EFFECTS OF SUBSTRATE CONCENTRATION AND AGITATION RATE ON BUTANOL PRODUCTION FROM PALM OIL MILL EFFLUENT USING *CLOSTRIDIUM ACETOBUTYLICUM*

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BORANG PENGESAHAN STATUS TESIS♦

JUDUL : EFFECTS OF SUBSTRATE CONCENTRATION AND AGITATION RATE ON BUTANOL PRODUCTION FROM PALM OIL MILL EFFLUENT USING *CLOSTRIDIUM ACETOBUTYLICUM*.

SESI PENGAJIAN : 2010/2011

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

Faculty of Chemical Engineering and Natural Resources
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DECEMBER 2010

I declare that this thesis "Effects of Substrate Concentration and Agitation Rate on Butanol Production from Palm Oil Mill Effluent using *Clostridium acetobutylicum*" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Special dedication to:

Beloved mother and father,

Mr Maslan bin Mohamed and Mrs Siti Hafsah binti Ismail

Loving brothers,

Kamarrul Ariffin, Mohamad Firdaus, Muhammad Hanis and Muhammad Luqman

family members, friends, lecturers and everyone who knows me.

For their loves, supports and best wishes.

ACKNOWLEDGEMENT

In the name of Allah S.W.T, the Most Gracious and Most Merciful

First of all, the most thankful to Allah S.W.T for grants me the knowledge, strength and determination to accomplish my final year project. Praise to His Messenger, Muhammad S.A.W, his companions and to those on the path as what He preached upon, might Allah Almighty keep us His blessings and tenders.

I would like to take this opportunity to express my appreciation and thankful to my supervisor, Miss Shariza Jamek for her guidance, advices, patience and support in my final year project. Without her supervision, this thesis would not have been the same as presented here.

I would like to thank to Mr Mohd Anuar bin Ramli, Mr Mohd Zaki bin Sahad and all the technical academics for their guidance, help, support, advices and encouragement during conducting the experiments for my research at laboratory.

I wish also to convey my sincere gratitude to Miss Norhazimah binti Abdul Halim for her valuable knowledge and time that help me to accomplish my final year project. I am very much indebted to my group members, Ellie Fazlin binti Mohd Sarian, Siti Nadiah binti Mustafa Kamal and Siti Hazriah binti Hamzah for helping, continuous support, sharing their knowledge and concern towards my work.

My special appreciation goes to my beloved parents, Mr Maslan bin Mohamed and Mrs Siti Hafsah binti Ismail and brothers for love, understanding, encouragement and prayers for me. To those who involved directly or indirectly throughout my final year project, an additional measure of thanks is due.

ABSTRACT

This study is mainly focusing on butanol production from palm oil mill effluent (POME) by anaerobic fermentation using *Clostridium acetobutylicum.* Despite that untreated POME could bring severe effects to environment, POME also can be used as the main substrate due to abundant supply and it potentiality to be utilised by saccharolytic clostridia in butanol fermentation. Reinforced Clostridia Medium (RCM) was functioned as control medium. This study was also to investigate the growth profile rate and the consumption of glucose by *C. acetobutylicum* during fermentation for 72 hour at 37°C. Fermentation was carried out in 250 mL Schott bottle at a working volume of 150 mL. Other parameters were kept constant at pH 5.8 for POME, pH 6.8 for RCM and 10% inoculum. The effects of substrate concentration and agitation rate in producing butanol were studied. Substrate concentrations used were 70%, 80% and 90% while for agitation rates were 0 rpm, 100 rpm, 175 rpm and 250 rpm. Butanol produced from the fermentation was analyzed using gas chromatography equipped with flame ionization detector (GC-FID). Growth profile rate of *C. acetobutylicum* in POME and RCM were measured using UV-Vis spectrophotometer. Glucose concentration was measured from the calculation of the amount of glucose consumed by dinitrosalicylic acid (DNS) method which monitored using UV-Vis spectrophotometer. This experiment were started by clostridia cultivation and then followed by fermentation medium preparation, inoculum preparation, fermentation process for 72 hours and fermentation product analysis. The highest butanol yield in POME was 0.3485 g/L at 70% concentration and 175 rpm while maximum butanol production was produced in 90% RCM at 175 rpm which was 0.5034 g/L. In conclusion, lots of hard work and precaution steps need to be taken in order to make sure higher butanol can be produced at theoretically substrate concentration which is 90% and agitation rate at 200 rpm.

ABSTRAK

Kajian ini memfokuskan kepada penghasilan butanol daripada bahan buangan kilang minyak sawit (POME) oleh *C. acetobutylicum* melalui fermentasi anaerobik. Selain faktor POME yang tidak dirawat akan mendatangkan kesan buruk terhadap alam sekitar, POME boleh digunakan sebagai substrat utama dalam fermentasi kerana ianya dihasilkan dalam jumlah yang banyak dan mempunyai potensi untuk digunakan dalam fermentasi butanol. 'Reinforced Clostridia Medium' (RCM) bertindak sebagai media kawalan. Kajian ini juga dilakukan untuk mengkaji kadar profil pertumbuhan dan pengambilan glukosa oleh *C. acetobutylicum* semasa proses fermentasi selama 72 jam pada suhu 37ºC. Proses ini dijalankan di dalam botol Schott dengan isipadu sebanyak 150 mL. Parameter lain seperti pH 5.8 untuk POME, pH 6.8 untuk RCM dan kepekatan 'inoculum' sebanyak 10% dikekalkan pada keadaan yang optimum. Kesan yang diperolehi terhadap penghasilan butanol daripada kepekatan substrat dan kadar kocakan dikaji. Kepekatan substrat yang digunakan adalah 70%, 80% dan 90% manakala kelajuan yang digunakan adalah 0 rpm, 100 rpm, 175 rpm dan 250 rpm. Butanol yang terhasil daripada fermentasi dianalisis menggunakan GC-FID. Kadar profil pertumbuhan *C. acetobutylicum* dalam POME dan RCM diukur dengan menggunakan spektrofotometer ultra lembayung-nampak. Kepekatan glukosa ditentukan dengan mengira kuantiti glukosa yang digunakan melalui kaedah asid 'dinitrosalicyclic' (DNS) dan diukur menggunakan spektrofotometer ultra lembayung-nampak. Kadar penghasilan butanol tertinggi dihasilkan di POME adalah 0.3485 g/L pada kepekatan substrat 70% dan kelajuan 175 rpm manakala kadar penghasilan butanol yang maksimum terhasil di RCM adalah pada kepekatan substrat 90% dan kelajuan 175 rpm iaitu 0.5034 g/L. Secara kesimpulannya, pelbagai usaha dan langkah-langkah pencegahan perlu diambil untuk memastikan butanol dapat dihasilkan dengan kuantiti yang lebih tinggi pada kepekatan substrat dan kadar kocakan yang sesuai seperti yang dinyatakan dalam teori iaitu pada kepekatan substrat 90% dan kelajuan 200 rpm.

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LIST OF SYMBOLS/ABBREVIATIONS

ABE	-	Acetone-Butanol-Ethanol
BOD	-	Biological Oxygen Demand
C. acetobutylicum	-	*Clostridium acetobutylicum*
DNS reagent	-	Dinitrosalicylic Colorimetric Method
E. coli	-	*Escherichia coli*
GC-FID	-	Gas Chromatography equipped with Flame Ionization Detector
g	-	Gram
L	-	Liter
mL	-	Milliliter
µm	-	Micrometer
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
OD	-	Optical density
POME	-	Palm oil mill effluent
RCM	-	Reinforced Clostridia Medium
S. cerevisiae	-	*Saccharomyces cerevisiae*
UV-Vis	-	Ultra violet vision

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Biofuel derived from biomass has been renewed interest after the so-called oil crisis and tremendous demand in worldwide due to increasing in population. Against a backdrop of rising crude oil prices, depletion resources, political instability in producing countries and environmental challenges, only biomass has the potential to replace the supply of an energy hungry civilisation by producing biofuel.

Butanol fermentation (or also called as acetone, butanol and ethanol fermentation or solvent fermentation), a historical process because it is one of the oldest known industrial fermentations and ranked second to ethanol. Butanol can be produced from a variety of renewable biomass resources as glucose. The most commonly used microorganism which converts these sugars into butanol is *Clostridium acetobutylicum.* Butanol fermentation also is a potential path to upgrade biomass into valuable liquid fuels.

Butanol can be produced from biomass and mineral fuel. Butanol from biomass is denoted as biobutanol to make it differ from butanol produced from petroleum. Primarily used as an industrial solvent, it is now known as other alternative for fuel. Butanol can be used instead of gasoline even in higher degree than ethanol due to its physical properties, economy, safety and because it can be applied without the needs to modify the engine of vehicles. Biobutanol is

environmental friendly as it does not produce sulphur and its by-product is carbon dioxide (CO_2) which is complete combustion.

The key problems associated with the production of biobutanol are the cost of substrate and butanol toxicity or inhibition of the fermenting microorganisms, resulting in a low butanol filter in the fermentation broth. However, recent interest in the production of biobutanol from biomass such as POME has led to the re-examination of acetone-butanol-ethanol (ABE) fermentation. This situation initiated and has sustained interest in identifying and channelling renewable (biomass) raw materials into the manufacture of liquid fuel alternatives because development of such biomass-based power would ensure that new technologies are available to keep pace with society's need for new renewable power alternatives for the future.

Palm oil production is one of the major industries in Malaysia and this country ranks as one of the largest productions in the world. In Malaysia, the total production of crude palm oil in 2008 is 17,734,331 tonnes. However, the production of this amount of crude palm oil results in even larger amounts of palm oil mill effluent (POME). In the year 2008 alone, at least 44 million tonnes of POME was generated in Malaysia. POME consists of water soluble components of palm fruits as well as suspended materials like palm fibre and oil. POME is selected as a substrate in this study because of its abundant supply and low-cost. The availability of supply and cost in previous research by Lee *et al.* (1995) is identified as the main factor in acetone-butanol-ethanol (ABE) fermentation. Compared to other substrates, it has no limitation resources as it produced in huge quantities which make it cheap. Thus, POME is sustainable resources. However, untreated POME have a significant impact on the environment if they are not dealt properly because despite its biodegradability, POME cannot be discharged without first being treated because POME is acidic and has a very high biochemical oxygen demand (BOD) and chemical oxygen demand (COD).

Anaerobic bacteria such as the solventogenic clostridia are capable of converting a wide range of carbon sources (e.g. glucose, galactose, cellobiose, mannose, xylose and arabinose) to fuels and chemicals such as butanol, acetone and

ethanol. Hence, it permits direct fermentation of POME to ABE. This fermentation process is facilitated by *Clostridium acetobutylicum.*

1.2 Problem Statement

This study needs to be conducted due to the undeniable evidence that world is running out of fossil fuel which is non-renewable sources in the next few years. For more than two centuries, the world's energy supply has relied on non-renewable crude oil-derived (fossil) liquid fuels. In addition, worldwide energy demand is bound to increase. Although biofuel as renewable source has been recognised to overcome this problem, many of these alternatives are made from food crops. Several experts have voiced similar concerns that creating biofuel i.e. bioethanol from food crops, such as corn, grains and whey, is considered as stealing food right out of people mouths. Food crisis or shortage also could become crucial due to this.

A number of studies reported that the cost of substrate was identified as the main factor that influences economic viable. Some of fermentation media showed that it is compatible to produce butanol in a huge amount but they are expensive and limited source. Thus, POME is selected as fermentation medium which could help to cut the cost as it is from waste and unlimited source in this country. Hence, producing butanol from abundant waste i.e. POME could replace bioethanol and help to obtain sustainable, inexpensive and suitable substrate.

Untreated POME discharged to water could bring environmental problem due to its high biochemical oxygen demand (BOD), chemical oxygen demand (COD), oil and grease, total solids and suspended solids. Other than that, emissions from the combustion of fossil fuel such as carbon dioxide (CO_2), carbon monoxide (CO), nitrogen oxide (NO_x) and sulphur-containing residues are the principal causes of global warming and its incomplete combustion are harmful to human health.

Butanol is chosen from ABE fermentation because ethanol is regarded to be less superior to butanol as a renewable source of fuel. Ethanol is known for its high volatility because of high vapour pressure and engine modification is needed if want to consume it.

1.3 Objective of Study

This study is proposed with the aim to study the effects of substrate concentration and agitation rate on butanol production from POME in anaerobic condition using *C. acetobutylicum.*

1.4 Scope of Study

The main scopes of study are:

(i) To study the growth profile of *Clostridium acetobutylicum* in POME and Reinforced Clostridia Medium (RCM).

(ii) To study the effects of substrate concentration of 70, 80 and 90% to the butanol production.

(iii) To study the effects of agitation rate at 0, 100, 175 and 250 rpm in producing butanol.

(iv) To study the glucose consumption in fermentation medium.

1.5 Rationale of Study

The main rationale and significance of this study includes:

(i) This study applies concept of ‘waste to wealth’ due to abundant supply of POME as a substrate to yield butanol in huge amount.

(ii) Biobutanol is environmental friendly due to its complete combustion. It is known to contain “green” carbon.

(iii) Another alternative to overcome depleting source petroleum and fossil fuels.

(iv) Butanol produced from POME could help to reduce the production of biofuel from food crops.

(v) Enhance the usage of POME as fermentation media to produce butanol.

CHAPTER 2

LITERATURE REVIEW

2.1 Palm Oil Mill Effluent (POME)

The Malaysian palm oil industry is growing rapidly and quickly becoming a significant agriculture-based industry in this country. Table 2.1 shows that the total productions of crude-palm oil in 2008 and 2009 are 17,734,441 and 16,044,874 tonnes, respectively (MPOB, 2008a, 2009). The palm oil industry provides a source of livelihood to rural families in government land schemes and private small holders, as well as employment opportunities to agricultural workers in estates (Wu *et al.,* 2010). In Malaysia, palm oil is even utilized in the production of biodiesel (palm oil methylester or palm oil diesel) for buses and cars (Wu *et al.,* 2010).

The number of palm oil mills in Malaysia has increased tremendously, i.e. from about 10 mills in 1960 (Ma *et al.,* 1993) to 410 operated mills in 2008 (MPOB, 2008b), in order to meet the crude palm oil demands both logically and internationally. However, the production of such large amounts of crude palm oil results in even larger amounts of palm oil mill effluent (POME) in which cases in the year 2008 alone, at least 44 million tonnes of POME was generated in Malaysia and the figures are expected to rise every year. This alarming figure caused the palm oil industry in Malaysia to be identified as the one generating the largest pollution load in rivers throughout the country (Wu *et al.,* 2010).

Table 2.1 Malaysian production of crude palm oil in 2008 and 2009 (MPOB, 2008a, 2009; Wu *et al.*, 2010)

Month	2008 (tonnes)	2009 (tonnes)
January	1,424,244	1330,195
February	1,227,969	1187,381
March	1,294,710	1275,822
April	1,327,591	1281,852
May	1,457,878	1395,275
June	1,468,921	1447,926
July	1,560,215	1492,958
August	1,600,214	1496,073
September	1,579,442	1557,764
October	1,652,071	1984,036
November	1,658,417	1595,592
December	1,482,769	Data not available
Total	17,734,441	16,044,874

From environmental perspective, fresh POME is a hot and acidic brownish colloidal suspension, characterized by high amounts of total solids (40,500 mg/l), oil and grease (4000 mg/l), COD (50,000 mg/l) and BOD (25,000 mg/l). POME has been identified as one of the major sources of aquatic pollution in Malaysia. The characteristic of a typical POME is shown in Table 2.2. Despite its biodegradability, POME cannot be discharged without first being treated because POME is acidic and has a very high biochemical oxygen demand (BOD). Raw POME is high in BOD and acidic with pH of around 4.0. After treatment, the pH is raised to around 8 and BOD is lowered. In terms of nutrient value, anaerobic sludge of treated POME contains high plant nutrients (Lorestani, 2006).

Table 2.2 Typical characteristics of POME (Ma, 2000; Lorestani, 2006)

Parameter	***Average**	**Metal**	***Average**
pH	4.7	Phosphorus	180
Oil and Grease	4000	Potasium	2270
Biochemical Oxygen Demand (BOD)	25000	Magnesium	615
Chemical Oxygen Demand (COD)	50000	Calcium	439
Total Solids	40500	Boron	7.6
Suspended Solids	18000	Iron	46.5
Total Volatile Solids	34000	Manganese	2.0
Ammonical Nitrogen	35	Copper	0.89
Total Nitrogen	750	Zinc	2.3

*****All in mg/l except pH

It is generally accepted that surplus starchy grains and effluents generated from agro-industrial processes are cheap substrate that could serve as potential fermentation feedstock (Hipolito *et al.,* 2008). In any fermentation process, the cost of the substrate (fermentation medium) will be about 60% of the overall cost (Ross, 1961; Kalil *et al.,* 2003). The availability of an abundant supply of a low-cost, lignocellulosic, agricultural waste substrate is essential if acetone-butanol-ethanol (ABE) fermentation is to become economically viable (Lee *et al.,* 1995). This is due to the cost of the substrate was identified as the main factor that influences the economic viability of ABE fermentation (Lee *et al.,* 1995; Durre, 1998; Ezeji *et al.,* 2004). Furthermore, POME was produced in vast amounts throughout the year could be a kind of sustainable resources (Kalil *et al.,* 2003; Ngan *et al.,* 2004; Wu *et al.,* 2009; 2010).

POME is a thick, brownish liquid with a discharged temperature in the range of 80 to 90^{O}C. In palm oil mills, POME is generated from three major sources, sterilizer condensate, separator sludge and hydrocyclone operation where the broken shells are separated from the kernels (Vijayaraghavan *et al.,* 2005; Takriff *et al.,* 2009). POME consists of various suspended components including cell walls,

organelles and short fibers, a spectrum of carbohydrates ranging from hemicellulose to simple sugar, a range of nitrogenous compound from proteins to amino-acids and free organic acids (Ugoji, 1997; Takriff *et al.,* 2009) and an assembly of minor organic and mineral constituents (Ugoji, 1997; Lorestani, 2006). This entire feature has made POME a potential substrate for ABE fermentation (Somrutai *et al.,* 1996; Kalil *et al.,* 2003, Takriff et *al.,* 2009) and can be utilized by saccharolytic clostridia in ABE fermentation (Kwon *et al.,* 1989; Lee *et al.,* 1995). Such utilization would further increase profitability of palm oil industry besides solving an environmental problem (Kalil *et al.,* 2003).

2.2 Butanol-producing Clostridia

Clostridia have a long history of being employed in several biotechnological processes, for instance, *C. acetobutylicum* play role in the conversion of renewable biomass for butanol production; *C. perfringens* are significant for production of potent toxins such as enterotoxin; *C. botulinum* and *C. tetani* are used for neurotoxins; *C. histolyticum* and *C. oncolyticumto* produce agents for cancer therapy (Gheshlagi *et al.,* 2009) and *C. saccharoperbutylacetonicum* are proven to produce more butanol than *C. acetobutylicum* (Soni *et al.,* 1982). Solvent-producing clostridia were extensively used from the beginning of the 20th century for the industrial production of acetone and butanol and have remained a focus of research because of their potential applications in biotechnology (Keis *et al.,* 2001). Solvent-producing clostridia could produce acetone, butanol and ethanol from several biomass types such as palm oil waste (Lee *et al.,* 1995), domestic waste (Gheshlagi *et al.,* 2009), and abundant agricultural crops (Madihah *et al.,* 2001; Qureshi *et al.,* 2001; Shinto *et al.,* 2008). A number of studies has reported that the production of organic acids, alcohols, and other neutral solvents by the degradation of a wide range of polysaccharides by many species of clostridia (Gheshlagi *et al.* 2009). Saccharolytic mesophilic species that are able to form butyrate, however, are the only species that are capable of producing butanol along with different amounts of acetone, isopropanol, and ethanol (Jones and Woods, 1989; Gheshlagi *et al.* 2009).

Microbial butanol production was first reported by Louis Pasteur in 1861 and developed to an industrial production level by Chaim Weizmann using *Clostridium acetobutylicum* in the early 20th century (Gheshlagi *et al.* 2009). Hence, strains classified as *Clostridium acetobutylicum* were the first industrial cultures to be successfully isolated, patented and used for the large-scale production of solvents from starched-based substrates (Keis *et al.,* 2001). Followed by the switch (in the mid-1930s) to molasses as the preferred fermentation substrate, numerous new solvent-producing clostridial strains were isolated and patented, and each was given a novel species name (Jones and Keis, 1995; Keis *et al.*, 2001). However, none of these saccharolytic industrial strains were recognized as legitimate species, and when the acetone-butanol fermentation process went into decline these names fell into disuse. Subsequently, the majority of these solvent-producing clostridial strains were designated as *C. acetobutylicum* or *Clostridium beijerinckii* (Keis *et al.,* 2001).

C. acetobutylicum is an anaerobic and spore-forming bacterium (Gheshlagi *et al.*, 2009). It also is able to use polymeric substrates such as starch and xylan, but not cellulose, for growth (Durre, 1998). Anaerobic bacteria such as the solventogenic clostridia are capable of converting a wide range of carbon sources (e.g. glucose, galactose, cellobiose, mannose, xylose and arabinose) to fuels and chemicals such as butanol, acetone, and ethanol (Ezeji *et al.,* 2007; Masngut *et al.,* 2007). Hence, *C. acetobutylicum* is a known alcohol-producing microorganism (Alshiyab *et al.*, 2008).

Furthermore, this strain attracts a lot of attention when Finch *et al.,* (2011) reported that by consolidating the functions of waste management, renewable power generation, and solvent production, *C. acetobutylicum* fuel cells have the potential to reduce organic wastes and increases opportunities to convert those wastes to usable energy.

Butanol-producing clostridia such as *C. acetobutylicum, C. beijerinckii and C. pasteurianum* exhibit very similar metabolic pathways. During fermentation, C. acetobutylicum produces three major classes of products: (i) solvents (acetone, ethanol and butanol); (ii) organic acids (acetic acid, lactic acid and butyric acid); (iii)

gases (carbon dioxide, and hydrogen) (Zheng *et al.*, 2009). *C. acetobutylicum* exhibits a biphasic fermentation in which acetate and butyrate are produced initially, known as acidogenesis, followed by a switch to production of the solvents, acetone and butanol (Prescott and Dunn, 1959; Green *et al.*, 1994).

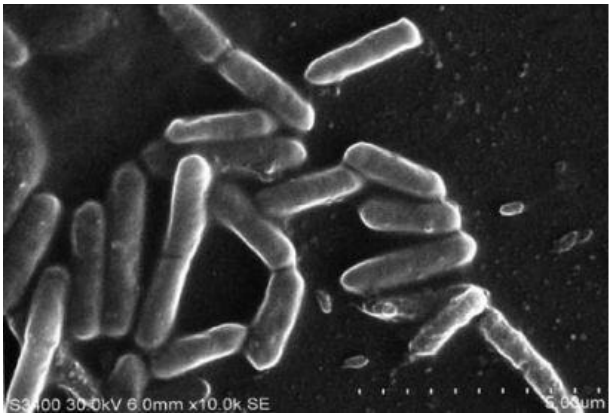

Figure 2.1 Scanning electron microscope (SEM) image of *C. acetobutylicum* cells for ABE production (Ni and Sun, 2009)

2.3 Fermentation

2.3.1 Anaerobic Fermentation

Anaerobic fermentation is the process of fermentation without using any oxygen. Durre (1998) mentioned that the first account of biological butanol synthesis stems from Louis Pasteur. In 1861 he isolated a butyric-acid-forming bacterium and named it *Vibrion butyrique.* This organism was unable to grow in the presence of air; later it became evident that oxygen was the proper toxic compound. This led to the term "anaerobic" to describe this type of metabolism. One of advantages of the anaerobic process is the recovery of the useful matters such as solvents (Hwang et.al. 2004). Anaerobic fermentation is a promising method of sustainable butanol production since organic matter, including waste products, can be used as a feedstock for the process (Alalayah *et al.*, 2009).

2.3.2 Acetone-butanol-ethanol (ABE) Fermentation

A great number of studies were performed in order to improve the process and fermentative process became competitive with chemical synthesis by the middle of the 20th century. Its application, however, declined during the 1950s and was overtaken by cheaper petrochemical-based processes by 1960 (Tashiro *et al.,* 2004; Kobayashi *et al.,* 2005; Gheshlagi *et al.* 2009). Furthermore, end-product inhibition, low product concentration and large volumes of fermentation broth, the requirements for large bioreactors, in addition to the high cost involved in generating the steam required to distil fermentation products from the broth largely contributed to the decline in fermentative acetone-butanol-ethanol (ABE) production (Ishizaki *et al.,* 1999). In the 1980s the reduced supply and escalating price of petroleum rekindled interest in fuel production by anaerobic bacteria including ABE fermentation by various clostridial species (Gheshlagi *et al.,* 2009). This is also due to a worldwide desire to identify and improve alternative but renewable sources of fuels as a safeguard against depleting reserves of fossil fuels have rekindled research into finding ways that would enhance solvent production by the ABE fermentation (Ishizaki *et al.,* 1999).

At present, considerable research has been conducted on the type of ABE fermentation system (Tashiro *et al.,* 2004), including batch culture (Qureshi and Blaschek, 1999; Ishizaki *et al.,* 1999; Tashiro *et al.,* 2004) or fed-batch culture (Ezeji *et al.,* 2004; Tashiro *et al.,* 2004) integrated with a butanol removal process, and continuous culture with concentrated cell mass or immobilized cell mass (Tashiro *et al.,* 2004). In previous studies, the yields of butanol to glucose were under 30%, and the residual glucose concentrations in broth were very high. To date, a highly efficient butanol production system has not yet been established (Tashiro *et al.,* 2004). New possibilities for more sustainable solvent production via ABE fermentation with less expensive substrates have been proposed. For instance, lignocellulosic materials such as domestic organic waste (Gheshlaghi *et al.,* 2009) or fibrous corn wastes (Qureshi *et al.,* 2006; Gheshlaghi *et al.,* 2009) can be used for ABE fermentation.

The metabolic pathways of solvent-producing clostridia consist of two distinct characteristic phases, namely, acidogenesis and solventogenesis (Green *et al.*, 1994; Badr *el al.*, 2001; Kalil *et al.* 2003; Tashiro *et al.*, 2004; Shinto *et al.*, 2008). Typically, during acidogenesis, cell growth is exponential and products are acetic acid and butyric acid with ATP formation. Accumulation of these organic acids results in a decrease in the pH of the broth. During solventogenesis, cell growth enters the stationary phase and the above organic acids are reutilized and acetone, butanol and ethanol are produced. This reutilization of organic acids results in a pH increase of the broth. It is reported that organic acid production is enhanced at higher pH, while solvents are mainly produced at lower pH (15 – 18). On the other hand, since the addition of organic acids to the growth medium has been shown to stimulate solvent production and protect against the degeneration of ABE-producing clostridia, it is suggested that organic acids in broth trigger a metabolic shift from acidogenesis to solventogenesis although the exact mechanism is still unknown. Thus, we noted that butanol could be produced effectively at lower pH by feeding organic acids such as acetic acid or butyric acid. Presently, there is no report on this feeding method in ABE fermentation (Tashiro *et al.*, 2004).

The production of ABE by solvent-producing strains of *Clostridium* was one of the first large-scale industrial fermentation process developed (Kalil *et al.*, 2003). Shinto *et al.* (2008) then mentioned that results of the simulation suggested that *C. saccharoperbutylacetonicum* N1-4 has a robust metabolic network in acid- and solvent-producing pathways (Shinto *et al.*, 2008).

2.4 Butanol

Butanol is a higher alcohol with a four carbon atom structure and a general formula of $C_4H_{10}O$. Butanol can be produced from biomass and from mineral fuel. The butanol from biomass is conventionally denoted as biobutanol despite the fact that it has the same characteristics as the butanol from petroleum (Shapovalov and Ashkinazi, 2008).

Often mis-described as a “new” fuel, biobutanol has been in almost continuous production since 1916, and most of the time as a solvent as well as a basic chemical. Today, new uses for butanol are emerging, e.g. as a diesel and kerosene replacement, as silage preserver, biocide and C4 compound for chemical industry. n-Butanol has many advantageous characteristics which make it a superior gasoline replacement (Schwarz and Gapes 2006) (Antoni *et al.*, 2007).

Butanol is used as an industrial solvent (Shapovalov and Ashkinazi, 2008), chemical feedstock, and particularly liquid fuel (Durre, 2008; Gheshlaghi *et al.*, 2009). Since butanol has remarkable features such as hydrophobicity, high energy content, and ease of storage and transportation, it has been proposed as a substitute and supplement of gasoline as a transportation fuel (Shinto *et al.*, 2008). It also can be used instead of gasoline even in higher degree than ethanol due to its physical properties, economy, safety and because it can be applied without remodelling car engine (Shapovalov and Ashkinazi, 2008). Butanol can be but does not have to be blended with fossil fuel.

While current utilization strategies for biomass have focused on ethanol production, producing butanol instead of ethanol offers several advantages for biofuel-gasoline blending. Butanol has a lower vapour pressure but higher energy content than ethanol, which makes the former safer for blending with gasoline as well as offering better fuel economy than ethanol-gasoline blends. In addition, butanol has a higher tolerance to water contamination in gasoline blends and therefore butanol-gasoline blends are less susceptible to separation and that facilitates its use in existing gasoline supply and distribution channels. Furthermore, butanol can be blended with gasoline at higher concentrations than ethanol without the need to retrofit vehicles. Therefore, optimizing ABE fermentation to enhance butanol production over ethanol appears to be more commercially and technologically attractive option (Hipolito *et al.*, 2008).

Butanol is more safe to handle because is evaporated six times less than ethanol and by factor 13.5 less volatile than gasoline. Its vapour pressure by Reid is 0.33 pounds per square inch, of ethanol 2.0 pounds per square inch. This makes

butanol safe at its application as oxygenate, and need no significant changes in the mixture proportion at summer and winter application.

Butanol is much less aggressive and less corrosive substance than ethanol and therefore it can be transported with currently used fuel pipes, while ethanol should be transported by rail way transport (Shapovalov and Ashkinazi, 2008).

Like ethanol, fuel biobutanol contains “green” carbon that allows decreasing emission of hotbed gasses (Shapovalov and Ashkinazi, 2008) which means butanol when consumed in an internal combustion engine yields no carbon monoxide or all environmentally harmful by products of combustion (Elshahed, 2010). CO_2 is the combustion by product of butanol, and is considered environmentally 'green’. At combustion, butanol does not produce sulphur and nitrogen oxides that are advantageous from the ecology viewpoint (Shapovalov and Ashkinazi, 2008).

Producing butanol can simultaneously solve problems connected with the infrastructure of supplying hydrogen (Shapovalov and Ashkinazi, 2008). Reformed butanol has four more hydrogen atoms than ethanol, resulting in a higher energy output which is 10 Wt-h/g compared to ethanol i.e. 8 10 Wt-h/g (Shapovalov and Ashkinazi, 2008).

2.5 Advantages of Butanol

Butanol has 1/3 higher energy density (36 vs.27 kJ/g) than ethanol (Demain, 2009) and its energy is similar to gasoline (Ha *et al.*, 2010). Also, butanol use in cars does not require engine modification until it reaches 40% of total fuel; ethanol requires it at concentrations of over 15%. At one time, the acetone–butanol–ethanol (ABE) fermentation was used commercially to produce the solvents acetone and butanol, but the fermentation was replaced by less expensive chemical procedures. Today, there is renewed interest in this fermentation to produce biobutanol (Demain,

2009). Furthermore, it could be used as a solvent of quick-drying lacquer for automobile coating (Ni and Sun, 2009).

Due to extensive oil consumption and its increasing price trend over the past decades, the use of biofuel as a partial replacement for fossil fuels has gained great attention worldwide. Among alternative fuels, bio-based butanol (biobutanol) is expected to play a major role in the next generation of biofuel (Ni and Sun, 2009).

Butanol is also widely used as a solvent for acid-curable lacquers and baking finishes. Other important applications of butanol and its derivatives include paint thinners, solvent for dyes, brake fluids, and as an extractant in the production of drugs and natural substances such as antibiotics hormones, vitamins, etc. In a similar vein of interest in ethanol and bio-diesel, an important application has emerged for butanol as a renewable energy carrier since it can be used directly as a liquid fuel. Compared with ethanol, butanol has many chemical and physical features that are particular attractive for application as a biofuel (Ni and Sun, 2009).

Furthermore, the bioethanol industry in Brazil was criticized as environmentally hazardous, as large area is being used for monocultures. A similar discussion was sparked in North America and Europe where starch production for biofuel competes over land with the food industry and environmental issues. The sky rocketing price for starch is already hindering the start-up of new bioethanol plants (Antoni *et al.*, 2007).

Butanol is not miscible with water and is therefore noncorrosive, and has a lower vapour pressure and a nearly 50% higher energy content than ethanol. It can be blended with gasoline fuel at any ratio without the necessity for modification of vehicle engines, and can be transported and stored in the existing pipeline and tank system infrastructure (Ni and Sun, 2009).

In addition, butanol is less hazardous to handle, less flammable, less hygroscopic (thus does not pick up water) and can easily mix with gasoline in any

proportion (Ha *et al.*, 2010). It was recognised that gasoline additives, such as tetra-ethyl-lead, required for high compressions in ethanol free engines, were environmental and health hazards (Antoni *et al.*, 2007). Summarize comparison of gasoline to biofuels can be observed in Table 2.3.

Table 2.3 Comparison of gasoline to biofuel (Ha *et al.*, 2010)

Parameter	Gasoline	Ethanol	Butanol
RON (research octane number)	91 – 99	129	94
MON (motor octane number)	85	103	81
Anti-knock index	90	116	87
Boiling point (°C)	< 210	78.3	118
Heating value (MJ/L)	32.2	21.1	27.0
Air-fuel ratio	14.6	3.0	11.2

All biofuels have to exhibit defined chemical and physical properties, meeting the demands of engine application such as stability and predictable combustion at high pressures as well as the demands of transportation such as safety and energy density (Antoni *et al.*, 2007).

2.6 Summary on Previous Research

Butanol has received more attention since it can be produced from a renewable resource via ABE fermentation process by *C. acetobutylicum* (Chuichulcherm and Chutmanop, 2004). Table 2.4 shows the review on previous research on the same finding in ABE fermentation using *C. acetobutylicum* and POME as fermentation medium.

Table 2.4 Various products or metabolites produced in bioprocess during the reuse of POME or its derivatives as substrate (Wu *et al.*, 2009)

Product	**Microorganism**	**Fermentation medium based on POME**	**Fermentation conditions**	**Fermentation time (h)**	**Maximum production A=acetone, B=butanol, E=ethanol**	**Reference**
ABE	*Clostridium acetobutylicum* NCIMB 13357	90% (v/v) particulate fraction of raw POME	35 °C, 10% inoculum, initial pH=5.8, flask fermentation	48 (for ABE)	A=1.97g/l, B=1.74 g/l, E=0.3 g/l	Kalil *et al*. (2003), Pang *et al*. (2004)
ABE	*Clostridium acetobutylicum* NCIMB 13357	Particulate fraction of raw POME	35 °C, 10% inoculum, initial pH=6, flask fermentation	≈30 (for A) ≈24 (for E)	A=1.2 g/l, B=0 g/l, E=0.5 g/l	Takriff *et al*. (2005)
ABE	*Clostridium acetobutylicum* NCIMB 13357	Particulate fraction of raw POME	Oscillated at 0.45 Hz, 35 °C, 10% inoculum, initial pH=6, oscillatory flow bioreactor fermentation	≈42 (for A) ≈30 (for E)	A=0.7 g/l, B=0 g/l, E=0.6 g/l	Takriff *et al*. (2005)

ABE	*Clostridium acetobutylicum* NCIMB 13357	Particulate fraction of raw POME	Oscillated at 0.78 Hz, 35 °C, 10% inoculum, initial pH=5.8, oscillatory flow bioreactor fermentation	48 (for ABE)	A=0.05 g/l, B=1.54 g/l, E=0 g/l	Masngut *et al.* (2006, 2007)
ABE	*Clostridium acetobutylicum* NCIMB 13357	Particulate fraction of raw POME	250 rpm, 35 °C, 10% inoculum, initial pH=5.8, stirred tank bioreactor fermentation	60 (for ABE)	A=0.13 g/l, B=0.50 g/l, E=0.24 g/l	Masngut *et al.* (2007)
ABE	*Clostridium acetobutylicum* NCIMB 13357	90% (v/v) particulate fraction of raw POME	35 °C, 10% (v/v) inoculum, initial pH=5.8, flask fermentation	48 (for ABE)	A=1.97g/l, B=1.74 g/l, E=0.3 g/l	Kalil *et al*. (2003), Pang *et al.* (2004)
ABE	*Clostridium acetobutylicum* NCIMB 13357	Particulate fraction of raw POME	35 °C, 10% (v/v) inoculum, initial pH=6, flask fermentation	≈30 (for A) ≈24 (for E)	A=1.2 g/l, B=0 g/l, E=0.5 g/l	Takriff *et al.* (2005)

ABE	*Clostridium acetobutylicum* NCIMB 13357	Particulate fraction of raw POME	Oscillated at 0.45 Hz, 35 °C, 10% inoculum, initial pH=6, oscillatory flow bioreactor fermentation	≈42 (for A) ≈30 (for E)	A=0.7 g/l, B=0 g/l, E=0.6 g/l	Takriff *et al*. (2005)
ABE	*Clostridium acetobutylicum* NCIMB 13357	Particulate fraction of raw POME	Oscillated at 0.78 Hz, 35 °C, 10% inoculum, initial pH=5.8, oscillatory flow bioreactor fermentation	48 (for ABE)	A=0.05 g/l, B=1.54 g/l, E=0 g/l	Masngut *et al.* (2006, 2007)
ABE	*Clostridium acetobutylicum* NCIMB 13357	Particulate fraction of raw POME	250 rpm, 35 °C, 10% (v/v) inoculum, initial pH=5.8, stirred tank bioreactor fermentation	60 (for ABE)	A=0.13 g/l, B=0.50 g/l, E=0.24 g/l	Masngut *et al.* (2007)

CHAPTER 3

METHODOLOGY

3.1 Introduction

The overview of methods in this study as followed:

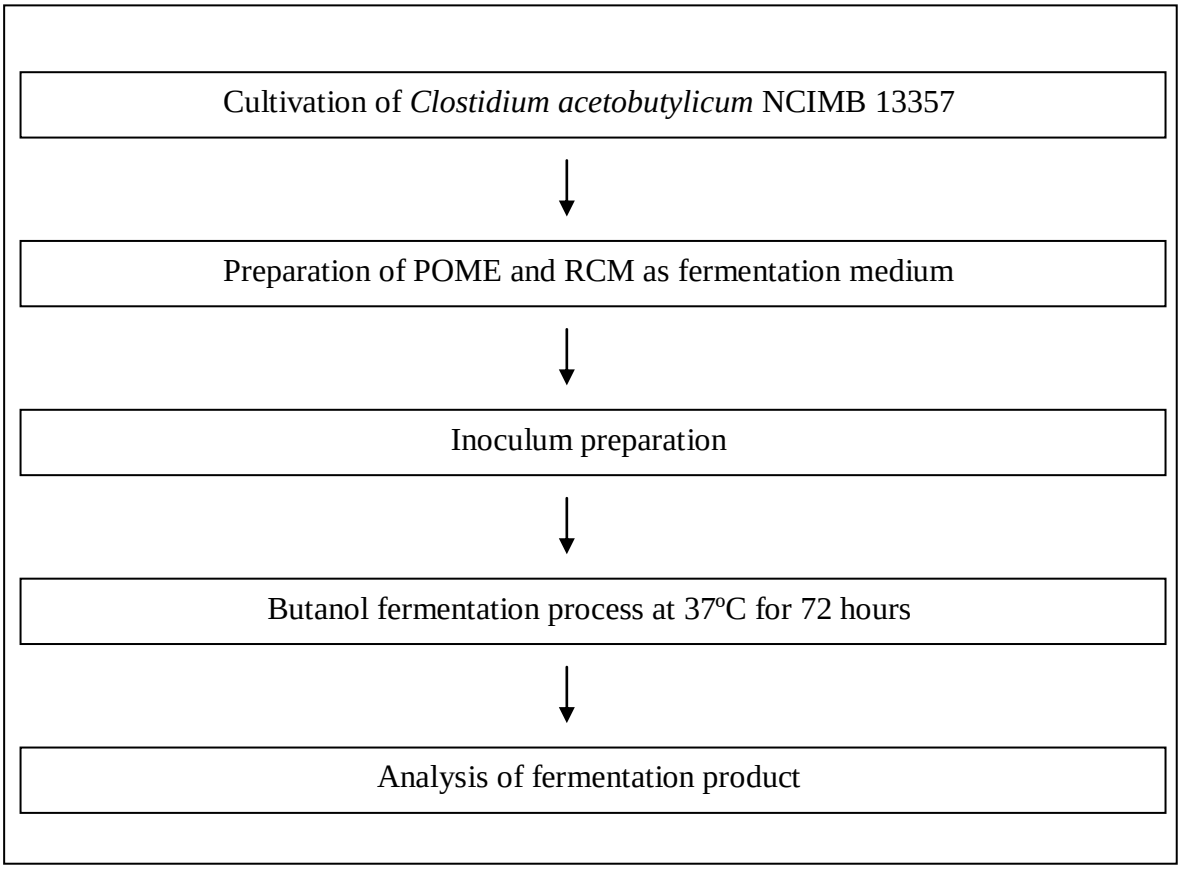

Figure 3.1 Flow chart of experimental procedures

3.2 Materials and Equipments

3.2.1 Strain

Clostridium acetobutylicum NCIMB 13357 was used throughout this study. This strain was obtained from Universiti Kebangsaan Malaysia (UKM) and kept in the freezer with temperature at - 4°C.

The pure stock of *C. acetobutylicum* was inoculated onto both petri plate and universal bottle contained with Reinforced Clostridia Agar by streaking method. Cultivation of *C. acetobutylicum* in petri plate resulted as agar plate while in universal bottle resulted as agar slant. Anaerobic and sterilised conditions were maintained during this procedure at 37°C in anaerobic chamber (Bactron, Sheldon Manufacturing Inc., USA). Then, both petri plate and agar slant were sealed with parafilm before incubated at 37°C for 24 hours in incubator.

3.2.2 Fermentation Media

3.2.2.1 Agar Medium

52.5 grams of Reinforced Clostridial Agar powder was dissolved completely in 1 liter of distilled water and then transferred into a Schott bottle. The agar medium was sterilised at 121°C for 20 minutes using Autoclave HVE-50 (Hirayama HVE-50). Then, it was cooled down approximately 50°C before poured onto a sterile petri plate and a universal bottle. Both were cooled at room temperature in order to obtain solid agar medium. The universal bottle was put leaned over for about 45° to one side while cooling down. Then, the agar medium was kept in freezer at 4°C.

3.2.2.2 Reinforced Clostridia Medium (RCM)

Reinforced Clostridia Medium (RCM) was used as the control medium. 38 grams of RCM powder was dissolved completely in 1 liter of distilled water and then transferred into a Schott bottle. RCM was autoclaved at 121°C for 20 minutes.

3.2.2.3 Palm Oil Mill Effluent (POME)

Fresh POME was obtained from Kilang Sawit Felda Lepar Hilir, Lepar Hilir, Pahang and kept in heat resistant bottle. POME was preserved and passively sedimented in a cool room temperature less than 4°C but above freezing over a period of 24 hours before used in order to prevent the wastewater from undergoing biodegradation due to microbial action. The supernatant layer (upper layer) was decanted and sedimented POME sludge (lower part) was used as the fermentation medium. Initial pH of POME sludge was adjusted to pH 5.8 by the addition of 5.0M NaOH. The pH 5.8 was optimum for solvent production from POME. Then, POME was sterilised at 121°C for 20 minutes using Autoclave HVE-50 (Hirayama HVE-50) and used directly as fermentation medium without additional nutrient.

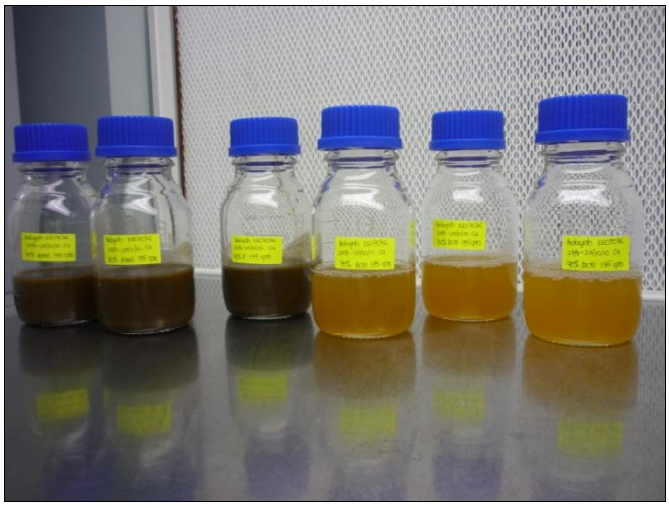

Figure 3.2 Palm oil mill effluents (POME) and Reinforced Clostridia Medium (RCM) as fermentation medium

3.2.3 Equipments

The fermentation process were carried out using Autoclave HVE-50 (Hirayama HVE-50), anaerobic chamber (Bactron, Sheldon Manufacturing Inc., USA), UV-Vis spectrophotometer (Hitachi U-1800, Hitachi, Japan), Refrigerated Centrifuge (Eppendorf), hybridization incubator (FinePCR) and Gas Chromatography Agilent 6890 equipped with flame ionization detector (Agilent Technology, USA).

3.3 Experimental Procedures

3.3.1 Inoculum Preparation

Liquid medium of RCM was used for inoculum preparation. Toothpicks were used to take a portion of incubated culture and put into the sterile deoxygenated Reinforced Clostridial Medium (RCM) and incubated for 24 hours at 37°C in anaerobic chamber. Then, broth was centrifuged using Refrigerated Centrifuge (Eppendorf) at 10000 rpm for 4 minutes. The supernatant was decanted and cell was re-suspended with sterile normal saline solution, 0.85% sodium chloride. The growth of culture in RCM was monitored by measuring an optical density (OD) at 660 nm (Barbeau *et al.*, 1988) using UV-Vis spectrophotometer (Hitachi U-1800). Only inoculum with OD values greater than 0.7 after 18 hours cultivation was used as inoculum which formed 10% (v/v) of the total of culture. No shaking was required during this process.

Figure 3.3 Reinforced Clostridia Medium (RCM) broth

3.3.2 Butanol Fermentation

Butanol production by *C. acetobutylicum* was studied and conducted anaerobically in 250 mL Schott bottles. 150 mL of working volume was used for this fermentation contained with 10% or 15 mL of inoculum. The effects of substrate concentrations (RCM as control medium and sedimented POME) were studied at 70, 80 and 90% v/v while the effects of agitation rates were studied at 0, 100, 175 and 250 rpm.

Sedimented POME and RCM were diluted with distilled water to obtain required substrate concentrations before deoxidizing by gassing with nitrogen gas for few minutes to minimize the introduction of oxygen into the culture. These fermentation media were put in hybridization shaker (FinePCR) at the speed of 0, 100, 175 and 250 rpm. It was noted that each speed was run at different time. Other parameters were kept constant at optimal condition i.e. pH 5.8 for POME, pH 6.8 for RCM, temperature at 37^{o}C, fermentation time for 72 hours and inoculum 10% v/v.

Samples also were taken for every 18 hours and assayed using 3, 5-dinitrosalicyclic acid (DNS) method using UV-Vis spectrophotometer. These for observing the growth profile rate and glucose consumption determination in POME and RCM media. After samples were taken, maintenance of constant stream nitrogen gas over the culture headspace was done in order to enhance anaerobic fermentation environment (Alalayah *et al.*, 2009).

3.3.3 Fermentation Product Recovery Process

After 72 hours, fermentation product was centrifuged for five minutes at 5000 rpm to separate between pellet and supernatant. Pellet was decanted and supernatant was used for next process. Then, supernatant of POME and RCM were filtered using vacuum filtration. Then, fermentation product was filtered using syringe filter of 0.2µm

Figure 3.4 Sampling after product recovery process

3.4 Analysis of Fermentation Product

3.4.1 Growth Profile of *Clostridium acetobutylicum*

Growth profile of *C. acetobutylicum* was carried out in 500 mL Schott bottle at a working volume of 300 mL. Substrate concentration used was 90%. The profile growth rate of culture in RCM and sedimented was monitored by measuring an optical density (OD) at 660 nm using UV-Vis spectrophotometer (Hitachi U-1800).

3.4.2 3, 5-Dinitrosalicyclic acid (DNS) reagent Preparation

10 g 3, 5-dinitrosalicyclic acid (DNS) acid, 2 g phenol, 0.5 g sodium sulfide, 10 g sodium hydroxide and 182 g potassium sodium tartrate (Rochelle salts) were mixed together in 1 L of sterile distilled water. This mixture was placed in conical flask which covered with aluminium foil. It was then kept in freezer at 4°C.

3.4.2.1 Glucose Consumption Determination

1 mL of sample was mixed together in a vial with 1 mL of DNS reagent and 0.1 mL of 0.1 M NaOH. The mixtures were placed in a boiling water bath for 5 min at 90°C. Then, it was diluted with 10 mL of distilled water and has been shaken vigorously. The absorbances were monitored by measuring an OD at 540 nm using UV-Vis spectrophotometer for all samples and recorded. The glucose concentration was calculated from a standard curve (Alalayah *et al.,* 2009).

It was noted that sample of sedimented POME was diluted before mixed with DNS reagent. 0.1 mL of sedimented POME was diluted with 9.9 mL of deionised water. Then, 1 mL of this dilution was used for determination of glucose concentration.

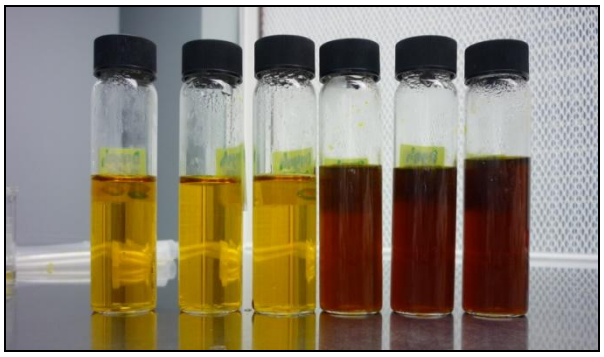

Figure 3.5 Samples are mixed with 3, 5-dinitrosalicyclic acid

3.4.3 Butanol Production

Butanol concentrations in the supernatant were determined with a gas chromatograph (Agilent 6890N) equipped with a flame ionization detector and a 15 m capillary column (HP Inowax). The oven temperature was programmed to increase from 50°C to 170°C at the rate of 10°C/min. The injector and detector temperature were set at 250°C. Nitrogen was the carrier gas and was set at a flow rate 72 mL/min. Iso-butanol was used as the internal standard.

In order to prepare for determination of butanol concentration, 0.1 mL of sample was filtered using 0.2µm filter and mixed together with 0.9 mL iso-butanol into gas chromatograph vial.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Introduction

Butanol production by *Clostridium acetobutylicum* was conducted in anaerobic fermentation condition using Palm Oil Mill Effluent (POME) and Reinforced Clostridia Medium (RCM) as fermentation medium. POME was the main substrate in this study while RCM acted as control medium. Fermentations were carried out in 250 mL Schott bottles at a working volume of 150 mL. This fermentation process was controlled at pH 5.8 for POME, pH 6.8 for RCM, temperature at 37°C, inoculum 10% v/v and 72 hours of fermentation time. The effects of substrate concentration were studied at 70%, 80% and 90% while the effects of agitation rates were studied at 0, 100, 175 and 250 rpm. Butanol production was the major focus of fermentation end-product in this study along with the growth profile rate and glucose consumption by *C. acetobutylicum* during fermentation process. Butanol produced from the fermentation was analyzed using gas chromatography equipped with flame ionization detector (GC-FID). Growth profile rates of *C. acetobutylicum* in POME and RCM were measured using UV-Vis spectrophotometer. Glucose concentration was measured for the calculation of the amount of glucose consumed by dinitrosalicylic acid (DNS) method using UV-Vis spectrophotometer.

4.2 Growth Profiles of *C. acetobutylicum* Analysis

Table 4.1 Comparisons on growth of *Clostridium acetobutylicum* in POME and RCM (optical density at 660 nm)

Time (h)	Optical density (abs)	
	POME	RCM
0	0.8873	0.5530
6	1.4590	0.5940
12	2.1750	1.4783
18	2.1840	1.6567
24	2.1720	1.9610
30	2.3780	1.6910
36	2.5633	1.7037
42	2.7530	1.8110
48	2.8430	2.0780
54	2.1560	1.9870
60	2.0847	1.9650
66	2.3643	1.6433
72	2.2680	1.6880

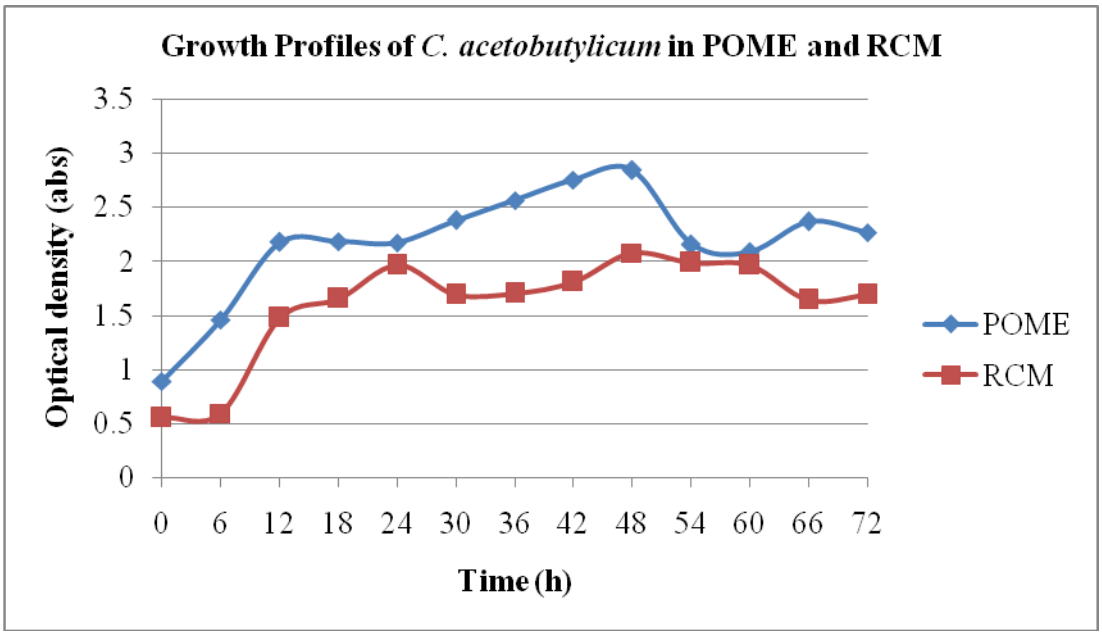

Figure 4.1 Comparisons on growth of *C. acetobutylicum* in POME and RCM

Figure 4.1 shows that the growth profile curves of *C. acetobutylicum* in POME and RCM are different from each other. It shows that absorbances of *C. acetobutylicum* in POME are higher than in RCM. This graph can be divided into four different phases which are lag phase, exponential or log phase, stationary phase and death phase. These phases in growth profiles of *C. acetobutylicum* are related to two distinct characteristic phases in energy acquiring pathway, specifically acidogenesis and solventogenesis.

The lag phases for *C. acetobutylicum* in both fermentation mediums occur from 0 to 6 hours. During lag phase, bacteria are adapting themselves to growth conditions. They are maturing and not yet able to divide in this period. Then, *C. acetobutylicum* enters exponential phase in RCM from 6 to 24 hours while in POME from 6 to 12 hours. In this period, cell in bacteria are doubling and grow rapidly. Barbeau *et al.* (1988); Evans and Wang (1988); Durre (1998), Tashiro *et al.* (2004) and Shinto *et al.* (2008) reported that typically, in acetone-butanol-ethanol (ABE) fermentation, this period also known as acidogenesis. During acidogenesis, cell growth is exponential and this is where acetic acid and butyric acid produced with ATP formation. These organic acids attain their maximal concentrations and accumulation of these acids resulting in a decrease in the pH of the broth.

Stationary phase occurs as a result of the nutrient depletion, accumulation of toxic product and the rate of bacterial growth is equal to the rate of bacterial death. This phase also claimed as solventogenesis where cell growth enters the stationary phase and the organic acids are reutilised and butanol, acetone and ethanol are produced. This reutilisation of organic acids results in a pH increase of the broth. In other words, reassimilation of organic acids occurs and forming the solvents. Stationary phase for RCM occurs from 24 to 48 hours. However, for POME, stationary phase is considered to occur from 12 to 48 hours due to there is increasing of absorbances from 24 to 48 hours. This condition is resulting from contents in POME or glucose metabolism.

Death phase occurs for both fermentation medium from 48 to 72 hours. At death phase, bacteria are running out of nutrients and die. However, there is fluctuation in POME whereas absorbance increases from 60 to 66 hours. This condition also is resulting from contents in POME.

Ezeji *et al.* (2010) proposed that the acetic and butyric acid pathway reactions have important roles in solventogenic clostridia metabolism because synthesis of these acids is accompanied by generation of ATP, which is important for cell growth and metabolism.

4.3 Butanol Production Analysis

Fermentation process was carried out in Schott bottles at working volume of 150 mL. Butanol production was studied at different substrate concentrations of 70%, 80% and 90% with agitation rate at 0, 100, 175 and 250 rpm. The results of butanol are analysed at the end of fermentation time which is after 72 hours.

4.3.1 Effects of Substrate Concentrations on Butanol Production

4.3.1.1 Effects of Substrate Concentration at 0 rpm

Table 4.2 Effects of different substrate concentration on butanol productivity at 0 rpm

Substrate concentration (%)	Butanol concentration (g/L)	
	POME	RCM
70	0.2154	0
80	0.3605	0
90	0.2431	0

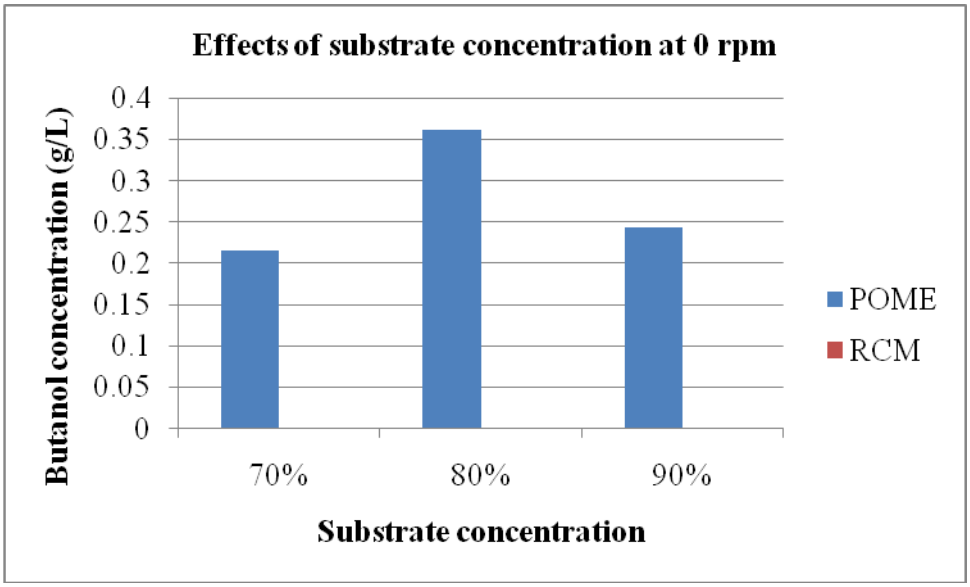

Figure 4.2 Effects of different substrate concentration on butanol productivity at 0 rpm

Table 4.2 and Figure 4.2 show that butanol production at the end of fermentation time only occurs from POME but none in RCM at 0 rpm. The highest value in butanol production is 0.3605 g/L from 80% substrate concentration of POME and followed by 0.2431 g/L from 90% v/v and 0.2154 g/L from 70% v/v.

4.3.1.2 Effects of Substrate Concentration at 100 rpm

Table 4.3 Effects of different substrate concentration on butanol productivity at 100 rpm

Substrate concentration (%)	Butanol concentration (g/L)	
	POME	RCM
70	0	0
80	0	0
90	0	0.2923

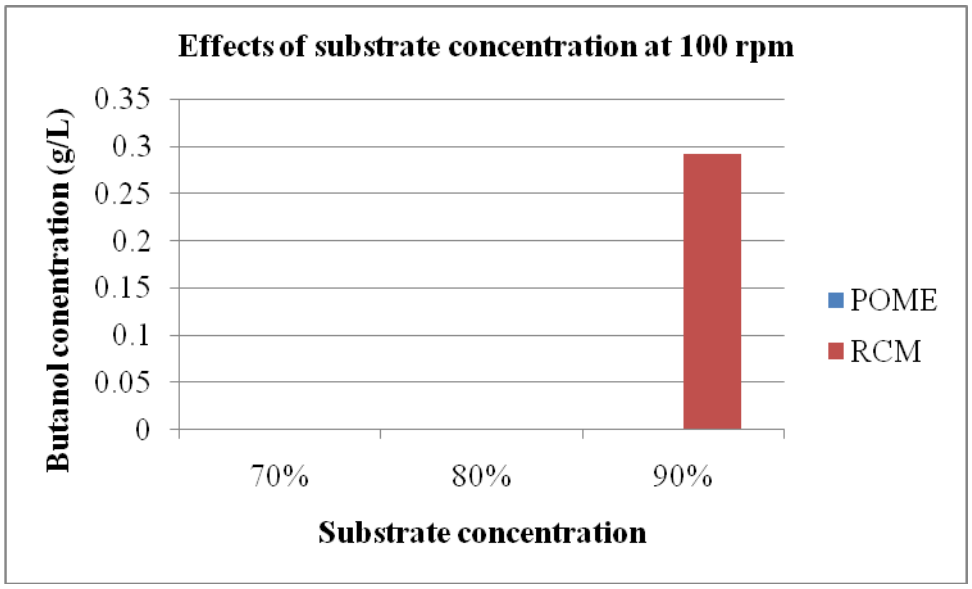

Figure 4.3 Effects of different substrate concentration on butanol productivity at 100 rpm

From Table 4.3 and Figure 4.3, it shows that butanol production at the end of fermentation time only occurs in RCM at 90% of substrate concentration. The value is 0.2923 g/L. There is no butanol produced in other substrate concentrations other than 90% of RCM.

4.3.1.3 Effects of Substrate Concentration at 175 rpm

Table 4.4 Effects of different substrate concentration on butanol productivity at 175 rpm

Substrate concentration (%)	Butanol concentration (g/L)	
	POME	RCM
70	0.3485	0.3038
80	0.1952	0.5007
90	0	0.5034

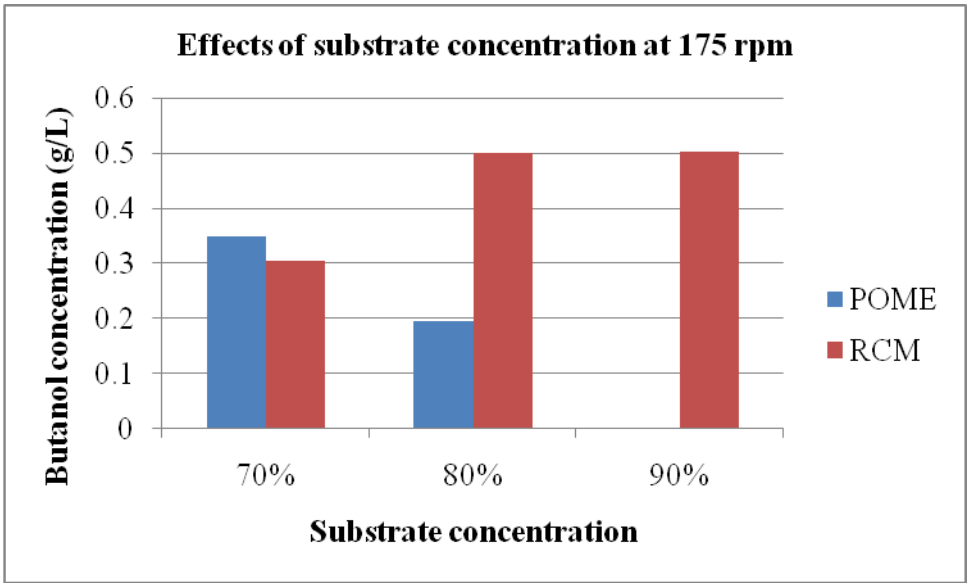

Figure 4.4 Effects of different substrate concentration on butanol productivity at 175 rpm

From Table 4.4 and Figure 4.4, it can be observed that the butanol concentrations in RCM are higher compared to the butanol concentrations in POME. Both table and figure above also shown that the values of butanol in POME dropped while for RCM, values of butanol increased by substrate concentration. Butanol production at substrate concentrations of 70% and 80% are 0.3485 g/L and 0.1952 g/L respectively. There is no butanol production in POME at 90% v/v which clarifies that value of butanol decreases rapidly. Values of butanol in RCM at 70%, 80% and 90% are 0.3038 g/L, 0.5007 g/L and 0.5034 g/L respectively. At 175 rpm, the

maximum butanol production is 0.5034 g/L which produced at substrate concentration of 90% in RCM.

4.3.1.4 Effects of Substrate Concentration at 250 rpm

Table 4.5 Effects of different substrate concentration on butanol productivity at 250 rpm

Substrate concentration (%)	Butanol concentration (g/L)	
	POME	RCM
70	0	0
80	0	0
90	0	0

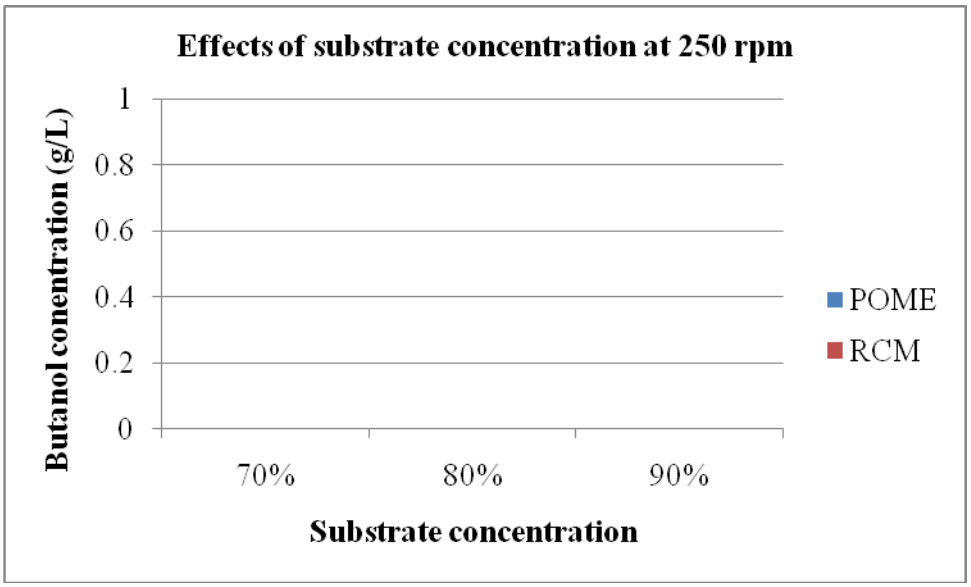

Figure 4.5 Effects of different substrate concentration on butanol productivity at 250 rpm

Table 4.5 and Figure 4.5 show no butanol produced at 250 rpm for both POME and RCM in the final product after 72 hours of fermentation. There is no butanol concentration are detected at 70%, 80% and 90% of substrate concentrations

while using 250 rpm. 250 rpm is the highest speed used in agitation rate for mixing of fermentation broth.

4.3.2 Effects of Agitation Rate on Butanol Production

4.3.2.1 Effects of Agitation Rate at 70%

Table 4.6 Effects of different agitation rate on butanol productivity at 70%

Agitation rate (rpm)	Butanol concentration (g/L)	
	POME	RCM
0	0.2154	0
100	0	0
175	0.3485	0.3038
250	0	0

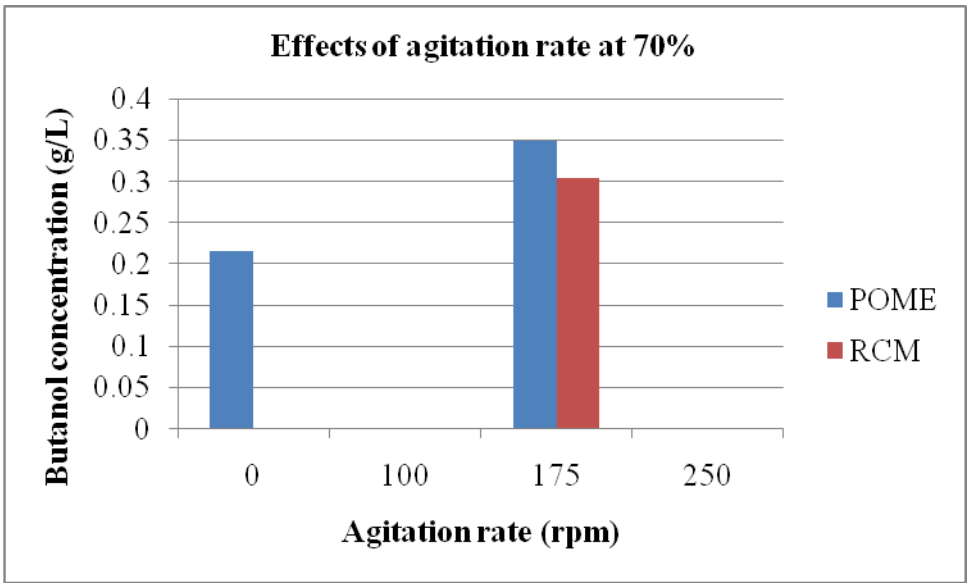

Figure 4.6 Effects of different agitation rate on butanol productivity at 70%

From Table 4.6 and Figure 4.6, it can be observed that the highest butanol is produced at 175 rpm from POME. Furthermore, butanol production occurs at 0 and 175 rpm for POME and 175 rpm for RCM. At 0 rpm, butanol is produced in POME

with value of 0.2154 g/L while at 175 rpm; butanol is produced in POME with value of 0.3485 g/L and 0.3038 g/L from RCM.

4.3.2.2 Effects of Agitation Rate at 80%

Table 4.7 Effects of different agitation rate on butanol productivity at 80%

Agitation rate (rpm)	Butanol concentration (g/L)	
	POME	RCM
0	0.3605	0
100	0	0
175	0.1952	0.5007
250	0	0

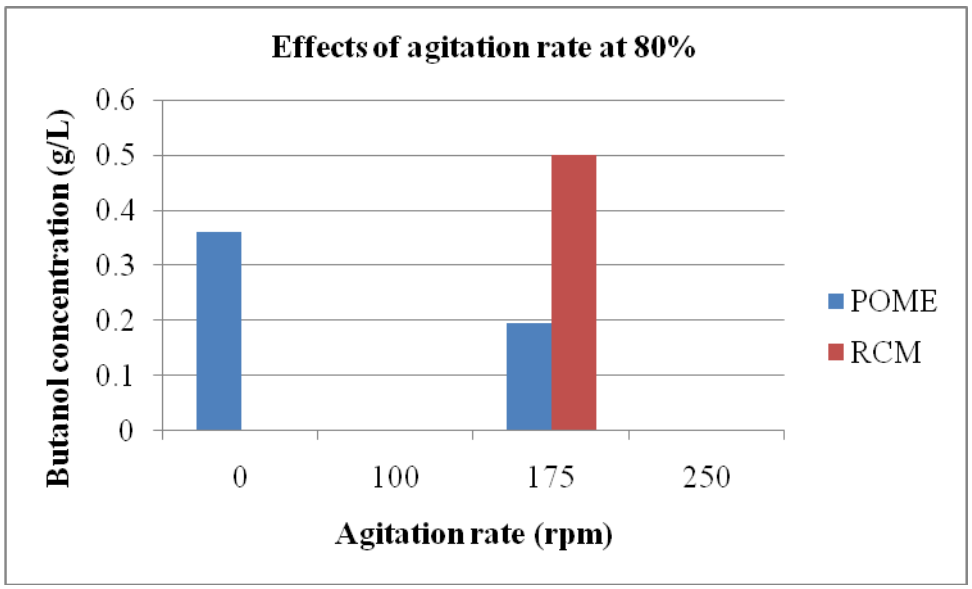

Figure 4.7 Effects of different agitation rate on butanol productivity at 80%

As showed in Table 4.7 and Figure 4.7, RCM produced highest butanol at 175 rpm. Butanol production only occurs at 0 and 175 rpm in substrate concentration of 80% v/v. Values of butanol at 0 rpm in POME is 0.3605 g/L. Butanol concentration at 175 rpm in POME is 0.1952 g/L and butanol concentration in RCM is 0.5007 g/L.

4.3.2.3 Effects of Agitation Rate at 90%

Table 4.8 Effects of different agitation rate on butanol productivity at 90%

Agitation rate (rpm)	Butanol concentration (g/L)	
	POME	RCM
0	0.2431	0
100	0	0.2923
175	0.5034	0
250	0	0

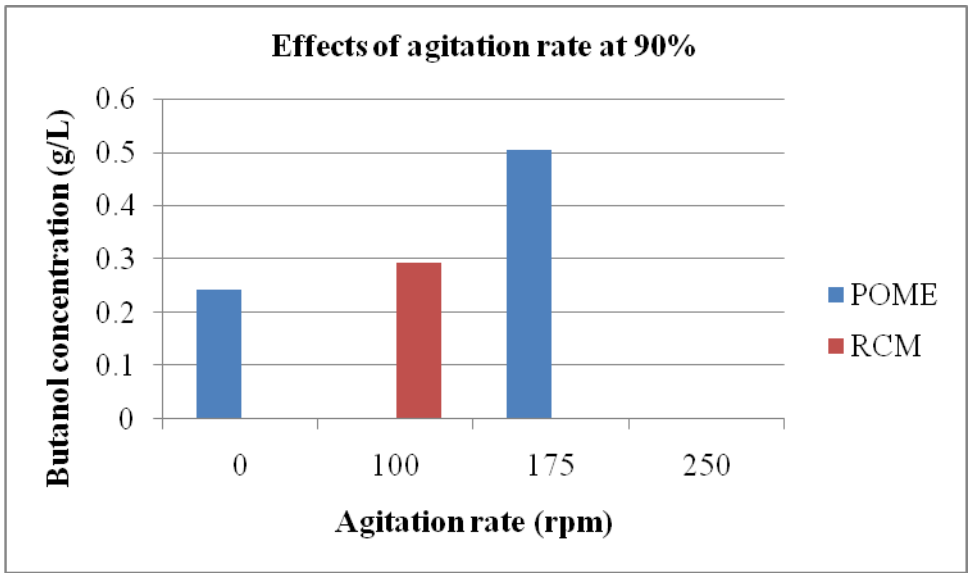

Figure 4.8 Effects of different agitation rate on butanol productivity at 90%

Table 4.8 and Figure 4.8 show that the highest butanol in substrate concentration at 90% v/v is produced by POME at 175 rpm and followed by butanol production from RCM at 100 rpm. The least butanol has been produced is 0.2431 g/L at 0 rpm by POME. The maximum butanol production at 90% substrate concentration is 0.5034 g/L by POME. Butanol produced at 100 rpm by RCM is 0.2923 g/L.

4.3.3 Effects of Substrate Concentration and Agitation Rate on butanol production

Table 4.9 Effects of both parameters on butanol production

Agitation rate (rpm)	Butanol concentration (g/L)					
	POME			RCM		
	70%	80%	90%	70%	80%	90%
0	0.2154	0.3605	0.2431	0	0	0
100	0	0	0	0	0	0.2923
175	0.3485	0.1952	0	0.3038	0.5007	0.5034
250	0	0	0	0	0	0

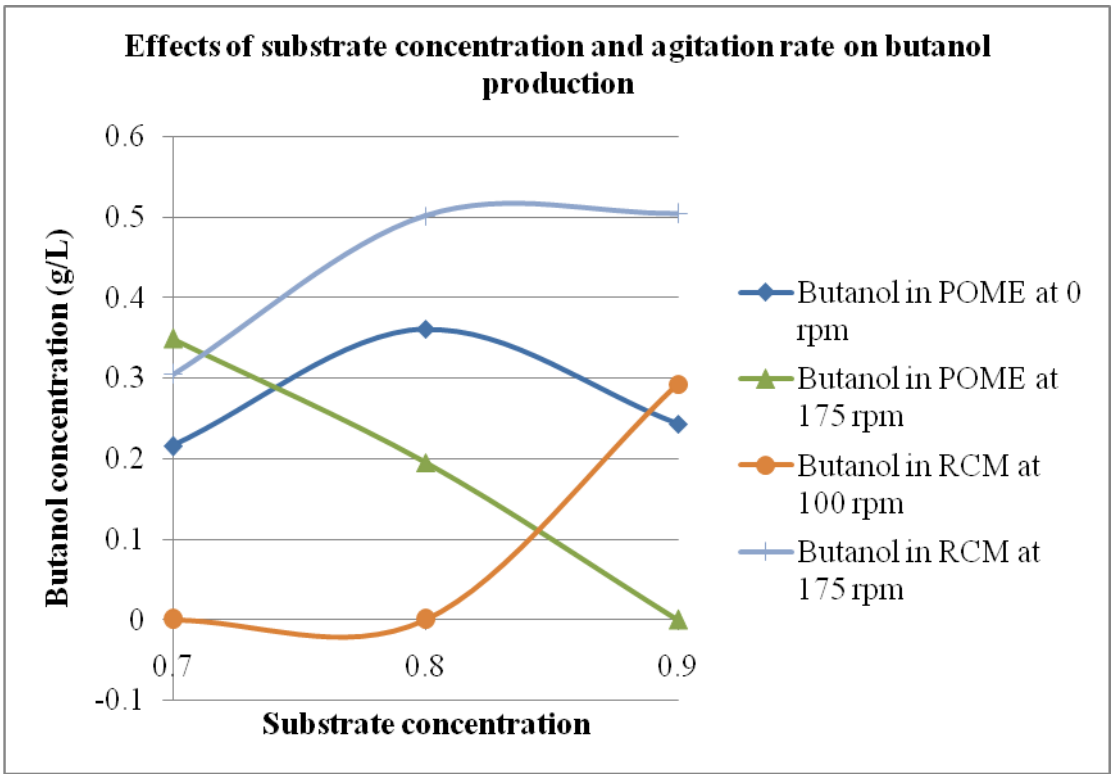

Figure 4.9 Effects of substrate concentration and agitation rate on butanol production

Table 4.9 shows the overall values of butanol produced from POME and RCM after fermentation process. It can be observed that most of the butanol is produced from POME compared to RCM. Other than that, butanol production most

likely to occur at 175 rpm compared to other agitation rate and each substrate concentration managed to produce butanol. Maximum butanol production is 0.5034 g/L which detected in 90% RCM at 175 rpm and the highest value of butanol produced by POME is 0.3485 g/L in 70% v/v at 175 rpm. Lowest butanol produced in RCM is 0.2923 g/L at 90% substrate concentration using 100 rpm and 0.1952 g/L for POME at 80% substrate concentration using 175 rpm. There is no butanol production for POME at 100 and 250 rpm. Meanwhile, for RCM, no butanol produced at 0 and 250 rpm. Three main factors are identified to cause low butanol production and no butanol production at all which are severe product inhibition, strain degeneration and contamination.

Figure 4.9 shows the effects of both parameters, substrate concentration and agitation rate, on butanol production. It clearly shows butanol produced by RCM at 100 and 175 rpm increased compared to butanol produced by POME which shows a decline. Substrate concentration at 70% produced highest butanol in POME at 175 rpm while substrate concentration at 80% and 90% produced highest butanol in RCM at 175 rpm.

In butanol production, solventogenic clostridia produce two major types of products, solvent (acetone, butanol and ethanol) and gases (carbon dioxide and hydrogen), and one major type of fermentation intermediate product, organic acids (acetic and butyric acid). More butyric acid than acetic acid is produced both during acidogenic (exponential growth phase) and solventogenic (stationary growth phase). Butyric acid is quickly re-assimilated for butanol production during the solventogenic phase (Ezeji *et al.*, 2010).

Kalil *et al.* (2003) found out that *C. acetobutylicum* produced highest total ABE in 90% v/v particulate fraction of raw POME after 48 hours of fermentation at an initial pH of 5.8 compared to POME 70% and POME 80%. It means that sedimented POME at 90% concentration is suitable for butanol production by fermenting *Clostridia*. This is due to sedimentation of POME helped to remove traces of oil and soluble toxic substances leaving less inhibitory POME which is more suitable for growth of *Clostridia.* With reduction of water content, sedimented

POME contains higher concentrations of lignocellulose and other insoluble materials which supported growth of *C. acetobutylicum*. However, this study supported that the highest butanol production (0.5034 g/L) occurs from RCM 90% not from POME. This might be due to the use of glucose derived from POME instead of the whole POME as the medium.

A study from Andrade and Vasconcelos (2003) used the operating condition for agitation rate is 200 rpm in continuous cultures of *C. acetobutylicum* to produce acetone, butanol and ethanol. Another study by Takriff *et al.* (2009) attributed that fermentation process requires mixing to enhance substrate interface with the bacteria to produce high yield and in the absence of mixing, the various of components of POME which made-up the substrate for fermentation will sediment to the bottom of the fermenter thus lowering the bacteria-substrate interface. Combination of gentle mixing and excellent heat and mass transport provide a means for efficient mixing in the utilisation of POME as fermentation media. Both studies clarify that the suitable agitation rate for mixing in order to enhance substrate interfaces with bacteria is 200 rpm which lead to high yield of butanol. Lower agitation rate than 200 rpm such as 0 and 100 rpm could cause mixture not mixed well or no contact at all between bacteria and substrate. However, higher than 200 rpm such as 250 rpm could cause cell to burst and die due to too high speed. Hence, no fermentation process could occur if agitation rate used is out of range. From this study explains that no butanol production occurs at 250 rpm because of its speed is too high. Butanol are most likely to produce at 175 rpm due to its speed is almost near to 200 rpm and make it the most favourable agitation rate in this study.

However, as mentioned before, the main reason for no production of butanol at 250 rpm is not due to high agitation rate as butanol production is related to glucose consumption. The declining in glucose concentration in glucose consumption analysis clarifies that there is butanol produced. In other words, examination of the results for butanol production at 250 rpm showed that no butanol detected after 72 hours even though glucose consumption was relatively decreased.

Therefore, the primary limitation of butanol fermentation in this study could be related to butanol toxicity or end-product inhibition to the fermenting bacteria, limits its concentration in the fermentation broth, resulting in low butanol yields. Toxicity is defined here as the inhibition of growth of *C. acetobutylicum.* Ishii *et al.* (1985) confirmed that butanol, which is a main product in fermentation, exhibited a greater toxic effect on *C. acetobutylicum* than did acetone. Bowles and Ellefson (1985) reported that butanol at higher concentration would inhibit the *C. acetobutylicum* growth, would destroy the ability of the cell to maintain the internal pH, lowered the intracellular level of ATP and finally to inhibit the glucose uptake. These statements not only explains no butanol produced at 250 rpm, it also explains no butanol production at 0 rpm for RCM; 100 rpm for POME; 70% and 80% of substrate concentration in RCM at 100 rpm; and 90% of substrate concentration in POME at 175 rpm. There is probability that butanol was produced during fermentation process. However, due to butanol toxicity, butanol produced at a time would inhibit the production of butanol for the afterward.

Previous study by Kalil *et al.* (2003) reported that sedimented POME at 90% concentration is suitable for solvent production by *C. acetobutylicum* as it produced the maximum value of butanol. On the other hand, in opposite from that, this study reports that the highest butanol concentration is produced at 90% concentration of RCM while the maximum butanol produced by POME is at 70% concentration. Fresh POME to be used as fermentation medium could affect the results in butanol production. This is because sample was collected at different times of production. Wu *et al.* (2010) reported that normally, the characteristics of POME may vary considerably for different batches, days and factories, depending on the processing techniques and the age or type of fruit as well as the discharge limit of the factory, climate and condition of the palm oil processing. Seasonal oil palm cropping, activities of the palm oil mill (such as occasional public holidays, closure of the mill, operation and quality control of individual mills) will also influence the quality and quantity of the discharged POME which in turn affect the biological treatment process of POME.

Contamination probably occurs during fermentation even though extra precaution steps are taken. Bacteria probably died at the end of fermentation process due to inefficient of nitrogen supply to eliminate oxygen. Oxygen is toxic to anaerobe bacteria. Other than that, there is possibility that bacteria was infected which makes its growth was impossible. According to Qureshi *et al.* (2001), the presence of inactive or dead cells, accumulation of inhibitory macromolecules (like polysaccharides) and deficiency of nutrients were possible reasons to this phenomenon.

pH in fermentation process also can be another factor that contributes to low product concentration. Van Ginkel and Logan (2005b) mentioned that the concentrations of the undissociated forms of acetic or butyric acid are ten times greater at a pH of 4.5 than at pH of 5.5 and thus higher amounts of the undissociated form are present at the lower pH to cause inhibition. Tashiro *et al.* (2004) confirmed that growth pattern in bacteria influences pH value. Accumulation of acetic and butyric acids during acidogenesis results in a decrease in the pH while reutilisation of these organic acids during solventogenesis results in a pH increase of the broth. Initial pH used in this study is 5.8 for POME and 6.8 for RCM.

Strain used throughout this study was considered as secondary strain because it has been used for previous studies. Hence, strain degeneration occurring upon subculturing and inoculum preparation in batch fermentation when cultures are transferred in a stage of rapid growth and acid production (Barbeau *et al.*, 1988). There is improvement in sugar utilisation, solvent concentration and numbers of cell at initial subculture of bacteria. Unfortunately, Gapes *et al.* (1983) attributed that after third subculture, degeneration was rapid and by the time reached at sixth, no solvent was detected.

Another factor that contributes to low yield of butanol is a sudden cessation of butanol production due to an apparent failure of a “switch” from acidogenic to solventogenic culture, a phenomenon known as “acid crash”, which occasionally occurs in pH-uncontrolled batch fermentations (Ezeji *et al.*, 2004). It is proposed that “acid crash” occurs when the acid concentration in the fermentation broth exceeds

the maximum tolerable limit, causing cessation of glucose uptake and rapid termination of solventogenesis after the switch has occurred. In experiments where "acid crash" occurred, the pH value was less than 5.0. Therefore, "acid crash" may not be the real cause for the cessation of butanol fermentation but may have contributed to the premature termination of the fermentation because of high amount of butyric acid, which is more toxic than acetic acid (Ezeji *et al.*, 2004).

4.4 Glucose Consumption Analysis

Fermentation process was carried out in Schott bottles at working volume of 150 mL. Glucose consumption was studied in order to determine the glucose uptake by bacteria throughout fermentation process. The results of glucose consumption are analysed every 18 hour period of fermentation time.

4.4.1 Effects of Substrate Concentration on Glucose Consumption

4.4.1.1 Effects of Substrate Concentration at 0 rpm

Table 4.10 Glucose consumption of 18 hour period in POME and RCM at 0 rpm

Time (h)	Glucose concentration (g/L)					
	POME			RCM		
	70%	80%	90%	70%	80%	90%
18	4.2857	3.5924	7.0798	0.4107	0.5809	0.6807
36	3.4384	5.5777	5.9944	0.3204	0.6208	0.5196
54	3.2038	3.9496	5.8403	0.2542	0.5420	0.4664
72	2.6576	3.4349	5.6933	0.1943	0.44333	0.4191

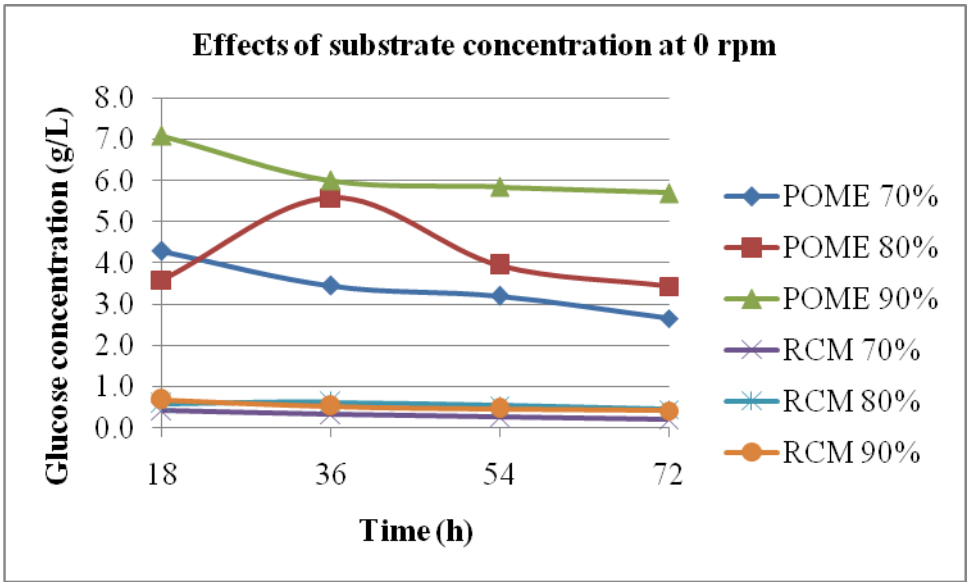

Figure 4.10 Glucose consumption of 18 hour period in POME and RCM at 0 rpm

Table and Figure 4.10 showed the value of glucose was much higher in the POME medium compared to RCM while the consumption of glucose was decreasing throughout the fermentation. The highest glucose was found to be as 7.0798 g/L for POME and 0.6807 g/L for RCM. Both values at substrate concentration of 90%. These values dropped to 5.6933 g/L and 0.4191 g/L respectively after 72 hours of fermentation.

4.4.1.2 Effects of Substrate Concentration at 100 rpm

Table 4.11 Glucose consumption of 18 hour period in POME and RCM at 100 rpm

Time (h)	Glucose concentration (g/L)					
	POME			RCM		
	70%	80%	90%	70%	80%	90%
18	3.4139	7.7731	8.6239	0.2910	0.2584	0.3718
36	3.2668	5.3466	6.4496	0.2752	0.2626	0.3298
54	3.2248	5.5567	6.4286	0.2384	0.2447	0.3393
72	3.1197	5.1576	6.1134	0.1838	0.2122	0.3172

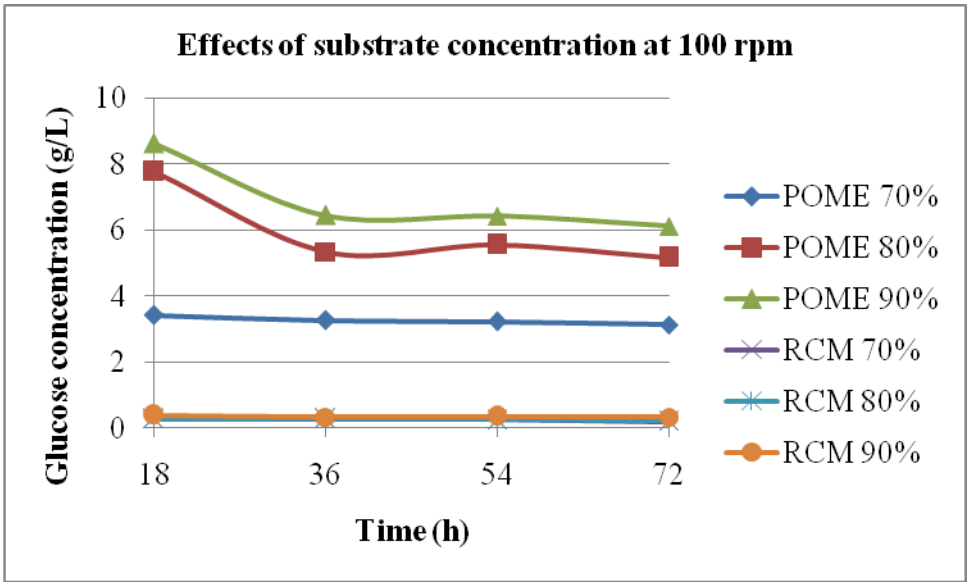

Figure 4.11 Glucose consumption of 18 hour period in POME and RCM at 100 rpm

In Table and Figure 4.11, it can be observed that values of glucose in POME are higher compared to RCM. The graph shows similar patterns of decreasing values in the glucose concentration throughout time. Glucose concentration in POME is higher (8.6239 g/L) compared to RCM (0.3718 g/L) at substrate concentration of 90%. Obtained results showed that lowest values of glucose in both POME and RCM are at 70% substrate concentration.

4.4.1.3 Effects of Substrate Concentration at 175 rpm

Table 4.12 Glucose consumption of 18 hour period in POME and RCM at 175 rpm

Time (h)	Glucose concentration (g/L)					
	POME			RCM		
	70%	80%	90%	70%	80%	90%
18	20.9979	24.7374	25.0525	0.2174	0.3445	0.3487
36	10.4832	17.3529	18.8761	0.1922	0.2405	0.3046
54	9.4433	16.9223	18.1618	0.1796	0.2321	0.2794
72	9.2857	16.1765	17.0693	0.1702	0.2143	0.2511

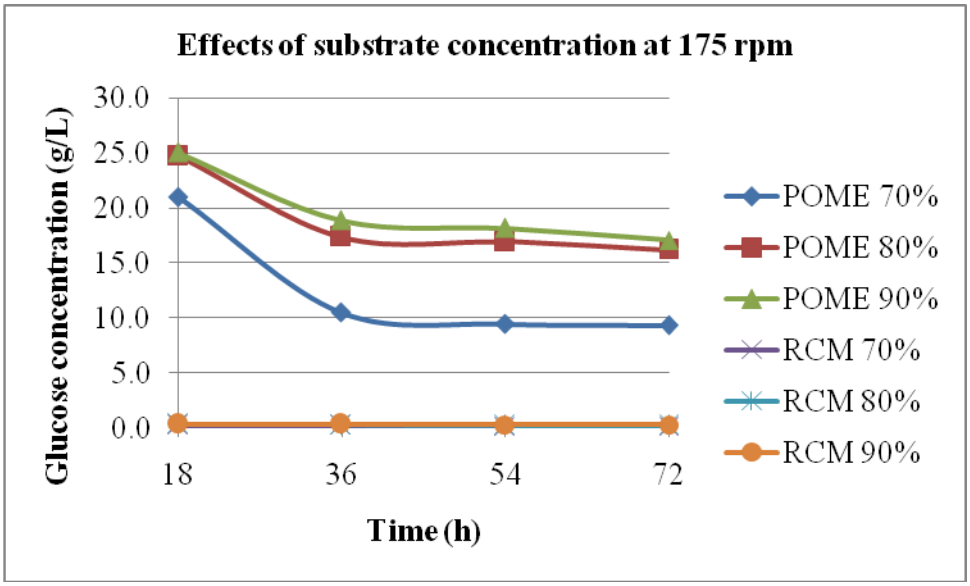

Figure 4.12 Glucose consumption of 18 hour period in POME and RCM at 175 rpm

Table and Figure 4.12 show a similar graph in which the glucose values in POME are higher compared to RCM. For the highest glucose concentration in POME 90%, the initial glucose values decrease from 25.052 g/L to 17.0693 g/L. Meanwhile, for RCM, highest glucose values decrease from 0.3487 g/L to 0.2511 g/L.

4.4.1.4 Effects of Substrate Concentration at 250 rpm

Table 4.13 Glucose consumption of 18 hour period in POME and RCM at 250 rpm

Time (h)	Glucose concentration (g/L)					
	POME			RCM		
	70%	80%	90%	70%	80%	90%
18	9.7059	12.2059	13.4769	0.8256	1.3845	1.4370
36	8.5084	12.0903	13.2983	0.8036	1.3740	1.3109
54	9.6744	7.0378	8.5504	0.6408	0.7269	0.8025
72	8.7815	6.5651	7.0483	0.5683	0.6502	0.7311

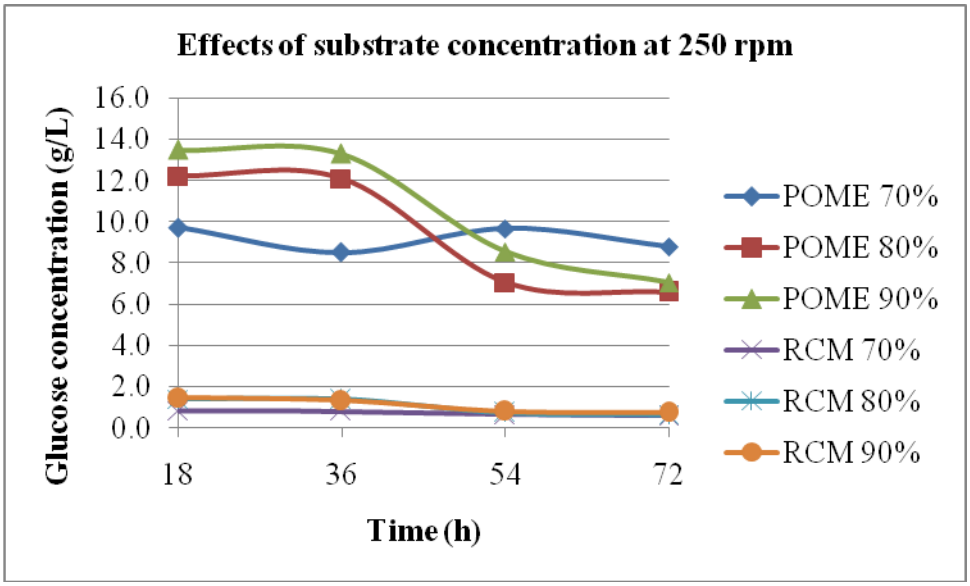

Figure 4.13 Glucose consumption of 18 hour period in POME and RCM at 250 rpm

Table and Figure 4.13 also showed the dropped values from higher glucose concentration to lower glucose concentration. POME at substrate concentration of 90% has the highest glucose values compared to others. Its initial glucose (13.4769 g/L) dropped to 7.0483 g/L. On the other hand, maximum glucose value in RCM is 1.4370 g/L. This value decreases to 0.7311 g/L at the end of fermentation time.

In general, Tables and Figures 4.10, 4.11, 4.12 and 4.13 shows the same patterns which are declining in glucose concentration by time throughout fermentation. Each tables and figures show the values of glucose consumed by *C. acetobutylicum* to produce butanol in POME and RCM. This condition happened due to glucose was utilised in fermentation process as a nutrient or food to bacteria. Therefore, it concludes that fermentation occurs.

In overall, at agitation rates of 0, 100, 175 and 250 rpm, the highest glucose concentration were produced at 90% POME whiles the lowest glucose concentration in 70% RCM. Maximum glucose consumed for POME is 25.0525 g/L in 90% substrate concentration at 175 rpm and 1.4370 g/L in 90% RCM at 250 rpm. It also can be concluded that at higher substrate concentration, bacteria consumed higher amounts of glucose by time but yields low glucose concentration.

4.4.2 Effects of Agitation Rate on Glucose Consumption

4.4.2.1 Effects on Agitation Rate at 70%

Table 4.14 Glucose consumption of 18 hour period in POME and RCM at 70%

Time (h)	Glucose concentration (g/L)							
	POME				RCM			
	0 rpm	100 rpm	175 rpm	250 rpm	0 rpm	100 rpm	175 rpm	250 rpm
18	4.2857	3.4139	20.9979	9.7059	0.4107	0.2910	0.2174	1.4370
36	3.4384	3.2668	10.4832	8.5084	0.3204	0.2752	0.1922	1.3109
54	3.2038	3.2248	9.4433	9.6744	0.2542	0.2384	0.1796	0.8025
72	2.6576	3.1197	9.2857	8.7815	0.1943	0.1838	0.1702	0.7311

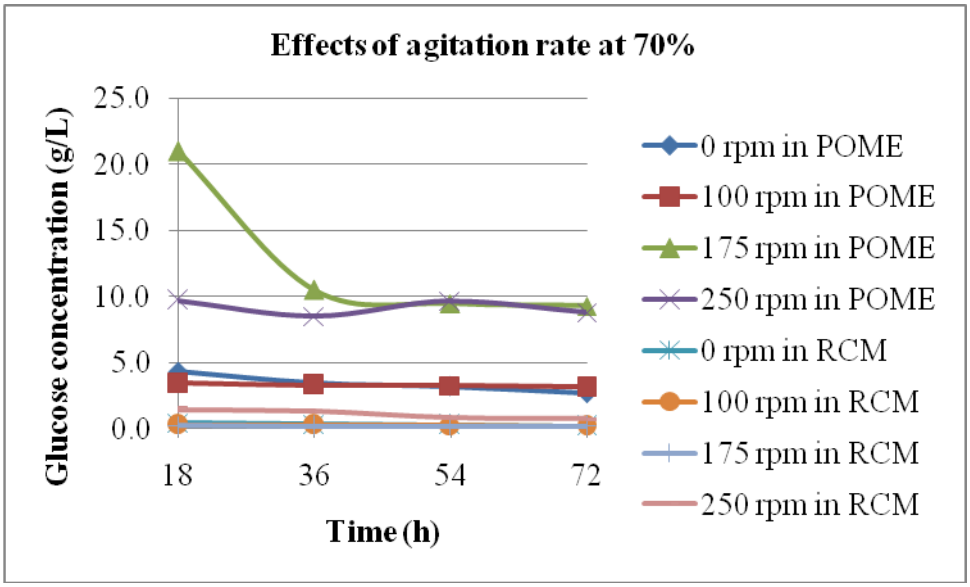

Figure 4.14 Glucose consumption of 18 hour period in POME and RCM at 70%

Table and Figure 4.14 show the identical pattern in every glucose concentration graph which is glucose concentration decreases by time. Maximum glucose concentration is produced at 175 rpm in POME (20.9979 g/L) in this substrate concentration. In opposite, as for RCM, its highest glucose value is produced at 250 rpm (1.4370 g/L).

4.4.2.2 Effects on Agitation Rate at 80%

Table 4.15 Glucose consumption of 18 hour period in POME and RCM at 80%

Time (h)	Glucose concentration (g/L)							
	POME				RCM			
	0 rpm	100 rpm	175 rpm	250 rpm	0 rpm	100 rpm	175 rpm	250 rpm
18	3.5924	7.7731	24.7374	12.2059	0.5809	0.2584	0.3445	1.3845
36	5.5777	5.3466	17.3529	12.0903	0.6208	0.2626	0.2405	1.3739
54	3.9496	5.5567	16.9223	7.0378	0.5420	0.2447	0.2321	0.7269
72	3.4349	5.1576	16.1765	6.5651	0.4433	0.2122	0.2143	0.6502

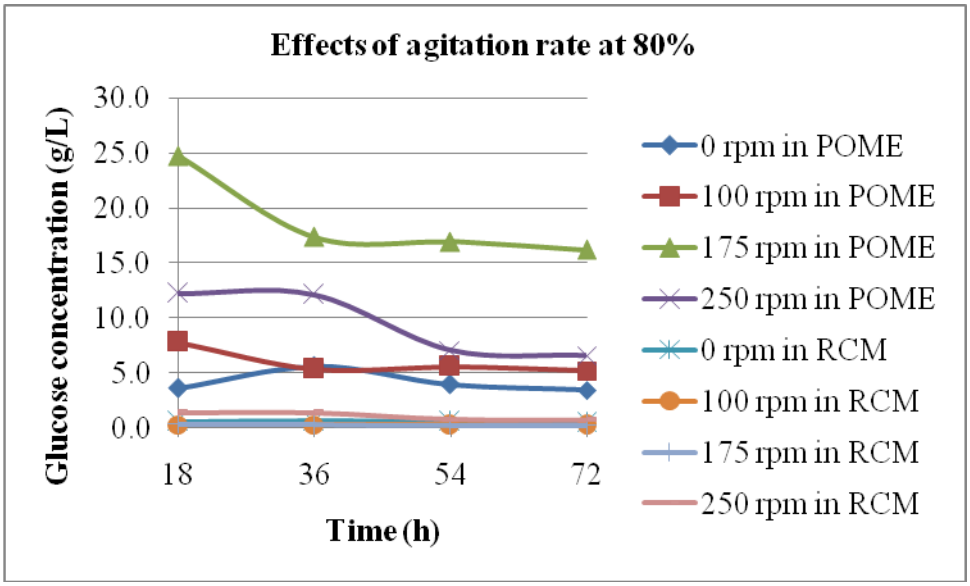

Figure 4.15 Glucose consumption of 18 hour period in POME and RCM at 80%

From Table 4.14 and Figure 4.14, it can be observed that the maximum glucose is produced at 175 rpm from POME with value of 12.2059 g/L while for RCM, highest glucose concentration is produced with value of 1.3845 g/L at 250 rpm.

4.4.2.3 Effects of Agitation Rate at 90%

Table 4.16 Glucose consumption of 18 hour period in POME and RCM at 90%

Time (h)	Glucose concentration (g/L)							
	POME				RCM			
	0 rpm	100 rpm	175 rpm	250 rpm	0 rpm	100 rpm	175 rpm	250 rpm
18	7.0798	8.6239	25.0525	13.4769	0.6807	0.3718	0.3487	1.4370
36	5.9944	6.4496	18.8761	13.2983	0.5196	0.3298	0.3046	1.3109
54	5.8403	6.4286	18.1618	8.5504	0.4664	0.3393	0.2794	0.8025
72	5.6933	6.1134	17.0693	7.0483	0.4191	0.3172	0.2511	0.7311

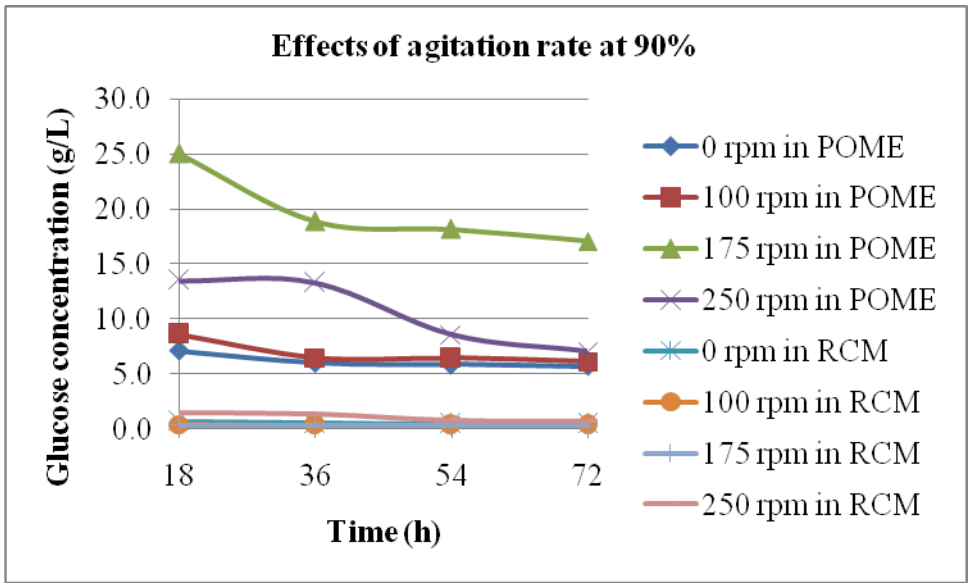

Figure 4.16 Glucose consumption of 18 hour period in POME and RCM at 90%

From Tables and Figures 4.14, 4.15 and 4.16, it can be observed that glucose concentration decreases by time. These tables and figures also show that maximum glucose concentration was produced at 175 rpm in POME. Obtained results show that the lowest glucose concentration is produced at 100 and 175 rpm in RCM. Glucose concentration increases as the agitation rate increases but after reached at optimal agitation rate which is 175 rpm, glucose concentration declines. Produced butanol affects inversely on the glucose metabolism.

Glucose is fermented via the Embden-Meyerhof-Parnas pathway to pyruvate (Ezeji *et al.,* 2010). Glucose consumption also plays an important role in acidogenesis and solventogenesis. During acidogenic or active growth phase, glucose concentration decreased owing to its consumption for intermediate acetic acid and butyric acid production. At the end of fermentation, all glucose values were dropped from its initial. This result indicates that glucose was used during fermentation to produce butanol. In other words, butanol produced throughout fermentation. During solventogenic phase, the acetic acid and butyric acid were consumed concomitantly with glucose to produce the butanol.

Obtained results also indicate that the fermentation was subject to product (butanol and organic acids) inhibition. Alshiyab *et al.* (2008) mentioned that it has been reported that butanol inhibition gets predominant at higher glucose concentration because this modifies the metabolic pathway. Further increased of substrate was shown to enforce the bacteria to make shift from acid phase to solvent phase and stop the first metabolites production, suggested that lower glucose concentration was necessary for maximum bacterial productivity.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Results from this study show that fermentation process consumed glucose in order to produce butanol. Growth profile is important to determine the period of when production of butanol and consumption of glucose happened.

Based on the experiments, it can be concluded that POME has considerable potential as a substrate for butanol fermentation and such fermentation can help to control pollution by POME. Furthermore, POME requires mixing to enhance substrate interface with the bacteria in order to produce high yield of butanol.

From the analysis of glucose consumption, it can be concluded that butanol production in this anaerobic fermentation process is accompanied by the breakdown of glucose. Glucose is used as 'food' by *C. acetobutylicum* for its growth. It also plays its role in acidogenic and solventogenic phases during growth of *C. acetobutylicum.*

Taken as a whole from obtained results, the highest butanol yield in POME was 0.3485 g/L at 70% concentration and 175 rpm while maximum butanol production was produced in 90% RCM at 175 rpm which was 0.5034 g/L. However, previous studies reported that butanol would produce highest in POME at substrate concentration of 90% and agitation rate of 200 rpm (≈175 rpm). It can be concluded

butanol production decreased during fermentation and many factors can be considered to contribute in this results.

5.2 Recommendations

Butanol is the center of attention in this study because it is the desired end product of fermentation. However, it gives effects in the fermentation process on *C. acetobutylicum.* Despite from that, POME as the fermentation medium also contributes in the low yield of butanol. Therefore, several practices and factors should be considered to improve the fermentation performance.

Wu *et al.* (2009) reported that Suwandi (1991) and Wu *et al.* (2006b) proposed that the possibility of recovering and concentrating the availability of bioresources in POME by an ultrafiltration process in order for the concentrated bioresources to be reused more effectively as fermentation media.

Hipolito *et al.* (2008) proposed that nitrogen sparging shortened the lag phase and improved solvent yield. Hence, besides eliminating oxygen in fermentation process, nitrogen also can help in altering the growth of *C. acetobutylicum*.

A number of studies by Evans and Wang (1998), Durre (1998), Keis *et al*. (2001), Andrade and Vasconcelos (2003) and Ezeji *et al.* (2007; 2010) proposed that genetic strain improvement or modification for overcoming butanol toxicity. Butanol toxicity results in a lower butanol concentration and negatively impacts fermentation time, productivity, and yield when compared to the ethanol fermentation.

Ezeji *et al.* (2010) attributed that development of butanol-tolerant strains to ameliorate butanol toxicity has typically followed one of two approaches: (1) enhancement of butanol toxicity defenses in solventogenic clostridia and (2) metabolic engineering of well-characterized microorganisms (*E. coli* and *S.*

cerevisiae) for ABE production. The recent availability of genomic sequence information for two of the solvent-producing clostridia, namely, *C. acetobutylicum* ATCC 824 and *C. beijerinckii* 8052, enables global strategies for improving resistance of these microbes to butanol and acetone. These strategies may involve the use of gene expression microarrays as well as various synthetic biology-based approaches. Additionally, investigations with the aim of improving ABE tolerance and productivity are ongoing for the identification of solvent-resistant microorganisms as alternative solvent production hosts.

Maddox *et al.* (1995) suggested that continuous product removal technique can be used so that inhibitory product concentrations are never reached. Other than that, product recovery using liquid – liquid extraction with oleyl alcohol is the most suitable for ABE recovery from POME-ABE solution (Takriff *et al.*, 2008). This is because more energy efficient recovery process is desired and liquid – liquid extraction appears to be particularly promising compared conventional distillation process, centrifugation and filtration due to its high energy consumption for product purification.

In this study, it is wise to subculture *C. acetobutylicum* less than three times because more than that can cause strain degeneration. Subculturing is a method that can increase and improve the sugar utilization, butanol concentration and cell numbers. However, repeating of subculturing will degenerate the bacteria and until one stage, it will not be effective in fermentation.

Lastly, lots of hard work and precaution steps need to be taken in order to conduct this fermentation under sterile condition. This is because contamination gives severe effects to bacteria and bacteria probably died at the end of fermentation process due to inefficient of nitrogen supply to eliminate oxygen. Oxygen is toxic to anaerobe bacteria. Other than that, there is possibility that bacteria was infected which makes its growth was impossible.

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APPENDIX A

STANDARD FOR BUTANOL

Table A 1 Concentration of butanol versus are using GCFID

Concentration (g/L)	Area
1.0	197.7136
2.0	397.1444
3.0	614.6014
4.0	839.517
5.0	990.3093

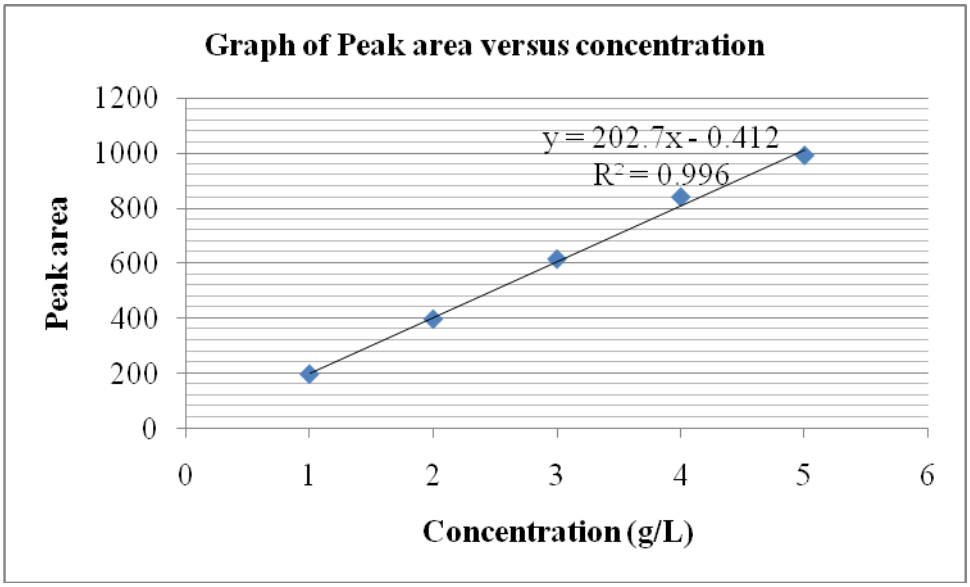

Figure A 1 Standard curve for butanol

APPENDIX B

STANDARD FOR GLUCOSE CONSUMPTION

Table B 1 Standard for glucose consumption

Concentration (g/L)	Optical density
0	0
0.2	0.092
0.4	0.368
0.6	0.524
0.8	0.71

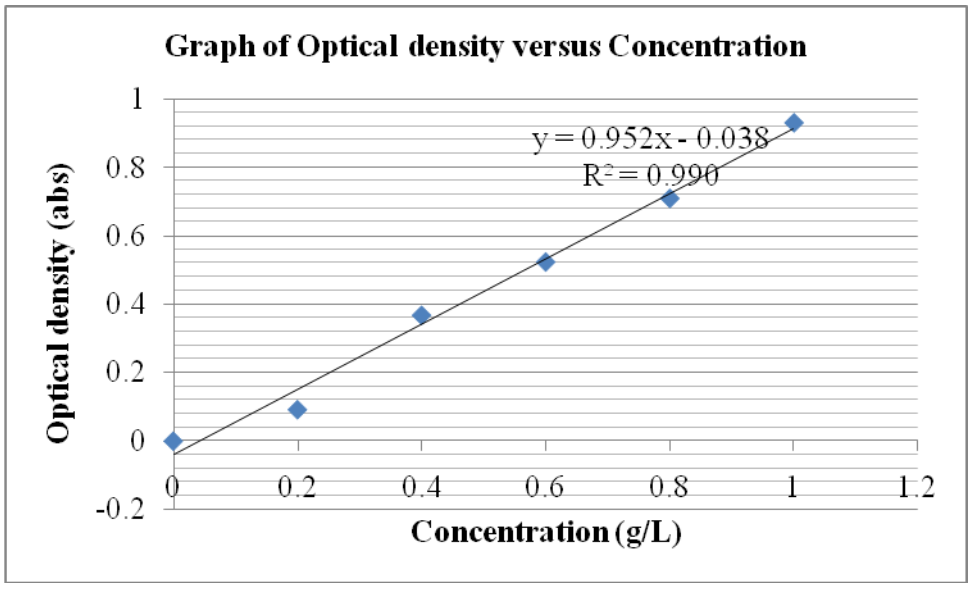

Figure B 1 Standard curve for concentration of glucose consumption