EFFECT OF INOCULUM CONCENTRATION ON BUTANOL AND ETHANOL PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM*

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EFFECT OF INOCULUM CONCENTRATION ON BUTANOL AND ETHANOL PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM*

BAIYINAH BTE HAJ A. TALIP

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

Faculty of Chemical & Natural Resources Engineering
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APRIL 2010

I declare that this thesis entitled "*Effect Of Inoculum Concentration On Butanol And Ethanol Production by Clostridium acetobutylicum"* is the result of my own research except as cited in the 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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Special Dedication to:

My mom, Hjh Zaharah bte Yaamat,
My dad, Hj.A. Talip bin Md.Noh,
My family members,
My beloved,
My fellow lecturers,
My friends and
My fellow colleague

For all your care, support and believe in me.

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ABSTRACT

In Malaysia, the government has implement policy instruments aimed at promoting the production of butanol and ethanol as an alternative to renewable energy. The objective was to study the effect of inoculum concentration on butanol and ethanol production and besides that, the study also focused on growth profile of *Clostridium acetobutylicum* and glucose consumption in POME and RCM. The methods begin with palm oil mill effluent (POME) analysis using High Performance Liquid Chromatography (HPLC). The process continued with the microbial fermentation and each inoculums concentration at 5% v/v, 10 % v/v and 15 % v/v were prepared in Reinforced Clostridia Media (RCM). POME as an alternative medium was sedimented for 24 hours, and has been prepared at pH 5.8, diluted with 90 % substrate, deoxygenated the POME using nitrogen gas. Both medium POME and RCM were seeded with different inoculums concentration and after 3 days fermentation, the fermentation broth were undergo liquid-liquid extraction and prepared for Gas Chromatography (GC) analysis. The result obtained from POME analysis showed that galactose was the main component in POME. Moreover, the maximum butanol production for both POME and RCM medium were 0.18533% at 15% v/v (POME) and 0.00801% at 5% v/v (RCM). Result showed that increasing the inoculum concentration would reduce the fermentation time, increased the total glucose consumption and increased the butanol production. While the maximum production of ethanol were, 1.87593% at 15% v/v during 40 hours (POME) and 2.36754% at 5% v/v during 60 hours (RCM) of fermentation time. The result showed that there were significant correlation between inoculum concentration and butanol and ethanol production. Thus, it could be concluded that inoculum concentration does affect the butanol and ethanol production, besides, POME could be an alternative medium for butanol and ethanol fermentation.

ABSTRAK

Kerajaan Malaysia telah mengaplikasikan polisi untuk menghasilkan butanol dan etanol sebagai sumber bahan bakar alternatif. Objektif kajian ini adalah untuk mengkaji kesan kepekatan inokulum terhadap penghasilan butanol dan etanol, dan di samping itu, kajian turut memfokuskan kepada pertumbuhan *Clostridium acetobutylicum* dan penggunaan glukosa dalam proses fermentasi menggunakan media" palm oil mill effluent (POME)"dan "Reinforced Clostridia Media (RCM)". Penghasilan butanol dan etanol bermula dengan analisis "POME" dengan menggunakan "High Performance Liquid Chromatography (HPLC)". Proses fermentasi diteruskan dengan menyediakan kepekatan inokulum yang berbeza iaitu 5 % v/v, 10 % v/v dan 15 % v/v di dalam pati RCM. POME telah dienapkan selama 1 hari, pH POME turut diubah kepada 5.8, POME juga dilarutkan kepada 90 % substrat dan proses pengnyahkan oksigen dengan menggunakan gas nitrogen. Kedua-dua medium RCM dan POME telah dimasukkan dengan kepekatan inokulum yang berbeza dan proses fermentasi adalah selama 3 hari. Selepas 3 hari, butanol dan etanol telah diekstrak daripada POME and RCM media dan digunakan untuk analisis "Gas Chromatography (GC)". Keputusan yang diperolehi daripada analisis POME menunjukkan galaktosa merupakan komponen utama di dalam POME. Sementara itu, penghasilan butanol yang maksimum adalah pada 15 % v/v dengan peratusan 0.18533 % (POME) dan pada 5 % v/v dengan peratusan 000801 % (RCM). Manakala, maksimum penghasilan etanol adalah ketika 15 % dengan peratusan 1.87593 % (POME) dan pada 5 % v/v dengan peratusan 2.36754 % (RCM) . Hasil kajian menunjukkan wujudnya korelasi anatara kepekatan inokulum dengan penghasilan butanol dan etanol. Kesimpulannya, kepakatan inokulum member kesan terhadapan penghasilan butanol dan etanol.

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LIST OF SYMBOLS / ABBREVIATIONS

µm	-	micrometer
mL	-	millimeter
µL	-	micro liter
RCM	-	Reinforced Clostridia Media
POME	-	palm oil mill effluent
Vs	-	versus
v/v	-	volume per volume
HPLC	-	High Performance Liquid Chromatography
GC-FID	-	Gas Chromatography-Flame Ionization Detector
UV-Vis spectrophotometer	-	Ultraviolet-Visible spectrophotometer
DNS	-	DiNitroSalicylic

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

Nowadays, fuel is the major topic had been discussed over these decades, because of the population growth is increasing while percentage of fuel available is decreasing. Thompson, (2003) claimed that public has enjoyed using up the gasoline, heating oil, plastic and countless other oil products for decades. They did not notice that petroleum kept flowing generously and had been announced by environmentalists who reported that fossil fuels would run out. Even though media keep constantly convinced public that, there will be a new oil discoveries and increasing stocks oil, but still fuel is running empty. Moreover, petroleum is a basic human needs primarily used for transportation to do their own routine. In Malaysia, the development of automotive industry has cause increase in the numbers of cars, vans, buses, lorries and etc on the road which needs petrol to move (Jailani and Jaafar, 1999). Thus, this issue will force on Malaysian Government to provide others alternative fuel for the future appears that petroleum is running out.

Then, researchers begin to take an opportunity to resolve the scarcity of fossil fuels problems which starting late of 1970s and early 1980s they found that alcohol fuel can be an alternative fuel for the future and easily being produced from renewable resources was very promising (Qureshi and Blaschek, 2000). Butanol,

ethanol, and acetone were classified as alcohol and because of researchers found that those alcohol can be produced using biologically substrate, thus they called it as bioalcohol. Furthermore, studies showed that the most suitable and powered bioalcohol was butanol or already known as biobutanol suitable used for combustion engine for cars and other transportations. Biobutanol can be produced from renewable resources such as molasses, corn, wheat straw, corn stove, corn fiber, and other agricultural byproducts in the process named as acetone-butanol-ethanol (ABE) fermentation.

For decades, public only noticed presence of ethanol as an alternative fuel for the next potential biofuel, but researcher had found that butanol was a chemical which had excellent fuel characteristics, for example, butanol had a higher calorific value than ethanol according to their numbers of carbon atoms (Qureshi and Blaschek, 2000). ABE fermentation was the oldest fermentation technique to produce bioalcohol from renewable resources. Similar to others fermentation method, ABE fermentation was still utilize the substrate containing carbon sources from glucose using microbes but commonly used anaerobic bacteria which was obligate no oxygen during the process. The microbial fermentation of carbohydrates to butanol was well known and had potentially attractive for several economic reasons (Syed *et al.*, 2008). Most reasons in alternative energy were the values were very concerned on the product being produced in terms of pricing and quality of the products. This means that people started trusted on the presence of butanol as another alternative energy for their transportation fuel since biobutanol was being introduced for ages.

Although, in production of biofuel had several alternative of raw materials but researcher had found that palm oil mill effluent (POME) had a greater potential used as renewable resources in production of biobutanol. POME was known as wastes generated in the vast amount at palm oil industry which POME can affect the watercourse if did not treated very well. In addition, Kalil *et al*. (2003) and Wu *et al*. (2007) showed that in their studies that POME contain high concentration of lignocelluloses that can be utilized by *Clostridium acetobutylicum* ad converted it into valuable product such as butanol, ethanol and acetone.

1.2 Problem Statement

The sustainability of the petroleum industry in Malaysia was increasingly becoming an issue because world was currently in an energy crisis. Fossil fuels were the lifeblood of our society and for many others around the world. Furthermore, fossil fuels, coal, oil and natural gas, were a non-renewable source of energy. Formed from plants and animals that lived up to 300 million years ago, fossil fuels are found in deposits beneath the earth. The main problem was petroleum was from non renewable energy and it took so many years to produce oil and gas.

Alternative forms of energy were currently under development even though most of them were only in their initial stages. In addition, conventional process only utilized food products as our renewable sources but society will deal with food crisis during producing an alternative energy. The key factors which determined the economic viability of the ABE fermentation were the costs of raw material. For example researchers were using maize as the substrate in ABE fermentation, if the price of maize at particular time increased thus automatically the price of butanol also increases, because cost of substrate made about 60% of the overall cost of production.

Furthermore, POME was abundance and worse waste water in Malaysia. More than 40 million tons of POME was generated in Malaysia. Therefore, the palm oil mill industry in Malaysia was identified as the one that produces the largest pollution load into the rivers throughout the country. Another aspect of pollution was from environmental impacts, such as drilling, generation of polluting wastes, greenhouse gases and climate change not produced by renewable energy produced from non-renewable energy. In addition, petroleum can create incomplete combustion thus releasing carbon monoxide and becoming threat to public health.

So, concluded that POME is a low cost substrate that can be used to in ABE fermentation and converting it into valuable product used as a combustion engine. Furthermore, utilizing POME as a substrate for fermentation can be one of method to treat the waste.

1.3 Research Objective

The research was proposed to achieve an objective in investigated production of butanol which was;

1. To study the effect of inoculums concentration on butanol and ethanol production by *Clostridium acetobutylicum*.

1.4 Research Scopes

In order to achieve following objectives, several research scopes have been identified;

1. To study on growth profile of *Clostridium acetobutylicum* in different batch medium (Reinforced Clostridia Media (RCM) and Palm Oil Mill Effluent (POME)).
2. To complete composition analysis of batch fresh POME using High Performance Liquid Chromatography (HPLC).
3. To study the effect of different inoculums concentration in the range of 5% v/v, 10% v/v and 15% v/v on anaerobic fermentation.
4. To study on glucose consumption of different batch of fresh POME and RCM using Ultraviolet and Visible Spectroscopy (UV - VIS).

1.5 Significance of Study

Pollution from residual oil content in the wastewater was one of the serious environmental problems. Due to the rapid development of the palm oil industry in Malaysia, a large volume of palm oil mill effluent (POME) had been produced. Thus, Environmental Act regulated that, POME must be treated first before discharge into the river. In connection with the issue of pollution, this study could shows to the public that POME can be fully utilized in the production of valuable product such as biofuel through microbial fermentation. POME was one of abundance wastes in Malaysia which 50% of water from palm oil mill factory will end up as palm oil mill effluent (POME). In addition, POME contains higher concentration of biological oxygen demand (BOD) and chemical oxygen demand (COD) which will harm aquatic life if there was no pretreatment before being released into the river. Hence, by using POME as a source for valuable product thus this study was applicable for converting "Waste to Wealth", which "Waste" was the palm oil mill effluent (POME) while "Wealth" was the valuable product, butanol used as biofuel for the future.

Moreover, the number of human population in the country increased thus will increase the numbers of car users. Development of high growth, encourage the population to represent the vehicle for them. As a result, it wills effects of the liberation of carbon monoxide from incomplete combustion when using the petrol, which then lead to the air pollution problem. In 1990, the number of motor vehicles on the peninsula only surpassed by 4, 249, 758 units. . These reliable figures will increase by 17 % per year and extension was estimated over 500, 000 tons of pollutants produced in the atmosphere at each year affected by increasing numbers of vehicle (Jailani and Jaafar, 1999). Immediate action was taken from this study where butanol was used to replace the utilization of petroleum as transportation fuel. Butanol has similar characteristics with gasoline and petrol known as biofuel will undergo complete combustion and finally will produced carbon dioxide that less hazardous than carbon monoxide. Thus, this study will overcome the air pollution problem by applying biobutanol as biofuel since it helps to reduce pollution in this country.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter is about combining new and old interpretation in this recent study which will summarize the important information regarding butanol production by using solventogenic clostridia. In addition, detailed discussion on each particular topic such as, raw material, fermentation process, process selection and product determination will be presented in this chapter.

2.2 Fermentation Substrate

In the fermentation process, the basic need is glucose which act as carbon source then converted it into products. This study shows that substrate being used that compatible with the *Clostridium acetobutylicum* are varies. Takriff et al. (2009) demonstrated that Reinforced Clostridium Medium (RCM) which is an establish medium for clostridia while Palm Oil Mill Effluent (POME) is an alternative medium for the fermentation. In addition, there are a lots of establish medium for

clostridia such as Clostridia Basal Medium (CBM), Cooked Meat Medium (CMM), Potato Medium and etc. Those medium differ in composition but similar usage for clostridia. This study only focus on RCM as an establish medium for *Clostridium acetobutylicum*, while POME is selected for an alternative medium because of their characteristics suitable for butanol production, even though there are another alternative such as wheat straw and corn but POME has a best characteristics for this process.

2.2.1 Reinforced Clostridia Media (RCM)

Reinforced Clostridia Media (RCM) is used for cultivating and enumerating clostridia, other anaerobes, and other species of bacteria from foods and clinical specimens. RCM provides enough necessity for clostridia and other anaerobe to growth and regenerate. In addition RCM can be used as fermentation medium, preparation of inoculums and to obtain pure culture by streaking method. Furthermore, RCM is used to suspend the preserved spores and activate the vegetative cell of clostridia (Castano, 2003). Moreover, RCM used for fermentation media compared to other alternative medium or so called as "control experiment" (Takriff *et al.*, 2009).

There are two types of RCM for cultivation of clostridia, RCM broth and RCM agar. Both has similar composition but agar content in RCM agar is much higher than RCM broth and mostly suitable for plating media. Table 1.1 is the summarization composition of RCM broth with the particular formula and Table 1.2 is the summarization composition of RCM agar with different formula.

Table 2.1: Formulation of Reinforced Clostridia Media broth

Approximate Formula / Liter	Mass / gram
Pancreatic Digest of Casein	5.0
Protease Peptone No 3	5.0
Beef Extract	10.0
Yeast Extract	3.0
Dextrose	5.0
Sodium Chloride	5.0
Soluble Starch	1.0
Cysteine Hydrochloride	0.5
Sodium Acetate	3.0
Agar	0.5

Table 2.2: Formulation of Reinforced Clostridia Media agar

Approximate Formula / Liter	Mass / gram
Yeast Extract	3.0
Meat Extract	10.0
Meat Peptone	5.0
Starch	1.0
D(+) - Glucose	5.0
L-Cysteine hydrochloride	0.5
Sodium Chloride	5.0
Sodium Acetate	3.0
Agar	12.5

Reinforced Clostridial Medium contains peptones and beef extract as sources of carbon, nitrogen, vitamins and minerals. While yeast extract supplies B-complex vitamins which stimulate bacterial growth. Dextrose is the carbohydrate source. Sodium Chloride maintains the osmotic balance. Cysteine Hydrochloride is the reducing agent. Sodium Acetate acts as a buffer and finally small amount of agar in RCM broth makes the medium semisolid but higher amount of agar in RCM agar makes the medium solid phase.

2.2.2 Palm Oil Mill Effluent (POME)

The palm oil industry is the successful story in Malaysia because, Malaysia is the second biggest exporter in the world after Indonesia. About 60% of the agriculture land in this country occupied with palm oil estate (Sulaiman and Ling, 2001). The improvement of this plantation lead to other environmental problem which is waste generated from palm oil mill industry called as palm oil mill effluent (POME). In the year 2004, more than 40 million tons of POME was generated from 372 mills in Malaysia (Hassan and Puteh, 2007). Wah and Sulaiman (2002) reported that in the May 2001, the production of 985, 063 tons of crude palm oil means total of 1, 477, 595 m^3 of water was used, and 738, 797 m^3 was released as POME. Wu, *et al.* (2007) reported that palm oil milling consumes large amount of process water and 50% ended as palm oil mill effluent (POME). In connection with those facts, POME can be catogarized as the most abundance waste in Malaysia and harmful to the environment because it contains high organic strenght wastewater which dangerous to the water bodies

2.2.2.1 Characteristics of Palm Oil Mill Effluent (POME)

Raw POME is a colloidal suspension containing 95-96% water, 0.6-0.7% oil and 4-5% total solids including 2-4% suspended solids that are mainly consisted of debris from palm fruit mesocarp generated from three main sources, namely sterilizer condensate, separator sludge and hydrocyclone wastewater (Wu *et al.,* 2007). It is also claimed that 1.5 m^3 of wastewater is generated from separator sludge which waste that produced from separator sludge is thick, brownish liquid with discharged temperature in the range of 80 to 90°C (Takriff *et al.,* 2009). In addition, different palm oil mill factory will generate different composition of POME but still the composition contains similar parameters such as oil & grease, BOD, COD, total solids, suspended solid, and total volatile solids. Takriff *et al*, (2009) also reported that POME contains 20000-25000 mg/L of BOD and 40000-50000 mg/L of COD which means causes environmental problems such as heavy stench in the area.

2.2.2.2 Potential of Palm Oil Mill Effluent (POME): Renewable Resources

The most important economic factor in solvent fermentation is the cost of raw material, which makes up 60% of the overall cost (Tsuey *et al.*, 2006). Castano (2003) reported that POME has high concentrations of hemicellulose, cellulose and soluble carbohydrate that makes POME is much appropriate medium for clostridia to utilize and converted into alcohol. The richness of carbon source in POME has made it as good candidate for use as culture medium for microbial fermentation. Moreover, this fact is supported by Rha *et al.*(2002) where their studies on POME composition by using HPLC showed that, the composition of Palm Oil Mill Effluent (POME) consists of various monosaccharides such as glucose, xylose, galactose and others. While other method such as using ultrafiltration membrane by Wu *et al.* (2007) showed that rententate from pretreated POME consists of protein and carbohydrates and claimed that both useful materials using microbial fermentation. Eventhough, based from POME characteristics consists high volume of water but (Kalil *et al.*, 2003) claimed that sedimented POME helped to remoce other toxic materials and contains higher concentrations of lignocellulose and other insoluble materials which supported growth of *Clostridium acetobutylicum*. In addition lignocellulose is the most abundant renewable resource and have a great potential for fermentation which lignocellulose will be hydrolyzed into hmicellulose, cellulose and lignin (Jones and Woods, 1986). Hemicellulose will be converted to other simple sugars such as xylose, arabinose and galactose, while cellulose will converted into glucose and then will be utilized in fermentation process to produce acetone-butanol-ethanol.

2.3 Microbial Fermentation

Fermentation process is the process that required carbon source and convert it into products. Fermentation can be divided into two types of fermentation, which are aerobic fermentation and anaerobic fermentation. Both process undergoes different requirement, aerobic fermentation required oxygen throughout the process while, anaerobic fermentation not required any oxygen. Microbial fermentation is using microbes to utilize the substrate and divided based on the types of fermentation. In this study, only anaerobic fermentation was consumed since, clostridium species are used for acetone-butanol-ethanol (ABE) fermentation, which ABE fermentation does not required oxygen during the process. ABE fermentation is the most economic process to produce valuable product especially butanol that can used as combustion engine in transportation.

2.3.1 Acetone-Butanol-Ethanol fermentation (ABE fermentation): Anaerobic Process

Production of butanol from microbial fermentation was first determined by Pasteur in 1861 and in latter part of the 19th century the production of butanol by anaerobic bacteria was studied by several investigators (Jones and Woods, 1986). ABE fermentation can undergoes several of raw materials since clostridia species could utilize variety of starchy substances to produce better yield of butanol. Moreover, ABE fermentation was first carried out during first half of last century but cannot compete with the economically with the petrochemical industry (Kobayashi, *et al.*, 2005). However, they claimed that there has been revival of interest in ABE fermentation, since renewable resources as such domestic and agro-industrial wastes have become possible alternative for the production of chemicals. Anaerobic process for ABE fermentation could be a complicated process since *Clostridium acetobutylicum* is a strictly anaerobic bacteria, which will be contaminated with oxygen if they exposed too long with air. During ABE fermentation, anaerobe

bacteria will convert the substrate into three products acetone, butanol and ethanol after undergoes acidogenesis phase and finally shifted into solventogenesis phase.

2.3.1.1 Declined of Acetone-Butanol-Ethanol Fermentation (ABE Fermentation)

Butanol production suffered from end-product inhibition thus affected the cost of solvent recovery in industrial plants because of low concentration of solvents (Qureshi and Blaschek, 2000). The cost of recovery of butanol is high due to the fact that is concentration in the fermentation broth is low because of product inhibition (Qureshi and Blaschek, 2000). In addition, between 1950s and 1960s, fermentative produced ABE was unable to compete economically with petro chemically produced ABE (Qureshi and Blaschek, 2000). This resulted in the virtual elimination of this fermentation. As a result, the following factors which severely affect the economics of butanol fermentation were identified ; (i) high cost substrate; (ii) low product concentration (<20 g l^{-1}); (iii) low reactor productivities (<0.3 g l^{-1} h^{-1}); (iv) low ABE yields (0.28-0.33); and (v) an escalated cost of butanol recovery by distillation which was the only technique for recovery at that time (Qureshi and Blaschek, 2000). In order to reduce the cost of production, attempts were made to utilize cheaper substrates such as molasses, whey permeates, and corn depending upon availability and the region of the world where they are produced.

However, the decline in ABE fermentation was twofold. First, the petrochemical industry grew at unprecedented rate, and by late 1950s competition between the fermentation and chemical process had become very acute (Jones and Woods, 1986). Second, particularly in the United States, molasses began to be used in substantial amounts in cattle feed (Jones and Woods, 1986). Kalil *et al.* (2003) claimed that the lower production of ABE might due to the use of glucose derived from substrate (POME) instead of the whole POME as the medium.

Although many chemicals can be produced by fermentation, the accumulation of toxic or inhibitory metabolites in the fermentation broth often inhibits cell growth and product formation. Final product concentrations are low, fermenter productivity is reduced, and wastewater treatment and product separation costs are high. The acetone-butanol-ethanol fermentation is characterized by strong product inhibition. This strong butanol inhibition adversely affects the economics of the ABE fermentation in three main ways; butanol accumulation in the broth lowers fermenter productivity so that large fermenter are required; butanol inhibition limits the concentration of substrate that can be completely consumed, and thus large volumes of wastewater are produced and product recovery is expensive due to the low final product concentrations in the fermenter (Jones and Woods, 1986).

Another reason of product inhibition is process selected between continuous and fed batch fermentation. Productivity in the continuous is lower than fed-batch fermentation because of, first some bacteria may have been damaged during circulation through the extraction loop; the bacteria had to pass through a diapgram pump and several valves on each pass through the extraction (Roffler *et al.*, 1988). They also added that there will be any air entered the extraction system through the many valves present could have inhibited the strictly anaerobic cells of *Clostridium acetobutylicum*.

2.3.2 *Clostridium acetobutylicum*

Among the saccharolytic butyric acid-producing clostridia, there are number of species capable of producing significant amounts of neutral solvents during the later stages of batch fermentation under the appropriate conditions (Jones and Woods, 1986). The strains used most extensively for the production of acetone, ethanol and butanol are now generally classified as *Clostridium acetobutylicum* which that there are numbers of different species of butanol-producing clostridia that had been recognized. These strains categorized based on differences type and ratio the solvents produced. For example *Clostridium beijerinckii* (*Clostridum butylicum*)

produces solvents in approximately the same ratio as *Clostridium acetobutylicum*, but isopropanol is produced in replacing acetone.

Clostridium acetobutylicum is an anaerobe microorganism which lacks the metabolic enzyme systems for using oxygen in respiration. Because strict, obligate, anaerobes also lack of enzyme for processing toxic oxygen, they cannot tolerate any free oxygen in the immediate environment. Growing anaerobic bacteria usually requires special media, methods of incubation, and handling chamber (Cowan and Talaro, 2006). Figure 2.1 shows the morphology of *Clostridium acetobutylicum* under microscopic technique.

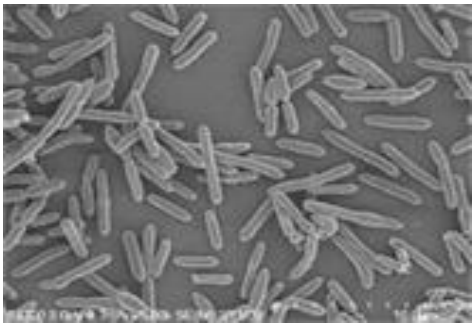

Figure 2.1 Figure of *Clostridium acetobutylicum*

Furthermore, *Clostridium acetobutylicum* is a Gram positive bacillus which can break down sugar and capable of producing a number of different commercially useful products which most notably are acetone, ethanol and butanol. In addition, *Clostridium acetobutylicum* required anaerobic conditions in order to grow in its vegetative state. Vegetative state means that clostridia is in actively grow and prepared to utilize substrate. Jones and Woods (1986) reported that *Clostridium acetobutylicum* is most commonly associated with living plant material rather than with decaying plant material or soil. Potatoes, the roots of nitrogen-fixing legumes, and other root crops have been reported to be excellent material for the isolation of these bacteria

2.3.2.1 Acidogenesis and Solventogenesis Phases

In ABE fermentation the most popular studies is about the phase shifting from acidogenic to the solventogenic phase. Schuster *et al.* (1998) examined the characteristics of *Clostridium acetobutylicum* which was during exponential growth of *Clostridium acetobutylicum*, the major fermentation products are acetate and butyrate called as acidogenic phase. As the acids accumulate, the growth ceases, and sugar is converted into acetone, butanol and ethanol and called as solventogenic phase. Takriff *et al.* (2009) also showed that *Clostridium acetobutylicum* undergoes biphasic phase of fermentation, where at the initial period,a rapid microbial growth occurred and then accumlated the acetic and butyric acid then pH will reduce to acidic medium. They also added that net acid production ceased and synthesis of solvent began thus acetone, butanol and ethanol will be produced.

Jones and Woods (1986) supported those facts is by claiming that in a normal batch culture, *Clostridium acetobutylicum* produce hydrogen, carbon dioxide, acetate, and butyrate during the initial growth phase (acidogenic phase) which results in a decrease in the pH of the culture medium. As the culture enters the stationary growth phase, the metabolism of the cells undergoes a shift solvent production (solventogenic phase). Fond *et al.* (1984) found that residual glucose concentration is weak during the 50 first hours whereas the level of butyric acid reaches a maximum. They also succeed knowing that solvent and glucose concentration increase in the culture medium simultaneously to a partially reassimilation of the butyric acids. He also claimed that total glucose consumption and butanol formation are related to the butyric acid concentration.

In additon, the concentration of acetic acid is almost constant throughout the process. Based from metabolic pathway of *Clostridium acetobutylicum*, acetic acid will converted into acetone and ethanol whereby, butyric acid will convert into butanol and finally it can be conclude that, higher butyric acid at the acidogenic phase will produce higher butanol concentration in solventogenic phase.

2.4 Analytical Procedure

Recent studies by Harvey (2000) using analytical procedure to determine the product quantitatively or qualitatively using different analytical equipment based on the characteristics of analyte. Analytes defined as the constituents of interest in a sample. There are two different analyzing techniques which are determination of monosachharides in palm oil mill effluent (POME) using High Performance Liquid Chromatography (HPLC) and for butanol and ethanol percentage are qualitatively anayzed by using Gas Chromatography – Flame Ionization Detector (GC – FID)

2.4.1 High Performance Liquid Chromatography (HPLC)

Of all the chromatographic techniques whose mobile phase is a liquid, High Performance Liquid Chromatography (HPLC) is perhaps the best knowns (Rouessac & Rouessac, 2007). HPLC consists two different phases which are mobile phase and stationary phase. Stationary phase (SP) in contact with the mobile phase (MP) is the second medium with which the compounds initially dissolved in the mobile phase will interact. The heart of HPLC assays is the column selection. Different type of column will give different result based on the elution time. In addition, HPLC consists of two types of phase such as normal phase (polar stationary phase and non-polar solvent phase) and reverse phase (non-polar stationary phase and polar solvent). In this study normal phase is being used and carbohydrate column with RI detector is used to determine reducing sugars in POME.

2.4.1.1 Analyzing of reducing sugars in palm oil mill effluent (POME)

In determining the composition of reducing sugars in palm oil mill effluent (POME) using analytical equipment such as HPLC is important to study the phase of the analyte. POME is a liquid based analyte that suitable for HPLC assays since

Liew *et al*. (2006) reported that Gas Chromatography (GC) will only used to analyze solvents, whereas HPLC is used to analyze organic acids and reducing sugars. The HPLC assays technique is supported by Rha et al. (2002) which proved that POME could be run using HPLC to determine the other monosachharides using particular preparation method. The research shows that palm essence sample resulting various reducing sugars such as xylose, glucose and mannose quantitatively from calibration curve equation from external standard run. The results shows that xylose has the higher concentration 1.285 x 10^{-2} mg/ml followed by glucose 1.03 x 10-^{2} mg/ml and finally mannose is 8.5 x 10^{-4}. Ngan *et al.* (2004) decided to determine the components of POME by using two different methods which are Liquid Chromatography (LC) and Gas Chromatography – Flame Ionization Detector (GC – FID) and found that the main components are starch, glucose, fructose and glycerine in POME. In addition, since this study choose the Supelcosil LC-NH_2 column, it will separate monosaccharides, disaccharides, and some trisaccharides. Sugar retentation decreases as the proportion of water:acetonitrile in the mobile phase is increased. Sugars generally will be eluted in order of increasing molecular weight. Table 2.3 shows the retentation time index for carbohydrate columns by Sigma-Aldrich:

Table 2.3: Retentation time for different components of sugars to elute

No.	Components	Retentation Time / min
1	Arabinose	7.5
2	Galactose	10.3
3	Glucose	9.8
4	Xylose	6.8

2.4.2 Liquid-Liquid Extraction (LLE)

A liquid-liquid extraction is one of the most important separation techniques to separate two different solution. In a simple liquid-liquid extraction the solute is partitioned between two immicisble phases. In most case one of the phases is aqueous, and other phase is an organic solvent such as chloroform. Because the

phases are immisicble, they form two layers, with the denser phase on the bottom (Harvey, 2000). Liquid-Liquid Extraction (LLE) used as preparation for GC analysis which to eliminate the water content in the solution. Roffler *et al*, (1988) investigated that in batch extractive fermentation, an immisicible organic solvent is added directly to a batch culture of microorganism because butanol is toxic to the clostridia thus organic solvent used to extract butanol from the medium. In addition, the organic solvent being used are oleyl alcohol and benzyl benzoate which both are are strong extractant that can extract butanol in higher concentration (Roffler *et al*, 1988). But, differ from Chuichulcherm and Chutmanop (2000) result which they compared the organic solvent between oleyl alcohol, 2-ethyl-1-hexanol and palm oil methyl ester. The result showed that 2-ethyl-1-hexanol has the greatest potential in extraction of butanol because oleyl alcohol is much expensive while 2-ethyl-1-hexanol is more economic. In this study, toluene is used as organic solvent for LLE process which toluene is best known as universal solvent which applicable to extract most alcohol form immisicible solution. In addition, butanol, ethanol and tolune has lesser density (0.810 g/ml, 0.789 g/ml, 0.867 g/ml) than water (0.932 g/ml), thus water which more denser than solvent will be at the bottom.

2.4.2.1 Drying Agents

Even though Liquid-Liquid Extraction (LLE) could separates water and solvent, it does not mean there 100% being removed during the process. Some small amount of water will dissolve in the organic solvent. Drying agent used when an organic liquid has been exposed to water . Molecules that make hydrates have cavities in their molecular structure that will accommodate a certain number of water molecule. One of an example of drying agent is sodium sulphate (Na_2SO_4) which will be hydrated when been contact with water molecule. The water molecule will be clumped together and decanted after a few moment. Drying agent probabily used for the small volume of water content in organic solution. Figure 2.2 shows that the water molecule being “clumped” after mixed with drying agent.

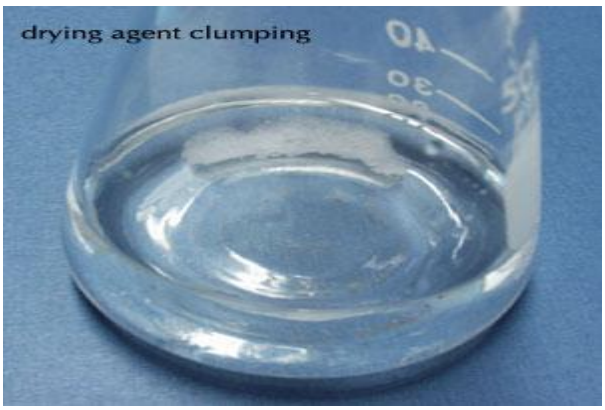

Figure 2.2: Drying Agent Clumping the water molecule and formed precipitate on the bottom of the beaker

2.4.3 Gas Chromatography – Flame Ionization Detector (GC – FID)

Gas Chromatography (GC) is a very common technique for both qualitative and quantitavie analysis. Samples are separated in GC base on differences in vapor pressure (boiling point) and interaction with a stationary phase. Samples are injected, vaporized and separated on a column that contains the stationary phase. The samples travel thorugh the column via an inert carrier gas. Depending upon their boiling points and relative affinities for the stationary phase, they move through the column at different rates and so ideally each analyte has its own retentation time, the time required to move through the column. While Flame Ionization Detector (FID) is suitable for volatile component such as alcohol. Table 2.4 shows boiling point of each analyte, ethanol, butanol, hexane, and toluene. Hexane is the solvent carrier used to carry analyte through the capillary collumn.

Table 2.4: Properties table for different solvent according to their boiling point

No	Solvent	Boiling Point / °C	Chemical Formula
1	Butanol	118	CH_3-CH_2-CH_2-CH_2-OH
2	Ethanol	79	CH_3-CH_2-OH
3	Hexane	69	CH_3-CH_2-CH_2-CH_2-CH_2-CH_3
4	Toluene	111	C_6H_5-CH_3

Recently, most of the researchers used GC analysis for butanol and ethanol determination. Kalil *et al.* (2003) showed that the concentration of ABE is measured by gas chromatography (Shidmazu 17-A) fitted with a flame ionization detector (FID) using capillary column BP1 with nitrogen as the carrier gas while the temperature is programmed at 40°C to 170°C. In addition, with similar equipment GC-FID, Chuichulcherm and Chutmanop (2000) claimed that they used (Shidmazu GC7AG) with temperature is programmed at 210°C to 300°C respectively. Moreover,acetone-butanol-ethanol and acids (butyric and acetic) could be analyzed using GC-FID (Hewlett-Packard, Avondale, PA) (Ezeji *et al.*, 2005). Finally, solvent can be analyzed using GC-FID (Agillent 6890) with temperature starting from 20°C to 270°C (Liew *et al.*, 2006). Although, solvent analyzed techniqued using similar equipment but, with different temperature setting will effect the retentation time for the peak of chromatogram to be eluted. Figure 2.3 shows GC-FID used in FKKSA Laboratory.

Figure 2.3: Gas Chromatography-Flame Ionization Detector (GC-FID)

2.4.3.1 Qualitative Analysis

Qualitative analysis defined as one approach to spike the sample by adding an aliquot of a suspected analyte and looking for an increase in peak height. Furthermore, retentation time also can be compared with values measured for standards, provided that the operating condition are indentical. This study more concern only on qualitative analysis since validation approached has been proven by standardization method which the greater the concentration of an alcohol, the larger its peak area, thus the greater the area percent (A%) of the correspond peak. The analysis is then supported by Pavia *et al.* (1999) explained that the quantity of a substance present is directly proportional to the area under the peak caused by the substance on the chromatogram so the percent composition can be approximated by comparing relative peak area. Below is the calculation of area percentage:

% Area of the component = {(Area under peak) / (total area)} / 100%

2.5 Fermentation Product: Butanol and Ethanol

During ABE fermentation there are three different product will be produce after fermentation process which are, acetone, butanol and ethanol. But only ethanol and butanol been compared after the GC analysis. Commonly both product will appeared after undergoes acidogenesis and solventogenesis phases.

2.5.1 Butanol

Nowadays, federal governments were concern to avoid overly dependent in oil and gas production, where it has been estimated that the wells will emptied in 10 to 20 years later. A new "green" fuel namely as butanol has been found as a new biofuel replacing ethanol. Butanol is a four carbon alcohol. It has double the amount of carbon of ethanol, which equates to a 25 percent increase in harvestable energy (Btu`s). Butanol is produced by fermentation, from corn, grass, leaves, agricultural waste and other biomass. Butanol is the one of first generation biofuel as known as biogasoline.

Butanol is a chemical which has excellent fuel characteristics. It has a higher calorific value than ethanol, and a low freezing point (Qureshi and Blaschek, 2000). Moreover, butanol is an alcohol that does not have to be blended with fossil fuels. The most important is butanol consumed in an internal combustion engine to held no carbon monoxide which is environmentally harmful byproducts of combustion, carbon dioxide (CO_2) is the combustion byproduct of butanol, and is considered environmentally 'green'

More or less, butanol is far less corrosive than ethanol and can be shipped and distributed through existing pipelines and filling stations. Butanol solves the safety problems associated with the infrastructure of the hydrogen supply. Reformed butanol has four more hydrogen atoms than ethanol, resulting in a higher energy output and is used as fuel. Butanol is a superior fuel to ethanol and an industrial

solvent that can be produced from renewable resources employing a number of organisms including *Clostridium acetobutylicum* and /or *Clostridium beijerinckii* (Qureshi and Blaschek, 2000).

2.5.2 Ethanol

Ethanol or ethyl alcohol is a clear, colorless liquid with a characteristic, agreeable odor and for ages applied as alcoholic beverages industry. Bioethanol is well established chemical in the world, hence most people in the foreign country preferred to use ethanol as for the fuel transportation. Bioethanol can be produced from renewable resources such as agricultural byproduct and undergoes microbial fermentation. Ethanol having 2 numbers of atoms in chemical structure makes ethanol less preferable than butanol which having 4 carbon atoms hence has high calorific values the bioethanol (Qureshi and Blaschek, 2000). In addition, ethanol has its own advantages which ethanol does burn cleaner than gasoline. This is proven when car user used 85/15 percent blend of ethanol to gasoline, do create fewer toxic emissions. Furthermore, by lowering the amount of greenhouse gases and ozone created by car exhaust, the use of ethanol is believed to be much better alternative to gasoline.

Ethanol has its disadvantages which are ethanol could be too corrosive because ethanol can absorb water and dirt very easily, and if those contaminants are not filtered out successfully, they can cause damage and corrosion inside the engine block. Moreover, ethanol seems not fulfill the characteristics of gasoline, which means car users need more ethanol to drive the same distance, and ethanol prices are expected to be higher than gasoline prices when it is implemented on a national scale.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter was about method being used to study the effect of inoculum concentration on butanol and ethanol production. The method begun with palm oil mill effluent (POME) analysis which to determine the quantity of monosaccharide found in batch fresh POME by using High Performance Liquid Chromatography (HPLC). Next, the method continued with cultivation of bacteria, fermentation process and finally butanol, ethanol and glucose consumption analysis by using Gas Chromatography-Flame Ionization Detector (GC-FID) and Ultraviolet-Visible Spectrophotometer (UV-VIS). Figure 3.1 showed the overview of the overall process of butanol and ethanol production.

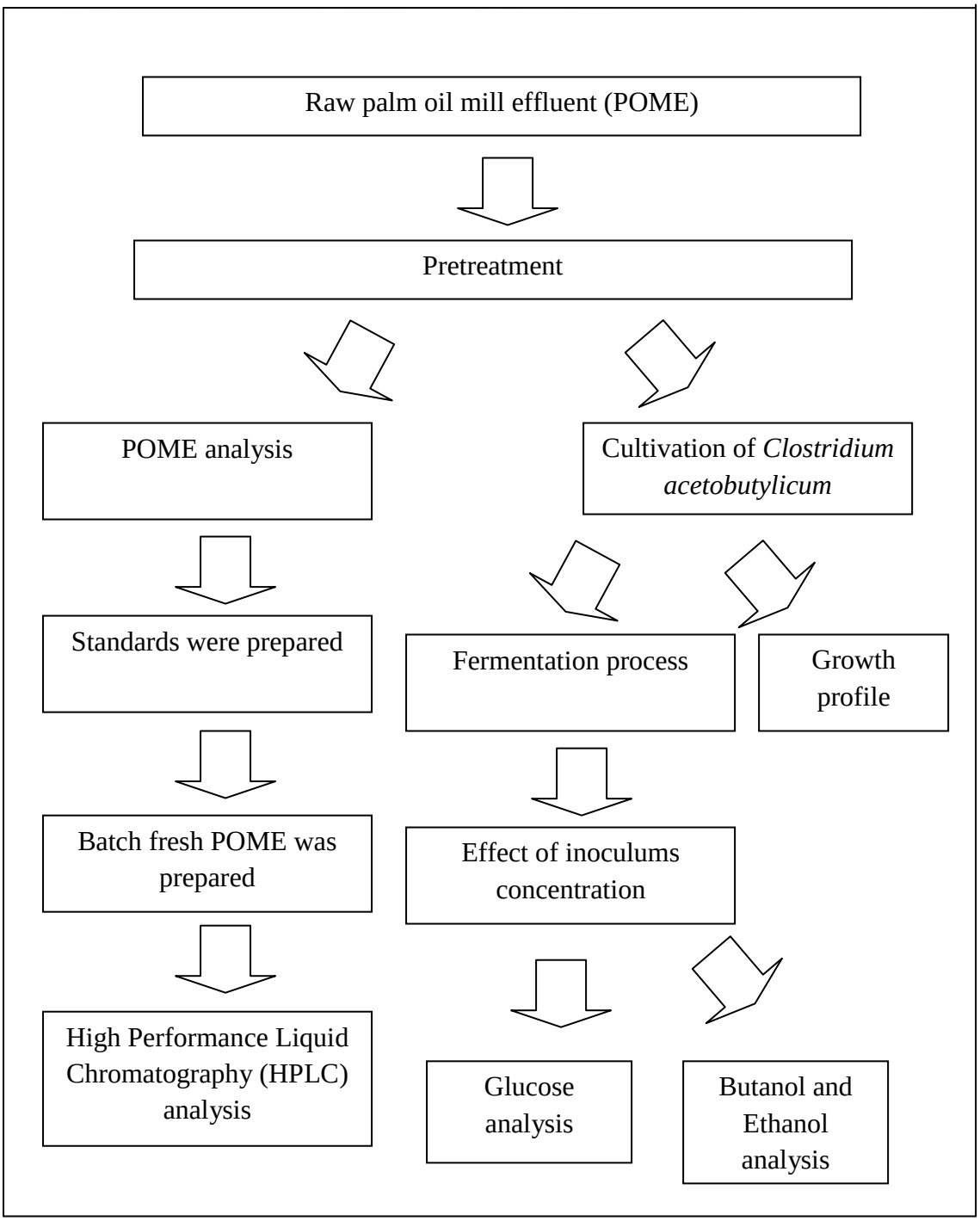

Figure 3.1: Overview of the overall process on butanol and ethanol production

3.2 Materials

Palm oil mill effluent (POME) obtained from Palm Oil Mill Factory Lepar Hilir, Pahang. *Clostridium acetobutylicum* preserved in glycerol stock was obtained from University Kebangsaan Malaysia (UKM), Bangi, Selangor. Sodium hydroxide (NaOH) was from Sigma. Chromatographic standards for HPLC such as glucose (99% purity), xylose (99% purity), arabinose (99% purity) and galactose (99% purity) were come from Sigma. Chromatographic standards for GC-FID such as hexane (99% purity), ethanol (99% purity), butanol and toluene were purchased from Sigma. Another material being used was Reinforced Clostridia Media (RCM) broth and Reinforced Clostridia Media (RCM) agar which were weighed at 38 gram and 52.5 gram using analytical balance. Then, RCM agar and RCM broth were autoclaved at 121°C for 15 minutes. Sodium sulphate (Na_2SO_4) was came from Sigma similar with acetonitrile was purchased from same chemical company. Ultrapure water, distilled water and nitrogen gas were provided in Faculty of Chemical Engineering and Natural Resources (FKKSA) laboratory. 70% ethanol also purchased from Sigma. Moreover, Dinitroalicyclic reagent (DNS reagent) was prepared using standard methods.

3.3 Equipment and apparatus

Equipment used such as refrigerated centrifuge, clamp incubator shaker, anaerobic chamber, laminar flow cabinet, chiller at 4°C, refrigerator at -20°C, UV-Vis Spectrophotometer, water bath at 90°C, autoclave, hot plate, HPLC, GC-FID, analytical balance, and pH meter were provided in FKKSA laboratory. Furthermore, apparatus used during experimental procedure were sample bottles, beakers, separator funnels, stirrer, spatula, syringe filter (0.20 µm), scotch bottles (250 mL and 500 mL), aluminum foils, petri dish, Bunsen burner, sterilized tooth picks, cuvette, sterilized syringe, modified bottle (will be discussed later), parafilm, measuring cylinder, test tubes, test tubes rack, micropipette, volumetric flask,

centrifuge bottle, conical flask, funnels, 0.45µm filter and retort stands borrowed from FKKSA laboratory.

3.3.1 Fermentation Process: Modified Bottle

Modified bottle referred to the fermentation process where strictly needed to use modified bottle to preserve the anaerobic condition. Modified bottle required two 500 mL of scotch bottles for RCM and POME medium, metal clips or binder clips, silicone tubes, stainless steel rod cut into 3 cm, and driller. Only the bottles capped were being modified, which two stainless steel rod that had been cut into 3cm were placed on the top of the bottle capped for each inlet and outlet. Drilling process was carefully done, so the rod will compatible with the hole which had been drilled earlier. Next, the silicone tubes were used for inlet and outlet, where for the inlet, the length of the tubes has to be longer going down to the bottom of the bottles because nitrogen gas has to be flow through the medium during purged with the nitrogen gas for preparation of anaerobic condition. Furthermore, metal clips or book binders were used to clip the outsides silicone as to avoid any air came into the bottles. Finally, as to make sure the rods were stacked onto the capped, silicone gum was used for further precaution. Figure 3.2 shows the sketch of modified bottle prepared for anaerobic fermentation. Since, nitrogen gas available at FKKSA laboratory was not sterilized, syringe filter (0.20 µm) used for both inlet and outlet exits.

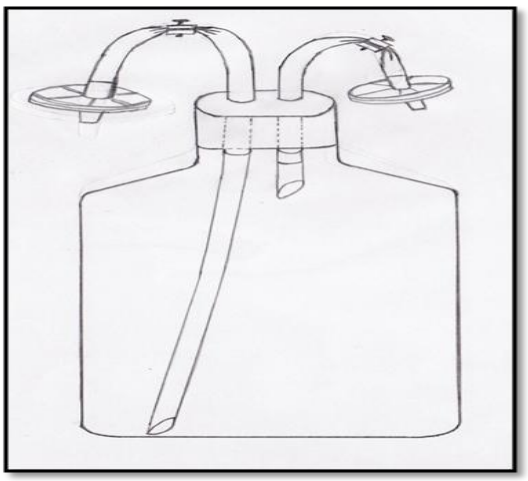

Figure 3.2: A sketch of complete modified bottle

3.4 Experimental Procedure

3.4.1 Palm oil mill effluent (POME) Analysis

3.4.1.1 Preparations of Standards

Standards used in POME analysis; glucose, xylose, arabinose, and galactose had been prepared at concentration of 10 g/L, 20 g/L, 30 g/L 40 g/L and 50 g/L respectively. In order to prepare those standards, a stock solution containing 50 g/L of each standard must be prepared carefully. 5 gram of each standard was weighed using analytical balance accurately. Then, after weighed four different standards, each of them had to be diluted with ultrapure water. Ultrapure water used since, HPLC required particular diluents for the analysis. The solution had to be mixed thoroughly using stirrer till all the particles were dissolved. Then each 2 mL, 4 mL, 6 mL, and 8 mL were taken out using 100 µL micropipette from stock solution to prepared 10 g/L, 20 g/L, 30 g/L and 50 g/L and diluted with different 10 mL volumetric flask and finally ultrapure water was top upped with the solution and mixed thoroughly. Each standard had to be filtered using 0.45 µm filter before transferred into HPLC vials. Finally, each standard was then transferred into HPLC vials till 1µL for calibration glucose, xylose, galactose and arabinose determination.

Figure 3.3: High Performance Liquid Chromatography (HPLC) used for POME analysis

3.4.1.2 Preparation of palm oil mill effluent (POME)

Since, palm oil mill effluent (POME) contains a lot of particles that could be clogged the column of HPLC, hence, a pretreatment process needed to be taken. A batch fresh POME were taken out from chiller at 4°C then 35 mL of POME was transferred into centrifuged bottle, and refrigerated centrifuge was set 10 000 rpm for 30 minutes. After that, supernatant was decanted and debris was removed from the centrifuge bottle. Next, supernatant containing small amount of other particles were then undergoes gravitational filtration using filter paper. Figure 3.5 shows that 30 hours filtration process using funnels and conical flasks.

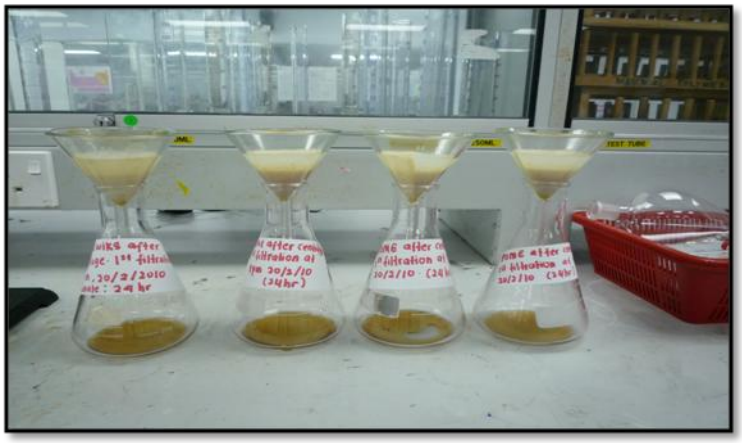

Figure 3.4: Gravity filtration process after POME was centrifuged

Finally, preparation for POME analysis ended when filtrate after gravity filtration was then filtered again using 0.45 µm syringe filter. Liquid POME that had been passed several filtration processes was then transferred into HPLC vials without diluted with ultrapure water.

3.4.1.3 High Performance Liquid Chromatography (HPLC) analysis

POME analysis finally entered the final stage of monosaccharide analysis. This analysis required standards and sample run together to get better result. Specification of properties of HPLC were, column used was SUPELCOSIL LC-NH2, 25 cm x 4.6 mm, detector used was RI detector, flow rate was at 2 mL/min, injection was 10-100 µL, 150 µg each sugar, ambient temperature and mobile phase used was acetonitrile:water (75:25). Analyte that suitable were sugars. Analysis ended after determination of calibration curve for external standards and sample chromatograms. Figure 3.5 below shows the summary of overall process in POME analysis using HPLC.

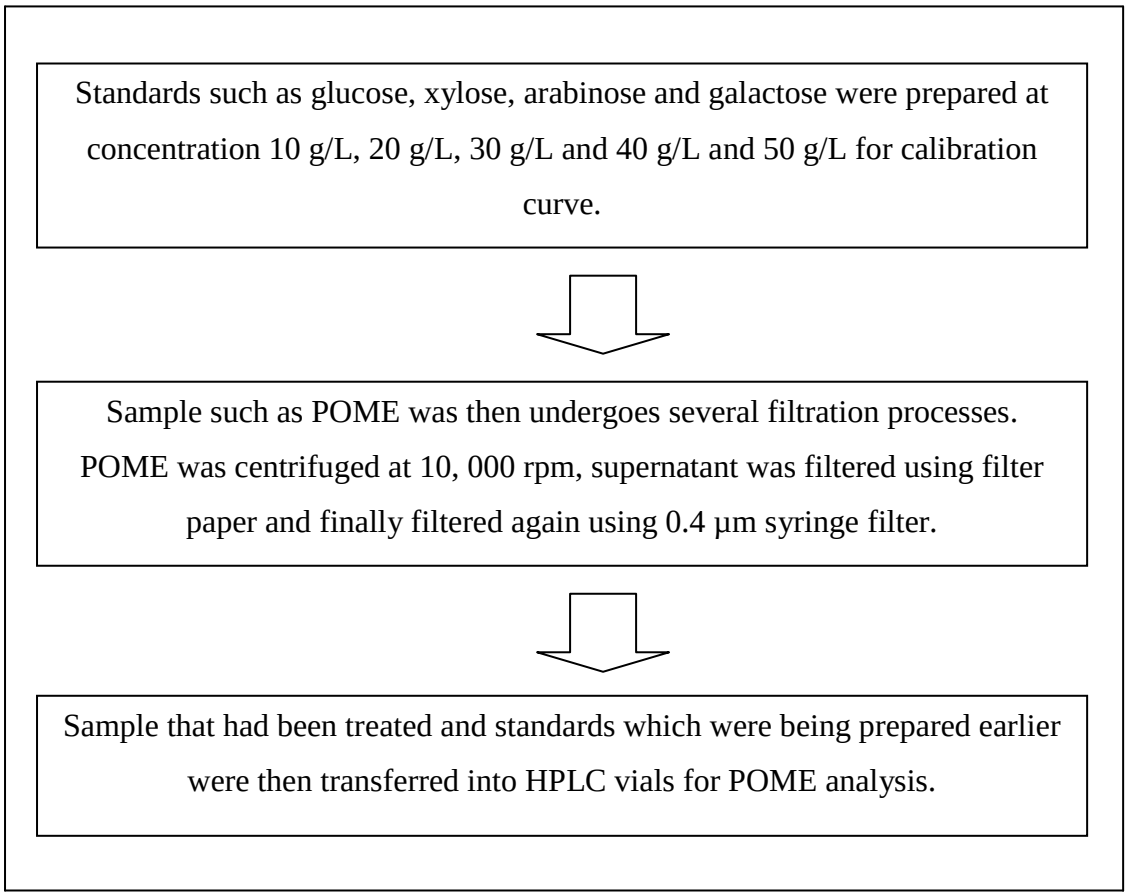

Figure 3.5: Summary of flow methodology for POME analysis using HPLC

3.4.2 Bacterial Cultivation

All procedures must have done in laminar flow cabinet with septic technique to avoid any contamination. Besides that all apparatus that have been contacted with the biological worked must be sterilized before used especially measuring cylinder used to transfer RCM broth and tooth picks used for inoculating procedure. RCM broth was then seeded with *Clostridium acetobutylicum* in the anaerobic chamber at open flask from glycerol stock. Inoculated medium was incubated in the anaerobic chamber for 24 to 30 hours at temperature 37°C. Besides that, RCM agar that has been prepared earlier was used to pour onto petri dish for sub-cultured technique. After incubation process, petri dish was inoculated using sterilized tooth picks and was incubated in the anaerobic chamber for 48 hours.

3.4.2.1 Inoculum Preparation

After plate media was incubated, and obtained a single colony which have similar color and size compared to other inoculated plate media, a new sterilized tooth pick was used to inoculate a single colony from the plate media into the RCM broth for inoculums preparation. Most of the works were done in anaerobic chamber at open flask. The liquid medium that has been seeded with single colony was then incubating in anaerobic chamber for 18 hours. The growth of culture in RCM medium was monitored by measuring and OD at 680 nm using UV-Vis Spectrophotometer. Only OD approximately 0.7 was used as inoculum. Figure 3.6 showed the summary of flow methodology for bacteria cultivation and inoculums concentration.

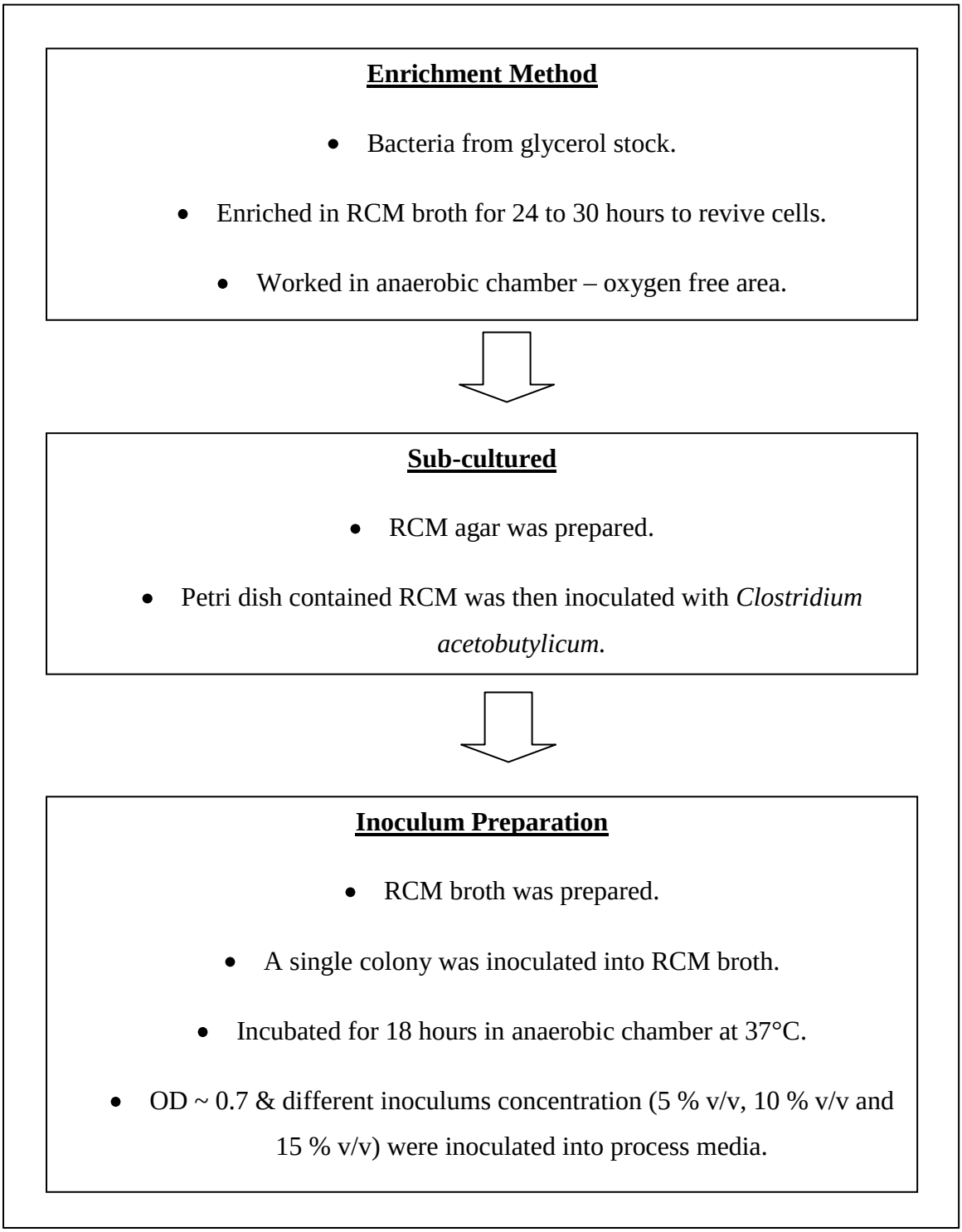

Figure 3.6: Summary of flow methodology for bacterial cultivation

3.4.3 Preparation of Fermentation Media

Fresh POME was sedimented in the chiller at 4°C for 24 hours while RCM broth was weighed at 38 gram using analytical balance and diluted with 1 liter distilled water. The supernatant layer (upper part) was decanted while POME sludge (lower part) was then diluted with distilled water to obtained desired concentration. Initial pH was increased by added sodium hydroxide (NaOH) to 5.8. Both medium were sterilized at 121°C for 15 minutes. After that, both medium were cooled to the room temperature. Then, nitrogen gas was purged for 10 minutes for each medium to provide anaerobic condition for *Clostridium acetobutylicum*. Both medium, POME and RCM broth were seeded with inoculums that had been prepared earlier in the anaerobic chamber. After that, both medium were clipped using metal clip before transferred out from the anaerobic chamber. Both medium were incubated in clamp incubator shaker at optimal condition, 200 rpm, 35°C, 90 % POME, 10 % v/v, and fermentation time was 60 hours. Figure 3.7 showed the summary of flow methodology for fermentation process.

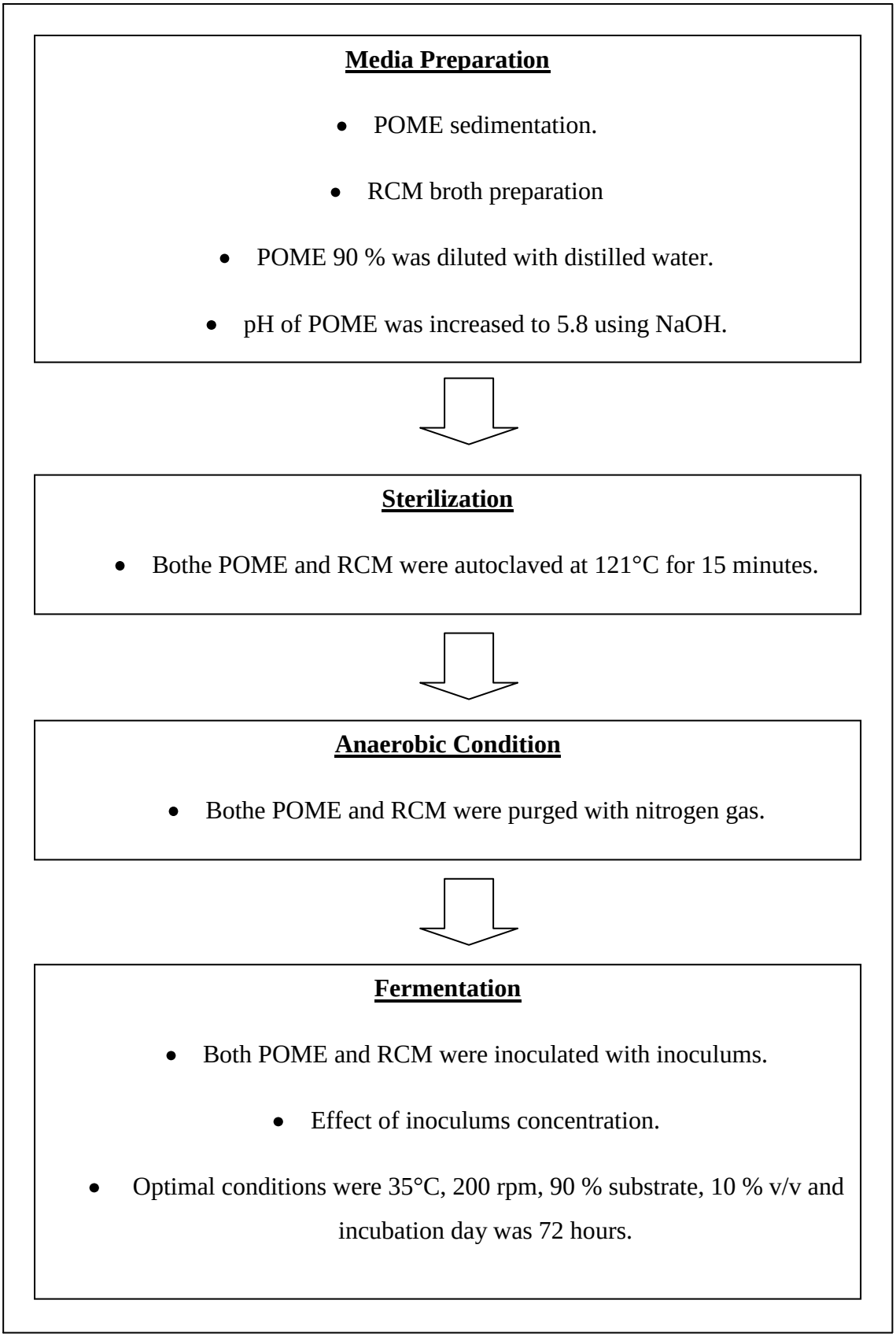

Figure 3.7: Summary of flow methodology for fermentation process

3.4.4 Effect of Inoculum Concentration

Effect of inoculums concentration was done by manipulating the percentage volume per volume of inoculums. This study only covered three inoculums concentration which were 5 % v/v, 10 % v/v and 15 % v/v which then inoculated into both fermentation media.

3.4.5 Growth Profile

Growth profile of *Clostridium acetobutylicum* was done by prepared the inoculums in RCM broth. Different time interval for each 6, 12, 18, 24, 30, 36, 42 and finally was 48 hours. This method was done by measuring the Optical Density (OD) of the inoculums for each time interval by using wavelength at 680 nm examined using UV-Vis. Finally OD obtained used to construct growth profile by plotting Optical Density (OD) versus Time (hours).

3.4.6 Analysis Procedure

Fermentation broths for both POME and RCM were taken for each 20, 40, 60 and 72 hours for butanol, ethanol and glucose analysis. Glucose assay was done by examined the concentration of glucose before and after fermentation using DNS method. Fermentation broths were centrifuged using refrigerated centrifuge for 30 minutes at 10,000 rpm. The supernatant were then used for butanol, ethanol and glucose analysis.

3.4.6.1 Liquid-Liquid Extraction

Supernatant obtained was used for liquid-liquid extraction and the volume of supernatant used for each different inoculums concentration time interval must be constant. The ratio between solvent extracting (toluene) and sample was 1:1, and both of them were mixed in separator funnel. Separator funnel needed to be shaking for the solvent extracting could mix with the samples. The top of the separator funnel needed to be opened after shaking, because to release pressure. Finally placed the separator funnel using retort stand and leaved it for 24 hours. Figure 3.8 shows the supernatant from POME and RCM in the separator funnel after mixed with the toluene.

Figure 3.8: Palm oil mill effluent (POME) (left) and Reinforced Clostridia Media (RCM) (right) after mixed with toluene (solvent extracting)

After 24 hours, the bottom liquid at the separator funnel was decanted carefully, and the upper layer was added with the sodium sulphate (Na_2SO_4) and stored in screwed capped bottle and leave both samples for 24 hours in the chiller at 4°C.

3.4.6.2 Butanol and Ethanol Analysis

Butanol and ethanol (99% purity) standards were prepared by using method of 1% sample. 10µL samples which are butanol, ethanol and toluene were taken using 10-100µL micropipette and moved in different beakers. Then, 990 µL hexane with purity was 99.9% GC grade was taken using 100-1000 µL micropipette and mixed with each samples. Each sample then mixed thoroughly and filtered using syringe filter (0.2 µm). After that, the mixture was transferred into GC vials. Samples were then proceeding with GC-FID analysis. Specification of parameter used in GC-FID must be similar for standards run and samples analysis. The specification was Gas Chromatography (GC) installed with Flame Ionization Detector (FID) and HP-5 capillary column. (30 m x0.25 mm, 0.25 µm); carrier gas was helium at 33 cm/sec and 1.5 mL/min at constant flow; temperature limit at 60°C to 325°C (350°C); detector was FID at 280°C and sample injected was 1 µL. Figure 3.9 shows the GC-FID vials used for butanol and ethanol analysis.

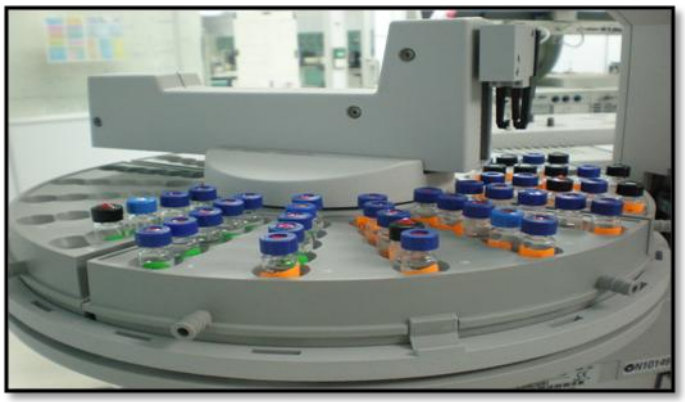

Figure 3.9: GC-FID vials used for preparation standards and butanol/ethanol analysis

After both samples were stored with Na_2SO_4, they used for preparation in GC-FID analysis to determine butanol and ethanol production qualitatively. 10 µL samples were mixed with hexane 990 µl and filtered using syringe filter (0.2µm) and proceed with GC-FID analysis for butanol and ethanol production. Figure 3.10 shows the POME and RCM samples after fermentation and pretreated using LLE and stored with sodium hydroxide which ready for GC-FID analysis.

Figure 3.10: POME and RCM samples after liquid-liquid extraction, stored with sodium hydroxide and ready for GC-FID analysis

3.4.6.3 Glucose Assay

Glucose calibration curve was determined using DNS method at different concentration by using commercial D-Glucose (99% purity). A 50 mg commercial D-Glucose was weighed using analytical balance and diluted with the 50 mL of distilled water to obtain 1 mg/mL as a stock solution. Then, different glucose concentrations were prepared at 0.0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, and 0.8 mg/mL were taken from the prepared stock solution. After that, 3 mL of each concentration was then mixed with 3 mL of DNS reagent by using 100-1000µL micropipette for accurate measurement in the test tube.

The mixture of sample and DNS reagent were then swirled and heated up at 90°C using water bath without shaking for 5 to 15 minutes. For precaution step, the test tube containing solution needed to be covered using aluminum foil to avoid any contamination. During heating using water bath, the lid had to be closed as to maintain temperature of water bath at 90°C. After 5 to 15 minutes, the test tube was then removed from water bath and cooled to ambient temperature at test tube rack. The physical changes needed to be observed and optical density (OD) was measured using UV-Vis at wavelength 540 nm. The OD for each concentration must be

recorded and calibration curve Optical Density (OD) versus Glucose Concentration (mg/mL) could be plotted. Figure 3.11 shows the type of UV-Vis used for OD determination.

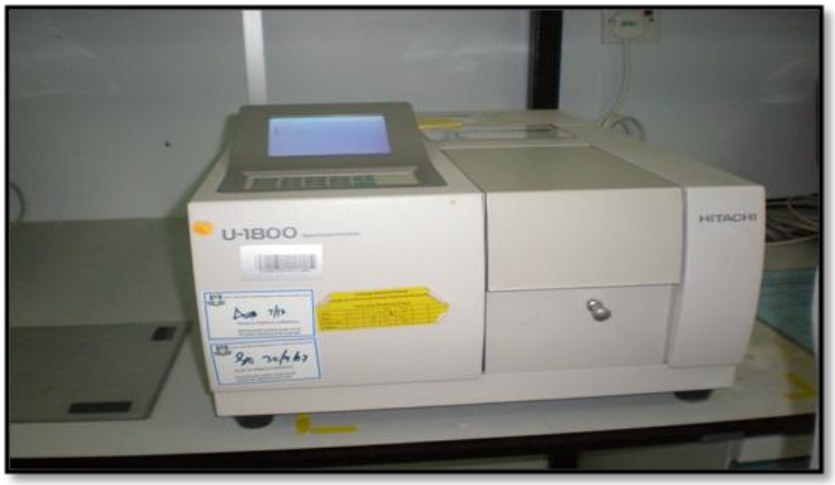

Figure 3.11: Ultraviolet-Visible Spectrophotometer (UV-Vis)

After determined the glucose calibration, the balance from supernatant before liquid-liquid extraction for both samples POME and RCM were used in glucose assay using DNS method. 3 mL of each sample from different time interval 0, 20, 40, 60 and 72 hours for both RCM and POME were mixed with 3 mL DNS reagent Before heating process, the dilution factor for POME was DF = 10 means 1:10, while RCM does not required any dilution. Next, mixed solutions were heated at temperature 90°C in the water bath for 5-15 minutes. After that, samples that had been reacted were cooled to the room temperature and examined their optical density (OD) using UV-Vis to determine their glucose concentration based from glucose calibration curve. Figure 3.12 showed a summary of butanol, ethanol analysis and glucose assays.

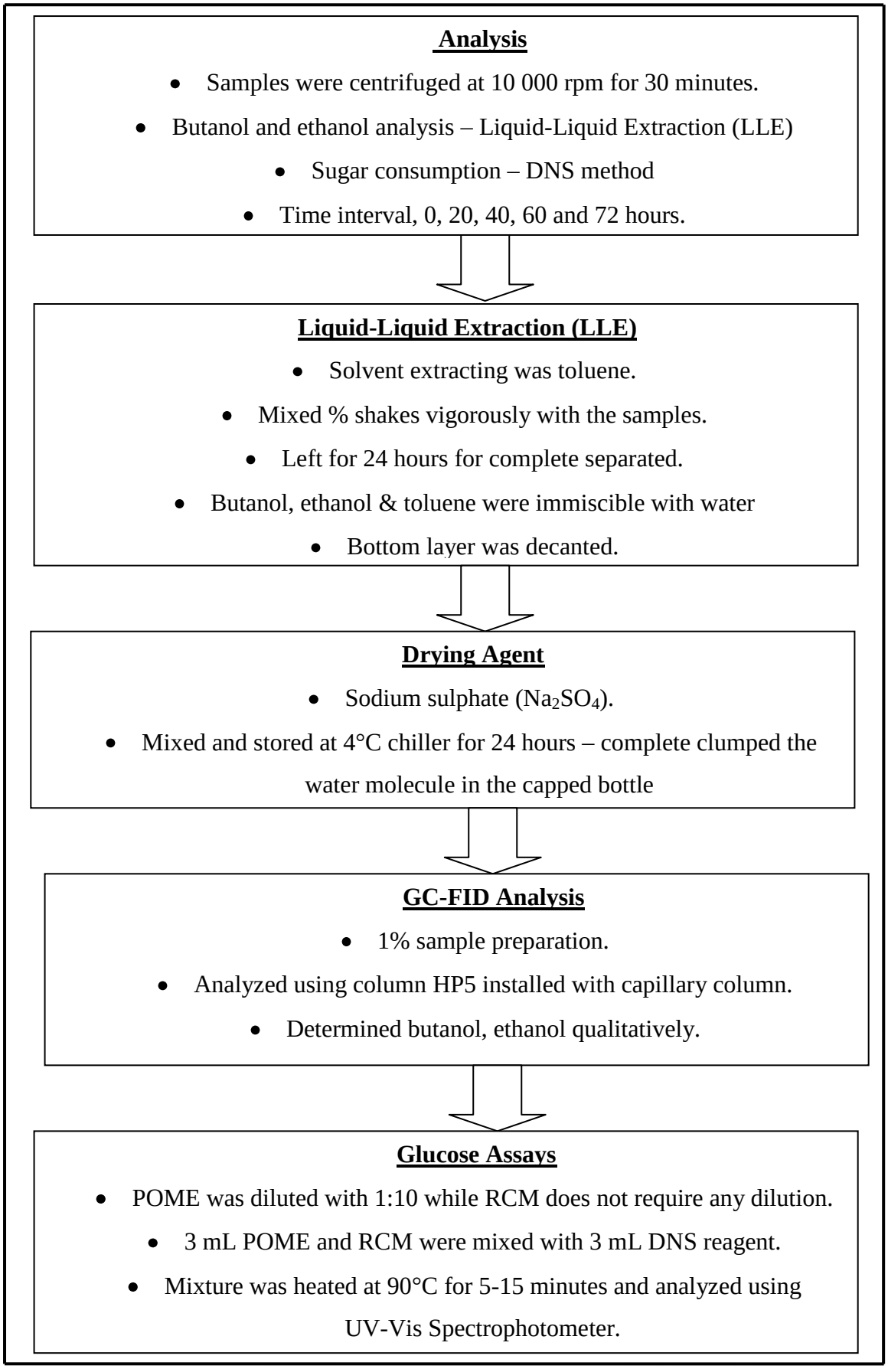

Figure 3.12: Summary of butanol, ethanol analysis and glucose assays

CHAPTER 4

RESULT AND DISCUSSION

4.1 Composition of palm oil mill effluent (POME)

Table 4.1: Result of sugars obtained after separation using HPLC

Monosaccharide	Time of elution	Concentration (g/L)	Molecular Weight (g/mol)
Glucose	7.751	3.842	180.16
Galactose	8.250	11.3847	180.16
Xylose	5.961	2.5524	150.13
Arabinose	6.235	3.0349	150.14

From the Table 4.1, the main component in palm oil mill effluent (POME) was galactose because it has the highest concentration among others. Galactose concentration was 11.3847 g/L followed with glucose at 3.842 g/L, thirdly was arabinose at 3.0349 g/L and finally was xylose at 2.5524 g/L. The concentration of each sugar was determined from calibration curve using equation obtained. (Appendix C). The most important factor was operating condition of samples must be similar to the standards thus, the result could be used to compare between standard and samples (Harvey, 2000).

The SUPELCOSEL LC-NH2 separated the monosaccharide in POME based on their molecular weight. Monosaccharide with higher molecular weight will elute later than monosaccharide with lower molecular weight. Based from Table 4.1, xylose was eluted first followed by arabinose, glucose and galactose.

Xylose > Arabinose > Glucose > Galactose

$\longrightarrow$

According to the increase of molecular weight and elution time

The composition of palm oil mill effluent (POME) obtained from Lepar Hilir, Pahang consists of galactose, glucose, arabinose and xylose. Rha *et al.* (2002) also proved that POME contains several reducing sugars such as glucose, galactose and mannose. Furthermore, POME was an agricultural byproduct which contained high concentration of lignocellulose which suitable for *Clostridium acetobutylicum* to grow (Kalil *et al.*, 2003). Lignocellulose was an abundance component in renewable resources which hydrolyzed into lignin, hemicellulose and celluose. Hemicellulose contained arabinose, xylose and galactose while cellulose contained glucose which then made POME consists of various of educing sugars (Jones and Woods, 1986). Ngan *et al.* (2004) also showed that from their studies, the main component of POME were starch, glucose, fructose and glycerine.

Even though POME analysis showed that those sugars were presence in the batch fresh POME from Lepar Hilir,Pahang, but (Rha *et al.*, 2002) examined that xylose was the highest abundance in sugar composition in POME followed by glucose . Based on Table 4.1, showed that xylose was the lowest concentration in POME this might due to the separation process on xylose within POME during HPLC analysis. Xylose chromatogram was not very well separated using SUPELCOSIL LC-NH2 and by comparing to the previous study, they used Reverse Phase (RP) C-18 in determining composition in POME (Rha *et al*, 2002). However, the peak was not well separated, but the method was feasible because, different column will give different performance of separation in HPLC.

4.2 Growth Profile

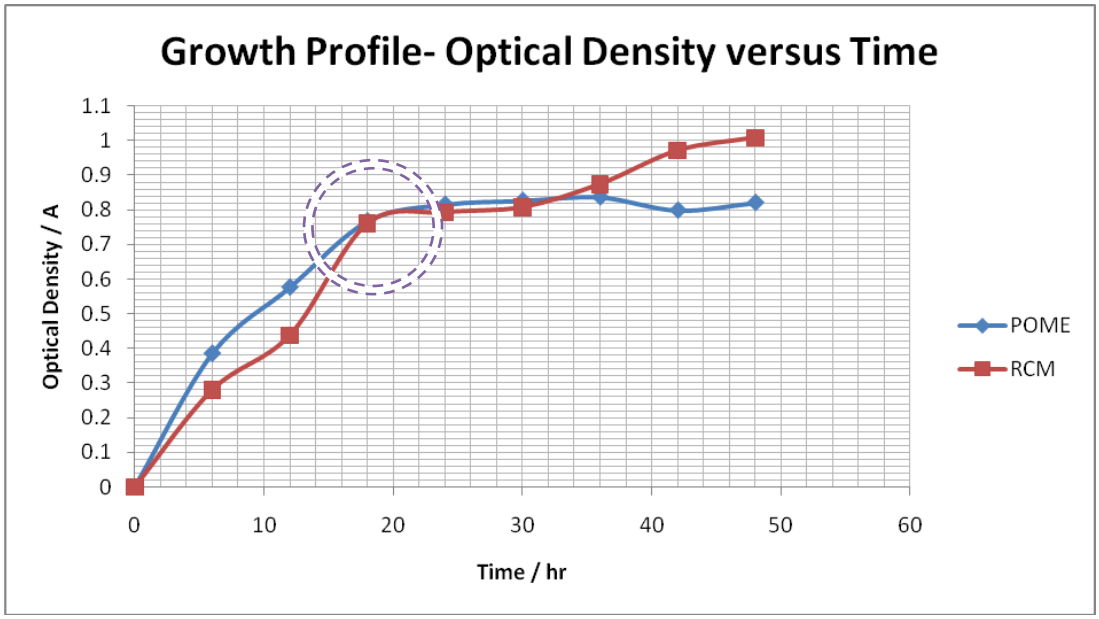

Figure 4.1: Growth Profile of *Clostridium acetobutylicum*

Population of bacteria does not maintain its potential growth rate and does not double endlessly. Quantitative laboratory studies indicate that a population typically displays a predictable pattern, or growth curve, over time. Figure 4.1 shows the growth curve of *Clostridium acetobutylicum* in different fermentation medium. Red line shows the curve of bacteria in Reinforced Clostridia Media (RCM) has a similar pattern with the Blue line indicates growth curve of bacteria in palm oil mill effluent (POME).

From the graph (Figure 4.1), it showed that at 0 to 6 hours illustrated that *Clostridium acetobutylicum* was at lag phase or relatively "flat" period. The period happen when, newly inoculated cells required a period of adjustment, enlargement and synthesis. POME growth curve shows at the first lag phase was slightly higher than RCM growth curve, this was because, clostridia at the vegetative cell was rapidly dividing themselves and quickly adapted into a new environment. The length of lag phase was varies for different culture. Clostridia shows better performance for both medium when their length of lag period much shorter and rapidly entered exponential phase.

Investigation on exponential phase entered between time interval was at 6 to 18 hours demonstrated that, the cells reach the maximum rate of cell division during the exponential growth. This phase will continue as long as cells have adequate nutrients and the environment was favorable (Cowan and Talaro, 2006). Performance of clostridia seems supported the previous dicussion because, from figure 4.1 showed that within the time interval of 6 to 18 hours, the curve increased rapidly for both growth rate in POME and RCM medium. This explained that, the maximum growth in RCM and POME medium was at after 18 hours with optical density (OD) was approximately 0.7. (Shown in the purple circle). The physical changes showed, the medium in RCM was cloudy but physical changes in POME cannot be seen. Based from the Figure 4.1, the maximum growth of *Clostridium acetobutylicum* was at OD 0.7, thus it was suitable used for inoculum in fermentation media since more clostridia cells were produced at OD 0.7. Both studies by Kalil *et al.* (2003) and Takriff *et al.* (2009) were also used inoculum with OD was 0.7 after 18 hours of incubation period

Similar to basic growth curve of others microbes, clostridia growth curve for both POME and RCM will entered stationery phase. The population entered survival mode in which cells stop growing or grow slowly. After 18 to 30 hours time interval, clostridia growth curve was decreased gradually. But after 30 hours to 72 hours, curve of both medium were gave different result. After stationary phase, POME clostrida growth curve seem became slower explained that, due to the use of glucose derived from POME instead of the whole POME as the medium (Kalil *et al.,* 2003).

4.3. Comparison of glucose consumption between 5 % v/v, 10 % v/v and 15 % v/v and efficiency of each inoculums size in POME

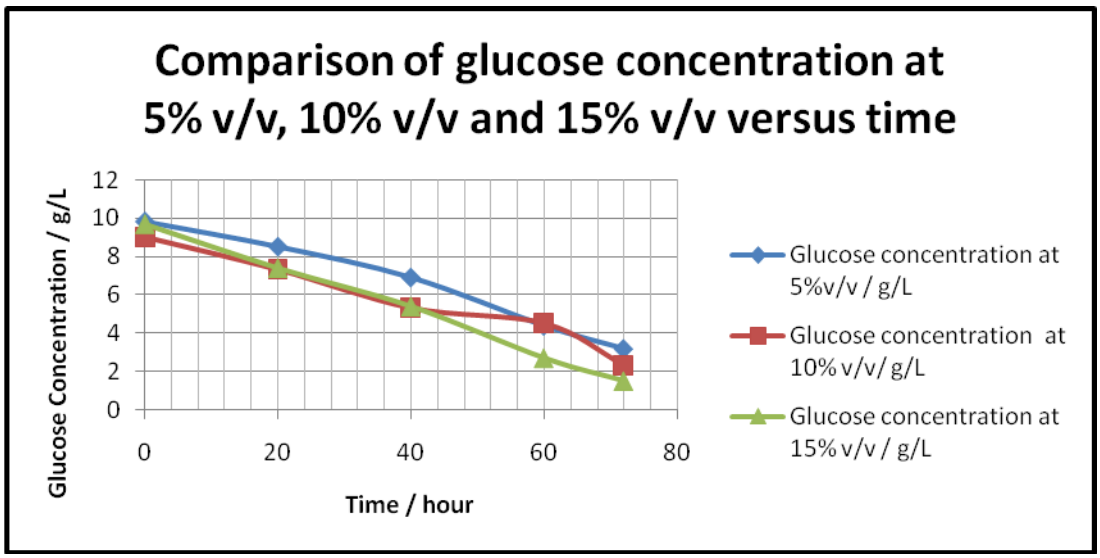

Figure 4.2: Graph of comparison of glucose concentration for 5 % v/v, 10 % v/v and 15 % v/v of inoculum concentration versus time in POME medium

Figure 4.2 (shown in blue line), showed that glucose concentration at 5 % v/v in POME was utilized by *Clostridium acetobutylicum* since there was a declined curve in the graph. Palm oil mill effluent (POME) consists of several reducing sugars such as galactose, glucose, arabinose and xylose that can be fully utilized by *Clostridium acetobutylicum* (Kalil *et al.*, 2003 and Jones and Woods, 1986). *Clostridium acetobutylicum* was a unique strain that could utilized variety of strachy substrate (Jones and Woods, 1986). The declining of glucose concentration in POME showed that *Clostridium acetobutylicum* has consumed glucose and other reducing sugars in POME. Furthermore, Figure 4.2 showed that the degree of declined in glucose concentration at 5 % v/v was the least compared to 10% v/v and 15% v/v. The percentage of glucose consumption in POME for each time interval were 13.27%, 29.59%, 55.10% and 67.35 % and when compared with percentage glucose consumption with 10 % v/v and 15 % v/v in POME medium, the glucose consumption at 5 % v/v inoculum concentration was the least consumed of glucose by *Clostridium acetobutylicum*. This was because, inoculum concentration at 5 % v/v consists smaller volume of cell concentration to utilize the glucose and other reducing sugars.

Figure 4.2 (shown in red line) illustrated the glucose consumption at 10% v/v of inoculum concentration with the degree of decreased was much higher than glucose consumption at 5% v/v of inoculum concentration. This was because of size of inoculum increased thus increased the quantitiy of *Clostridium acetobutylicum.* Sharma *et al.,* (2007) reported that glucose consumption in fermentation media was linearly increased as the cell concentration was increased. In addition, time required for glucose and other reducing sugars accomplished was faster at 10 % v/v compared to 5 % v/v inoculum concentration. Cheng *et al.* (2007) also claimed that increased the inoculum concentration affected the fermentation time to be completed earlier. The percentage of glucose consumption were given as 18.89%, 41.11%, 50.00% and 74.44% and this explained the degree of declined happen in 10% v/v was much faster decreasing, because more *Clostridium acetobutylicum* cells could utilized sugars in POME respectively.

Finally, Figure 4.2 (shown in green line) showed the glucose concentration was rapidly declined as the inoculum concentration increased to 15% v/v. Sharma *et al.* (2007) reported that the amount of sugar consumed was increased with increased of cell conncentration. Even though, different medium used (POME and RCM), but as long as there was glucose available, thus there was a possibility for glucose concentration consumed by *Clostridium acetobutylicum* and converted it into a renewable energy (Qureshi and Blaschek, 2000). The percentage of glucose consumption at 15 % v/v inoculum concentration, 23.47%, 44.43%,71.43% and 84.54% showed that the total glucose consumption in POME medium was higher than 5% and 10% thus explained the rapid declined in glucose concentration because of the cultivation of clostridia cell was increased in POME and illustrated that POME was suitable as an alternative medium for *Clostridium acetobutylicum* to grow (Ejezi *et al.,* 2005; Kalil *et al.,* 2003 and Takriff *et al.,* 2009).

Table 4.2: Efficiency of total glucose consumption in POME at 5 % v/v, 10 % v/v and 15 % v/v inoculum concentration

Inoculum Concentration / % v/v	**Efficiency / %**	**Total Glucose Consumption / g/L**
5	13.47	67.35
10	7.44	74.44
15	5.63	84.54

Table 4.2 showed the efficiency of total glucose consumption in POME for each inoculum concentration. The efficiency was decreased as the inoculum concentration increased from 5 % v/v to 15 % v/v inoculum concentration. The lowest inoculum concentration at 5 % v/v showed the most efficient in total glucose consumption compared to 10 % v/v and 15 % v/v inoculum concentration. Cheng *et al.* (2007) reported that glucose was the growth limiting factor which made up the efficiency of 5 % v/v inoculum concentration was the most competent to utilize glucose in POME medium compared to 10 % v/v and 15 % v/v. The higher inoculum concentration showed the lowest efficiency in utilized the glucose because, the quantity of glucose available in POME was fixed while the cell concentration was increased, thus made the glucose consumption in 15 % v/v inoculum concentration not competent compared to 5 % v/v inoculum concentration.

4.4 Comparison of glucose consumption between 5% v/v, 10% v/v and 15% v/v and efficiency of each inoculums size in RCM

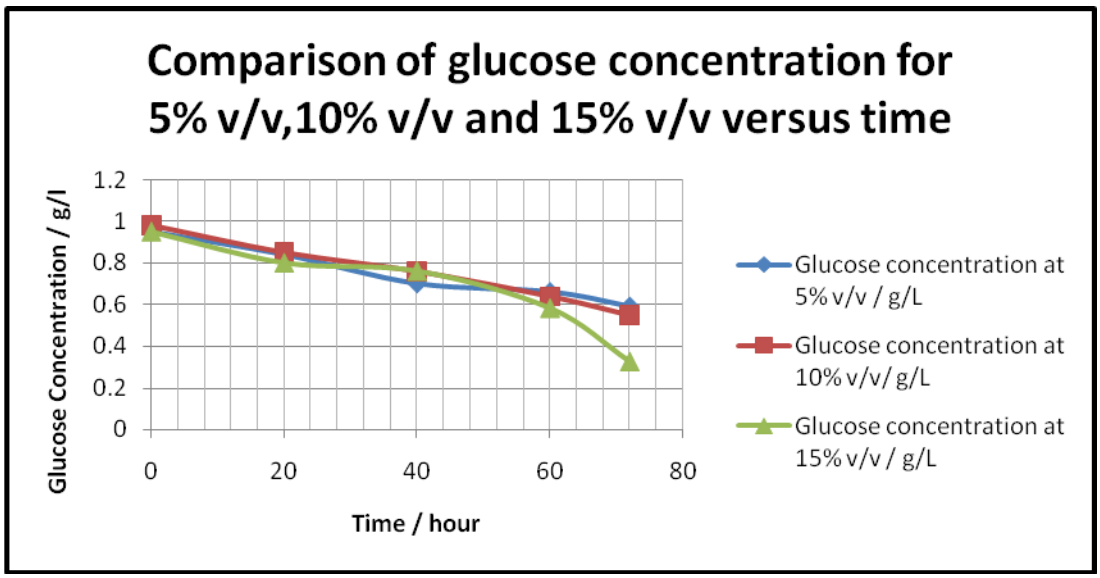

Figure 4.3: Graph of comparison of glucose concentration for 5 % v/v, 10 % v/v and 15 % v/v of inoculum concentration versus time in RCM medium

Figure 4.3 (shown in blue line) showed the glucose consumption at 5% v/v of inoculum concentration in RCM was decreased as time of incubation increased. RCM was an established medium consists of soluble starch and other carbon sources used for growth of *Clostridium acetobutylicum*. The percentage of glucose consumption for 5 % v/v, 10% v/v and 15 % v/v inoculum concentration were declined as the the fermentation time was increased. *Clostridium aacetobutylicum* was utilized the carbon sources in RCM medium and converted it into alcohol (butanol and ethanol). The percetage of glucose consumption at 5 % v/v inoculum concentration showed the least percentage which were 26.32 %, 30.53 % and 37.89 % compared to 10 % v/v and 15 % v/v inoculum concentration. The percentage of glucose consumption were increased for both 10 % v/v and 15 % v/v. The 10 % v/v inoculum concentration has higher percentage glucose consumption (shown in red line) than 5 % v/v inoculum concentration, 13.27 %, 22.45 %, 34.69 % and 43.87 %. Figure 4.1 (shown in green line) illustrated the higher degree of declined for 15 % v/v inoculum concentration. The percentage of glucose consumption at 15 % v/v inoculum concentration were 15.79 %, 19.89 %, 38.47 % and 65.79 %.

Similar behavior as shown in palm oil mill effluent (POME) medium which demonstrated that increased the inoculum concentration, thus increased the percentage of glucose consumption. This was because the higher inoculum concentration at 15 % v/v has more *Clostridium acetobutylicum* cells compared to the 5% v/v inoculum concentration which has less *Clostridium acetobutylicum* cells to utilize carbon sources in RCM medium.

Table 4.3: Efficiency of total glucose consumption in RCM at 5 % v/v, 10 % v/v and 15 % v/v inoculum concentration

Inoculum Concentration / % v/v	**Efficiency / %**	**Total Glucose Consumption / g/L**
5	7.58	37.89
10	4.39	43.87
15	4.39	65.79

Table 4.3 showed the efficiency of total glucose consumption in RCM for each inoculum concentration. Since, RCM medium was an established medium, so the pattern shown in glucose consumption in POME was similar for both POME and RCM. Table 4.4 illustrated that the lowest inoculum concentration at 5 % v/v was the higher efficiency compared to 10 % v/v and 15 % v/v. Glucose in RCM medium was a growth limiting factor thus increased the inoculum concentration will only affected the fermentation time which more *Clostridium acetobutylicum* cells were utilized the glucose in RCM which supported by (Cheng *et al.*, 2007). The lowest inoculum concentration 5 % v/v gave the most efficient compared to 10 % v/v and 15 % v/v inoculum concentration, in utilized the glucose in RCM medium

4.5 Effect of different inoculum concentration on butanol and ethanol production

Butanol and ethanol production presented in this work was in area percentage (A%), since, area under the peak of chromatogram indicates the quantity of butanol and ethanol after compared with the standard of butanol and ethanol (Harvey, 2000).

4.5.1 Effect of 5%, 10% and 15% v/v of inoculum concentration on butanol production in POME

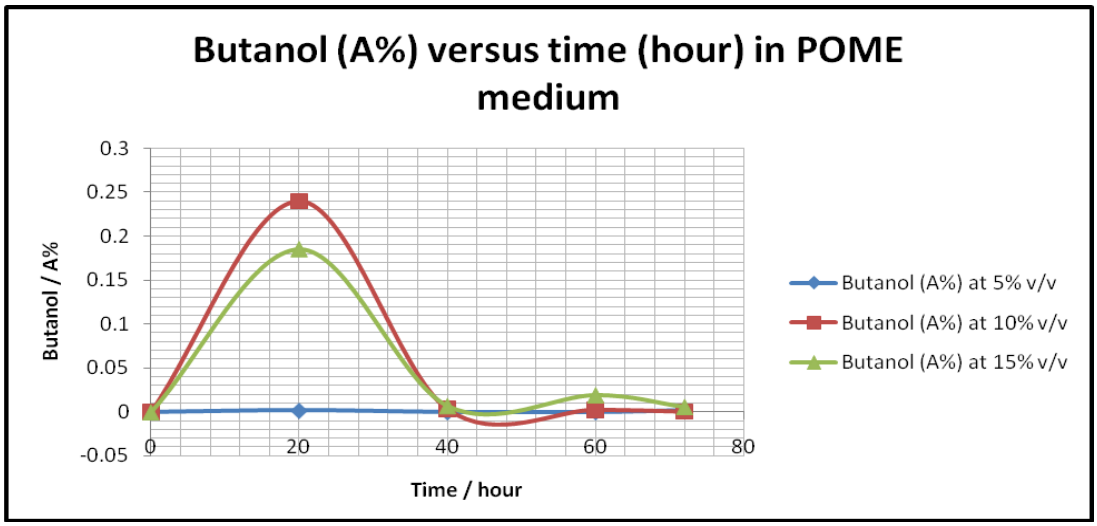

Figure 4.4: Graph of butanol production (A%) at different inoculum concentration (5%, 10% and 15%) v/v versus time (hour) in POME medium

Figure 4.4 showed the butanol production at different inoculum concentration, 5 % v/v, 10 % v/v and 15 % v/v in palm oil mill effluent (POME) medium. Results was presented in area percentage (A%) showed area under peak of butanol production. Based from the graph in Figure 4.4, The higest butanol production was at 10 % v/v inoculum concentration during 20 hour fermentation with 0.23954 %. Then, followed by butanol production was at 15 % v/v inoculum concentration at 20 hour with 0.18533 %. Finally, the lowest butanol production was at 5 % v/v inoculum concentration which approximately to zero production.

The higher butanol production was . Figure 4.4 showed that, after 40 hours fermentation, the butanol production curves were gradually decreased.

4.5.2 Effect of 5%, 10% and 15% v/v of inoculum concentration on ethanol production in POME

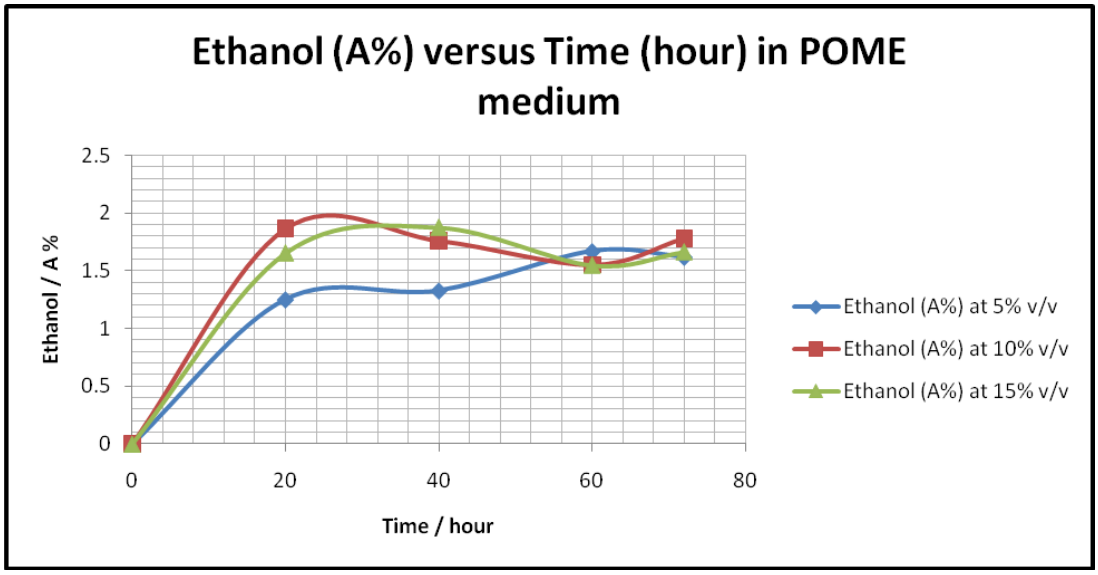

Figure 4.5: Graph of ethanol production (A%) at different inoculum concentration (5%, 10% and 15%) v/v versus time (hour) in POME medium

Figure 4.5 showed the ethanol production at different inoculum concentration, 5 % v/v, 10 % v/v and 15 % v/v in palm oil mill effluent (POME) medium. Results was presented in area percentage (A%) showed area under peak of ethanol production. Figure 4.5, showed the higher ethanol production was at 10 % v/v with 1.87593 % during 20 hours fermentation. Then, thes second higher was at 15 % v/v with 1.86309 % during 40 hours fermentation. Finally, the lowest ethanol production was at 5 % v/v with 1.66791 % during 60 hours fermentation. The ethanol production curves in Figure 4.5 were gradually decreased after 60 hours fermentation.

Production of butanol and ethanol in POME and RCM medium were undergo two different phases such as acidogenic phases and solventogenic phase. Jones and Woods (1986) reported that *Clostridium acetobutylicum* was converted the glucose molecule into butyric and acetic acid in acidogenic phase and shifted into butanol, ethanol and acetone in solventogenic phase. Shuster *et al*. (1998) claimed that, during exponential phase, the major product produced by *Clostridium acetobutylicum* were acetate and butyrate in acidogenic phase. The presence of acetate and butyrate in fermentation broth, decreased the pH because acetate and butyrate were both acidic (Takrif *et al.,* 2009). After the net acid ceased, the synthesis of solvent begun and fermentation was entered solventogenic phase, which acetic acid converted into ethanol and butyric acid converted into butanol (Takrif *et al.,* 2009 and Jones and Woods., 1986). Furthermore, Fond *et al.,* (1984) showed that butyric acid was the main component thus produced more butanol, while acetic acid was at constant production during the fermentation process. Thus, it could be showed that, the butanol production increased, as the butyric acid increased in fermentation medium.

Based from Figure 4.4 and Figure 4.5, butanol and ethanol production were increased as the inoculum concentration increased. Sharma *et al.* (2007) reported that the increased of inoculum concentration was linearly increased with ethanol and butanol production. Furthermore, butanol and ethanol production in palm oil mill effluent (POME) were gave a positive results, showed that, POME could be an alternative medium for ethanol and butanol production. The percentage of butanol produced was much lower than ethanol after compared between Figure 4.4 and Figure 4.5. There were several factors that affected the butanol production. Firstly, it might due to the butanol inhibition. Roffler *et al.* (1988) reported that, the major problem in butanol fermentation, was butanol inhibition which made the cost for recovery of butanol was increased. Butanol was toxic to the *Clostridium acetobutylicum* thus inhibit the butanol production (Jones and Woods, 1986). Figure 4.4 showed that after 20 hours fermentation the butanol percentage was decreased because, the butanol was accumulated in the POME medium and became toxic to *Clostridium acetobutylicum*, thus inhibit the butanol production. While there was no studies showed that ethanol could inhibit *Clostridium acetobutylicum*, thus the ethanol production increased as the inoculum concentration increased.

Secondly, the percentage of butanol was lesser than percentage of ethanol because during pretreatment of fermentation broth in POME, toluene was used as solvent extracting in Liquid-Liquid Extraction (LLE), thus affected the quantity of butanol being extract from POME broth.. Toulene was known as an universal solvent extracting which could extract most of the organic solvent. Previous studies by Roffler *et al.* (1987) and Sinshupa *et al.* (2004) used benzyl benzoate and 2-ethyl-1 hexanol as solvent extracting for butanol extraction. Even though toluene could be the extractant for ethanol and butanol and immisicble with water, but Gas Chromatography (GC) analysis showed that, the percentage of ethanol was much higher compared to butanol production. Toluene was not an appropriate solvent extracting for butanol but suitable for ethanol extraction from fermentation broth.

4.5.3 Effect of 5%, 10% and 15% v/v of inoculum concentration on butanol production in RCM

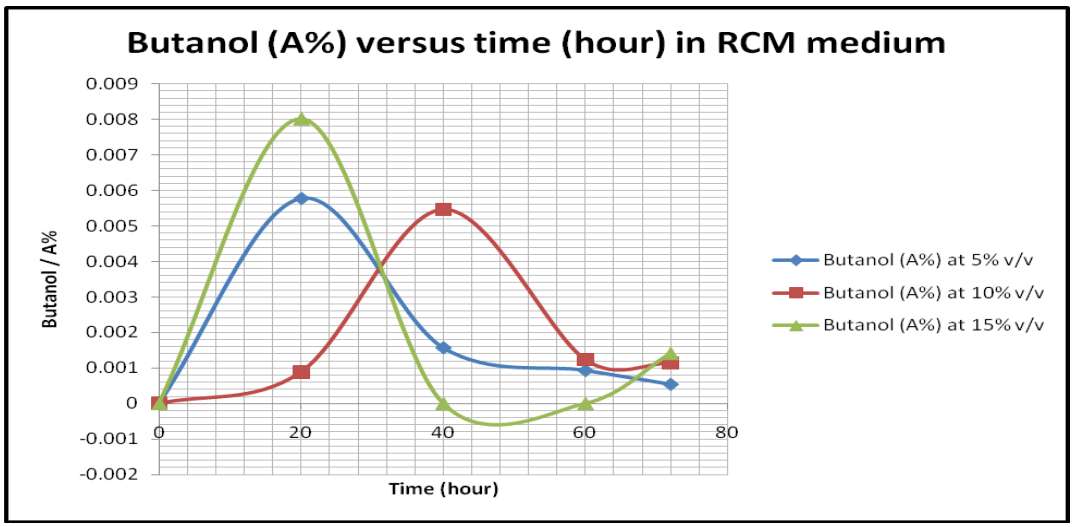

Figure 4.6: Graph of butanol production (A%) at different inoculum concentration (5%, 10% and 15%) v/v versus time (hour) in RCM medium

Figure 4.6 showed the butanol production at different inoculum concentration, 5 % v/v, 10 % v/v and 15 % v/v in Reinforced Clostridia Media (RCM). The results were presented in area percentage (A%) showed area under peak of butanol production. From the graph at Figure 4.6, showed that the higher butanol production was at 15 % v/v during 20 hours fermentation with 0.00801 %. Then, followed by the second higher butanol production was at 5 % v/v at 0.00577 % during 20 hour fermentation. Finally, the lowest butanol production was at 10 % v/v at 0.00546 % during 40 hours fermentation. The butanol production was decreased gradually after 40 hours fermentation.

4.5.4 Effect of 5%, 10% and 15% v/v of inoculum concentration on ethanol production in RCM

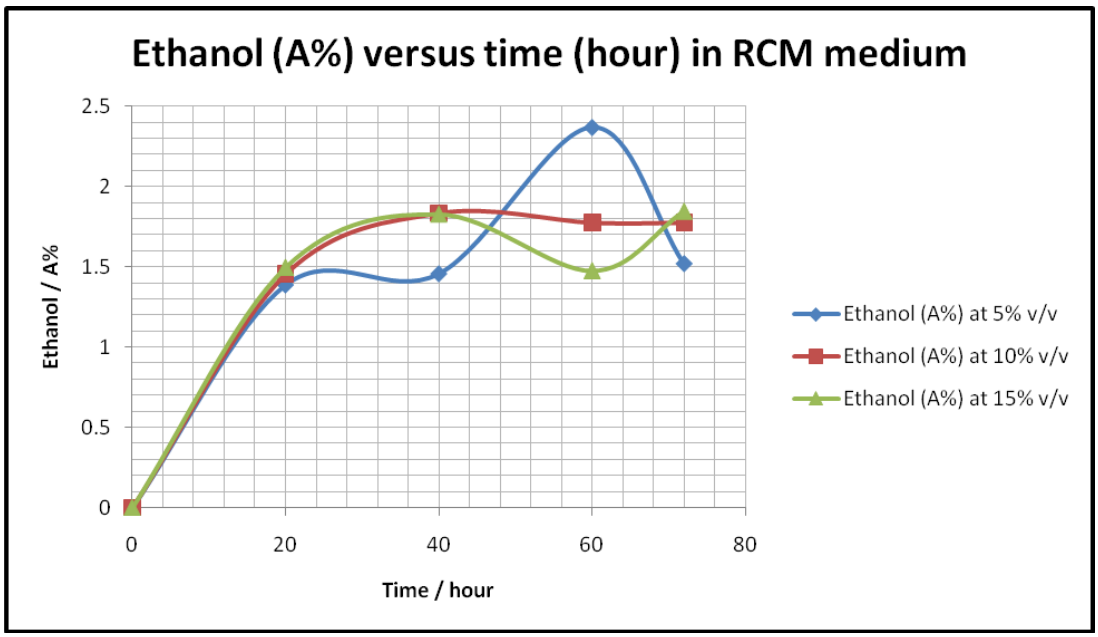

Figure 4.7: Graph of ethanol production (A%) at different inoculum concentration (5%, 10% and 15%) v/v versus time (hour) in RCM medium

Figure 4.7 showed the ethanol production at different inoculum concentration, 5 % v/v, 10 % v/v and 15 % v/v in Reinforced Clostridia Media (RCM). The results were presented in area percentage (A%) showed area under peak of ethanol production. The higher ethanol production was at 5 % v/v with 2.36754 % at 60 hours fermentation. Then, followed by ethanol production at 10 % v/v during 40 hours fermentation with 1.8348 %. Finally, the lowest ethanol production was at 15 % v/v inoculum concentration during 40 hours fermentation with 1.82463 %. The ethanol production was decreased gradually after 60 hours fermentation.

Reinforced Clostridia Media (RCM) was an established medium for *Clostridium acetobutylicum* to grow and produced butanol and ethanol during microbila fermentation. Figure 4.6 showed butanol production increased as the inoculum concentration increased and supported by Sharma *et al*. (2007) showed that butanol and ethanol production were linearly increased with increased of inoculum concentration. 15 % v/v inoculum concentration consists higher *Clostridium acetobutylicum* cells compared to 5 % v/v inoculum concentration which has less *Clostridium acetobutylicum* cells, which utilized the carbon sources in RCM and POME medium. Figure 4.7 of ethanol production does not supported the finding by Sharma *et al* (2007) because, the the ethanol production was decreased as the inoculum concentration increased . This was a new finding which showed that, ethanol production was optimal at 5 % v/v inoculum concentration, further increased the inoculum concentration will decreased the ethanol production.

Takriff et al. (2009) claimed that POME is rich with natural carbon source and dissolve complex substance requires mixing to enhance substrate interface with the microorganisms to produce high yield. In the absence of mixing, the various components of POME which made-up the substrate for fermentation will sediment to the bottom of the fermenter thus lowering the microorganisms-substrate interface (Takriff et al., 2009). In addition the decline of ABE fermentation might be due to the deficiency of sugar when needed (Qureshi and Blaschek., 2000).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Biobutanol and bioethanol were successfully produced from Reinforced Clostridia Media (RCM) as an established clostridia media and an alternative media was palm oil mill effluent (POME). Butanol and ethanol fermentation was anaerobic fermentation which Clostridium acetobutylicum was obligate anaerobic condition to utilize the carbon sources in POME and RCM. POME analysis was done by employed HPLC analysis and result showed that POME consist four types of reducing sugars which were galactose, glucose, arabinose and xylose. This showed that POME could be used as substrate that was required for supported clostridia growth. In addition the maximum butanol production for both POME and RCM medium were 0.18533% at 15% v/v (POME) and 0.00801% at 5% v/v (RCM) and. Result showed that increasing the inoculum concentration would reduce the fermentation time, increased the total glucose consumption and increased the butanol production. While the maximum production of ethanol in both POME and RCM media were, 1.87593% at 15% v/v during 40 hours of fermentation time (POME) and 2.36754% at 5% v/v during 60 hours (RCM) . Result showed that ethanol required more fermentation time to produce at higher amount of ethanol and inoculum concentration does affect the production.

Although, the concentration of butanol and ethanol produced from both POME and RCM medium was quite low, but still an interesting process since the process only less chemicals required and uses biomass as raw material. Evaluation on economic potential would be carried out for further studies.

5.2 Recommendation

Throughout this research there are several recommendation regarding to improve the production of butanol and analysis of palm oil mill effluent (POME) using High Performance Liquid Chromatography (HPLC).

Based from result obtained in this research shows that, the production of butanol and ethanol were oscillated for both POME and RCM medium and this is might due to the Liquid-Liquid Extraction Method (LLE). The selection of solvent extracting was not suitable for butanol, thus it is recommended that, the solvent extracting suitable for extracting butanol are, (Sinshupa *et al.,* 2005) or benzyl benzoate (Roffler *et al.,* 1988). In addition, before implementing LLE, it was suggested by (Sinshupa *et al.,* 2005) to undergo a mass transfer between the solvent extracting and the fermentation broth for 3 hours before do the LLE method.

Moreover, the resolution peak on butanol chromatogram showed less efficient compared to ethanol production. This is might be the selection of column for butanol separation. Different column will give different performance, thus Liew *et al*., 2006) suggested to implement BP column for GC analysis on butanol because it gave good separation in butanol analysis.

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APPENDIX A

CALCULATION

A1. Calculation on preparing of 2/3 of fermentation medium

Fermentation medium was prepared in 500 mL of Schott bottle. Thus total volume was 500 mL while the ***working volume*** was calculated as below:

2/3 x Total Volume = 2/3 x 500 mL = 333.33 mL, approximately took 300 mL

Similar to liquid medium was prepared in 250 mL of Schott bottle. The ***working volume*** was calculated as below:

2/3 x Total Volume = 2/3 x 250 mL = 166.66 mL, approximately took 167 mL

A2. Calculation on preparing the inoculums concentration

Inoculum concentration was calculated based on volume per volume (v/v). The further detailed on calculating inoculums concentration shown as below:

5% v/v inoculum concentration

5/100 x working volume of fermenter = 5/100 x 300 mL = 15 mL of inoculum needed to be seeded into production medium

10% v/v inoculum concentration

10/100 x working volume of fermenter = 10/100 x 300 mL = 30 mL of inoculum needed to be seeded into production medium

15% v/v inoculum concentration

15/100 x working volume of fermenter = 15/100 x 300 mL = 45 mL of inoculums needed to be seeded into production medium

A3. Calculation on preparing dilution factor (DF) on POME

Since POME was very concentrated with other particles thus POME needed to be diluted with distilled water to obtain required glucose concentration. Formula that to be used was $M_1V_1 = M_2V_2$;

M_1 = Initial concentration
V_1 = Volume initial
M_2 = Concentration that will needed
V_2 = Final volume

Dilution was conducted in 1/10 , thus 1 mL sample top up with 9 mL of distilled water. Thus, the final volume was 10 mL. To calculate the Dilution Factor (DF) which used to get actual glucose concentration was calculated as formula below;

Dilution Factor (DF) = final volume / aliquot volume

DF = 10 mL / 1mL of sample = 10
Thus, DF is 10

A4. Calculation on preparing sample for GC analysis

Butanol and ethanol analysis has to be undergoes GC preparation sample similar for both sample and standards. The preparation needed to be done at 1%, 2% and so on samples. Calculation will be demonstrated on 1% and 2% sample where the hexane volume needed to be diluted with sample was differ for each sample preparation;

1% sample preparation

10 µL of sample + 990 µL of hexane or organic solvent used for GC analysis

2% sample preparation

20 µL of sample + 980 µL of hexane or organic solvent used for GC analysis

A5. Calculation on percentage of total glucose consumption

For example in 5% v/v inoculums concentration the percentage of glucose consumption was;

Initial glucose concentration in RCM medium was 0.95 g/L. Thus, the 20 hour glucose consumption was 0.84 g/L;

% of glucose concentration = {(0.95 – 0.84) g/L / 0.95 g/L} x 100 = 11.58%

A6. Calculation on efficiency of total glucose consumption

5% v/v will consumed 37.89%, thus 1% v/v will gave 7.578% of efficiency on total glucose consumption

APPENDIX B

TABLE ON GROWTH PROFILE OF *CLOSTRIDIUM ACETOBUTYLICUM*

B1. Tables on Figure 4.1: Growth Profile of *Clostridium acetobutylicum* versus time

Time/hr	POME / OD	RCM / OD
0	0	0
6	0.385	0.28
12	0.576	0.438
18	0.767	0.76
24	0.815	0.792
30	0.826	0.807
36	0.836	0.874
42	0.798	0.971
48	0.82	1.008

APPENDIX C

TABLES OF GLUCOSE CONSUMPTION

C1. Table on Figure 4.2 and Figure 4.3: Graph of comparison of glucose consumption (g/L) in POME and RCM medium versus time (hour)

	Glucose Concentration at different inoculums concentration in RCM and POME / %					
Time/hour	**RCM**			**POME**		
	5% v/v	**10% v/v**	**15% v/v**	**5% v/v**	**10% v/v**	**15% v/v**
0	0.95	0.98	0.95	9.8	9.0	9.7
20	0.84	0.85	0.80	8.5	7.3	7.4
40	0.70	0.76	0.76	6.9	5.3	5.4
60	0.66	0.64	0.58	4.4	4.5	2.7
72	0.59	0.55	0.33	3.2	2.3	1.5

C2. Table on total glucose consumption in RCM and POME

	Percentage of glucose consumption at different inoculums concentration in RCM and POME / %					
Time/hour	**RCM**			**POME**		
	5% v/v	**10% v/v**	**15% v/v**	**5% v/v**	**10% v/v**	**15% v/v**
0	0	0	0	0	0	0
20	11.58	13.27	15.79	13.27	18.89	23.47
40	26.32	22.45	19.89	55.10	50.00	44.43
60	30.53	34.69	38.47	29.59	41.11	71.43
72	37.89	43.87	65.79	67.35	74.44	84.54

APPENDIX D

TABLES OF BUTANOL AND ETHANOL PRODUCTION

D1. Table on Figure 4.4: Graph of butanol production (A%) at different inoculum concentration (5% v/v, 10% and 15%) v/v versus time (hour) in POME medium

Time / hour	Butanol (A%) at 5% v/v	Butanol (A%) at 10% v/v	Butanol (A%) at 15% v/v
0	0	0	0
20	0.00168	0.23954	0.18533
40	0	0.00316	0.00718
60	0	0.00242	0.0194
72	0.00158	0.000441	0.00601

D2. Table on Figure 4.5: Graph of ethanol production (A%) at different inoculum concentration (5% v/v, 10% and 15%) v/v versus time (hour) in POME medium

Time / hour	Ethanol (A%) at 5% v/v	Ethanol (A%) at 10% v/v	Ethanol (A%) at 15% v/v
0	0	0	0
20	1.25017	1.87593	1.65461
40	1.3269	1.75952	1.86309
60	1.66791	1.55053	1.54827
72	1.61893	1.77946	1.66383

D3. Table on Figure 4.6: Graph of butanol production (A%) at different inoculum concentration (5% v/v, 10% and 15%) v/v versus time (hour) in RCM medium

Time / hour	Butanol (A%) at 5% v/v	Butanol (A%) at 10% v/v	Butanol (A%) at 15% v/v
0	0	0	0
20	0.00577	0.00089	0.00801
40	0.00157	0.00546	0
60	0.00093	0.00124	0
72	0.00054	0.00114	0.00141

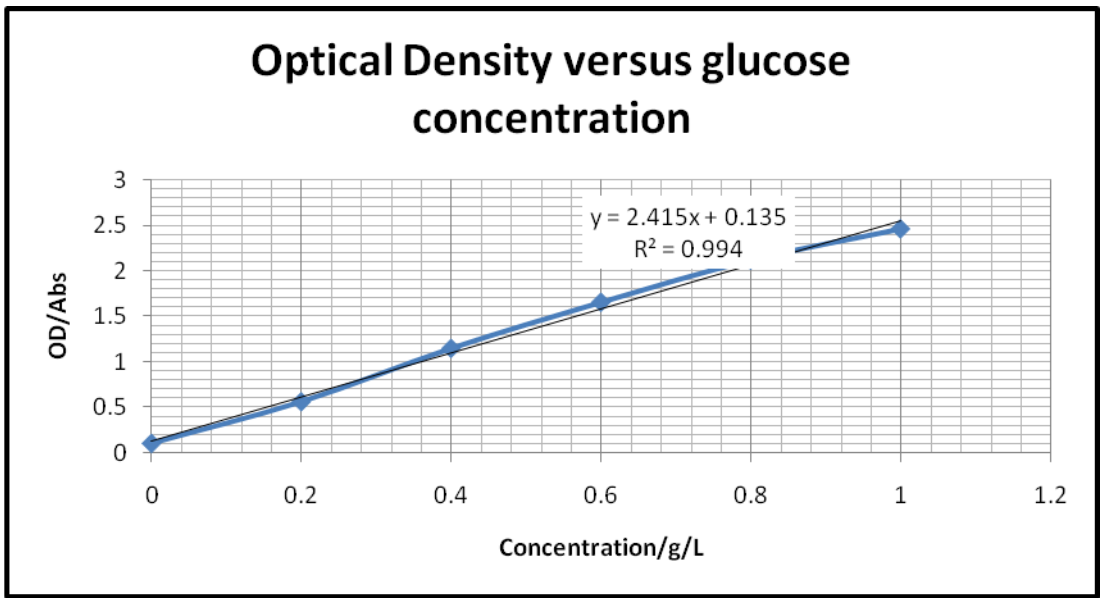
D4. Table on Figure 4.6: Graph of ethanol production (A%) at different inoculum concentration (5% v/v, 10% and 15%) v/v versus time (hour) in RCM medium

Time / hour	Ethanol (A%) at 5% v/v	Ethanol (A%) at 10% v/v	Ethanol (A%) at 15% v/v
0	0	0	0
20	1.3865	1.45459	1.49352
40	1.45593	1.8348	1.82463
60	2.36754	1.77605	1.47316
72	1.51891	1.7757	1.8431

APPENDIX E

CALIBRATION CURVE OF GLUCOSE ANALYSIS BY DNS METHOD

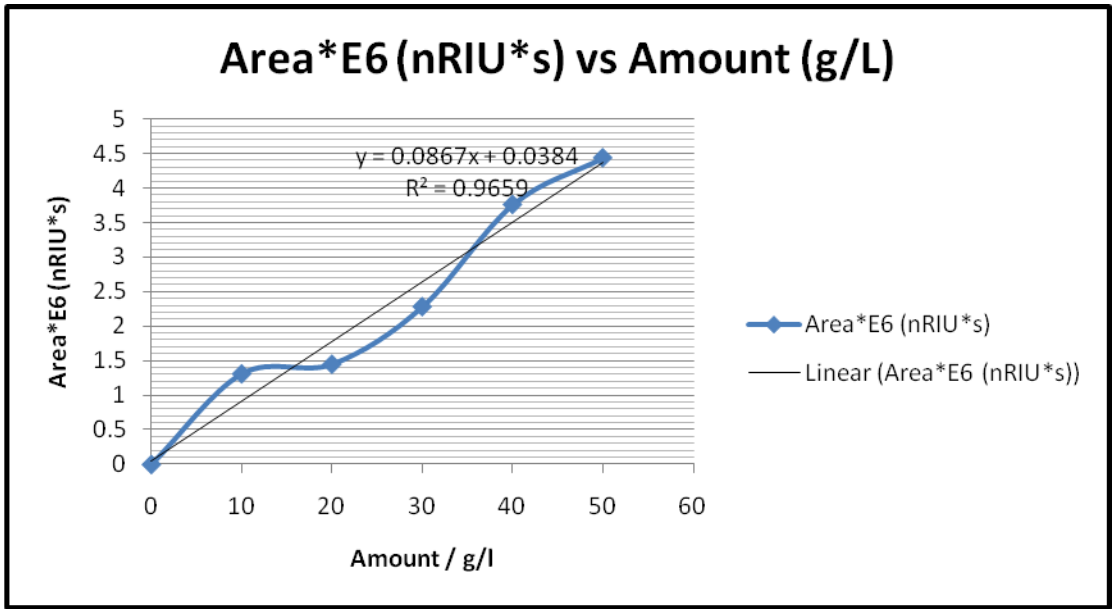
E1. Glucose calibration curve on DNS method



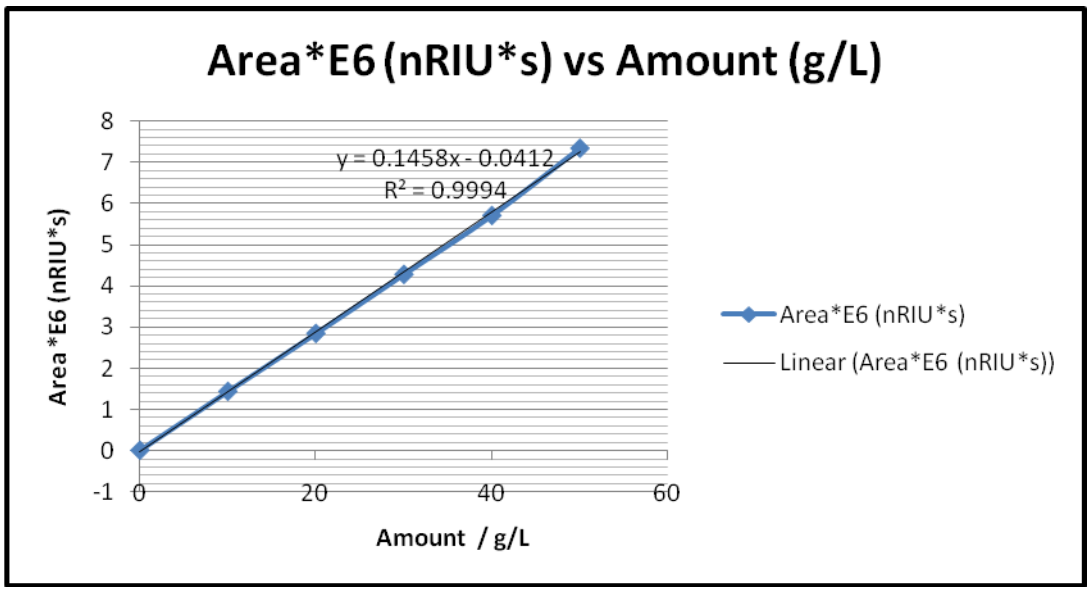
APPENDIX F

CALIBRATION CURVE OF ARABINOSE, XYLOSE, GALACTOSE AND GLUCOSE

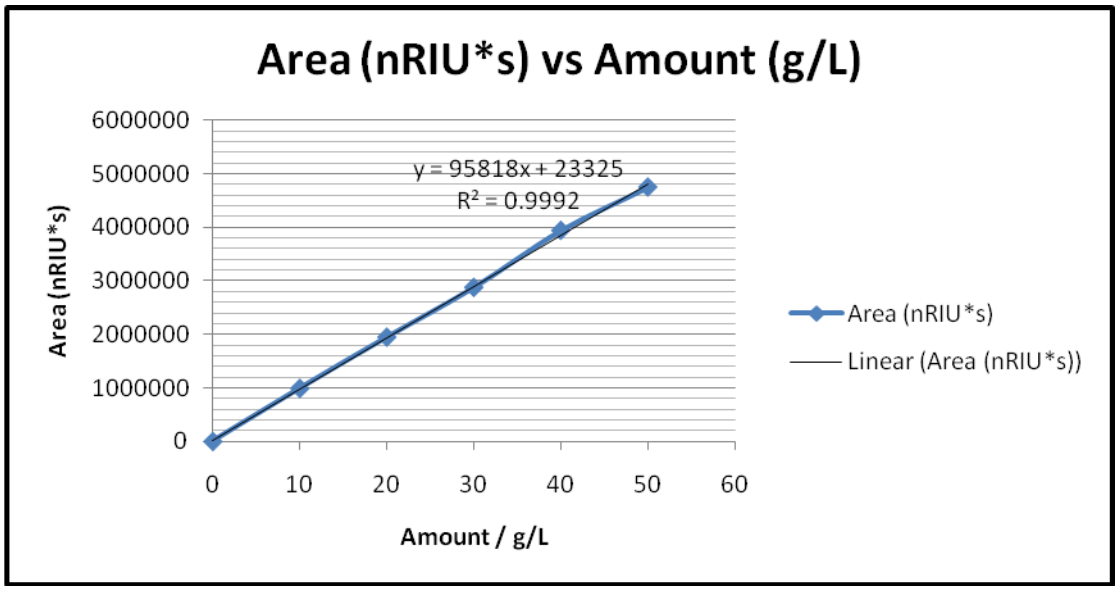
F1. Arabinose calibration curve fom HPLC assays



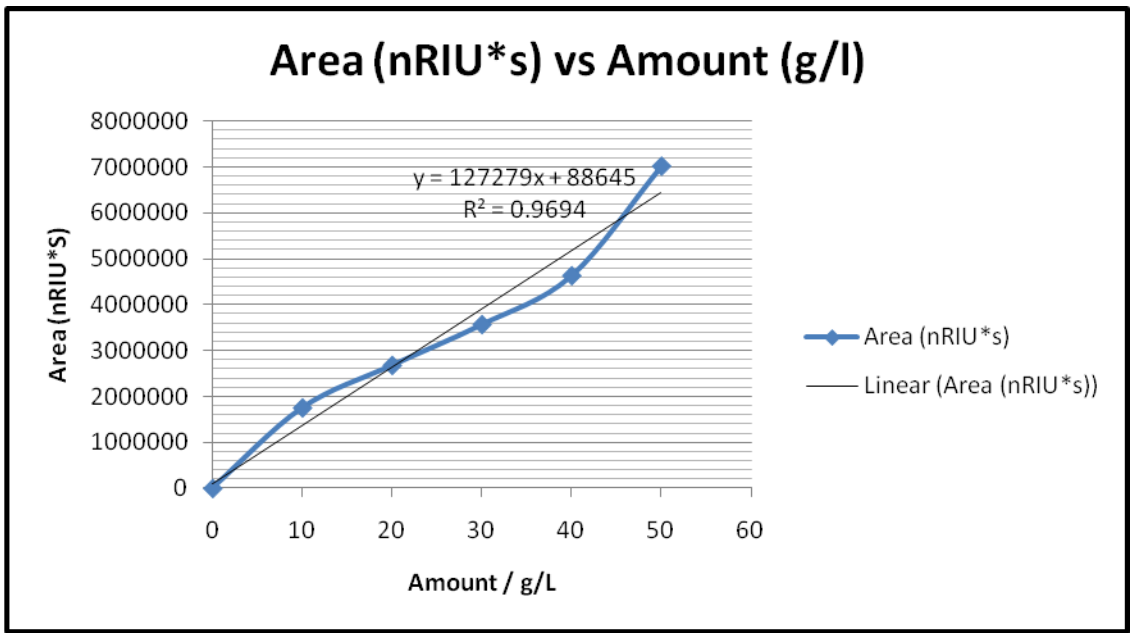
F2. Xylose calibration curve from HPLC assays



F3. Galactose calibration curve from HPLC assays



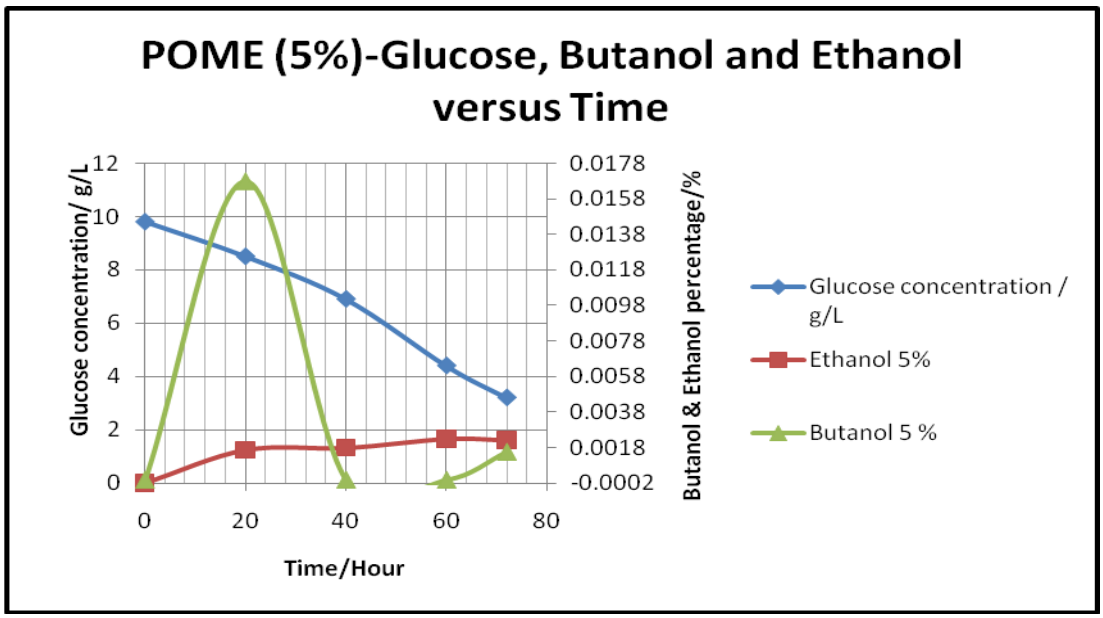
F4. Glucose calibration curve from HPLC assays



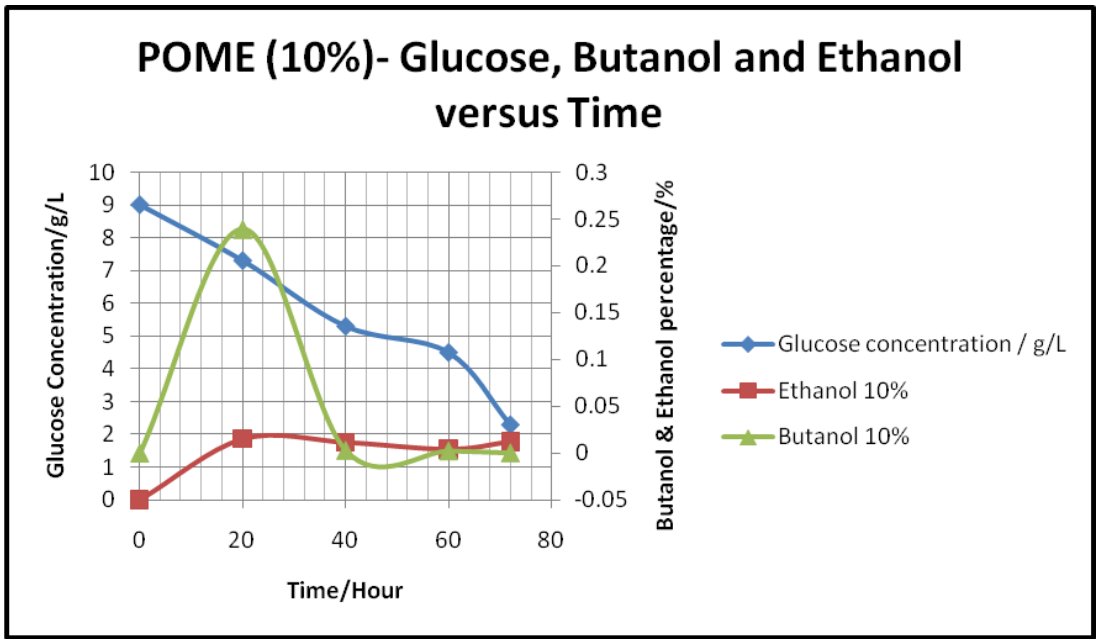
APPENDIX G

GRAPHS OF GLUCOSE, BUTANOL AND ETHANOL VERSUS TIME FOR POME AND RCM

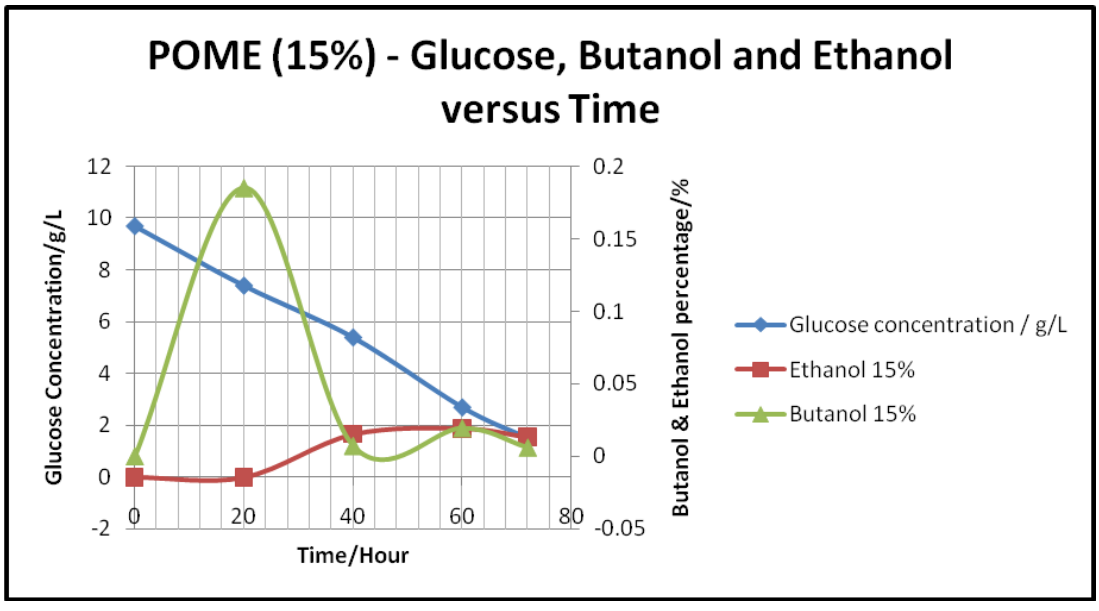
G1. Graph butanol, ethanol and glucose concentration versus time at 5 % v/v inoculums concentration in POME medium



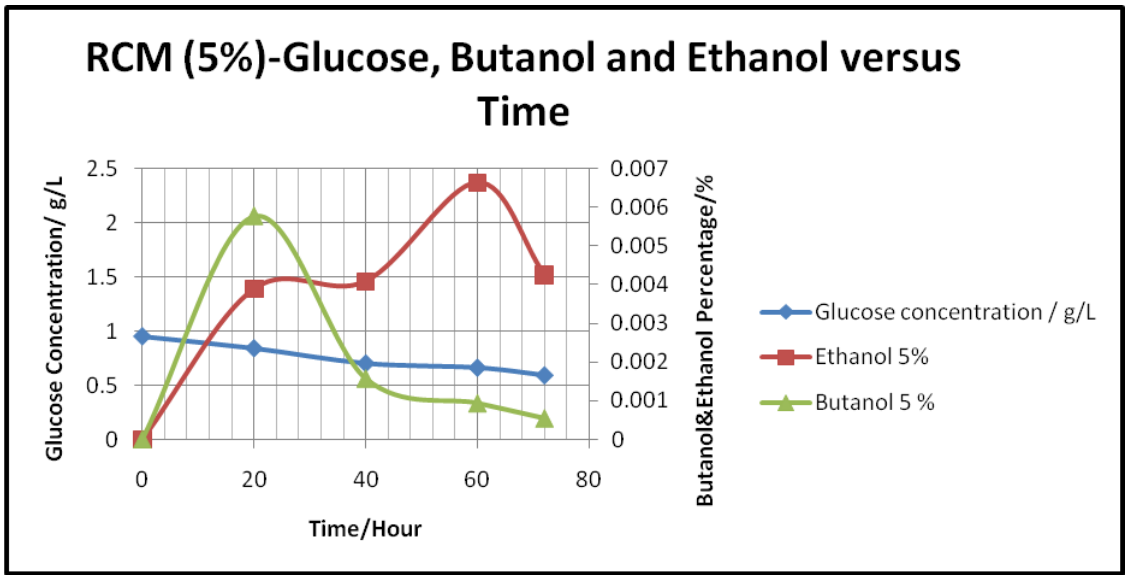
G2. Graph butanol, ethanol and glucose concentration versus time at 10 % v/v inoculums concentration in POME medium



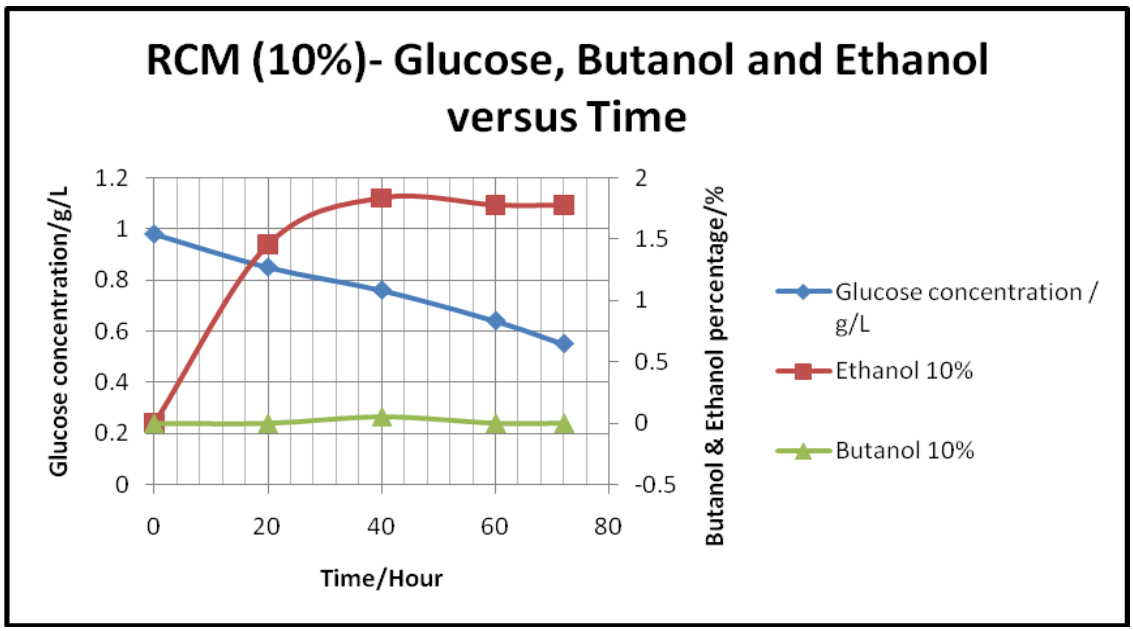
G3. Graph butanol, ethanol and glucose concentration versus time at 15 % v/v inoculums concentration in POME medium



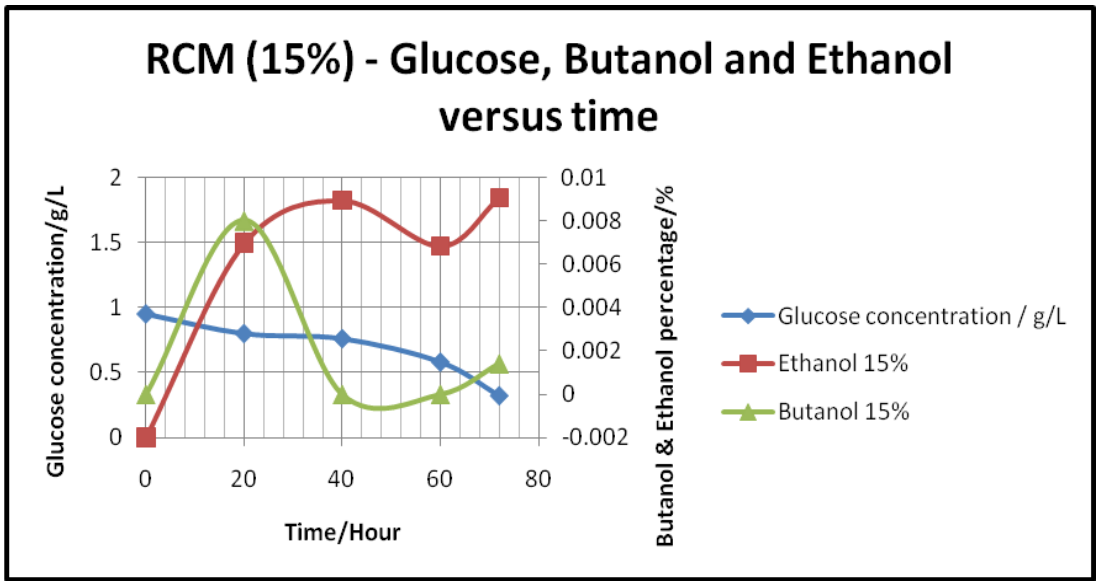
G4. Graph butanol, ethanol and glucose concentration versus time at 5 % v/v inoculums concentration in RCM medium



G5. Graph butanol, ethanol and glucose concentration versus time at 10 % v/v inoculums concentration in RCM medium



G6. Graph butanol, ethanol and glucose concentration versus time at 15 % v/v inoculums concentration in RCM medium



APPENDIX H

EXPERIMENTAL PICTURES

Figure H1: Palm oil mill effluent (POME) broth after centrifuged

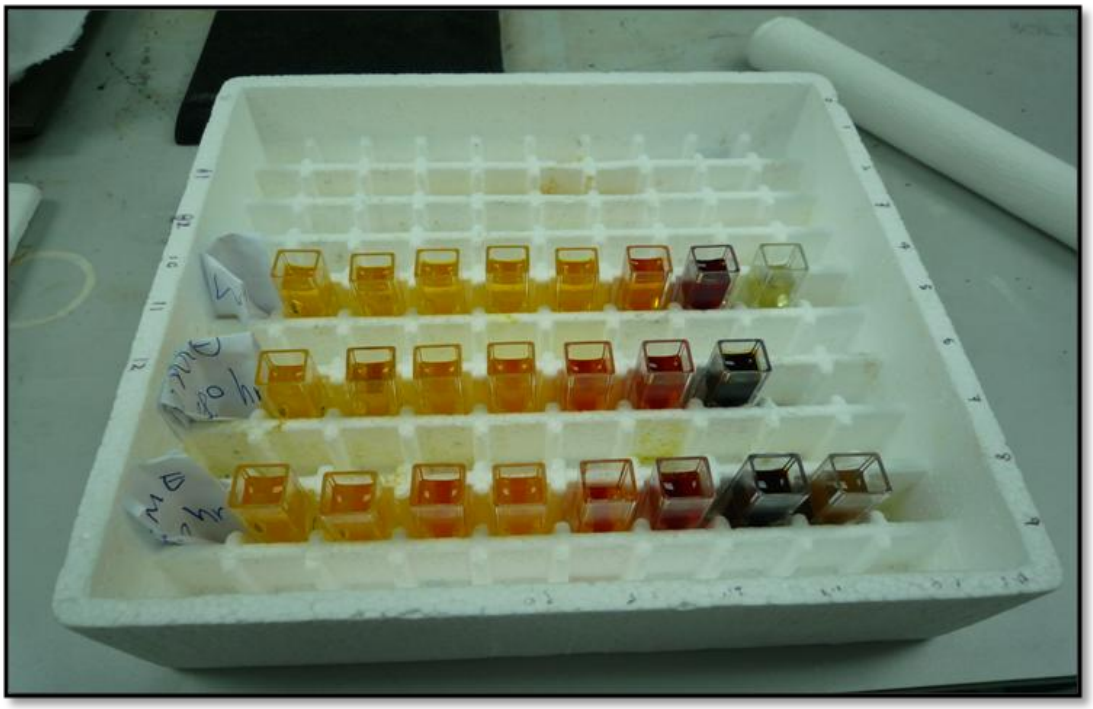


Figure H2: POME and RCM samples after reaction with DNS reagent

Figure H3: Anaerobic Chamber