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

ZURIANA BT SIDI AHMAD

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Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

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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)

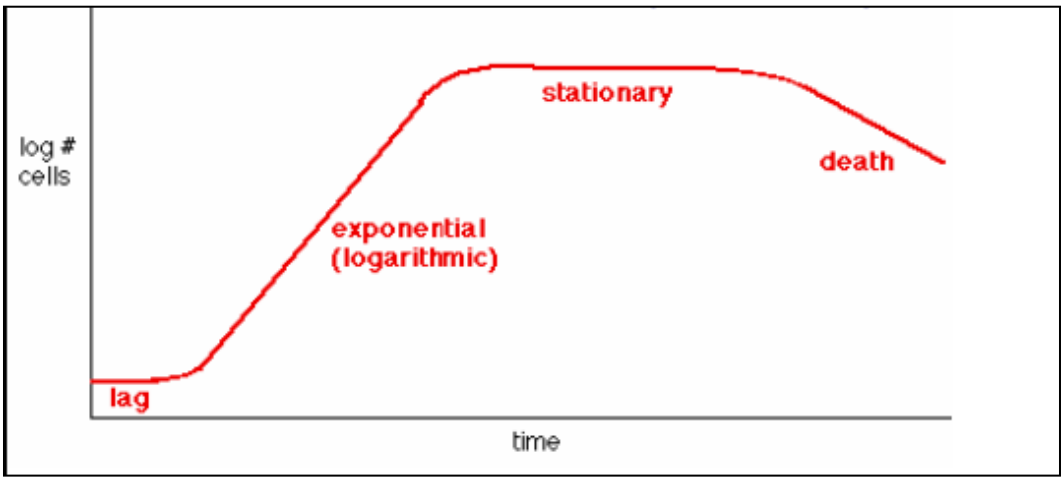

Figure 2.1: Phase of Microbial Growth in Batch Culture

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.