EFFECT OF AGITATION RATE ON BUTANOL AND ETHANOL PRODUCTION FROM PALM OIL MILL EFFLUENT BY *CLOSTRIDIUM ACETOBUTYLICUM*

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

Faculty of Chemical & Natural Resources Engineering
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I declare that this thesis entitled "Effect of agitation rate on butanol and ethanol production from palm oil mill effluent by *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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Date : 27 April 2010

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

For all your care, support and believe in me.

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ABSTRACT

Malaysia is world largest palm oil producing country. The crude palm oil mill releases liquid effluent known as palm oil mill effluent (POME). These waste result in a significant environmental problem if not dispose in proper manner. The objective of this research is to study the effect of agitation rate on butanol and ethanol production from palm oil mill effluent by *Clostridium acetobutylicum.* This study also investigated the type of sugar that contain in POME, the profile growth rate of *C.acetobutylicum* and then to investigate consumption of glucose by *C. acetobutylicum* during fermentation. The experiment works were conducted in schott bottle in anaerobic chamber to maintain anaerobic condition. Fermentation were carried out for 72 hours at 35^{o}C and maintain the concentration of POME (90%) and inoculums concentration (10%) using palm oil mill effluent and reinforced clostridia medium as a substrate in batch culture. The speed that used in this investigation are, 100, 150, 175 and 200 rpm. The result showed that the yield of butanol and ethanol production will increase as the speed increase until it reaches the optimum point (200 rpm). From this study, it was observed that optimum condition for butanol and ethanol fermentation by *C. acetobutylicum* is at 200 rpm. The concentration of butanol and ethanol from POME for 200 rpm at optimum time (72 hours) is 0.137 g/l and 68.958 g/l respectively. The result of this experiment showed that fresh POME is a viable media for butanol and ethanol fermentation by *C. acetobutylicum*

ABSTRAK

Malaysia adalah sebuah negara pengeluar minyak kelapa sawit yang terbesar. Minyak kelapa sawit yang mentah akan menghasilkan cecair yang dipanggil POME. Cecair ini perlu dirawat untuk mengelakan daripada berlakunya masalah persekitaran. Objektif kajian ini dilakukan bertujuan untuk mengkaji kesan halaju adukkan untuk menghasilkan butanol dan ethanol melalui proses fermentasi menggunakan mikroorganisma yang dikenali sebagai *Clostridia acetobutylicum* dengan menggunakan POME sebagai medium. Kajian juga dilalukan untuk mengetahui kandungan gula dan jenis gula yang terdapat dalam POME, untuk melihat kadar pertumbuhan *C.acetobutylicum* dan mengkaji penggunaan glukosa oleh *C.acetobutylicum* semasa fermentasi. Ujikaji makmal telah dijalankan dalam schott botol dan proses dilakukan dalam 'anaerobic chamber' untuk memastikan gas oksigen tiada semasa proses fermentasi. Fermentasi ini dilakukan selama 72 jam dan pada suhu 35^{o}C dengan kepekatan POME adalah 90% dan kepekatan inoculums adalah 10% menggunakan POME dan RCM. Halaju adukkan yang hendak dikaji adalah 100, 150, 175 dan 200 rpm. Keputusan kajian menunjukkan kandungan butanol dan ethanol akan meningkat sekiranya halaju adukkan meningkat. Daripada kajian ini, didapati keadaan optimum untuk fermentasi butanol dan ethanol oleh *C.acetobutylicum* adalah pada 200 rpm. Daripada keputusan experimen, penghasilan butanol dan ethanol pada 200 rpm dan pada masa optimum (72 jam) adalah 0.137 g/l dan 68.958 g/l. Keputusan kajian ini menunjukkan POME adalah media tumbesaran yang sesuai bagi fermentasi butanol dan ethanol.

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LIST OF SYMBOLS/ABBREVIATIONS

CO_2	= Carbon dioxide
C	= Carbon
CO	= Carbon monoxide
^oC	= Degree Celcius
DNS	= DiNitroSalicylic acid
GC-FID	= Gas chromatography with flame ionization detector
g	= gram
H_2	= Hydrogen
hr	= Hour
HPLC	= High performance liquid chromatography
ml	= mililitre
N_2	= Nitrogen gas
O_2	= Oxygen
OD	= Optical density
POME	= Palm Oil mill effluent
µm	= micrometer
µl	= micro liter
UV-Vis	= Ultra violet visible
vol %	= Percentage of volume

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

Malaysia is the world largest producer of palm oil. Its production generates various wastes chief among which is palm oil mill effluent (POME). POME is generated from three major sources such as sterilizer condensate, separator sludge and hydrocyclone operation where the broken shells are separated from kernels. POME consist of various suspended component including cell wall, organelles, and short fibers, a spectrum of carbohydrates ranging from hemicelluloses to simple sugar, a range of nitrogenous compound from protein to amino acid and free organic acid (Takriff *et al*.,2009). POME has an extremely high content of degradable organic matter, which is due in part to the presence of unrecovered palm oil, thus, POME should be treated before discharge to avoid serious environmental pollution (Okwute *et al*., 2007).

The acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fermentations and one of the largest fermentation processes ever developed in industry. However, since the 1950's industrial ABE fermentation has declined continuously, and almost all butanol is now produced via

petrochemical routes. Production of acetone, butanol and ethanol are important in industrial chemical, it can be produced by fermentation of carbohydrates using various solventogenic clostridia. In a typical ABE fermentation, butyric and acetic acid are produced first by *C. acetobutylicum*, the culture then undergoes a metabolic shift and solvent (butanol, acetone and ethanol) are formed Current butanol prices as a chemical are at $3.75 per gallon, with a worldwide market of 370 million gallons per year. The market demand is expected to increase dramatically if green butanol can be produced economically from low cost biomass.

Fermentation process using anaerobic microorganisms provide a promising path for converting biomass and agricultural waste into chemical and fuel. However, anaerobic fermentation process for production of fuel and chemical, usually suffer from a number of serious limitations including low yield, low productivity, and low final product concentration (David and Shang, 2004). The high yielding butanol process eliminates production of all ancillary by-products. The butanol's first market is not as fuel but as an industrial solvent where it sells for 2.5 to 3 times the price of gasoline and it is the entry point for Environmental Energy Inc. Butanol has many characteristic that make it a better fuel than ethanol, now used in the formulation of gasohol. Butanol can solve many problems associated with the use of ethanol. Then, butanol has many advantages over ethanol such as butanol is safer than ethanol because of its higher flash point and lover vapor pressure, butanol has a higher octane rating, more miscible with gasoline and diesel fuel but less miscible with water (David and Shang, 2004).

The present study on the butanol and ethanol fermentation has been focused primarily on the effect of agitation rate in batch cultures of strain from *C.acetobutylicum.* To overcome the problems of low productivity and yield of butanol and ethanol, the agitation rate was investigate in the fermentation process. By changing and varying the rate of agitation speed, the optimal conditions for high productivity and butanol yield were investigated.

1.2 Problem Statement

During the early twentieth century, the primary method of butanol production was anaerobic fermentation with *C.acetobutylicum* to produce mixture of acetone, butanol and ethanol. The butanol yields were low at that time.

The fermentation produce ABE was unable to complete economically with petrochemically produce ABE. This resulted in virtual elimination of this fermentation. When the oil price increases, the following factor which severely effect the economic of butanol and ethanol fermentation were identified such as high cost of substrate. To economize the butanol and ethanol production, palm oil mill effluent (POME) has been subjected to be the substrate medium for ABE fermentation.

Malaysia is world largest palm oil producing country. Therefore, a large quantity of waste is produced due to the huge amount of palm oil production. The crude palm oil mill releases liquid effluent known as palm oil mill effluent (POME). These waste result in a significant environmental problem if not dispose in proper manner. The palm oil mill effluent (POME) has great potential as a substrate for ABE fermentation because it contains a mixture of carbohydrate including starch, hemicelluloses, sucrose and other carbohydrates that can be utilized by clostridia. Such utilization would further increase profitability of palm oil industry besides solving an environmental problem.

1.3 Research Objective

The main objective of this project is to study the effect of agitation rate on butanol and ethanol production from palm oil mill effluent by *Clostridium acetobutylicum*

1.4 Research Scope

The main research scopes of this project are:

- To study the growth profile of *C.acetobutylicum* by using palm oil mill effluent (POME) as a medium.
- To complete the composition analysis of selected batch fresh POME by using HPLC
- To study the effect of agitation rate (100 rpm,150 rpm,175 rpm,200 rpm) on the butanol and ethanol production by using fresh POME
- To study the glucose consumption during the fermentation by DNS analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Acetone-butanol-ethanol (ABE) fermentation

2.1.1 Historical Background

Fermentation of acetone-butanol-ethanol (ABE) producing solvents, that is acetone, butanol, and ethanol in various proportions. Since the 1950's industrial ABE fermentation has declined continuously, and almost all butanol is now produced via petrochemical routes. Beginning (as with fuel ethanol) with the oil crisis of the 1970s, renewed interest was evinced in the technology, aided greatly by the accelerating advance of microbial physiology and genetic at that time.

The microbial species capable of this multiproduct biosynthesis are clostridia, which also have remarkable appetites for cellulosic and hemicellulosic polymers, able to metabolize hexose sugars and pentoses. This again parallels the derive to produce ethanol from lignocellulosic biomass substrates. Therefore, when the neologism 'biobutanol' (for n-butanol, C_4H_9OH) appeared, DuPont, Wilmington, Delawere, and British Petroleum are

the companies most associated with the development of butanol as advanced biofuel and which aim to market biobutanol (Qing *et al*., 2005).

The butanol and ethanol, an industrially important chemical, can be produced by fermentation of carbohydrates using various solventogenic clostridia. In fact, this fermentation was commercially viable until after World War II when petrochemically produced butanol and ethanol became available at competitive prices (David and Shang, 2004).

2.1.2 Butanol production

Butanol or butyl alcohol (sometimes also called *biobutanol* when produced biologically), is a primary alcohol with a 4 carbon structure and the molecular formula of C_4H_9OH. It belongs to the higher alcohols and branched-chain alcohols. Butanol is also a potential substitute for fossil fuel and considered a superior fuel to ethanol for several reasons: more favorable physical properties, better economics, and safety.

Butanol is produced by fermentative bacteria including *Clostridium acetobutylicum and Clostridium beijerinkii.* The ratio of acetone, butanol and ethanol (ABE) is 3:6:1, with butanol being the major fermentation byproduct. The solvent production particularly, butanol, take place during the solventogenesis and is directly correlated to the spore-forming ability of culture. Low butanol yield through fermentation couple with cheap petroleum feedstock is the major impediment to the widespread development of butanol fuel. (Man *et al*., 2003).

Table2.1: Properties of Butanol

Common synonyms	n-butanol, butanol, butyl alcohol, 1-butyl acohol, n-butyl alcohol, butan-1-ol
Formula	C_4H_9OH
Physical properties	Form: colourless liquid Stability: Stable Melting point: -89 ^oC Boiling point: 118 ^oC Water solubility: high Specific gravity: 0.81
Principal hazards	*** Butanol is harmful if you swallow or inhale it. *** This material is very flammable
Safe handling	-Wear safety glasses. -Ensure adequate ventilation. -Do not work near a source of ignition. Very flammable chemicals may be ignited by contact with a hot plate or even a hot water pipe - a naked flame is not required.
Emergency	-Eye contact: Immediately flush the eye with plenty of water. If irritation persists call for medical help. -Skin contact: Wash off with water. Remove any contaminated clothing. If the skin reddens or appears damaged, call for medical aid. Be aware that clothes soaked in butanol present a serious fire risk, so ensure that clothes (and anybody in them) are kept well away from sources of ignition.

2.1.3 Ethanol Production

Ethanol, C_2H_5OH, (also called Ethyl Alcohol) is the second member of the aliphatic alcohol series. It is a clear colourless liquid with a pleasant smell. Except for alcoholic beverages, nearly all the ethanol used industrially is a mixture of 95% ethanol and 5% water, which is known simply as 95% alcohol. Although pure ethyl alcohol (known as absolute alcohol) is available, it is much more expensive and is used only when definitely required.

Table 2.2: Properties of Ethanol

Molecular formula	C_2H_6O
Molar mass	46.07 g mol^{-1}
Melting point	−114.3 °C, 159 K, -174 °F
Boiling point	78.4 °C, 352 K, 173 °F
Density	0.789 g/cm^3
Appearance	Colorless liquid
Dipole moment	1.69 D (gas)
Acidity (pK_a)	15.9
Refractive index (n_D)	1.36 (25 °C)

2.1.4 Advantages of Butanol

Butanol has many advantages than ethanol. Butanol has higher energy content than ethanol and can be blended with gasoline at higher concentrations for use in standard vehicle engine. It also suitable for transport in pipelines, butanol has the potential to be introduced into gasoline easily and without additional supply infrastructure.

Then butanol/gasoline mixtures are less susceptible to separate in the present of water than ethanol/gasoline blends, demanding no essential modifications to blending facilities, storage tanks, or retail station pumps. Butanol's low vapor pressure (lower than gasoline) means that vapor pressure specifications do not need to be compromised. Production routes from conventional agricultural feedstock (corn, wheat, sugarcane, beet sugar, cassava, and sorghum) are all possible, supporting global implementation. Lignocellulosics from fast-growing energy crops for example grasses or agricultural waste are also feasible feed stocks (Qing *et al*., 2005).

Beside that it is safer than ethanol because of its higher flash point and lower vapor pressure. The butanol also has a higher octane rating, more miscible with gasoline and diesel fuel but less miscible with water. Butanol has the propensity to solve some infrastructure problems associated with fuel cell use. Dispersed through existing pipelines and filling stations and then reformed onboard the fuel cell vehicle, butanol offers a safer fuel with more hydrogen (Annapurna *et al*., 2009).

2.1.5 Application of Butanol

Butanol has many characteristics that make it a better fuel than ethanol, now used in the formulation of gasohol. Beside that, butanol's application as a replacement for gasoline will outpace ethanol, biodiesel and hydrogen when it safety. The butanol's application for the Department of Defense as a clean-safe replacement for batteries when used in conjunction with fuel cell technology is seen as an application for the future. The butanol also used to generate electricity for computers, night vision and stealth equipment. Many new uses will occur in these field as ''green'' butanol became available to the market. Others uses include current industrial application in solvent (paint thinner), rubber monomers and break fluids (Man *et al*., 2003).

2.2 Anaerobic Fermentation

Anaerobic processes are defined as a biological process in which organic matter is metabolized in an environmental free of dissolved oxygen or it precursors. In an anaerobic fermentation, organic matter is catabolized in the absence of an external electron acceptor by strict or facultative anaerobes through internally balanced oxidation-reduction reaction under dark conditions. The product generated during the process accepts the electrons released during the breakdown of organic matter. Thus, organic matter acts as both electron donor and acceptor. In fermentation the substrate only partially oxidized, and therefore, only a small amount of the energy stored in the substrate is conserved. The major portion of the adenosine triphosphate (ATP) or energy is generated by substrate-level phosphorylation (Man *et al*., 2003).

2.3 *Clostridia*

Butanol (and acetone, ethanol, and isopropanol) are naturally formed by a number of clostridia. In addition, clostridia can produce chiral products which are difficult to make by chemical synthesis and degrade a number of toxic chemicals. 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. Complex nitrogen sources such as yeast extract are generally required for good growth and solvent production, but otherwise the nutrient requirements for the growth of clostridia are rather simple. The clostridia require high redox potential to produce butanol and ethanol and the supply of additional reducing power results in increased butanol and ethanol formation with reduced acetone formation (Mitchell, 1998).

A typical feature of the clostridial solvent production is biphasic fermentation. The first phase is the acidogenic phase, during which the acids forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products. This acidogenic phase usually occurs during the exponential growth phase. The second phase is the solventogenic phase during which acids are reassimilated and used in the production of acetone, butanol and ethanol. The transition from acidogenic to solventogenic phase is the result of a dramatic change in gene expression pattern. Solventogenesis is closely coupled to sporulation. The transcription factor responsible for initiation of sporulation also initiates solvent production in *C. acetobutylicum* by activating transcription of acetoacetate decarboxylase (adc), alcohol dehydrogenase (adhE), and CoA transferase (ctfAB) genes (Sang *et al*., 2008).

For commercial purposes, clostridium species are preferred for butyric acid or butanol production. They can form resistant endospores under harsh environments. These

strains can be isolated from soil, wastewater, animal digestion systems, contaminated dairy products, etc. Optimal cultivation conditions are as follows: 30-37°C, pH 6.5-7.0, an atmosphere of pure CO_2 or N_2, or N_2 and CO_2 in the ratio of 1:9. Common carbon sources include glucose, but lactose from whey, sucrose from molasses, starch, potato wastes, cellulose, and xylose are also utilizable or suitable and commercially interesting. 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. The solventogenic clostridia have received much attention in recent years, because of their ability to produce industrially relevant chemicals such as butanol.

An important advantage of the solventogenic clostridia is the variety of fermentation products (acetone, butanol, ethanol, acetic, butyric, lactic acids, etc.) that can be synthesized by this group of microorganisms (Kalil *et al*., 2009). Anaerobic organisms are averse to air. They are used in biodegradation, breaking down organic chemicals into smaller compound, producing methane and carbon dioxide. Some anaerobic organisms can break down organic chemicals by fermentation. Such organisms are useful at hazardous waste sites. Clostridia are well-known obligatory anaerobic bacteria which cannot utilize oxygen, or otherwise die in oxygenated environments (Kalil *et al*., 2003).

2.3.1 Batch Culture

Batch culture is a closed culture system which contains an initial, limited amount of nutrient. Cell suspension in a system increases in biomass by cell division and cell growth until a nutrient availability becomes limiting. During this period, the inoculated

cell culture will pass through a number of phases. After inoculation there is a period during which it seems that no growth take place. This is a phase referred to as the lag phase and may be considered as a time of adaptation. In a commercial process the length of the lag phase should be reduced as much as possible and this may be achieved by using a suitable inoculums. The next phase is exponential or log phase when the growth rate of the cells gradually increases. The cells grow at the constant, maximum rate.

During the exponential phase nutrients are in excess and the organism is growing at its maximum specific growth rate. During stationary phase, the growth rate slows as a result of nutrient depletion and accumulation of toxic products. This phase is reached as the bacteria begin to exhaust resources that are available to them. This phase is a constant value as the rate of bacterial growth is equal to the rate of bacterial death. At death phase, bacteria run out of nutrients and die. After this phase, the fermentation process is considered to be completed (Nasratun *et al*., 2007)

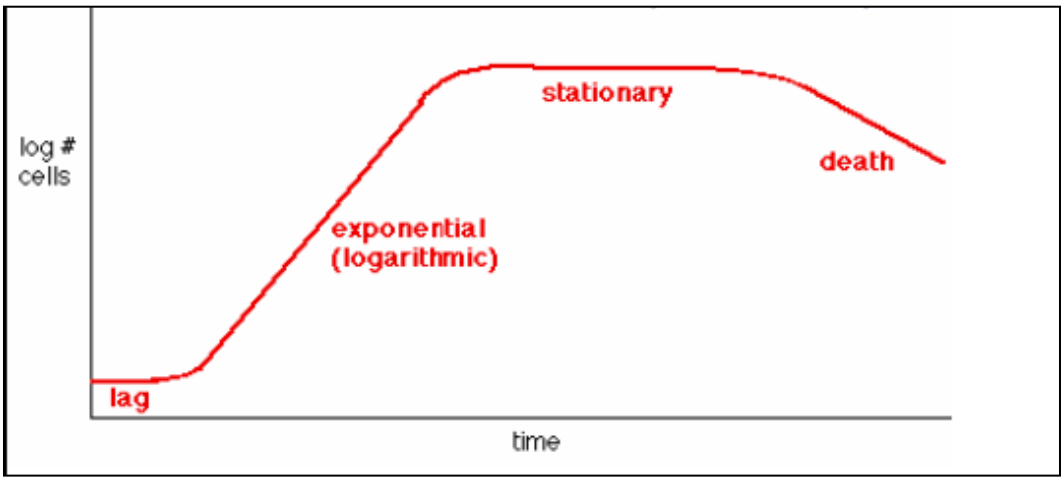

Figure 2.1: Phase of Microbial Growth in Batch Culture

2.3.2 Log phase

When population get a new climate at initial is followed by active growth phase follow rapid divisions called log phase or exponential phase. Then growth gradually comes down either by the use-up of nutrients of toxic metabolic products or some other growth limited factors and finally stops and this period is static and termed stationary phase, and lead the cells die and leads to loss of capacity to reproduce, however death rate differ with organisms and the environment factors. Some bacteria die and consequently few viable cells remain after 72 hours

2.3.3 Exponential Growth Phase

Exponential phase (sometimes called the log phase) is a period characterized by cell doubling. The living bacteria population increases rapidly with time at an exponential growth in numbers, and the growth rate increasing with time. Conditions are optimal for growth.

The number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time.

The actual rate of this growth depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

2.3.4 Stationary Phase

The third phase of bacterial cell growth is called the "stationary phase". With the exhaustion of nutrients and build-up of waste and secondary metabolic products, the growth rate has slowed to the point where the growth rate equals the death rate. Effectively, there is no net growth in the bacteria population. As resources are used, the rate of cell death begins to match the rate of cell division. Thus, the entire colony slows its growth.

The stationary phase is often referred to as a being in a state of equilibrium. This simply means that the colony of bacteria is not getting any bigger or smaller, it is simply living. In addition to limited resources, the build-up of bacterial waste products can limit the growth of a bacterial colony.

2.3.5 Death Phase

The living bacteria population decreases with time, due to a lack of nutrients and toxic metabolic by-products

2.4 Palm Oil Mill Effluent (POME)

Palm oil mill effluent (POME), is produced from production of crude palm oil which involve extraction process where the fresh palm oil fruit bunches undergo sterilization, digestion and extraction of the oil, which is then clarified. POME is just an only negative byproduct that might cause pollution of environment. POME is thick, brownish liquid with the discharged temperature in the range of 80 to 90^{o}C.

In the palm oil mills, POME is generated from three major sources, sterilizer condensate, separator sludge and hydrocyclone operation where the broken shell are separated from kernels. POME consist of various suspended component including cell walls, organelles and short fibers, a spectrum of carbohydrates ranging from hemicelluloses to simple sugar, a range of nitrogenous compound from protein to amino acid and free organic acid (Takriff *et al*., 2009).

POME can be utilised directly as the sole substrate in the anaerobic fermentation of acetone-butanol-ethanol (ABE) and hydrogen by *C. acetobutylicum* in a submerged batch system. Such utilization would further increase profitability of palm oil industry

besides solving an environmental problem. The current treatment technology for POME is biological digestion, which is combination of aerobic and anaerobic ponds.

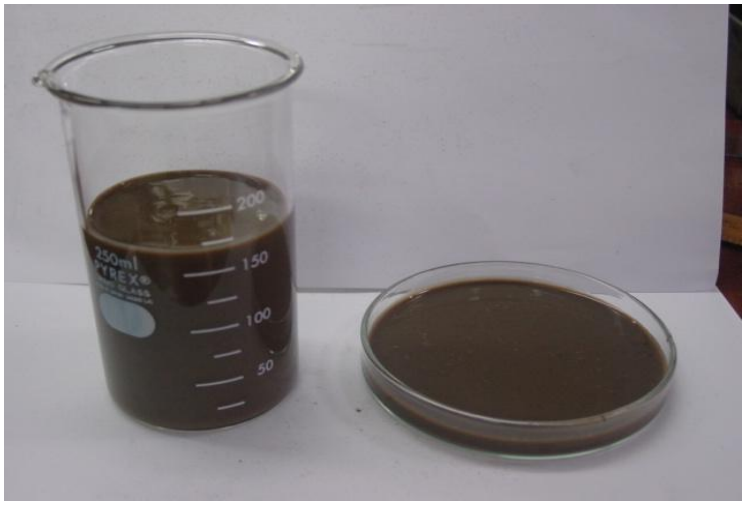

Figure 2.2: Palm Oil Mill Effluent

2.4.1 Characteristic of Palm Oil Mill Effluent

The raw or partially treated POME has an extremely high content of degradable organic matter, which is due in part to the presence of unrecovered palm oil. It consist of 95-96% water, 0.6-0.7% oil and 4-5% total solid, 2-4% suspended solid and extremely high content of degradable organic matter originating from the mixture of a sterilizer condensate, separator sludge, and hydrocyclone wastewater. This highly polluting

wastewater can therefore cause several pollution of water ways due to oxygen depletion and other related effects.

The oil droplet of POME can be found in two phases. They either suspended in the supernatant or float on the upper layer of the suspension. The residue oil droplets in POME were solvent extractable. The effluent is also characterized by high temperature of approximately 80-90^{o}C and acidic. The characteristic of POME are highly dependent on the operation and quality control of individual mill. The typical characteristics of POME and standard discharge limit set by Malaysian Department of Environment (DOE) are given in table below (Shakila,2008).

Table 2.3: Characteristic of POME

Parameter	Range, mg/L	Standard limit, mg/L
pH	3.5-5.2	5-9
Oil and grease	150-18,000	50
BOD	10,250-43,750	100
COD	15,000-100,000	-
Total Solid	11,500-78,000	-
Suspended solid	5000-54,000	400
Total nitrogen	180-1400	150
Ammoniacal Nitrogen	4-80	-

CHAPTER 3

METHODOLOGY

3.1 Materials

In order to determine the effect of agitation rate on solvent production of butanol and ethanol, several important material are used. In this study, materials that used are fresh palm oil mill effluent (POME) as a substrate and reinforce clostridia medium (RCM). The strain that used is *Clostridia acetobutylicum.* Reinforced clostridia medium is a semisolid medium formulated by Hirsch and Grinstead. The RCM is used for cultivating and enumerating clostridia, other anaerobes, and other species of bacteria from food and clinical specimens. Reinforced clostridia agar contains peptones and beef extract as a sources of carbon, nitrogen, vitamins and minerals.

3.2 Equipments

3.2.1 High Performance Liquid Chromatography (HPLC)

High pressure liquid chromatography (or high pressure liquid chromatography, HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds based on their idiosyncratic polarities and interactions with the column's stationary phase. HPLC utilizes different types of stationary phase (typically, hydrophobic saturated carbon chains), a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. To analyze the component of POME, the high performance liquid chromatography (HPLC) (Agilent Technology, USA) is used by using capillary column Supelcosil LC-NH_2. The flow rate that used is 1ml/minute and the retention time around 15 minutes. The mobile phase for this HPLC is acetonitrile and water. The ratio of mobile phase is 75% of acetonitrile and 25% of water.

Figure 3.1: Partition Chromatography

3.2.2 Gas Chromatography

Gas chromatography (GC) is a common type of chromatography used in analytic chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined).

In some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture. The flame ionization detector (FID) is a non-selective detector used in conjunction with gas chromatography. In this study, for GC-FID (Agilent Technology, USA) the Gas chromatography column that used is HP-inowax. The temperature for the operation is 50^{o}C-180^{o}C and the carrier gas is hydrogen.

Figure 3.2: Gas Chromatography with Flame ionization detector

3.2.3 Separating Funnel for liquid-liquid extraction (LLE)

Liquid-liquid extraction is a basic technique in chemical laboratories, where it is performed using a separating funnel. Liquid-liquid extraction, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubility in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase

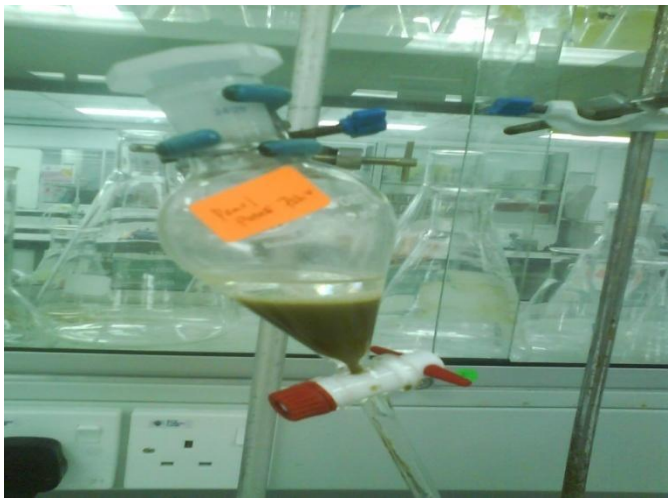

Figure 3.3: Separating Funnel

3.2.4 Anaerobic Chamber

Anaerobic chamber (Sheldon Manufacturing Inc, USA) is designed to allow efficient and dexterous glove-free handling and inspection of samples. Modular systems within the bactron anaerobic chamber facilitate the completion of procedures from unpacking material to inoculation, incubation, inspection and recovery all without a single exposure to oxygen.

Figure 3.4: Anaerobic Chamber

3.2.5 UV-Vis Spectrophotometer

The UV-Visible spectrophotometer (HITACHI, Japan) uses two light sources, a deuterium (D_2) lamp for ultraviolet light and a tungsten (W) lamp for visible light. After bouncing off a mirror (mirror 1), the light beam passes through a slit and hits a diffraction grating. The grating can be rotated allowing for a specific wavelength to be selected. At any specific orientation of the grating, only monochromatic (single wavelength) successfully passes through a slit. A filter is used to remove unwanted higher orders of diffraction. The light beam hits a second mirror before it gets split by a half mirror (half of the light is reflected, the other half passes through). One of the beams is allowed to pass through a reference cuvette (which contains the solvent only), the other passes through the sample cuvette. The intensities of the light beams are then measured at the end. The wavelength that used for measure optical density growth culture is 680nm.

Figure 3.5: UV-Vis Spectrophotometer

3.2.6 Autoclave

An autoclave is a device to sterilize equipment and supplies by subjecting them to high pressure steam at 121 °C or more, typically for 15 to 20 minutes depending on the size of the load and the contents.

Figure 3.6: Autoclave

3.2.7 Incubator Shaker

Incubator shaker is appropriate for bacterial cultures and enzyme reactions. The temperature that used is 35^{o}C and the speeds are 100 rpm, 150 rpm, 175 rpm and 200 rpm.

Figure 3.7: Incubator Shaker

3.3 Experimental Procedure

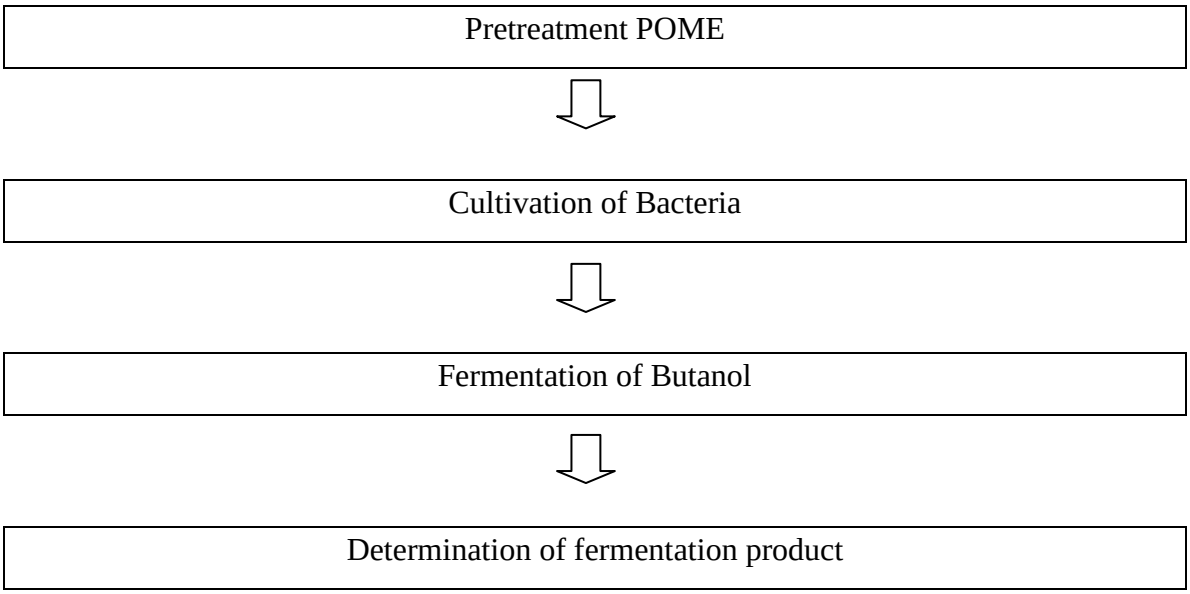

Figure 3.8: Flow chart of experimental procedures

3.3.1 Pretreatment Palm Oil Mill Effluent (POME)

Raw POME was obtained from a local palm oil mill factory (Felda Palm Industries Sdn Bhd at Lepar Hilir, Gambang, Pahang). Fresh POME was sediment passively in a cool room at 4^{o}C for 24 hours before use. The supernatant layer (upper part) was decanted and sediment POME sludge (lower part) was used and sterilized. Then the sediment POME was dilute with distilled water and maintains the pH at 5.8. After that the medium was autoclaved at 121^{o}C for 20 minutes. After the process of autoclaving, the sample was centrifuge to separate the particle in the sample. The supernatant from the sample of POME was taken to check the component of POME.

3.3.2 Growth Profile of *C.acetobutylicum* by using Palm Oil mill Effluent (POME) And Reinforced clostridia medium (RCM)

Before proceed fermentation done, the growth profile of bacteria (*C.acetobutylicum*) should be do first to know the rate of growth *C. acetobutylicum*. In this experiment, the *C.acetobutylicum* was culture in palm oil mill effluent and reinforced clostridia medium. After culture the bacteria on agar plate, the bacteria was transfer into POME and RCM medium.

This process was done in anaerobic chamber to maintain the anaerobic condition. Then, every 6 hours the sample was taken to measure the optical density. The sample was taken every 6 hours until 72 hours. This process was done in order to know the growth rate of bacteria in POME and RCM medium.

3.3.3 Preparation of Fermentation Media

Fresh POME was obtained from Lepar Hilir (Felda Palm Industries Sdn Bhd) Gambang, Pahang. POME medium was prepared from fresh POME that was passively sediment at 4^{o}C over a period of 24 hours. The initial pH of the POME was adjusted to pH 5.8 by the addition of 5M NaOH and pH 5.8 is optimum for solvent production from POME. Then reinforce clostridia media (RCM) medium was prepared by dissolving 38 g of the powder into 1L distilled water. Both mediums were autoclaved at 121^{o}C for 20 minutes

3.3.4 Preparation of Agar Medium

Reinforce Clostridia Agar was prepared by dissolving 52.5 g of the powder in 1 liter of distill water and then transferred it into schott bottle. After that, it was boiled to dissolve completely. Then the agar medium was sterilized by autoclaved at 121^{o}C for 20 minutes. After autoclaved the medium, the medium was cooling at room temperature. Then the tube was pour with melted agar into a sterile petri dish. Keep the dish level until the agar has hardened.

3.3.5 Process Enrichment of Bacteria

Pure bacteria in the glycerol stock was transferred by using inoculating loop into reinforced clostridia medium (RCM) or call as broth media for 3 days or 1 week. After that, broth media was transferred into agar slant store by inoculating loop. Then the agar slant was incubated in incubators for 24 hours at 37^{o}C

3.3.6 Striking Bacteria on Petri Dish

To strike the bacteria on petri dish, the anaerobic chamber was used to maintain anaerobic condition. The agar slant after 24 hours incubation was used to strike the bacteria on the agar plate in petri dish. Then the plate was inoculated in three parallel lines about 5 mm distance from each others. After strike the bacteria on petri dish, the

petri dish was seal with parafilm. Then it was incubation in the incubator at 37^{o}C for 2 days

3.3.7 Inoculums preparation

The bacterium was cultivated in anaerobic condition in reinforced clostridia medium (RCM) for 18 hours at 37^{o}C in incubators. The liquid medium of RCM was used for inoculums preparation. 167 ml of RCM medium was transferred into 250 ml schott bottle, and the single colony of bacteria (*C. acetobutylicum*) from agar plate was transferred into 167 ml of RCM medium. After that, it was kept in incubators at 37^{o}C. Then after 18 hours, checked the optical density (OD).The growth of culture in RCM was monitored by measuring an optical density at 680 nm using spectrophotometer. Only the inoculums with optical density (OD) value greater than 0.7 after 18 hours cultivation was used as inoculums. An inoculums of 10% v/v was used throughout this work.

3.3.8 Fermentation Process

Fresh POME was sediment passively in a cool room at 4^{o}C for 24 hours before use. The supernatant layer (upper part) was decanted and sediment POME sludge (lower part) was use as substrate. 270 ml of POME sludge was transferred into 500 ml of schoot bottle. Then POME sludge was diluted with 30 ml distilled water to obtain the required concentration. The initial pH of the POME was adjusted to pH 5.8 by the addition of 5M NaOH. The reinforce clostridia medium (RCM) is also used as a positive control which is

300 ml of RCM medium was transferred into schott bottle. After that, the medium was sterilized at 121^{o}C for 20 minutes and then it was used directly as fermentation medium without additional nutrient.

After autoclaving the medium of POME and RCM medium, the medium was deoxidizing by gassing with the nitrogen gas for 10 minutes. Then the medium was entered in anaerobic chamber before making any process transferred such as inoculums transformations. After that, 10% v/v (16 ml) of inoculums was transferred into the POME medium and RCM medium.

After the process transferred inoculums, it was incubate at optimum temperature 35^{o}C and at optimum speed 200 rpm. For run 1, 2 and 3, used the different of speed to measure the yield of butanol. For run1 the agitation speed that used is 100 rpm, run2:150 rpm and the run3: 175 rpm. Then every 20 hours of fermentation, the sample was taken to measure the optical density (OD). The sample was taken every 20 hours until 72 hours in order to check the glucose concentration in the POME medium and RCM medium. For run 4 of fermentation, it was maintained at optimal condition.

3.3.9 DNS assay

The glucose concentration in the medium was measured by using 3,5 dinitrosalicylic acid (DNS) assay for total reducing sugars. 1 ml of POME and RCM sample during fermentation and 2 ml of the DNS reagent mixture were mixed together in the test tube. Then the mixtures were placed in the water bath for 5 minutes and then diluted with 10 ml of distill water. After that the sample was measured by using UV-vis

spectrophotometer. The absorbance at OD 550 nm for all samples was recorded and the glucose concentration was calculated from standard curve.

3.3.10 Liquid-liquid extraction (LLE) process

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

After that, the sample was mix and leaves the process in the separating funnel for 24 hours. Then after 24 hours, the sample from the separating funnel was taken and transferred into the biker. The sample in the biker was added with Na_2SO_4. The Na_2SO_4 was used to absorb the molecule of water that have in the sample. After added the Na_2SO_4 the biker was closed tightly with aluminum fold and it was stored in chiller at 4^oC for 24 hours. Then after 24 hours, the sample was filtered by using syringe filter of 0.2µm and then prepared for GC analysis.

3.3.11 Analysis of Process

3.3.11.1 POME Analysis

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

The standard that is used to detect glucose, galactose, fructose, xylose, and lactose in the sample of POME is the pure solution of glucose, galactose, fructose, xylose, and Lactose. The calibration curve for each sugar (glucose, galactose, fructose, xylose, and Lactose) was prepare for 20 g/L,40 g/L,60 g/L,80 g/L and 100 g/L. Then filter the solution of sample and standard solution by using the filter 0.45µm. Then the sample was entered into the vial to analyze by HPLC.

3.3.10.2 Butanol and Ethanol Analysis

To analyze the composition of butanol and ethanol, the gas chromatography (GC-FID) was used. 10µl of sample POME and RCM was mix with 990 µl of hexane. After process mixture between the sample and hexane the sample was transferred into vial by using filter of 0.2µm. The standard that used to detect concentration of butanol and ethanol in the sample is pure butanol and ethanol solution. The standard solution of butanol and ethanol also was filtered by using 0.2 µm filter before it entered into the vial.

The gas chromatography column that used is HP-inowax. The temperature for the operation is 50^{o}C -180^{o}C and the carrier gas is hydrogen.

CHAPTER 4

RESULT AND DISCUSSION

4.0 Results

4.1 Concentration of Sugar in POME

Table 4.1: Concentration of Sugar in POME

Sample	Sugar Group	Concentration(g/L)
POME	Fructose	3.023
	Glucose	6.116
	Galactose	5.848
	Sucrose	3.57
	Lactose	1.0198

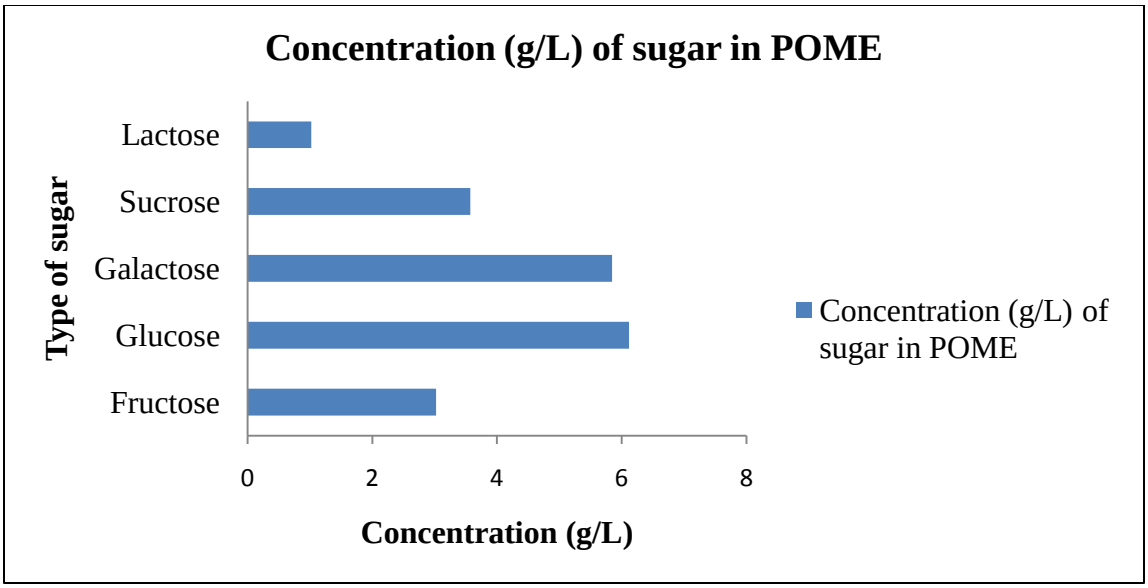

Figure 4.1: The concentration of sugar in POME

The sediment of POME helped to remove trace of oil and soluble toxic substances leaving less inhibitory POME which more suitable for growth of Clostridia. The sediment POME contain high concentration of lignocellulase and others soluble materials that supported the growth of *C. acetobutylicum*.

From the analysis POME, analysis the result (Figure 4.1) showed that certain type of sugar that contain in the POME. The types of sugar that contain in the POME are lactose, sucrose, glucose, galactose and fructose.

From the result (Table 4.1), the concentration of lactose is 1.0198 g//l, sucrose 3.57 g/l, galactose 5.848 g/l, glucose 6.116g/l and fructose 3.023 g/l. The higher concentration of sugar in POME is glucose where the value of glucose is 6.116 g/l and then the sugar that has lower value in POME is lactose where it value is 1.0198 g/l.

4.2 Growth Profile

Table 4.2: Growth profile of *C. acetobutylicum* in POME

Time (hour)	Average Optical Density value in POME
0	0
6	0.345
12	0.576
18	0.767
24	0.815
30	0.826
36	0.836
42	0.798
48	0.82
54	0.857
60	0.827
66	0.823
72	0.825

Table 4.3: Growth profile of *C. acetobutylicum* in RCM

Time (hour)	Average Optical Density value in RCM
0	0
6	0.28
12	0.438
18	0.76
24	0.792
30	0.807
36	0.874
42	0.971
48	1.008

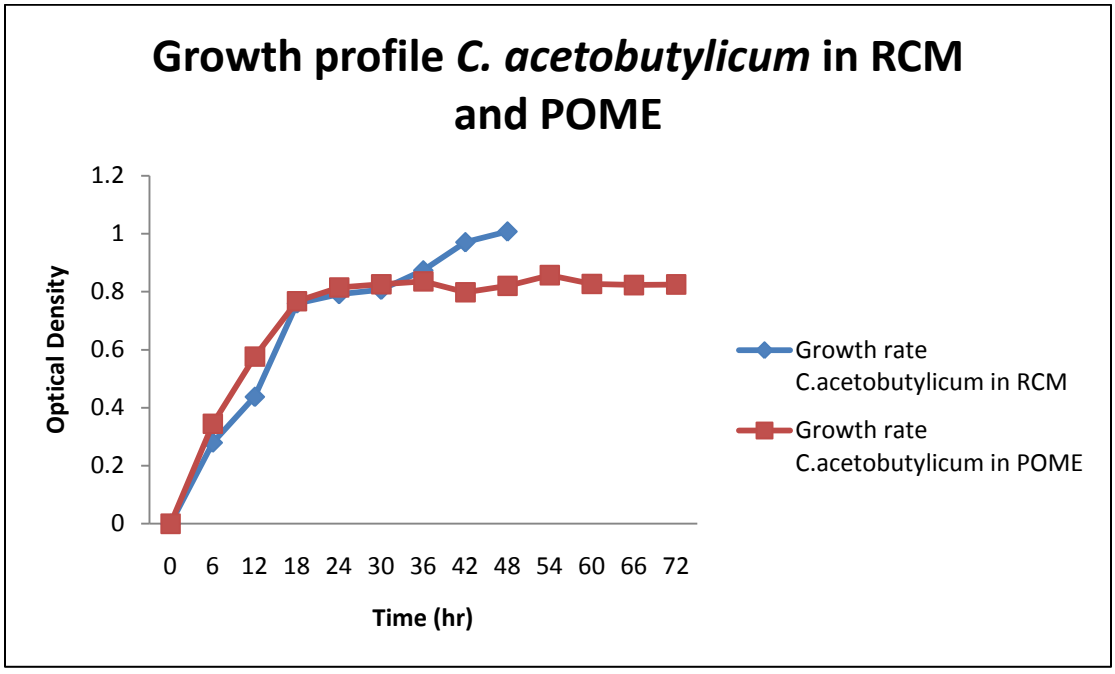

Figure 4.2: Growth profile *C. acetobutylicum* in RCM and POME

The growth profile of *C. acetobutylicum* utilizing POME and reinforce clostridia medium (RCM) grown in a schott bottle is shown in Figure 4.2. The Fresh POME contains major carbon sources, namely sugar. The types of sugar that contain in fresh POME are glucose, galactose, sucrose and lactose.

The growth curve of *C. acetobutylicum* utilizing carbon sources can be divided into two phases, acidogenic phase and solventogenic phase. An acidogenic phase where organic acid (acetic and butyric acid) were actively produced was observed during the first 12 hours of fermentation which cause the reduction in culture pH. At the same time glucose is also actively consumed to accommodate the high growth rate in the culture between 0 to 36 hours.

From the graph (Figure 4.2), the bacteria *C.acetobutylicum* in POME and RCM are growth actively from 0 to 36 hours. From time 0 to 36 hours, the phase that call is exponential phase (sometimes called the log phase) is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population.

Then from the result, after 36 hours to 66 hours the growth rate bacteria is low. This phase is reached as the bacteria begin to exhaust the resources that are available to them. This phase is call as stationery phase. This phase is a constant value as the rate of bacterial growth is equal to the rate of bacterial death. Then, after 66 hours to 72 hour the growth rate of bacteria *C.acetobutylicum* are not active where the bacteria run out of nutrients and die. This phase is call death phase

4.3 Glucose Consumption for each Fermentation

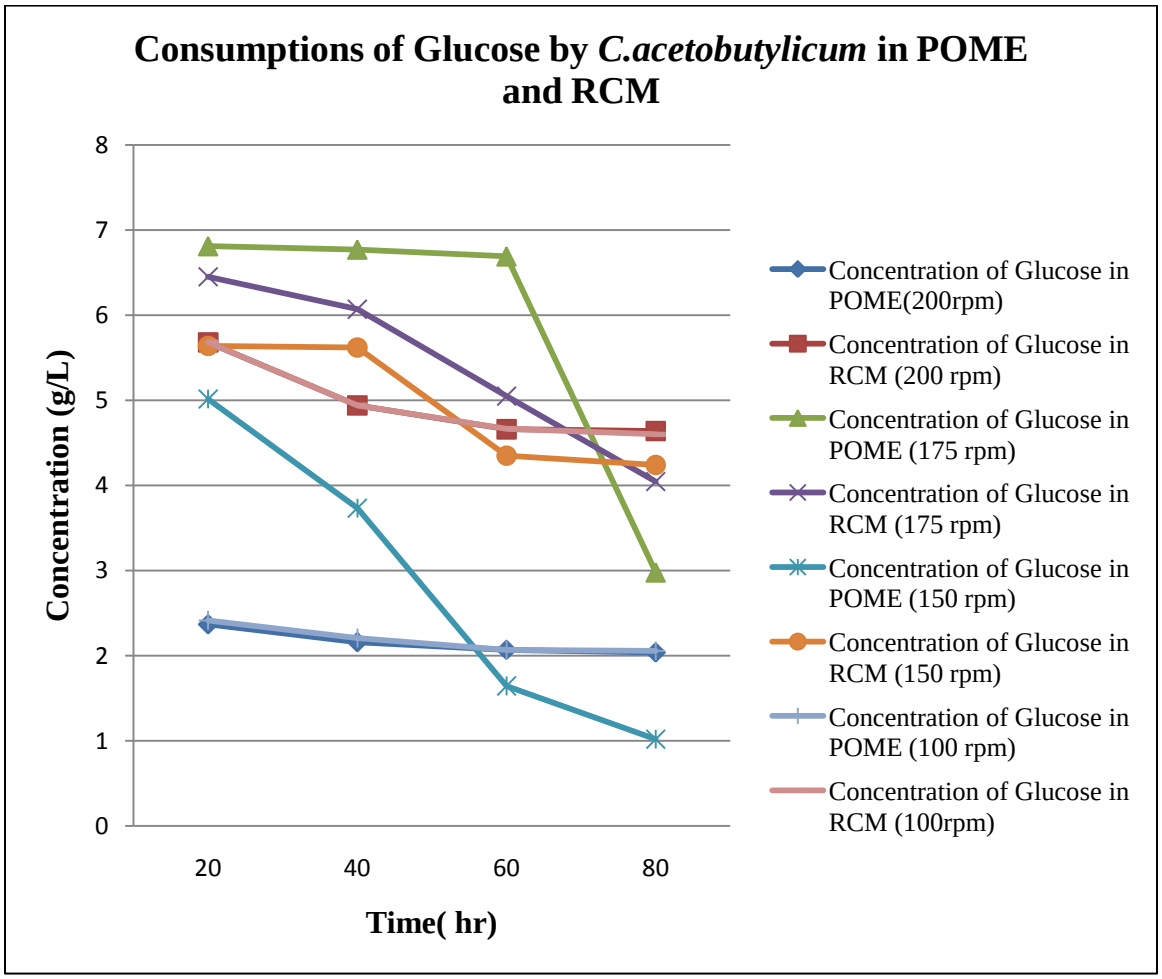

Figure 4.3: The glucose consumption in each fermentation by *C.acetobutylicum*

The sample of POME and RCM for each 20 hours until 80 hours was taken and then checked the optical density. From the result, the glucose consumption by bacteria is increase with the time from 20 hours, 40 hours, 60 hours and 72 hours. From the graph

(Figure 4.3) it is showed that, at time 20 hours to 40 hours, the consumption of glucose was very high. This showed that the growth rate of bacteria *C. acetobutylicum* very active.

From the result, the concentration of glucose in POME for 200 rpm was higher at 20 hours after fermentation where it value is 5.68 g/l. The lower concentration of glucose for 200 rpm of speed was at time 80 hours after fermentation where it value is 4.942 g/l. Then, the glucose concentration in POME at 175 rpm is higher at 20 hours after fermentation where it concentration is 6.81 g/l and the lower concentration is 5.41 g/l. For 150 rpm and 100 rpm the higher concentration of glucose in POME at that speed is 5.01 g/l and 2.416 g/l. Then the lower concentration glucose of both speed are 1.02 g/l and 2.07 g/l.

4.4 Butanol and ethanol production

Lab scale fermentation using schott bottle was used to produce the butanol and ethanol from *Clostridum acetobutylicum*. All the condition or parameter have been controlled and set constant. Butanol have been produced at different rate of agitation speed (100 rpm,150 rpm,175 rpm and 200 rpm). The results are shown below:

First Fermentation:

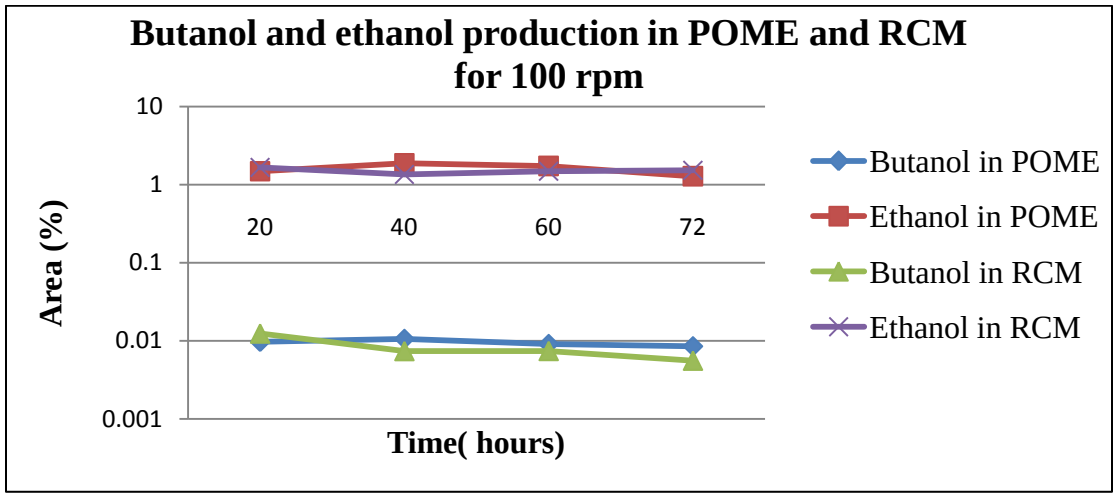

Figure 4.4:Butanol and ethanol production in POME and RCM for 100 rpm

Second fermentation:

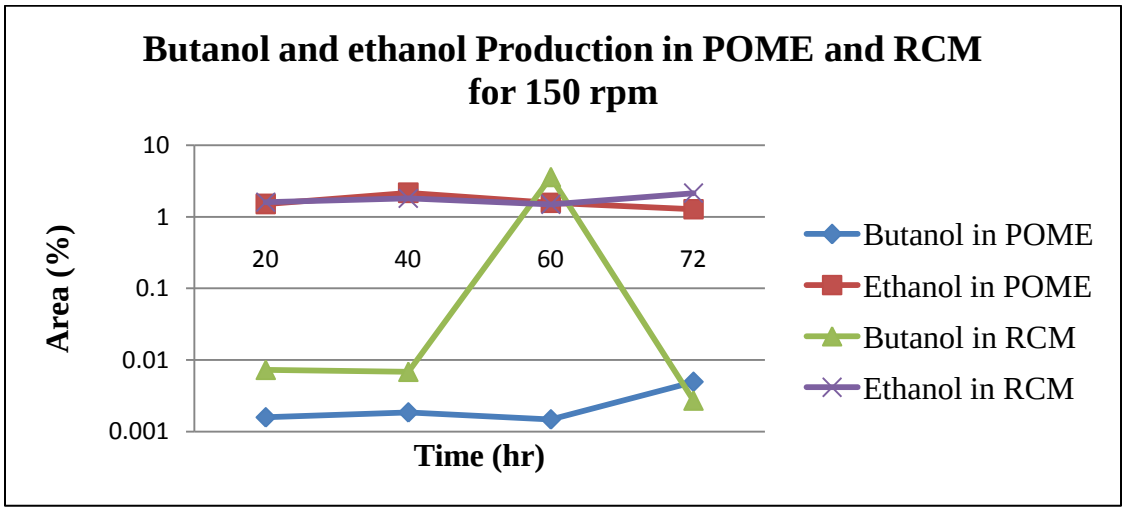

Figure 4.5: Butanol and ethanol production in POME and RCM for 150 rpm

Third Fermentation:

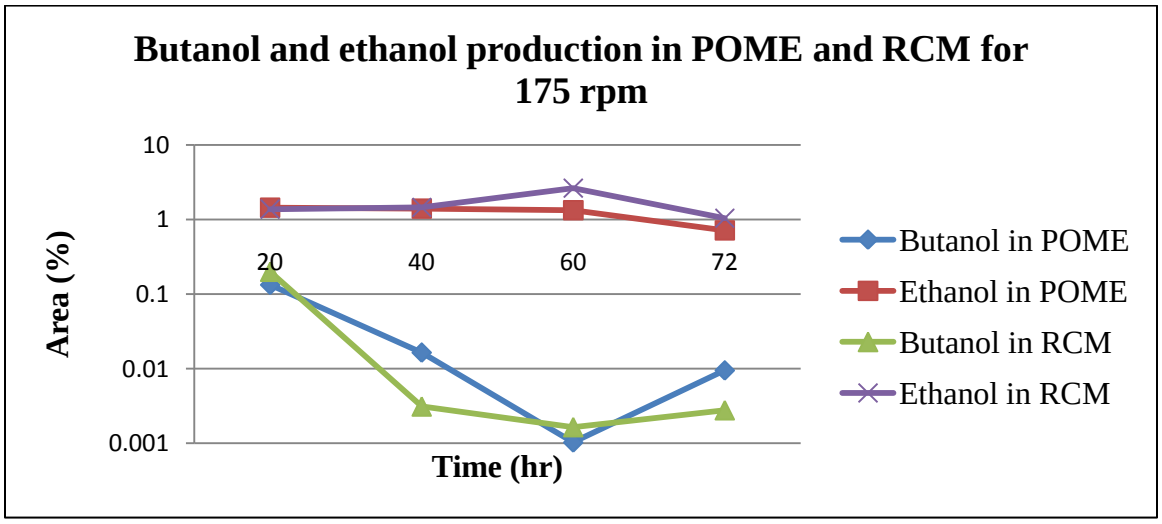

Figure 4.6: Butanol and ethanol production in POME and RCM for 175 rpm

Fourth fermentation:

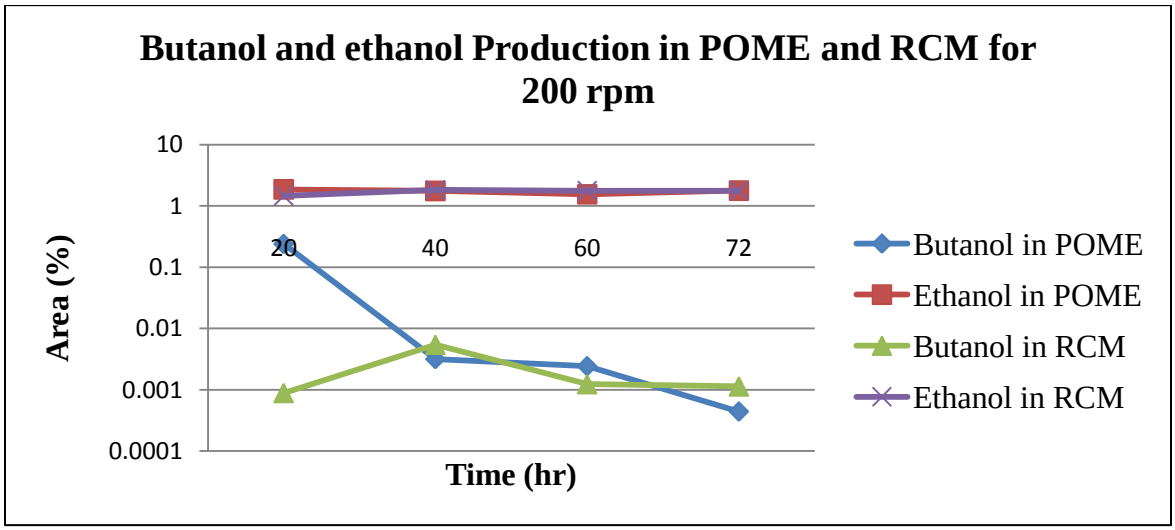

Figure 4.7: Butanol and ethanol production in POME and RCM for 200 rpm

Table 4.4: Butanol and ethanol at optimum time with their speed in POME

Speed (rpm)	Butanol Concentration at optimum time (g/L)	Ethanol Concentration at optimum time (g/L)
100	0.0602	68.77
150	0.111	57.193
175	0.117	62.83
200	0.137	68.958

Table 4.5: Butanol and ethanol at optimum time with their speed in RCM

Speed (rpm)	Butanol Concentration at optimum time (g/L)	Ethanol Concentration at optimum time (g/L)
100	0.2176	59.43
150	0.208	89.55
175	0.12156	109.99
200	0.11578	79.072

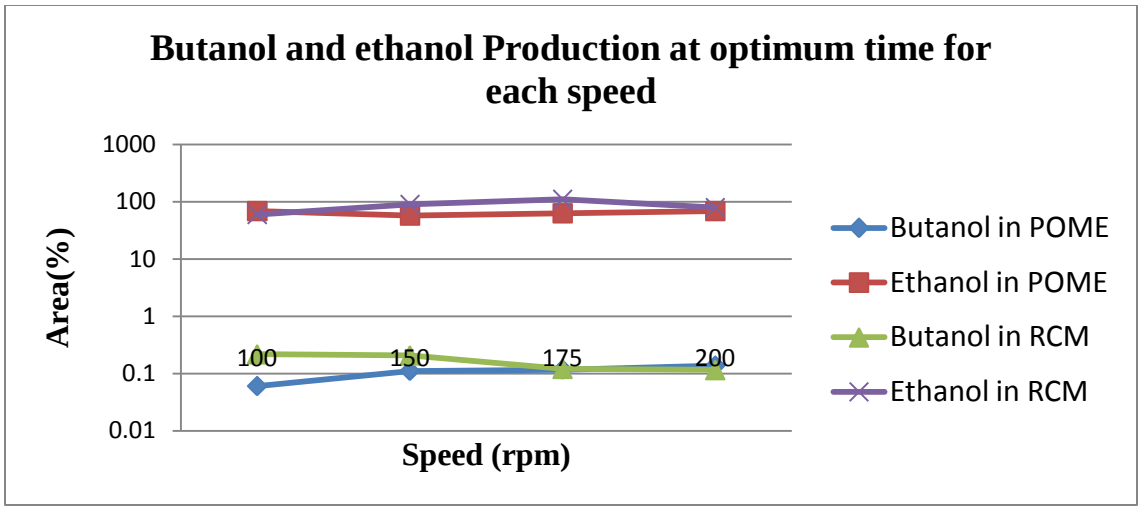

Figure 4.8: Butanol and Ethanol production in POME and RCM at optimum time for each run

From the result, an acidogenic phase was observed during the first 24 hours of fermentation in where *C. acetobutylicum* grew rapidly with high production of acids (acetic and butyric) which cause the reduction in pH of the culture. The fermentation entered the solventogenic phase when cell growth reached a deceleration phase after 24 hours. During this phase, the metabolism of cells undergoes a shift to produce solvent by reasimilation of organic acid (acetic and butyric acids) caused slight increased in pH of the culture.

The initial pH of the POME must be increase to pH 5.8 before used for ABE fermentation medium in order to get high yield of solvent. From the result, the production of butanol and ethanol in POME and RCM medium increase by increase the rate of agitation speed. The result also showed that at 200 rpm (optimum speed) of speed the production of butanol and ethanol are higher compare to others rate agitation speed. The concentration of butanol for 200 rpm in POME is 0.137 g/l.

Then for concentration of ethanol at 200 rpm is 68.958 g/l in POME. The rate agitation play role important to produced high yield of butanol and ethanol. From the result, the speed at 100 rpm will be produced low concentration butanol and ethanol in POME, where the value is 0.0602 g/l for butanol and 68.77 g/l for ethanol. In the RCM medium, the concentration of butanol and ethanol at 200 rpm are 0.11578 g/l and 79.072 g/l.

The production of butanol is lower than ethanol may be cause by several reasons. Firstly, they may be cause by substrate inhibition that occurred during the fermentation. Then, the second one is cause by down time for cleaning, sterilizing and filling during the process fermentation. One of the critical problems in butanol and ethanol fermentation is solvent toxicity. The lipophilic solvent butanol is more toxic than others as it disrupt the phospholipid component of cell membrane fluidity (Bowles and Ellefson, 1985).from the result in this fermentation process, the solvent toxicity was occured and inhibit the solvent production and furthermore the production of butanol will be decrease than ethanol production.

Study by Qureshi *et al*. (2006) showed that, the overall competitiveness of the bioprocess highly depends on the strain performance. The strain of *C. acetobutylicum* are play important role in enhance butanol production. From the result, the production of butanol lower maybe because of to many subcultures that has been done during the inoculums preparation.

From the result, it is showed that concentration of yielded butanol and ethanol are low. The culture utilized glucose, xylose, galactose and arabinose and produced different amounts of butanol and ethanol. Qureshi *et al*. (2006) reported that uses of glucose resulted in the production of the greatest amount of butanol and ethanol followed by arabinose. In the mixed sugar fermentation glucose was the preferred sugar followed by

arabinose. In this fermentation, significant amounts of residual galactose and xylose remained in the fermentation. The value of concentration is low because the fresh POME not containing sugar xylose. The contain of xylose in POME are also give the effect to butanol and ethanol production. POME that contain xylose will be produce high yield of butanol and ethanol.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The main objective of this study is to study the effect of agitation rate on butanol and ethanol production from palm oil mill effluent by *Clostridium acetobutylicum*. The parameter such as rate agitation speed is important in control high yield of butanol and ethanol productions. An analysis of the data was conducted to obtain the comprehensive conclusion towards achieving the objective of the study. The result of this experiment showed that fresh POME is a viable media for butanol and ethanol fermentation by *C. acetobutylicum*. The sediment of POME contain high concentration of lignocellulase and others sugar that supported the growth of *C. acetobutylicum*.

The types of sugar that contain in POME are sucrose, lactose, fructose, glucose and galactose. Fresh POME which is rich with natural carbon sources and dissolved complex substance requires mixing to enhance substrate interface with the microorganism to produce high yield. The optimum speed (200 rpm) in fermentation will be produce high yield of butanol and ethanol. The production of butanol and ethanol also highly depend on the strain performance.

5.2 Recommendations

From the experimental results, the following recommendations are proposed:

1. A further study on investigation, fed-batch fermentation should be used for the butanol and ethanol production to avoid substrate inhibition and to increase cell mass.

2. For the next research, the continuous culture should be used to eliminate the preparation time and lag phase. If used the batch culture, the products will inhibition as well as down time for cleaning, sterilizing and filling during fermentation process.

3. To avoid the decreasing of inoculums productivity, the subcultures that to be done during inoculums process should be decrease.

4. The solvent extraction that used to absorb butanol and ethanol during the liquid-liquid extraction process should be good solvent.

5. Development of strategies for strain improvement, fermentation and purification

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APPENDIX A

COMPOSITION ANALYSIS OF SELECTED FRESH POME

Xylose (Retention time = 5.902)

Table A-1:The peak area and concentration of xylose

Amount concentration (g/l)	Area
20	1.43341e6
40	2.84440e6
60	4.28133e6
80	5.71472e6
100	7.3507e6

Fructose (Retention time = 7.052)

Table A-2:The peak area and concentration of Fructose

Amount concentration (g/l)	Area
20	1.13320e6
40	2.36668e6
80	4.37532e6
100	5.19066e6

Glucose (Retention time = 7.702)

Table A-3:The peak area and concentration of Glucose

Amount concentration (g/l)	Area
20	1.69828e6
40	3.27955e6
60	5.05296e6
80	6.64497e6
100	8.23004e6

Galactose (Retention time = 8.633)

Table A-4:The peak area and concentration of Galactose

Amount concentration (g/l)	Area
20	9.92166e5
40	1.94887e6
60	2.87786e6
80	3.94253e6
100	4.75126e6

Sucrose (Retention time = 10.381 min)

Table A-5:The peak area and concentration of Sucrose

Amount concentration (g/l)	Area
20	1.86245e6
40	3.26337e6
60	4.89866e6
80	6.36837e6
100	7.84323e6

Lactose (Retention time = 13.127 min)

Table A-6:The peak area and concentration of Lactose

Amount concentration (g/l)	Area
20	1.30290e6
40	2.50230e6
60	3.21022e6
80	4.19806e6
100	5.35126e6

Sample Analysis 1

Table A-7:The peak area and retention time of sample analysis 1

Peak	Retention Time (min)	Area
1	6.761	2.59147e5
2	7.311	2.64544e5
3	7.769	5.29701e5
4	8.756	3.03513e5
5	8.922	4.43881e5
6	10.190	4.33496e5
7	13.290	2.31336e5

Sample Analysis 2

Table A-8:The peak area and retention time of sample analysis 2

Peak	Retention Time (min)	Area
1	6.764	1.29103e5
2	7.297	1.96194e5
3	7.766	2.42428e5
4	8.771	4.76619e5
5	10.216	1.45158e5
6	13.347	8.30884e4

Sample Analysis 3

Table A-9: The peak area and retention time of sample analysis 3

Peak	Retention Time	Area
1	6.779	1.67508e5
2	7.302	1.84783e5
3	7.770	2.11423e5
4	8.774	5.63179e5
5	10.232	1.48901e5
6	10.994	7.46828e4
7	11.593	7.73765e4
8	13.370	1.06895e5

APPENDIX B

GLUCOSE CONSUMPTION BY *C.ACETOBUTYLICUM*

Fourth Fermentation (200rpm)

20 hours (POME)

Blank = -0.00

Table B-1: Concentration of Glucose for 20hr (POME) Run1

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	0.80	0.800	0.800	0.800	2.37
1/10	-0.20	-0.20	-0.20	-0.20	-
1/20	-0.770	-0.770	-0.770	-0.770	-

40 hours (POME)

Blank = -0.00

Table B-2: Concentration of Glucose for 40hr (POME) Run1

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc (g/L)
X	0.70	0.70	0.70	0.70	2.16
1/10	-0.265	-0.265	-0.265	-0.265	-
1/20	-0.779	-0.779	-0.779	-0.779	-

60 hours (POME)

Blank = -0.00

Table B-3: Concentration of Glucose for 60hr (POME) Run1

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	0.653	0.653	0.653	0.653	2.07
1/10	-0.047	-0.047	-0.047	-0.047	-
1/20	-0.779	-0.779	-0.779	-0.779	-

80 hours (POME)

Blank = -0.00

Table B-4: Concentration of Glucose for 80hr (POME) Run1

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	0.640	0.640	0.640	0.640	2.04
1/10	-0.779	-0.779	-0.779	-0.779	-
1/20	-0.779	-0.779	-0.779	-0.779	-

20 hours (RCM)

Blank = -0.00

Table B-5: Concentration of Glucose for 20hr (RCM) Run1

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.396	2.396	2.396	2.396	5.68

40hours (RCM)

Blank = -0.00

Table B-6: Concentration of Glucose for 40hr (RCM) Run1

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.041	2.040	2.041	2.0405	4.94

60hours (RCM)

Blank = -0.00

Table B-7: Concentration of Glucose for 60hr (RCM) Run1

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.906	1.906	1.906	1.9060	4.66

80 hours (RCM)

Blank = -0.00

Table B-9: Concentration of Glucose for 80hr (RCM) Run1

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.90	1.90	1.90	1.90	4.64

First Fermentation (100rpm)

20 hours (POME)

Blank = -0.00

Table B-10: Concentration of Glucose for 20hr (POME) Run2

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	0.820	0.820	0.820	0.820	2.416
1/10	-0.265	-0.265	-0.265	-0.265	-
1/20	-0.779	-0.779	-0.779	-0.779	-

40 hours (POME)

Blank = -0.00

Table B-11: Concentration of Glucose for 40hr (POME) Run2

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc (g/L)
X	0.720	0.720	0.720	0.720	2.209
1/10	-0.265	-0.265	-0.265	-0.265	-
1/20	-0.779	-0.779	-0.779	-0.779	-

60 hours (POME)

Blank = -0.00

Table B-12: Concentration of Glucose for 60hr (POME) Run2

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	0.656	0.656	0.656	0.656	2.07
1/10	-0.047	-0.047	-0.047	-0.047	-
1/20	-0.779	-0.779	-0.779	-0.779	-

80 hours (POME)

Blank = -0.00

Table B-13: Concentration of Glucose for 80hr (POME) Run2

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	0.647	0.647	0.647	0.647	2.057
1/10	-0.779	-0.779	-0.779	-0.779	-
1/20	-0.779	-0.779	-0.779	-0.779	-

20 hours (RCM)

Blank = -0.00

Table B-14: Concentration of Glucose for 80hr (RCM) Run2

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.398	2.398	2.398	2.398	5.68

40hours (RCM)

Blank = -0.00

Table B-15: Concentration of Glucose for 40hr (RCM) Run2

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.041	2.040	2.041	2.0405	4.94

60hours (RCM)

Blank = -0.00

Table B-16: Concentration of Glucose for 60hr (RCM) Run2

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.907	1.907	1.907	1.9070	4.667

80 hours (RCM)

Blank = -0.00

Table B-17: Concentration of Glucose for 80hr (RCM) Run2

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.90	1.90	1.90	1.90	4.60

Second Fermentation(150 rpm)

Before fermentation

POME

Blank = -0.00

Table B-18: Concentration of Glucose before fermentation (POME) Run3

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	3.0	3.0	3.0	3.0	-
1/10	3.0	3.0	3.0	3.0	-
1/20	1.329	1.329	1.329	1.329	69.399

Before Fermentation

RCM

Blank = -0.00

Table B-19: Concentration of Glucose before fermentation (RCM) Run3

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.854	2.854	2.854	2.854	6.627

20 hours (POME)

Blank = -0.00

Table B-20: Concentration of Glucose for 20hr (POME) Run3

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.076	2.076	2.076	2.076	5.016
1/10	1.836	1.836	1.836	1.836	45.19
1/20	0.764	0.764	0.764	0.764	46.00
1/40	-0.017	-0.017	-0.017	-0.017	-
1/60	-0.235	-0.235	-0.235	-0.235	-
1/80	-0.389	-0.389	-0.389	-0.389	-

40 hours (POME)

Blank = -0.00

Table B-21: Concentration of Glucose for 40hr (POME) Run3

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.456	1.456	1.456	1.456	3.733
1/10	1.450	1.450	1.450	1.450	37.20
1/20	0.799	0.799	0..799	0.799	47.45
1/40	0.768	0.768	0.768	0.768	92.33

60 hours (POME)

Table B-22:Concentration of Glucose for 60hr (POME) Run3

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	0.448	0.448	0.448	0.448	1.645
1/10	-0.309	-0.309	-0.309	-0.309	-
1/20	-0.779	-0.779	-0.779	-0.779	-
1/40	-0.779	-0.779	-0.779	-0.779	-

80 hours (POME)

Table B-23: Concentration of Glucose for 80hr (POME) Run3

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	0.146	0.146	0.146	0.146	1.02
1/10	-0.779	-0.779	-0.779	-0.779	-
1/20	-0.779	-0.779	-0.779	-0.779	-
1/40	-0.779	-0.779	-0.779	-0.779	-

20 hours (RCM)

Blank = -0.00

Table B-24: Concentration of Glucose for 20hr (RCM) Run3

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.377	2.377	2.377	2.377	5.64
1/10	-0.261	-0.261	-0.261	-0.261	-

40hours (RCM)

Blank = -0.00

Table B-25: Concentration of Glucose for 40hr (RCM) Run3

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.366	2.366	2.366	2.366	5.62
1/10	0.306	0.306	0.306	0.306	13.52

60hours (RCM)

Blank = -0.00

Table B-26: Concentration of Glucose for 60hr (RCM) Run3

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.753	1.753	1.753	1.753	4.35
1/10	0.18	0.18	0.18	0.18	10.91

80 hour

Blank = -0.0

Table B-27: Concentration of Glucose for 80hr (RCM) Run3

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.70	1.70	1.70	1.70	4.24
1/10	0.087	0.087	0.087	0.087	8.985

Third Fermentation (175 rpm)

Before fermentation

POME

Blank = 3.

Table B-28: Concentration of Glucose before fermentation (POME) Run4

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.553	2.553	2.553	2.553	6.004
1/10	1.979	1.979	1.979	1.979	48.16
1/20	1.08	1.08	1.08	1.08	59.09
1/40	1.02	1.02	1.02	1.02	113.21

Before Fermentation

RCM

Blank = -0.00

Table B-29: Concentration of Glucose before fermentation (RCM) Run4

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.432	2.432	2.432	2.432	5.75
1/10	1.765	1.765	1.765	1.765	43.726

20 hours (POME)

Blank = 3.00

Table B-30: Concentration of Glucose for 20hr (POME) Run4

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.94	2.94	2.94	2.94	6.81
1/10	2.308	2.308	2.308	2.308	54.96
1/20	2.083	2.083	2.083	2.083	100.62
1/40	-0.779	-0.779	-0.779	-0.779	-

40 hours (POME)

Blank = 3.0

Table B-31: Concentration of Glucose for 40hr (POME) Run4

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.921	2.921	2.921	2.921	6.77
1/10	2.620	2.620	2.620	2.620	61.4
1/20	1.797	1.797	1.797	1.797	88.78
1/40	0.927	0.926	0.926	0.926	105.42

60 hours (POME)

Blank = 3.0

Table B-32: Concentration of Glucose for 60hr (POME) Run4

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.886	2.886	2.886	2.886	6.69
1/10	2.149	2.208	2.208	2.208	52.89
1/20	1.095	1.094	1.093	1.093	59.63
1/40	0.395	0.395	0.395	0.395	61.45

80 hours (POME)

Table B-33: Concentration of Glucose for 80hr (POME) Run4

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.095	1.094	1.093	1.093	2.98
1/10	0.395	0.395	0.395	0.395	15.36
1/20	0.20	0.20	0.20	0.20	22.65

20 hours (RCM)

Blank = -0.00

Table B-34: Concentration of Glucose for 20hr (RCM) Run4

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.769	2.769	2.769	2.769	6.45
1/10	0.682	0.682	0.682	0.682	21.31

40hours (RCM)

Blank = -0.00

Table B-35: Concentration of Glucose for 40hr (RCM) Run4

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.585	2.585	2.585	2.585	6.07
1/10	0.860	0.860	0.860	0.860	24.98

60hours (RCM)

Blank = 0.00

Table B-36: Concentration of Glucose for 60hr (RCM) Run4

Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	2.092	2.092	2.092	2.092	5.049
1/10	1.611	1.611	1.607	1.607	40.45

80 hours (RCM)

Table B-37: Concentration of Glucose for 80hr (RCM) Run4

Blank = -0.00					
Dilution	Reading 1	Reading 2	Reading 3	Average	Conc(g/L)
X	1.611	1.611	1.607	1.607	4.045
1/10	1.50	1.50	1.50	1.50	38.24

APPENDIX C

BUTANOL AND ETHANOL PRODUCTION

First Fermentation

Table C-1: Butanol production for agitation speed=100rpm (POME)

Time (hour)	Area (pA*s)	Area (%)
20	45.89959	0.00970
40	50.11993	0.01061
60	42.95192	0.00911
72	40.55407	0.00851

Table C-2: Ethanol production for agitation speed= 100rpm POME

Time (hour)	Area (pA*s)	Area (%)
20	7021.67139	1.48394
40	8887.55664	1.88205
60	8192.26367	1.73711
72	6122.52o51	1.28460

Table C-3: Butanol production for agitation speed=100rpm (RCM)

Time (hour)	Area (pA*s)	Area (%)
20	58.56466	0.01239
40	35.07168	0.00738
60	35.26833	0.00740
72	26.57292	0.00556

Table C-4: Ethanol production for agitation speed= 100rpm (RCM)

Time (hour)	Area (pA*s)	Area (%)
20	7864.29688	1.66389
40	6421.75781	1.35101
60	7078.58838	1.48578
72	7291.83057	1.52679

Second Fermentation

Table C-5: Butanol production for agitation speed=150rpm (POME)

Time (hour)	Area (pA*s)	Area (%)
20	7.41327	0.00158
40	8.80647	0.00185
60	7.05261	0.00148
72	23.41251	0.00494

Table C-6: Ethanol production for agitation speed= 150rpm (POME)

Time (hour)	Area (pA*s)	Area (%)
20	7098.49756	1.51026
40	1.02847×10^4	2.16507
60	7482.92480	1.56786
72	6020.24268	1.27142

Table C-7: Butanol production for agitation speed=150rpm (RCM)

Time (hour)	Area (pA*s)	Area (%)
20	35.07235	0.00730
40	32.67218	0.00686
60	32.75944	3.59702
72	13.01505	0.00272

Table C-8: Ethanol production for agitation speed= 150rpm (RCM)

Time (hour)	Area (pA*s)	Area (%)
20	7686.25635	1.60002
40	8609.38770	1.80859
60	13.60515	1.49386
72	1.02415×10^4	2.14168

Third Fermentation

Table C-9: Butanol production for agitation speed=175rpm (POME)

Time (hour)	Area (pA*s)	Area (%)
20	671.69989	0.13338
40	83.13669	0.01649
60	5.28250	0.00103
72	47.83181	0.00951

Table C-10: Ethanol production for agitation speed= 175rpm (POME)

Time (hour)	Area (pA*s)	Area (%)
20	7261.04150	1.44181
40	7069.87207	1.40253
60	6811.91846	1.33256
72	3597.11108	0.71484

Table C-11: Butanol production for agitation speed=175rpm (RCM)

Time (hour)	Area (pA*s)	Area (%
20	1008.23096	0.19905
40	15.34507	0.00312
60	8.21955	0.00165
72	13.83557	0.00278

Table C-12: Ethanol production for agitation speed= 175rpm (RCM)

Time (hour)	Area (pA*s)	Area (%)
20	6979.95557	1.37802
40	7150.53076	1.45453
60	1.31056×10^4	2.62815
72	5190.49414	1.04246

Fourth fermentation

Table C-13: Butanol production for agitation speed=200rpm (POME)

Time (hour)	Area (pA*s)	Area (%)
20	1278.20630	0.23954
40	16.80263	0.00316
60	12.81426	0.00242
72	2.34620	0.00044

Table C-14: Ethanol production for agitation speed= 200rpm (POME)

Time (hour)	Area (pA*s)	Area (%)
20	9941.50293	1.86309
40	9355.22949	1.75952
60	8214.27051	1.55053
72	9447.41016	1.77946

Table C-15: Butanol production for agitation speed=200rpm (RCM)

Time (hour)	Area (pA*s)	Area (%)
20	4.72908	0.00089
40	28.81533	0.00546
60	6.59068	0.00124
72	6.04680	0.00114

Table C-16: Ethanol production for agitation speed= 200rpm (RCM)

Time (hour)	Area (pA*s)	Area (%)
20	7732.61621	1.45459
40	9690.20410	1.83480
60	9419.82031	1.77605
72	9407.25879	1.77570