PRODUCTION OF ABE (ACETONE-BUTANOL-ETHANOL) FROM POME BY *Clostridium beijerinckii*

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PRODUCTION OF ABE (ACETONE-BUTANOL-ETHANOL) FROM POME BY *Clostridium beijerinckii*

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Thesis submitted in partial fulfilment of the requirements
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SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis was adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

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STUDENT'S DECLARATION

I hereby declare that the work in this thesis was my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and was not concurrently submitted for award of ot
her degree.

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ABSTRACT

Malaysia was one of the biggest producers and exporters of palm oil and palm oil products (Malaysian Palm Oil Council, 2012). Thus, the total oil palm cover has increased with a corresponding increase in palm oil production. As a result, palm oil waste which was a by-product of the milling process will also increase. Due to the presence of high total solids in palm oil mill effluent (POME), attempts have been made to convert this waste into valuable products such as feed stock (Rupani *et al.*, 2010). Consequently, purpose of this research was to study about the production of acetone-butanol-ethanol (ABE) solvents using POME by *Clostridia beijerinckii*. These ABE solvents which are consist of acetone, butanol and ethanol have each one benefits such as butanol used for biofuel, acetone used for making plastic and ethanol can be used as disinfectant. The culture was grown in RCA and then 10% v/v of the culture was transferred to RCM for preparation of inoculum medium. After that, 10% v/v of inoculum medium was transferred into fermentation medium which consists of 90% of POME. In this research, POME would be used as substrate medium. Then, the fermentation medium was run to find the optimum pH for fermentation medium (initial pH 5.4 until 6.2), concentration of POME (80% to 100% v/v) and temperature of fermentation (27°C to 47°C). The highest value of total ABE obtained was 0.771 g/L with 90% concentration at condition of pH 5.8 of 37°C.

ABSTRAK

Malaysia merupakan salah satu pengeluar dan pengeksport terbesar minyak sawit dan produk minyak sawit (Majlis Minyak Sawit Malaysia, 2012). Oleh itu, jumlah perlindungan kelapa sawit telah meningkat dengan peningkatan dalam pengeluaran minyak kelapa sawit. Kesannya, sisa kelapa sawit yang merupakan hasil sampingan proses pengilangan juga akan meningkat. Kerana kehadiran jumlah pepejal yang tinggi di kilang minyak sawit (POME), percubaan telah dibuat untuk menukar sisa ini ke dalam produk berharga seperti stok makanan (Rupani *et al*.,2010). Oleh itu, tujuan kajian ini adalah untuk mengkaji mengenai pengeluaran aseton-butanol-etanol (ABE) pelarut menggunakan POME oleh *Clostridia beijerinckii*. Pelarut ABE ini yang terdiri daripada aseton, butanol dan etanol mempunyai setiap satu faedah seperti butanol digunakan untuk biofuel, aseton digunakan untuk membuat plastik dan etanol boleh digunakan sebagai disinfektan. Budaya ini telah ditanam di RCA dan kemudian 10% v/ v budaya telah dipindahkan ke RCM untuk penyediaan medium inokulum. Selepas itu, 10% v/ v inokulum telah dipindahkan ke dalam medium fermentasi yang terdiri daripada 90% daripada POME. Dalam kajian ini, POME akan digunakan sebagai medium substrat. Kemudian, sederhana penapaian telah dijalankan untuk mencari pH optimum untuk medium penapaian (pH awal 5.4 sehingga 6.2), kepekatan POME (80% kepada 100% v/v) dan suhu fermentasi (27 °C hingga 47 °C). Nilai tertinggi daripada jumlah ABE yang diperolehi ialah 0.771 g/L dengan kepekatan 90% pada keadaan pH 5.8 daripada 37° C.

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LIST OF ABBREVIATIONS

ABE	Acetone-Butanol-Ethanol
CPO	Crude Palm Oil
DNS	Dinitrosalicylic Acid
FID	Flame Ionization Detector
GC	Gas Chromatography
min	Minute
OD	Optical Density
POME	Palm Oil Mill Effluent
RCA	Reinforced Clostridia Agar
RCM	Reinforced Clostridia Medium
UV-VW.	Ultraviolet-Vis Spectroscopy

1 INTRODUCTION

1.1 Background of research

The revival of A–B–E fermentation was currently being inspired by the consideration of butanol for biofuel. (Lee *et al*, 2008a). During early 20th century through World Wars I and II, ABE fermentation was significant for the production of butanol and acetone solvents. However, its use has declined since the 1950s due to increasing costs of the substrate molasses and unable to compete economically with petrochemically produced ABE.

As the result, the following factors which severely affect the economics of ABE fermentation were identified which high cost of substrate, low product concentration (<20 g/L), low reactor productivities (<0.3 g/L/h), low ABE yields (0.28–0.33); and an escalated cost of butanol recovery by distillation which was the only technique for recovery at that time. Additional factors such as bioreactor costs, interest rate on the borrowed capital, and rate of return on the investment were also identified as factors which affect the price of fuels derived from renewable resource (Qureshi & Blaschek, 2001). Nevertheless, in 1973, because of oil price increases, this crisis led to renewed interest in solvent production by ABE fermentation (Tashiro & Sonomoto, 2010).

Recently, there has been increased interest on using renewable resources as starting materials. Biomass is a widely available substrate which considered an environmentally friendly process (Lo´pez-Contreras, 2001). Kalil *et al*. (2003) mentions that palm oil is one of the world's leader edible oils produced by South East Asian and African countries. Palm oil was used for producing various products of food, pharmaceutical and oleo-chemicals. Its production generates various wastes and one of it was palm oil mill effluent (POME). POME was selected as substrate for ABE fermentation because of cheap raw material and to solve environmental problem. Furthermore, its mixture contents were suitable for growth of *C. beijerinckii*.

1.2 Motivation and problem statement

As the natural resources such as oil decreasing over the time; the researchers have been looking for other alternatives by producing biofuel from renewable sources that can be updated through fermentation. The acetone-butanol-ethanol (ABE) fermentation has attracted the attention of the researchers because it has the potential to produce chemicals and liquid fuels. Due to the cost of materials for medium preparation which was most expensive, ABE fermentation could not survived. The other solution was to find another substrate (fermentation medium) that can carry on the fermentation process.

Malaysia is the world largest Palm Oil Producing Country. Approximately 99.85 MT/yr of Palm Oil Mill were produced in 2011. Because of POME gave pollution of water ways, it had an impact on the environment. Thus, the industry faced a major problem, as it virtually lacked any proven technology to treat POME (MPOB, 2012).

Therefore, some ways to solve this problem was found. Because of sedimented POME with reduction of water content contains higher concentrations of lignocelluloses and other insoluble materials; it could support growth of *Clostridia beijerinckii* for ABE fermentation (Kalil *et al*., 2003). Palm oil mill effluent (POME) also seems has a 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 saccharolytic *clostridia*.

Other than that, POME was a sustainable resources and it was a cheap raw material that can be easily obtained. (Kalil, 2003). Lorestani (2006) estimated that in Malaysia about 53 million m^3 POME was being produced every year based on palm oil production in 2005 (14.8 million tonnes). While Yacob *et al.* (2005) estimated that about 0.5-0.75 tonnes of POME will be discharged from mill for every tonne of fresh fruit bunch. If there is no strategic mechanism on reusing the waste, it could further create other problems which would harm the environment.

1.3 Objectives

The following are the objectives of this research:

- To study the effect of substrate concentration, temperature and initial pH of substance on solvent production by *Clostridium beijerinckii*.

1.4 Scope of research

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

i) Effect of substrate concentration on solvent production: 80%, 85%, 90%, 95% and 100%

ii) Effect of temperature on solvent production: 27°C, 32°C, 37°C, 42°C and 47°C

iii) Effect of initial pH on solvent production: 5.4, 5.6, 5.8, 6.0 and 6.2

iv) Glucose consumption by *Clostridium beijerinckii* along the fermentation process.

1.5 Rationale and significance

1. This research focused on the production of solvents which are ABE (acetone-butanol-ethanol) through fermentation process. In this research, POME was used as the substrate fermentation for ABE production.

2. The revival of ABE fermentation was presently being inspired by the consideration of butanol for biofuel. Butanol has been proposed as a gasoline additive or even as a complete gasoline replacement. (Li *et al.*, 2011). Butanol can also be used as a blended additive to diesel fuel to reduce soot emissions.

3. Acetone was a first-class solvent for most plastics and synthetic fibers including of polystyrene, polycarbonate and some types of polypropylene. For ethanol, it was used as fuel same as butanol. It also used as a solvent for various organic compounds and as disinfectant.

2 LITERATURE REVIEW

2.1 A-B-E (Acetone-Butanol-Ethanol)

According to Lee *et. al* (2008), biological production of acetone-butanol-ethanol was one of the largest industrial fermentation processes early in the 20th century. In 1861 butanol production through microbial fermentation was reported for the first time by Pasteur. This was followed by Schardinger in 1905 reporting production of acetone by fermentation. From 1912 to 1914 strains of Clostridium acetobutylicum were isolated by Chaim Weizmann which had the ability of fermenting starchy substrate. These cultures produced higher butanol yields than the cultures of Fernbach.

During World War I and II the ABE fermentation industry had the largest growth in Europe and USA. Acetone was used for manufacturing cordite, a smokeless powder used in ammunition. Butanol was also used as a solvent for quick drying lacquers used in the automobile industry for painting cars. 2/3 of the overall butanol and 1/10 of acetone was produced by fermentation in USA at the end of World War II in 1945. At this time, large-scale production of acetone and butanol through ABE fermentation was implemented in the former Soviet Union. Maize, wheat and rye were used as major substrates. Large-scale fermentation processes were also operated in countries such as China, Japan, Australia and South Africa. (Lee *et. al*, 2008)

After World War II the petrochemical industry flourished at an unprecedented rate and so a huge decline in the ABE fermentation industry was observed. Through petrochemical industry large quantities of much cheaper acetone und butanol were available on the market. This fact resulted in an uneconomical ABE fermentation process. Most of the fermentation industry in western countries ceased to exist by 1960. In South Africa, Russia and China the ABE fermentation was carried out until 1980 to 1990 because of cheap supply of molasses as substrate and a relatively small availability of acetone and butanol from petrochemical industry (Lee *et. al*, 2008).

Nowadays, the interest in this fermentation process has come back due to depleting oil reserves and high oil price. The main aims in today's research are to improve the complete process by using genetically modified strains and cheaper renewable substrates. Some other important renewals must be the research into better cultivation and efficient product removal techniques.

2.1.1Industrial used of ABE fermentation

Acetone with formula OC $(CH_3)_2$ was the organic compound. Acetone is a colorless, mobile and flammable liquid of the ketones. Acetone was miscible with water and virtually all organic solvents, it serves as an important solvent in its own right, typically the solvent of choice for cleaning purposes in the laboratory. Acetone was also used as solvents and in the production of the rubber monomers, butadiene and dimethyl butadiene (Ezeji *et al*., 2003). In industrial solvent, acetone was found in the paint, lacquer & varnish industry, rubber industry, plastics industry, dyeing industry, celluloid industry, photographic & explosives industry & in the manufacture of artificial silk & synthetic leather.

Butanol is a chemical which has excellent fuel characteristics. It has a higher calorific value than ethanol, and a low freezing point (Qureshi & Blaschek, 2000). Butanol has recently been proposed as a gasoline additive, or even as a complete gasoline replacement (Lee *et al.*, 2008a). Butanol was the most promising solvent compared to acetone and ethanol due to its higher price, better fuel extender than ethanol, low vapor pressure; low miscibility with water and it was completely miscible with diesel fuel even at low temperatures (Qureshi, 2001).

Butanol or Bio-butanol has been recognized as a potential fuel from renewable resources. Bio-butanol was formed via ABE fermentation from renewable feedstocks using *Clostridium beijerinckii* in anaerobic conditions. Butanol gives several benefits compared to ethanol as a bio-fuel such as higher energy content, lower vapor pressure, and lower hygroscopy (Kraemer, 2010). Butanol was also used as a solvent for quick drying lacquers used in the automobile industry for painting cars. Butanol with acetone was used in the making of explosive materials. N-butanol was used in the manufacture of plasticizers, brake fluids, urea-formaldehyde, extractants and petrol additives (Priya, 2009).

Ethanol is a clear, colorless, very mobile liquid, a clean-burning and high-octane motor fuel that is produced from renewable sources. Other than that, it was used as a solvent, extractant, and antifreeze. At its most basic, ethanol is grain alcohol, produced from crops such as corn. Because it is domestically produced, ethanol helps reduce America's dependence upon foreign sources of energy. Unblended 100% ethanol is not used as a motor fuel; instead, a percentage of ethanol is combined with unleaded gasoline (American Coalition for Ethanol).

2.2 Palm oil

After being one of the biggest producers and exporters of palm oil and palm oil products, Malaysia has an important role to play in fulfilling the growing global need for oils and fats sustainably. Malaysia currently accounts for 39 % of world palm oil production and 44% of world exports. Today, 4.49 million hectares of land in Malaysia was under oil palm cultivation; producing 17.73 million tonnes of palm oil and 2.13 tonnes of palm kernel oil (“MPOC”, 2012).

Oil palm bears both male and female flowers on the same tree as it was a monoecious crop. Each tree produces 1000 to 3000 fruitlets per bunch weighing between 10 and 25 kilograms. Each fruitlet was almost spherical or elongated in shape. Generally, the fruitlet was dark purple, almost black and the colour turns to orange red when ripe. Each fruitlet consists of a hard kernel (seed) enclosed in a shell (endocarp) which was surrounded by a fleshy mesocarp ("MPOC", 2012).

An oil palm plantation was capable of assimilating up to 36.5 t of dry matter per hectare per year, which was higher than the 25.7 t assimilated by natural rainforest. The oil palm was thus more effective than the rain forest in generating new biomass, which has wide uses for wood-replacement and was also a potential source of renewable biofuel. ("MPOC", 2012).

Figure 2–1: Illustration Oil palm tree

2.2.1 Palm oil mill effluent (POME)

Characteristics of palm oil mill effluent depend on the quality of the raw material and palm oil production processes in palm oil mills. There has categorized three major processing operations responsible for producing the POME. Sterilization of FFB, clarification of the extracted CPO, and hydrocyclone separation of cracked mixture of kernel and shell hydrocyclone contributes about 36, 60 and 4% of POME respectively in the mills (Rupani *et. al*, 2010).

According to Hii *et al* (2012), palm oil mill effluent (POME) was rich in carbohydrates, proteins, nitrogenous compounds, lipids, minerals, cellulose, hemicelluloses and lignin. It can be used naturally as a fermentation medium, either for cellulase or other value-added product fermentation. Ma (2000), stated that because of the organic acids produced in the fermentation process, palm oil mill effluent was low in pH about 4-5. It also contains large amounts of total solids (40,500 mg/ l), oil and grease (4000 mg/ l).

POME can be sustainably reused as a fermentation substrate in the production of various metabolites, fertilizers and animal feeds through biotechnological advances (Wu, 2009). Hii (2012) said that several researchers was determined the proximate composition of POME found that POME was very rich in carbohydrates, proteins, nitrogenous compounds, lipids and minerals. Kalil (2003) also agreed and said that sedimented POME with reduction of water content contains higher concentrations of lignocelluloses and other insoluble materials which supported growth of *Clostridia*. Moreover, sedimentation of POME assisted to eliminate traces of oil and soluble toxic substances leaving less inhibitory POME. (Kalil, 2003)

Table 2-1: Characteristic and composition of POME

Components	Range/average*
pH	3.5-4.7
Oil and grease	4000
Biochemical oxygen demand (BOD_5)	10 250-43 750
Chemical oxygen demand (COD)	16 000-100 000
Total solids	11 450-164 950
Suspended solids	410-60 360
Volatile solids	8670-154 720
Total volatile solids	34 000
Ammoniacal nitrogen	35
Total nitrogen	200-500
Lignin	2900-7890
Cellulose	250-8000
Reducing sugar	4230-6720

*All units in mg/L except pH (Source: Hii, 2012)

2.3 Fermentation

2.3.1 *Clostridium beijerinckii*

Clostridia are rod-shaped, spore-forming Gram positive bacteria and typically strict anaerobes. Solventogenic clostridia can utilize a large variety of substrates from monosaccharides including many pentoses and hexoses to polysaccharides (Jones and Woods, 1986). In *Clostridium beijerinckii* (and probably also other butanologenic strains), the solventogenic genes are located on the chromosome (Zverlov *et al*., 2006)

Hiu *et al.* (1987) mentions that strains of *Clostridium beijerinckii* which formerly known as *Clostridium butylicum* can produce isopropanol in addition to acetone, butanol and ethanol. *C. beijerinckii* was the gram-positive, spore forming, mesophilic, motile, rod-shaped bacteria with oval, sub-terminal spores and anaerobic clostridia constitute a diverse group of species (Shi & Blaschek, 2008).

2.3.2 *Anaerobic Fermentation*

Anaerobic was composting without oxygen which results in fermentation. "Anaerobic composting" describes the process of putrefactive (cause odor nuisance) breakdown of organic matter by reduction in the absence of oxygen where end products such as CH_4 and hydrogen sulfide (H_2S) are released. This condition causes organic compounds to break down by the action of living anaerobic organisms. Anaerobic composting may be accomplished in large and well composting systems. These should contain 40% to 75% moisture, into which little oxygen can penetrate, or 80% to 99% moisture so that the organic material was a suspension in the liquid. When materials are composted anaerobically, the odor nuisance may be quite severe. ("Compost fundamentals")

2.3.3 *Batch Fermentation*

It takes 2–6 days to complete batch fermentation depending on the condition and the type of substrate employed. The final total concentration of solvents produced ranges from 12 to 20 g/L in batch fermentation, which can be separated from the fermentation broth by distillation (Lee *et. al*, 2008).

2.3.4 ABE Fermentation

ABE hetero-fermentation produces acetate, butyrate, ethanol, and acetone, as well as butanol. The metabolism of ABE producing clostridia can be divided into the following 2 distinct phases: acidogenesis (acid-production) and solventogenesis (solvent-production) during the exponential and stationary phases of growth. The metabolic pathways of ABE-producing clostridia are summarized in **Figure 2.2**.

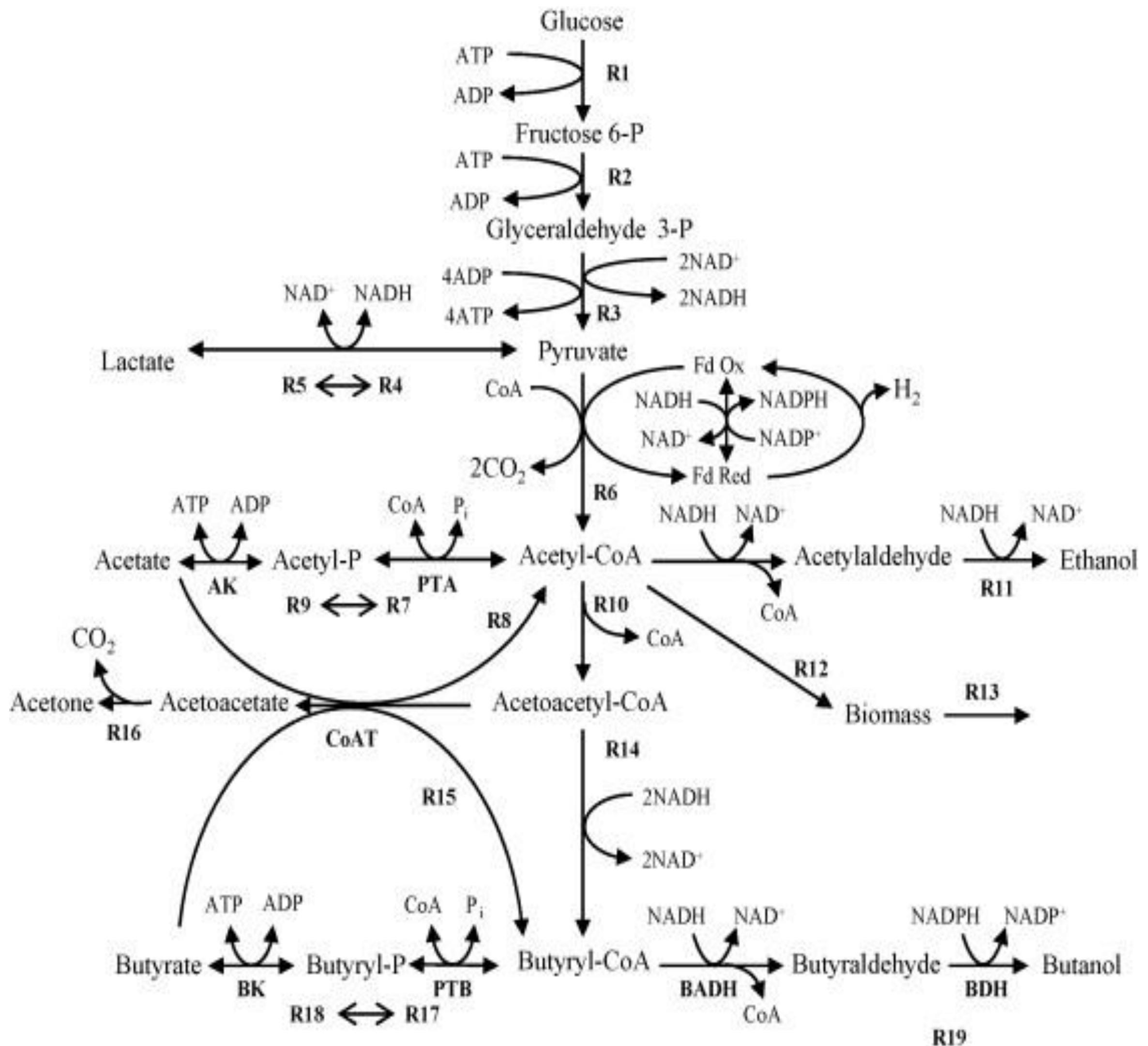

Figure 2-2: Metabolic pathway of acetone-butanol-ethanol fermentation

(Source: Run *et. al*, 1988)

C. beijerinckii produces hydrogen, carbon dioxide, acetate and butyrate through a first growth phase (acidogenic phase) in carbohydrate batch culture. During acidogenesis, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA, respectively; ATP was also produced. The four primary enzymes involved in the formation of butyryl Co-A are thiolase, B-hydroxy butyryl Co-A dehydrogenase, crotonase and butyryl Co-A dehyrogenase.

Accumulation of these organic acids reduces the culture pH. Medkor (2010) mentions that a decrease in the pH of the culture medium was occurred on acidogenic phase. Decrease in pH was needed to start solventogenesis. Enough acids have to be formed before pH decrease or otherwise the solventogenesis will be unproductive ("REBEL WP7", 2009). The organic acids are then re-utilized insolventogenesis, when the culture pH begins to rise. The re-utilization of acetate and butyrate was generally considered to occur via the acetoacetyl-CoA: acetate/butyrate: CoA transferase (CoAT) pathway and the reverse pathway generates the organic acids.

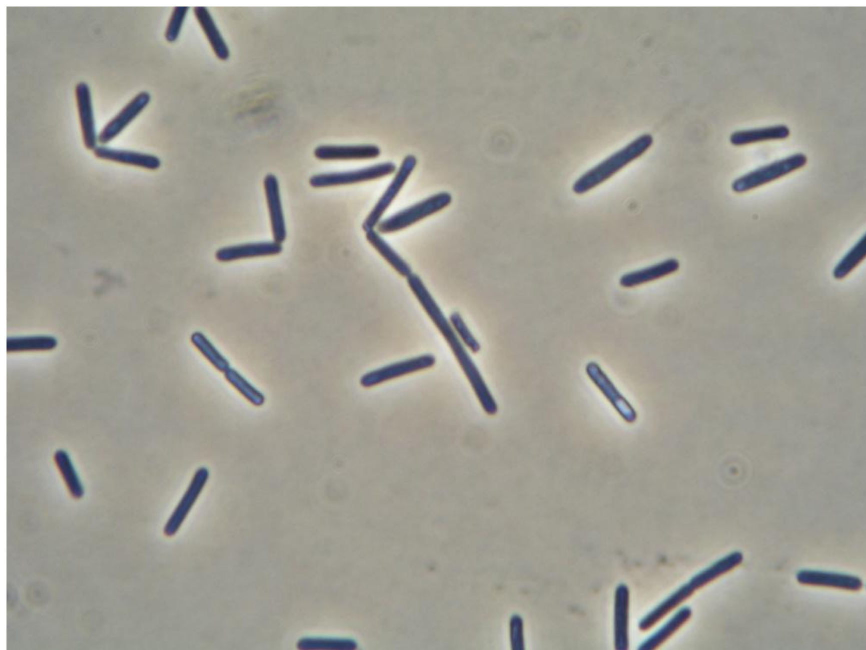

Figure 2-3: Growth phase (acid production)

(Source: Melzoch *et. al,* 2010)

During solvent production, acetyl Co-A and butyryl Co-A become main intermediates for ethanol and butanol production. These pathways produce acetaldehyde and butraldehyde, respectively as intermediates. The reduction of butryl Co-A to butanol was mediated by butraldehyde dehydrogenases and butanol dehydrogenase. Acetoacetyl Co-A transferases convert the acetoacetyl Co-A to acetoacetate.

Acetone, n-butanol, ethanol, and isopropanol (solvents) are characteristic products of several Clostridium species (Run *et. al*, 1988). Recently, ABE producing clostridia were reclassified into 4 species which are *Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* by using the molecular biological methods of 16S rRNA sequencing, DNA fingerprinting, and DNA-DNA hybridization (Tashiro and Sanomoto, 2010).

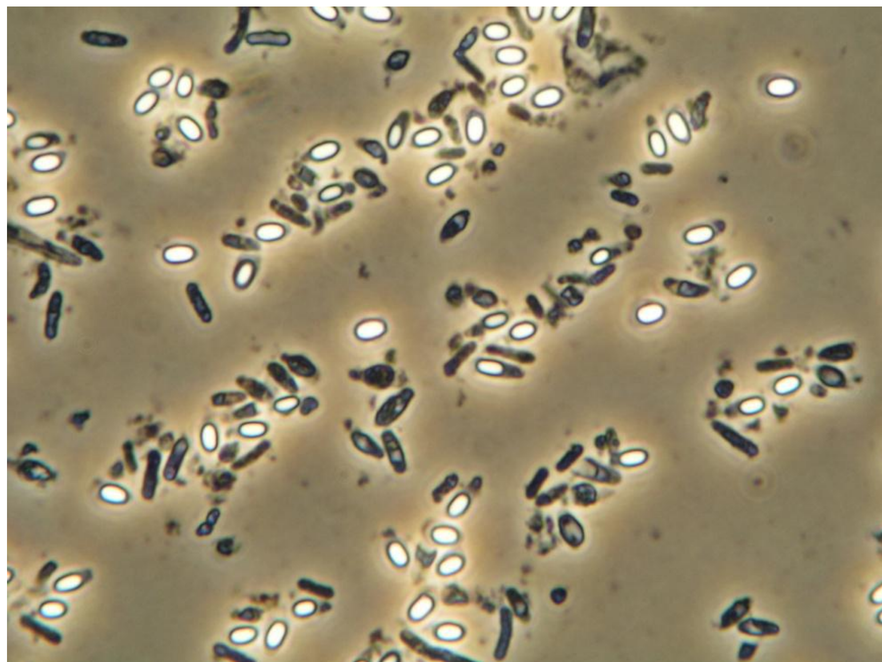

Figure 2-4: Sporulation (solvent production)

(Source: Melzoch *et. al,* 2010)

2.4 Effect of Fermentation Parameters

2.4.1 pH

According to Kim *et al*. (1984), the pH of the medium was very important to the biphasic acetone–butanol fermentation. In acidogenesis, rapid formation of acetic and butyric acids causes a decrease in pH. Solventogenesis starts when pH reaches a critical point, beyond which acids are reassimilated and butanol and acetone are produced. Therefore, low pH is a prerequisite for solvent production. However, if the pH decreases below 4.5 before enough acids were formed, solventogenesis would be brief and unproductive. Ahmed *et al.* (1988) conclude that *C. beijerinckii* produces butanol at more rapid growth rates in acid medium and at slower growth rates in neutral medium. Ethanol was produced at all growth rates and at both pH levels.

POME cultures that work at lower pH compared to RCM culture produced more solvent and this agreed with the findings of Girbal and Soucaille (1998) who found that culture with high pH produced more acids than solvent itself. According to Kalil *et al*. (2003), clostridial strains other than *C. acetobutylicum* showed that production of ABE was optimum at the initial pH values of 5.0 to 6.2. The initial pH of POME concentration was increased to pH 5.8 before used for ABE fermentation medium in order to get high yield of solvent.

2.4.2 Temperature

According to Al-Shorgani *et al.* (2012), the yield of ABE was higher when POME treated by autoclaving was used. This might be due to the effect of high temperature, resulting in hemicellulosic degradation and lignin transformation. It shows that, the effect of temperature was important to produce high ABE solvent. Other than that, Boonsombuti *et al.* (2013) mentioned the incubation temperature was maintained at 37°C. Lopez-Contreras *et al.* (2001) also agreed that the optimal temperature for incubation of the cultures was suitable at 37°C.

2.4.3 POME concentration

Kalil *et al*. (2003) agreed that sedimented POME at 90% concentration was the optimum concentration for ABE production by fermenting *Clostridia*. According to previous result, 90% POME concentration (sedimented) seen produced the highest ABE compared to POME 70% and POME 80%. Sedimentation of POME assisted to eliminate traces of oil and soluble toxic substances leaving less inhibitory POME. Sedimented POME was suitable to be used because of high lignocellulose concentrations and other insoluble materials needed for production of ABE solvent.

Figure 2-5: Palm oil mill effluent

3 MATERIAL AND METHODS

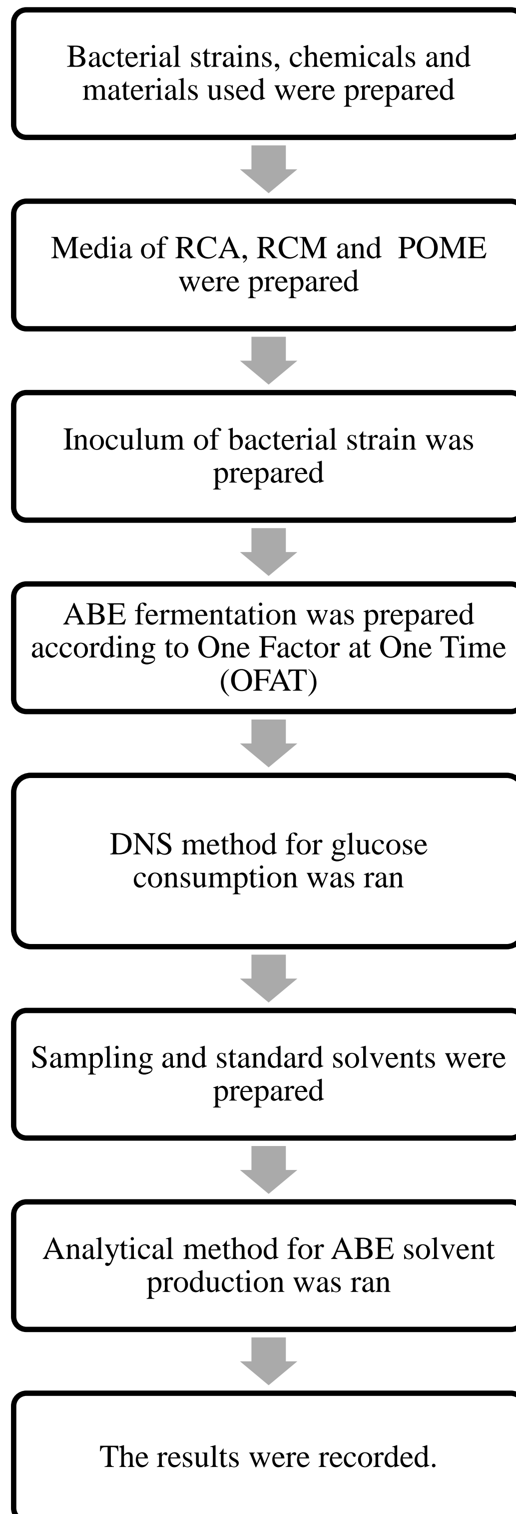

Figure 3-1: Flow chart of methodology

3.1 Equipment

3.1.1 Autoclave

Autoclave model Hirayama HVE-50 was used to sterilize equipment by subjecting them to high pressure saturated steam at 121°C for 15 to 20 minutes. Mode that common being used for autoclave was 2 which was for culture medium. Before use the equipment, water level of that equipment must be checked first and used an appropriate water type whether de-ionized water or distilled water depend on what type of autoclave used. In this research, this equipment was the main equipment for sterilization.

3.1.2 Biohazard Safety Cabinet

Biohazard safety cabinet brand ESCO, model AC2-4EI was used as the place where the experiment was conducted. This Biohazard safety cabinet provide ideal partical-free, bacteria free, clean air environment that needed for laboratory work testing. Other than that, it also does sterilization of various microorganisms in the interior of laminar flow. UV lamp was also used as a germicidal lamp.

3.1.3 Incubator shaker

The incubator shaker used was brand Infors Multitron 11. This incubator was used to incubate the Reinforced Clostridial Medium and POME for ABE production. The optimum incubation temperature used was at 37 °C with a 150 rpm orbital shaking rate without pH control for RCM (Boonsombuti *et al*., 2013).

3.1.4 UV-Vis Spectrophotometer

In this experiment, UV-Vis Spectrophotometer brand Hitachi, model U-1800 was used to observe the concentration of growth profile and glucose consumption of *C. beijerinckii*. Other applications of UV-Vis Spectrophotometer are to define nucleic acid quantification, protein quantification and cell quantification. The wavelength of this equipment is around 190 – 1100 nm. The detector used was Silicon Photodiode. Cell concentration was measured by an optical density method at 540 nm and was presented as cell dry weight concentration (g/L). (Qureshi *et al*., 2011)

3.1.5 Gas Chromatography-Flame Ionization Detector (GC-FID)

Gas Chromatography brand Agilent, model 6890 Series equipped with a flame ionization detector (FID) was set up to determine acetone, butanol and ethanol concentration. The column used was HP-INNOWax Polyethylene Glycol (30 m x 250 µm x 0.25 µm nominal). Oven temperature was set at 80°C while injector and detector used temperature at 150°C. The initial temperature was 80°C held for 4 min, increased rate of 20C/min until 120C min was held for 2 minutes. The carrier gas used was nitrogen at a flow rate of 30 mL/min (Yan *et al*., 1988).

Other than that, GC- FID also used to test the purity of particular substance. Sample chemical compound on this experiment was measured using GC with flame ionization detector (FID) because it was a useful detector for the analysis of organic compounds that has high sensitivity, a large linear response range, and low noise.

Table 3-1: Brand/model of the equipment

Equipment	Brand/Model
Biohazard safety cabinet	ESCO AC2-4EI
Autoclave	HIRAYAMA HVE-50
Incubator shaker	Infors Multitron 11
UV-Vis spectrophotometer	Hitachi U-1800
Water bath	Memmert
Refrigerated centrifuge	Eppendorf 5810 R
Microcentrifuge	BIOFUGE pico
Gas chromatography	Agilent 6890 N

2.3 Materials

3.2.1 Bacterial strains and chemicals

Bacterial strains used for this research was *Clostridium beijerinckii*. Whereas, chemicals used for this research were acetone (99.98%), butanol (99.98%) and ethanol (99.99%). These chemicals were used for preparation of standard curve and n-propanol (99%) was used as internal standard. Other than that, Dinitrosalicylic acid (DNS) was used for DNS method. Otherwhile, Sodium hydroxide (NaOH) and Hydrochloric acid (HCl) were used to adjust the initial pH for the ABE fermentation.

Figure 3-2: Chemicals (acetone, butanol and ethanol)

Table 3-2: Chemical and media used in the experiment

Chemical	Media
Acetone (99.98%) Butanol (99.98%) Ethanol (99.99%) N-propanol (99%) Dinitrosalicylic acid (DNS) Sodium hydroxide (NaOH) Hydrochloric acid (HCl)	Reinforced Clostridial Agar (RCA) Reinforced Clostridial Medium (RCM) Palm Oil Mill Effluent (POME)

3.2.2 Cultivation media

3.2.2.1 Reinforced clostridia agar (RCA)

Reinforce Clostridia Agar was used as medium for inoculate single colony of strain after growth in medium. RCA was prepared by dissolving 52.5g of the powder in 1 Liter of distilled water and then was transferred it into a 1 Liter schott bottle on a hotplate in order to completely dissolved. Then, the agar medium was sterilized at 121°C for 20 minutes. Then, the medium was left to cool at room temperature. Then agar was poured into a sterile Petri dish and universal bottle. Wait few minutes to make sure agar was hardened before kept in freezer under 4°C.

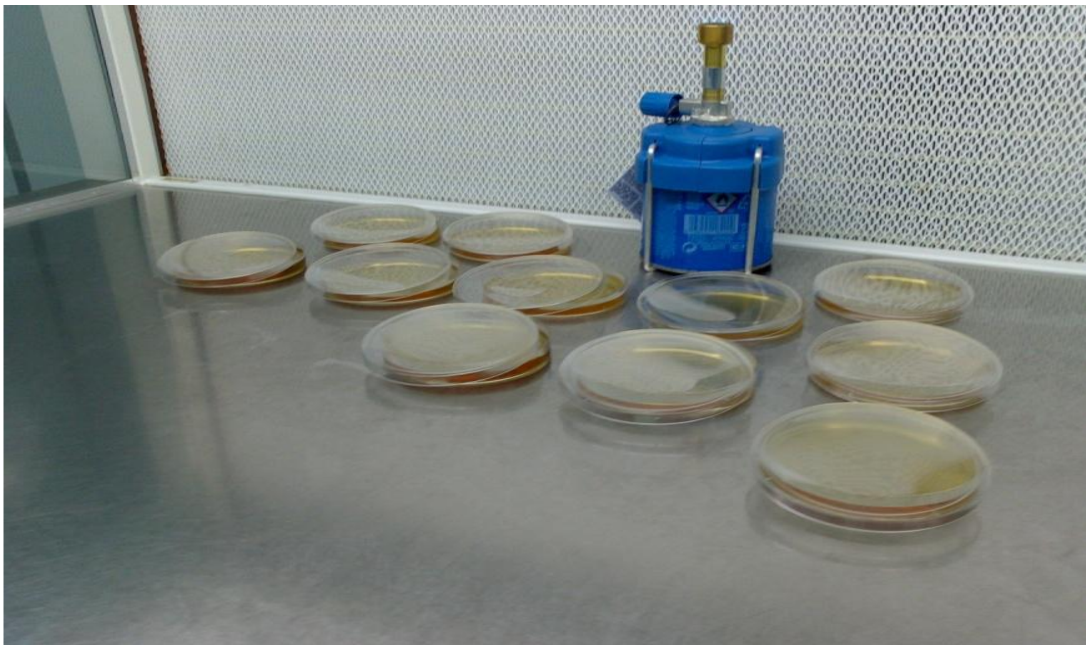

Figure 3-3: Preparation of RCA

3.2.2.2 Reinforced clostridia medium (RCM)

Reinforced Clostridia Medium was used as the control medium during fermentation. The medium was prepared by dissolving 38 g of the RCM powder in 1 Liter of distilled water and stirred to dissolve mixture completely. Mixture was transferred to 1 Liter scotch bottle and heat up on a hotplate. Then, RCM was autoclaved at 121°C for 20 minutes. After autoclaving process, medium was kept in freezer under 4°C.

3.2.2.3 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 24hours using beaker covered with aluminium foil. Then, the supernatant layer (upper part) was decanted and sediment POME sludge (lower part) was taken. The sediment of POME was sterilized at 121°C for 20 minutes and used directly as fermentation medium without additional nutrient. Sedimentation of POME helped remove traces of oil and soluble substances leaving less inhibitory POME which was more suitable for *Clostridia* growth. Sedimented POME was diluted with distilled water to obtain required concentration and also calibrated with NaOH and HCl to maintain at pH 5.8 before deoxidizing by gassing with nitrogen gas for few minutes (Kalil *et al*., 2003).

Figure 3-4: Palm oil mill pond

3.3 Experimental procedures

3.3.1 Preparation of Inoculum

C. beijerinckii strain was streak on the agar plate and being incubated for 3 days to distinguish visible colonies. After 3 days, few loops of the colonies of *C. beijerinckii* were taken from the incubated agar plate and were transferred to 150 mL of RCM in a 250 mL schott bottle. Liew *et al*. (2006) mentioned that the RCM was used for the preparation of inoculum. Then, place the schott bottle in anaerobic condition for 18 to 24 hours at 37°C for inoculum development. After the incubation time, the culture broth was prepared for fermentation process. The culture broth was centrifuge for 10 minutes at 10 000 rpm in microcentrifuge. The supernatant was decanted and the cell was resuspended with 100 mL of sterile saline solution (0.85% w/v NaCl) for cell washing. Optical density value of 0.7 at 680 nm was read as the OD value for final cell suspension. More saline water was added when OD value exceeds 0.7 but if the OD value less than 0.7, more cells needs to be added. 10% v/v of culture with the OD value of 0.7 ± 0.1 was used as the inoculum (Kalil *et al*., 2003)

3.3.2 Batch fermentation

500 mL of schott bottle (250 mL of medium) was used and was put in an incubator shaker brand Infors, model Multitron 11. The medium of RCM and 90% concentration of POME were autoclaved at 121°C for 20 minutes. After sterilized, the final cell suspension at inoculation rate of 10% was added into the POME which was the substrate for ABE fermentation. POME was used directly as fermentation medium without any additional nutrient. Sedimented POME was diluted with distilled water to obtained required concentration before deoxidizing by gassing with nitrogen gas (Kalil *et al*., 2003). Then, the broth was purge with nitrogen gas for 15 minutes to maintain anaerobic conditions.

One-factor-at-one-time (OFAT) was used to identify ABE production from the temperature of fermentation, concentration of substrate (POME) and initial pH rate. The best range in each parameter was performed by making other parameters become constant. The effect of substrate (POME) concentration was studied at temperature 37°C with initial pH of 5.8 using POME concentration at 80, 85, 90, 95 and 100%. While effect of temperature at 27, 32, 37, 42 and 47°C were determined with 90% substrate concentration and pH 5.8 and effect of initial pH at 5.4, 5.6, 5.8, 6.0 and 6.2 with 90% substrate concentration at 37°C. Fermentation was continued till 72 hours and 10 mL of sample was collected at interval of time 48-60 hours for ABE production identification. Dilution was done from the collected sample to get the accuracy OD value.

Table 3-3: One Factor at One Time (OFAT)

Parameter	Value
Temperature, °C	27, 32, 37, 42 and 47
Substrate concentration, %	80, 85, 90, 95 and 100
Initial pH	5.4, 5.6,5.8, 6.0, 6.2

Figure 3-5: RCM medium and POME medium

3.4 Analytical method

3.4.1 Gas Chromatography Analysis

3.4.1.1 Preparation of solvent standard curve

Standard for calibration curve were prepared for acetone, butanol and ethanol. n-propanol was used as internal standard at different concentration. 0.3 mL of acetone/butanol/ethanol was mixed with 2.7 mL of n-propanol to prepared standard solution. The concentrations used for acetone were 0.5, 1.0, 1.5, 2.0 and 2.5 %. While, for butanol, the concentration were 1, 2, 3, 4 and 5% and the concentration for ethanol were 0.2, 0.4, 0.6 0.8 and 1.0%. All standard solutions were prepared according these concentrations. Ultrapure water was used for dilution and these standard solutions were run using gas chromatography with flame ionized detector (FID).

3.4.1.2 Preparation of sample solutions

Sample was taken in interval time between 72 hours for analysis. 5 mL of sample was taken and centrifuge using 10000 rpm for 10 minutes to separate cell with solvent. The supernatant was taken to analyze the solvent production in gas chromatography.

Sample solutions were prepared by taking 0.3 mL of each sample and added with 2.7 mL of n-propanol. Then, the mixture solution was filtered by using 0.22 µm nylon microfilter and was injected into vial (Al-Shorgani *et al.*, 2012). Nitrogen as a carrier gas was purged through samples during analysis at 30ml/min flow rate. Temperature initial was 40C was maintain for 10 minutes and increase to 170°C when rate of oven was 10°C/min.

Figure 3-6: Sample solutions and standard curve solutions

3.4.2 Glucose consumption Analysis

3.4.2.1 Preparation of Dinitrosalicyclic Acid (DNS)

300 g of potassium sodium tartrate tetrahydrate was weighed into 1 L conical flask. 16 g of sodium hydroxide and 500 ml of water were added into that conical flask and it was dissolves by heating gently. When the solution was clear, 10 g of 3, 5-dinitrosalicylic acid (DNS) was added slowly. The conical flask was cool to room temperature and was making up to 1 L with distilled water.

3.4.2.2 DNS method

The reducing sugars were measured using the 3, 5-dinitrosalicylic acid (DNS) method. 3 mL of sample was taken and mixed properly with 3 mL of DNS solution in the test tube with cap. All the test tubes were closed with cap and placed into boiling water bath at temperature of 90°C for 5 minutes. After that, all the test tubes were cool at room temperature. The samples were measured at absorbance at 540 nm by using UV-Vis spectrophotometer (Boonsombuti *et al*., 2013).

4 RESULTS AND DISCUSSIONS

4.1 Introduction

ABE fermentation using palm oil mill effluent (POME) by *Clostridium beijerinckii* was accomplished in 500 mL schott bottle with 250 mL working volume. One factor at one time (OFAT) was applied in order to get the optimum parameter in ABE fermentation production. In this research, there were 3 parameters used which were temperature, POME concentration and initial pH.

4.2 Growth profile

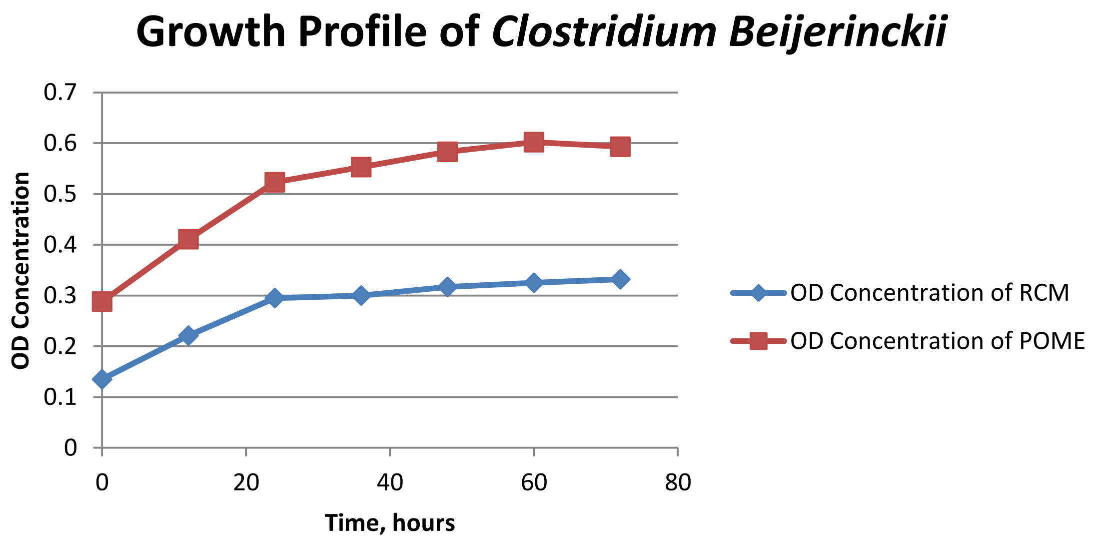

Figure 4-1: Graph of growth profile of *C. beijerinckii*

By analyzing the growth curve of *C. beijerinckii* strain, the ability of bacteria growth can be studied in certain medium. Growth profile for bacteria *Clostridium beijerinckii* in two different medium, Reinforced Clostridium Medium (RCM) and Palm Oil Mill Effluent (POME) medium were done.

The acidogenic phase was observed during the first 16 hours where cell biomass and organic acids (acetic acid and butyric acid) were faster produced. Rapid secretion of organic acids from *C. beijerinckii* caused a reduction of culture pH and only small amount of solvent was produced at this stage. When growth reached a stationary phase, solventogenic phase was started where intermediate acids were reassimilated for solvent production. During this phase, organic acids were utilized parallel with reducing sugars for the production of solvent. Active uptakes of organic acids caused the rise of pH. Increased in solvent concentration was observed during this phase where total solvent concentration reached had their maximum value (Liew *et al*., 2006).

According to Ahmed *et al*. (1988), investigators that examined the batch growth curves of C. *beijerinckii* shown that the shift to solventogenesis occurred around 20 and 25 h at a mass doubling time. Which it resembled other batch-grown solventogenic clostridia in that the shift occurred at slow growth rates during the later stages of the culture. While, Run *et al.* (1988) said that the level could increased when cells switch from acid production to solvent production, or the level could decrease or remain unchanged when cells at the solvent-producing stage are transferred into fresh growth medium. Takriff *et al*. (2009) agreed and also mentions that lower microbial growth might be due to the use of glucose derived from POME instead of the whole POME as the medium.

From **Figure 4-1**, it shown that two medium have slightly similar curve of bacteria *C. beijerinckii* growth. Bacteria *C. beijerinckii* in RCM takes less than 24 hours to adapting with medium in lag phase rather than bacteria *C. beijerinckii* in POME. After lag phase, the bacteria growth goes to the exponential or log phase. According to Shi & Blaschek (2008), cells in the early exponential growth phase, the primary fermentation products included acetic and butyric acids. The initiation of solvent formation was observed during mid-exponential growth, typically at 7 to 8 h after the start of the fermentation time course. After 24 hours, both medium seems to be at stationary phase.

4.3 Effect of Cultivation Process

4.3.1 Effect of POME concentration on solvent production

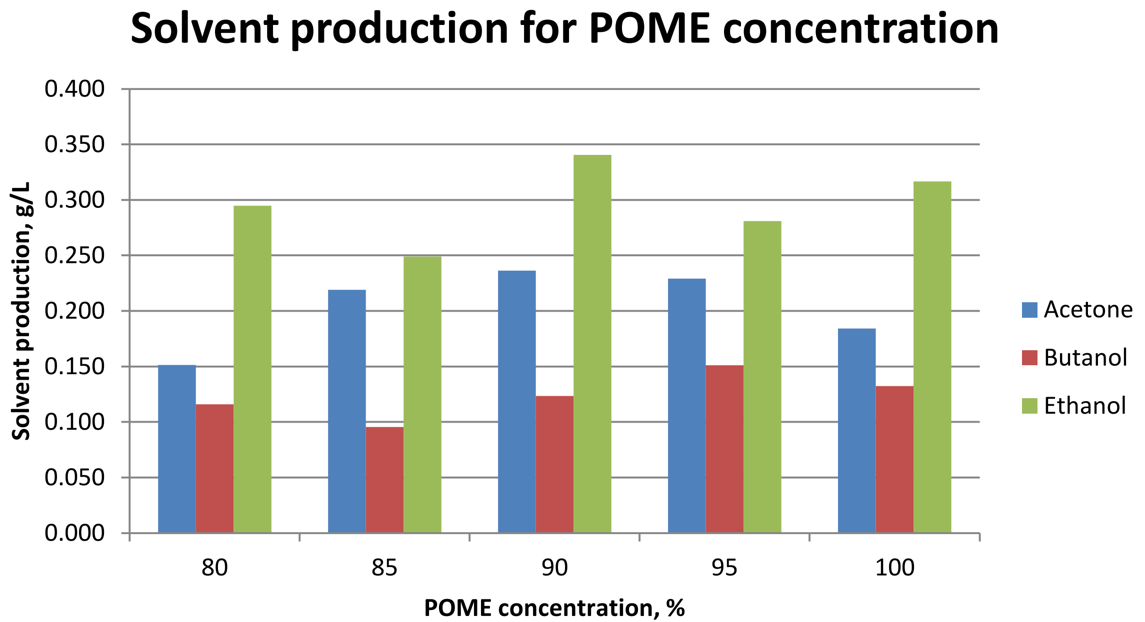

Figure 4-2: Solvent production for POME concentration

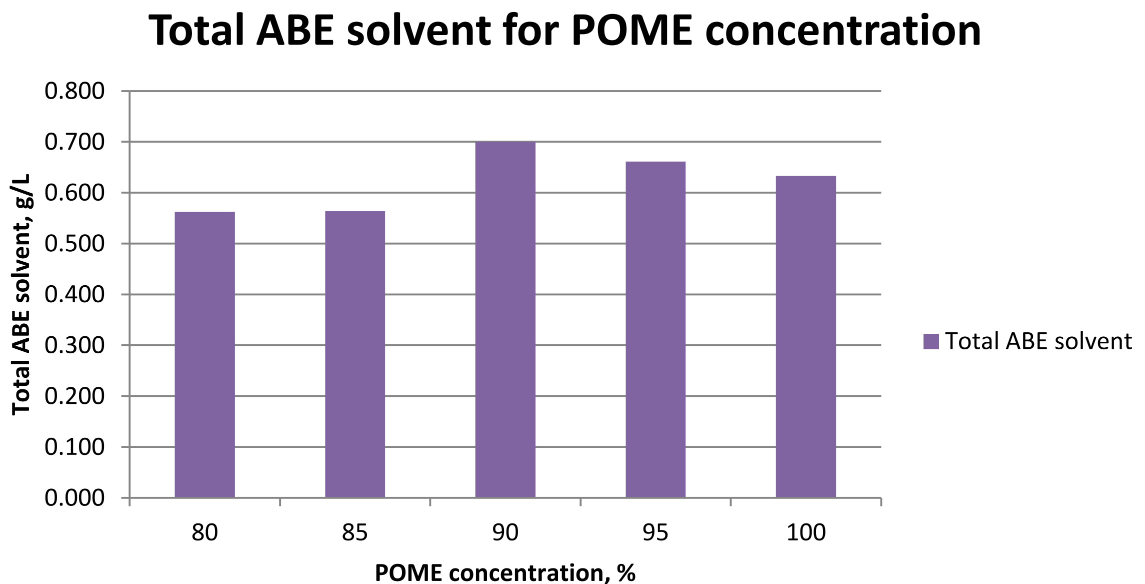

Figure 4-3: Total ABE solvent produced for POME concentration

This experiment was performed using a different POME concentration as manipulated variable. The objective was to identify the effect of POME concentration on ABE production. Regarding past research, different POME concentration resulted in different total of ABE production. Based on previous studies, it was found that *C. beijerinckii* had the ability to grow in POME without addition of nutrients and produced nearly 1 g/L ABE after 48 hours incubation. However, the lower production of ABE in this experiment might be due to the used of glucose derived from POME instead of the whole POME as a medium (Kalil *et al.*, 2003).

First, the experiment was conducted by using POME concentration range about 80% until 100% with temperature 37°C and pH 5.8 were served as a control. The concentrations of POME used were 80%, 85%, 90%, 95% and 100%. The result of ABE production from the experiment was illustrated in **Figure 4-2** and **Figure 4-3**.

From **Figure 4-2**, it shown that butanol production was lower than acetone and ethanol. Ahmed *et al.* (1988) mentioned that about 29 hours after the culture entered the growth-rate range, solventogenesis was increased. First, ethanol production rates increased and after 6 hours, butanol began to be produced. That was the reason why butanol was lowered than acetone and ethanol. After that, the rate of ethanol and butanol production continued to increase until sporulation ended the experiment. From that figure also, it was revealed that POME concentration of 95% produced the highest butanol with value 0.151 g/L. While for acetone and ethanol were obtained the highest on 90% of POME concentration with value of 0.236 g/L and 0.341 g/L.

According to **Figure 4-3**, it was proven that the highest total ABE solvent produced was at 90% of POME concentration with value 0.700 g/L. It was followed by 95% and 100% of POME concentration with value 0.661 g/L and 0.633 g/L. While for the rest 85% and 80% of POME concentration were produced about 0.564 g/L and 0.562 g/L. The optimum substrate concentration was provided better solvent production which leaded to maximum activity of the bacteria.

4.3.2 *Effect of POME concentration on glucose consumption*

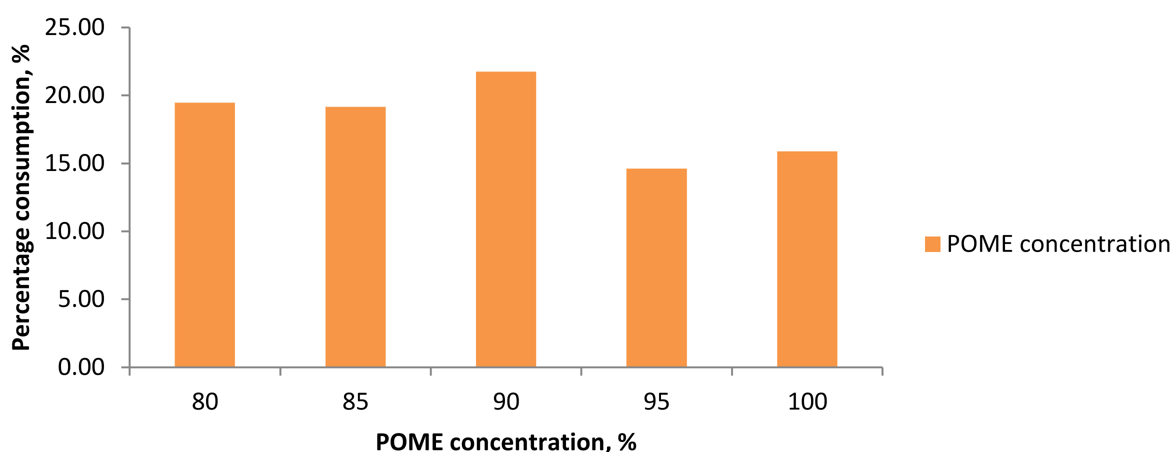

Figure 4-4: Percentage consumption for POME concentration

The following experiment was done by using DNS method to find out the glucose consumption on POME concentration. Based on the **Figure 4-4**, it was shown that the percentage of glucose consumption rate was at the highest at POME concentration of 90% with the 21.74% of consumption. It was followed by 19.47% for 80% of POME concentration. For 85% of POME concentration, the percentage of glucose consumption was 19.16%. The second lowest for glucose consumption was 15.90% by using 100% of POME concentration. Meanwhile, the lowest percentage for the glucose consumption with 14.61% of consumption was at 95% of POME concentration.

4.3.3 Effect of temperature on solvent production

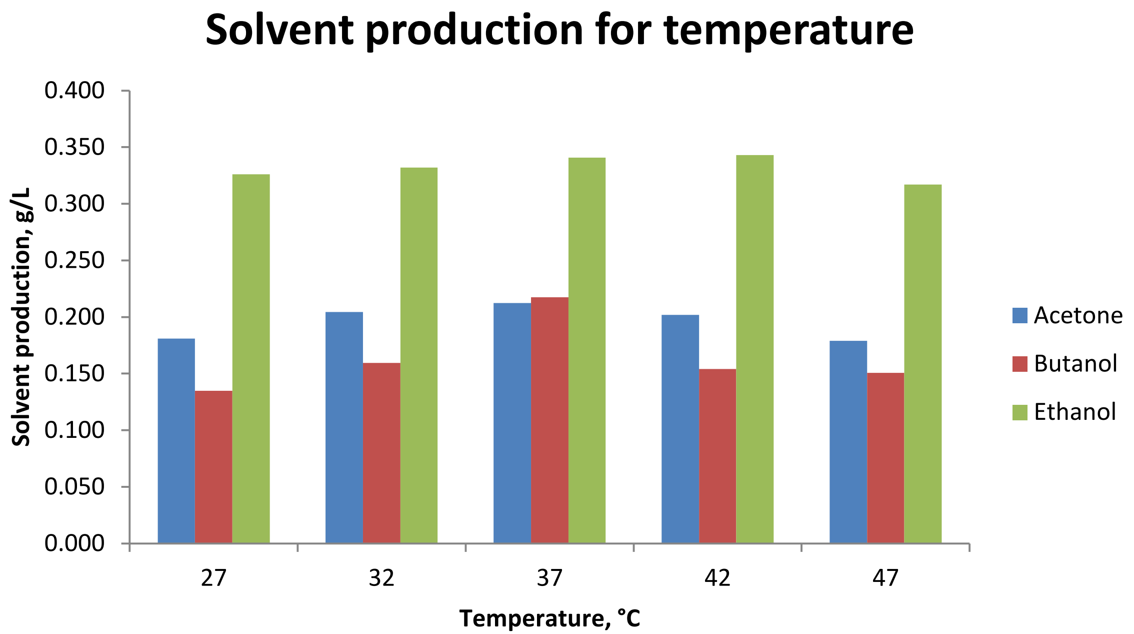

Figure 4-5: Solvent production for temperature

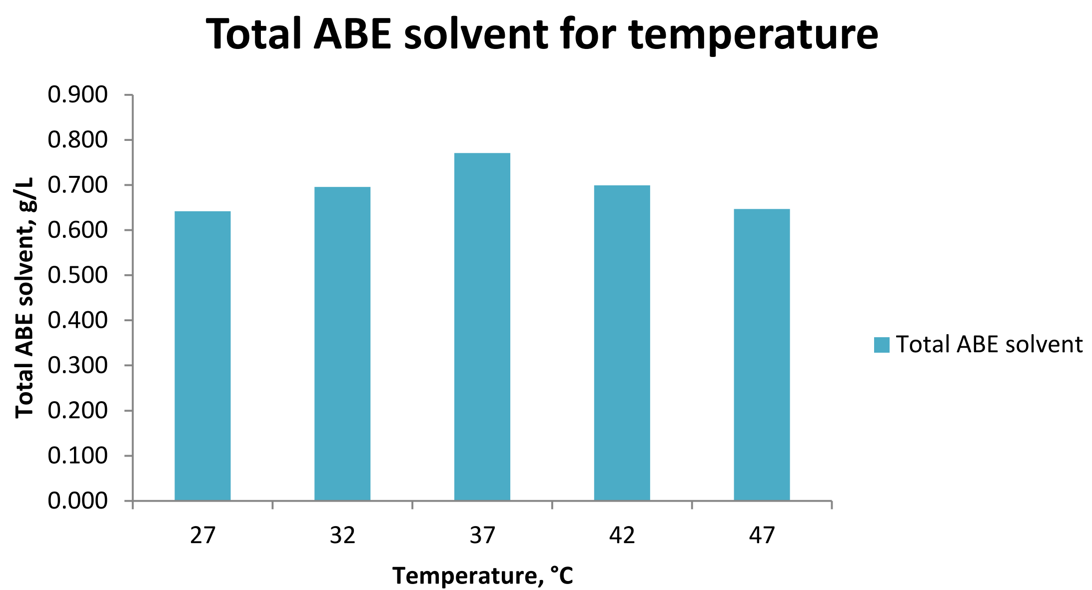

Figure 4-6: Total ABE solvent produced for temperature

In this research, the effect of temperature was carried out to determine the optimum temperature for ABE production. Based on the **Figure 4-5**, ethanol production always precedes than acetone and butanol. For each temperature that had been run, it showed that the production of acetone were higher than butanol. However, at temperature 37°C, there were slightly different value on acetone and butanol production which were 0.212 g/L and 0.218 g/L. This was because the temperature had reached the optimum production for acetone and butanol. According to Nakayama *et al.* (2011), butanol production was decreased with increasing culture temperatures. Thus, it can be seen that the butanol production was decreased as the temperature became increased after 37°C. This was also same with acetone as increasing in temperature would lower the production of acetone. However, for the ethanol production, the variation of temperature did not show much effect on that solvent production.

From **Figure 4-6** it shown that the trend for total production of ABE by different value of temperature. It showed that the highest total of ABE solvent was produced at temperature 37°C with value 0.771 g/L. At that temperature, solvent produced for acetone was 0.212 g/L, while for butanol was 0.218 g/L and ethanol was 0.341 g/L. This can be claimed as the optimum temperature for ABE production This were supported by Boonsombuti *et al*. (2013) and Lopez-Contreras *et al*. (2001).They both agreed that the optimal temperature for incubation of the cultures was 37°C. The bacteria activity was at the highest rate on that temperature (37°C). The total production of ABE increased as the temperature increased from 27°C-37°C. However, further increases temperature will reduce total solvent production where at temperature 37°C until 47°C. This finding showed that, the optimum temperature had effect to optimize the solvent production where suitable temperature will leads to maximums bacterial activity.

4.3.4 Glucose consumption for temperature effect

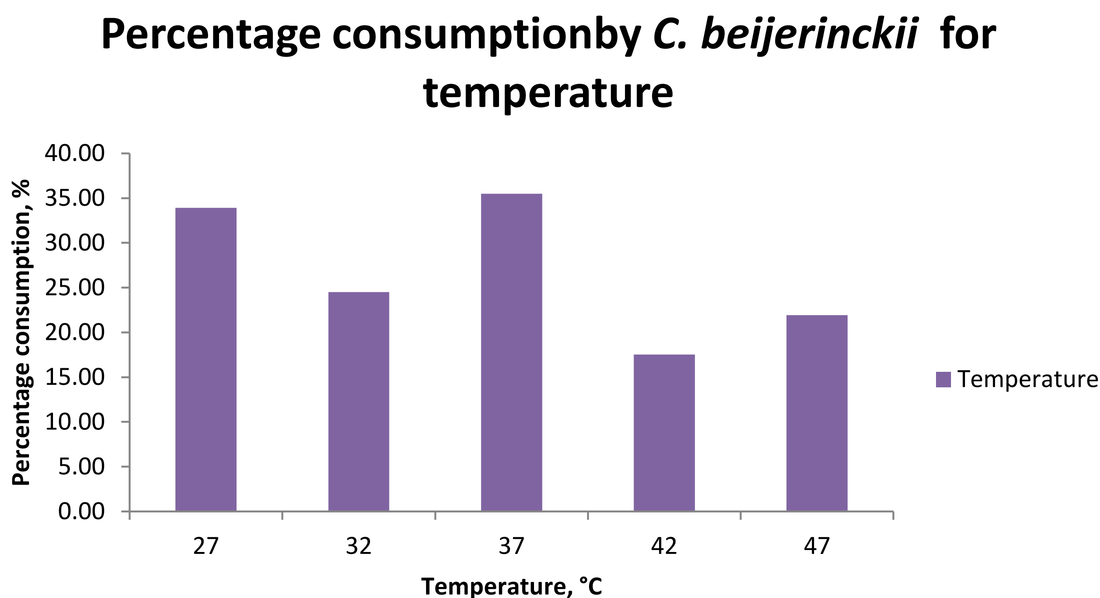

Figure 4-7: Percentage consumption for temperature

According to **Figure 4-7**, was shown that significant amount of glucose was consumed by bacteria for cell growth and ABE production. The highest glucose consumed was at 37°C with value 35.47%. Then, it was followed by temperature of 27°C and 32°C with value 33.91% and 24.50%. As the temperature was increased, the glucose consumed by the bacteria became lower as the bacteria activity lowered. Especially at temperature 42°C and 47°C, the glucose consumed were lower with value 17.53% and 21.94%. From the figure also, glucose concentration at 37°C was seen as the most stable glucose concentration with optimum temperature.

4.3.5 Effect of initial pH on solvent production

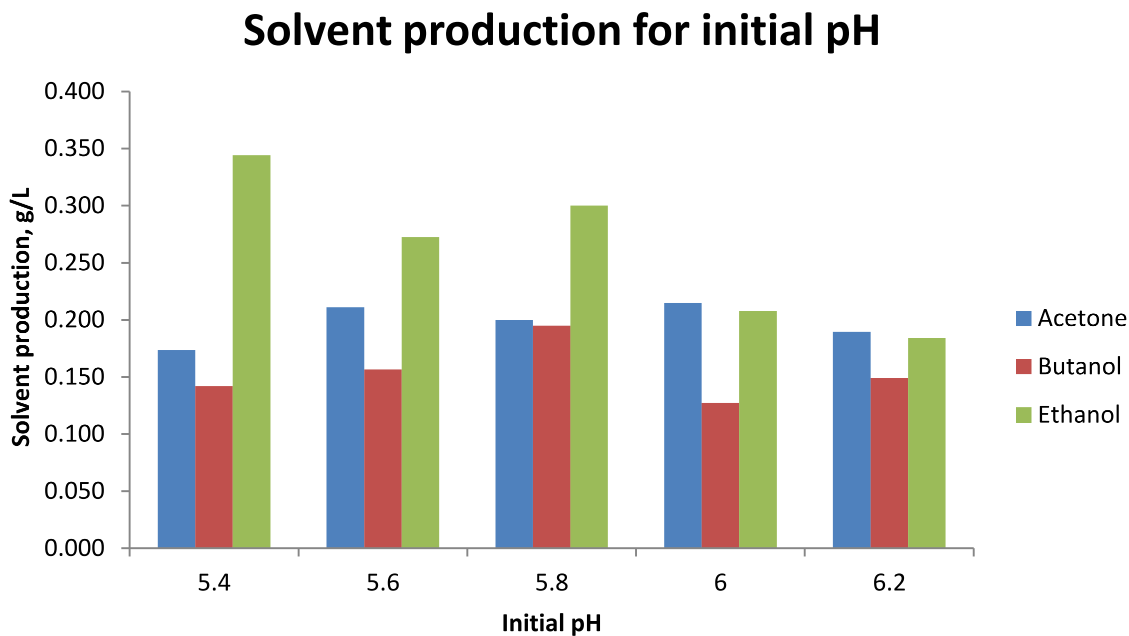

Figure 4-8: Solvent production for initial pH

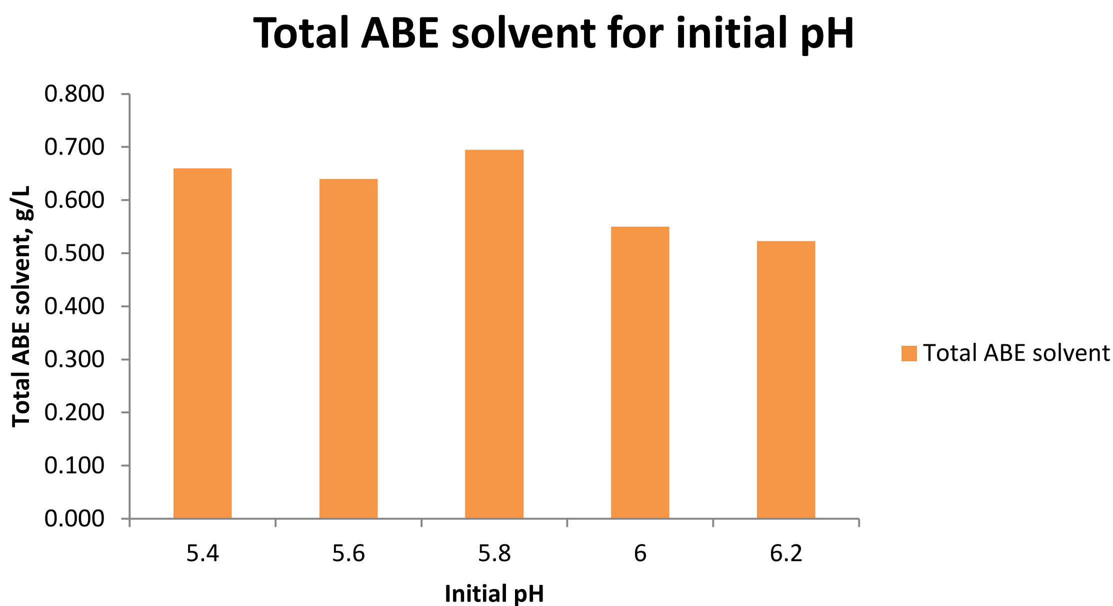

Figure 4-9: Total ABE solvent produced for initial pH

In this experiment, the study for effect of initial pH on the ABE production was carried out. There were five different initial pH used which were 5.4, 5.6, 5.8, 6, and 6.2 to determine the optimum initial pH to produce ABE solvent. Based on the **Figure 4-8,** it can be seen that the production of ethanol were the highest among the ABE solvent produced for all initial pH used. It was followed by acetone and lastly the butanol as the lowest production concentration. The highest production of ethanol was at pH 5.4 with 0.344g/L. Then followed by pH of 5.8 at 0.300g/L, pH of 5.6 at 0.272g/L, pH of 6 at 0.208g/L and lowest at pH 6.2 with 0.184g/L. However, the trend was little bit different for acetone as the highest production was at pH of 6 with 0.215g/L. It was then followed by pH of 5.6 with 0.211g/L, pH of 5.8 at 0.200g/L, pH of 6.2 at 0.190g/L. The trend for production of butanol is slightly different with acetone. The highest production of butanol was at pH of 5.8 with 0.195g/L. Meanwhile, the lowest one was at pH of 6 with 0.127g/L.

The total ABE solvent produced at initial pH 5.8 seems to be the highest value based on which produced 0.695 g/L**.** Then, it was followed by the initial pH of 5.4 with the total ABE of 0.660g/L**.** The total ABE decreased more at initial pH 6 with total concentration of 0.640g/L. The figure also shown that acetone and butanol production started to slow down while butanol was started to increasing. The concentration of ABE then decreased a lot at pH 6 with total concentration of 0.550g/L. The lowest production of ABE was at initial pH 6.2 with total concentration of 0.523g/L. According to Takriff *et al*. (2009) it was found that culture with high pH will produced more acids than solvent itself. Based on **Figure 4-9**, the ABE solvent produced were lower at initial pH of 6 and 6.2. It was because acids were produced more that the solvent.

4.3.6 Effect of initial pH on glucose consumption

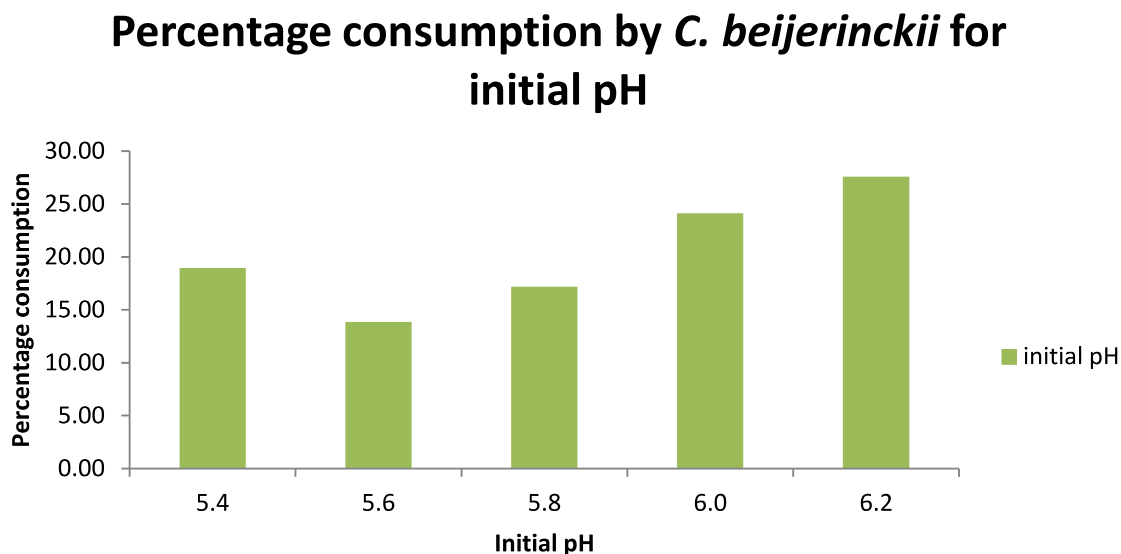

Figure 4-10: Percentage consumption for initial pH

Based on **Figure 4-10**, the initial pH affects the most on the percentage of glucose consumption at 6.2 because of other factor that trigger the growth bacteria activity. The percentage consumption on initial pH of 6.2 was 27.59%. Then, it was followed by pH 6.0 and 5.8 which consumed the percentage of 24.12% and 17.19%. The lowest percentage consumption was at pH 5.6 with value 13.87% and the second lowest was 18.95% for pH 5.4.

For overall result, it could be seen that the ABE solvent produced was less than 0.8 g/L. According to Nakayama *et al.* (2011), the poor production of ABE by *C. beijerinckii* from POME may not be due to the low concentration of sugars but due to the inhibitory compounds and the high content of salts in POME.

5 CONCLUSION AND RECOMMENDATION

As the conclusion, all the effect of parameters was studied. For the first parameter, which using variation of initial pH, the highest total ABE solvent produced was 0.695 g/L at initial pH of 5.8. While for the second parameter, by using different POME concentration, total ABE solvent was produced the highest at 90% with value 0.700 g/L. Lastly, parameter used was different temperature which produced the highest ABE solvent at 37°C with value 0.771 g/L. Therefore, the objectives of this research were achieved.

The low concentration of solvents was caused by end-product inhibition. In order to overcome the problem of product inhibition (butanol toxicity), in situ product recovery (ISPR) techniques need to be developed with an aim to improve the process performance by reducing the product toxicity. Several ISPR techniques including adsorption, liquid–liquid extraction, reverse osmosis, pervaporation, and gas stripping required to be evaluated for their efficiency and performance with respect to butanol production and recovery during ABE fermentation.

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