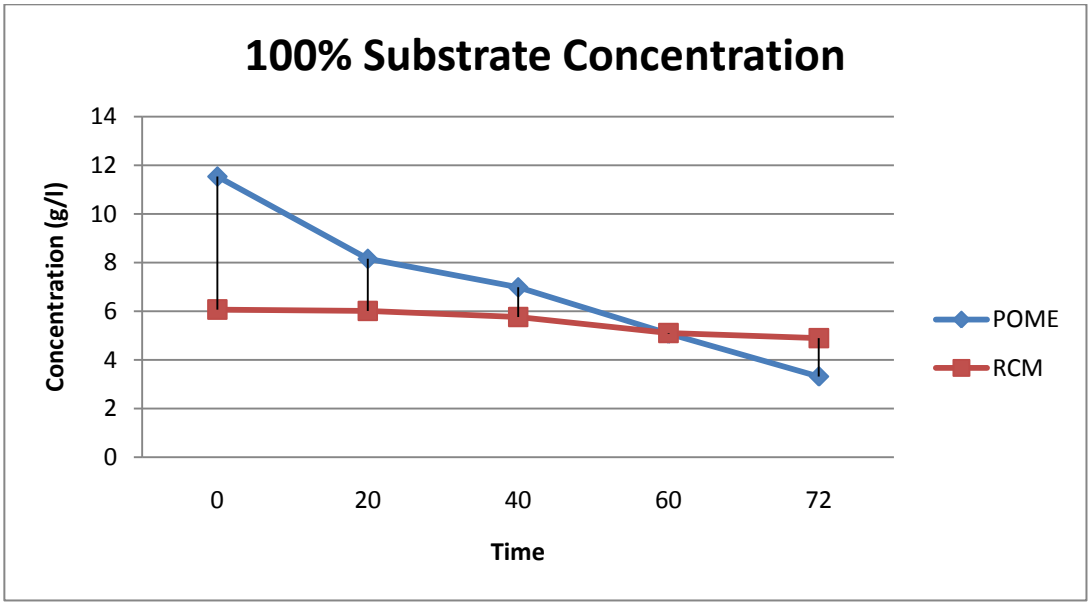

Figure 4.16: The concentration of glucose consumption in g/l versus time during 100% POME substrate concentration.

In overall, all the four Figures, 4.13, 4.14, 4.15 and 4.16 shows the same pattern of decreasing value in the glucose concentration throughout the fermentation. Each figure shows the consumption of glucose in both POME and RCM substrate in g/l by the bacteria (*C. acetobutylicum*). The values taken are in the range of 20 hours after the start of fermentation until the 72 hours of fermentation.

In Figure 4.13, it can observed that the glucose in RCM is much higher (7.179 g/l) compared to POME as the glucose value (5.545g/l). The main factor for this reason could have been the sample of POME itself which was collected from the palm oil mill, Lepar Hilir. The sugar or hemicelluloses in POME could have been in a very dilute form before being diluted again to achieve 70% substrate concentration. In this figure however it can be clearly seen that, the graph shows a decline value in the consumption of sugar throughout the period of fermentation by *C acetobutylicum.* In POME the initial value decreased from 5.545 g/l to an amount below than 1.755 g/l whereas in RCM it decreased from 7.9865 g/l to 5.122 g/l.

Figure 4.14, 4.15 and 4.16 however showed a similar graph in which the value of glucose was much higher in the POME medium compared to RCM while the consumption of glucose was decreasing throughout the fermentation as there was consumption on the glucose intake by the *C.acetobutylicum* to produce the solvents (butanol and ethanol). In Figure 4.14 for the 80% substrate concentration, the highest glucose was found to be 11.535 g/l for POME and 9.386 g/l for RCM. This value decreased to 4.741 g/l and 3.484 g/l respectively after 72 hours of fermentation.

For the substrate concentration of 90% in Figure 4.15 showed that the glucose decreased from 10.478g/l to 4.741g/l in the POME while the value of glucose decreased 5.079g/l to 2.926g/l in RCM substrate. On the other hand, value of 11.535g/l decreased to 3.322g/l for POME in 100% substrate concentration while value of glucose decreased from 6.071g/l to 4.895g/l for RCM as shown in Figure 4.16.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

At this chapter the conclusion from the experimental analysis and the results obtained will be discussed. With different substrate concentration and keeping the other parameters in the optimum value, fermentation was conducted to achieve the highest value of butanol production.

5.1.1 Growth Profile of *C. acetobutylicum*

The importance of the profile growth is to determine the most suitable time in the growth of the bacteria for fermentation to produce the highest solvent production. From the experiment, it can be concluded that POME is a potential utility substrate to produce solvents (butanol and ethanol). It can be seen that *C.acetobutylicum* displays a characteristic three-phase pattern of growth in both medium. The lag phase occurs

from 0-6 hours for both POME and RCM followed by a log phase where for RCM, replication occurs from the 6^{th} hour to 42 hours. On the other hand, for POME replication occurs from the 6^{th} hour to 18 hours.

Stationary phase occurs for RCM from 42 hours to 54 hours while for POME, the stationary phase starts from the 18^{th} hour to 54^{th} hour and finally will continue with the decline phase. Both the mediums, undergo logarithmic decline phase after that when cells die faster than they are replaced.

5.1.2 Composition Analysis of selected fresh POME

Composition analysis of selected fresh POME is conducted to identify the component of reducing sugar in POME for the usage of fermentation. From the analysis it can be concluded that the POME contains a number of reducing sugar including fructose, glucose, galactose, sucrose and lactose. POME has showed that components of the hemicelluloses contains the essential reducing sugars which is very useful for the conversion to solvents as the variety of sugar components in the separator sludge will increase the chances of the solvent production.

5.1.3 Butanol and Ethanol Production

In this experiment, it has been successfully shown that POME is a substrate which can actually change its negative effect and negative money on the environment towards a better product with less pollution besides producing positive money. Butanol and ethanol can be produced successfully from the conducted

fermentation. However, the production of butanol was much lower compared to the production of ethanol in the fermentation conducted.

The aim in this experiment to achieve a higher butanol production compared to ethanol was achieved only in the first 20 hours fermentation . Therefore, many improvement steps need to be taken into consideration to ensure the objective of this experiment can be achieved. Inhibition factor was definitely the main effect that has to be taken into consideration to ensure higher butanol value to be produced. This is because butanol is an inhibitor to the growth *C.acetobutylicum* while ethanol is a non inhibitory solvent towards *C.acetobutylicum*.

Not only that, phenolic component in POME sample, strain degeneration, pH of fermentation and sample of separator sludge from Lepar Hilir could have also contributed in the fermentation. Besides that pretreatment of POME, sedimentation also could have affected in the lower production of butanol compared to ethanol. To ensure a good extraction is done of the butanol to the aim to increase the butanol production, a better extractor must also be taken into consideration besides ensuring every safety and cleanliness steps have been taken to decrease contamination towards the fermentation.

5.1.4 Glucose Consumption

Glucose has high influence towards the production of butanol and ethanol in the fermentation. Glucose is the main substrate in the POME and RCM to be used in the conversion to produce the wanted solvent. This method used which is DNS method is a very popular and effective method to detect glucose. As the value of glucose in POME and RCM decrease throughout the fermentation, it can be concluded that *C.acetobutylicum* uses it for its growth to enhance the production of solvents. This enables the increase in the production of solvents through the period of 72 hours which was studied during the experiment was conducted.

5.2 Recommendation

Butanol, is the desired end product of the fermentation, was found to have several harmful effects on *C. acetobutylicum*. Butanol destroyed the ability of the cell to maintain internal pH (which consequently dissipates the pH gradient across the cell membrane), lowered the intracellular level of ATP, and inhibited glucose uptake (Bowles and Ellefson, 1985). Therefore, was desirable to develop an effective butanol recovery technique to improve the fermentation performance.

Yang and Tsao (1994) in their experiment has suggested and developed novel separation-coupled fermentation processes. It has been reported that fermentation operated continuously with separation could improve volumetric and specific butanol productivity. The study in this experiment hence should be focused on the development of increase in butanol production by using an adsorption- coupled fermentation process to overcome the shortcomings of inhibition effects.

Inhibition caused by the phenolic component from the POME should also be taken into consideration before starting a new fermentation in the process of producing solvents. Peroxidase could be applied to remove the model phenolic components and it has been proved for the detoxification of lignocellulosic hydrolysates. The detoxified solution has remarkly improved the cell growth and level of inhibition towards butanol (Cho *et al.*, 2009).

Besides that, it is also advised that to decrease the strain degeneration, heat shocking and sub-culturing the bacteria, *C. acetobutylicum* should not be done more than 3 times. Heat shocking and sub-culturing is a method that can increase and improve the sugar utilization, solvent concentration and cell numbers. However decreasing the heat shocking and sub-culturing should be done due the reason that, sub-culturing above the numbers stated will degenerate the bacteria and at one stage it will not be effective for the fermentation to occur.

Immobilization of the bacteria can be effective method to increases the progressive production of solvents in this fermentation. Immobilization of anaerobe

has two merits on the fermentation of palm oil mill effluent (POME). Firstly, anaerobe can proliferate even in the immobilized gel particles without any requirement of oxygen, therefore, the gel might reach to high cell population. Secondly, on the comparison between immobilized cell and hollow fiber unit both which will help to concentrate cell population, the insoluble material in POME will have no effect on the fermentation by immobilized cell particle, but it will affect on permeability of hollow fiber unit. On the basis of such ideas, then, fermentation system with immobilized cell particles should be used in order to achieve the objectives of this fermentation which is to produce higher butanol than ethanol.

Not only that, the improvements in the extraction method of both butanol and ethanol should also be taken into consideration. Many studies have been done by different researches to get the highest and best extractor for the products in the fermentation. Oleyl alcohol is the most common extractor used in fermentation to extract the solvents because of the high capacity towards the solvents. Study by Chuichulcherm and Chutmanop (2004) stated that 2-ethyl-1-hexanol is better extractant compared to oleyl alcohol crude palm oil ester. Hence, for this experiment this extractant can be used as replacement to toluene in ensuring highest value of solvents especially for butane can be produced.

Last but not least, since this fermentation process is quite complex, extra safety precaution needed to be taken in order run the fermentation under very sterile conditions. Contamination, particularly due to phage infections can cause problems making it impossible for the growth of wanted bacteria. Here each sample taken out for the reading is done in very sterile procedure and each time the sample is taken, it is advisable to purge the bottle with nitrogen to keep the bottle in anaerobic sample and not contaminating the fermentation process.

REFERENCES

Addison, K., (2009). Introduction to a Farmer's Fuel...Alcohol. *The Mother Earth News, 1980*. Retrieved July 30, 2009, from http://www.journeytoforever.org/biofuel_library/ethanol_motherearth/meCh1.html

Agustin, M. B., Sengpracha, P. and Phutdhawong, W. (2008). Electrocogulation of Palm Oil Mill Effluent. *International Journal of Environmental Research and Public Health*, 5(3), 177-180.

Bahl, H., Gottwald, T. M., Kuhn, A., Rale, V., Andersch, T. W. and Gottschalk, G., (1986). Nutritional Factors Affecting the Ratio of Solvents Produced by *Clostridium acetobutylicum*. *Applied and Environmental Microbiology*, 169-172.

Brekke, K. (2007, March). Butanol. *EthanolToday S*, 36-39.

Chuicuhlcherm, S., and Chutmanop, J., (2004).Butanol Separation from ABE model fermentation broth by liquid-liquid extraction. *Department of Chemical Engineering, SrinakharinwirotUniversity, Thailand.*

Dadger, A. M. and G. L. Foutch (1985) Evaluation of solvents for the recovery of *Clostridium*. Fermentation products by liquid-liquid extraction. *Biotechnol. Bioeng. Syrup.*,15, 611-620.

Durre, P. (1998), New insights and novel developments in Clostridial acetone/butanol/isopropanol fermentation. *Appl. Microbiol. Biotechnol*, 49, 639-648.

Fond, O., Matta-Ammouri, G., Petitdemange, H., and Engasser, J. M., (1985). The role of acids on the production of acetone and butanol by Clostridium acetobutylicum. *Appl. Microbiol. biotechnol.*, 22, 195-200.

Gapes, J. R., Larsen, V. F. and Maddox, I.S., (1983). A note on procedures for inoculums development for the production of solvents by a strain of C*lostridium butylicum*. *Journal of applied bacteriology*, 55, 363-365.

Hayasida, S. & Ahn, B. K. (1990). Isolation and Characteristics of an Acetone-Butanol-Negative, Ethanol-Isovaleric Acid Producing Mutant of Clostridium Saccharoperbutylacetonicum N1-4 (ATCC 13564). *Journal of Agricultural and Biological Chemistry*, 54(2), 343-351.

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

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

Mun, L.T, Ishizaki, A., Yoshino, S. & Furukawa, K. (1995). Production of Acetone, Butanol and Ethanol from Palm Oil Waste by Clostridium Saccharoperbutylacetonicum N1-4. *Biotechnology Letters*, 17(6), 649-654.

Maddox, I. S. (1989). The acetone-butanol-ethanol fermentation: recent progress in technology. *Biotechnol. Genet. Eng.*, 7, 189-220.

Maheswaran, A. & Singam, G. (1977). Pollution control in the palm oil industry–promulgation of regulations. *Planter*, 53, 470-476.

Masahito, T., Ohmiya, K., Kobayashi, T., and Shimizu, S., (1980). Monitoring and control of a cellulolytic anaerobe culture by using gas evolved as an indicator. Journal of Fermentation Technology, 58, 463-469.

Monot, F., Engasser, J. M., & Petitdemange, H. (1984). Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of Clostridium acetobutylicum. *Appl Microbial Biotechnology*, 19, 422-426.

Nag A. (2008). *Biofuels Refining and Performance*. New York: McGraw-Hill Professional.

Ngan, M. A., Tanisho, S., Marimoto, M., Yoshino, S. (2003). Conversion Technology of Biomass/Bioenergy. *Research report of the International Joint Research Grant Project for FY2003.*

Nielsen C. B., Singh G., Toh T.S. (1999). Bioremediation of palm oil mill effluent. In: Proceedings Porim International Palm Oil Congress 16th February 1999, Kuala Lumpur, Malaysia.

Pang, Kit, W., Yusoff, W. M. W., and Kalil, M. S., and Hassan, O., (2004). Production of Acetone-Butanol-Ethanol and Hydrogen of Palm Oil Mill Effluent Anaerobic Fermentation with Clostridium Acetobutylicum NCIMB 13357. *Sains Malaysiana*, 33, 73-81.

Papoutsakis, E. T. (2008). Engineering Solventogenic Clostridia. *Current Opinion in Biotechnology*, 19(5), 420-429.

Park, C. H., Okos, M. R., Wankat, P. C. (1991). Acetone-Butanol-Ethanol (ABE) fermentation and simultaneous separation in trickle bed reactor. *Biotechnology Prog*., 7, 185-194.

Ramey, D (2009). *Butanol is an alcohol that replaces gasoline!*. Retrieved July 30, 2009, from http://www.butanol.com/

Ramey, D. & Yang, S. T. (2004). Production of Butyric Acid and Butanol from Biomass (Final Report). U.S. Department of Energy Morgantown, WV.

Renewable Energy Source. (2009). *How Does Butanol Work?*. Retrieved July 28, 2009, from http://renewable-energy-future.com/how-does-it-work/butanol.php

Righelato, R. C. (1980). Anaerobic Fermentation: Alcohol Production. *Biological Sciences*, volume 290, Issue 1040, 303-310.

Takriff, M. S., Masngut, N., Khadum, A. A. H., Kalil, M. S., Mohammad, W. (2009). Solvent fermentation from plam oil mill effluent using *clostridium acetobutylicum* in oscillatory flow bioreactor bioreactor. *Sains Malaysiana*, 38(2), 191-196.

Wong, P.W., Sulaiman, N.M., Nachiappan, M. & Varadaraj, B. (2002). Pre-treatment and membrane ultrafiltration using treated palm oil mill effluent (POME) Songklanakarin *Journal of Science Technology*, 24, 891-898.

Wu, T. Y., Mohammad, A. 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.

Yang, X., and Tsao, G. T., (1984). Mathematical Modeling of Inhibition Kinetics in Acetone-Butanol Fermentation by *Clostridium acetobutylicum*. *Appl Microbiol Biotechnol*, 19, 422-426.

APPENDIX A

GROWTH PROFILE

Appendix A-1: Growth Profile of *Clostridium acetobutylicum* in Palm Oil Mill Effluent (POME)

Table A-1-1: 1st reading at 11.00 pm (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	2.99	2.99	2.99	2.99
1/10	1.638	1.638	1.638	1.638
1/20	0.989	0.989	0.989	0.989
1/40	0.385	0.385	0.385	0.385

Table A-1-2: 2nd reading at 5.00am (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.854	2.854	2.854	2.854
1/20	1.310	1.310	1.310	1.310
1/40	0.576	0.576	0.576	0.576

Table A-1-3: 3rd reading at 11.00am (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.678	2.678	2.678	2.678
1/20	1.674	1.674	1.674	1.674
1/40	0.765	0.768	0.768	0.767

Table A-1-4: 4th reading at 5.00pm (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.856	2.855	2.856	2.856
1/20	1.294	1.294	1.294	1.294
1/40	0.815	0.815	0.815	0.815

Table A-1-5: 5th reading at 11.00pm (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.685	2.685	2.685	2.685
1/20	1.356	1.356	1.356	1.356
1/40	0.826	0.826	0.826	0.826

Table A-1-6: 6th reading at 5.00am (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.898	2.898	2.898	2.898
1/20	1.456	1.456	1.456	1.456
1/40	0.836	0.836	0.836	0.836

Table A-1-7: 7th reading at 11.00am (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.678	2.678	2.678	2.678
1/20	1.674	1.674	1.674	1.674
1/40	0.798	0.798	0.798	0.798

Table A-1-8: 8th reading at 5.00pm (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.523	2.523	2.523	2.523
1/20	1.821	1.816	1.813	1.813
1/40	0.820	0.820	0.820	0.820

Table A-1-9: 9th reading at 11.00pm (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.569	2.569	2.569	2.569
1/20	1.865	1.865	1.865	1.865
1/40	0.857	0.857	0.857	0.857

Table A-1-10: 10th reading at 5.00am (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.824	2.824	2.824	2.824
1/20	1.975	1.975	1.975	1.975
1/40	0.827	0.827	0.827	0.827

Table A-1-11: 11th reading at 5.00pm (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	3.0	3.0	3.0	3.0
1/10	2.769	2.769	2.769	2.769
1/20	1.051	1.051	1.051	1.051
1/40	0.823	0.823	0.823	0.823

Table A-1-12: 12th reading at 5.00pm (blank = 3.0)

Dilution	Reading 1	Reading 2	Reading 3	Average
X	2.797	2.797	2.797	2.797
1/10	2.319	2.319	2.319	2.319
1/20	1.836	1.836	1.836	1.836
1/40	0.825	0.825	0.825	0.825

Appendix A-2: Growth Profile of *Clostridium acetobutylicum* in Reinforced Clostridia Media (RCM)

Table A-2-1: Optical density value for the growth profile of *C. acetobutylicum* in RCM

Time/ hr	1	2	3	Average
0	0	0	0	0
6	0.28	0.281	0.28	0.28
12	0.45	0.431	0.433	0.438
18	0.766	0.773	0.741	0.76
24	0.799	0.789	0.789	0.792
30	0.812	0.8	0.81	0.807
36	0.874	0.874	0.874	0.874
42	0.97	0.971	0.971	0.971
48	1.007	1.007	1.009	1.008
54	1.007	1.007	1.009	1.008
60	1.007	1.007	1.009	1.008
66	1.007	1.007	1.009	1.008
72	1.007	1.007	1.009	1.008

APPENDIX B

COMPOSITION ANALYSIS OF SELECTED FRESH POME

Appendix B-1: Standard for POME Composition

Table B-1-1: Xylose (Retention time = 5.902)

Amount concentration (g/l)	Area
20	1.43341e6
40	2.84440e6
60	4.28133e6
80	5.71472e6
100	7.3507e6

Formula from calibration curve;

y = mx + b,

Where,

m = 72906.51429 **x = amount (g/l)**

b = -41225.71429 **y = area**

Table B-1-2: Fructose (Retention time = 7.052)

Amount concentration (g/l)	Area
20	1.13320e6
40	2.36668e6
80	4.37532e6
100	5.19066e6

Formula from calibration curve;

y = mx + b

Where,

m = 52218.22769 **x = amount (g/l)**

b = 106696.09593 **y = area**

Table B-1-3: Glucose (Retention time = 7.702)

Amount concentration (g/l)	Area
20	1.69828e6
40	3.27955e6
60	5.05296e6
80	6.64497e6
100	8.23004e6

Formula from calibration curve;

y = mx + b

Where,

m = 82519.54286 **x = amount (g/l)**

b = 24989.54381 **y = area**

Table B-1-4: Galactose (Retention time = 8.633)

Amount concentration (g/l)	Area
20	9.92166e5
40	1.94887e6
60	2.87786e6
80	3.94253e6
100	4.75126e6

Formula from calibration curve;

y = mx + b

Where,

m = 47909.12205 **x = amount (g/l)**

b = 23325.22024 **y = area**

Table B-1-5: Sucrose (Retention time = 10.381)

Amount concentration (g/l)	Area
20	1.86245e6
40	3.26337e6
60	4.89866e6
80	6.36837e6
100	7.84323e6

Formula from calibration curve;

y = mx + b

Where,

m = 77670.26196 **x = amount (g/l)**

b = 155833.71429 **y = area**

Table B-1-6: Lactose (Retention time = 13.127)

Amount concentration (g/l)	Area
20	1.30290e6
40	2.50230e6
60	3.21022e6
80	4.19806e6
100	5.35126e6

Formula from calibration curve;

Y = mx + b

Where,

m = 51642.42268 **x = amount (g/l)**

b = 178669.59524 **y = area**

Appendix B-2: Sample Data of POME Composition

Table B-2-1: Sample Analysis 1

Peak	Retention Time	Sugar compound	Area	Concentration (g/l)
3	7.769	Glucose	5.29701e5	6.1163
4	8.756	Galactose	3.03513e5	5.8483
6	10.190	Sucrose	4.33496e5	3.5749
7	13.290	Lactose	2.31336e5	1.0198

Table B-2-2: Sample Analysis 2

Peak	Retention Time	Sugar compound	Area	Concentration (g/l)
3	7.766	Glucose	2.42428e5	2.6350
4	8.771	Galactose	4.76619e5	9.4615
5	10.216	Sucrose	1.45158e5	1.5588
6	13.347	Lactose	8.30884e4	1.2754

Table B-2-3: Sample Analysis 3

Peak	Retention Time	Sugar compound	Area	Concentration (g/l)
3	7.770	Glucose	2.11423e5	2.2593
4	8.774	Galactose	5.63179e5	11.2683
5	10.232	Sucrose	1.48901e5	1.5990
8	13.370	Lactose	1.06895e5	1.6409

APPENDIX C

BUTANOL AND ETHANOL PRODUCTION

Appendix C-1: Standard for Butanol

Table C-1-1: Standard Butanol Concentration Table

Concentration (g/l)	Area (pA*s)
0.1	27.94280
0.2	40.96081
0.4	106.17123
0.6	149.61745
1.0	217.72562
1.5	424.41580
2.0	472.72708
10.0	2798.79004

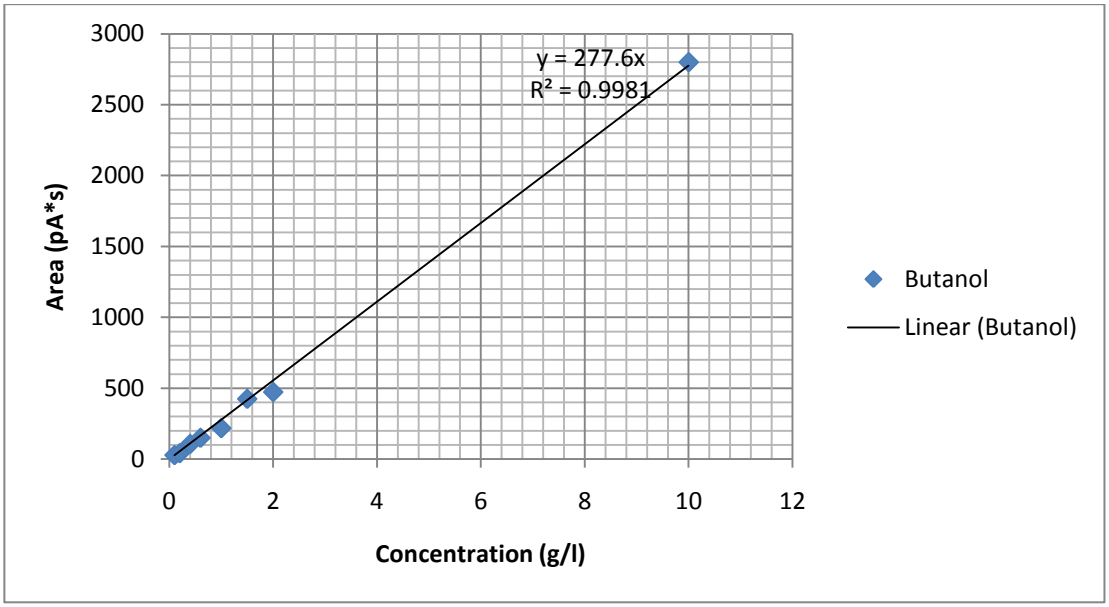

Figure C-1-1: Standard Curve for Butanol Production

Appendix C-2: Standard for Ethanol

Table C-2-1: Standard Ethanol Concentration Table

Concentration (g/l)	Area (pA*s)
0.2	24.37853
0.4	34.07329
0.8	52.41584
1.0	153.71109
1.6	183.79024
2.0	233.67892

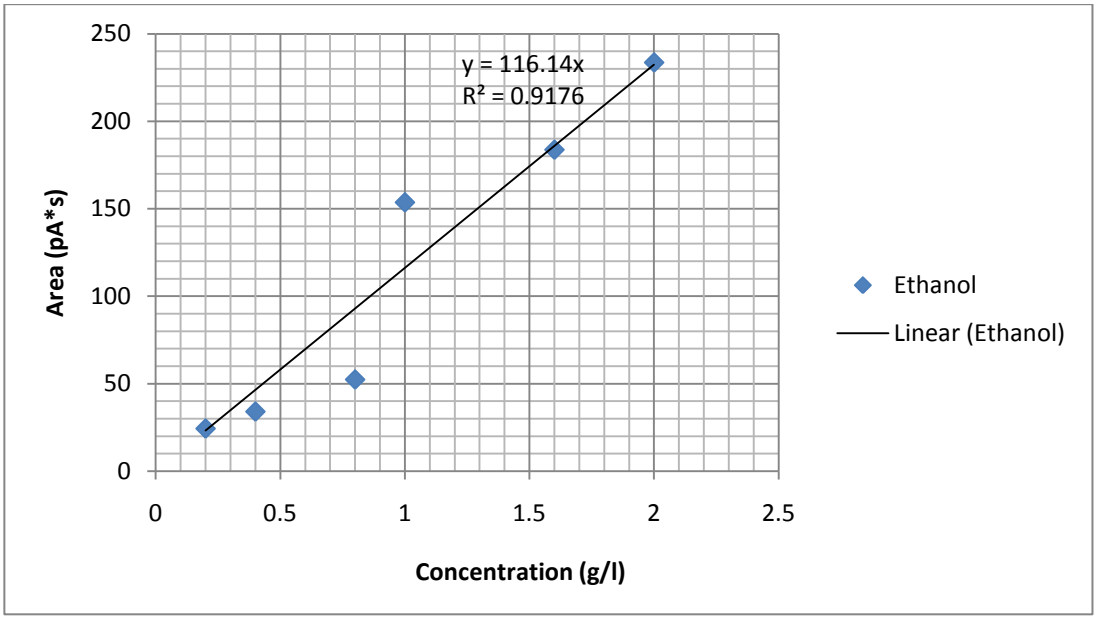

Figure C-2-1: Standard Curve for Ethanol Production

Appendix C-3: Sample Data of 70% substrate concentration

Table C-3-1: Butanol and ethanol production in substrate concentration of 70% POME

Time (hr)	Butanol POME		Ethanol POME	
	Area (pA*s)	Conc. (g/l)	Area (pA*s)	Conc. (g/l)
20	84.7727	0.3054	6755.9927	58.1711
40	82.5692	0.2974	6959.8994	59.9268
60	92.9975	0.3350	11219.7000	96.6050
72	99.4910	0.3584	7056.7710	60.7609

Table C-3-2: Butanol and Ethanol production in 70% RCM

Time (hr)	Butanol RCM		Ethanol RCM	
	Area (pA*s)	Conc. (g/l)	Area (pA*s)	Conc. (g/l)
20	76.3730	0.2751	5604.0288	48.2524
40	87.0629	0.3136	5466.1606	47.0653
60	69.3166	0.2497	6823.3618	58.7512
72	67.3656	0.2427	7395.3491	63.6762

APPENDIX C-4: Sample Data of 80% Substrate Concentrations

Table C-4-1: Butanol and ethanol production in substrate concentration of 80% POME

Time (hr)	Butanol POME		Ethanol POME	
	Area (pA*s)	Conc. (g/l)	Area (pA*s)	Conc. (g/l)
20	18.4795	0.0666	6463.4981	55.6526
40	9.2746	0.0334	7512.5947	64.6857
60	4.5390	0.0164	5349.0596	46.0570
72	3.4766	0.0125	7620.5698	65.6153

Table C-4-2: Butanol and ethanol production in 80% RCM

Time (hr)	Butanol RCM		Ethanol RCM	
	Area (pA*s)	Conc. (g/l)	Area (pA*s)	Conc. (g/l)
20	144.9852	0.5222	5908.6685	50.8754
40	10.9807	0.0395	7792.2748	67.0938
60	3.0788	0.0111	6216.7559	53.5281
72	-	-	19.8910	0.1713

APPENDIX C-5: Sample Data of 90% Substrate Concentrations

Table C-5-1: Butanol and ethanol production in substrate concentration of 90% POME

Time (hr)	Butanol POME		Ethanol POME	
	Area (pA*s)	Conc. (g/l)	Area (pA*s)	Conc. (g/l)
20	1278.2063	4.6045	9941.5029	85.5993
40	16.8026	0.0605	9355.2295	80.5513
60	12.8143	0.0462	8214.2705	70.7273
72	2.3462	0.0085	9447.4102	81.3450

Table C-5-2: Butanol and ethanol production in 90% RCM

Time (hr)	Butanol RCM		Ethanol RCM	
	Area (pA*s)	Conc. (g/l)	Area (pA*s)	Conc. (g/l)
20	9941.5029	35.8123	8358.2637	71.9671
40	9355.2295	33.7004	9690.2042	83.4355
60	8214.2705	29.5903	9429.8265	81.1936
72	9447.4102	34.0325	9407.2588	80.9993

APPENDIX C-6: Sample Data of 100% Substrate Concentrations

Table C-6-1: Butanol and ethanol production in substrate concentration of 100% POME

Time (hr)	Butanol POME		Ethanol POME	
	Area (pA*s)	Conc. (g/l)	Area (pA*s)	Conc. (g/l)
20	78.2572	0.2819	7687.3677	66.1905
40	78.5438	0.2829	7195.7344	61.9574
60	55.7752	0.2009	7196.5054	61.9641
72	56.3658	0.2030	7862.8955	67.7019

Table C-6-2: Butanol and Ethanol production in 100% RCM

Time (hr)	Butanol RCM		Ethanol RCM	
	Area (pA*s)	Conc. (g/l)	Area (pA*s)	Conc. (g/l)
20	52.4611	0.1890	7273.8950	62.6304
40	64.3544	0.2318	8414.3965	72.4505
60	47.6658	0.1717	7423.3823	63.9175
72	60.5835	0.2182	7449.9830	64.1466

APPENDIX D

GLUCOSE CONSUMPTION

Appendix D-1: Standard for Glucose Consumption

Table D-1-1: Standard for Glucose Consumption

concentration	OD			
	1	2	3	AVG
0.0	0.109	0.116	0.106	0.1103
0.2	0.571	0.566	0.562	0.5663
0.4	1.156	1.150	1.146	1.1507
0.6	1.658	1.662	1.648	1.6560
0.8	2.097	2.149	2.108	2.1180
1.0	2.456	2.468	2.456	2.4600

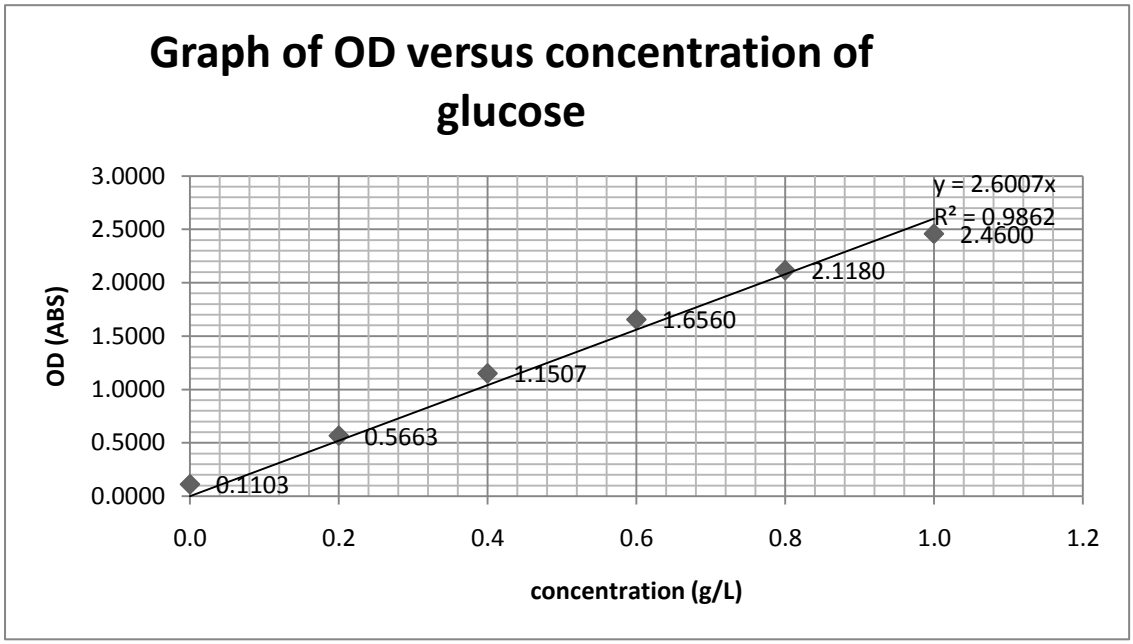

Figure D-1-1: Standard curve for concentration of glucose consumption in g/l

APPENDIX D-2: Sample data of 70% substrate concentration

Table D-2-1: Glucose consumption in 70% POME Concentration at 0 hours

Blank = 3.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.051	2.051	2.051	2.051	7.886
1/10	1.442	1.442	1.442	1.442	5.545

Table D-2-2: Glucose consumption in 70% POME concentration after 20 hour

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	1.597	1.597	1.597	1.597	6.141
1/10	0.675	0.675	0.675	0.675	2.595

Table D-2-3: Glucose consumption in 70% POME concentration after 40 hour

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	0.678	0.678	0.678	0.678	2.607
1/10	0.513	0.513	0.513	0.513	1.973

Table D-2-4: Glucose consumption in 70% POME concentration after 60 hour

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	0.647	0.647	0.647	0.647	2.488
1/10	0.453	0.455	0.455	0.454	1.755

Table D-2-5: Glucose consumption in 70% POME concentration after 72hour

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	0.589	0.589	0.589	0.589	2.265
1/10	-0.779	-0.779	-0.779	-0.779	-

Table D-2-6: Glucose consumption in 70% RCM at 0 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	3.0	3.0	3.0	3.0	11.535
1/10	2.076	2.077	2.077	2.077	7.986

Table D-2-7: Glucose consumption in 70% RCM after 20 hour

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.194	2.194	2.194	2.194	8.436
1/10	1.876	1.867	1.878	1.874	7.179

Table D-2-8: Glucose consumption in 70% RCM after 40 hour

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.092	2.092	2.092	2.092	8.044
1/10	1.631	1.631	1.633	1.632	6.275

Table D-2-9: Glucose consumption in 70% RCM after 60 hour

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.076	2.076	2.076	2.076	7.982
1/10	1.543	1.545	1.543	1.544	5.947

Table D-2-10: Glucose consumption in 70% RCM after 72 hour

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.036	2.036	2.036	2.036	7.829
1/10	1.332	1.311	1.332	1.325	5.122

APPENDIX D-3: Sample Data of 80% substrate concentration

Table D-3-1: Glucose consumption in 80% POME concentration at 0 hours

Blank = 3.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	3.0	3.0	3.0	3.0	11.535
1/10	3.0	3.0	3.0	3.0	11.535

Table D-3-2: Glucose consumption in 80% POME concentration after 20 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	3.0	3.0	3.0	3.0	11.535
1/10	2.432	2.432	2.432	2.432	9.351

Table D-3-3: Glucose consumption in 80% POME concentration after 40 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.477	2.477	2.477	2.477	9.524
1/10	2.201	2.201	2.201	2.201	8.463

Table D-3-4: Glucose consumption in 80% POME concentration after 60 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.310	2.310	2.310	2.310	8.882
1/10	2.022	2.022	2.022	2.022	7.775

Table D-3-5: Glucose consumption in 80% POME concentration after 72 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.034	2.034	2.034	2.034	7.821
1/10	1.233	1.233	1.233	1.233	4.741

Table D-3-6: Glucose consumption in 80% RCM at 0 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	3.0	3.0	3.0	3.0	11.535
1/10	2.441	2.441	2.441	2.441	9.386

Table D-3-7: Glucose consumption in 80% RCM after 20 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.768	2.768	2.768	2.768	10.643
1/10	1.311	1.311	1.311	1.311	5.041

Table D-3-8: Glucose consumption in 80% RCM after 40 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.699	2.721	2.721	2.721	10.463
1/10	0.976	0.976	0.976	0.976	3.753

Table D-3-9: Glucose consumption in 80% RCM after 60 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.658	2.658	2.658	2.658	10.220
1/10	0.923	0.923	0.923	0.923	3.549

Table D-3-10: Glucose consumption in 80% RCM after 72 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.501	2.501	2.501	2.501	9.617
1/10	0.906	0.906	0.906	0.906	3.484

APPENDIX D-4: Sample Data of 90% Substrate concentration

Table D-4-1: Glucose consumption in 90% POME concentration at 0 hours

Blank = 3.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	3.0	3.0	3.0	3.0	11.535
1/10	3.0	3.0	3.0	3.0	11.535

Table D-4-2: Glucose consumption in 90% POME concentration after 20 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	3.0	3.0	3.0	3.0	11.535
1/10	2.725	2.725	2.725	2.725	10.478

Table D-4-3: Glucose consumption in 90% POME concentration after 40 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.489	2.489	2.489	2.489	9.571
1/10	2.201	2.201	2.201	2.201	8.463

Table D-4-4: Glucose consumption in 90% POME concentration after 60 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.303	2.303	2.303	2.303	8.855
1/10	2.026	2.026	2.026	2.026	7.790

Table D-4-5: Glucose consumption in 90% POME concentration after 72 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.034	2.034	2.034	2.034	7.821
1/10	1.233	1.233	1.233	1.233	4.741

Table D-4-6: Glucose consumption in RCM at 0 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	3.0	3.0	3.0	3.0	11.535
1/10	2.441	2.441	2.441	2.441	9.385

Table D-4-7: Glucose consumption in 90% RCM after 20 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.194	2.194	2.194	2.194	8.436
1/10	1.321	1.321	1.321	1.321	5.079

Table D-4-8: Glucose consumption in 90% RCM after 40 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.092	2.092	2.092	2.092	8.044
1/10	0.981	0.980	0.981	0.981	3.772

Table D-4-9: Glucose consumption 90% RCM after 60 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.076	2.076	2.076	2.076	7.982
1/10	0.953	0.953	0.953	0.953	3.664

Table D-4-10: Glucose consumption in 90% RCM after 72 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.036	2.036	2.036	2.036	7.829
1/10	0.762	0.760	0.761	0.761	2.926

APPENDIX D-5: Sample Data of 100 % Substrate Concentration

Table D-5-1: Glucose consumption in 100% POME concentration at 0 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	3.0	3.0	3.0	3.0	11.535
1/10	3.0	3.0	3.0	3.0	11.535

Table D-5-2: Glucose consumption in 100% POME concentration after 20 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.276	2.276	2.276	2.276	8.751
1/10	2.121	2.121	2.121	2.121	8.155

Table D-5-3: Glucose consumption in 100% POME concentration after 40 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.229	2.229	2.229	2.229	8.571
1/10	1.816	1.816	1.816	1.816	6.983

Table D-5-4: Glucose consumption in 100% POME concentration after 60 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	1.773	1.773	1.773	1.773	6.817
1/10	1.321	1.321	1.321	1.321	5.079

Table D-5-5: Glucose consumption in 100% POME concentration after 72 hours

Blank = -0.00					
Dilution	Reading 1	Readin g 2	Readin g 3	Average	Glucose concentration (g/l)
X	1.441	1.440	1.442	1.441	5.541
1/10	0.864	0.865	0.863	0.864	3.322

Table D-5-6: Glucose consumption in 100% RCM at 0 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.432	2.432	2.432	2.432	9.351
1/10	1.579	1.578	1.579	1.579	6.071

Table D-5-7: Glucose consumption in 100% RCM after 20 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.699	2.699	2.699	2.699	10.378
1/10	1.564	1.564	1.565	1.564	6.014

Table D-5-8: Glucose consumption in 100% RCM after 40 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	2.587	2.587	2.587	2.587	9.947
1/10	1.499	1.499	1.499	1.499	5.764

Table D-5-9: Glucose consumption in 90% RCM after 60 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	1.914	1.914	1.914	1.914	7.360
1/10	1.327	1.327	1.327	1.327	5.102

Table D-5-10: Glucose consumption in RCM (100% POME) after 72 hours

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Glucose concentration (g/l)
X	1.733	1.733	1.733	1.733	6.664
1/10	1.273	1.273	1.273	1.273	4.895