UNIVERSITI MALAYSIA PAHANG

BORANG PENGESAHAN STATUS THESIS♦

JUDUL: EFFECT OF INOCULUMS CONCENTRATION, TEMPERATURE AND AGITATION RATE ON SOLVENT PRODUCTION FROM PALM OIL MILL EFFLUENT(POME) BY *CLOSTRIDIUM BEIJERINCKII* ATCC 51743

SESI PENGAJIAN: 2011/2012

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FATIMAH AZZAHRA' BT MISEBAH

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"I hereby declare that I have read this thesis and in my opinion this thesis has fulfilled the qualities and requirements for the award of Degree of Bachelor of Chemical Engineering (Biotechnology)"

Signature :……..

Name of Supervisor :Pn.Shariza bt Jamek

Date :…….

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A thesis submitted in fulfillment
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Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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JANUARY 2012

I declare that this thesis entitled “Effect of Inoculums Concentration, Temperature and Agitation Rate on Solvent Production from Palm Oil Mill Effluent (POME) by *Clostridium Beijerinckii*” 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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ABSTRACT

Few decades ago, world is totally depends on fossil fuel as energy source that currently most economically available source for personnel and commercial used leads in diminishing interest on development fermentation of biofuel industry. Unfortunately, due to constant conflict in the oil-supply regions and depletion of fuels oils supply resulted in viable of solventogenic fermentation industry in the world. Besides that, combustion of fossil fuel will produce various particle and gas that contribute to crises of environmental problem such as acid rain as well as greenhouse effect which is key factor in global warming. Thus, pressing need in reviewing the solventogenic fermentation of biofuel from a viable alternative sustainable and renewable energy resources that more renewable, more efficient and safer for environment. Unfortunately, the solventogenic fermentation needs to overcome high cost of production substrate together with low fermentation yield in order to compete with solvent production from fossil fuel. Palm oil mill effluent (POME) is abundant agricultural waste in Malaysia, shows great potential in used directly as alternative fermentation substrate. The objective of this study is manly to study the effect of temperature, agitation rate and inoculums concentration as variable factors that significantly influenced solvent production from palm oil mill effluent (POME) by *Clostridium beijerinkii* ATCC 51743. Analysis of total solvent production was done by using gas chromatography and isobutanol has been used as internal standard. Results that obtain from the study effect of 5 to 25% inoculums concentration to total solvent concentration show that the total solvent concentration will decrease as inoculums concentration increase. The highest production of total solvent is at 10% of inoculums concentration with 0.719 g/L production. Besides that, the study effect of 25 to 35 °C temperature to the total solvent production shows that concentration of total solvent production will decreases as temperature increase. The highest production is 0.568g/L at 30°C. Meanwhile effect of 50 to 250rpm agitation rate to the total solvent production shows that as agitation increases, total solvent production will decrease where the highest total solvent production was recorded at 150rpm with 0.675g/L. It can be conclude that, the temperature, agitation rate and concentration inoculums will be the significant parameter that affected production solvent from POME by *Clostridium Beijerinckii* (ATCC 51743).In addition, applying the Response Surface Methodology (RSM) was recommended for this study in order to determine the optimum level in enhances the solvent production.

ABSTRAK

Beberapa dekad yang lalu, dunia bergantung sepenuhnya kepada bahan api fosil sebagai sumber tenaga kerana harga bahan api ketika itu yang murah menyebabkan penurunan minat terhadap perkembangan industry bahan api daripada proses fermentasi. Namun begitu, disebabkan konflik yang berterusan di negara pengeluar minyak dan pengurangan sumber minyak menyebabkan kemunculan semula industri fermentasi solventogenik menghasilkan bahan api di dunia. Selain itu, pembakaran bahan api menghasilkan pelbagai gas yang menyumbang kepada pencemaran alam seperti hujan acid dan kesan rumah hijau yang menjadi punca pemanasan global. Oleh itu, perlunya penekanan dalam mengembangkan proses fermentasi solventogenik menghasilkan bahan api daripada sumber alternative yang lebih baharu dan mesra alam. Tetapi fermentasi solventogenik menghadapi harga bahan mentah yang mahal dan juga kurangnya penghasilan produk akhir untuk bersaing dengan industry yang menghasilkan bahan api daripada bahan api fosil. Bahan buangan kelapa sawit atau dikenali sebagai (POME) ialah bahan buangan yang membebankan Malaysia dilihat mempunyai potensi untuk digunakan sebagai bahan fermentasi. Objective kajian ini adalah untuk mengkaji kesan kepekatan inoculums, suhu dan kadar pengoncangan terhadap penghasilan bahan pelarut daripada POME oleh bacteria *Clostridium Beijerinckii.* Produk yang dihasilkan akan di analisis menggunakan gas chromatography dilengkapi pengesan api ion (GC-FID) yang menggunakan isobutanol sebagai standard dalaman. Keputusan yang diperolehi melalui kajian 5-25% kepekatan inoculum terhadap penghasilan bahan pelarut dilihat bahawa penghasilan bahan pelarut semakin menurun dengan peningkatan kepekatan inoculum. Penghasilan bahan pelarut tertinggi ialah pada 10% kepekatan inoculums iaitu sebanyak 0.719 g/L. Selain itu, kajian suhu dari 25-35°C terhadap pengahasilan bahan larut mendapati pengahasilan bahan pelarut semakin menurun dengan peningkatan suhu. Penghasilan tertinggi direkodkan pada 30°C dengan pengasilan sebanyak 0.568 g/L. Sementara itu, kajian terhadap kadar pengoncangan 50-250rpm terhadap penghasilan

bahan larut mendapati penghasilan menurun dengan peningkatan kadar pengoncangan. Penghasilan tertinggi ialah pada 150 rpm dengan catatan 0.675 g/L. Ia boleh dirumuskan bahawa kepekatan inoculums, suhu dan kadar pengoncangan menunjukkan kesan signifikan kepada penghasilan bahan pelarut. Tambahan lagi, sebagai cadangan untuk meninggikan lagi penghasilan produk aplikasi dengan menggunkan cara tindakbalas permukaan atau RSM boleh digunakan dengan mendapatkan nilai pembolehubah yang optimum.

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NOMENCLATURE

ABE	Acetone – butanol – ethanol
BOD -	Biological oxygen demand
COD -	Chemical oxygen demand
°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

1.1 Background of Study

Historically, late 1970s there was increases international interest to review production of alcohol fuels from easily and renewable resources (Qureshi *et al.,* 2000). The main factor increasing interest among various government and scientific communities to develop solvent fermentation process is due to increasing cost of petrochemical feedstock and high demand of co-solvent gasoline as energy source to generate energy in industrial sector. Brazil is the first country that introduced its own biofuels, ethanol production program followed by USA in 1977. In early 1980s, fermentation production biofuels could not compete with the economical creation of solvents from cheap, plentiful petroleum due to reduction in oils price lead to diminishing interest. However, constant conflicts in the oil-supply regions and depletion of fuels oils supply resulted to oil price increases make research activities on this fermentation is revival.

Nowadays, increases demand in chemical additive and liquid fuels create a pressing need to find a viable alternative sustainable energy. The solvent (acetone-butanol-ethanol) was define has many commercial application in various industries such as in oleochemical industries and also in manufacture of lacquers, resins and also increase demand in blend with gasoline as energy sources. Unfortunately, there was certain issues occurs that must facing in developing renewable energy resources that

more renewable, more efficient and safer for environment. For the first generation in solvent fermentation research is in bioethanol fermentation by yeast whereby production is derived from sugar, starches, and oils. These solvent are creating from crops that are competing with food crops for the use of agricultural land and water.

As result, solvent product from solvent fermentation becomes another attractive sustainable alternative due to its ability in production that derived from high carbohydrate waste of industries that abundant in certain country especially in Malaysia. Its inherent chemical properties make it superior than bioethanol for use in combustion engines. Biobutanol is product from anaerobic fermentation of carbohydrate to solvent by *Clostridium* species and has four carbons chains, make it has more energy than bioethanol that is for about 25% more energy per unit volume. It's also has lower vapor pressure and higher flashpoint than bioethanol making it easier to store and safer to handle. Contrast to properties of ethanol that attract to water, butanol is hydrophobic that can be blended at a refinery without requiring modifications in blending facilities, storage tanks or retail station pumps that can run in unmodified engines at any blend with petrol and may also blended with diesel and biodiesel. Furthermore, butanol is less corrosive than ethanol and can be transported using existing infrastructures.

The commercial manufacture of butanol via Acetone- Butanol- Ethanol (ABE) fermentation from non-food sources by using solventogenic clostridia has almost completely replaced petrochemical routes. Current research in ABE fermentation is directed at genetically altering *Clostridium spp.* to enhance final butanol yield. Ian (1989) state that by using *Clostridium acetobutylicum or C.beijerinckii* can help to minimize problem of all solvent fermentation process such as lower reactor productivities and problem in product recovery. In previous study reported by Ezeji *et al.*(2007), *C.beijerinckii* has great biotechnological potential for production of butanol, acetone and isopropanol due to abilities in utilize of broad substrate range (pentose, hexose, starch and others) besides can sustained production of solvents well into log-phase with respects to strain degeneration and adaptability in continuous processes.

There have several effects that identified contribute to the economic solvent price in industry that is high cost of substrate, low product concentration, and low solvent yields (Qureshi *et al.,* 2001). The most effected problem that made up to 60% for overall cost solvent production is cost of substrate. Thus, the availability of variety low cost feedstock as an inexpensive raw material including whey permeate, soy molasses, barley straw and other agriculture biomass culture make economically viable for ABE fermentation. Unfortunately, this substrate need to hydrolyzed first into simple sugar before use as medium in fermentation process (Qurehi *et al.,* 2010).

Besides that, inhibitor directly occurs after fermentation process contributes to lower product concentration makes widely research was done to discover the most reliable substrate to use. In Malaysia, Palm Mill Oil Effluent (POME) represents an alternative raw material for fermentation process that is attractive in both economic and geographical consideration. POME is one of agricultural waste that produces form production of cruel oil and the most polluted organic residues that abundant in Malaysia show great potential to be used directly as raw material for production of butanol without need to hydrolyzed first into simple sugar (Sahaid *et al.,* 2003). In addition, POME is industrial waste that containing 95-96% water, 0.6-0.7 % oil and 4-5% total solids that if untreated effluent is discharged into water sources will cause environmental problem (Wu *et al*., 2008). On the other hand, POME was identified to be a potential source to generate renewable bioenergy such as biofuel, biomethane and biohydrogen (Lam *et al.,* 2010).

Traditionally, technique of ‘one-factor at a time’ was applied widely in optimizing production for multivariable system. This preliminary study is seen very important in order to give the basic data before proceed with the optimization step for the solvent production by *C.beijerinckii.* Thus, estimation of several main effect and factor interaction in solvent production from various renewable resources becomes favorable in ABE fermentation process review.

1.2 Problem Statement

In Malaysia, development of renewable energy resources is become favorable due to decreasing availability fossil fuels source that now can only survive for another 20 to 30 years. An alternatives source for fuels and other petroleum based products is extremely needed due to global demand and unstable condition in worldwide petroleum prices. Furthermore, negative impact to environment generates by the worldwide utilization of fossil fuels besides problem that contribute from agricultural waste that most abundant in Malaysia get attention to study on. According to Claassen *et al*. (2000), the ABE fermentation has resurfaced due to new global support for the exploitation of biomass as a sustainable source of energy. Therefore, solvent production should be focus towards the use of renewable energy that more friendly to environment.

Unfortunately, solvent fermentation process by *C.beijerinckii* suffered from high cost of solvent production from high cost medium component. In previous study by Mariam *et al.* (2007), solvent production from food based crops was widely used by researcher such as corn, potato and sugar cane. But, an issue occurs that give negative impact on global food supply due to substrate used is competing human food based material resulted to high demand and fluctuation price in market. This becomes the main factor causing why ABE fermentation could not survive due to high cost of raw material for medium preparation. Therefore, studies in POME an agricultural residue that contain high concentrated organic compound become favorable as suitable with an economical substrate for solvent production.

Since 1950's, solvent fermentation process by clostridia is very complicated and difficult to control resulted to the limitations on production such as lower yields and lower productivity of product. David (2004) reported that, low solvent yield and solvent concentration due to severe product inhibition made solvent production from glucose by ABE fermentation is uneconomical. According to Christine *et al.* (2009), the main problem in ABE fermentation that needs to overcome is the low conversion of glucose to

solvent. Thus, study the effect of different parameters on solvent production need to be focus in order to increase production yield during fermentation process.

1.3 Objective

The aim of this study is want to study effect of inoculums concentration, temperature and agitation rate on solvent production by *Clostridium Beijerinckii* from Palm Oil Mill Effluent (POME).

1.4 Scope Of Study

To achieve objective of this study, there are several scope that have been identified:

i) To study the growth profile of *Clostridium Beijerinckii* in Palm Oil Mill Effluent (POME) and Reinforce Clostridium Medium (RCM).
ii) To study the effect of inoculums concentration (5 to 20%) on solvent production.
iii) To study the effect of temperature (30 to 50°C) on solvent production.
iv) To study the effect of agitation rate (40 to 160 rpm) on solvent production.
v) To study the glucose consumption at different inoculums concentration, temperature and agitation rate.

1.5 Rational & Significant Of Study

The uncertainty and depletion petroleum supply resulted in viable interest in traditional solvent (Acetone Butanol Ethanol) fermentation by *Clostridium* strain (Parekh *et al.,* 1998). Recently, world is facing problem in developing renewable energy that more renewable, more efficient and more safe in environment in order to switching

dependence on fossil fuels supply. Thus, widely study on solvent production was developed to help world in indicating another solvent from renewable resources.

For more than three decades, critics have tried to cast raw materials that used in production biofuels such as corn that said disturbing food chain in human life being. But nowadays, the dominant route for making biofuels was turn to material that renewable and economical substrate that viable source or abundant in each country that interest in ABE fermentation. In Malaysia, agricultural waste that abundant in peninsular of Malaysia that is palm oil mill effluent (POME) was proposed to use in fermentation process to produce solvent. POME is high contaminant agricultural residue that contain high mixture of carbohydrate such as starch, cellulose and other carbohydrate but can harmful to environment if not properly manage. It can be utilized by *C. beijerinckii* and able to produce high production of solvent compare to other substrate used such as barley straw, corn fiber and switch grass (Qureshi *et al.,* 2007). By using POME as raw material also can help to reduce price of solvent at international market that influence from cost of substrate.

Over the years, fermentation process by using anaerobic microorganisms provides a dominant review for converting biomass and agricultural waste into chemicals and biofuels. One of the largest acetone-butanol-ethanol fermentation (ABE fermentation) process ever developed in process industry is in 1917-1955 where applied widely bacterium that strictly anaerobic condition that is *Clostridium acetobutylicum*. Unfortunately, this strain is facing problem that produce lower yield in final product due to lack tolerance in acid production in final process. In order to overcome this problem and slightly increase solvent production, study in applying strain that tolerant to higher acid production such as *Clostridium beijerinckii* is need to focusing more (Tashiro *et al.,* 2010). In addition, further study is needed to screen the factor that effects the solvent production.

CHAPTER 2

LITERATURE REVIEW

2.1 Acetone-Butanol-Ethanol (ABE) Fermentation

In present, solvent that manufactured from industrial scale by ABE fermentation is not longer competing with chemical synthesis from petrochemical feedstock. However, due to high demand for solvent is industry besides development in biotechnology and bioprocessing field, interest in fermentation of solvent production as renewable energy has returned and current research was focusing directed towards the development of better processes and microbial hyper producing- strains. On the other hand, the strong reason in a revival of research activities on this field is the depressed crude oil world prices due to limited supply of fossil fuels and constant conflicts in oil-supply region (*Qureshi et al., 2007*).

Fermentation of ABE (acetone, butanol, and ethanol) fermentation involve a two-step that is acid production phase carried out by *clostridium* species and followed by solvent production acetone, butanol and ethanol in 3:6:1 wt ratio composition production (Qureshi *et al*.,2007). The uncertainty associate with petrochemical supplied resulted in interest to traditional fermentation by *clostridia*. In 1914, first industrial ABE fermentation was commercialized from starcy raw material by *Clostridium acetobutylicum* has discovered. Second generation was developed for butanol production due to development modern technology in biotechnology and bioprocess field. Unfortunately, production of butanol hampered with low of product yield due to butanol

itself toxicity to bacteria resulted to lower solvent yield and also faced to high cost of raw material. In response to overcome this problem, production of butanol from cheap renewable resource such as sugar crops (*Atsadawut et al., 2006*), lignocelluloses feedstock and agricultural residue (*Qureshi et al., 2010*) was develop.

Metabolic pathway that demonstrates among clostridia species such as *C.acetobutylicum, C.beijerinckii* and *C. saccharoperbutylacetonicum* is similar (Zheng *et al.,* 2009). Figure 2.1 shows metabolic pathways to ABE fermentation by *C. acetobutylicum.* Clostridia have a diverse group of anaerobic bacteria that can utilize variety of substrates including monosaccharides and polysaccharides (Sakuragi *et al.,* 2010). Glucose is supply through the glycolytic (EMP) pathway that generates pyruvate and 2 molecules of ATP and NADH. During fermentation, *C.acetobutylicum* will produce 3 major product classes that are solvent (acetone, ethanol and n-butanol), organic acid (acetic acid, lactic acid and butyric acid) and gases (carbon dioxide and hydrogen) (Sakuragi *et al.,* 2010). Metabolic reaction of ABE fermentation is represent in two phase that is acidogenic phase and solventogenic phase.

At acidogenic phase, accumulation of organic acid primarily acetate and butyrate will start. The synthesis of acetate and butyrate is important for generation of ATP that needed for cell growth and further metabolism synthesis. When the concentration of undissociated acid reaches some threshold value, second phase that is solventogenesis is triggered. During solventogenesis is the second phase of ABE fermentation, the acid that produce during acidogenic phase is reused for production of Acetone, Butanol and Ethanol. Pathway in production ethanol is started with reaction of acetyl-CoA and acetaldehyde dehydrogenase that will produce acetaldehyde, by then reacts with ethanol dehydrogenase will produce ethanol. On the other hand, production of acetone is interaction reaction between acetate and butyrate with involve phosphotrans acetylase that produce Acetyl P by then, with involving acetate kinase enzyme will produce acetone. The butanol production pathway from acetyl-CoA requires 5 enzymes that is that is thiolase (thl), 3-hyroxybutyryl-CoA dehydrogenase (hbd), 3-hydroxybutyryl-CoA

dehyratase (corotonas, crt), butyryl-CoA dehydrogenase (bcd) and aldehyde dehydrogenase (adhE)/aldehyde-alcohol dehydrogenase (adhE1).

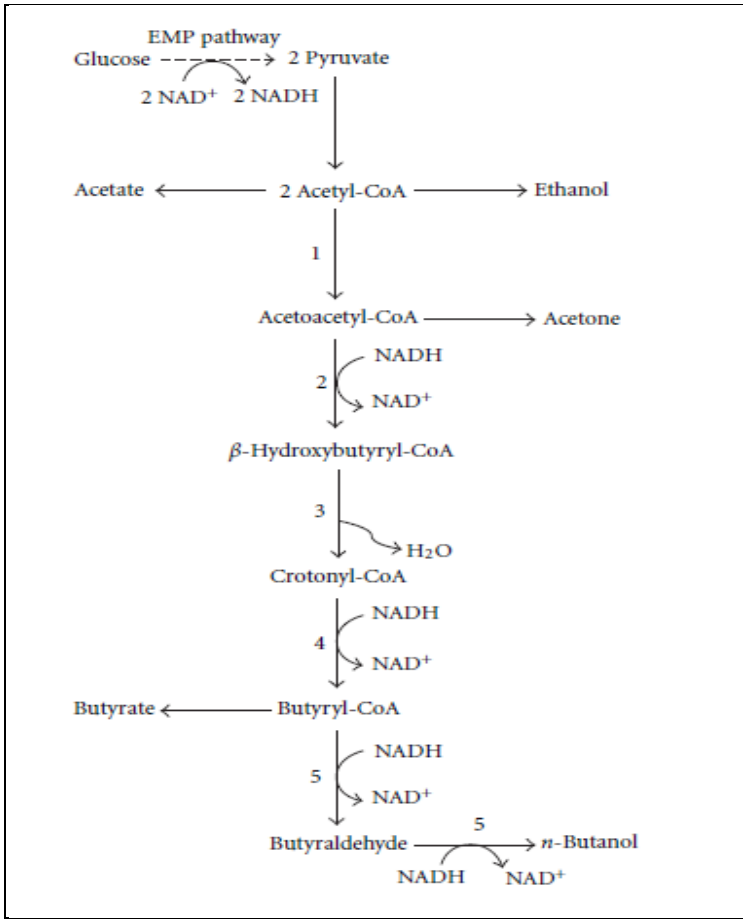

Figure 2.1: Metabolic pathways of Acetone-Butanol-Ethanol (ABE) Production by *Clostridium Acetobutylicum.* Numbers refer to the enzymes:1:acetyl-CoA acetyltransferase (thiolase; THL), 2: 3-hydroxybutyryl-CoA dehydrogenase (HBD), 3: 3-hydroxybutyryl-CoA dehydratase (crotonase; CRT), 4: butyryl-CoA dehydrogenase (BCD), and 5: aldehyde dehydrogenase (ADHE)/aldehyde-alcohol dehydrogenase (ADHE1) (Sakuragi *et al.*, 2010).

2.1.1 Acetone

Acetone or 2-propanone is colorless and flammable liquid organic compound with molecular formula $(CH_3)_2CO$ that represent in Figure 2.2. Molar mass of this chemical is 58.1 g/mol while –94.9°C melting point and has high boiling point that is 56.3°C.

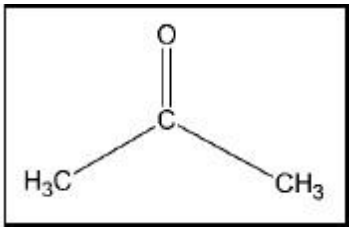

Figure 2.2: Chemical structure of Acetone

At the start 1st War World, demand for acetone was increased rapidly where acetone was applied in manufactured of cordite, a smokeless explosive (Alan, 2009). Due to the high demand in market that exceeds supply, results to the commercialization on biological process production of acetone by *Clostridium acetobutylicum*. During 2nd World War declined rapidly production of acetone-butanol production due to the both increases substrate prices and competition to the petrochemical industry based on the economical raw material (Nimcevic and Richard, 2000). Process production of acetone is primarily by cumene oxidation process where cumene hyroperoxide will decompose with acid to produce acetone and phenol. This kind of process was replacing fermentation process to produce acetone whereby acetone is other byproduct that process in ABE fermentation from bacterium *Clostridium Acetobutylicum* (Weizmann *et al.*, 1914).

Figure 2.5 shows percentage of application acetone in industrial. Roughly, 75% acetone was applied as chemical intermediate to produce other chemical such as manufacture of acetone cyanohydrin for methyl methacrylate, bisphenol A and aldol chemicals and 12 % acetone applied as direct solvent in industry. Other application of

acetone include as lacquer for automotive/furniture finishes, as chemical addition in various cosmetic product and others.

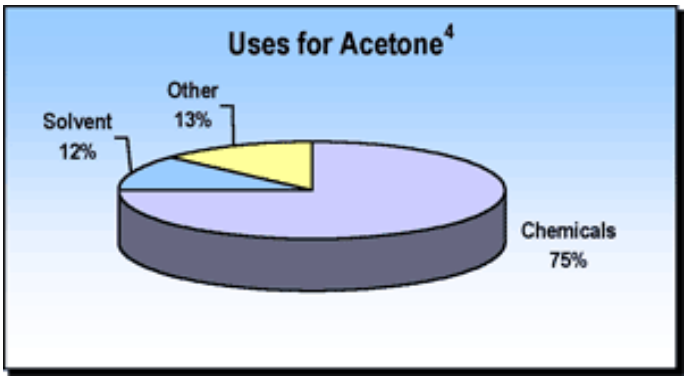

Figure 2.3: Percentage Acetone Industrial Application (Dow Acetone Safety Data Sheet, 2006)

2.1.2 Butanol

Few decades ago, butanol is producing chemically either by Oxo process from propylene or aldol process from acetaldehyde (*Sherman, 1979*). Currently, world market shows that United States become the largest producer that represents approximately 25% of the worldwide market (Yixiang *et al*., 2008). From this organic chemical production, fossil oil and natural gas served as the main material resources that applied seven basic building blocks that include syngas from methane, ethylene, butanes, propylene, butylenes, butadiene and BTX (a mixture of benzene, toluene and xylene). There was depressing supply on using fossil oil and natural gas as raw material resources for organic chemical production, that resulted to growing interest of the replacement fossil-based chemical with biochemical based product. Thus, production bio-butanol from renewable resource gain the great interest not only can use as renewable fuel that more advantages from ethanol but also has vast application as an organic chemical feedstock in industry. Recently, Acetone-Butanol-Ethanol (ABE) fermentation that produce butanol as major product, acetone and ethanol as by product from renewable resources substrate as medium such as potato, whey permeate, corn starch and employing

microbial strain such as *C. acetobutylicum* or *C.beijerinckii* were focused to study on (*Qureshi et al., 2007*).

In addition, genetic strain improvement also has discovered in order to get higher yield solvent and better tolerance to butanol. Advance fermentation technique was developing to enhance production of solvent such as applied immobilized cell reactor, continuous reactor fed (*Ezeji et al., 2004*) and recovery product improvement such as gas stripping, liquid-liquid extraction, adsorption and pervaporation (*Blaschek et al., 2001*).

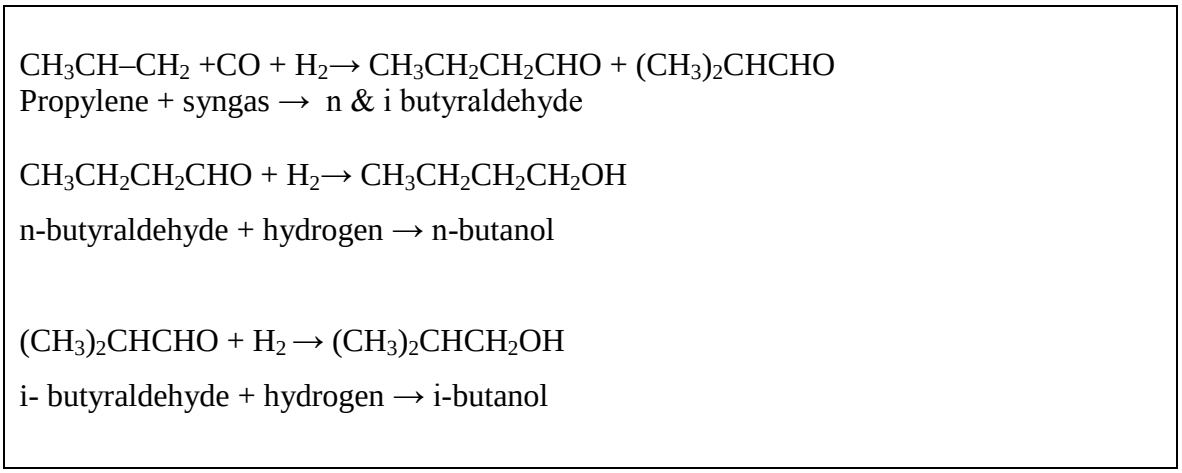

Figure 2.4: Production butanol by Oxo Process (Veronica *et al.,* 2010)

Butanol is four carbon alcohols $C_4H_{10}O$, a colorless liquid with a strong characteristic odor. Nowadays, organic chemicals such as butanol play important role in our daily life especially in production of polymer and plastics based materials. Butanol is widely used as direct solvent in detergent formulation, varnish, paint, resin and for cosmetics industry in makeup, nail care products and shaving products. Besides that, butanol is used as chemical intermediate in manufacturing other chemical such as butyl acrylate, glycol ether, butyl acetate and etc. In textile industry, butanol is applied as swellings agent from coated fabric and also applied in production of drugs and antibiotics, hormones, and vitamins.

The crises of energy and environmental in the world nowadays, has forcing whole world to re-evaluate the efficient utilization of waste or finding alternative from natural, renewable resources using clean technologies (Okonko *et al.,* 2009). Nowadays, reviewing alternative fossil fuels such as oils and gas derived from biofuels that produced from renewable resources such as plants, organic waste and etc become favorable. The combination of bio-based feedstock, bio-process and new products offers the high potential to revolutionized energy sector besides higher oil prices makes this business development profitable. Biodiesel, bioethanol and biogas are the main biofuels that produces today, but there is other such as biobutanol that still in research and development phase. Biofuels offers high energy resources without net increases carbon to atmosphere because plant that used production biofuels has remove CO_2 from atmosphere beside ability to produce complete combustion that commonly applied in vehicle fuels and cooking stoves. Thus, production biofuel such as biodiesel, biogas, bioethanol and biobutanol will ensuring the sustainability energy supply and environmental safety.

Butanol is an alcohol that produced by fermentation from same type of sugar sources that are same sugar that are used in ethanol production but when derived from biomass, it is called biobutanol. Biobutanol is identical to used as transport fuels due to its high energy contain 110,000 BTUs per gallon, closer to gasoline's 115,000 BTUs. As a biofuels, biobutanol has greater potential than bioethanol in transportation industry due to its containing 25% more energy than bioethanol (*Qureshi et al., 2007*).

In addition, butanol is suitable for fuel extender because its several attractive characteristic such as more hydrophobic than ethanol that able to mixes better with hydrocarbon fuels contribute in sustaining clean environment (Hsieh *et al.,* 2001). Sustaining a clean environment become important issues in industrial society whereby air pollution caused by automobiles and motorcycles contribute the highest environmental problem and need to be tackled (Yuksel *et al.,* 2003). Moreover, alcohol also has a much higher flash point and boiling point than gasoline which make safer for transportation usage. Alcohol also has 3-5 times higher vapor pressure and also less

corrosive, thus can be shipped and distributed through existing pipelines and filling stations beside easier to store and safer to handle (Aleksic *et al., 2009*). Unlike ethanol, butanol can run in unmodified engines at any blended at a refinery without requiring modification in engine vehicle and shows need lesser amount of air for complete combustion where, the stoichiometric air-fuel ratio (AFR) about 2/3-1/2 of alcohol with gasoline (Yuksel *et al.,* 2003).

Table 2.1: Properties of biofuel (*Blaschek et al.,* 2009)

Properties of fuels	Butanol	Gasoline	Ethanol	Methanol
Energy density (MJ/L)	29.2	32	19.6	16
Air-fuel ratio	11.2	14.6	9	6.5
Heat of vaporization (MJ/kg)	0.43	0.36	0.92	1.2
Research octane number	96	91-99	129	136
Motor octane number	78	81-89	102	104

2.1.3 Ethanol

Ethanol is by product in solvent fermentation process using *Clostridium* strain that successfully demonstrate from previous study (Syafiqa, 2009). In metabolic pathway of solvent-producing by *Clostridium spp,* solvent production is after cell growth entering stationary phase where organic acid will utilize to produce acetone, butanol and ethanol in ratio 3:6:1 (Jamilah *et al.*,2009). Production Ethanol also gains interest in used as a petrol substituent for alternative transport fuels. Bioethanol is mainly produce by fermentation of sugar besides can be produce by chemical process by reacting ethylene with steam (Hisham, 2009).

Ethanol or ethyl alcohol (C_2H_5OH) is an alcohol group of chemical compound where contain a hydroxyl group (-OH) that bonded with carbon compound. Ethanol is a clear and colorless liquid with lower toxicity level. Ethanol is miscible with water or other organic solvent. Beside that it has high octane number where help as enhancer fuel burning when blending with gasoline. Fuels that burns when blending of ethanol with gasoline contribute to emission gas that more environmental friendly due to its ability

oxygenate the fuel mixture so it burns more completely. In United State, ethanol fuel blend was widely sold with common blend in 10% ethanol with 90% petrol without modification of engine. It also use as solvent in making perfumes, paints, lacquer and explosive.

Currently, production ethanol that becomes favorable in biotechnology field study is either by direct glucose fermentation, enzymatic conversion starch-based crops such as corn and sweet potato, or by enzymatic hydrolysis of lignocelluloses feedstock (Kundiyana *et al.,* 2008).

2.2 *Clostridium Beijerinckii* ATCC 51743

According to Lassi (2009), microbial production of solvent by *clostridium* species is a cost effective if a cheap carbon sources was applied. In order to compete fermentation process with based petrochemicals solvent production, researchers was suggest to focusing on the development of superior cultures. Strain enhancement has tremendous potential for improving performance and economic in production of solvent. Studied for last century, approved that genus clostridium contains diverse physiology quite remarkable and provide a broad view into sporulation, energetic, regeneration of reducing power and pathway switching acid production to solvent production. Recent development of genetic manipulation paved the way for exploiting the enormous metabolic potential bacterial embracing biofuel production, lignocelluloses conversion and exiting new enzyme activities.

All members of genus Clostridium are gram positive, spore forming rods that are anaerobic. These motile bacteria are usually isolated from soil sample and shows optimum growth when plated on blood agar at human body temperature. However, these motile bacteria will form spore that tolerate with extreme condition and secrete powerful exotoxins. Therefore, there are responsible for such disease as tetanus, botulism and gas gangrene. The cell changes morphology occur over the growth cycle of organism. At

early exponential phase, the cells are long, filamentous and very motile but when stationary phase which culture approaches the solventogenic stage, the cell will shorten, become plumper and exhibit motility.

Several strains of solvent producing clostridia that attracting most for their ability in producing solvent in the acetone-butanol-ethanol (ABE) fermentation is *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Gutierrez *et al., 1998*) and will produce acetone, butanol, ethanol as co-product and organic acids (acetic and butyric acid), carbon dioxide, and hydrogen as by product (Chang, *1996*). History state that, the first isolated *C. acetobutylicum* strain was identified to generate acetone, which was needed for the production of artillery shells in World War I. The continuous studied in this solventogenic clostridia to improve the efficiency of solvent production resulted to new other strain that more efficient in fermentation that is *Clostridium beijerinckii*.

C.beijerinckii's morphology is same like other clostridium strain but it has great biotechnological potential for production butanol, acetone, and ethanol because of its broad substrate range which it sustained production of solvent well. This bacteria is a gram positive, motile bacterium, has rod-shaped bacteria with oval and sub-terminal spores. It is a saccharolytic metabolism and strictly anaerobic bacteria where usually isolate from soil sample. Applying *C.beijerinckii* in fermentation will produce a number of products including acetate, butyrate, lactate, hydrogen gas, carbon dioxide, acetone, ethanol, butanol and etc.

This strain successfully shows to continuous processes during fermentation due to its stability in respect to strain degeneration and the adaptability into log phase. Ezeji *et al.* (2005) demonstrate that *C.beijerickii* utilize starch efficiently compare to glucose during batch fermentation and operating at same dilution rate in long time without any sign of acid drift and morphological changes. Thus, this shows that *C.beijerinckii* able to sustain in continues process during solvent fermentation due to lower residual acid production. On the other hand, *C.beijerinckii* also grows well and easy to handle in simple, inexpensive media that is realistic to use in industrial (Blaschek *et al., 2007*).

Interestingly, *C.beijerinckii* has shows responsiveness for genetic improvement after only one episode of mutagenesis produce the enormous potential of derivative in solvent production. The sequences of *C.beijerinckii* will possible the application of DNA for gene expression profiling and comparative genomic in order to approaches strain construction and optimization of ABE fermentation. Figure 2.5 shows the picture of *C.beijerinckii* under Scanning Electron Microscope (SEM).

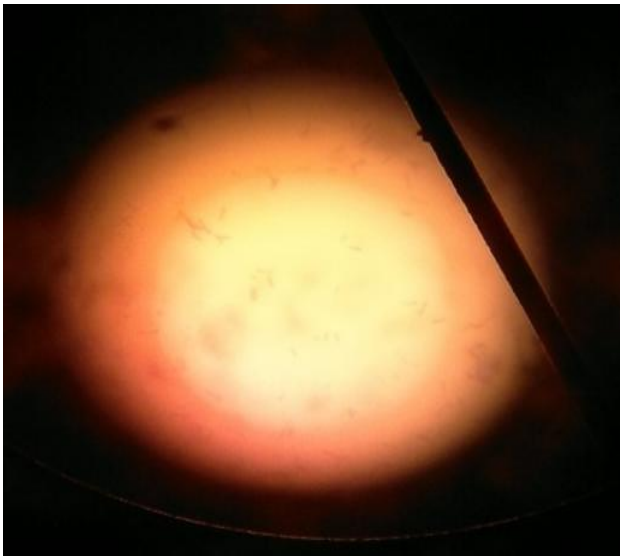

Figure 2.5: Scanning Electron Microscope (SEM) of *Clostridium Beijerinckii*

2.3 Palm Oil Mill Effluent (POME)

In order to produce butanol economically, biomass fermentation medium was focused more due its renewable energy resource besides to reduce competition with food product. Non-food substrate or feedstock is one of the area of biomass that preferred mostly by researcher in order to maintain a predictable cost. In addition, researches was interested in applying cheap renewable substrate for butanol production such as corn

(Prakash *et al.*, 1999), sweet sorghum (Atsadawat *et al.*, 2006), cassava flour (Leonard *et al.*, 2010), barley straw and switch grass hydrolyses (Qureshi *et al.*, 2010) depending upon availability and region where they produce.

Malaysia is the largest producer and exporter of palm oil product resulted from rapidly development of Malaysian palm oil milling and refining industries until has emerged Malaysia as one of the major oil commodities in the world oil and fats market (Shazrin *et al.*, 2004). Despite country earning high economic return, the industry also contribute large amount of waste. For every tonne of fresh fruit bunch (FFB) will produce empty fruit bunch (EFB) (23%), mesocarp fiber (12%), shell (5%) and palm oil mill effluent (POME) (60%) (Baharuddin *et al.*, 2010).

Figure 2.6 shows the process flow of palm oil production that involve large amount of water and steam that resulted to huge amount POME production (Lam *et al*.,2010). Commonly, palm oil refinery process involves three important stages that contribute large amount of POME. After harvest, the fresh fruit bunch (EFB) was transfer to the mills process where hundreds of fruits bunch will going through sterilization process with steam at a pressure 3 bars and a temperature 140°C for 75-90 minutes. Then, condensate that coming out from sterilizer contribute as one major source of POME. Second stage that contribute large amount of POME is during clarification process that has purpose to separate the oil from its entrained impurities. Hot water is added into clarifier and breaks the oil emulsion to act as barrier between insoluble solid that will settle to bottom clarifier and oil droplet that flow through water at the top. Bottom phase at clarifier will drain out as sludge or POME. The last stage that produces POME is during kernel oil recovery phase where nut from press machine residue is sent to nut cracker and send to hydrocyclone. Hydrocyclone has purpose to separate the kernel from empty shells after cracking the nut and POME will discharge at this phase.

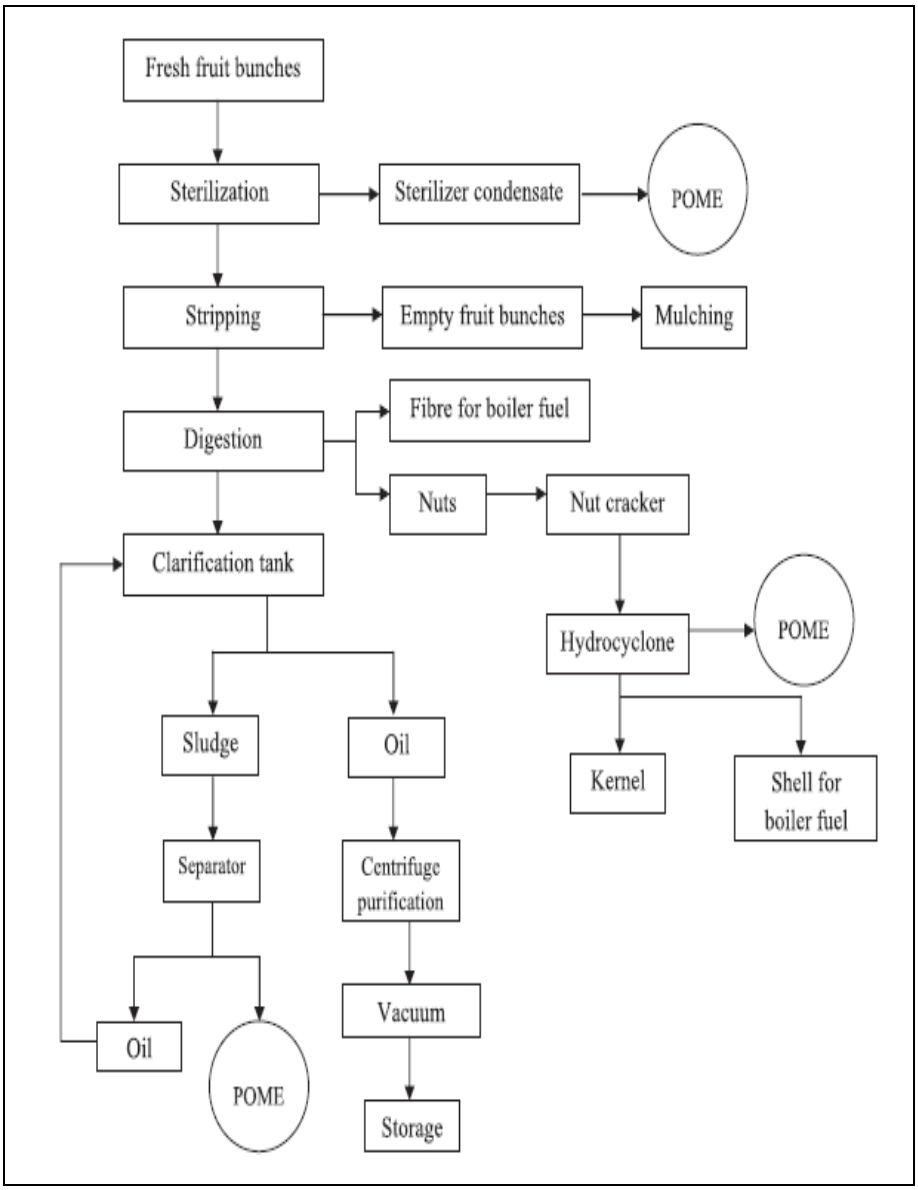

Figure 2.6: Process Flow POME Production (Lam *et al.*, 2011)

Table 2.2 shows the organic compound in POME that proven that POME is most polluted organic residue that compose of high organic content mainly oil and fatty acid state beside other organic matter.

Table 2.2: Characteristics of untreated palm oil mill effluent (POME)

Parameter	Concentration	Metal	Average
pH	4.7	Phosphorus	18
TEMPERATURE	80-90	Potassium	2,270
BOD 3-day, 30°C	25,000	Magnesium	615
COD	50,000	Calcium	439
Oil and Grease	4000	Boron	7.6
Total Solids	40,500	Iron	46.5
Suspended Solids	18,000	Manganese	2.0
Total Volatile Solids	34,000	Copper	0.89
Ammoniac-Nitrogen	35	Zinc	2.3

* All parameters in mg/l except pH and temperature (°C) Source: (Shazrin *et al.,* 2004)

Realizing to abundant of this waste, many researcher was take action in study about treatment of POME. In Malaysia, the most popular treatment method is by open pond system where utilized 85% of the mills (Baharuddin *et al.,* 2010). The composting treatment take until 80 days to completed because of composting various suspended components including organelles, short fibers, a spectrum of carbohydrates ranging from hemicellulose to simple sugars, a range of nitrogenous compounds from proteins to amino acids, free organic acids and an assembly of minor organic and mineral constituents in POME. Therefore, it is essential to provide another ways for better treatment of POME. In 2005, the anaerobic treatment of POME and methane production was developed at Felda Serting Hilir Palm Oil Mill, Malaysia in order to improve management waste of palm oil.

Recently, anaerobic treatment of POME was develop in production of solvent to reduce pollution strength of POME beside can help reduce cost production of solvent in industry. Previous report shows that the concentration solvent production using POME as medium is about 4 g L^{-1} (Sahaid *et al.*, 2003) compared to 3.2 g L^{-1} using corn steep water (Parekh *et al.*, 1998). Besides that, POME contain mixture of carbohydrate including starch, hemicelluloses, sucrose and other carbohydrate that can be utilized in ABE fermentation by anaerobic bacteria (Pang *et al.*, 2003). Thus, present study was applying POME as potential substrate in fermentation of solvent not only profitability to chemical industry but also to palm oil industry that help them to reduce their waste production.

CHAPTER 3

METHODOLOGY

3.1 Equipment

3.1.1 Anaerobic Chamber

This equipment is used to maintain anaerobic condition for cultivation of strictly anaerobic bacteria. In this experiment, anaerobic chamber is used when deal with bacteria *C.beijerinckii* either during bacterial growth phase or during inoculums development. It's equipped with mixture of gas in ratio 90% N_2, 5% CO_2, and 5% H_2 supply to help maintain anaerobic inside chamber. Temperature inside chamber is constant 37°C to incubate inoculums inside.

Figure 3.1: Anaerobic Chamber

3.1.2 Autoclave

Before experiment is performing in laboratory, equipment that used must clean and sterilize to avoid any contamination to sample. Thus, an autoclave is a device that used to sterilize equipment by subjecting them to high pressure saturated steam at 121 °C or more, typically for 15 to 20 minutes depending on the size of the load and the contents. In this experiment, autoclave is the main equipment for example to sterilize fermentation medium, preparation medium for cell growth and etc.

3.1.3 Uv-Vis Spectrophotometer

UV-Vis Spectrometer is used in the quantitative determination of solutions of transition metal ions highly conjugated organic compound and biological macromolecule. It also can used to determine concentration of absorbing materials based on developed calibration curves of material. In this experiment, this equipment is used to determine the optical density value of *C.beijerinckii* to use in medium fermentation.

3.1.4 Gas Chromatography (GC) with Flame Ionization (FID)

Gas Chromatography (GC) commonly used in analytical chemistry for separating and analyzing compounds that can vaporized without decomposition. GC application includes testing the purity of particular substance, or separating the different components of a mixture. Sample chemical compound on this experiment is measured using GC with flame ionization detector (FID) because it is a useful general detector for the analysis of organic compounds that has high sensitivity, a large linear response range, and low noise.

3.1.5 Incubator

In this study, incubator is used to place for growth bacteria due to its application that used to grow and maintain microbial cultures or cell cultures. The incubator is main equipment that to be used due to its ability to maintain optimum temperature, humidity and other condition that important for cell growth. This equipment is essential for lots of work in cell biology, microbiology and molecular biology.

3.1.6 Incubator Shaker

This equipment is to allow fermentation process under simultaneous incubation either sample is with or without shaking in flask or a constant and variable temperature environment. Experiment is run inside incubator shaker with one parameter variable and other parameter is constant. For example, in experiment effect of temperature process, temperature becomes variable while agitation rate and concentration inoculums will constant. This will continue with other variable.

3.2 Material

3.2.1 Strain and Inoculums Development

C.beijerinckii ATCC 51743 was used for solvent fermentation. Laboratory stocks of bacteria were maintained in glycerol stock vial at temperature -80°C. For inoculums preparation, 1mL of bacteria in glycerol stock was transferred into 150mL Reinforced Clostridium Media (RCM) and incubated at 37°C for 12 hours. Streaking method used to isolate single colony of *C. beijerinckii* from active culture to be grown in Reinforced Clostridia Agar plate. Streaking process was done in anaerobic chamber to maintain anaerobic condition. After streak the active strain on plate, the plates were incubated for more 24 hours at 37°C at anaerobic condition. Single colony was isolates and inoculated

into 100ml of RCM liquid medium. Culture incubated at 37°C until optical density at 680nm of 1.3 was obtained.

3.2.2 Preparation of medium

3.2.2.1 Reinforced Clostridia Medium (RCM)

Reinforced Clostridia Medium was used as the control medium during fermentation. 38 g of the RCM powder was dissolved in 1 liter of distilled water and continuous stirrer to dissolve the mixture completely. Then, mixture was transfer to 1 liter scotch bottle and heat up on hotplate to dissolve completely. The solution was autoclaved at 121°C for 20 minutes and medium was kept in freezer under 4°C after autoclaving.

3.2.2.2 Reinforce Clostridia Agar (RCA)

Reinforce Clostridia Agar was used as medium for inoculate single colony of strain after growth in medium. 52.5g of the powder was dissolved with 1 liter of distilled water in 1 liter scotch bottle and heat up on hotplate to dissolve completely. Then, the agar medium was autoclaved at 121°C for 20 minutes. Agar was poured immediately into a sterile Petri dish and universal bottle to avoid contamination. Make sure agar was hardened before keep in freezer at 4°C.

3.2.1.3 Palm Oil Mill Effluent (POME)

Fresh samples of POME were obtained from Kilang Kelapa Sawit Felda Lepar Hilir , Lepar Hilir, Pahang. POME was sediment passively in a cool room at 4°C for 24 hours before used. Then, the supernatant layer (upper part) was decanted and sediment POME sludge (lower part) was sterilized at 121°C for 20 minutes and used directly as fermentation medium without additional nutrient. Sediment POME was dilute with distilled water to obtain required concentration and also calibrate with Natrium

Hydroxide (NaOH) and Acid Hydrochloric (HCl) to maintain at pH 5.8 before deoxidizing by gassing with nitrogen gas for few minutes.

3.2.3 Preparation of 3, 5-Dinitrosalicyclic Acid (DNS) Reagent

300g of potassium sodium tartarate tetrahydrate was weight into 1L conical flask. Then, 16g of sodium hydroxide and 500ml of water was added into flask and heating gently until completely dissolved. After clear solution obtained, 10g of 3, 5-Dinitrosalicyclic acid was added into solution slowly. Make up solution to 1 liter with distilled water and cooled to room temperature. Wrap flask with aluminum foil to keep solution from exposure to light.

3.3 Experimental Procedure

3.3.1 Gram staining of bacteria

Gram staining method was used to confirmation morphology of *Clostridium Beijerinckii.* 3ml of growth bacteria in RCM medium was taken after 24 hours incubation in 37^0C. A loopful of the liquid culture was transferred to a surface of a clean glass slide and spread over a small area. Glass slide was dried over flame on the Bunsen burner. Then, the slide was flood with crystal violet solution for up to one minute. The slide was washing off briefly with tap water (not over 5 seconds) and drain before dry up on Bunsen burner. Gram's iodine solution was flood to the slaid and allows reacting for about one minute before wash off with tap water and drain. The slide was allowed to dry on Bunsen burner. The slide was flooded with acetone for 10 seconds and wash with tap water before dry up on Bunsen burner. Finally, safranin solution was flooded to slaid and allows counterstaining for 30 seconds and washing out with tap water. After slide is dried, bacteria were examined under the oil immersion lens.

3.3.2 Batch Fermentation (Screening Process)

One-factor-at-one-time (OFAT) is firstly employed to identify effect of variable on solvent production from different temperature, inoculums concentration and agitation rate. Batch fermentations were carried out in 500 mL screw-capped bottle (300 mL of medium) and placed in incubator shaker. The medium, POME at 243 mL adding up with 27 mL of sterilized distilled water for 90% concentration of substrate, and was autoclaved for sterilization condition. This was followed by the addition of inoculums solution and the broth was spurge with filtered oxygen-free nitrogen (OFN) gas for 5 minutes to maintain strict anaerobic conditions. The purpose of this experiment is wanted to study the effect of concentration inoculums concentration, initial temperature and effect agitation rate. Figure 3.1 shows the range of parameters that have been used in the study. Fermentation was carried out for 72 hours and 10ml of sample was collected at interval 12 hours for solvent production determination. Fermentation pH is not controlled in fermentation but initially adjusted to pH 5.8.

Sample was taken in interval time 12 hours between 74 hours for analysis. 5ml of sample was taken and centrifuge using 130000rpm for 20 minutes to separate cell with solvent. The supernatant was taken to analysis solvent production in gas chromatography.

Table 3.1: The range of Parameters

No.Run	Parameter		
	Temperature, °C	Agitation Rate, rpm	Inoculums Concentration,%
1	25	150	10
2	30		
3	35		
4	40		
5	45		
6	37	50	10
7		100	
8		150	
9		200	
10		250	
11	37	150	5
12			10
13			15
14			20
15			25

3.4 Methods

3.4.1 Bacterial Growth Profile

Bacterium growth population is studied by analyzing the growth curve of a microbial culture. Bacteria are cultivated in 250ml flask batch fermentation with optimum condition of ABE fermentation that is temperature at 37^0C, 10% inoculums concentration, 90% concentration POME and sample was taken within 72 hours interval of time. Bacterial growth was analysis by determine concentration in UV-Vis spectrophotometer at 680nm wavelength. Reading was recorded.

3.4.2 Preparation of Solvent standard curve

A calibration curve had to be constructed prior to sample analysis for determining the response factor. Standard for calibration curve was serving for ethanol, butanol, acetone and isobutanol as internal standard at different concentration. Solvent with different concentration was prepared as table 3.2 below. For internal standard, isobutanol constant concentration was used that is 2% v/v.

Table 3.2: Concentration of Standard Solvent

Solvent	Concentration (% v/v)
Acetone	0.5
	1
	1.5
	2
	2.5
Butanol	1
	2
	3
	4
	5
Ethanol	0.2
	0.4
	0.6
	0.8
	1

3.4.3 Solvent Analysis by Gas Chromatography equipped with a flame ionization detector (GC-FID)

1.5 ml of sample was taken at interval 12 hours for analysis and mixed with 1.5 ml of isobutanol as internal standard and incubated at 50°C for 10 minutes. Then, mixture solution will be injected into vial and filtrate using 0.2µm syringe filter before analysis using gas chromatography equipped with a flame ionization detector (FID). Table 3.3 shows method that used in GC-FID.

Table 3.3: Method for solvent analysis by GC-FID

Characteristic	Value
Nitrogen Flow Rate	30 ml/min
Temperature Initial	40^0C
Temperature Final	170^0C
Time Interval	10 minutes
Rate of oven	10^0C/min

3.4.4 DNS Method

3 ml of sample was added with 3ml of DNS solution in the test tube and mix properly. All tubes were covers and places in a boiling water bath at 90^0 C for 10 minutes. After that, sample was cool at room temperature. Sample was measure the absorbance at 540nm using UV-Vis spectrophotometer.

CHAPTER 4

RESULT & DISCUSSION

4.1 Introduction

Solvent fermentation (Acetone, Butanol, Ethanol) from palm oil mill effluent (POME) by *Clostridium Beijerinckii* ATCC 51743 was done in 500 ml Scott bottle with 300 ml working volume. One factor at one time (OFAT) was applied in order to screen the significant factors that affect the solvent production from POME. There are three different factors that had been studied; Inoculums concentration (5 to 25%), temperature (25°C to 45°C) and agitation rate (50 to 250 rpm).Throughout the fermentation process, the initial pH is set constant at 5.7 and only inoculums with optical density 1.3 were been used. In this chapter, three different analysis will be discussed which are growth profile of *Clostridium beijerinckii* ATCC 51743, total solvent production of POME by *C. beijerinckii* ATCC 51743 and analysis of glucose consumption during fermentation.

4.2 Growth Profile of *C. beijerinckii* ATCC 51743

Table 4.1: Growth Profile of *Clostridium Beijerinckii* in Reinforce Clostridium Medium (RCM) and Palm Oil Mill Effluent (POME) medium

Time,hr	OD (680nm)	
	RCM	POME
0	0.262	0.375
12	0.836	0.465
24	2.174	0.788
36	2.114	1.798
48	2.201	1.994
60	2.194	1.918
72	2.181	1.94

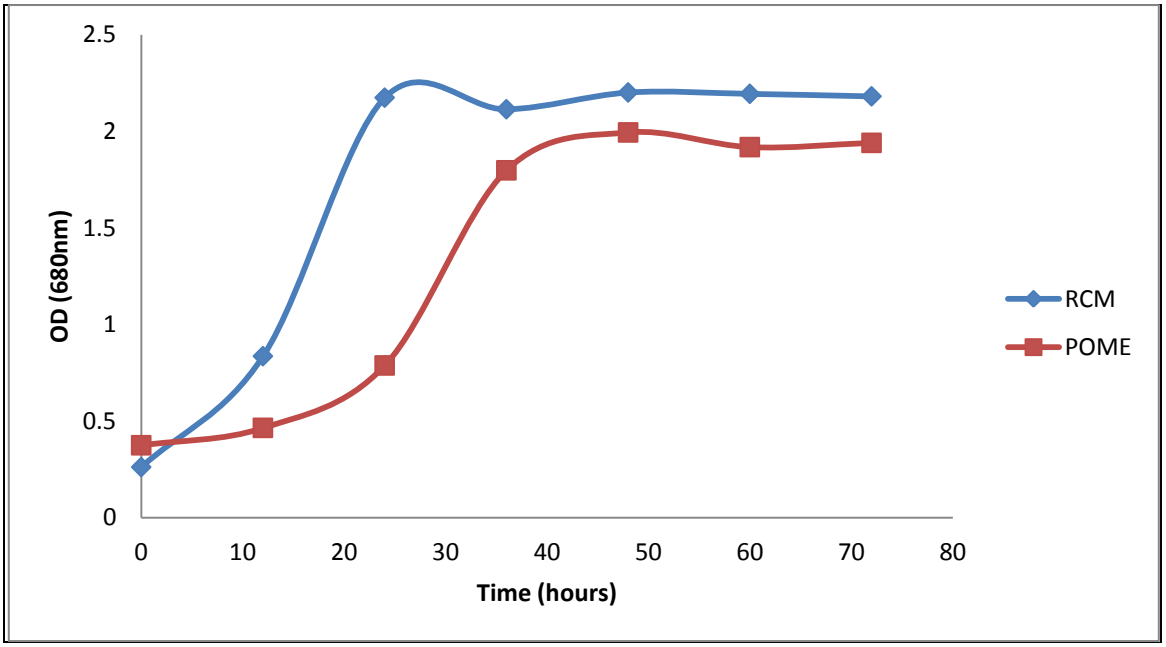

Figure 4.1: Growth Profile of *Clostridium Beijerinckii* in POME and RCM

The ability of bacteria growth in certain medium was studied by analyzing the growth curve of a microbial culture. Growth profile of bacteria *Clostridium Beijerinckii* was studied in two different medium that is synthetic medium, Reinforced Clostridium Medium (RCM) and natural medium, Palm Oil Mill Effluent (POME). Theoretically, bacteria will be reproducing by binary fission and their growth can be divided into four phases which are: lag phase, log phase, stationary phase and death phase.

From Figure 4.1, it is showed that *C. beijerinckii* have a slightly different on their growth pattern, where it take about 12 hours in adapting in RCM meanwhile take about 24 hours for adapting in POME medium before shifting, their lag phase to exponential or log phase. The lag phase occurs when the bacteria are introduces into fresh culture medium, where there will be in an immediate increase in cell number. According to Kenneth (2009), the length of the lag phase is dependent on several factors including the size of inoculums, time needs by bacteria to recover from physical damage or shock during transfer process, time required in synthesis of essential coenzyme and also time required for synthesis new enzyme that necessary for metabolism. Thus, this shows that bacteria *C.beijerinckii* is easily adapted in RCM medium rather than POME, where bacteria required less time to generate enzyme from nutrient in RCM medium to support bacteria growth.

In RCM medium, the bacteria enter the log phase and rapidly increase their number of cell after 12 hours until 36 hours while in POME medium, the bacteria enter the log phase after 24 hours until 48 hours. During this phase, the bacteria was adapting to their new environment, hence resulted in multiplying rapidly and the cell mass and cell number density will be increase exponentially with time. The bacteria growth rate are constant during this phase where the bacteria dividing and doubling in number at regular intervals. The curve rising smoothly due to ability of each individual bacterium divided at a slightly different moment. Biochemical and physiological of bacteria studies usually will be observed in this phase due to the balance growth that occurred when cellular constituents are manufactured at constant rate for each other between cell

bacteria. The cell will enhance protein and DNA synthesis by construct a new ribosome that finally reached expected reproductive rate and will entered stationary phase.

At the stationary phase, the population growth of bacteria remains constant and the growth curve become horizontal line. In RCM, bacteria will reach the stationary phase at early of 36 hours. On the other hand, the bacteria reach the stationary phase of 48 hours in POME medium. The fluctuation growth in log phase become constant because of the net growth rate become zero or the growth rates of bacterial equal to the death rate which than resulted to total number of viable bacterial cell balance with the number of death cell. Bacteria will entered this phase due to several reasons that cannot be avoided. One of the factors is a depletion of essential nutrients supply. In this phase, sugar limitation for *C.beijerinckii* becomes the main factors that make the population growth become slowly. Besides that, the reduction of bacteria growth also affected by an increases of toxic accumulation product from primary metabolite that release to the medium. *C.Beijeinckii* is an anaerobic bacterium that can utilize sugar that derived from cellulose and hemicelluloses in POME medium and produced so much butyric acid and other organic acid from sugar fermentation that will make the medium become acidic and hence the growth will be inhibited.

Finally, when the population of bacteria reached the critical level of limitation nutrient supply or toxicity medium it will result to death phase. The death phase occurred when some cell started to death and the dead cell will be lyses. The intracellular nutrients release into medium which then will be consumed by other viable bacteria cell in the stationary phase. The dead cell is defined to be the irreversible loss of the ability to reproduce a new cell and hence resulted the drastically reduce of the population.

The results also showed that, the growth of bacteria were higher in RCM medium compared to POME medium. This is because, POME medium contain less viable sugar group if compared with RCM medium that contain high glucose viable in medium. From this study, the growth phase that suitable to be used as an inoculums for solvent

fermentation is during, the late log phase between 24-36 hours of incubation with the OD reading at 680 nm, 0.788 to 1.788. In this phase, the bacteria are in their maximum growth rate. Thus, make them suitable to be used as inoculums.

4.3 Solvent Production (Acetone,Butanol,Ethanol) at Different Inoculums Concentration

Table 4.2: Solvent Production at Different Inoculums Concentration

Parameter		Solvent Production, g/L			Total Solvent Production (g/L)
		Acetone	Butanol	Ethanol	
Inoculums Concentration (%)	5	0.234	0.13	0.242	0.606
	10	0.216	0.16	0.343	0.719
	15	0.213	0.117	0.284	0.614
	20	0.147	0.108	0.266	0.497
	25	0.123	0.105	0.231	0.483

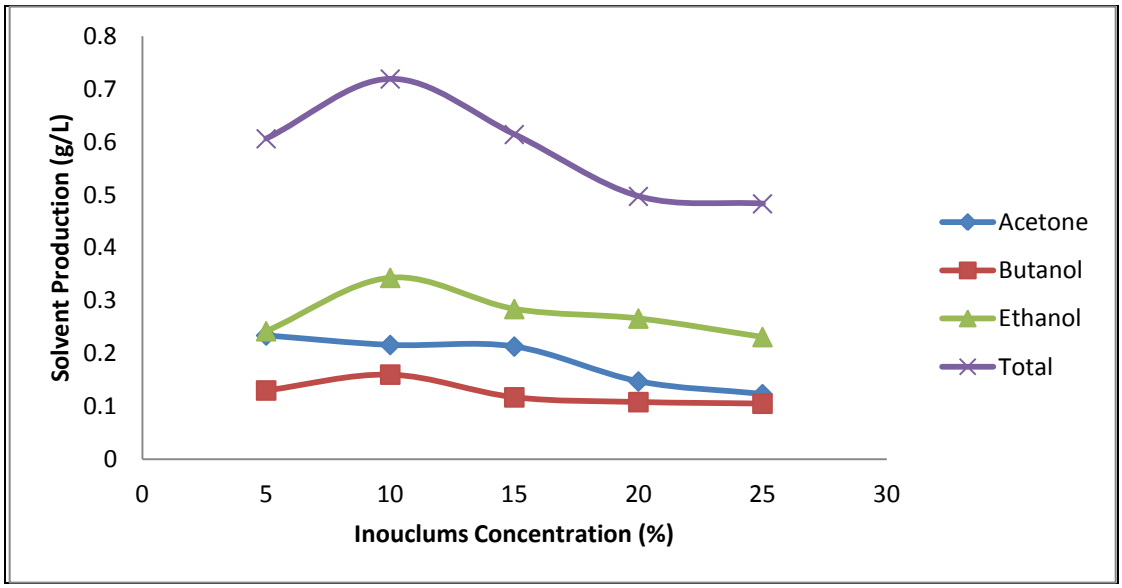

Figure 4.2: Solvent Production at Different Inoculums Concentration

Solvent-producing clostridia, *Clostridium Beijerinckii* gains an interest in solvent fermentation process because of the potential economic of their fermentation end products that is acetone, ethanol and butanol. Result in Table 4.2, showed the solvent production (acetone, ethanol, butanol) at different inoculums concentration. Result demonstrates there an inverse relationship between inoculums concentration with total solvent production. In Figure 4.2, it is showed that the highest total solvent production is at 10% inoculums concentration that is 0.719 g/L solvent production. On the other hand, with further increases inoculums concentration, will decrease total solvent production where the lowest total solvent production is recorded for about 0.483g/L at 25% inoculums concentration. According to Alshiyab *et al*. (2008), the inoculums concentration must be compatible with available substrate for maximum bacteria productivity due to substrate limitation would affect adversely on bacterial growth and finally to production. In addition, with increases inoculums concentration, each individual cell in population will perform less well due to competition for nutrient (Kanchanatawee *et al*., 1990). Thus, an appropriate inoculums concentration is important to achieve high solvent production that depends on the nutrient available for bacterial growth in substrate.

Throughout the fermentation, Figure 4.2 showed that ethanol produce more if compare with acetone and butanol. The highest production ethanol is at 10% inoculums concentration that recorded 0.343g/L of production compared to production of acetone is at 5% inoculums concentration with 0.234 g/L and butanol production is 0.13 g/L at 10% inoculums concentration. This pattern of production solvent can be analysis during solvent-producing phase. During this phase, acetyl-coA and butyryl-CoA produce as the key function to produce ethanol and butanol. Acetyl- CoA will reduce to acetaldehyde and acetoactyl-coA where, acetaldehyde will directly produce ethanol while acetoacytl-coA will produce acetone then reduce to butyryl CoA and produce butanol. This shows that, ethanol can produce independently from acetone and butanol that resulted produces high concentration rather than acetone and butanol. Besides that, according to Gregor *et al.* (1988) the low solvent yields are obtained owing to butanol toxicity and the short duration of solventogenic phase.

4.4 Solvent Production at Different Temperature

Table 4.3: Solvent Production at Different Temperature

Parameter		Solvent Production,g/L			Total Solvent Production (g/L)
		Acetone	Butanol	Ethanol	
Temperature(°C)	25	0.124	0.117	0.189	0.43
	30	0.165	0.12	0.283	0.568
	35	0.161	0.129	0.27	0.56
	40	0.151	0.106	0.265	0.482
	45	0.111	0	0.24	0.411

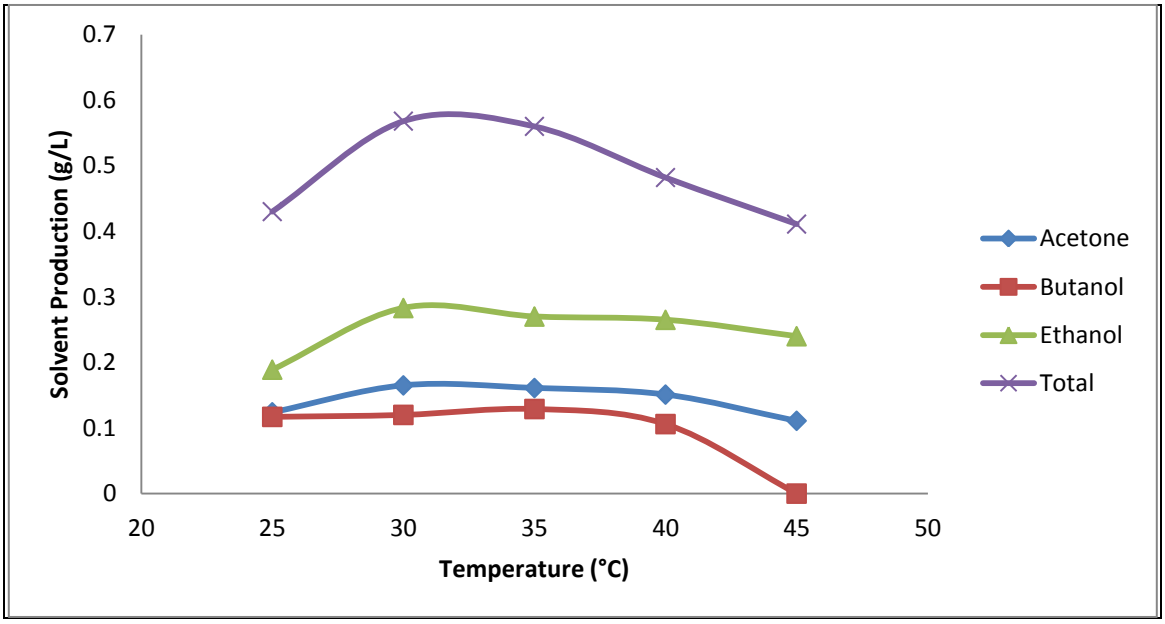

Figure 4.3: Solvent Production at Different Temperature

In this experiment, temperature effect was studied in order to determine optimum temperature of solvent production. Table 4.3 shows the solvent production will decrease with the increases temperature. Preliminary study from Saleha *et al*. (2006) on effect of temperature to solvent production also demonstrates that solvent productivity decrease with the increase in temperature. In Figure 4.4, shows total solvent production is higher at 30°C about 0.568 g/L solvent production. However, further increases temperature will reduce total solvent production where at temperature 35°C until 45°C total solvent production has observed will start to decrease from 0.560 g/L to 0.411 g/L. This finding showed that, at 30°C would enhance solvent production and these results are in agreement with the reported by Saleha *et al*. (2006). Thus, an optimum temperature was important to promote solvent production where suitable temperature will leads to maximums bacterial activity. On the other hand, with inappropriate temperature in fermentation process will obviously reduce solvent production due to diminishing of cell active.

This result demonstrate that in POME substrate, *Clostridium Beijerinckii* is unable to growth when temperature is higher than 40°C and resulted in reducing solvent production. Bacteria unable to tolerate with high temperature that lead to bacteria lysis and die. This result shows that temperature control is critical, since increases of fermentation temperature lead to reduction of solvent production. In addition, Perego (2003) reported that temperature effected to optimum growth of solventogenic bacteria during value within (30 to 39°C) while production solvent will decrease is observe at temperature higher than optimum value due to inactivation of biosystem temperature that inhibit growth of bacteria. This also supported with studied by Szewczyk and Myszka (1994), due to the deactivation effect of temperature on endogenous metabolism, for temperature above from 34°C, the maintenance coefficient of bacteria will decreases as the temperature increase.

Results from Figure 4.3 showed that maximum production of ethanol and acetone is at 30°C that produce 0.283 g/L production of ethanol while production acetone is 0.165 g/L. Meanwhile, the optimum temperature for production the highest of

butanol production is at 35°C for about 0.129 g/L. But, with further increases temperature, the production of butanol was dramatically decreases to 0.106g/L at 40°C and no production of butanol is obtain at 45°C. However, this result not exactly the same with result that demonstrate by Saleha *et al*. (2006), where state that fermentation with 35°C could induce acetone and ethanol production while temperature of 30°C would enhance butanol production. Contrast result obtain by Carlos (2010) in study effect temperature to butanol production, where an increases butanol production rising in temperature from 35°C to 40°C.

Results have showed that ethanol and acetone has produce more rather butanol at any point of temperature. This is because ethanol and acetone has lower optimum temperature production that is 30 °C if compared with optimum temperature of butanol production is 35°C. Bandaru *et al.,* (2005) observed that increase in the temperature up to optimum point will increased the ethanol production to maximum level and further increase in the temperature will decreased the ethanol production. This result demonstrate that, *C.beijerinckii* ATCC 51743 were unable to sustain longer at temperature more than 35°C thus resulted to the higher production of ethanol and acetone unfortunately, lower butanol production. Therefore, 30°C was considered as suitable temperature for ethanol and acetone production while butanol is 35°C.

4.5 Solvent Production at Different Agitation Rate

Table 4.4: Solvent Production at Different Agitation Rate

Parameter		Solvent Production, g/L			Total Solvent Production (g/L)
		Acetone	Butanol	Ethanol	
Agitation Rate (rpm)	50	0.114	0.120	0.176	0.410
	100	0.126	0.170	0.248	0.544
	150	0.201	0.190	0.284	0.675
	200	0.180	0.140	0.250	0.570
	250	0.100	0.005	0.220	0.325

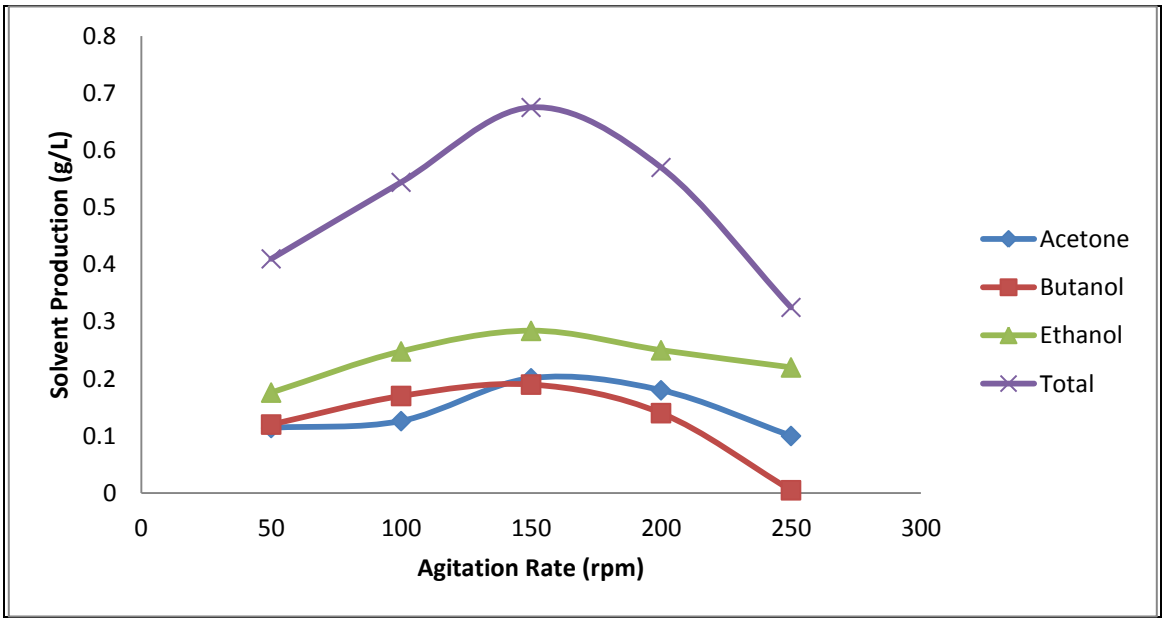

Figure 4.4: Solvent Production at Different Agitation Rate

Result in Table 4.4 showed that the solvent production was decreased significantly after 150 rpm. The presence of inactive or dead cell and also deficiency of nutrient were given earlier as possible reason for decreased total solvent production by *Clostridium Beijerinckii* during ABE fermentation (Ezeji *et al.*, 2003). Figure 4.4 showed that with the increases of agitation rate from 50 until 150 rpm will increase the

total solvent production that is from 0.41 to 0.675 g/L. However, with further increases of agitation rate from 200 to 250 rpm, the production of total solvent started to reduce from 0.57 to 0.325 g/L. Wang and Blascheck (2011) also demonstrate that with the increase of agitation rate from 0 to 150 rpm, the solvent yield gradually increase, and then will decrease slightly with further increases of agitation rate.

Regarding solvent production, ethanol showed the highest production compared butanol and acetone. Ethanol recorded production maximum 0.284 g/L at 150 rpm while acetone is 0.201 g/L and butanol production is 0.19 g/L. Further increases agitation rate obviously shows reduction of butanol concentration where the minimum butanol production is 0.005g/L recorded compared with ethanol 0.22 g/L and acetone 0.10 g/L. Batch fermentation with varying agitation rates will affect the level of dissolved hydrogen gas in media which also influence solvent production (Doremus *et al.,* 1985). The decreases ratio of butanol in solvent fermentation with higher agitation rate also contributes from the emission of CO_2 gas that facilitates carbon flow to production ethanol and acetone instead butanol production (Wang and blaschek, 2011). Thus, this investigation shows that maximum solvent production was at 150 rpm with further increases agitation rate will decrease the production of solvent.

According to Wang and blascheck (2011), a suitable agitation rate is need to facilitate the substrate mixing that enhance substrate accessibility to used during fermentation but high agitation rate lead to unnecessary waste of energy and poor economic industry. Mixing is important in minimizing local variation in concentration and temperature effect where it serve an adequate mass transfer trough out the suspension by arises the random redistribution elements of culture suspension in medium. However, high rate of agitation will increase the amount of foam production due to microbial protein or other component of the media. Foaming not only will causes deposited cell on the top of medium fermentation and removal of cell from media, it also will reduce the working volume in fermentation vessel that can disturb nutrient supply for bacterial growth.

4.6 Glucose Consumption

4.6.1 Glucose consumption at Different Inoculums Concentration

Table 4.5: Glucose consumption at Different Inoculums Concentration

Time(hours)	OD (540 nm) Inoculums Concentration (%)				
	5	10	15	20	25
0	2.27	2.94	2.57	2.42	2.52
12	2.25	2.44	2.52	2.4	2.43
24	2.24	2.43	2.49	2.36	2.37
36	2.21	2.37	2.39	2.29	2.17
48	1.66	2.27	2.36	2.05	2.03
60	1.6	1.57	2.26	1.83	1.91
72	1.41	1.48	1.71	1.77	1.56
Glucose Consumption (%)	37.9	50.0	33.5	27	38

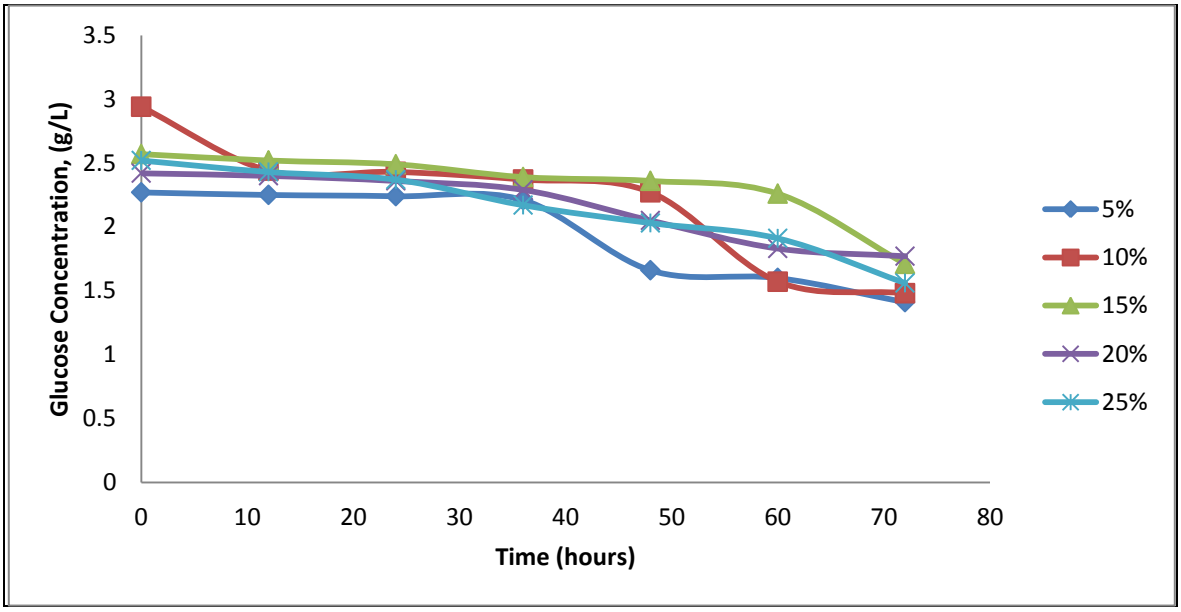

Figure 4.5: Glucose Consumption at Different Inoculums Concentration

Table 4.6: Relation between Glucose Consumption with Total Solvent Production at Different Inoculums Concentration

Inoculum Concentration (%)	Glucose Consumption (%)	Total Solvent Production (g/L)
5	37.9	0.43
10	50.0	0.568
15	38.0	0.56
20	33.5	0.482
25	27.0	0.411

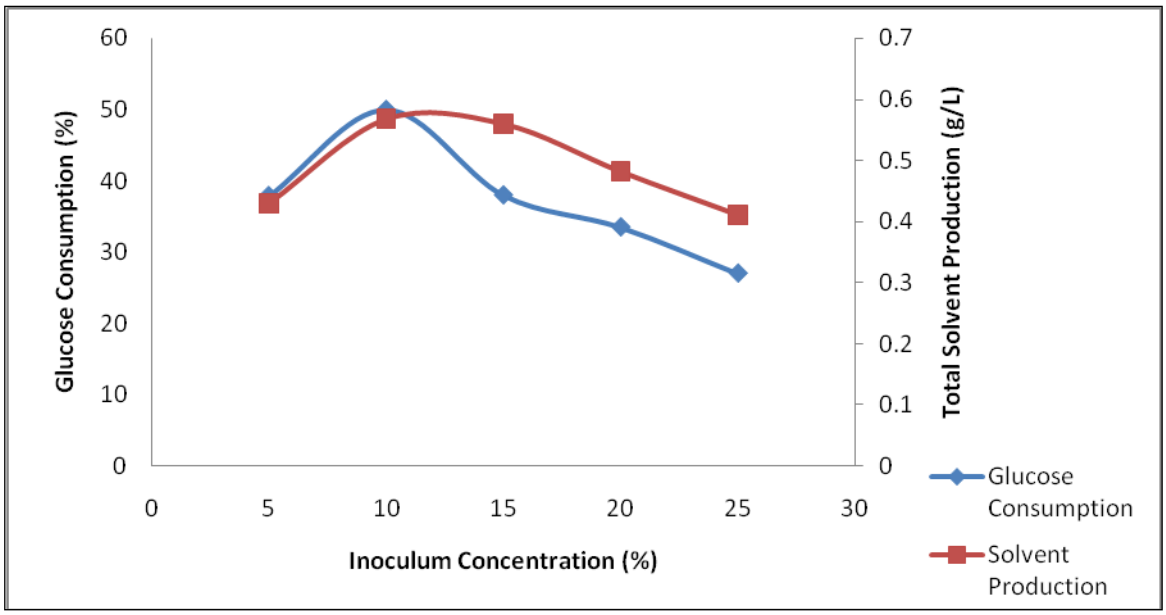

Figure 4.6: Relation between Glucose Consumtion with Total Solvent Production at Different Inoculums Concentration

Observation of residual glucose concentration obtained during batch fermentation process of *C.beijerinckii* in Palm Oil Mill Effluent (POME) medium was done by using DNS analysis. It was observed that significant amount of glucose consumption has been utilized by bacteria in order for cell growth and production of solvent. Table 4.5 showed the glucose concentration that left after 72 hours of fermentation process and the percentage glucose consumption at different inoculums concentration. The result obtain from fermentation of different inoculums concentration shows that *C.Beijerinckii* ATCC 51743 is less to utilize lignocelluloses biomass from POME medium into glucose directly for production solvent. This is because there still left high concentration of glucose after 72 hours fermentation even applied with high concentration of inoculums.

From Figure 4.5, the highest glucose consumption for 50% glucose consumption is at 10% inoculums concentration while the lowest glucose consumption is at 25% inoculums concentration for only 27% glucose consumption. This shows that, concentration inoculums at 10% is suitable for used as fermentation inoculums because the inoculums concentration was compatible with available substrate that encourage bacteria to utilize glucose viable at POME medium effectively. Compared to the 25% of inoculums concentration, bacteria and nutrient available is not balance thus resulted to the lowest percentage glucose consumption by *C. beijerinckii*.

The total solvent production was depend on ability of bacteria in utilize nutrient of medium and sustain longer to produce more production. Figure 4.9 shows relation between percentage of glucose consumption and total solvent production. At 10% inoculums concentration shows the maximum glucose consumption that is 50% resulted to the highest total solvent production that is 0.568 g/L. Otherwise, with further increases inoculums concentration from 15% to 25% inoculums concentration, percentage of glucose consumption was decrease resulted to slightly decreasing on total solvent production. Thus, in this study found that with the highest glucose consumption, production of solvent will increase due to ability of bacteria in utilizing carbon source for growth and solvent production efficiently.

4.6.2 Glucose Consumption at Different Temperature

Table 4.7: Glucose Consumption at Different Temperature

Time(hours)	OD (540 nm)				
	Temperature (°C)				
	25	35	30	40	45
0	2.77	2.66	2.62	2.79	2.63
12	2.74	2.64	2.55	2.66	2.57
24	2.66	2.58	2.45	2.63	2.51
36	2.56	2.55	2.43	2.57	2.38
48	2.54	2.52	2.03	2.23	2.29
60	2.37	1.56	1.56	1.91	2.04
72	1.63	1.21	1.27	1.37	1.91
Glucose Consumption (%)	41.2	54.5	51.5	50.9	27.3

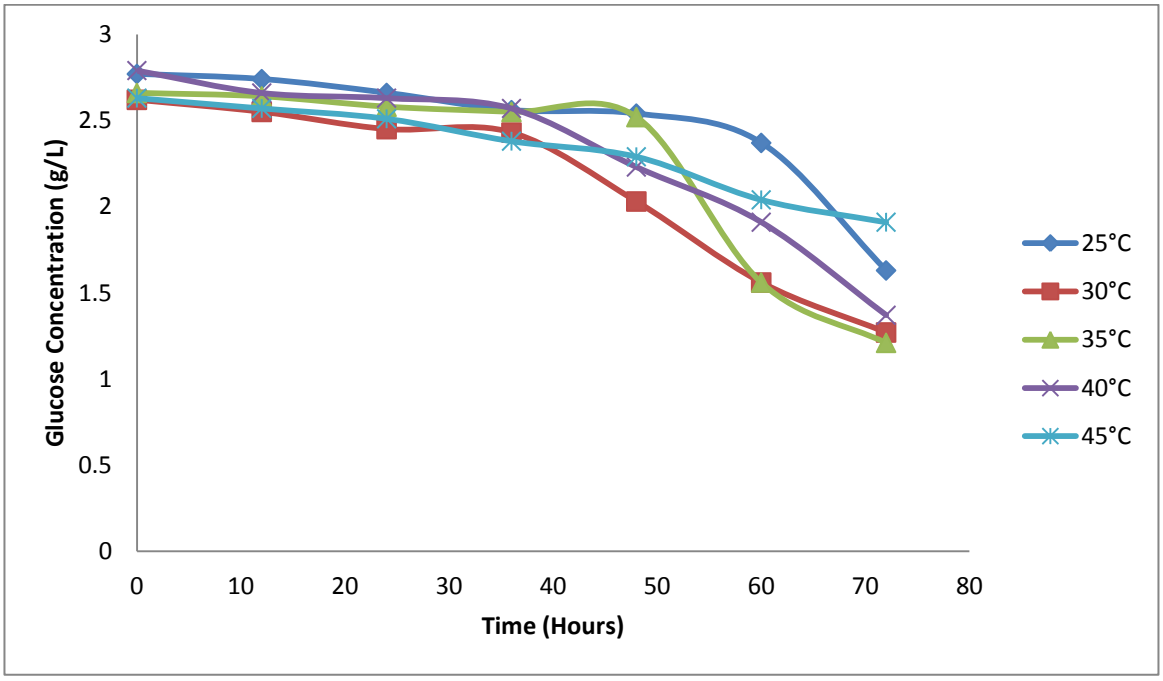

Figure 4.7: Glucose Consumption at Different Temperature

Table 4.8: Relation between Glucose Consumption with Total Solvent Production at Different Temperature

Temperature (°C)	Glucose Consumption (%)	Total Solvent Production (g/L)
25	41.2	0.43
30	54.5	0.568
35	51.5	0.56
40	50.9	0.482
45	27.3	0.411

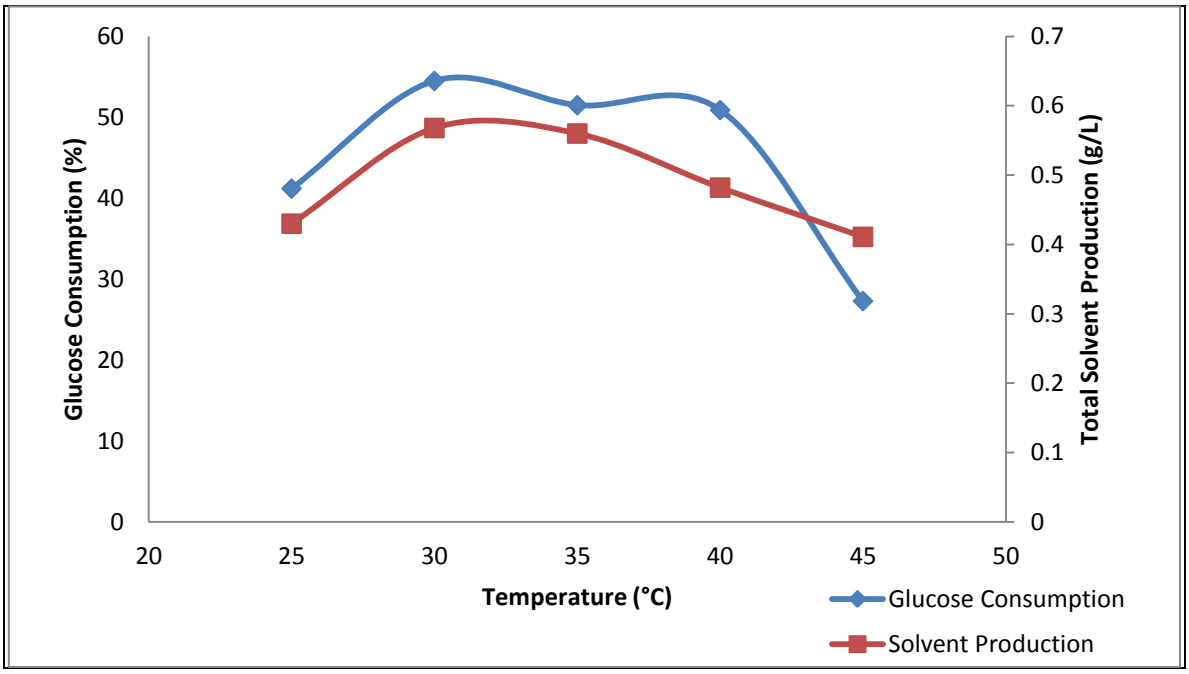

Figure 4.8: Relation Glucose Consumption with Total Solvent Production at different Temperature

Temperature is an important parameter to be optimized during fermentation in order to minimize energy cost of process. The results obtained from fermentation of different temperature showed that *C.beijerinckii* is capable to utilize glucose for

production of solvent efficiently at temperature range from 30°C until 40°C. This characteristic applied when *C.Beijeinckii* shows higher sugar utilization and total solvent production that demonstrated in Table 4.7 and 4.8.

Throughout the fermentation, Figure 4.7 shows that at low temperature, 25°C percentage of glucose consumption was at lower value, 27.3%. The lower percentage of glucose utilization because of at low temperature bacteria gain less energy resulted metabolic process is at low level. Following with temperature at 30°C, the culture is able to consume glucose reach to 54.5% of glucose. Meanwhile at 35°C, percentage of glucose consumption reduces slightly to 51.5%. At this range of temperature, optimum state was showed where bacteria actively to growth at this temperature and actively utilized glucose in medium. Metabolism activity of bacteria at optimum temperature was at maximum state due to activation energy that effected by optimum temperature. However at 40°C and 45°C, reduction of utilization glucose was observed that is 41.2% and 50.9%. At this stage, bacteria no longer able to growth and chance to lyses or death were high due to bacteria limitation. According to Rahman *et al.* (2005), temperature strongly influences the rates of biochemical reactions and also inducing or repressing production of solvent.

From Figure 4.8, it is found that percentage of glucose consumption is directly proportional to total solvent production. This can been seen at temperature 30°C where the total production is at the highest level production that is 0.568 g/L with the percentage of glucose consumption is at 54.5%. However, total solvent production was rapidly decrease at 45°C, where level of glucose consumption is at the lowest percentage that is 27.3% and reduction the total solvent production to 0.411 g/L was seen. This demonstrates that, glucose is utilized by bacteria to generate acid in producing solvent. Thus, production of solvent is at high level when glucose is consumed by bacteria at maximum rate. Ezeji *et al.*, (2005) state that the fermentation was active when the residual glucose concentration in fermentation is decrease where there was drift toward acid production.

4.6.3 Glucose Consumption at Different Agitation Rate

Table 4.9: Glucose Consumption at Different Agitation Rate

Time(Hours)	OD (540 nm)				
	Agitation Rate (rpm)				
	50	100	150	200	250
0	2.71	2.74	2.77	2.79	2.83
12	2.63	2.62	2.59	2.66	2.66
24	2.57	2.6	2.56	2.63	2.59
36	2.42	2.57	2.54	2.59	2.57
48	2.16	2.52	2.51	2.53	2.53
60	2.1	2.16	1.59	1.68	2.51
72	1.98	1.7	1.16	1.43	2.48
Glucose Consumption (%)	26.9	37.9	58.12	48.7	12.37

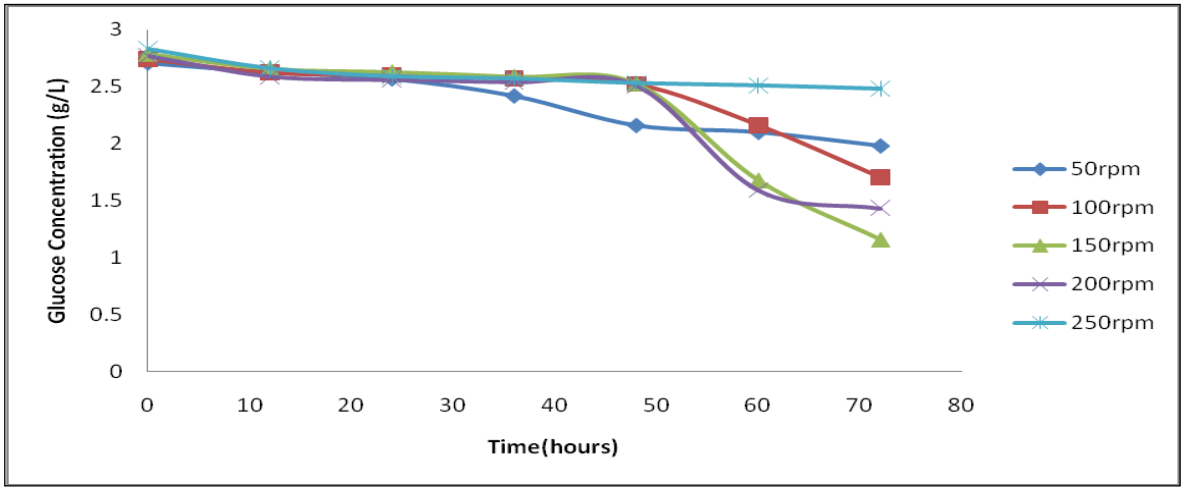

Figure 4.9: Glucose Consumption at Different Agitation Rate

Table 4.10: Relation between Glucose Consumption with Total Solvent Production at Different Agitation Rate

Agitation Rate (rpm)	Glucose Consumption (%)	Total Solvent Production (g/L)
50	26.9	0.41
100	37.9	0.544
150	58.12	0.675
200	48.7	0.57
250	12.37	0.325

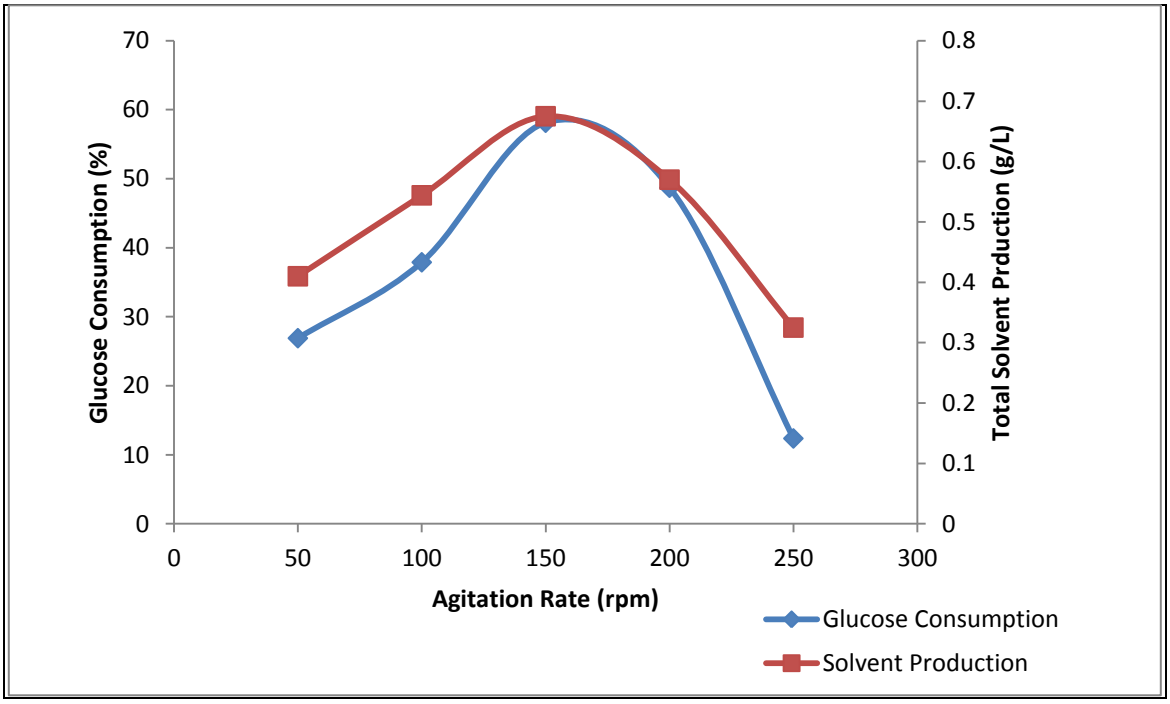

Figure 4.10: Relation between Glucose Consumption with Total Solvent Concentration at Different Agitation Rate

Prior to carrying out solvent fermentation, investigation was made to ascertain the ability of *C.beijerinckii* to adapt with different agitation rate during fermentation. Table 4.9 represents glucose consumption during solvent fermentation that has run for 72 hours with different agitation rate. Fermentation process was conducted in 500mL flask and condition of fermentation such as inoculums concentration, temperature and pH were fixed at 10%, 37°C and pH 5.7 respectively.

Based on Figure 4.9, as the agitation rate increased, the glucose consumption was increase. Wang *et al.* (2011), reported that effect of agitation rate to solvent production are not critical as effect of pH but it still contribute in enhance production of solvent during fermentation process. From the graph, with the increase agitation rate from 0 to 150 rpm, percentage glucose consumption was increase obviously from 26.9% to 58.12% then solvwnt production will decreases with increases agitation rate. Agitation rate has the influence to enhance the solvent production due to better mixing within medium with inoculums.

In Figure 4.10 shows the relation between total solvent production and glucose consumption with agitation rate effect. The highest total solvent production, 0.675 g/L was observed at 150 rpm with the highest glucose consumption value, 48.7%. Meanwhile, the lowest total production 0.375 g/L was observed at the lowest glucose consumption value 12.37% at 250 rpm. This finding suggests that with the increases of agitation rate, the percentage of glucose consumption will increase until it reached optimum value and decline obviously after that. Meanwhile, the total solvent production will decrease at high agitation rate due to excessively enzyme breakdown during fermentation process (Zaliha *et al.*, 2004).

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusion

From the result, it is showed that the solvent production will increase as temperature increase until reach optimum value, production of solvent will decrease. Optimum temperature for solvent production is in range 30 to 35°C. Besides that, appropriate inoculums concentration is important in the solvent production. Optimum inoculums concentration for solvent production is at 10%. Meanwhile, with the increase of agitation rate up 0 to 150 rpm, the solvent yield gradually increase, and then will decrease slightly with further increases of agitation rate. Thus, optimum agitation rate for solvent production is at 150 rpm. These results also conclude that, the glucose consumption is directly proportional to the solvent production.

5.2 Recommendation

Traditionally, technique of ‘one-factor at a time’ was applied widely in optimizing production for multivariable system. Unfortunately, this method is not only time consuming but also often easily misses the alternative effect between components. Thus, many statistical experimental design methods were developing in bioprocess optimization (Jianliang *et al.,* 2008). Respond surface methodology (RSM) is one of statistical method that becomes favorable to replace conventional method. RSM involve in designing experiment, building models through regression and evaluating optimum conditions for desirable response to increase production (Afshin *et al.,* 2008). This method was developing efficiently of complex process chemical engineering, waste treatment process and biological fermentation by performing minimum number of experiment (Wang *et al*., 2011). Thus, application RSM in solvent production from various renewable resources becomes favorable in solvent fermentation process review.

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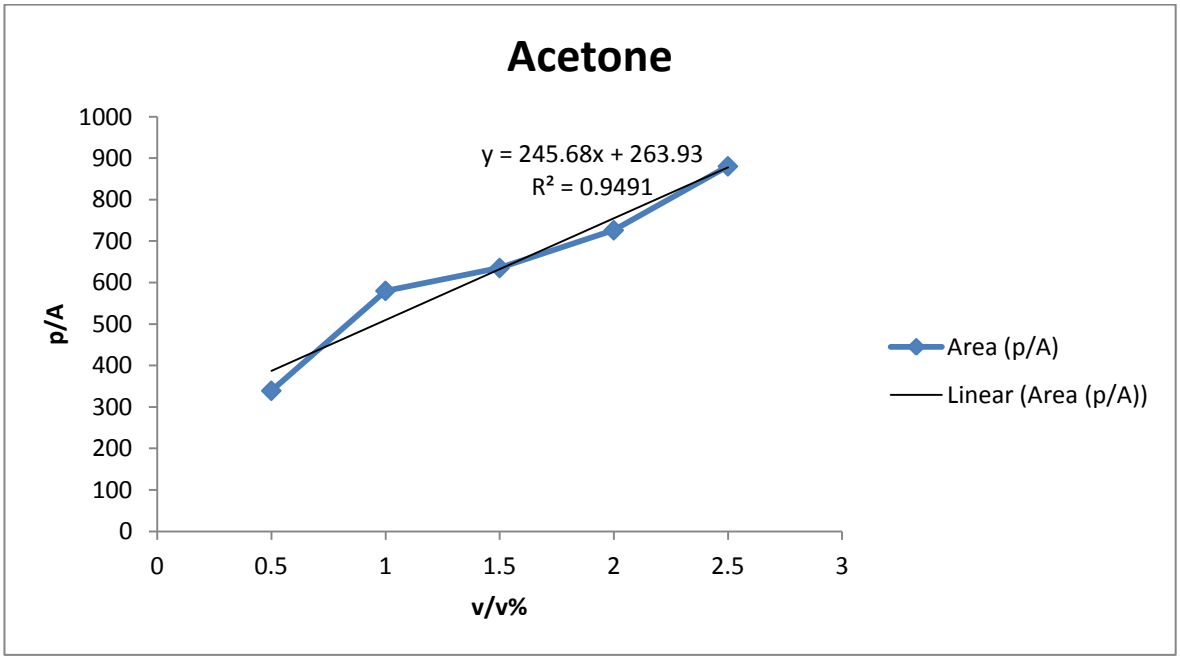
APPENDIX A

STANDARDS FOR ACETONE, BUTANOL AND ETHANOL

A1 Acetone

Table A1: Concentration of Acetone versus Area using GCFID

Concentration (g/L)	Area(p/A)
0.5	339.4
1	580.3
1.5	635.5
2	726.4
2.5	880.6



A2 Butanol

Figure A2: Concentration of Butanol versus Area using GCFID

Concentration (g/L)	Area(p/A)
1	324.4
2	1002
3	1419
4	1612
5	2094

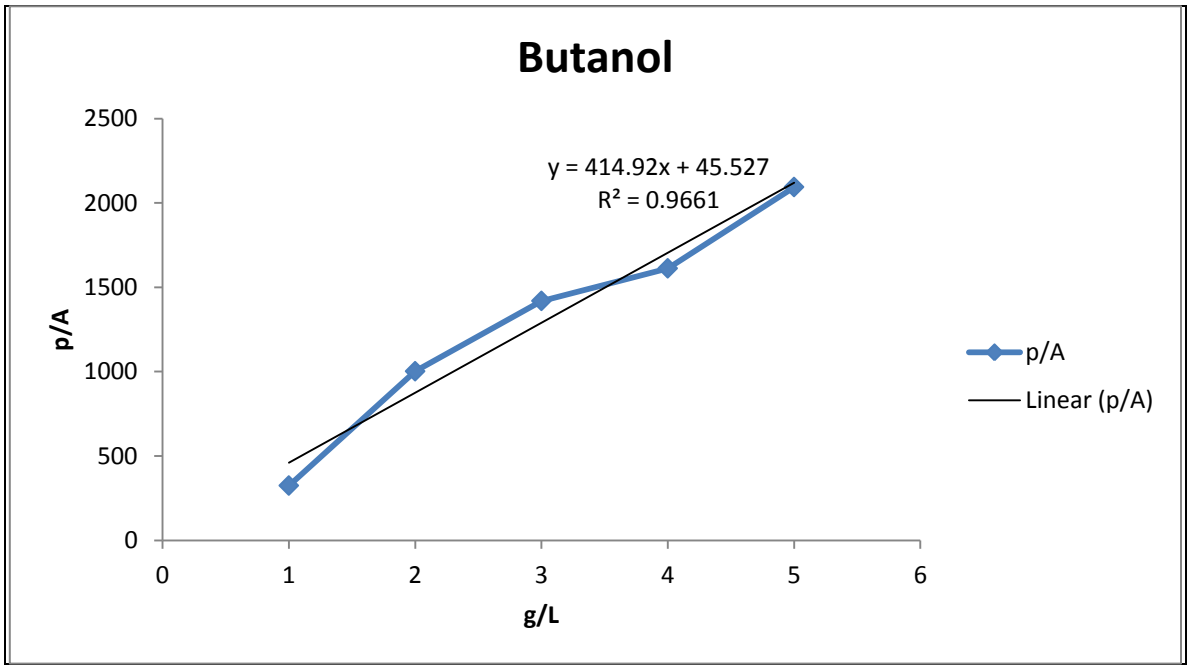


Figure A2: Butanol Concentration versus Area using GCFID

A3 Ethanol

Table A3: Ethanol Concentration versus Area using GCFID

Concentration (g/L)	Area(p/A)
0.2	155.8
0.4	307.3
0.6	292.5
0.8	403.9
1	459.22

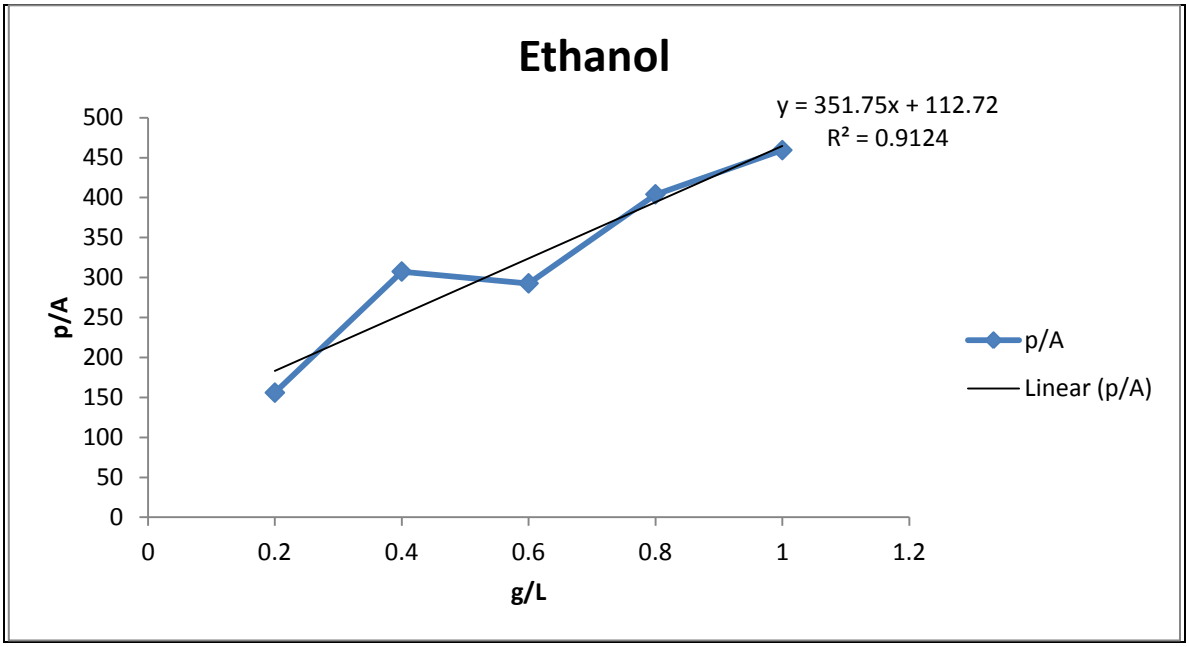


Figure A3: Ethanol Concentration versus Area using GCFID

APPENDIX B

RESULT GROWTH PROFILE

B1 Growth Profile *C.beijerinckii* in RCM medium

Table B1: Reading OD for Growth Profile *C.beijerinckii* in RCM medium

Time (hours)	OD (680nm)			
	Reading 1	Reading 2	Reading 3	Average
0	0.26	0.263	0.262	0.262
12	0.831	0.834	0.837	0.836
24	2.17	2.173	2.175	2.174
36	2.113	2.114	2.116	2.114
48	2.2	2.203	2.202	2.201
60	2.19	2.193	2.195	2.194
72	2.18	2.181	2.184	2.181

B2 Growth Profile *C.beijerinckii* in POME medium

Dilution Factor

$$= \frac{Total\ Volume\ solution\ (ml)}{Volume\ Sample\ (ml)}$$

$$= \frac{3ml}{0.4ml\ POME}$$

= 7.5

Table B2: Reading OD for Growth Profile *C.beijerinckii* in POME medium

Time (hours)	OD (680nm)			
	Reading 1	Reading 2	Reading 3	Average
0	0.374	0.373	0.377	0.375
12	0.461	0.463	0.466	0.465
24	0.788	0.786	0.788	0.788
36	1.79	1.798	1.798	1.798
48	1.99	1.994	1.994	1.994
60	1.918	1.916	1.918	1.918
72	1.94	1.94	1.93	1.94

APPENDIX C

DNS STANDARD CURVE

Table C1: Reading OD for DNS standarad curve

Concentration (g/L)	OD(540nm)
0	0
0.2	0.092
0.4	0.368
0.6	0.524
0.8	0.71
1	0.931

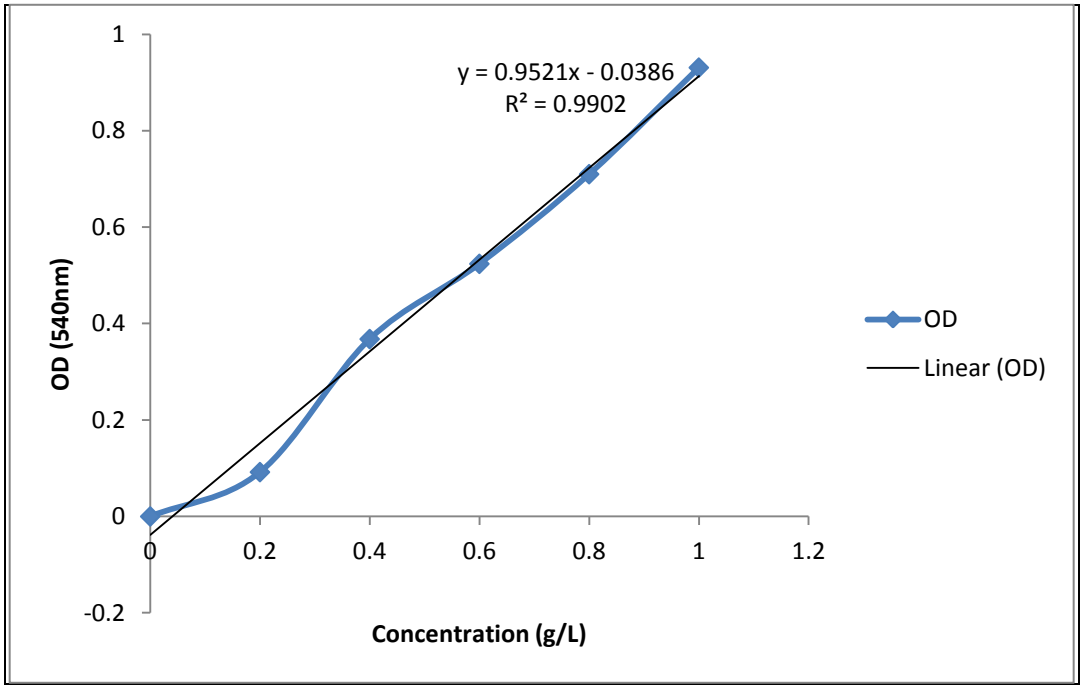


Figure C1: DNS standard curve for Glucose Consumption

APPENDIX D

PICTURE

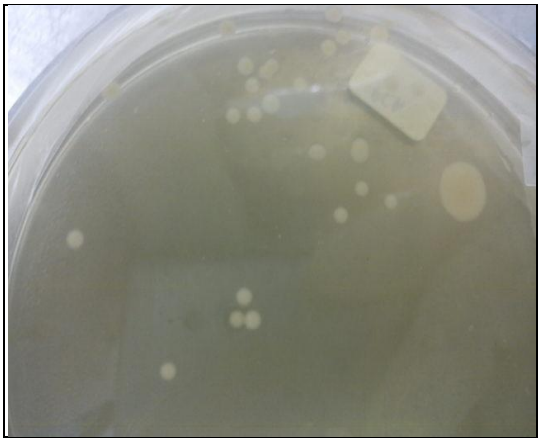


Figure E1: Single Colony for *Clostridium Beijerinckii*

Figure E2: Sedimentation of POME