EFFECT OF TEMPERATURE ON BUTANOL AND ETHANOL PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY *CLOSTRIDIUM ACETOBUTYLICUM*

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EFFECT OF TEMPERATURE ON BUTANOL AND ETHANOL PRODUCTION FROM PALM OIL MILL EFFLUENTS BY *CLOSTIDIUM ACETOBUTYLICUM*

EINAYAH BINTI KAMARUZAMAN

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
Universiti Malaysia Pahang

MEI 2010

I declare that this thesis entitled "Effect of temperature on butanol and ethanol production from Palm Oil Mill Effluent (POME) by *Clostridia Acetobutylicum***"** is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

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Special Dedication to my family members,
my friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

Palm oil mill effluents is a waste that produce in high quantity in Malaysia. Furthur studies would futher increase profitability of palm oil industries besides solving environmental problems. POME has great potential as substrate because it has low cost and also it contains a mixture of carbohydrates binding, hemicelluloses, sucrose, and other carbohydrates that can be utilized by saccharolytic clostria.This research mainly to study the effect of temperature to the solventogenic fermentation using c*lostridia.acetobutylicum* in producing butanol and ethanol by using palm oil mill effluent (POME) as a fermentation media. This research also to investigate the type of sugar that contain in POME, the profile growth rate of *clostridia acetobutylicum* and then to investigate consumption of glucose by *clostridia acetobutylicum* during fermentation. The experiment works were conducted in schott bottle in anaerobic chamber to maintain anaerobic condition. Fermentation was carried out for 72 hour at 35°C with the concentration of POME at 90% and inoculums concentration at 10%. Palm oil mill effluent and reinforced clostridia medium was used as a growth medium in batch culture. The temperature that used in this investigation is 33°C, 34°C, 35°C and 37°C. The result of this investigation showed that POME is a viable media for butanol and ethanol fermentation. The result also showed that the yield of butanol and ethanol production will decrease as the temperature increase. The results showed that the highest yield of butanol produced was 0.156 g/L. For ethanol production, the highest was 58.51 g/L. The experimental results also showed that the sugar groups that contains in POME are fructose, glucose, galactose, sucrose and lactose. From this study, it was observed that optimum condition for butanol and ethanol fermentation by *Clostridia acetobutylicum* at 35°C.

ABSTRAK

Bahan buangan daripada kilang kelapa sawit atau dikenali sebagai POME, merupakan bahan terbuang yang banyak terdapat di Malaysia. Kajian yang lebih lanjut boleh meningkatkan keuntungan kepada industri kelapa sawit selain daripada menyelesaikan masalah pencemaran alam sekitar. POME mempunyai potensi yang besar sebagai media fermentasi solventogenik kerana ia mempunyai harga yang rendah dan juga mempunyai campuran ikatan karbohidrat, hemicellulose, sukros dan lain – lain karbohidrat yang boleh digunakan oleh saccharolytic clostridia. Kajian ini bertujuan untuk mengkaji kesan suhu terhadap fermentasi solventogenik menggunakan *clostridia acetobutylicum* dalam menghasilkan butanol dan ethanol menggunakan POME sebagai media juga. Kajian ini dijlalankan untuk mengkaji kandungan gula yang terdapat di dalam POME, kadar pertumbuhan *clostridia acetobutylicum* dan mengkaji kadar penggunaan gula oleh *clostridia.acetobutylicum* semasa process fermentasi. Eksperimen ini dijalankan di dalam schott botol menggunakan ‘anaerobic chamber’ untuk mewujudkan keadaan tanpa oksigen. Proses fermentasi dijalankan selama 72 jam pada 35°C dengan kepekatan POME dikekalkan pada 90% dan kepekatan inoculum pada 10%. Suhu yang digunakan dalam kajian ini ialah 33°C, 34°C, 35°C dan 37°C. Keputusan daripada kajian ini menunjukkan POME merupakan medium yang sesuai untuk fermentasi butanol dan ethanol.Hasil daripada kajian juga menunjukkan kadar penghasilan butanol dan ethanol akan menurun apabila suhu meningkat. Keputusan juga menunjukkan kadar penghasilan butanol yang tertinggi adalah pada 0.156 g/L. Untuk penghasilan ethanol pula sebanyak 58.51 g/L. Keputusan kajian juga menunjukkan terdapat beberapa kumpulan gula yang terdapat di dalam POME iaitu fruktose, glukose, sukrose, dan laktose. Daripada kajian ini, ia menunjukkan keadaan optimum untuk fermentasi butanol dan ethanol dengan menggunakan *Clostridia acetobutylicum* adalah pada 35°C.

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LIST OF SYMBOLS/ABBREVIATIONS

ABE	-	Acetone – butanol – ethanol
BOD	-	Biological oxygen demand
COD	-	Chemical oxygen demand
^{o}C	-	degree Celcius
DNS	-	Dinitrosalicylic acid
eg	-	Example
GCFID	-	Gas Chromatography(Flame Ionization Detector0
g	-	gram
HPLC	-	High Performance Liquid Chromatoghraphy
hr	-	hour
L	-	Liter
MT	-	Metric tonne
ml	-	mililiter
µm	-	micrometer
min	-	minute
POME	-	Palm Oil Mill Effluents
UV – Vis	-	UltraViolet Vision

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CHAPTER 1

INTRODUCTION

1.0 Background of Study

Palm oil is one of the world's chief edible oils produced by South East Asian and African countries, used for producing various food products, cosmetics and pharmaceutical products and oleo chemicals. Palm oil production is one of the major industries in Malaysia. During the period between 1990 and 2002, palm oil production was nearly doubled from 6 094 622 to 1 188 000(MT) per year, making Malaysia the biggest palm oil producer worldwide. Its production generates various wastes chief among which is palm oil mill effluent (POME) (Khalil *et.al.*, 2003).

Malaysia is a world leader in palm oil industry, producing about 11.8 million tons of crude palm oil in 2001. Recycle uses of biomass generated from the production process of palm oil is an urgent subject to Malaysia, because, since the volume of palm oil mill effluent (POME) is proportional to the 3.6 times as much volume of the crude palm oil production, it gradually has become difficult to treat it only by natural evaporation at reservoirs. For this purpose, various technologies that recover useful materials and innovative organic materials from POME are being developed (Monot *et.al*, 1982). At an average, about 0.1 tonne of raw palm oil mill effluent (POME) is generated for every tonne of fresh fruit bunch processed. POME consists of water soluble components of palm fruits

as well as suspended materials like palm fiber and oil. Despite its biodegradability, POME cannot be discharged without first being treated because POME is acidic and has a very high biochemical oxygen demand (BOD) (Monot *et.al.*, 1982).

Concerns about the greenhouse effect, as well as legislation to reduce CO_2 emissions and to increase the use of renewable energy have been the main reasons for the increased production and use of biofuels. In addition to bioethanol and biodiesel production, the research on biobutanol production has also increased during the past years. Butanol can be produced by chemical or biochemical routes. Fuel properties of butanol are considered to be superior to ethanol because of higher energy content, and better air-to-fuel ratio. Butanol is also less volatile and explosive than ethanol, has higher flash point and lower vapor pressure which makes it safer to handle (Pakkila *et.a.l*, 2009).

Production of industrial butanol and acetone via fermentation, using *Clostridia acetobutylicum*, started in 1916, during World War I. Chime Weizmann, a student of Louis Pasture, isolated the microbe that made acetone. England approached the young microbiologist and asked for the rights to make acetone for cordite. Up until the 1920s acetone was the product sought, but for every pound of acetone fermented, two pounds of butanol were formed. A growing automotive paint industry turned the market around, and by 1927 butanol was primary and acetone became the byproduct (Monot *et.al.*, 1982).

Butanol, which is an excellent biofuel, has numerous other applications in the food, plastics, and chemical industries (Masngut *et.al.*, 2007). Clostridia acetone/butanol fermentation used to rank second only ethanol fermentation by yeast in its scale of production and thus are one of the largest biotechnological processes known.

Butanol (butyl alcohol) is an organic compound used largely as an industrial solvent. However, when it is produced using biological materials (biomass) for feedstocks, it is called biobutanol and is no different than butanol produced using fossil fuels like oil. Since it relies on sunlight and photosynthesis to contribute to the growth of that biomass (plants, grasses, corn, wheat, etc), biobutanol is a renewable fuel (Monot *et.al.*, 1982). The production of butanol for fuel was traditionally accomplished by fermenting biomass, such

as algae, corn, and other plant materials containing cellulose that could not be used for food and would otherwise go to waste.

Ethanol or ethyl alcohol (C_2H_5OH) is a clear colourless liquid; it is biodegradable, low in toxicity and causes little environmental pollution if spilt. Ethanol burns to produce carbon dioxide and water. Ethanol is a high octane fuel and has replaced lead as an octane enhancer in petrol. By blending ethanol with gasoline we can also oxygenate the fuel mixture so it burns more completely and reduces polluting emissions. Ethanol fuel blends are widely sold in the United States.

Bioethanol is one of the renewable energy source that is fast gaining foothold as potential fuel to power automotive engine. Contrary to gasoline which is refined through distilling crude fossil fuel, bioethanol can be synthesized from the starchy parts of natural plants or other biomass. Microscopic yeast cells break down the starch and water, creating the so called bioethanol and carbon dioxide as end products. Bioethanol burns to to produce carbon dioxide and water in complete combustion, a process akin to gasoline. It also possesses a high octane fuel. Bioethanol can be produced via traditional methods such as fermentation, and it can be distributed using the same petrol forecourts and transportation systems as before. In the biomass to bioethanol process, acids and enzymes are used to catalyze the reactions. (Cheng *et.al.*, 2007).

The fermentation process is facilitated mainly by a type of bacteria called *C. acetobutylicum*. Oddly enough, these bacteria are rather closely related to those which cause botulism. Other microorganisms are also able to ferment these materials, and research into these types of production techniques is ongoing (Ishizaki *et.al.*,1999).

The acetone – butanol – ethanol (ABE) fermentation was the largest scale bioindustry ever run second second to ethanol fermentation (Antoni *et.al.*, 2007). Acetone – butanol fermentation usually represents the first stage of growth and acid production followed by the second stage where solvents are produced with partial reutilization of the acids (Lepage *et.al.*, 1987). However, the traditional clostridial fermentation of butanol and acetone is suffering from the difficulties of switching the acidogenic fermentation stages to

the solventogenic stage, and thus, a discontinuous production mode, from common phage infections, the rising substrate costs and the effort required for downstream processing (Antoni *et.al.*, 2007). In 1927 butanol became the main product of ABE process. In the time of Second World War it was used for producing synthetic rubber. In the first half of 20th century biobutanol was produced from corn and molasses by fermentation. This process affords acetone, butanol and ethanol (therefore it is denoted as ABE process). However with growing demand for butanol its producing by fermentation began to fall because since 1954 the price of petroleum becomes below those of sugar because USA lost cheap sugar supply from Cuba. Now butanol is produced starting with petroleum via hydrolysis of haloalkanes or hydration of alkenes (Shapovalov *et.al.*, 2008).

1.1 Problem Statement

Concerns about the greenhouse effect, as well as legislation to reduce CO_2 emissions and to increase the use of renewable energy have been the main reasons for the increased production and use of biofuels. Although the butanol price is higher than the ethanol, but production of butanol comes with many advantages. Also, traditional fuel pipelines cannot be used with ethanol since water mixes into it, but butanol does this to a lesser extent and so could be used with more existing infrastructure.

Palm oil mill effluents are one of the most abundant wastes in Malaysia. They are mainly organic in nature and are highly polluting. The biological oxygen demand and chemical oxygen demand (BOD and COD) of this effluent is very high and also acidic. Thus, if such an effluent with its quality and quantity were to be discharged into the rivers, all the aquatic life will perish. POME has great potential as substrate because is has low cost and also it contains a mixture of carbohydrates binding starch, hemicelluloses,sucrose, and other carbohydrates that can be utilized by saccharolytic clostria. Such utilization would futher increase profitability of palm oil industries besides solving environmental problems.

Petroleum resources are the only major mineral commodities where many experts fear resource depletion will produce significant scarcities over the next several decades. By producing solvents such a ethanol or butanol, it can overcome this problems. Progress in the area of biotechnology allows using corn and other biomass as economically effective source of biobutanol. The alcohol based fuel including butanol and ethanol
is partially oxidized (compared to hydrocarbons) and therefore mixture for engine should be more enriched than in the case of gasoline.

1.3 Objective of Study

To study the effects of temperature (33°C to 37°C) on solvent production (butanol and ethanol) from palm oil mill effluent (POME) by *Clostridium acetobutylicum*.

1.4 Scope of Study

1. To study the growth profile of *Clostridia acetobutylicum* in POME and (Reinforced Clostridia Medium).
2. To analyze the composition of POME using High Performance Liquid Chromatography (HPLC).
3. To study the effect of temperature (33°C to 37°C) to the solvents productions using POME.
4. To study glucose consumption in the fermentation broth.

CHAPTER 2

LITERATURE REVIEW

2.1 Palm Oil Mill Effluents (POME)

The palm oil industry, apart from being a major foreign exchange earner for Malaysia,is also identified as the single largest source of water pollution. It produces a large volume of highly polluting effluents, for instance, 2.5 tonnes of Palm Oil Mill Effluent (POME) is generated for every tonne of crude palm oil produced (Abdullah *et.al*,2004).The improvement of processing technology will lead to further increase in world's palm oil supply. However, the rapid development of the industry has had serious consequences on the natural environment, which mainly related to water pollution due to a large discharge of untreated or partially treated palm oil mill effluent (POME) into watercourses. In the year 2004, more than 40 million tonnes of POME was generated from 372 mills in Malaysia (Abu *et.al.*, 2007).

POME is high volume liquid wastes which are non toxic but have an unpleasant odour. They are predominantly organic in nature and are highly polluting. The biological oxyegen demand and chemical oxygen demand (BOD & COD) of this effluent is very high and so goes for the total nitrogen, ammonical nitrogen and oil and grease. The effluent is also acidic. Other than this the raw effluent is made up of a few anions and cations. As for the physical nature of the raw effluents, it is hot, has a bad aroma and is brown in color.

Thus, if such an effluent with its quality and quantity were to be discharged into our aquatic life will perish (Khatiravale *et.al.*, 1997).

POME is produced from production of crude oil which involves extraction process where the fresh palm oil friuit bunches undergo sterilization, digestion and extraction of the oil, which is then clarified. POME is produced in vast amounts through the year.palm oil mill effluents has great potential as a substrate for ABE fermentation because it contains a mixture of carbohydrates including starch, hemicellulose, sucrose and other carbohydrates that can be utilized by saccharolytic clostridia (Khalil *et.al.*,2003).

Fresh POME is a colloidal suspension containing about 95% water, 0.6-0.7% oil, and 4-5% total solids including 2-4% suspended solids that are mainly debris from palm fruit (Ahmad *et al.*, 2005). It is acidic with pH 4-5 and discharged at temperature about 80-90°C. Although the effluent is non toxic, it has a very high concentration of biochemical oxygen demand (BOD) (25 000 mg/L) which becomes a serious threat to aquatic life when discharged in relatively large quantities into watercourses. Furthermore, POME contributes 83% of the industrial organic pollution load in Malaysia (Abu *et.al.*,2007).Palm oil mill effluents (POME) has great potential as a substrate for ABE fermentation because it contains a mixture of carbohydrates including starch, hemicelluloses, sucrose and other carbohydrates that can be utilized by saccarolytic clostridia(Khalil *et.a.l*,2003).

2.2 Solventogenic Clostridia

The solventogenic clostridia have received much attention in recent years, because of their ability to produce industrially relevant chemicals such as butanol and 1, 3-propanediol. The clostridia secrete numerous enzymes that facilitate the breakdown of polymeric carbohydrates into monomers (Ezeji,T.C *et.al.*, 2007). Clostridia have a long history of being employed in several biotechnological processes, for instance, *C.*

acetobutylicum in the conversion of renewable biomass for acetone/butanol production (Patova *et.al.*, 2009).

C. acetobutylicum is an anaerobic, zeterofermentative organism. It also is able to use polymeric substrates such as starch and xylan, but not cellulose, for growth (Durre, 1998). Anaerobic bacteria such as the solventogenic clostridia are capable of converting a wide range of carbon sources (e.g.glucose, galactose, cellobiose, mannose, xylose and arabinose) to fuels and chemicals such as butanol, acetone, and ethanol (Ezeji *et.al.*, 2007) (Masngut *et.al.*, 2007).

Studies on the production of solvents have always been done on natural media; yet, it is well known that *C. acetobutylicum* can grow on a sugar-salt-vitamin medium (Masngut *et.al.*, 2007). Several species of Clostridium bacteria are capable to metabolize different sugars, amino and organic acids, polyalcohols and other organic compounds to butanol and other solvents (Nik *et.al.*, 2004).

The growth of *C. acetobutylicum* utilizing both carbon sources can be divided into two phases, acidogenic phase and solventogenic phase. An acidogenic phase where organic acid (acetic and butyric acid) were actively produced which cause the reduction in culture pH. At the same time glucose is also actively consumed to accommodate the high growth rate in the culture (Liew *et.al.*, 2006).

In recent studies, the intracellular pH of *C. acetobutylicum* cells grown in a chemostat decreased with decreasing external pH during acetogenic fermentation and then become stabilized after solventogenesis was initiated (Huang *et.al.*,1986).

In clostridia fermentation, the sporulation occurs concomitantly with the solventogenesis. Sporulation makes the bacterial cells enter a dormant state where they lose the ability to produce solvents. It is likely that there is a relationship between sporulation and solventogenesis, given that many early molecular events connected with

sporulation appear in the initiation of solventogenesis.If this relationship is revealed, it may be possible to produce more solvents, including butanol, by preventing the clostridia from forming spores. (Wu *et.al.*, 2003).

During fermentation, *C. acetobutylicum* produces three major classes of products; solvents (acetone, ethanol and butanol); organic acids (acetic acid, lactic acid and butyric acid); gases (carbon dioxide, and hydrogen) (Wu *et.al.*, 2003).

In batch culture exponentially growing cells produce organic acids which lower the pH of the medium. As the culture enters the stationary phase the metabolism of the organism changes: carbohydrate and the preformed organic acids are converted into organic solvents (Bowles *et.al.*, 1985).

The biobutanol fermentation suffers from several limitations (e.g. low titer, yield and productivity) and improvements in the performance of the solventogenic clostridia are necessary to move biobutanol fermentation research to a competitive commercial position. Several approaches have been employed to improve the performance of solventogenic clostridia with the aim of generating strains that can be used in industrial biobutanol production. Recombinant DNA technology, in addition to traditional mutagenesis and selection, have been employed to modify targeted metabolic pathways in the solventogenic clostridia (Ezeji *et.al.*, 2007).

An important advantage of the solventogenic clostridia is the variety of fermentation products (acetone, butanol, ethanol, acetic, butyric, lactic acids, etc.) that can be synthesized by this group of microorganisms. However, the loss of available carbon as a result of the formation of unwanted products is an undesirable property of the solventogenic clostridia. Clearly, enzyme synthesis and control of electron flow in the glycolytic pathway are vital with respect to the regulation of the butanol fermentation pathways. (Ezeji *et.al.*, 2007).

In addition, the ability of the solventogenic clostridia to grow under a low redox potential enables them to undertake a variety of stereospecific reductions, yielding chiral products that are difficult to synthesize chemically (Bowles *et.al*, 1985). As the electron flow can be reversed, butanol yield should respond to factors that influence the direction of electron flow (Ezeji *et.al.*, 2007).

2.3 Fermentation

2.3.1 Anaerobic Fermentation

Anaerobic fermentation is the process of fermentation without using any oxygen. One of advantages of the anaerobic process is the recovery of the useful matters such as solvents (Hwang *et.al.* 2004).

The most important economic factor in solvent fermentation is the cost of substrate, which made up about 60% of the overall cost of production. (Liew *et.al.*,2006). Biobutanol production is an anaerobic two-stage fermentation process where acetic and butyric acids, carbon dioxide and hydrogen are first produced in the acidogenic phase. Then the culture undergoes metabolic shift to solventogenic phase and acids are converted into acetone, ethanol and butanol. At the end of the fermentation, products are recovered from the cell mass, other suspended solids, and by-products (Pakkila *et.al.*, 2009).

2.3.2 ABE Fermentation

The ABE fermentation was the largest scale bioindustry ever second run to ethanol fermentation. (Khalil *et.al.*, 2003) Its decline since about 1950 has been caused by increasing substrate costs and the availability of much cheaper feedstock for chemical solvent synthesis by the petrochemical industry (Durre., 1998).

In particular, biobutanol results a good candidate as transportation fuel and ABE fermentation is a potential path to upgrade biomass into valuable liquid fuels (Napoli *et.al.*, 2009).

Acetone-butanol fermentation by *C. acetobutylicum* has been characterized as biphasic batch-culture fermentation (Huang *et.al.*, 1986) (Kanouni *et.al.*, 1998). The first phase is characterized by rapid growth and by the formation of acetic and butyric acids which are excreted into the medium, thereby lowering the medium pH. The second phase commences after the pH of the medium has fallen below approximately 5.0. During this period, butanol and acetone become the major fermentation products. The fatty acids, previously accumulated in the medium, pass through the cell membrane in their undissociated form and are converted to solvents. At the end of the fermentation, the metabolic activity ceases primarily because the concentrations of the solvents have reached toxic levels (Huang *et.al.*, 1986).

The major end product of the fermentation is butanol, with acetone and ethanol being minor products (Bowles *et.al.*, 1985). All the butanol that have been produced along with acetone was considered a by – product and kept in large storage tanks (Durre., 1998). The production of the byproducts could be reduced or terminated, and the process could greatly benefit from new and cheaper substrates derived by hydrolysis from lignocelluloses biomass, as well as from advanced sterile fermentation and downstream possessing technology (Khalil *et.al.*, 2003).

Batch fermentation by *C. acetobutylicum* is characterized by two phases. During the first phase, or acidogenesis, *C. acetobutylicum* grows and produces acetate and butyrate from glucose. These acids attain their maximal concentrations and are consumed in the second phase, which is known as solventogenesis. The acids are reduced, and neutral solvents including butanol, acetone, ethanol, and acetone are produced (Evans *et.al.*, 1988).

2.4 Butanol

First of all, butanol is used as industrial solvent.World market for this product is estimately 350 million gallons per year, of which 220 million gallon/year is the fraction consumed by USA. Butanol can be used instead of gasoline even in higher degree than ethanol due to its physical properties, economy, safety and because it can be applied without remodeling car engine. (Shapovalov *et.al.*, 2008).

By 1927 butanol was increasingly used for the production of the lacquer solvent butyl acetate and for the synthetic rubber industry. Japan, and other possible combatants, used butanol as an aviation fuel during WWII when they exhausted their fossil fuel supply (Khalil *et.al.*, 2003).

The factors which severely affect the economics of butanol fermentation were high cost of substrate; low product concentration, low reactor productivity, low ABE yield, and escalated cost of butanol recovery by distillation which was the only techniques for recovery at old time. The cost of recovery of butanol is high due to the fact that its concentration in the fermentation broth is low because of the product inhibition (Qureshi *et.al.*, 2001)

Today, new uses for butanol are emerging, as a diesel and kerosene replacement, as silage preserver, biocide and C4 compound for chemical industries (Khalil *et.al*, 2003).

Butanol, which is an excellent biofuels, has numerous other applications in the food, plastics, and chemical industries (Ezeji *et.al.*, 2005).

The main reason that nobody knows butanol as an alternative fuel is the fact that its producing has never been suggested economically reasonable. As mentioned above, this product is used mainly as industrial solvent and costs triple compared to gas (Shapovalov *et.al.*, 2008). Butanol has a heat of combustion sufficiently high to allow for their use in high thrust – to – weight applications such as airplanes. For safe and environmentally friendly storage, vapor pressure and ignition temperature are important factors (Khalil *et.al.*, 2003).

Starch and starch based co – products can be used for conversions to fuels and chemicals such as butanol. The production of amylolytic enzymes (α – amylases and amyloglucosidases) by the production microorganism, known as saccharification fermentation, has an advantage over systems where hydrolysis and subsequent fermentation.(Ezeji *et.al*, 2005). Butanol is generally produced in concentrations of no greater than 12 g/liter .This limitation is thought to be due to the toxicity of butanol to *C.acetobutylicum* (Evans *et.al*, 1988).

Butanol toxicity to the fermenting microorganisms limits its concentration in the fermentation broth, resulting in low butanol yields and a high cost for butanol recovery from the dilute solutions.During the past decade, the application of molecular techniques to the solventogenic clostridia—combined with recent advances in fermentation techniques — have resulted in the development of a hyper-butanol-producing strain and an integrated ABE fermentation system for the simultaneous production and removal of butanol from the fermentation broth (Ezeji *et.al.*, 2007). One technique for increasing butanol production is genetic alteration of *C. acetobutylicum* to make it more tolerant to butanol (Evans *et.al.*, 1988).

2.5 Ethanol

Ethanol, with its high (C+H) to O ratio, retains most of the original energy content in combustion. Ethanol does not need to be rectified to high purity if it is to be used as a fuel (Bowles *et.al.*, 1985). Ethanol also called ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol is a volatile, flammable, colorless liquid. It is a powerful psychoactive drug, best known as the type of alcohol found in alcoholic beverages and in modern thermometers.

Ethanol is one of the oldest recreational drugs. In common usage, it is often referred to simply as alcohol or spirits. Ethanol, a traditional biofuel, is not an ideal fuel due to its high hygroscopic and low energy density, which increased the difficulty involved in and expense of its storage and distribution (Wu *et.al.*, 2003).

The fermentation if sugar into ethanol is one of the earliest organic reactions employed by humanity. The intoxicating effects of ethanol consumption have been known since ancient time. In modern times, ethanol intended for industrial use is also produced from byproducts of petroleum refining. Ethanol is a volatile, colorless liquid that has a strong characteristics odor. It burns with smokeless blue flames that are not always visible in normal light.

2.6 Advantages of butanol from ethanol

The alcohol based fuel including butanol and ethanol is partially oxidized (compared to hydrocarbons) and therefore mixture for engine should be more enriched than in the case of gasoline. As compared with ethanol, butanol can be used as a mixure with gasoline in higher proportion and thus can be used in currently working cars without modifi cation of their system for the formation of air–fuel mixture. The alcohol based fuel

contains less energy per unit of weight or volume than gasoline and its mixture with air should be more enriched. Per one cycle of engine running, butanol liberates more pure energy than ethanol (Hwang *et.al*, 2004) (R.Gheshlaghi *et.al*, 2009).

There are several advantages of butanol over ethanol:butanol contains 25% more energy than erthanol : 110 000 Britain heat units per gallons as compared with 84 000. Britain heat units per one gallons of ethanol. Energy content of gasoline is about 115 000 Britain heat units per gallons. Butanol is safer because is evaporated six times less than ethanol and by factor 13.5 less volatile than gasoline. Its vapor pressure by Reid is 0.33 pounds per squire inh,the same characteristic of gasoline is 4.5 pounds per square inch, of ethanol 2.0 pounds per square inch. This makes butanol safe at its application as oxygenate, and need no significant changes in the mixture proportion at summer and winter application. Butanol is much less aggressive substances than ethanol and therefore it can be transported with the currently used fuel pipes, while ethanol should be transported by rail way transport (Hwang *et.al*, 2004).

Butanol also can be mixed with gasoline. Butanol can be used instead of gasoline, while ethanol can be used as additive to benzene with content of the latter in the mixture not less than 85% and requires significant modernization of engine. Currently, predominantly are used mixtures with content of ethanol 10%. Production of butanol can simultaneously solve problems that connected with the infrastructures of supplying hydrogen. Besides that, butanol provides higher yield (10Wt-h/g) than ethanol (8Wt-h/g). At combustion, butanol does not produce sulfur and nitrogen oxides that are advantages from the ecology viewpoint.

Thus, biobutanol allows solving a part of problems retarding the fast application of biofuel over the world, in part; it is compatible with existing car fuel and with infrastructure of logistics. Low vapor pressure of biobutanol and its low sensitivity to the presence of water in its mixture with gasoline extend its versatility. It can be mixed with gasoline to higher concentration than existing biofuels without modernization of engines, economically it is preferred than mixture of ethanol with gasoline, it improves efficiency of cars in respects of fuels and increases run per a unit of consumed fuel.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Strain

The solvent – producing – bacterium, *C.acetobutylicum* (NCIMB 13357) was used throughout this study. This strain was obtained from Universiti Kebangsaan Malaysia (UKM). The culture was kept in the freezer.

To strike the bacteria on Petri dish, the anaerobic chamber was used to maintain anaerobic condition. Then from the pure stock *C acetobutylicum* was inoculated to the plate in three parallel lines about 5mm distance from each others. After striking the bacteria on Petri dish, it was sealed parafilm. Then it will incubated in the incubator at 37°C for 2 days.

3.1.2 Preparation of Medium

3.1.2.1 POME

Samples of POME were obtained from Kilang Sawit Felda Lepar Hilir,Lepar Hilir, Pahang. Fresh POME was sediment passively in a chiller at 4°C for 24 hours before use. The supernatant layer (upper layer) was decanted and sediment POME sludge (lower part) was use as the medium of the fermentation. The POME pH must be maintained at pH 5.8 before the fermentation process start. POME was autoclaved at 121°C for 20 minutes.

The sample was centrifuge to separate the particle in the sample. The supernatant from the sample of POME was taken to check the component of POME.

3.1.3.2 RCM

Reinforced Clostridia Medium was used as the control medium. The medium was prepared by dissolving 38 g of the powder in 1 liter of distil water and then mix it completely. RCM also must be autoclaved at 121°C for 20 minutes.

3.1.3.3 Agar Medium

Reinforce Clostridia Agar was prepare by dissolving 52.5g of the powder in 1 liter of distilled water and then transfer it into schott bottle. After that, bring to the boil to

dissolve completely. Then, the agar medium was sterilized by autoclaving at 121°C for 20 minutes. After autoclave the medium, the medium was cooling at room temperature. Then a tube of melted agar was poured into a sterile petri dish. The dish level was kept until the agar has hardened. Then agar medium was prepared by dissolving 38g of the powder into 1L distilled water.

3.2 Inoculums

The bacterium was cultivated in anaerobic condition in Reinforced Clostridia Media (RCM). The strain was in the glycerol stock. From the glycerol stock vial, the toothpicks was used and dipped it into the stock. Then, 1 portion of the stock was transferred into the broth medium. The broth will be incubated for 24 hours in incubator at 37°C. After a day, the broth medium will inoculate on the agar plate. Only the inoculums with OD values that greater than 0.7 will be used as inoculums. 10% v/v from the inoculums will be used in the fermentation process.

3.3 Fermentation

ABE production of *C.acetobutylicum* was studied in 500 mL schott bottles. Working volume for this fermentation were 90%. Sediment POME was diluted with distilled water to obtain required concentration before deoxidizing by gassing with nitrogen gas for a few minutes. The RCM and POME used directly as fermentation medium without additional nutrients. After autoclaving, the fermentation broth was spurge with oxygen-free nitrogen. Flushing by the N_2 gas was limited to the headspace of the fermenter before and after inoculation and was terminated as soon as the cells started to

produce the fermentation gases.10% v/v of inoculum will transfer to 300mL of RCM (control medium) and 300 mL of POME in anaerobic chamber.

After the process transferred of inoculums, it was incubated at optimum temperature 35°C and at optimum speed 200rpm. For run 1 of the fermentation, it was maintained at optimal condition. Then every 20 hours of fermentation till to 72 hours, the sample will be taken to measure the optical density (OD). This is as for the determination of glucose concentration in the POME and RCM medium respectively.

For the next run which is run 2, 3 and 4, different temperatures were used in order to study the effect of temperature on the yielded butanol and ethanol. The temperatures that were used are 33^0C (run 2), 34^0C (run 3) and the 37^0C (run 4).

3.4 Liquid-liquid extraction (LLE) process

The sample of POME medium and RCM medium was taken for every 20 hours, 40 hours, 60 hours and 72 hours The samples were then centrifuge at 10,000rpm for 30minute to separate between solid and liquid. The liquid call as supernatant and solid call as pellet. The supernatant was used in this process. Then the supernatant of RCM and POME was entered in the different of separating funnel. Then each the sample was mix with universal solvent (toluene). The toluene used to absorb the butanol in the sample of POME and RCM. The process mixture was used the ratio 1:1 where the 15ml of supernatant POME entered in separating funnel and the 15ml of toluene entered in the same separating funnel. The 15ml supernatant of RCM also entered in different separating funnel follow by entered the 15ml of toluene in the same separating funnel.

After that, the sample was mixed and left the process in the separating funnel for 24 hours. Then after 24 hours, the sample from the separating funnel was taken and transfer into the biker. The the sample in the biker was adding by Na_2SO_4. The Na_2SO_4 was used to absorb the molecule of water that have in the sample. After adding the Na_2SO_4 the biker was closed tightly with aluminum fold and the sample was stored or that solution in chiller at 4°C for 24 hours. Then after 24hours the sample was filtered using syringe filter of 0.2µm and then the process for GC analysis was prepared.

3.5 Analysis of Fermentation Product

3.5.1 Growth Profile

Growth profile was done to check the growth of the medium (POME and RCM). The step was repeated as mentioned in 3.1.3.1 and 3.1.3.2. First, the both medium was prepared by the same method for preparation of POME and RCM. Then, the inoculums will be transferred to the POME and RCM medium. Next, the mix sample will be incubated in anaerobic chamber at 37°C for 72 hours. Every 6 hours, the sample will be taken and will be analyze using UV – Vis to check the growth. The OD value is obtained by using UV – Vis at 680nm of wavelengths. The graph for its growth then plotted.

3.5.2 Composition in POME

To analyze the component of POME high performance liquid chromatography (HPLC) using capillary column Supelcosil LC-NH_2. The flow rate that used is 1ml/minute and the retention time around 15 minutes. The mobile phase for this HPLC is Acetonitrile and water. The ratio of mobile phase is 75% of Acetonitrile and 25% of water. The

standard that used to detect Glucose, Galactose, Fructose, Xylose, and Lactose in the sample of POME is the pure solution of Glucose, Galactose, Fructose, Xylose, and Lactose. The calibration curve for each sugar (Glucose, Galactose, Fructose, Xylose, and Lactose) was prepare for 20g/L,40g/L,60g/L,80 g/L and 100g/L.

3.5.3 Solvents Production

During the fermentation samples were withdrawn at appropriate time intervals for analysis. The concentrations of solvent and organic acids were determined by using gas chromatography equipped with the flame ionization detector (FID).

To analyze the composition of butanol and ethanol Gas Chromatography (GC-FID) was used. 10 micro liter of sample POME and RCM was mix with 990 micro liter of Hexane. After mixing the sample with thehexane, the sample was transferred into vial by using filter of 0.2µm. The standard that used to detect concentration of butanol in the sample is butanol solution. The standard solution of butanol also was filter by using 0.2µm filter before it entered into the vial.

3.5.4 Preparation of 3, 5 – Dinitrosalicylic acid (DNS) reagent

The 3,5 - Dinitrosalicylic acid(DNS) reagent is prepared in the following way, 20gram 3,5 – Dinitrosalicylic acid is suspended in 400 ml water. While continuously stirring 300 ml sodium hydroxide solution is added (32g/300 ml water) and the volume is subsequently adjusted to 1.5 L by the addition of water. Stirring is continued until a clear solution is obtained. Next 600 Rochelle salt (sodium potassium tartrate) is added and stirring (and, if necessary, heating) is continued until dissolution. The volume is adjusted to

2 L and the solution filtered, if necessary. The solution is kept at room temperature in the dark and protected against carbon dioxide absorption. The reagent is stable for at least one month.

3.5.4.1 Determination of the amounts of reducing sugar

After enzymatic hydrolysis was stopped, 3 ml DNS was added with 3 ml of sample (POME and RCM). The amount of reducing sugar groups were determined by heating the test tubes in boiling water at 90°C for 10 minutes, cooling to room temperature, and recording the OD at 540nm.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

Fermentation process was done in schott bottle in order to produce the butanol and ethanol from *C. acetobutylicum*. All the condition or parameter have been controlled and set constant. Butanol has been produced at different temperature of 33°C, 34°C, 35°C and 37 °C by adding distilled water to dilute the substrate concentration according to its percentage. In this study, it consist 4 types of analysis –for the profile growth rate, UV – Vis was used, for POME analysis, using High Performance Liquid Chromatoghraphy(HPLC), butanol and ethanol production using Gas Chromatoghraphy Flame Ion Detector (GCFID), and glucose concentration using Uv-Vis.

4.1.1 Analysis 1: Growth Profile

Table 4.1 OD reading for growth profile of *C. acetobutylicum* in POME

Hours	OD Readings
6	0.345
12	0.576
18	0.767
24	0.815
30	0.826
36	0.836
42	0.798
48	0.82
54	0.857
60	0.827
66	0.823
72	0.825

Table 4.2 OD reading for growth profile of *C.acetobutylicum* in RCM

Hours	OD Reading
6	0.28
12	0.438
18	0.76
24	0.792
30	0.807
36	0.874
42	0.971
48	1.008

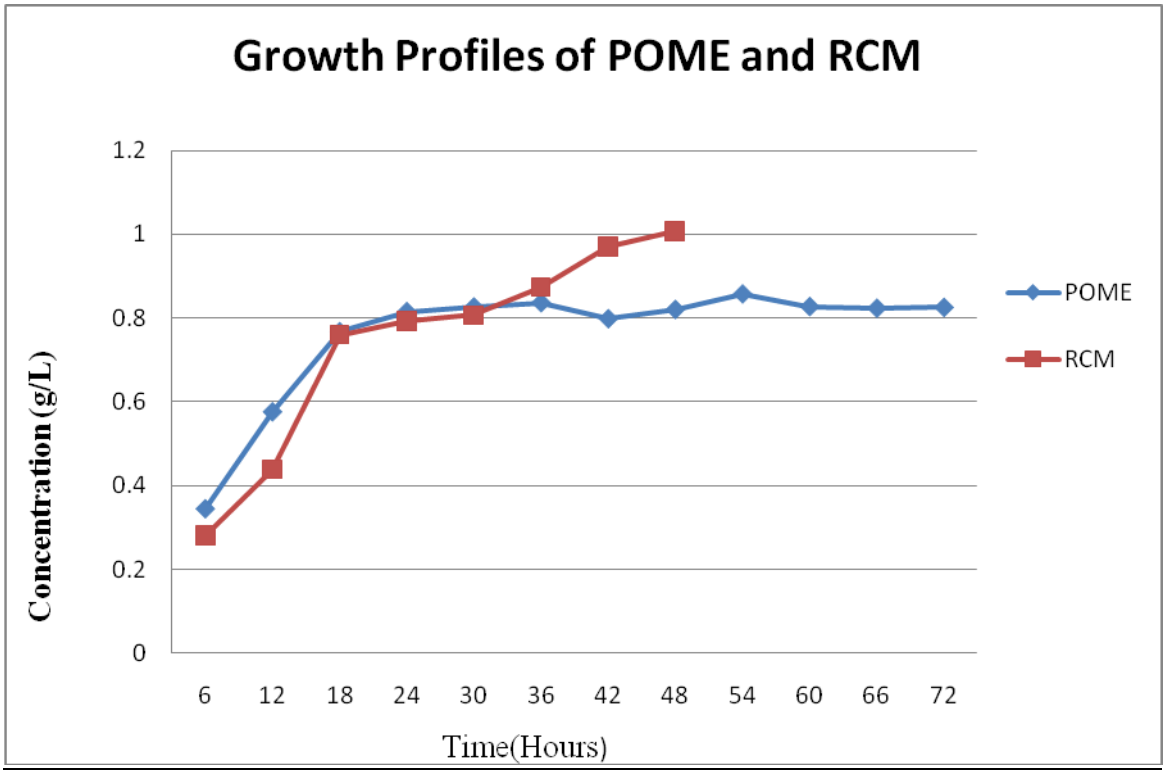

Figure 4.1 Growth profiles of POME and RCM

The analysis of the growth profile for each medium was done to see the patterns of the bacteria growth. From the Figure 4.1, the patterns of the reading are slightly different for the both of the samples. The graph is divided into three phases which are exponential, stationery and death phase. The exponential phase starts from 0 hour to 18th hours. After that, the graph is fluctuating before reaching the stationary. The graph entering the final phase, death phase after 72 hours.

The *C.acetobutylicum* becomes active for 18 hours for the POME medium. This is where during that time; *C.acetobutylicum* fully utilizes the availability of nutrients. This phase is called as exponential phase because during this period of time the microorganisms are growing and dividing at the maximum rate possible given their genetic potential, the nature of the medium and the conditions under which they are growing. The rate of growth is constant during the exponential phase that is the microorganisms are dividing and doubling in number at regular intervals. The population is most uniform in terms of chemical and physiological properties during this phase

After that, the graph is fluctuating before entering the stationary phase. Eventually the population growth ceases and the growth curve becomes horizontal. The population size depends on nutrient availability and other factors. In the stationary phase the total number of viable microorganisms remains constant. This may result from a balance between cell division and cell death or the population may simply cease to divide though remaining metabolically active. Microbial populations enter the stationary phase for several reasons. One of the factors is nutrient limitation. When the availability of the nutrients is severely depleted, population growth will slow. For the aerobic microorganisms, oxygen limitation is also one of the factors. Oxygen is not very soluble and may be depleted so quickly that only the surface of a culture will have an oxygen concentration adequate for growth. Population growth is also cease due to the accumulation of toxic waste products

The last phase is death phase. Detrimental environment changes like nutrient deprivation and the buildup of toxic wastes lead to the decline in the number of viable cell characteristics of the death phase.

The growth of bacteria in RCM were more higher if compared to POME.For the RCM, the growth also active at 18 hours. The growth increasingly until 48 hours. The graph might be stabale after 48 hours because if referred from previous studies, (Stephens *et.al.,* 1985) shows that the value of the OD for growth profile of *C.acetobutylicum* were in range 0.6 to 0.99. After that range, the bacteria will face the dead lag phase where most of them will be dead.

4.1.2 Analysis 2: POME Analysis

Table 4.3 Concentration of each sugar groups in POME

Sample	Sugar Group	Concentration(g/L)
POME	Fructose	1.967
	Glucose	3.668
	Galactose	8.859
	Sucrose	2.24
	Lactose	1.313

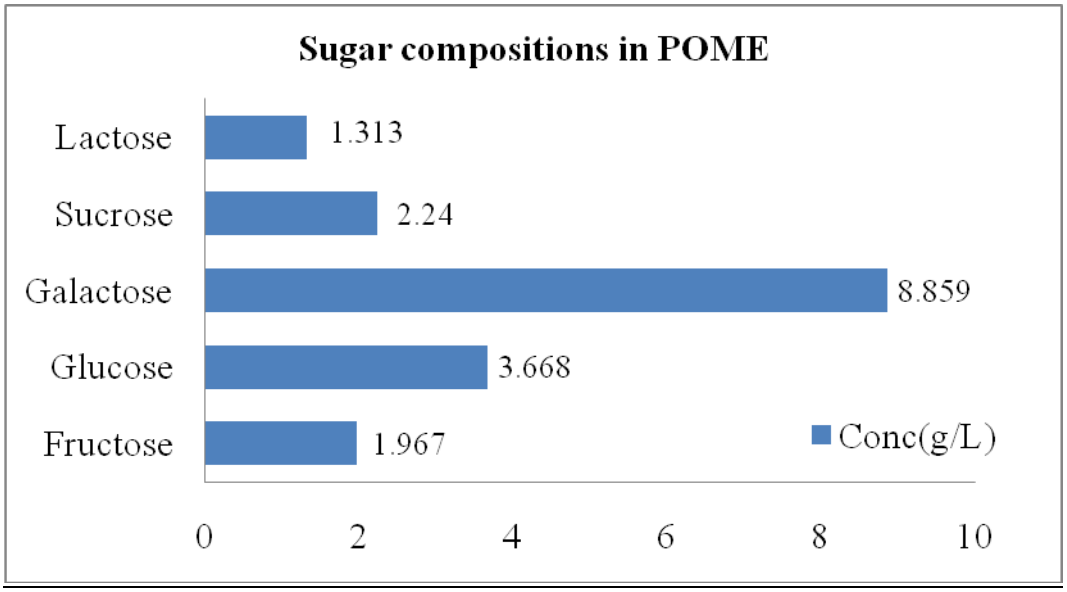

Figure 4.2 Composition of each sugar group in POME

From the HPLC analysis, it showed that fructose, glucose, galactose, sucrose, lactose are the sugars group that were obtains in POME. During these fementations glucose, fructose, galactose, sucrose, and lactose utilizations were 3.668, 1.967, 8.859,2.24, 1 and 1.313 g / L. respectively. In the glucose fermentation. all of the glucose present in the system was used. The highest components that this sample has are glucose which is consumed 6.116 g / L and the lowest is lactose – 1.0198 g / L.

4.1.3 Analysis 3: Solvents Production

Table 4.4 Results for butanol and ethanol production

TEMP (°C)	BUTANOL IN POME (g/L)	ETHANOL IN POME (g/L)	BUTANOL IN RCM (g/L)	ETHANOL IN RCM (g/L)
33	0.156	58.51	0.52	49.65
34	0.143	51.6	0.31	51.52
35	0.137	68.93	0.116	79.046
37	0.133	59.63	0.103	92.42

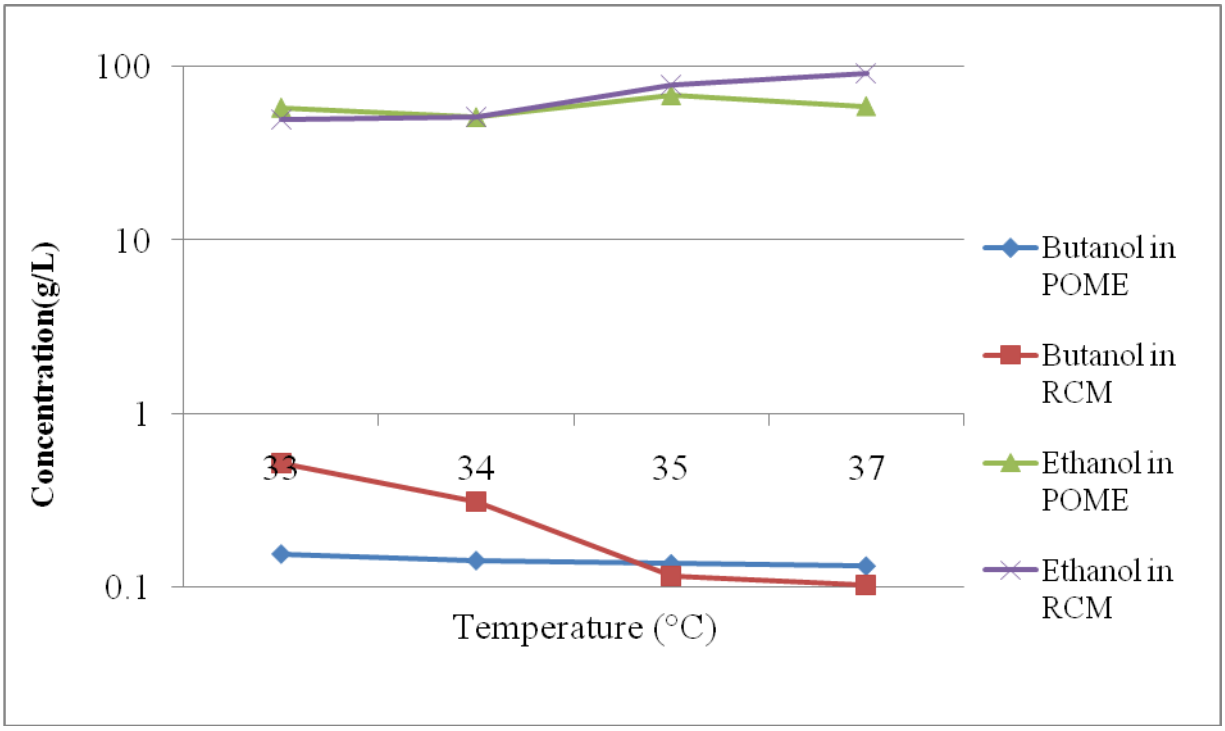

Figure 4.3 Butanol and ethanol production

Yielded butanol and ethanol was determined by Gas Chromatography equipped with flame – ionization detector (GCFID). From the Figure 4.3, it is showed that butanol production decreased when the temperature increased. The higher concentration for butanol in POME is only 0.156 g/ L. Butanol was found to be the primary toxic substances in the acetone – butanol fermentation and it inhibited cell growth by 50% (Huang *et.al.*, 1986). This is also maybe because of the product inhibition. Organic acid can be as inhibitory as butanol if they are present in the same concentration ranges – because acids are not easily removed by separation. Toxicity is defined here as the inhibition of growth of *C. acetobutylicum*. Circumvention of extractant toxicity has resulted in the use of nontoxic extractants that have the highest distribution coefficients or the use of membranes to separate toxic organic liquids from the broth (Evans *et.al.,* 1988).

End-product inhibition, low product concentration and large volumes of fermentation broth, the requirements for large bioreactors, in addition to the high cost involved in generating the steam required to distil fermentation products from the broth largely contributed to the decline in fermentative acetone-butanol-ethanol (ABE) production has been shown to be one technique that enhances solvent production in ABE fermentation by reducing end product inhibition by butanol (Ishizaki *et.al.*, 1999).

Too many subcultures will decrease the inoculums productivity. The strain used up less sugar and produced progressively less butanol and isopropyl alcohol and more butyric acid. In clostridia fermentation, the sporulation occurs concominatly with the solventogenesis (Gapes *et.al.*,1983). Sporulation makes the bacterial cells enter a dormant state where they lose the ability to produce solvents. It is likely that there is a relationship between sporulation and solventogenesis, given that many early molecular connected with sporulation appear in the initiation of solventogenensis. If this relationship is revealed, it may be possible to produce more solvents, including butanol by preventing the clostridia from forming spores (Zheng *et.al.,* 2009). When repeated serial subculturing was done, the cells abruptly lost their ability to use up the glucose in the medium and to accumulate a normal level of solvents the culture also will failed to form spores and even after shock heating, no growth will occurred (Assobhei *et.al.*, 1998).

4.1.4 Analysis 4: Glucose consumption

Table 4.5 Glucose consumption during fermentation within certain interval hours

Temp (°C)	POME				RCM			
	Concentration (g/L)				Concentration (g/L)			
	20 hr	40 hr	60 hr	72 hr	20 hr	40 hr	60 hr	72 hr
33	4.2	1.9	1.8	1.5	6.49	6.04	5.85	5.3
34	9.6	9.0	8.5	6.5	6.3	4.7	3.8	3.2
35	6.9	6.0	7.1	6.7	9.0	8.5	6.5	6.7
37	3.1	2.7	1.6	0.3	3.1	1.8	0.6	0.2

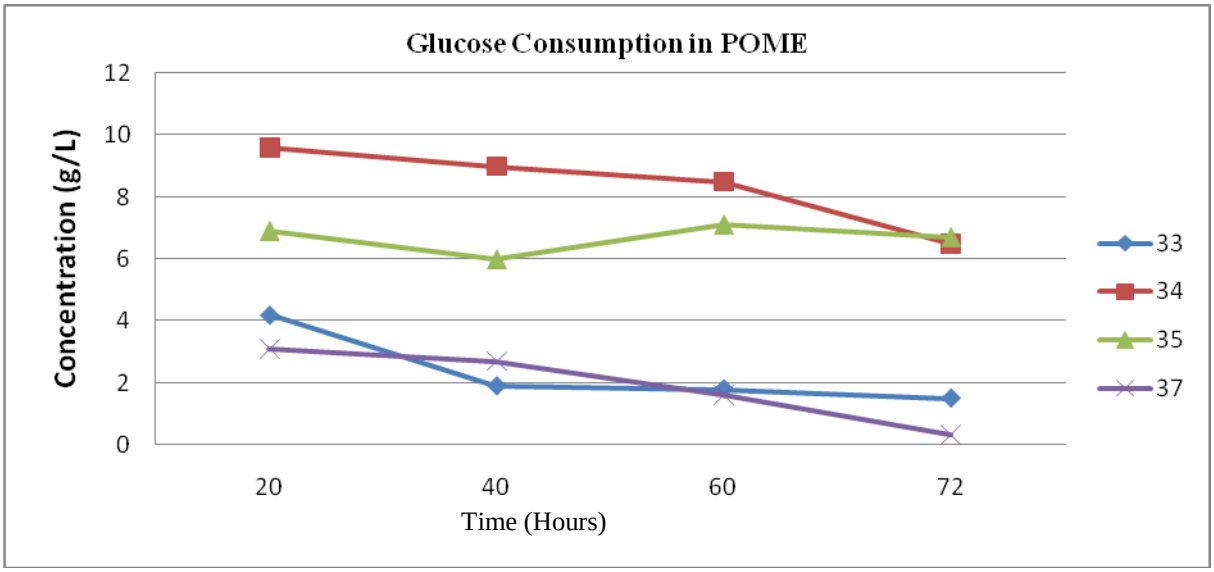

Figure 4.4 Glucose consumption in POME during fermentation within certain interval hours

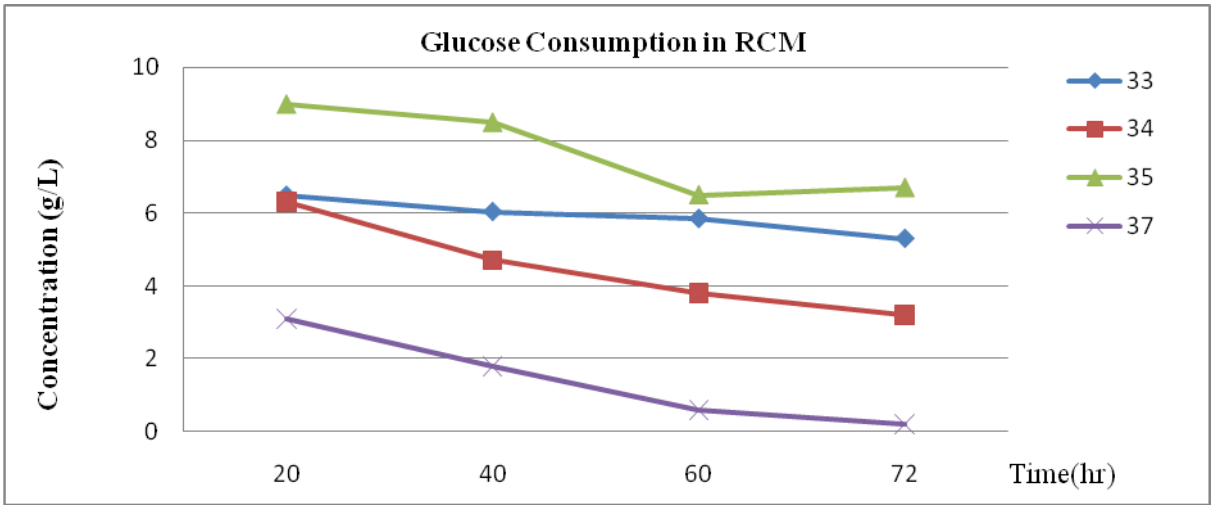

Figure 4.5 Glucose consumption in RCM during fermentation within certain interval hours

From Figure 4.4 and Figure 4.5, the concentration of the glucose that left will be decreased. It is because the glucose was used in the fermentation process as a nutrient growth. It also showed that the fermentation process had occurred. Cell growth was complete in 20 to 30 hours and solvent production was completed within 35 to 55 hours. In batch fermentation, there will be no addition of glucose or nutrient addition. This also can give effects to the butanol and ethanol production. A high sugar concentration will give the toxicity for the solvent (Evans *et.al.*, 1988).

The sugar concentration in the batch can medium becomes limiting, which results in a slower cellular growth, a slower metabolic transition from an acid to a solvent fermentation and, thus, a higher accumulation of acids. It is only at sufficiently high feeding rates that fed-batch fermentations yield kinetic results comparable to those of batch fermentations.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the result, it is showed that the fermentation process utilized glucose, galactose, and sucrose that produced different amounts of ABE. The highest sugar group within the POME has is galactose (8.859 g/L). It is showed that butanol and ethanol production were decreased when the temperatures increased.The highest yielded butanol is 0.156 g/L and for ethanol, the higher yielded is 68.93 g/L. Biobutanol production still has some limitations including butanol toxicity to culture leading to low butanol yields. On the other hand, growth at lower temperature can be improved the ability of the both strains to tolerate with butanol and produced higher yield. The optimum temperature that suitable for this process is 35°C.These results also conclude that POME is the viable substrate for ABE fermentation.

5.2 Recommendations

A high sugar concentration is toxic to the culture. Fed – batch fermentation is an industrial technique – concentrated sugar solution can be fed into the reactor while keeping the sugar concentration inside the reactor below the toxic level (Qureshi *et.al.*, 2001). During inoculums process – the strain need to be subcultured only twice. (Kutzenok *et.al.*, 1985).

One technique for increasing butanol production is genetic alteration of *C. acetobutylicum* to make it more tolerant to butanol. A number of attempts have been made in this regard with varying degrees of success. How much improvement can be accomplished is questionable though, because low-molecular-weight compounds such as butanol may not have specific sites of toxicity. Butanol may localize in the plasma membrane and disrupt a number of physiological processes including membrane permeability, solute transport, maintenance of the proton motive force, and conformation and activity of intrinsic membrane proteins.

Another technique is alteration of the environment rather than the cell. The toxic or inhibitory product can be removed simultaneously from the broth as it is formed. This technique is known as extractive fermentation. One approach is to add to the broth an immiscible organic liquid that has a high distribution coefficient for butanol. A general problem inherent with liquid extractants is that liquids with high butanol distribution coefficients are toxic, and nontoxic liquids have low distribution coefficients. Toxicity is defined here as the inhibition of growth of *C. acetobutylicum*. Circumvention of extractant toxicity has resulted in the use of nontoxic extractants that have the highest distribution coefficients or the use of membranes to separate toxic organic liquids from
the broth

In situ solvent extraction during fermentation can also become one of the technique that enhances solvent production in ABE fermentation by reducing end product inhibition by butanol (Ishizaki *et.al*, 1999). Increased productivity could be achieved in a single-stage continuous fermentation when operated at low pH and dilution rate (Lepage *et.al.*, 1998).

In order to increase product yield, one of the several options that may be tried is using the immobilized cells. This might be due to cells being at late log phase when harvested, where most of them were in the solventogenic phase (ready for solvent production). Acidogenic phase will occur first where cells produce organic acids (acetic and butyric acids) followed by solventogenic phase where cells assimilate the organic acids formed to produce solvents. (Khalil *et.al.*, 2003) The same beads also can be used at least 5 times for the ABE fermentation compared to liquid broth that we used in this project that can be use at least twice.

The other possibility to improve the traditional process is to reduce costs of product recovery. Membrane – based systems, such as reverse osmosis, per traction, evaporation, and membrane evaporation, as well as liquid/liquid extraction, adsorption and gas stripping. Membrane – based systems showed a high selectivity for solvents, but might suffer from clogging and fouling and seem to be more suited for use with immobilized cells. Liquid / liquid extraction also has a high selectivity, but emulsions might form that render the process less suitable. On the other hand, gas stripping does not lead to complete removal of solvents from clogging or fouling by biomass. All these procedures can be designed to allow on – line product recovery, so that butanol toxicity is reduced.

It is suggested that the organism further be developed to hydrolyze xylose and utilize all of its sugars simultaneously. This would further increase productivity of the reactor. It is also suggested that hydrolysis of concentrated POME followed by fermentation and recovery be performed in a single reactor (Qureshi *et.al.*, 2006).

Last but not least, the process development to achieve an economical and efficient can be done so that days by days, we can find the solution and way to produce the higher yield for butanol and ethanol production.

REFERENCES:

Abdullah, A.Z, Ibrahim,M.H, and A.KAdir,M.O (Jun 2004), Treatment of Palm Oil Mill Effluent (POME) supernatant using Aerobic Attached – Growth System: Trickling Filter as a Case Study, *Jurnal Teknologi*, 40(F) : 77 – 90.

Abu H,M.A and Puteh,M.H (2007), Pre – treatment of Palm Oil Mill Effluent (POME) : A Comparison Study using Chytosan and Alum. *Malaysian Jurnal of Civil Engineering* 19(2) : 128 – 141.

Antoni D, V.Zverlov V, and H. Schwarz W, (2007) Biofuels from Microbes, *Appl Microbiol Bioehanol* 77:23 – 35.

Assobhei,O and Kanouni,A.E, (1998) Effects of Acetic Acid and Butyric Acids on the Stability of Solvents and Spore Formation by *Clostridium acetobutylicum* ATCC 824 during Repeated Subculturing, *Journal of Fermentation and Bioengineering*, Vol. 85, No 2, 209 – 212.

Baut,F, Fick,M, Viriot,M.L, Andre,J.C, and Donner,M (1994), Investigation of Acetone – Butanol – Ethanol (ABE) Fermentation by Fluorescense, *Journal of Flourescence*, Vol 4, No 1.

Bowles,L.K and Ellefson,W.L(1985), Effects of Butanol on *Clostridium Acetobutylicum*. *Applied and Environmental Microbiology*,, p 1165 – 1170.

Chen,W.M, Tseng,Z.J, Lee,K.S and Chang,J.S(2005), Fermentative Hydrogen Production with *Clostridium Butyricum* CGS5 Isolated from Anaerobic Sewage Sludge, *International Journal of Hydrogen Energy* 30 1063 – 1070.

Cheng,C.K, Hajar H.H, and Ku,S.K.I, (2007) Production of Bioethnaol from Oil Palm Empty Fruit Bunch,

Durre,P (1998), New Insights and Novel Developments in Clostridial Acetone – Butanol – Isopropanol Fermentation.*Appl Microbiol Biotechnol* 49 : 639 – 648.

Evans,P.J and Wang,H.Y (1988), Enhancement of Butanol Formation by *Clostridium acetobutylicum* in the presence of Decanol – Oleyl Mixed Extractants. *Applied and Environmental Microbiology*,p 1662 – 1667.

Ezeji,T.C., Qureshi,N and Blaschack,H.P (2005), Continous Butanol Fermentation and Feed Starch Retrogradation : Butanol Fermentation Sustainability using *Clostridium Beijerinckii* BA 101.*Journal of Biotechnology* 115 179 – 187.

Ezeji,T.C, Qureshi,N, and Blaschack,H.P (2007), Bioproduction of Butanol from Biomass : from Genes to Bioreactors. Current Opinion in Biotechnolgy 18 : 220 – 227.

Guo-qing,H, Ding,K, He,C.Q, and Hui,R (13 July 2005), Batch and Fed Batch Production of Butyric Acid by *Clostridium Butyricum* ZJUCB. Journal of Zheijang University Science.

Huang,L, Forsberg,C.W, Gibbins,L.N (June 1986), Influence of External pH and Fermentation Products on *Clostridium Acetobutylicum* Intracellular pH and Cellular Distribution of Fermentation Products. Applied and Environmental Microbiology, p 1230 – 1234.

Hwang,M.H, Jang,N.J, Hyun,S.H, and Kim,I.S (2004), Anaerobic Biohydrogen Production from Ethanol Fermentation : The Role of pH. *Journal of Biotechnology* 111 297 – 309.

Ishizaki,A, Michiwaki,S, Crabbe,E, Kobayashi,G, Sonomoto,K, and Yoshino,S (1999), Extractive Acetone – Butanol – Ethanol Fermentation using Methylated Crude Palm Oil as Extractant in Batch Culture of *Clostridium Saccharoperbutylicum* N1 – 4 (ATCC 13564). *Journal of Bioscience and Bioengineering*.

Gapes,J.R,(2000), The Economics of Acetone – Butanol Fermentation : Theoritical and Market Considerations, *J. Mol. Micribiol. Biotechnol* 2(1):27 – 32

Khalil,M.S, Kit,P.W, W.Yusoff,W,M, Sadazo,Y, and Ab Rahaman, R (2003), Direct Fermentation of Palm Oil Mill Effluents to Acetone – Butanol – Ethanol by Solvent Producing Clostridia. *Pakistan Journal of Biological Sciences* 6 (14) : 1273 – 1275.

Kanouni,E.A, Zerdani,I, Zaafa, S, Znasnni,M, Loutfi,M,and Boudama,M,(1998), The Impovement of Glucose / Xylose Fermentation by *Clostridium acetobutylicum* using Calcium Carbonate.

Khathiravale,S,and Ripin,A (July 1997) , Palm Oil Mill Effluent Treatment Towards Zero Discharge. *National Science and Technology Conference*.

L.A.R Sallam, El – Refai,A.H, El – Zanati,A.E, Allam,R.F, Shafei,M.S, and El – Ardi,O.A.M (2003), Mathematical Modelling and Simulation of Batch Ab Fermentation with immobilized Cells of *C.Acetobutylicum.*

Liew,S.T, Arbakariya, Rosfarizan,M,and Raha,A.R (2006), Production of Solvent (ABE) in Continous Fermentation by using *C.Saccharobutylicum* DSM 13864 using Gelatinized Sago Strach as a Carbon Source. *Malaysian Journal of Microbiology*, Vol 2(2),pp 42-50.

Masngut,N, Takriff,M.S, Mohamad,A.W, Kamil,,M.S, and Khadum,A.A.H (Dis 2007). Performance of Oscilatory Flow Reactor and Stirred Tank Reactor in Solvent Fermentation from Palm Oil Mill Effluents. *Journal Technology*, 47 (F) Keluaran Khas,: 45 – 54.

Monot,F, Martin,J.R, Pttidemage,H, and Gay,R(June 1982), Acetone and Butanol Production by *Clostridium Acetobutylicum* in Synthetic Medium.*Applied and Environmental Microbiology*, p 1318 – 1324.

Napoli, F, Oliveri,G, Russo,M.E, and Marzocchella,A,(2009) Production of Biobutanol by *Clostridium Acetobutylicum*, *Universita Degli Studi Di Napoli "Federico II*" – April 26 – 28..

Ngam,M.A, Morimoto,M(Malaysian Palm Oil Berhad), Tanisho,S(Yokohama National

University), and Yoshino,S(Kyushu University),(2004), Conversion Technology of Biomass into Bioenergy.

Nik Sulaiman,N.M, and Ling,C.K (2004),Membrane Ultrafiltration of Treated Palm Oil Mill Effluents(POME).*Journal Technology, 41(F) Keluaran Khas*: 113 – 120.

Pakkila,J, Hillukkala,T, Myllykoski,L, and Keiski,R.L (June 2009), An anerobic Bioreactor System for Biobutanol Production.

Qureshi,N, and Blaschack, H.P, (2001), Recent Advances in ABE Fermentation : Hyper-Butanol Producing *C.Beijerinckii* BA 101. *Journal of Industrial Microbiology & Biotechnology* (2001) 27, 287 – 291.

Patova.P, J.Lipovsky, M.Linhova, P.fribert, M.Rychtera, and K.Melzoch, Development of Butanol Production Process using *Clostridium* Fermentation. *New Biotechnology*,Volume 255,September 2009.

R.Gheshlaghi, (2009) J.M. Scharrer, M.Moo Young, and C.P,Chou. Metabolic Pathways of *Clostridia* for Producing Butanol. *Biotechnology Advances* 27, 764 – 781.

Shapovalov.O.I, and l.A. Ashkinazi. Biobutanol : Biofuel of Secong Generation. Russian *Journal of Applied Chemistry*, 2008. Vol 81, No 12, pp. 2232 – 2236.

Shinto,H, Tashiro,Y, Yamashita,M, Kobayashi,G, Sekiguchi,T, Hanai,T, Okomato,M, and Sonomoto,K, Kinetic Modelling of Metabolic Pathways on ABE fermentation using Novel Simulator.

Somrutai,W, Takagi,M, and Yoshida,T, (1996), Acetone – Butanol Fermentation by *C.Aurantybutyricum* ATCC 17777 from a Model Medium for Palm Oil Mill Effluents. *Journal of Fermentation and Bioengineering*.

Stephens,G.M, Holt,R.A, Gottschal,J.C, and Morris,J.G, (1985), Studies on the Stability of Solvent Production by *C.Acetobutylicum* in Continous Culture, *Journal of Applied Biotechnology* 1985,59,597 – 605.

Wu, A.W.Mohammad, J.Md Jahim, and N.Anuar. Palm Oil Mill Effluent (POME) Treatment and Bioresources Reovery Using Ultrafiltration Membrane: Effects of Pressure on Membrane Fouling.

Zinadeth A.A.L, Mohamed,A.R, Mashitah,M.D, Abdullah,A.Z, and HAsnain,M.I (2007), Optimization of Pre – Treated Palm Oil Mill Effluents Digestion in an Up – Flow Anaerobic Sludge Fixed Film Bioreactor : A Comparative Study.*Biochemical Engineering Journal* 35, 226 – 237.

Zheng,Y.N, Li,L.Z, Xian,M, MA,Y.J, Yang,J.M, Xu,X, and De,D.Z, (2009), Problems with Microbial Production of Butanol. *J Ind Microbiol Biotechnol*.

APPENDIX A:

GROWTH PROFILE

Table A.1 : First reading at 11pm (Blank = 3.0)

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

Table A.2 : Second reading at 5am (Blank = 3.0)

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

Table A.3 : Third reading at 11am (Blank = 3.0)

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

Table A.4 : Fourth reading at 5pm (Blank = 3.0)

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

Table A.5 : Fifth reading at 11pm (Blank = 3.0)

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

Table A.6 : Sixth reading at 5am (Blank = 3.0)

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

Table A.7 : Seventh reading at 11am (Blank = 3.0)

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

Table A.8: Eighth reading at 5pm (Blank = 3.0)

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

Table A.9: Nineth reading at 11pm (Blank = 3.0)

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

Table A.10: Tenth reading at 5am (Blank = 3.0)

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

Table A.11: Eleventh reading at 11am (Blank = 3.0)

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

Table A.12: Tweleveth reading at 5pm (Blank = 3.0)

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

APPENDIX B:

STANDARDS FOR BUTANOL AND ETHANOL

B1 Ethanol

Table B 1: Concentration of ethanol versus area using GCFID

CONCENTRATION(g/L)	AREA
0.2	24.37853
0.4	52.41584
0.8	34.07329
1.0	153.71109
1.6	183.79024
2.0	233.67892

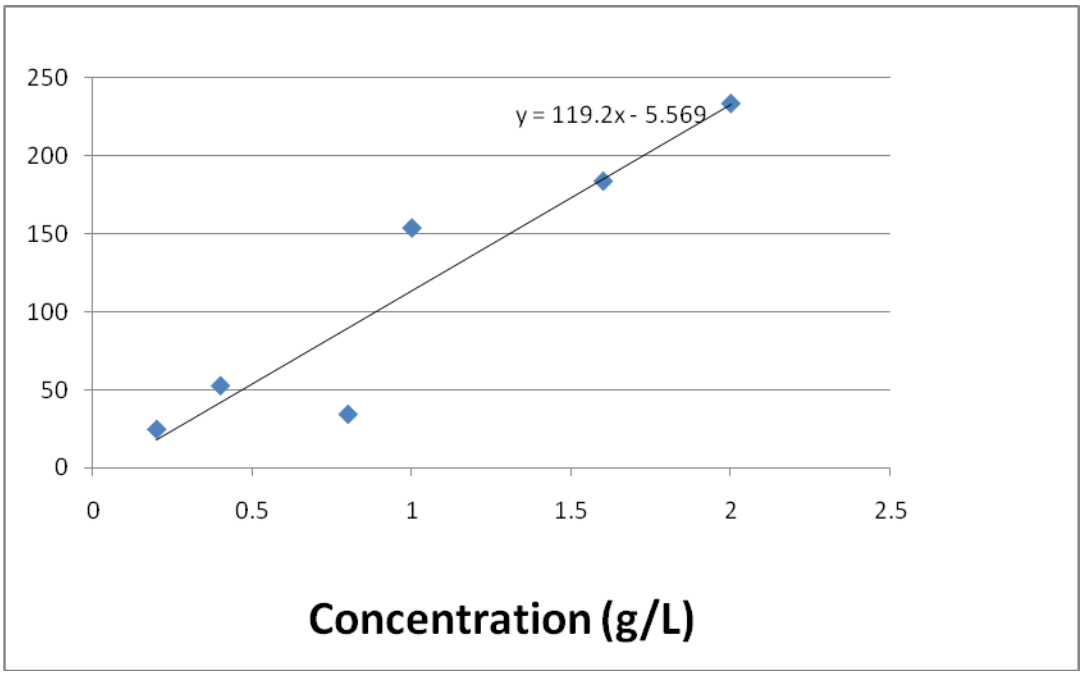

Figure B.1 Standard Curve for Ethanol

B2 Butanol

Table B 2: Concentration of butanol versus area using GCFID

CONCENTRATION(g/L)	AREA
0.1	27.94280
0.2	40.96081
0.4	106.17123

0.6	149.61745
1.0	217.72562
1.5	424.41580
2.0	472.72708
10	2798.79004

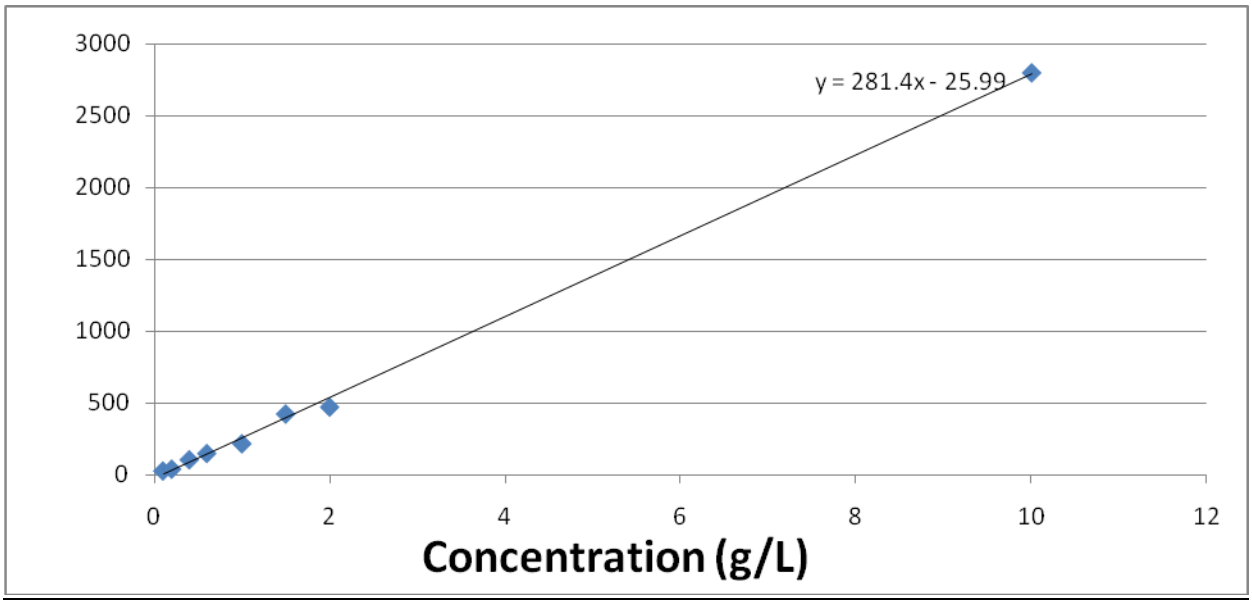

Figure B.2 Standard Curve for Butanol

APPENDIX C :

DNS ANALYSIS

C 1 : POME(Fermentation at 33°C)

Table C 1 1 Reading at 20 hours (Blank = 0.0)

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

Table C 1.2 Reading at 40 hours (Blank = 0.0)

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc (g/L)
X	2.366	2.387	2.387	2.38	0.96
1/10	0.522	0.513	0.517	0.517	0.18
1/20	0.158	0.155	0.154	0.155	0.03

Table C 1 3 Reading at 60 hours (Blank = 0.0)

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

Table C 1 4 Reading at 72 hours (Blank = 0.0)

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

C 2 : RCM (Fermentation at 33°C)

Table C 2.1 Reading at 20 hours (Blank = 0.0)

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X					

Table C 2 2 Reading at 40 hours (Blank = 0.0)

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

Table C 2 3 Reading at 60 hours (Blank = 0.0)

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

Table C 2.4 Reading at 72 hours (Blank = 0.0)

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

C 3 : POME (Fermentation at 34°C)

Table C 3. 1 Reading at 20 hours (Blank = 0.0)

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

Table C 3. 2 Reading at 40 hours (Blank = 0.0)

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

Table C 3. 3 Reading at 60 hours (Blank = 0.0)

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

Table C 3. 4 Reading at 72 hours (Blank = 0.0)

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

C 4 : RCM (Fermentation at 35°C)

Table C 4.1 Reading at 20 hours (Blank = 0.0)

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

Table C 4.2 Reading at 40 hours (Blank = 0.0)

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

Table C 4.3 Reading at 60 hours (Blank = 0.0)

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

Table C 4.4 Reading at 72 hours (Blank = 0.0)

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

C 5 : POME (Fermentation at 35°C)

Table C 5.1 Reading at 20 hours (Blank = 0.0)

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

Table C 5.2 Reading at 40 hours (Blank = 0.0)

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

Table C 5.3 Reading at 60 hours (Blank = 0.0)

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

Table C 5.3 Reading at 60 hours (Blank = 0.0)

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.599	2.599	2.599	2.599	-
1/10	2.049	2.049	2.049	2.049	0.71

1/20	0.6	0.6	0.61	0.6	0.14

Table C 5.4 Reading at 72 hours (Blank = 0.0)

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.617	2.617	2.617	-	-
1/10	2.015	2.015	2.015	2.015	0.67
1/20	0.7	0.7	0.7	0.7	0.11

C 6 : RCM (Fermentation at 35°C)

Table C 6.1 Reading at 20 hours (Blank = 0.0)

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

Table C 6.2 Reading at 40 hours (Blank = 0.0)

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

Table C 6.3 Reading at 60 hours (Blank = 0.0)

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

Table C 6.4 Reading at 72 hours (Blank = 0.0)

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.409	2.409	2.409	2.409	0.94
1/10	0.216	0.216	0.216	0.216	0.02

C 7 : POME (Fermentation at 37°C)

Table C 7.1 Reading at 20 hours (Blank = 0.0)4th Fermentation

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

Table C 7.2 Reading at 40 hours (Blank = 0.0)

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	3.0	3.0	3.0	3.0	-
1/10	2.319	2.319	2.319	2.319	0.9
1/20	1.779	1.779	1.779	1.779	0.68
1/40	-0.779	-0.779	-0.779	-0.779	-

Table C 7.3 Reading at 60 hours (Blank = 0.0)

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

Table C 7.4 Reading at 72 hours (Blank = 0.0)

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.699	2.699	2.699	2.699	-
1/10	2.149	2.208	2.208	2.208	0.85
1/20	1.095	1.094	1.093	1.094	0.31

1/40	0.5	0.499	0.499	0.4993	0.17

C 8 : RCM(Fermentation at 37°C)

Table C 8.1 Reading at 20 hours (Blank = 0.0)

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.517	2.517	2.517	-	-
1/10	2.015	2.015	2.015	2.015	0.67
1/20	0.8	0.8	0.8	0.8	0.08

Table C 8.2 Reading at 40 hours (Blank = 0.0)

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

Table C 8.3 Reading at 60 hours (Blank = 0.0)4th Fermentation

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

Table C 8.4 Reading at 72 hours (Blank = 0.0)4th Fermentation

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

APPENDIX D

STANDARD FOR OD VERSUS CONCENTRATION OF GLUCOSE

Table D 1 Reading concentration versus OD reading

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

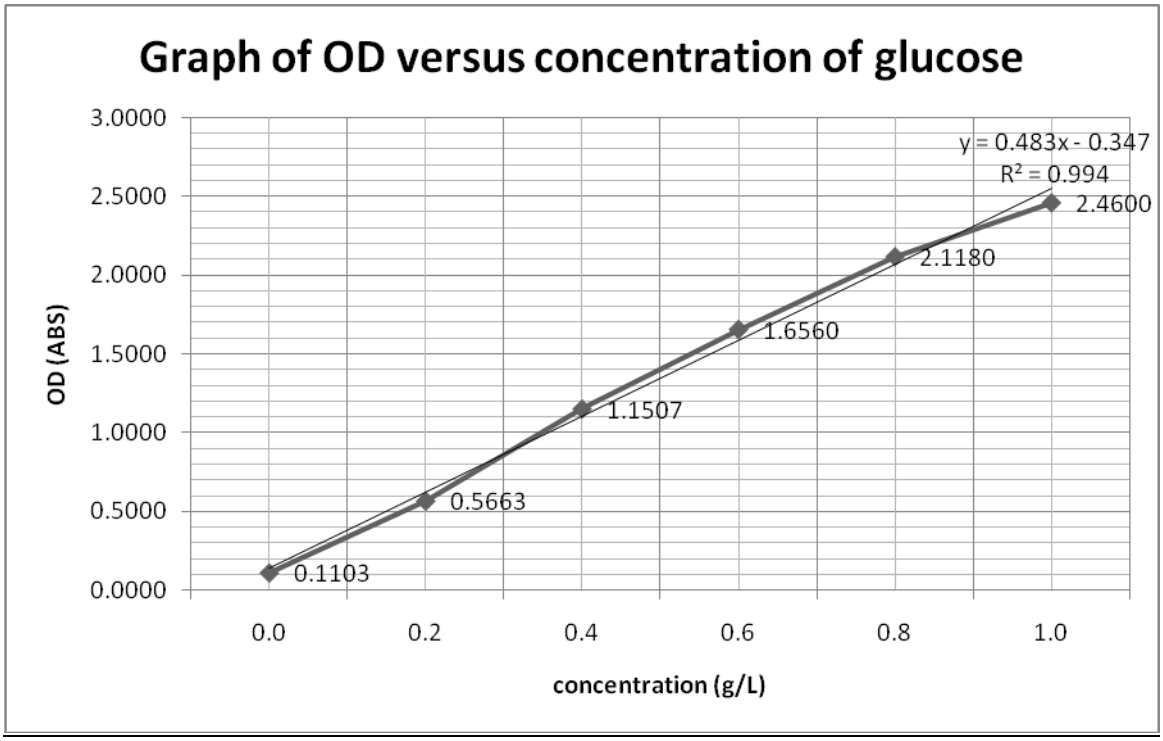

Figure D.1 Graph OD versus concentration of glucose

APPENDIX E

PICTURES

Figure E.1 POME medium

Figure E.2 RCM medium

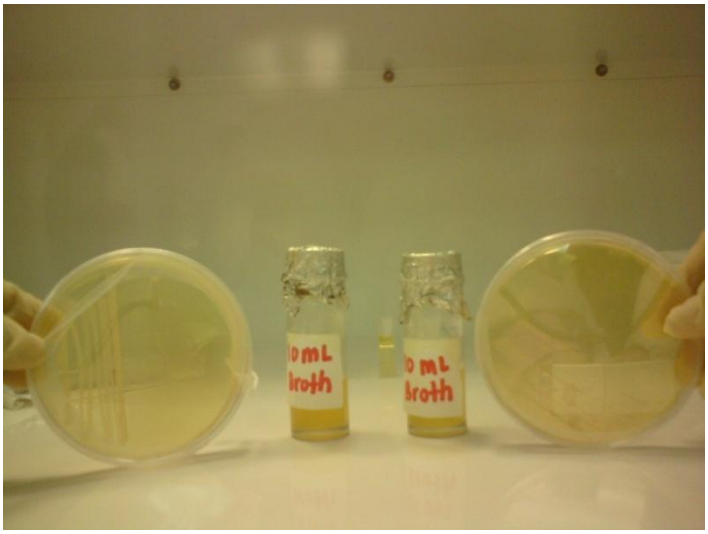

Figure E.3 Inoculated plate and broth media

Figure E.4 Sample vial before check the OD reading

Figure E.5: UV-Visible Single Beam Spectrophotometer

Figure E.6: Incubator Shaker

Figure E.7: Refrigerated Centrifuge 5810 R

Figure E.8 Anaerobic Chamber