EFFECT OF FERMENTATION TIME ON BUTANOL AND ETHANOL PRODUCTION FROM PALM OIL MILL EFFLUENT BY *CLOSTRIDIUM ACETOBUTYLICUM*.

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Signature : ……

Name of Supervisor : Miss Shariza Jamek

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

Faculty of Chemical and Natural Resources Engineering

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I declare that this thesis entitled “Effect of Fermentation Time on Butanol and Ethanol Production from Palm Oil Mill Effluent by *Clostridium Acetobutylicum* ” is the result of my own research except as cited as references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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DEDICATION

"I'm proudly dedicated this thesis to my most beloved parent and siblings, even word won't able to express the love. To my supportive friends, PSM team and my supervisor in hard and joy time, thank you so much for the continuous assist and encouragement."

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ABSTRACT

Biofuels production through fermentation from renewable waste has been put more to focus in mid 20th century till now. Main focus of the research is to determine optimum fermentation time to obtain maximum concentration of butanol and ethanol via ABE fermentation. The ABE fermentation is a two phase chemical production which is from acidogenesis into solventogenesis. The fermentation is caused by clostridia bacteria and in this case of study is *Clostridium acetobutylicum* pattered NCIMB 13357. The main substrate for nutrient in this study is Palm Oil Mill Effluent (POME) which freshly taken from Lepar Hilir, Pahang. Through the research, POME was found to contain majorly of pentose and carbohydrate substance such as galactose, glucose, fructose, sucrose and lactose. The research parameter is fermentation time at 72, 80, 48 and 60 hours. The experiments start from medium preparation, cultivation of bacteria, inoculation, fermentation and lastly analysis of product. All standard solution is made in order to standardize the reading of concentration of product and substance analyzed. The condition for bacteria environment is set to anaerobic and optimum pH at 5.8, temperature at 37 °C and agitation speed is set at 200 rpm along ABE fermentation. Final result shown that at fermentation time of 40 hours, the maximum value of 0.07 g/L of butanol was produced, while for ethanol is 83.499 g/L at 20 hour fermentation time. POME is encouragingly valid for use as fermentation media as it contain many utilizable substrates for *Clostridium acetobutylicum*.

ABSTRAK

Penghasilan biofuel melalui fermentasi sisa buangan telah menjadi fokus pada pertengahan abad ke-21 sehingga kini. Fokus kajian ini adalah untuk menentukan masa fermentasi yang mana kepekatan maksimum butanol dan etanol menerusi fermentasi ABE. Fermentasi ABE adalah produksi kimia dua fasa yang mana dari fasa asidogenasis kepada solventogenesis. Proses ini dirangsangkan oleh bacteria clostridia yang mana dalam kajian ini adalah *Clostridia acetobutylicum* berpaten NCIMP 13357. Substrat utama sebagai nutrisi dalam kajian ini adalah sisa kelapa sawit hancuran (POME) yang diambil segar dari Lepar Hilir, Pahang. Menerusi kajian, didapati bahawa POME mengandungi pentos dan rangkain karbohidrat seperti galaktos, glukos, fruktos, sukros dan laktos. Parameter kajian adalah masa fermentasi iaitu pada 72, 80, 48 dan 60 jam. Eksperimen dimulakan dengan penyediaan media, mengkultur bacteria, inokulum, proses fermentasi dan akhirnya analisis produk. Semua larutan standad dibuat bertujuan menyelaraskan bacaan kepekatan produk dan substrat yang dianalisis. Persekitaran bakteria untuk fermentasi adalah anaerobik dengan pH optimum pada bacaan 5.8, suhu pada 37 °C, dan kelajuan goncangan pada 200 rpm sepanjang fermentasi ABE .Hasil kajian menunjukkan pada masa fermentasi 40 jam, maksimum sebanyak 0.07 g/L butanol dihasilkan, manakala untuk etanol adalah sebanyak 83.499 g/L pada 20 jam masa fermentasi. Didapati bahawa POME adalah sangat bersesuaian dan amat digalakkan sebagai media fermentasi kerana ia mengandungi pelbagai substrat yang boleh di gunakan oleh *C. acetobutylicum.*

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LIST OF SYMBOLS / ABBREVIATIONS

ºC	-	Degree Celsius
%	-	Percentage
ABE	-	Acetone-butanol-ethanol
DNS	-	DiNitroSalicyclic acids
GC-FID	-	Gas Chromatography Flame Ionized Detector
g/L	-	gram per liter
g	-	Gram
HPLC	-	High Performance Liquid Chromatography
hr	-	hour
L	-	Liter
mL	-	milliliter
POME	-	Palm Mill Oil Effluent
rpm	-	Revolution Per Minute
w/v	-	Weight per volume
w/v %	-	Weight per volume in percentage

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

The conversion of plant biomass into solvents for fuel and chemical industry is regarded as old technology founded in the early 20th century (Zverlov *et al*., 2006). Fermentation of sugar into ethanol is in fact the oldest biotechnology in beer and wine production and regarded as the largest biotechnology process until now (Berezeina *et al.,* 2006). Biological production of solvent such as acetone, butanol and ethanol by fermentation in industrial scale has start in the early part of this century (Ranad *et al.*,2000). An early attempt L. Pasteur and others, the fermentation of starch to the solvents acetone, butanol and ethanol was developed by C. Weizman in 1912 at Manchester University into an industrial process (Velokodvorskaya *et al.,* 2006). The strain isolated and used was then called *clostridium acetobutylicum* and the first production plant was run for acetone large scale production from starch. The plant was erected in Canada and USA during World War I and during that time butanol was regarded as unnecessary by-product (Zverlov *et al.,* 2006). After war, butanol need was increased and the fermentation industry changed the substrate to molasses and the search for more applicable strain and clostridia were in highly demand (Berezeina *et al.,* 2006).

The commercial acetone-butanol fermentation process was established by the Commercial Solvent Corporation (CSC), USA which predominant until 1930's when the widespread availability of cheap molasses from the sugar industry provided a strong incentive to switch substrate (Shaheen *et al.,* 2000). Considerable effort was invested in attempting to utilize the existing starch-fermenting for use was never showing great result (Hastings *et al.,* 1971). It was then until mid 1950s, *C. acetobutylicum* was used in the profitable acetone-butanol-ethanol (ABE) fermentation in respond to the increasing demands for butanol and ethanol as biofuel an acetone as industrial chemical solvent.

Clostridium acetobutylicum has a long history of fermentation industry due to its biphasic fermentation of sugars to produce acetic and butyric acids in acidogenesis phase and then converted into solvent (acetone, butanol and ethanol) during solventogenesis (Xue *et al.,* 2000: Jones and Woods, 1989). Isolated by Chaim Weizmann in 1912 till 1914 at Manchester University, strains with high solvent yield are then named as *Clostridium acetobutylicum* (Velikodvorskaya *et al.,* 2006). *C. acetobutylicum* has the ability to utilize a variety of starchy substances as fermentation media (Jones and Woods, 1986). Early 20th century, starch and molasses are the main substrate for fermentation (Zverlov *et al.,* 2006), due to increase demand for solvent such as butanol, acetone and ethanol for many application (Kwang *et al.,* 2008), the cost for substrate has a dramatic influence on the economic viability of fermentation for ABE fermentation production (Nasratun et al., 2007). An interest has been taken in considering waste as substrate such as palm oil mill effluent (POME) due to the typical characteristic of the POME itself (Takriff *et al.,* 2007).

Malaysia was the largest producer of palm oil after Indonesia (Nasratun *et al.,* 2007). Its production generates various wastes chief among which is POME from production of crude palm oil involving extraction process (Sahaid *et al.,* 2003). The large amount production along the years has solved the availability of POME as substrate for ABE fermentation and can be considered as sustainable resources. POME

is classify as highly concentrated industrial waste water with BOD up to 40,000 mg/L thus may result in serious pollution if waste management is at critical point stand-up (Pang *et al.,* 2003). From the result of a research by Khaw, 1999, the report showed that *C. acetobutylicum* produced a total of ABE up to 0.94 g/L when grown in POME as the fermentation medium. Not only is POME contain mixture of carbohydrates including starch, hemicelluloses, sucrose and other carbohydrates which can be utilize by saccharolytic clostridia such as *C. acetobutylicum* (Kwon *et al*., 1989; Mohtar *et al.,* 2003), such utilization can result large increase profitability of palm oil industry, solving environmental problem and reducing the cost for economical solvent production via ABE fermentation (Yoshino *et al.,* 2003).

1.2 Problem Statement

Currently, ABE fermentation is a value-added fermentation process as it attractive for several economics and environmental factors (Formanek *et al.,* 1997). Environmental and global energy problems have resulted in increase effort towards producing biofuels such as butanol and ethanol from renewable resources (Youngsoon *et al.,* 2009) such as agriculture residue or waste. Agriculture waste is produced in a great amount and sometimes has effect on environment. For example is POME as it contains highly concentration of BOD of 40,000 mg/L (Pang *et al.,* 2003). On an average a palm oil mill, for each tonne of fresh fruit bunch (FFB) processed, 1 tonne of liquid or waste water with biochemical oxygen demand (BOD) 37.5kg, chemical oxygen demand (COD) 75kg, suspended solids (SS) 27kg and oil and grease 8kg was generated alongside (Zinatizadeh *et al.,* 2007). In an aspect of biofuels economy, although biofuels such as biodiesel and bioethanol represent secure, renewable and environmentally safe alternative compared to fossil fuel, their economic viability in term of costing has become a major concern (Gonzalez *et al.,* 2007). As a result, application of waste-to-wealth concept as main ideas for more profitable and safer strategy is in run. Usage of POME as fermentation media has

become recent interest research on Malaysia and other palm oil country as it may help add-on economy profit, reducing waste management issues and costing and also contribute on green house effect reduction. Other than environmental and economy viability, health aspect also been considered as the main focus. Butanol from petro-derivatives carries carcinogen effect (Thaddeus *et al.,* 2007) thus fermentation derived butanol is preferred specifically usage on food and flavor industry (Ezeji *et al.,* 2007). Some strain (Clostridia strain family) pattern later after 1940s allowed fermentation times to be as short as 30 hour fermentation time (Shaheen *et al.,* 2000). With some strain, long fermentation time such as up to 72 hour resulted in lower solvent productivity (Matt *et al*., 2000). These two fact has up rise the issues of effective fermentation time in obtaining the target solvent namely butanol and ethanol.

1.3 Objectives of Researches

The objective of the research is to study the effect of fermentation time on butanol and ethanol production by *C. acetobutylicum*.

1.4 Scope of Research

There are three scope of the research. The scopes are as follow:

1. To complete the composition analysis of selected batch fresh POME by using HPLC analysis.

2. To analyze the glucose consumption throughout the fermentation using the UV-Vis and glucose calibration curve by concentration estimation.

3. To study the effect of fermentation time at 72, 80, 48 and 60 hours of ABE fermentation by *C. acetobutylicum*.

CHAPTER 2

LITERATURE REVIEW

2.1 Fermentation

Fermentation is describes a form of energy-yielding microbial metabolism in which carbohydrate acts as the electron acceptor (Adams, 1990). In a meaning is a process involving ethanol production by yeast or organic acids by certain bacteria such as clostridia family bacteria (Sahlin, 1999). Generally fermentation is applied in food industry to produce fermented food containing lactic acids for solving dehydration health problem (Peter, 1999) by children mainly. Fermentation can be aerobic or anaerobic. For anaerobic fermentation, ABE fermentation is the most in research at 21st century.

2.1.1 Anaerobic Fermentation

Anaerobic fermentation is a biological processes where organic matter is metabolized in and environment free of dissolved oxygen (Kumar *et al.*, 2008). Certain microorganism can produce adenosine triphosphate (ATP) by utilizing

metabolic pathways that do not require the participation of molecular oxygen. The process is named as anaerobic glycolysis or pathways of fermentation and can be simplify as anaerobic fermentation. One type of anaerobic condition derived bacteria is clostridia family strain as it can utilize sugars into organic acids and further fermentation can produce solvents (Blaschek, 2005). Anaerobic fermentation has recently focused more on waste water treatment with the recovery of useful byproducts and renewable biofuels by using anaerobic biotechnology (Samir, 2008).

2.1.2 ABE Fermentation

The acetone-butanol-ethanol (ABE) fermentation of *C. acetobutylicum* was a classical method to produce the commercially important solvents such as acetone, butanol and ethanol and operated successfully at an industrial scale in many countries during the first half of 20^{th} century (Yang *et al.*, 2007) process has received considerable attention in recent years as a method to produce commodity chemicals, such as butanol and acetone, from biomass. It was carried out industrially throughout the United State during the half of last century, but was discontinued in the early 1960s due to unfavorable economic conditions brought about the competition with the petrochemical industry (Ezeji *et al.*, 2000). The ABE fermentation is the most widely studied among the anaerobic fermentation processes and is a model for complex primary metabolism fermentations (Blascheck *et al.*, 2000). Below are the pathways of how does the fermentation proceed throughout the fermentation (Thaddeus *et al.*, 2007). The ABE fermentation is round-up mainly on biphasic concept and the change between. The phase shifts is from acidogenesis to solventogenesis and happen upon certain specific time interval throughout the fermentation. On early stage, it started with acidogenesis where the main product is butyric acid, acetic acid and small amount of other organic acid (Ahmed *et al.*, 1988). After a few hour, the fermentation process proceed to the next step where the phase are then change into solventogenesis,

where solvent such as butanol, ethanol, acetone and phenol are produce. At this process, almost all of the initial acids produced were to be converted by the Clostridia Acetobutylicum into solvent at certain ratio depending on the condition and many other factors such as fermentation time, temperature, agitation speed and concentration of substrate.

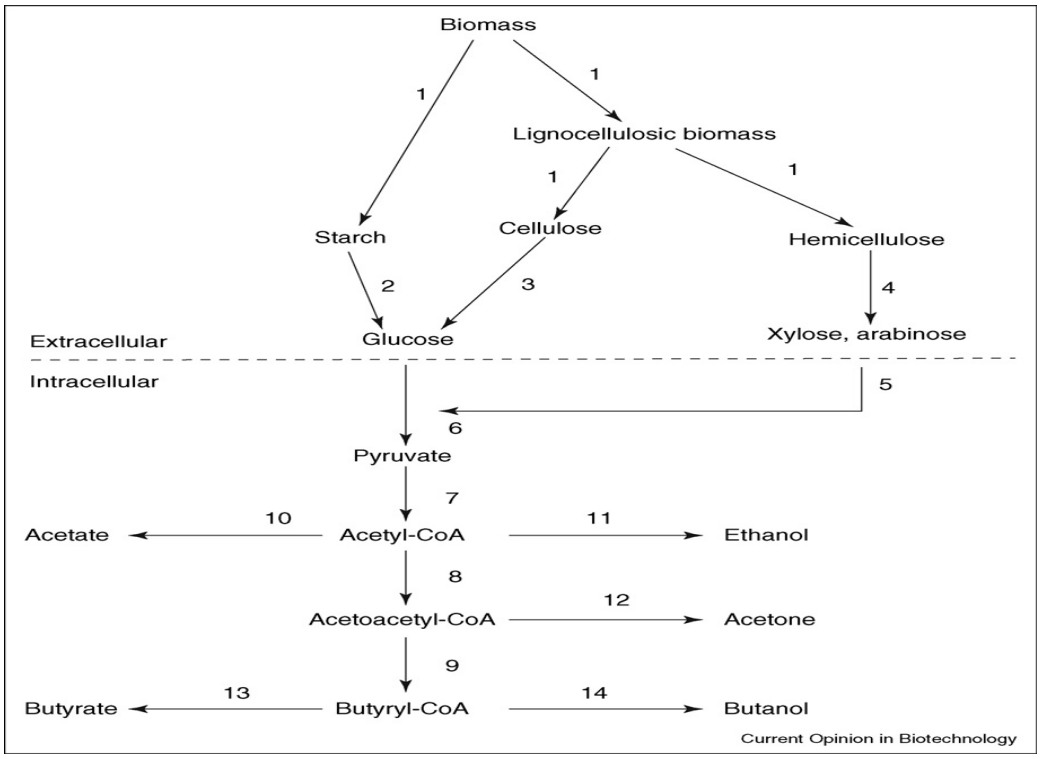

Figure 2.1 : Simplified metabolism of biomass by solventogenic clostridia. 1, Pretreatment of corn and lignocellulose; 2, starch hydrolysis (a-amylase,b-amylase, pullulanase, glucoamylase, a-glucosidase); 3, cellulose hydrolysis (cellulases, b-glucosidase); 4, hemicellulose hydrolysis; 5, xylose/arabinose uptake and subsequent breakdown via the transketolase-transaldolase sequence producing fructose 6-phosphate and glyceraldehydes 3-phosphate with subsequent metabolism by the Embden-Meyerhof-Parnas (EMP) pathway; 6, glucose uptake by the phosphotransferase system (PTS) and conversion to pyruvate by the EMP pathway; 7, pyruvate-ferrodoxin oxidoreductase; 8, thiolase; 9, 3- hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase; 10, phosphate acetyltransferase and acetate kinase; 11, acetaldehyde dehydrogenase and ethanol dehydrogenase; 12, acetoacetyl-CoA:acetate/butyrate:CoA transferase and acetoacetate decarboxylase; 13, phosphate butyltransferase and butyrate kinase; 14, butyraldehyde dehydrogenase and butanol dehydrogenase (Thaddeus *et al.*, 2007).

2.2 Butanol and Ethanol

Butanol and ethanol are the main solvent targeted in ABE fermentation as these two are the most potential biofuels. These two alcohol is the major solvents produced during fermentation at high ratio compare to other side product and these has become the targeted beneficial in finding the alternative fuels.

2.2.1 Butanol

Butanol is widely used as an industrial chemical. Butanol also exhibit a range of physical properties, including high energy content, water immiscibility low vapor pressure, and octane-enhancing power, which provide it with potential as a liquid fuel (Zhen *et al.*, 2008). The development of fermentation processes based on the solventogenic clostridia offers the prospect of butanol production from agriculture feedstock as an alternative to the petrochemical route (Blaschek *et al.*, 2008)

2.2.2 Ethanol

Ethanol (ethyl alcohol, grain alcohol) is a clear, colorless liquid with a characteristic, agreeable odor. It has low freezing characteristic which made it useful as the fluid in thermometers for temperature below -40 ^{o}C, the freezing point of mercury, and for other low-temperature purposes, such as for antifreeze in automobile radiators. Ethanol has been made since ancient times by fermentation of sugar (glucose, fructose etc). All beverage ethanol and more than half of industrial ethanol is still made by this process. The ethanol produced by fermentation ranges in concentration from percent up to 14 percent (Shakhashiri *et al.*,2001).

2.3 Clostridia Acetobutylicum

The main clostridia used for the experiment is *Clostridium acetobutylicum* coding pattern NCIMB 13357. Below are the table of the strain characteristic and identification.

Table 2.1 : Characteristic and pattern for *Clostridium acetobutylicum* NCIMB 13357 (Keis *et al* 2001). The incubation and also the fermentation temperature as shown are at 37^0C; Gram strain is gram positive.

Clostridium acetobutylicum	
Accession Number:	13357
Species Name:	Clostridium acetobutylicum
Other Collection:	ATCC824 DSM792 NRRLB527 VKMB1787
Date of Accession:	19/05/1995 00:00:00
Depositor Name:	H. Hippe
Type Strain:	Yes
Isolated by:	E.R.Weyer (Granulobacter pectinovorum)
Growth	
Growth Medium:	Clostridium acetobutylicum medium
Incubation Temp:	37°C
Gas Regime:	anaerobic
Colony Morphology	
Gram Strain:	gram positive
Preservation Information	
Method:	lyophilized

2.4 Palm Oil Mill Effluent (POME) Potential as Fermentation Medium.

POME has a great potential as alternative fermentation media due to its rich content of sugar and carbohydrate which can be utilize by C.Acetobutylicum for ABE fermentation. POME consist of various suspended components including cell walls, organelles and short fibers, a spectrum of carbohydrates ranging from hemicelluloses to simple sugar, a range of nitrogenous compound from proteins to amino-acids and free organic acids (Ugoji ,1997). The simple sugars contained within POME are fructose, glucose, galactose, sucrose and lactose. Due to the flexibility of *C. acetobutylicum* in using variety of starchy substances for media of fermentation (Jones and Wood, 1986), in these past 20 year, continuous experiment and research has been conducted in trying variety of starchy substance such as glucose, molasses, starch and corn. However, the cost of substrate has been the major consideration in determining the economy viability of ABE fermentation through anaerobic fermentation (Ezeji *et al.,* 2007). This means that availability of cheaper, abundant and readily available sources of substrates such as POME should be taken as alternative media for fermentation (Nasratun *et al.,* 2007) rather than other expansive material such as corn. Also, this can solve environment and water pollution issues since POME is greatly produced and can polluted the environment. This way also, economy viability can be enhanced via ABE fermentation process. Shown here is the table showing the typical characteristic of POME (Ahmad *et al.* 2006).

Table 2.2 : Characteristics of POME (palm oil mill effluent) (Ahmad *et al.* 2006). (As shown below, the BOD and COD reach a highly concentrated value and pose threat into environment (Pang *et al.*, 2003). Not to mention the total solid and suspended solid value and the difficulties of disposing the solid waste issues).

Parameter	Concentration (mg/L)	Element	Concentration (mg/L)
Oil & Grease	4000-6000	Phosphorus	180
BOD	20,000-25,000	Potassium	2270
COD	40,000-50,000	Calcium	439
Total Solids	40,500	Boron	7.6
Suspended Solid	18,000	Iron	46.5
Total volatile solids	34,000	Manganese	2.0
Ammoniccals nitrogen	35	Copper	0.89
Total	750	Magnesium zinc	615 2.3

CHAPTER 3

METHODOLOGY

3.1 Material

In this research, the main materials used in conducting the experiment is RCM powder, for inoculation and fermentation media (RCM powder) and agar (RCM agar powder); 5M NaOH for titration on POME media in maintaining pH at 5.8 (Kalil *et al.,* 2003); DNS reagent stored at 4°C (glucose presence analysis); distilled water (for media, sterilization in autoclave, sanitization); Toluene solution (LLE process); sugars solution for standard calibration curve (glucose, galactose, fructose, sucrose, and lactose); Nitrogen gas for anaerobic condition and media sparging; ethanol 70% for sterilization and sanitization; Acetonitrile solution for HPLC mobile phase; Hexane solution for GC-FID; Na_2SO_4 for LLE water absorption; POME media for fermentation media in studies. As for apparatus, test tubes; modified schott bottle; retort stand and holder; metal clipper; syringe 10 and 20mL, syringe filter 0.5 and 0.2mm; syringe needles; 60 vials; 40 GC-FID and HPLC vials; water bath; silica tube and lastly the Nitrogen gas supply.

3.2 Equipments

Equipment used majorly in this research is HPLC, GC-FID, autoclave, centrifuge, anaerobic chamber, incubator shaker and UV-Vis spectrophotometer.

3.2.1 HPLC

High performance liquid chromatography (HPLC) is a powerful tool in analysis for multi component in a solution. It is basically a highly improved form of column chromatography. Rather than solvent/solution being allowed to drip through a column under gravity, it is forced through under high pressure of up o 400 atmospheres, making it much faster. It also allows a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows better separation of the component of the mixture. The column is filled with tiny silica particles, and the solvent is non-polar such as hexane. A typical column has an internal diameter of 4.6 mm or less and length of 150 to 250 mm. The time taken for particular compound to travel through the column to the detector is known as its retention time. The time measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound (Jim, 2007). In this study, the HPLC was set to use capillary column Supelcosil LC-NH2. The flow rate used is 1ml/minute and the retention time around 15 minutes.

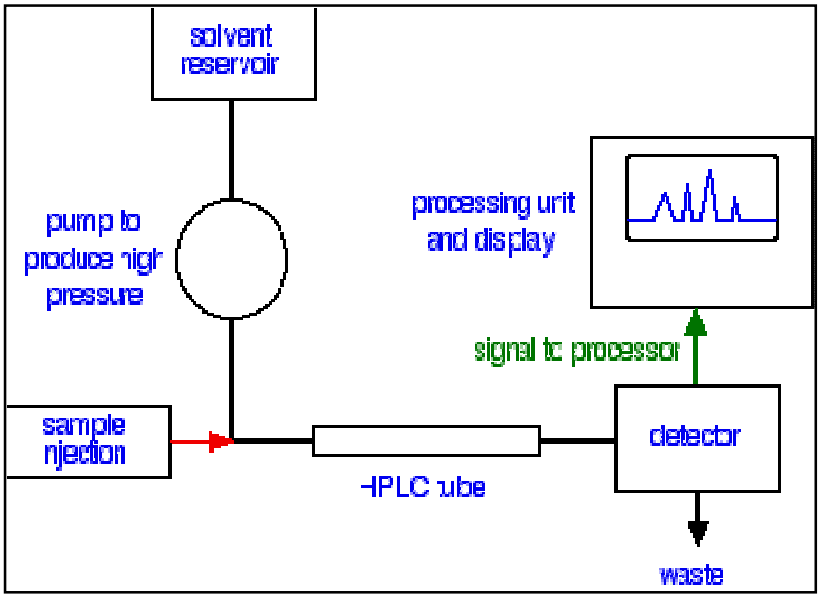

Figure 3.1 : Flow scheme for HPLC, the sample was injected and pass through HPLC tube (column) and after separation it pass through in separate retention time and detected by detector. After that, the value (area unit) was displayed and the waste was collected.

3.2.2 GC-FID

Gas chromatography flame ionization detector (GC-FID) is a non-selective detectoused in conjunction with gas chromatography. The FID works by directing the gas phase output from the column into a hydrogen flame. A voltage of 100 to 200 volt is applied between the flame and an electrode located away from the flame. The increased current due to electron emitted by burning carbon particles is then measured. The FID detect all carbon containing compound .The detector also has an extremely wide linear dynamic range that extends over, at least five orders of magnitude with a response index between 0.98 to 1.02.

3.2.3 Autoclave

An autoclave is a device to sterilize equipment and supplies by subjecting them with high pressure steam as 121 °C for 15 to 20 minutes depending on the 'MODE' and the content for sterilization. The mode available is mode 1, 2 and 3. For sterilization of liquid media, culture and waste mode 1 is used. For liquid and solid media (agar) mode 2 is used. For glassware and apparatus sterilization, mode 3 is used. The content and load for sterilization is covered by aluminums foil to cover the need-to-be-sterilized part. The working phase of autoclave by order is started by standby, heating, sterilization, exheating, warm and lastly complete mode. On sterilization phase, for 15-20 minute is the time for sterilization set-up beforehand. The media for fermentation was taken out only when the autoclave temperature reaches the 60 °C value. Apparatus sterilized by autoclave can be stored for 2 weeks period as long as the aluminum foil is not taken off.

3.2.4 Centrifuge

Centrifuge is use to separate solid particle and liquid resulting in 2 phase separation. The upper layer is called supernatant and the below layer is the solid particle. The principle work of centrifuge is by using centrifugal force to separate 2 phase component. For RCM media after fermentation, the supernatant is the solvent while the solid layer is the cell bodies. For POME media after fermentation and centrifuged, the supernatant is the solvents while the solid layer the hemicelluloses and other cellulose, cell bodies and suspended solid. Both of the media are centrifuge at 25 °C, for 30 minute and at 10,000 rpm speed rotation.

3.2.5 Anaerobic Chamber

Anaerobic chamber used in this researched is SHEL LAB Bactron Anaerobic Workstations (Glove Boxes type). It is design to allow efficient and dexterous glove-free handling and inspection of samples. It is used in procedures such as unpacking material to inoculation, incubation, inspection and recovery at one place and without exposure to oxygen. The inner temperature was set to 37 °C for incubation in inoculation of *C. acetobutylicum* and cell culture steps. All transfer process and striking bacteria also done in the anaerobic chamber.

3.2.6 Incubator Shaker

Incubator shaker is used in this study as platform for fermentation and agitation speed control. It also used to control the temperature for fermentation by controlling the temperature inside the incubator while heating the fermentation media to its temperature controlled. In this experiment, the temperature was set to 37°C and agitation speed is set up to 200 rpm which is the optimum condition for ABE fermentation using *C. acetobutylicum* (Nasratun *et al*., 2007). The fermentation time is set as parameter told beforehand.

3.2.7 UV-Vis Spectrophotometer

Ultraviolet-Visible (UV-Vis) spectrophotometer (HITACHI, Japan) is a type of spectrophotometer which uses two light sources for concentration analysis procedure. It uses Deuterium (D_2) lamp for ultraviolet and Tungsten (W) lamp for visible light generation. After bouncing off a mirror (mirror 1), the light beam passes through a slit and hits a diffraction grating. The grating functioned for specific

wavelength selection by rotation. At any specific orientation of the grating, only single wavelength successfully passes through the slit. A filter is used to remove unwanted higher orders of diffraction. The light beam hits a second mirror before it gets split by a half mirror (half of light passes through and the other half are reflected). One of the beams is allowed to passes through a reference cuvette (sample container), the other passes through the sample cuvette .The intensities of the light are the measured at the end. The wavelength used for concentration analysis in this study is 680 nm.

3.3 Order of Method

To be summarized, the steps of the experiment conducted were divided into seven steps .The steps are POME treatment, preparation of fermentation media and agar medium, enrichment process of *C. acetobutylicum*, striking clostridia onto Petri dish agar, inoculums preparation, fermentation process and lastly analysis of sample and product.

3.3.1 POME Pretreatment

Fresh POME was obtained from Felda Palm Industries Sdn.Bhd at Lepar Hilir, Gambang, Pahang. It is then treated by sedimentation process (Nasratun *et al.*, 2007) at 4 °C for 24 hour in a chiller. Supernatant were decanted and separated so that only the slurry left. Only the slurry were needed as fermentation media as the slurry contain all the substrates. After decantation, the slurry is then placed within schott bottle, diluted with distilled water and maintained at pH level of 5.8. Then, the slurry (now has become medium) being covered with aluminum foil is then autoclaved at 121 °C for 20 minute using Mode 2 of the autoclave. After 20 minutes autoclaving, small amounts of the medium were taken for POME component analysis.

3.3.2 Fermentation Media Preparation

The fermentation mediums in the research are POME medium and Reinforced Clostridia Media (RCM) medium (as control). 270 mL of POME media were added with 30 mL distilled water (preparing 90v/v %). By using 5M Sodium Hydroxide, NaOH were titrated into the POME medium so as to adjust the pH of the POME medium into 5.8. It was found that pH 5.8 was the optimum pH level for solvent production using POME as medium (Kalil *et al.*, 2003). As for RCM media, 2 types of media were prepared, one is the liquid type media and the other one is the agar type media. The liquid is prepared by dissolving 11.4 g of RCM powder into 300 mL distilled water in a Schott's bottle. This media is for the control fermentation. Next is the agar type media. It is prepared by dissolving 26.25 g of the RCM (agar) powder in a Schott's bottle containing 500 mL of distilled water. All three media are then autoclave using Mode 2 at 121°C for 20 minutes sterilization period until cool down at 60°C.

3.3.3 Enrichment of Clostridia

Pure bacteria (*C. acetobutylicum*) from glycerol stock was transferred into broth media (RCM agar media prepared before) and incubated at 37 °C for 3 to 7 days in the anaerobic chamber. The transfer process is done in a laminar flow fume board. After the incubating time end, from broth media, the Clostridia were transferred onto agar slant and incubated again at 37 °C for 24 hour. These processes are necessary for enriching the stock of bacteria by cultivation and ensure the bacterium is active enough to function during ABE fermentation.

3.3.4 Striking Clostridia onto Rich Clostridia Agar (RCA)

Using inoculating loop and within anaerobic chamber, clostridia on agar slant were strike onto petri dish (agar plate) with RCA by using zigzag pattern. Afterwards, the plate was covered and sealed using parafilm and incubated for 48 hour at 37 °C and 1 atmosphere pressure within the anaerobic chamber.

3.3.5 Inoculums Preparation

After incubation for 2 days, the bacterium is ready for inoculation process. Working in the anaerobic chamber, single colony of *C .acetobutylicum* from the agar plate was transferred into 167 mL RCM medium, and then incubated at 37 °C for 18 hours. Within 18 hours, a little amount of the sample were taken for OD measurement using UV-Vis and the medium is determined to be valid for further steps if the OD reading is within range 0.7 to 0.9. At this point, the bacterium is already at lag phase of growth where is at optimum activation life span and suitable for ABE fermentation process.

3.3.6 Fermentation Process

After autoclaving both POME and RCM medium, the mediums were deoxidized by using Nitrogen (N_2) gas for approximately 10 minutes. Then, both medium in schott bottle were taken into anaerobic chamber and inoculums were taken out from within the incubator of the anaerobic chamber for transfer process. The transfer process was only done in an anaerobic condition within the anaerobic chamber. 10% v/v of the inoculums (16.7 ml) were taken and inserted into POME media. The same was done towards the RCM media.

After finishing the transfer process, both media are then incubated within Rotating Incubator. The incubator was set up at 37 °C with agitation of 200 rpm (optimum temperature and agitation speed). The fermentation was run for 72 hours and the experiment is repeated for 80, 48 and 60 hours of fermentation time. Sample was taken at interval time and the time for sample to be taken for analysis is depending on the fermentation time. The initial sample at 0 to 20 hours and final sample of POME media were taken for glucose measurement to analyze glucose consumption.

3.4 Analysis Method

There are three analysis done in order to fulfill the requirement of the research scope .The analysis is glucose consumption analysis, POME component analysis and lastly butanol and ethanol production. The analysis method for the experiment is Liquid-liquid Extraction, DNS method and reading by using GC-FID, HPLC, comparison and plotting using standard calibration curve of component/product.

3.4.1 DNS Method

DNS method is used to clarify the presence of glucose within the fermentation media (POME and RCM) .Sample are prepared into 3 dilution, which is no dilution, dilution 1/10 and dilution 1/20 of initial concentration with distilled water. For sample with no dilution, 3 mL of sample are added with 3 mL DNS reagent. For 1/10 dilution, 1mL sample are added with 9 mL of distilled water. As for dilution 1/20, 2 mL of dilution 1/10 are added with 2 mL distilled water. The ratio for DNS reagent add-up is 1:1. 3 mL dilution of 1/10 are added with 3 mL DNS reagent and for dilution 1/20, the same quantity is also applied. All mixing are done in a test tube, and shacked to have well mixed solution and then put into water bath with temperature of 90 °C for 10

minute. At end result, the color changed into either red butter, blood red or reddish color, the result shown is positive that the samples contain glucose.

3.4.2 Liquid-liquid Extraction (LLE)

The entire sample which taken out was centrifuge at 10,000 rpm for 30 minutes and 25 °C. This is to separate the solid and liquid phase component. Using the supernatant separated through the centrifugation, it is stored in vials and labeled. The supernatant are to be further process using separating funnel. Each of the supernatant (sample) was mixed with Toluene (universal solvent) to separate water component. The ratio of sample towards toluene added is 1:1. The separating funnel was shakes to homogenize the mixture and sealed. It was left for 24 hours sedimentation process. The toluene function is to absorb the solvent (butanol and ethanol) and resulting in 2 immiscible liquid phase. The steps are applied onto both POME and RCM fermentation sample.

After 24 hour, the below level liquid is flow out and only the upper level liquid is need since it is the separated solvent. The solvent are then pour into biker, and added with sodium sulphite (Na_2SO_4). The Na_2SO_4 was used to absorb the remaining molecule of water that still left in the sample. After adding the Na_2SO_4 close the biker tightly with aluminum foil and the sample was stored in chiller at 4 ^{O}C for 24 hours. Then, after 24 hours, the sample was filter by using syringe filter of 0.2 μm and then prepared for GC-FID analysis.

3.4.3 Butanol and Ethanol Analysis

To analyze the composition of butanol and ethanol, Gas Chromatography Flame Ionized Detector (GC-FID) was used. 10 μL of sample POME / RCM was mix with 990 μL of Hexane. After mixing process between the sample and hexane, the sample was transfer into vial by using filter of 0.2 μm. The standard that used to detect concentration of butanol in the sample is butanol standard solution. The standard that used to detect concentration of ethanol in the sample is ethanol standard solution. The standard solution of butanol also was fitter by using 0.2 μm filter before it entered into the vial.

3.4.4 POME Component Analysis

After the centrifugation process to separate the solid particle in the sample, the supernatant from the sample of POME was taken to check the component of POME. To analyze the component of POME High performance liquid chromatography (HPLC) was used. The HPLC was set to use capillary column Supelcosil LC-NH2. The flow rate used is 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 standards that are used to detect glucose, galactose, fructose, sucrose, and lactose in the sample of POME are the pure solution of glucose, galactose, fructose, sucrose, and lactose (standard calibration curve). The calibration curve for each sugar (glucose, galactose, fructose, sucrose, and lactose) was prepare for 20 g/L,40 g/L,60 g/L,80 g/L and 100 g/L. Then filter the solution of sample and standard solution by using the filter 0.45 μm. Afterwards. the sample was entered into the vial to analyze by HPLC.

Table 3.1: Table of Analysis Summarize. Summarize of each analysis, method, reagent and major equipment used in proceedings the experiment.

Analysis	**Method**	**Reagent**	**Major Equipment**
Glucose Consumption Analysis	OD reading and calibration curve comparison	DNS reagent	• UV-Vis Spectrophotometer • Water-bath
POME component analysis	High performance liquid-chromatography reading	Hexane	HPLC
Butanol and Ethanol production	• Liquid-liquid extraction • Gas Chromatography	• Toluene • Na_2SO_4	• Separation Funnel • GC-FID

CHAPTER 4

RESULT AND DISCUSSION

4.1 Result

The result for this is study is focus more on POME component analysis, glucose consumption, butanol and ethanol production during fermentation and finally fermentation time effect towards butanol and ethanol production.

4.1.1 POME Component Analysis

The POME was analyzed using HPLC without dilution. The reason POME component is to be analyzed is to confirm the sugars such as fructose, glucose, galactose, sucrose and lactose are presence within POME (Mohamed *et al*., 2005) and from the result, it was proven that these sugars do exist within POME in an abundant. All of these sugars can be utilized as fermentation substrate (Matt *et al*., 2000; Ezeji *et al*., 2003; Joseph *et al*., 1997; Blaschek *et al*., 1997). (The target is to determine the sugars presence and to solidify the foundation of making the POME as alternative substrate for ABE fermentation in producing butanol and ethanol). It was the main target to make use of the POME which is a highly produce waste in Malaysia as

something profitable and at the same time reduce the environment issues and costing in waste management. The table for sugars and concentration chart are as shown.

Table 4.1 : Sugars (fructose, glucose, galactose, sucrose and lactose) concentration in POME in unit gram per liter. As shown from the table, galactose shown the greatest concentration presence within POME and followed by glucose which is usually used for ABE fermentation. The sugars are confirmed to be presence within POME on Lepar Hilir palm oil plantation waste.

Sugar	concentration (g/L)
Fructose	1.967
Glucose	3.668
Galactose	8.859
Sucrose	2.24
Lactose	1.313

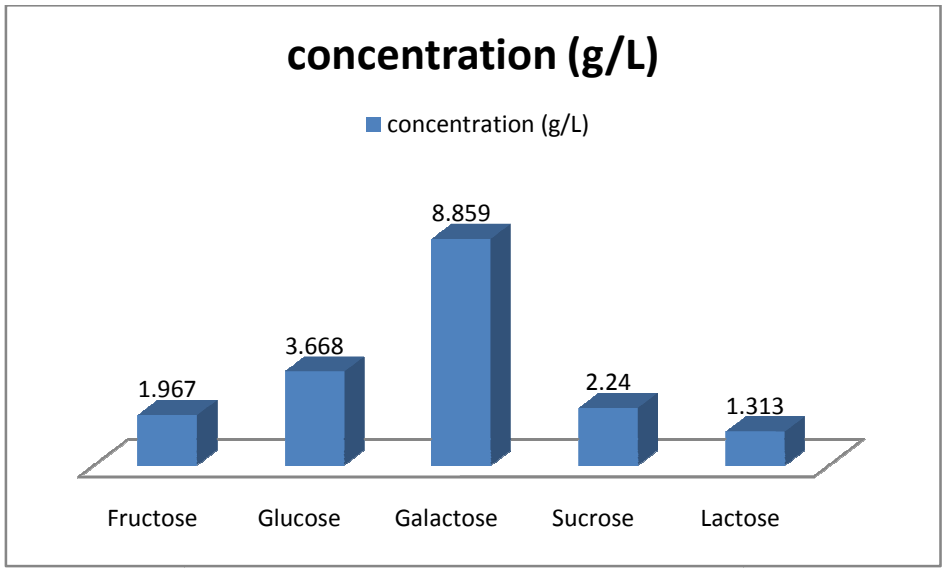

Figure 4.1 : Sugar concentration chart. Galactose is the highest concentration presence in POME

As the sugars presence and concentration confirmed, it can be firmly decide that the POME is positively suitable to be used as ABE fermentation substrates. With these sugars presence in POME, *C. acetobutylicum* is able to utilize POME for ABE fermentation process and solvent production is something that we can positively obtain at the end of the process (Nasratun *et al*., 2007). Further discussion on butanol and ethanol production by using POME will be discussed later on.

4.1.2 Growth Profile of *C. acetobutylicum.*

Before the bacteria are considered valid for fermentation process, the OD reading from inoculums must reach 0.7 to 0.9 reading. This reading indicates that the bacterium is in stationary phase of microbial growth for *C. acetobutylicum*. Microbial growth is the profile at which shown the phase of life for microorganism or bacteria. It starts from lag phase, continued by exponential phase (climbing state), stationary phase (constant rate state) and lastly death phase. Lag phase is where the bacteria in process of adapting to the media environment till it fully activate for metabolism process. Exponential phase is where the rate of specific growth rate of bacteria is maximum and the bacteria are growing by availability of excess of nutrient. Stationary phase is phase at which the growth of bacteria is at maximum constant rate and the nutrient uptake also constant. In death phase, the nutrient run out and the bacteria start to died. To be valid as inoculums, the OD reading must reach within range 0.7 to 0.9 where the bacterium has reached its stationary phase and the microbial metabolism is at max. At maximum microbial metabolism, we will get the best result as the product produce at maximum activation of the *C. acetobutylicum*. The profile growth in RCM and POME media will be shown.

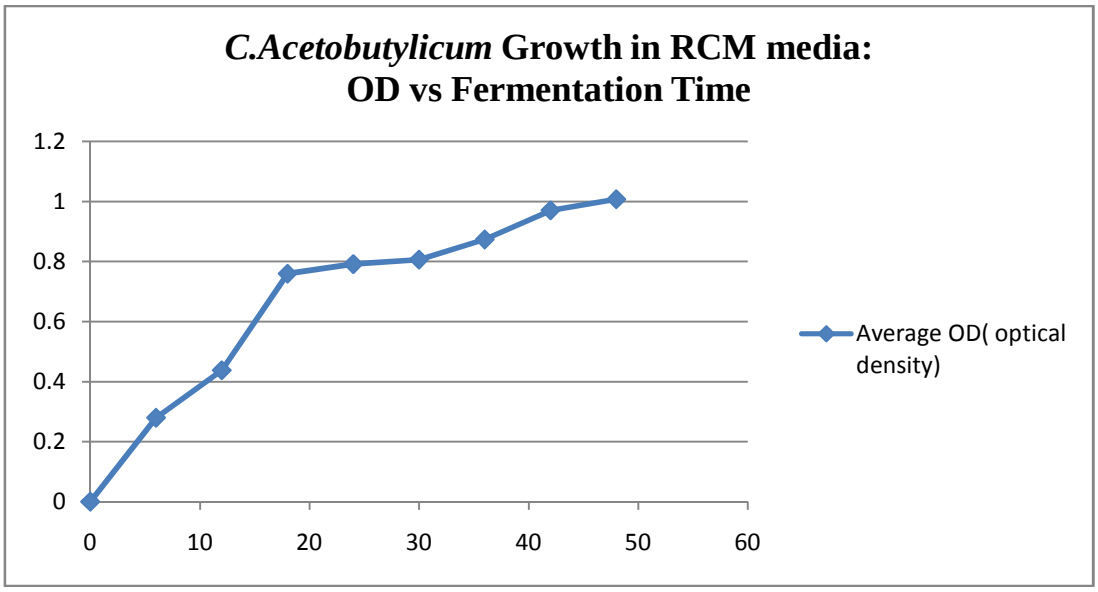

Figure 4.2 :Growth profile of *C. acetobutylicum* in RCM media.

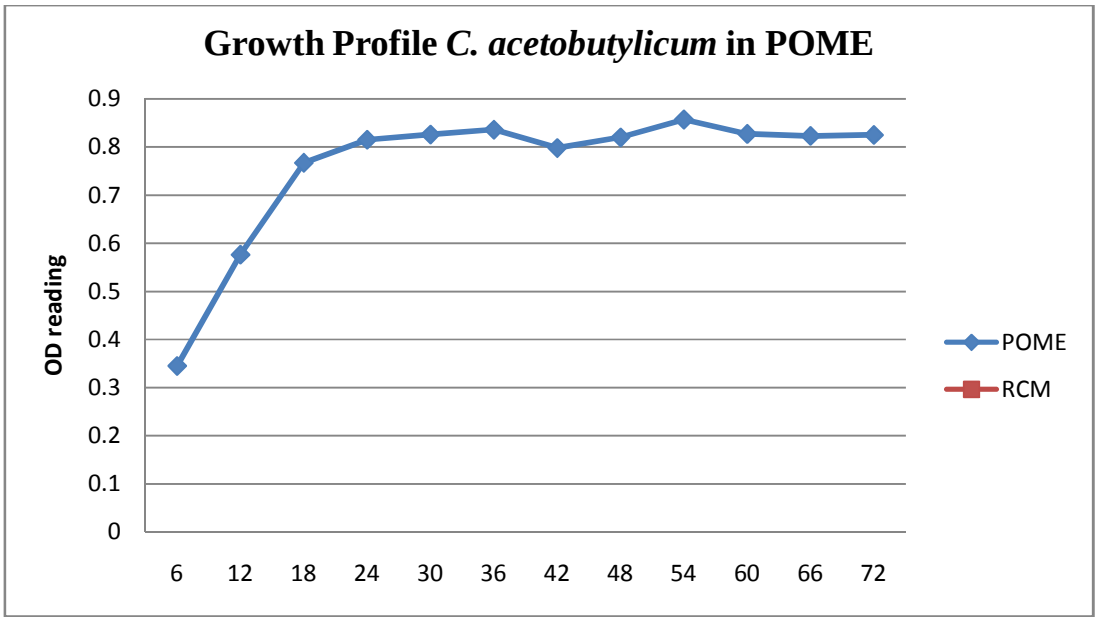

Figure 4.3 : Growth profile of *C. acetobutylicum* in POME media.

4.1.3 Glucose Consumption

In order to analyze the glucose consumption in POME during fermentation, the OD (optical density) reading was taken and graphs glucose concentration versus fermentation time was plotted. The dilution was set to be 1/10 of the actual concentration due to high concentration of POME and limit of UV-Vis reading at 3.000 only. Below is the table and plotting for all fermentation:

4.1.3.1 72 Hours Fermentation

Table 4.2 : Glucose reading for 72 hours fermentation. The readings show reduction of glucose within POME media throughout the fermentation.

Time (Hour)	Concentration (g/L)
20	0.96
40	0.9
60	0.83
72	0.73

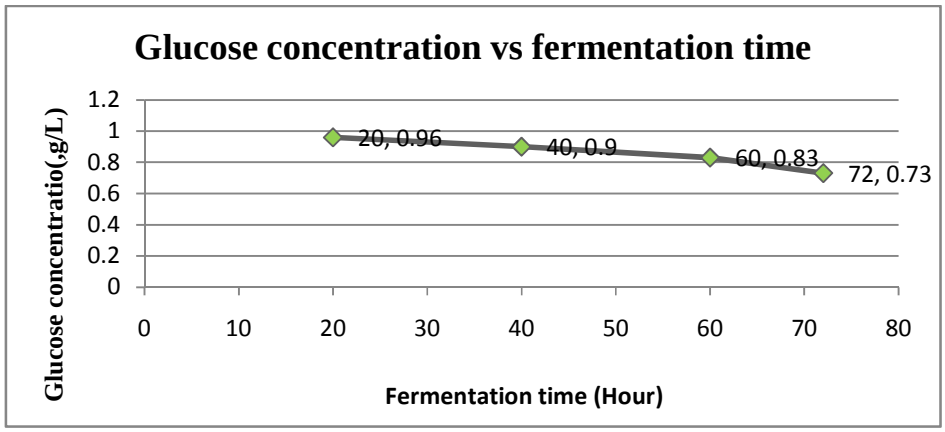

Figure 4.4 : Glucose concentration versus 72 hours fermentation time.

4.1.3.2 80 Hours Fermentation

Table 4.3 : Glucose reading for 80 hours fermentation. The readings show reduction of glucose within POME media throughout the fermentation.

Time (Hour)	Concentration (g/L)
20	0.33
40	0.28
60	0.245
80	0.2

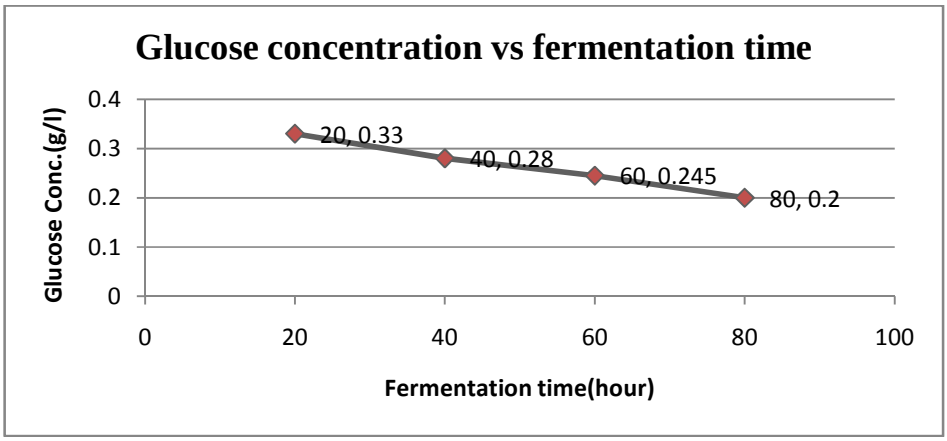

Figure 4.5 : Glucose concentration versus 80 hours fermentation time.

4.1.3.3 48 Hours Fermentation

Table 4.4 : Glucose reading for 48 hours fermentation. The readings show reduction of glucose within POME media throughout the fermentation.

Time (Hour)	Concentration (g/L)
16	0.21
45	0.16
48	0.14

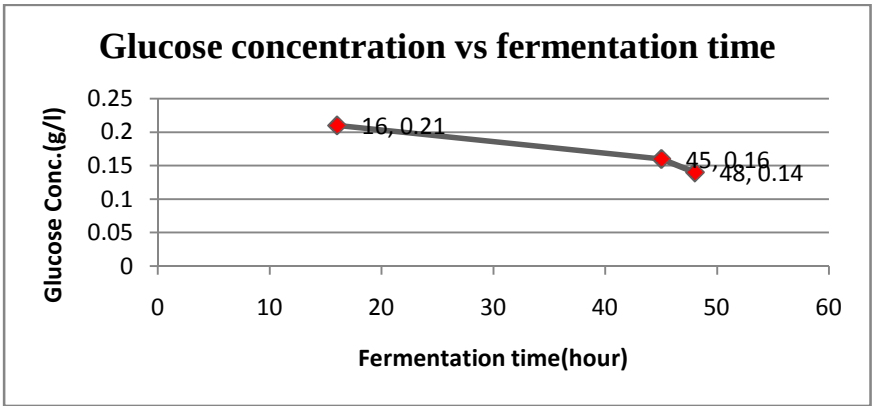

Figure 4.6 : Glucose concentration versus 48 hours fermentation time.

4.1.3.4 60 Hours Fermentation

Table 4.5 : Glucose reading for 60 hours fermentation. The readings show reduction of glucose within POME media throughout the fermentation.

Time (hour)	Concentration (g/l)
20	0.68
48	0.65
60	0.31

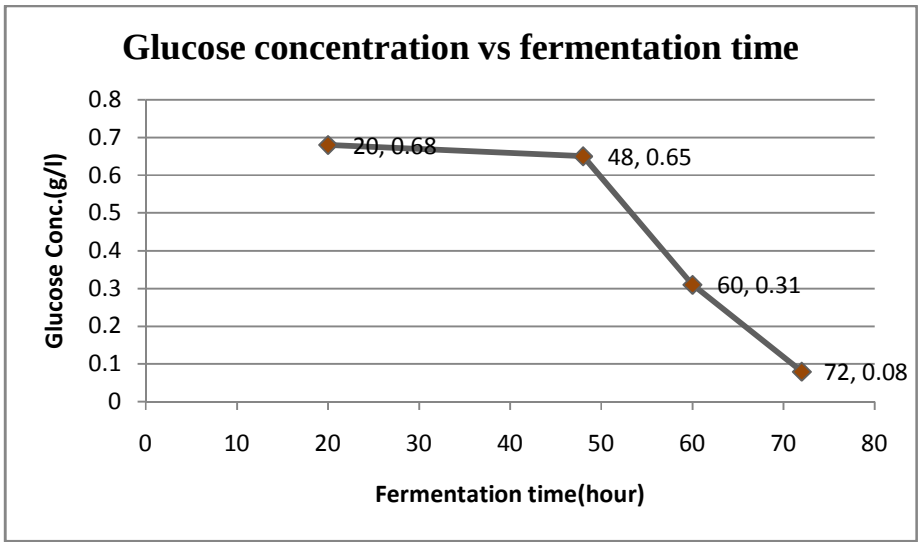

Figure 4.7 : Glucose concentration versus 60 hours fermentation time.

4.1.3.5 Summarize on Glucose Consumption

From the entire graph, the total consumption of glucose was calculated and by considering the dilution of 1/10 of actual concentration, the values were to be times with 10. Below is the table of total glucose consumption after calculation. 60 hours fermentation time is the highest glucose consumption rate shown.

Table 4.6 : Total glucose consumption .The total glucose was calculated and result shown that fermentation time of 60 hours is the highest rate of glucose consumption with 6.0 g/L throughout the fermentation.

Fermentation Time (Hour)	Glucose Concentration (g/L)
72	2.3
80	1.3
48	0.7
60	6.0

4.1.4 Butanol and Ethanol Production

The production of ethanol and butanol were determined from the butanol and ethanol standards curve and GC-FID reading. The plotting was plot for RCM and POME media as comparison for the unit is in g/L. The butanol and ethanol concentration table and plot as below.

4.1.4.1 72 Hours Fermentation Analysis

Table 4.7 : Butanol production during 72 hours fermentation (in POME and RCM (control) media).

Fermentation Time (hour)	Concentration (g/L)[POME]	Concentration (g/L)[RCM]
20	-	0.021
40	0.07	0.118
60	0.054	0.029
72	0.012	0.027

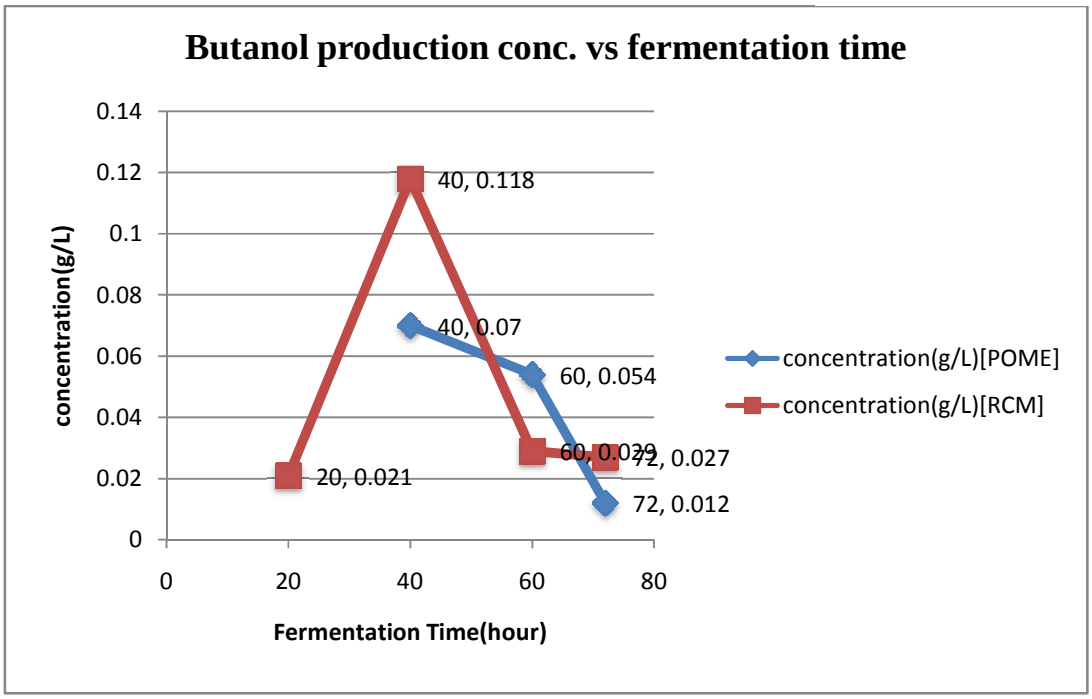

Figure 4.8 : Butanol production during 72 hours fermentation. Butanol is at highest production during 40 hours fermentation time in POME and RCM before gradually decreasing.

Table 4.8 Ethanol production during 72 hours fermentation (in POME and RCM (control) media)

Fermentation Time (hour)	Concentration (g/L)[POME]	Concentration (g/L)[RCM]
20	83.449	64.918
40	78.53	81.34
60	68.958	79.072
72	79.304	78.967

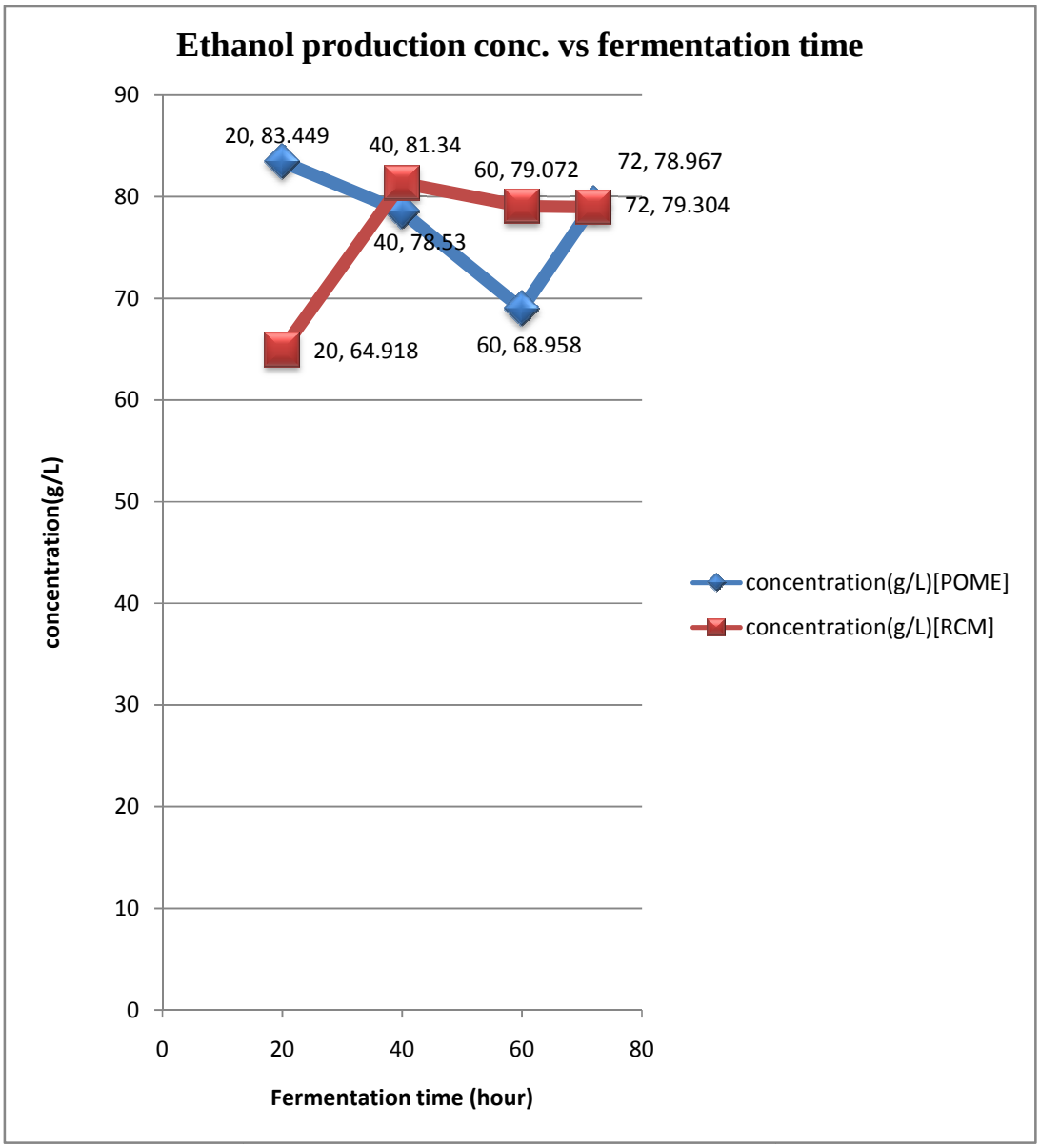

Figure 4.9 : Ethanol production during 72 hours fermentation. Ethanol is highly produced at 20 hours fermentation time for POME media and for RCM media is at 40 hours fermentation time.

4.1.4.2 80 hours fermentation analysis

The butanol and ethanol concentration for 80 hours fermentation table and plot was as shown:

Table 4.9 : Butanol production during 80 hours fermentation (in POME and RCM (control) media).

Fermentation Time (hour)	Concentration (g/L)[POME]	Concentration (g/L)[RCM]
20	0.033	0.038
40	0.031	0.035
60	0.037	0.038
80	0.035	-

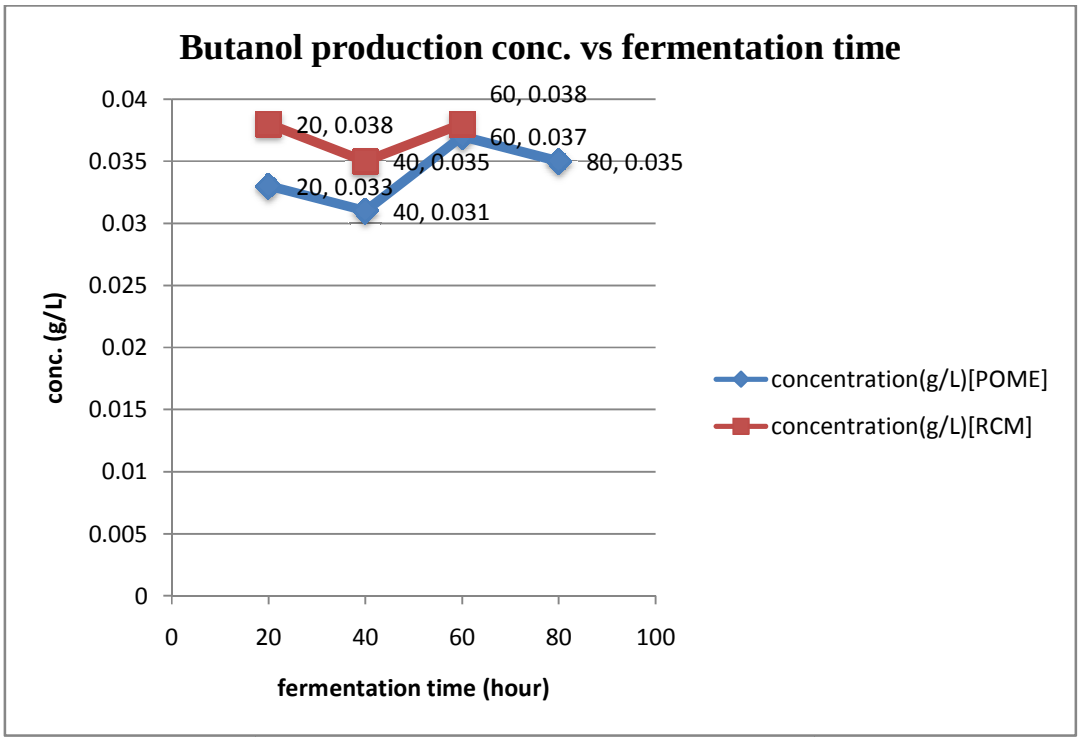

Figure 4.10 : Butanol production during 80 hours fermentation. For POME media, the highest butanol production is during 60 hours fermentation time, while for RCM media is at 20 and 60 hours.

Table 4.10 : Ethanol production during 80 hours fermentation in POME and RCM (control) media.

Fermentation Time (hour)	Concentration (g/L)[POME]	Concentration (g/L)[RCM]
20	48.372	68.088
40	56.567	52.048
60	59.501	54.475
80	56.995	56.129

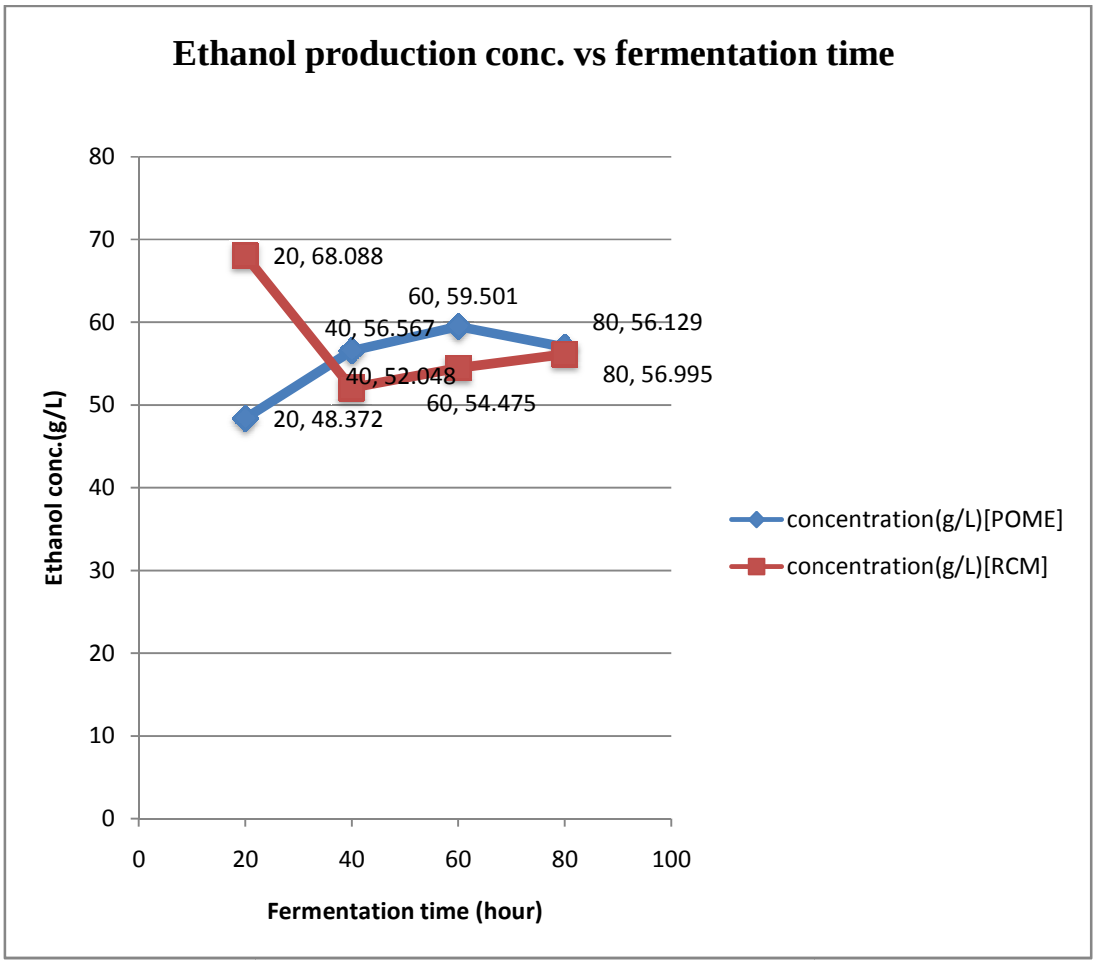

Figure 4.11 : Ethanol production during 80 hours fermentation. For POME media, the highest ethanol production is during 60 hours and for RCM media is at 20 hours fermentation time.

4.1.4.3 48 hours fermentation analysis

The butanol and ethanol concentration for 48 hours fermentation table and plot was as below:

Table 4.11 : Butanol production during 48 hours fermentation in POME and RCM (control) media

Fermentation Time (hour)	Concentration (g/L)[POME]	Concentration (g/L)[RCM]
16	0.037	0.039
45	0.035	-
48	0.036	0.043

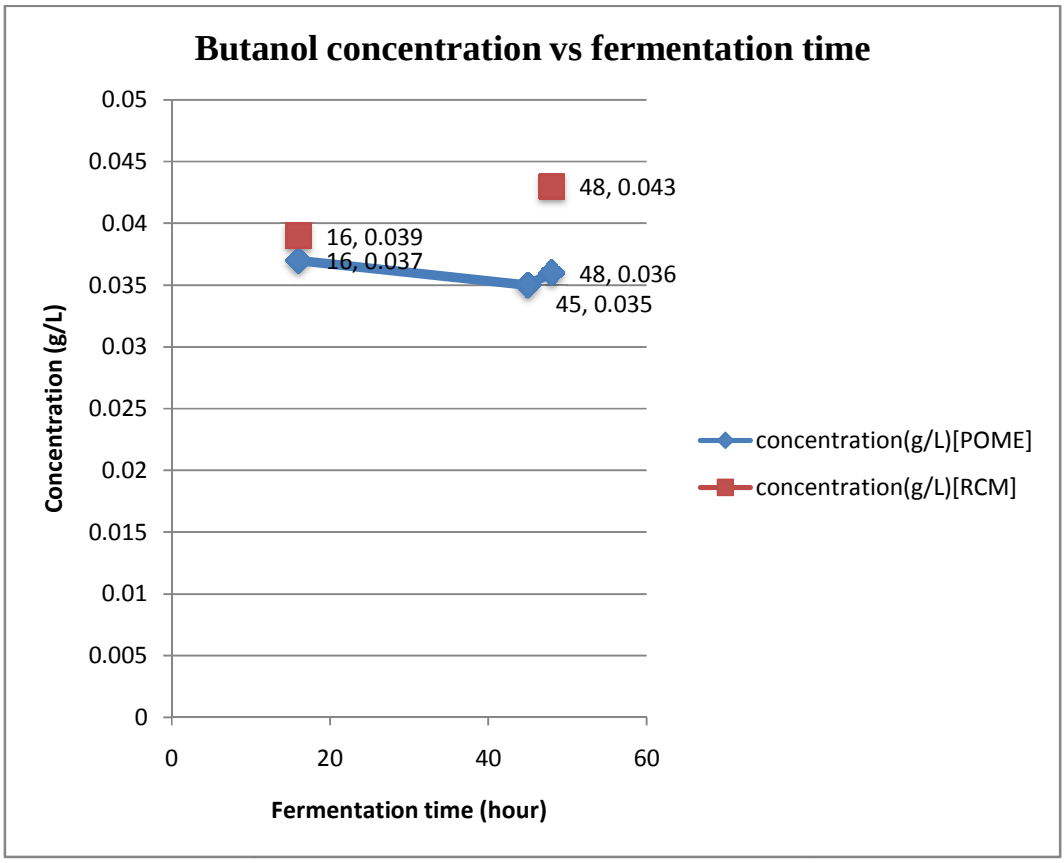

Figure 4.12 : Butanol production during 48 hours fermentation. For POME media, the highest butanol production is during 16 hours while for RCM media is at 48 hours.

Table 4.12 : Ethanol production during 48 hours fermentation in POME and RCM (control) media.

Fermentation Time (hour)	Concentration (g/L)[POME]	Concentration (g/L)[RCM]
16	59.655	57.754
45	82.193	62.075
48	62.469	61.553

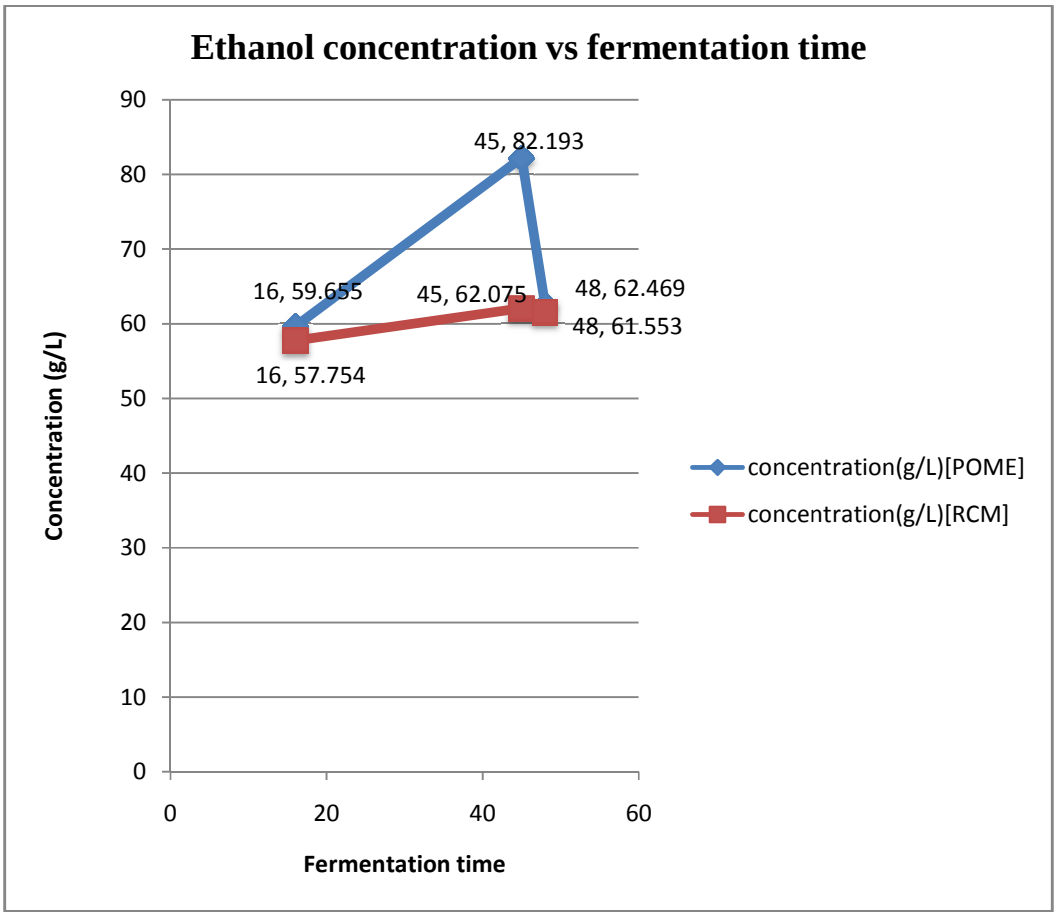

Figure 4.13 : Ethanol production during 48 hours fermentation. For POME media, ethanol is the highest production during 45 hours fermentation time while for RCM media is also at 45 hours.

4.1.4.4 60 hours fermentation analysis

The butanol and ethanol concentration for 60 hours fermentation table and plot was as below:

Table 4.13 : Butanol production during 60 hours fermentation in POME and RCM (control) media.

Fermentation Time (hour)	Concentration (g/L)[POME]	Concentration (g/L)[RCM]
20	0.72	0.095
48	0.028	-
60	0.026	0.022

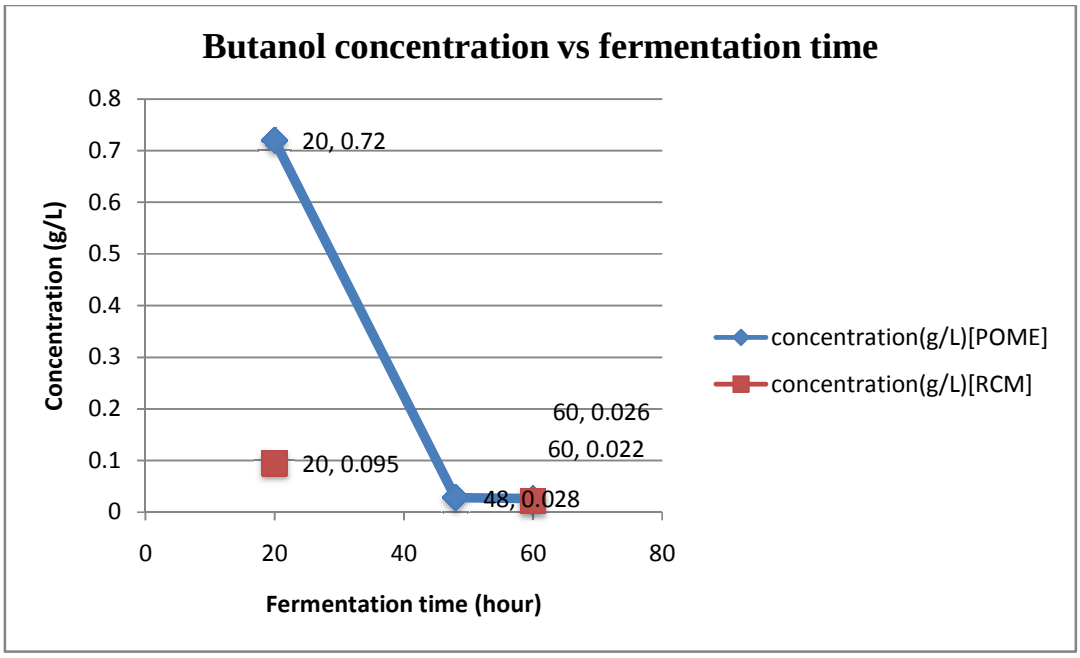

Figure 4.14 : Butanol production during 60 hours fermentation. For POME media, the highest is at 20 hours fermentation time while for RCM media is also at 20 hours fermentation time.

Table 4.14 : Ethanol production during 60 hours fermentation in POME and RCM (control) media. 72 hours reading were taken also to see the lower productivity resulted by long fermentation time in other research statement (Matt *et al*., 2000).

Fermentation Time (hour)	Concentration (g/L)[POME]	Concentration (g/L)[RCM]
20	69.6	63.864
48	59.561	22.515
60	68.058	71.905
72	0.122	58.315

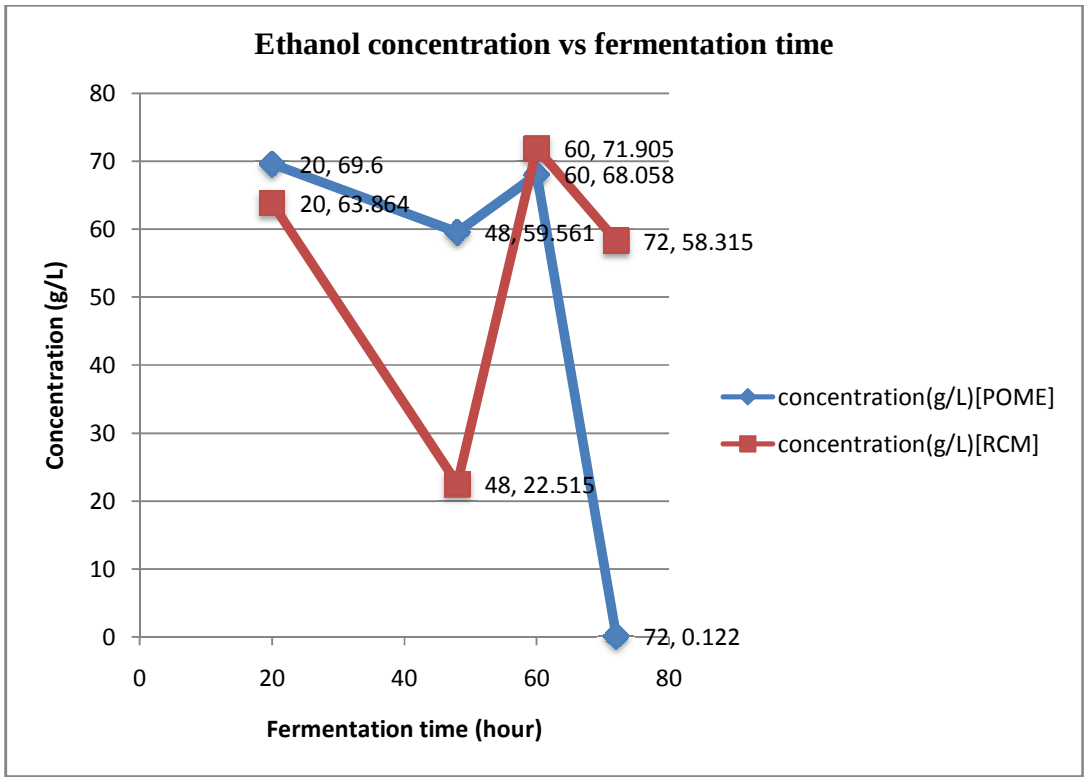

Figure 4.15 : Ethanol production during 60 hours fermentation. For POME media, the highest ethanol production is during 20 hours while for RCM media is at 60 hours fermentation time.

4.1.5 Fermentation Time Effect upon Butanol Production

Butanol production was plotted together to analyze the pattern of solvent production and to see which time gives the highest production

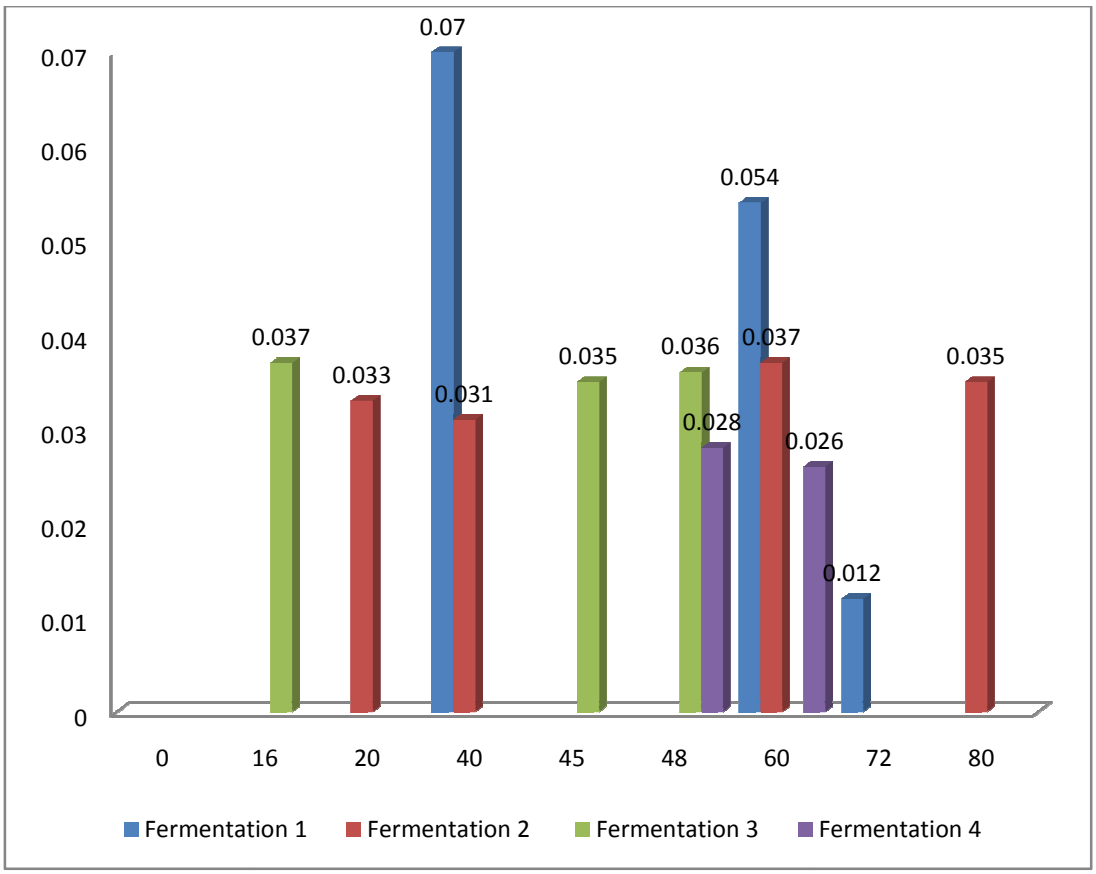

Figure 4.16 : Butanol concentration (g/L) versus fermentation time (hour). Fermentation 1 (72 hours); Fermentation 2 (80 hours); Fermentation 3 (48 hours); Fermentation 4 (60 hours). As shown, during 40 hours of fermentation time, butanol is the highest concentration produced with concentration of 0.07 g/L and gradually decreasing. The lowest value shown is during 72 hours fermentation time with concentration only 0.012 g/L butanol produced.

4.1.6 Fermentation Time Effect upon Ethanol Production

Ethanol production was plotted together to analyze the pattern of solvent production and to see which time gives the highest production

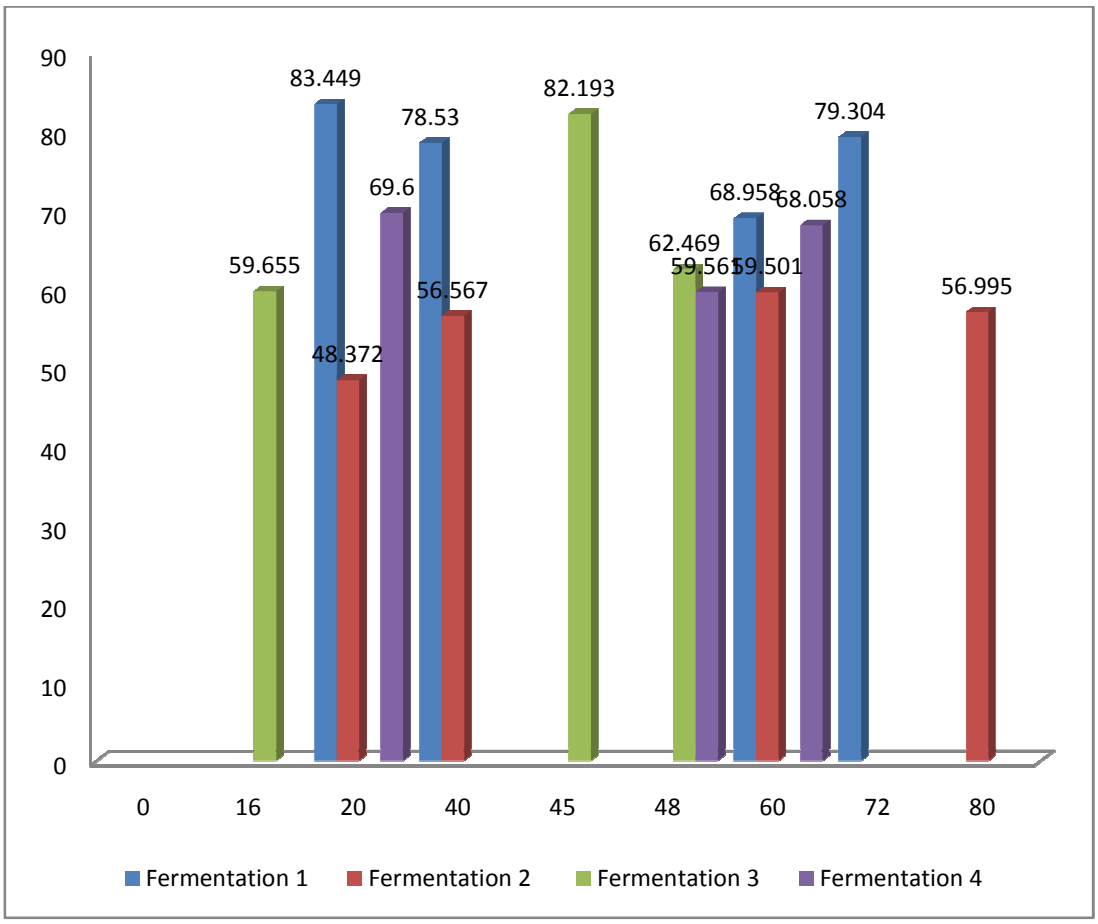

Figure 4.17 : Ethanol concentrations (g/L) versus fermentation time (hour). Fermentation 1 (72 hours); Fermentation 2 (80 hours); Fermentation 3 (48 hours); Fermentation 4 (60 hours). The highest value of ethanol produced is at 20 hours fermentation time which is 83.449 g/L.

4.2 Discussion

4.2.1 POME Component Analysis

From result, it shown that POME contains mainly 5 kinds of sugars compound named fructose, lactose, galactose, glucose and sucrose. For none dilution, galactose is the highest sugar contain within POME. From Table 4.1, the result shown POME contains total of 1.967 g/L fructose, 3.668 g/L glucose, 8.859 g/L galactose, 2.24 g/L sucrose and 1.313 g/L lactose. These five type of sugars can be fully utilize for ABE fermentation and able to pass through the cells membrane as all of this sugar build from same subs compound and available for ATP and ATPase formation (Huang *et al.*, 1986).

4.2.2 Growth Profile of *C. acetobutylicum*.

As shown from Figure 4.2, for RCM media, the OD reading reached stationary phase at 18 to 40 hours. During this interval, the inoculums are valid for further fermentation process.

Referred Figure 4.3, for POME media, the OD reading reached stationary phase at 18 to 72 hours. The readings stabilize for long interval showing that POME is very rich with substrate that *C. acetobutylicum* can maintain its stationary phase for long period. During this interval, the inoculums are valid for further fermentation process.

4.2.3 Glucose Consumption

Based from Figure 4.4, 4.5, 4.6 and 4.7, it is shown that as the time passed, more glucose is consumed and generally the total consumption is the highest at fermentation time of 60 hours with 6.0 g/L throughout the fermentation. From Figure 4.5, the glucose consumption rate is high from 48 to 60 hours fermentation with value calculated of 3.4 g/L glucose consumed. It was calculated by considering dilution of 1/10 of the actual concentration. This is shown within these time, *C. acetobutylicum* is actively converting glucose into acids and solvent as the number of bacteria increase with time and the changing of phase making the *C. acetobutylicum* able to consume and convert both sugars and acids into product.

4.2.4 Butanol and Ethanol Production

From the research, generally it was shown that for POME as fermentation media, we can speculate that solventogenesis start at 16 hours fermentation time and not necessarily at 36 hours as founded by Takriff, 2007. The result shown that ethanol is highly produced during the initial solventogenesis phase and is peak at 20 hours fermentation time which is 83.449 g/L of ethanol produced. After that, butanol are then started to be converted from butyric and other organic acids and achieve peak concentration produced with value of 0.07 g/L at 40 hours of fermentation time. The trends for butanol and ethanol production are then gradually decreasing. As the result shown, further detail will be discuss on the subject of butanol and phenol inhibition properties, phase shift during fermentation, sugars contained within POME, effect of fermentation time upon ABE fermentation and finally the glucose consumption throughout fermentation.

From result of butanol and ethanol production, it is shown that ethanol is produced far higher ratio than butanol, caused by butanol and phenol inhibiting factor

properties, resulting solvent toxicity (Haeng *et al* 2009). The toxicity resulting is based on repression upon gene expression pattern of the *C. acetobutylicum* which translate and produce solvent (Zhen and Blaschek, 2008) from sugars substrate. In aspect of butanol inhibition, at high enough concentration, growth is inhibited and butanol destroyed the cell ability to maintain internal pH, lowered the intracellular level of ATP and inhibit glucose uptake for further metabolism (Linda *et al.,* 1985). Research has been found that butanol inhibit the transport of sugar analog in *C. acetobutylicum* (Moriera *et al.,* 1981). At physiologically high concentrations of butanol, there were sudden decrease in the internal pH of the organism and also inhibit the ATPase activity. It was proved that butanol has shown to increase lipid fluidity and to some membrane related functions, such as ATPase and nutrient transport system in *C. acetobutylicum* (Guo *et al.,* 2005). On to the effect of butanol on glucose uptake, butanol in fact inhibit the rate of glucose uptake into cells and it was determined that greater-butanol-producing asporogenic strain was less sensitive to the butanol inhibition of glucose uptake than he parental strain (Wiliam *et al.,* 1985). As to summarize on effect of butanol toxicity, it can be concluded that butanol effect the cells by making it lost the ability to maintain internal pH, partially inhibit the membrane ATPase, collapsing intracellular ATP levels and lastly reducing glucose uptake for further metabolic process. It is related that ATP and ATPase is necessary for maintenance of internal pH and glucose is required for ATP formation within cells (Linda *et al.,* 1985).

In the research, POME is used as nutrient as it contain various lignocellulosic materials and sugars. Lignocellulosic materials are abundant and specifically sub by cellulose and hemicelluloses which liberate sugars by hydrolytic methods. Lignocellulosic hydrolysates contain 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 final product (Haeng *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.

4.2.5 Fermentation Time Effect

The parameter of fermentation was set to be more than 36 hours, since time interval of 0 to 36 hours, acidogenesis were on progression and up to 36 hours the shift phase takes charge which is solventogenesis (Nasratun *et al.,* 2007) and all acids produced before was converted into solvent mainly acetone, butanol, ethanol and small amount of phenol and other solvents. The time for fermentation as stated before is 48, 60, 72 and 80 hours. Even though 72 hours of fermentation time is enough to see the pattern of butanol (Takriff *et al.,* 2007) and ethanol production, 80 hours of fermentation time is taken in order to view the further pattern and confirm the butyric, solvent and glucose uptake. Also to view the limitation of 90% concentration of POME substrate contained at which it will last for further fermentation process. If the consume time availability of substrate within POME which can be utilize is longer, this means that the POME can become a valuable asset as medium for fermentation compared to other biomass in study such as corn and starch-based compound

In order to obtain solvent such as butanol and ethanol by using ABE fermentation in POME medium, it is proven that solventogenesis started at 16 hours of fermentation time as ethanol concentration is detected during that time. It is not necessarily need to consider 36 hours as solventogenesis initial starting point as stated by Takriff, 2007. It was also shown that as the fermentation time proceed longer, the solvents concentration decreased gradually as stated by Matt and co. (2000) that long fermentation time up to 72 hours resulted in lower solvent productivity. Shaheen, 2000 stated that some strain pattern later after 1940's allowed fermentation times to be as short as 30 hour fermentation time. As the total result, to obtain high concentration of butanol, the recommended fermentation time is 40 hours (0.07g/L butanol concentration) while to obtain ethanol at highest concentration, the recommended fermentation time is 20 hours (83.449 g/L ethanol concentration). For butanol, the highest production is at 40 hours. For ethanol, the highest is at 20 hours fermentation

time. These fermentation times are valid for a batch system like used in this study and for solvent recovery at the end of fermentation.

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusion

From all the result and discussion, it can be concluded that POME is applicable and recommended for ABE fermentation as it rich with utilizable sugars such as galactose, fructose, glucose, sucrose and lactose. From growth profile result, RCM medium inoculums is valid if it was taken at interval 18 to 40 hours incubation/fermentation, while for POME medium is within range 18 to 72 hours. To obtain high concentration of ethanol, the recommended fermentation time is at 20 hour with concentration of 83.449 g/L while for butanol is at 40 hour fermentation time with concentration of 0.07 g/L . It is clearly that butanol build-up at early stage of solventogenesis can affect greatly upon its next butanol batch production. It causing solvent toxicity may bring in great loss of substrate and resulting in poor income generation if the problem does not solve in industrial production.

5.2 Recommendation

Inhibition caused by the phenolic component from the POME should also be taken into consideration before starting the new fermentation in the process of producing solvent namely butanol and ethanol. It has been proved that using peroxidase to remove the model phenolic components could be applied towards the detoxification of lignocellulosic hydrolysates (Haeng *et al.,* 2009). The detoxified solution has remarkably improved the cell growth and the level of resistance towards butanol toxicity. Using fed-batch system rather than batch system for fermentation can produce better result as it can avoid high sugars build-up which may result in toxicity within the fermentation medium (Qureshi *et al.,* 2004). If sugars concentration is too high within medium, clostridia will convert the sugars into acids and then into solvent at such high pace that it accumulated and resulting in solvent toxicity even during the initial period of fermentation time. Thus, supplying or feeding substrate at control level and at which the amount is enough for fermentation process can ensure solvent toxicity does not happen. Recovery by gas stripping technology/system for solvent recovery (volatile product such as alcohol) may reduce butanol and ethanol build-up within fermentation media thus reducing solvent toxicity (Thaddeus *et al.,* 2004). Using robust biocatalysts can convert the mixed substrate into biofuels. The ideal biocatalysts able to tolerate inhibitory compounds released from biomass hydrolysis and increased concentrations of the final product such as butanol and ethanol (Liu *et al.,* 2009).

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APPENDIX A

Glucose Standard calibration Curve

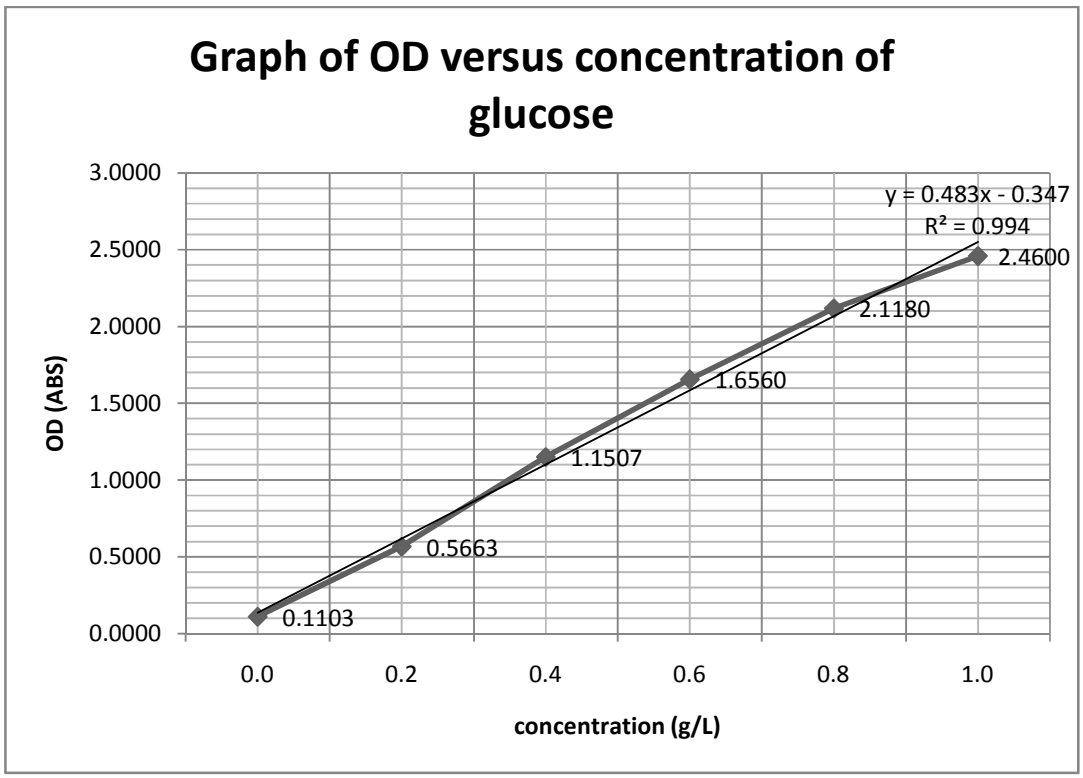

Figure A1 : Glucose Standard Calibration Curve

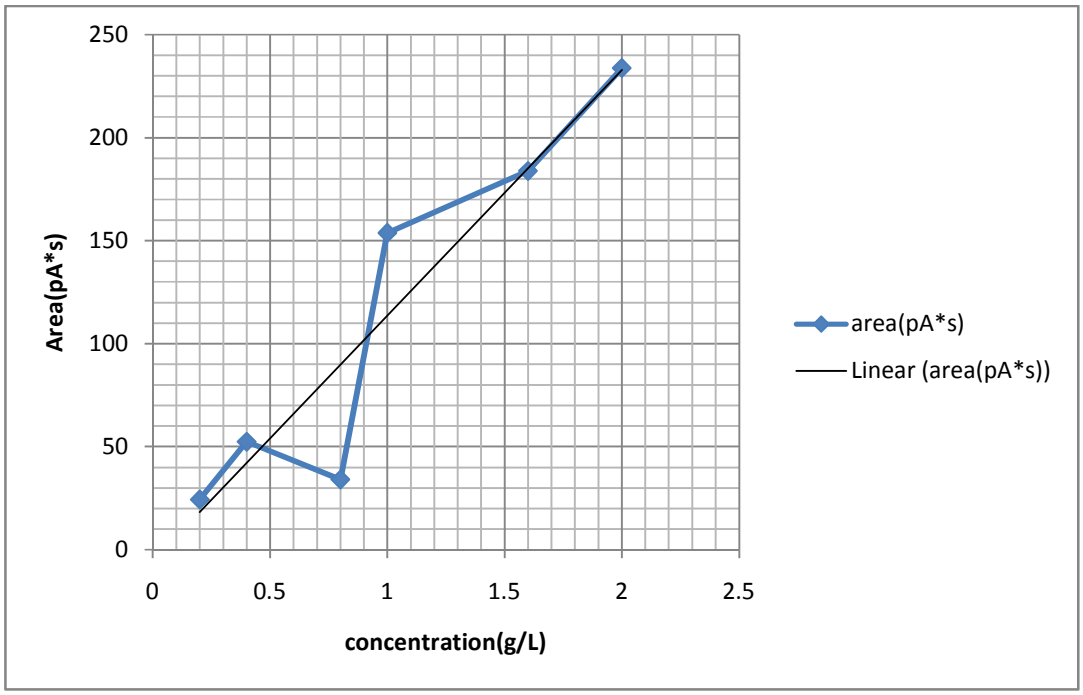

Figure A2 : Ethanol Calibration Curve

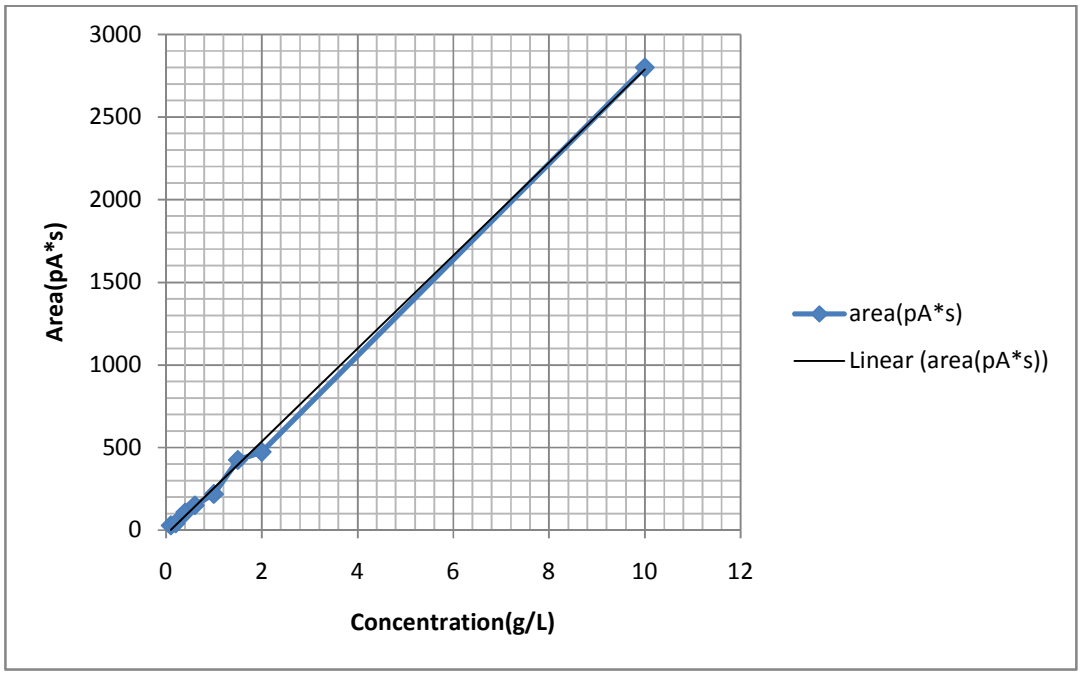

Figure A3 : Butanol Calibration Curve (Range: Y-axis:0 – 3000 pA*s; X-axis:0 – 12 g/L)

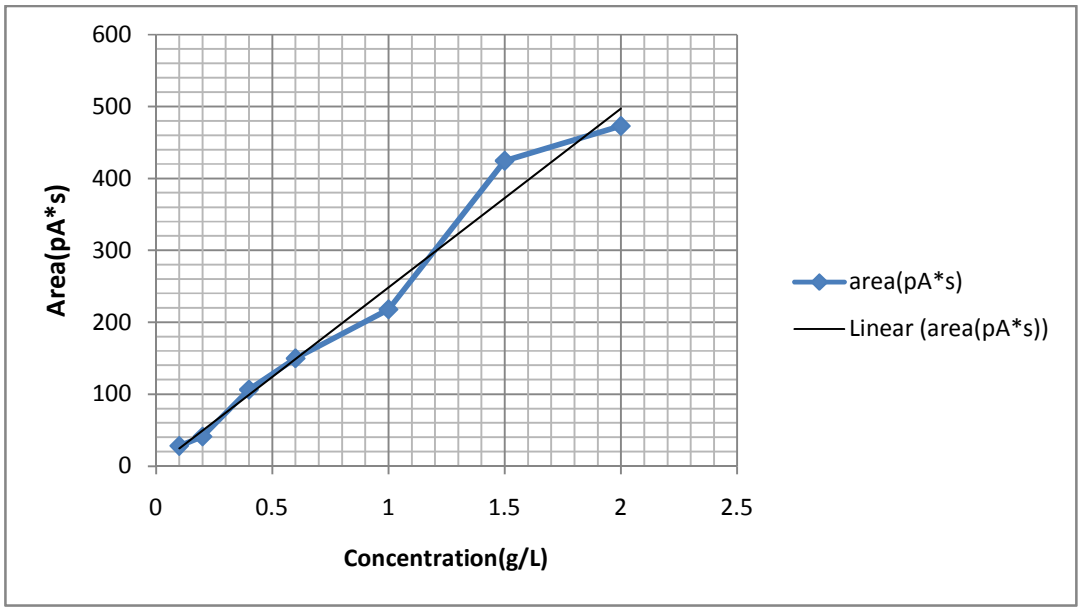

Figure A4 : Butanol Calibration Curve (Range: Y-axis:0 – 600 pA*s; X-axis:0 – 2.5 g/L).

APPENDIX B

ANALYSIS OF FRESH POME COMPOSITION

Fructose

Table B1 : Fructose concentration and peak area. Retention time is 7.052 minute

Fructose concentration (g/L)	Peak area
20	1.13320e6
40	2.36668e6
80	4.37532e6
100	5.19066e6

Glucose

Table B2 : Glucose concentration and peak area. Retention time is 7.702 minute

Glucose concentration (g/L)	Peak area
20	1.69828e6
40	3.27955e6
60	5.05296e6
80	6.64497e6
100	8.23004e6

Galactose

Table B3 : Galactose concentration and peak area. Retention time is 8.633 minute

Galactose concentration (g/L)	Peak area
20	9.92166e5
40	1.94887e6
60	2.87786e6
80	3.94253e6
100	4.75126e6

Sucrose

Table B4 : Sucrose concentration and peak area. Retention time is 10.381 minute

Sucrose concentration (g/L)	Peak area
20	1.86245e6
40	3.26337e6
60	4.89866e6
80	6.36837e6
100	7.84323e6

Lactose

Table B5 : Lactose concentration and peak area. Retention time is 13.127 minute

Lactose concentration (g/L)	Peak area
20	1.30290e6
40	2.50230e6
60	3.21022e6
80	4.19806e6
100	5.35126e6

POME sample analysis

Sample 1

Table B6 : The peak area and retention time of sample 1

Peak	Retention Time (minute)	Area
1	6.761	2.59147e5
2	7.311	2.64544 e5
3	7.769	5.29701 e5
4	8.756	3.03513 e5
5	8.922	4.43881 e5
6	10.190	4.33496 e5
7	13.290	2.31336 e5

Sample 2

Table B7 : The peak area and retention time of sample 2

Peak	Retention Time (minute)	Area
1	6.764	1.29103 e5
2	7.297	1.96194 e5
3	7.766	2.42428 e5
4	8.771	4.76619 e5
5	10.216	1.45158 e5
6	13.347	8.30884 e4

Sample 3

Table B8 : The peak area and retention time of sample 3

Peak	Retention Time (minute)	Area
1	6.779	1.67508e5
2	7.302	1.84783 e5
3	7.770	2.11423 e5
4	8.774	5.63179 e5
5	10.232	1.48901e5
6	10.994	7.46828e4
7	11.593	7.73765e4
8	13.370	1.06895e5

APPENDIX C

UV-VIS ANALYSIS

*reading in unit OD (Optical Density)

72 Hour Fermentation

Blank = -0.00

Table C1: Concentration of glucose at 20 hour for 72 hour fermentation time (POME)

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.387	2.387	2.387	2.387	0.96
1/10	0.385	0.387	0.38	1.152	0.42
1/20	0.08	0.075	0.077	0.0773	-

Blank = -0.00

Table C2: Concentration of glucose at 40 hour for 72 hour fermentation time (POME)

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.366	2.367	2.366	2.366	0.90
1/10	0.522	0.513	0.517	0.517	0.18
1/20	0.158	0.155	0.154	0.155	0.03

Blank = -0.00

Table C3: Concentration of glucose at 60 hour for 72 hour fermentation time (POME)

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.247	2.247	2.247	2.247	0.83
1/10	0.432	0.431	0.432	0.432	0.15
1/20	0.123	0.122	0.122	0.122	0.01

Blank = -0.00

Table C4: Concentration of glucose at 72 hour for 72 hour fermentation time (POME)

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.125	2.132	2.125	2.127	0.73
1/10	0.521	0.522	0.523	0.522	0.19
1/20	0.223	0.213	0.22	0.219	0.06

72 Hour Fermentation

Blank = -0.00

Table C5: Concentration of glucose at 40 hour for 72 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.569	2.569	2.569	2.569	-

Blank = -0.00

Table C6: Concentration of glucose at 60 hour for 72 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.456	2.456	2.455	2.456	1.0

Blank = -0.00

Table C7: Concentration of glucose at 72 hour for 72 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.211	2.212	2.212	2.212	0.83

80 Hour Fermentation

Blank = -0.00

Table C8: Concentration of glucose at 20 hour for 80 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	0.657	0.657	0.657	0.657	0.23
1/10	0.926	0.926	0.926	0.926	0.31
1/20	-0.779	-0.779	-0.779	-0.729	-

Blank = -0.00

Table C9: Concentration of glucose at 40 hour for 80 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	0.713	0.713	0.713	0.713	0.28
1/10	-0.162	-0.162	-0.162	-0.612	-
1/20	-0.779	-0.779	-0.779	-0.779	-

Blank = -0.00

Table C10: Concentration of glucose at 60 hour for 80 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	0.693	0.693	0.693	0.693	0.245
1/10	-0.31	-0.307	-0.31	-	-
1/20	-0.779	-0.779	-0.779	-	-

Blank = -0.00

Table C11: Concentration of glucose at 80 hour for 80 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	0.617	0.617	0.617	0.617	0.2
1/10	-0.779	-0.779	-0.779	-	-
1/20	-0.779	-0.779	-0.779	-	-

80 Hour Fermentation

Blank = -0.00

Table C12: Concentration of glucose at 20 hour for 80 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.398	2.398	2.398	2.398	0.913

Blank = -0.00

Table C13: Concentration of glucose at 40 hour for 80 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.420	2.398	2.398	2.398	0.913

Blank = -0.00

Table C14: Concentration of glucose at 60 hour for 80 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.289	2.289	2.289	2.289	0.89

Blank = -0.00

Table C15: Concentration of glucose at 80 hour for 80 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.678	2.678	2.678	2.678	-

48 Hour Fermentation

Blank = 3.00

Table C16: Concentration of glucose before fermentation for 48 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	3.0	3.0	3.0	3.0	-
1/10	3.0	3.0	3.0	3.0	-
1/20	1.329	1.329	1.329	1.329	0.54

Blank = -0.00

Table C17: Concentration of glucose before fermentation for 48 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.432	2.432	2.432	2.432	0.8

48 Hour Fermentation

Blank = 3.00

Table C18: Concentration of glucose at 16 hour for 48 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.161	2.161	2.161	2.161	0.87
1/10	1.836	1.836	1.836	1.836	0.69
1/20	0.571	0.571	0.571	0.571	0.21
1/40	-0.133	-0.133	-0.133	-	-
1/60	-0.309	-0.309	-0.309	-	-
1/80	-0.527	-0.527	-0.527	-	-

Blank = 3.00

Table C19: Concentration of glucose at 45 hour for 48 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.921	2.921	2.921	2.921	-
1/10	2.553	2.553	2.553	2.553	-
1/20	1.429	1.429	1.429	1.429	0.56
1/40	0.637	0.637	0.637	0.637	0.22

Blank = 3.00

Table C20: Concentration of glucose at 48 hour for 48 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.599	2.599	2.599	2.599	-
1/10	2.049	2.049	2.049	2.049	0.71
1/20	0.6	0.6	0.61	0.6	0.14

48 Hour Fermentation

Blank = -0.00

Table C21: Concentration of glucose at 16 hour for 48 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	1.595	1.595	1.595	1.595	0.57
1/10	-0.191	-0.191	-0.191	-0.191	-

Blank = -0.00

Table C22: Concentration of glucose at 45 hour for 48 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.602	2.602	2.602	2.602	-
1/10	0.386	0.386	0.386	0.386	0.18

Blank = -0.00

Table C23: Concentration of glucose at 48 hour for 48 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.260	2.260	2.260	2.260	0.81
1/10	0.103	0.103	0.103	0.103	0

60 Hour Fermentation

Blank = 3.0

Table C24: Concentration of glucose before fermentation for 60 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	3.0	3.0	3.0	-	-
1/10	2.921	2.921	2.921	-	-
1/20	2.568	2.568	2.568	-	-
1/40	1.848	1.848	1.848	1.848	0.7

Blank = -0.00

Table C25: Concentration of glucose before fermentation for 60 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.432	2.432	2.432	2.432	0.8
1/10	1.765	1.765	1.765	1.765	0.67

Blank = 3.00

Table C26: Concentration of glucose at 20 hour for 60 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	3.0	3.0	3.0	3.0	-
1/10	2.319	2.319	2.319	2.319	0.9
1/20	1.779	1.779	1.779	1.779	0.68

Blank = 3.00

Table C27: Concentration of glucose at 48 hour for 60 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.958	2.983	2.953	2.953	-
1/10	2.585	2.585	2.585	2.585	-
1/20	1.797	1.797	1.797	1.797	0.65
1/40	0.927	0.926	0.926	0.926	0.32

Blank = 3.0

Table C28: Concentration of glucose at 60 hour for 60 hour fermentation time (POME).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.699	2.699	2.699	2.699	-
1/10	2.149	2.208	2.208	2.208	0.85
1/20	1.095	1.094	1.093	1.094	0.31
1/40	0.5	0.499	0.499	0.4993	0.17

60 Hour Fermentation.

Blank = -0.00

Table C29: Concentration of glucose at 20 hour for 60 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.432	2.432	2.432	2.432	0.8
1/10	1.765	1.765	1.765	1.765	0.63

Blank = -0.00

Table C30: Concentration of glucose at 48 hour for 60 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	1.702	1.702	1.702	1.702	0.52
1/10	1.035	1.035	1.035	1.035	0.32

Blank = 0.00

Table C31: Concentration of glucose at 60 hour for 60 hour fermentation time (RCM).

Dilution	Reading 1	Reading 2	Reading 3	Average	Concentration(g/L)
X	2.585	2.585	2.585	2.585	0.73
1/10	1.122	1.121	1.120	1.120	0.38