EFFECT OF SUBSTRATE CONCENTRATION AND AGITATION RATE ON BUTANOL PRODUCTION FROM POME BY *CLOSTRIDIUM BEIJERINCKII*

ELLIE FAZLIN BINTI MOHD SARIAN

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

Faculty of Chemical Engineering & Natural Resources
University Malaysia Pahang

NOVEMBER 2010

ABSTRACT

Palm oil mill effluent (POME) is the largest by product generated from palm oil mills, about 50 million tonnes annually. POME has a potential substrate for acetone, butanol and ethanol production using *Clostridium beijerinckii*. This research mainly studied on the direct use of POME as fermentation medium for solvent fermentation by *Clostridium beijerinckii* ATCC 51743. This research also investigated the growth profile rate and the consumption of glucose by *Clostridium beijerinckii* during fermentation. Fermentation were conducted for 72 hours at 37^0C by using palm oil mill effluent and reinforced clostridia medium as a growth medium in batch culture. Investigations were carried out on the effect of subsrate (POME) concentration and the agitation rate into the production of butanol. The POME concentration that had been used in this investigation were 90%, 80% and 70% while the agitation rate that had been used were 0 rpm, 100 rpm, 175 rpm and 250 rpm respectively. The results showed that the highest yield of butanol produced was 3.12 g/L by 70% POME at 100 rpm of agitation rate.

ABSTRAK

Bahan buangan daripada kilang kelapa sawit atau POME adalah produk sampingan terbesar yang dihasilkan daripada kilang kelapa sawit iaitu kira-kira 50 juta tan setahun. POME mempunyai potensi sebagai bahan untuk penghasilan asiton, butanol dan etanol menggunakan *Clostridium beijerinckii*. Keutamaan kajian ini adalah untuk mengkaji tentang penggunaan POME sebagai media fermentasi untuk penghasilan larutan ABE oleh *Clostridium beijerinckii* ATCC 51743. Kajian ini juga mengkaji tentang kadar tumbesaran bakteria dan penggunaan sumber glukosa oleh *Clostridium beijerinckii* ketika fermantasi. Fermentasi dilakukan selama 72 jam pada suhu 37^0C menggunakan bahan buangan daripada kilang kelapa sawit dan media RCM sebagai media tumbesaran. Kajian juga dilakukan ke atas kesan kepekatan bahan (POME) dan kelajuan mengocak terhadap penghasilan ABE. Kepekatan POME yang digunakan dalam kajian ini adalah 90%, 80% dan 70% manakala kelajuan mengocak yang digunakan adalah 0rpm, 100rpm, 175rpm dan 250rpm. Keputusan menunjukkan butanol yang terhasil adalah paling banyak pada kepekatan POME 70% dan kelajuan mengocak 100rpm iaitu sebanyak 3.12 g/L.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/SHORT FORMS

POME	=	Palm Oil Mill Effluent
ABE	=	Acetone-butanol-ethanol
USA	=	United State of America
N_2	=	Nitrogen gas
O_2	=	Oxygen gas
CO_2	=	Carbon Dioxide
ABS	=	Absorption
%T	=	Transmittance
Conc.	=	Concentration
Rpm	=	rotation per minutes
RCM	=	Reinforced Clostridia Medium
NaOH	=	Sodium Hydroxide
HCl	=	Hydrocloride acid
UV-VIS	=	Ultra Violet Visible
OD	=	Optical Density
v/v	=	volume per volume
DNS	=	Dinitrosalicylic Acid
GC-FID	=	Gas Chromatography Flame Ionization Detector

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Research

Malaysia is the world's largest producer of palm oil. Its production generates various wastes chief among which is palm oil mill effluent (POME). POME is thick, brownish liquid with a discharged temperature in the range of 80^0C to 90^0C (Takriff *et al.*, 2009). POME is the largest by product generated from palm oil mills which is about 50 million tonnes annually. POME has a potential as a substrate for butanol production using *Clostridium beijerinckii*. Raw POME is a colloidal suspension containing 95–96%water, 0.6–0.7% oil and 4–5% total solids. Included in the total solids are 2–4% suspended solids, which are mainly constituted of debris from palm fruit mesocarp generated from three main sources, which is sterilizer condensate, separator sludge and hydrocyclone wastewater (Wu *et al.*, 2008). Compared to the currently popular fuel additive which is ethanol, butanol is more miscible with gasoline and diesel fuel. It also has a lower vapor pressure and less miscible with water. All of this make it a superior fuel extender. Butanol is currently used as a feedstock chemical in the plastic industry and as a food-grade extractant in the food and flavor industry (Formanek *et al.*, 1997).

The production of acetone, butanol, and ethanol (ABE) by fermentation is a process that had been used by industry for decades. However, this fermentation process for production of solvents was replaced by petrochemical methods in the 1920s, and by the 1960s. ABE fermentation was no longer in use industrially. There were several reasons for this shift including low ABE productivity (slow fermentation), low solvent yield and a substantially high cost of recovery, in particular, by distillation. Recent environmental concerns and the need to lessen the reliance on the diminishing petroleum supplies have renewed interest in obtaining solvents from renewable resources. The renewable resources is referring to include corn, whey permeate (a by-product of the dairy industry), soy molasses, cane molasses, and wood hydrolysate (Qureshi *et al*., 2000).

1.2 Identification of Problem

Nowdays world faced the waste to wealth concept to build a city with successful ventures providing solutions to environmental and social problems. Hence this research is to support the waste to wealth concept by taking palm oil mill effluent (POME) which is waste as substrate and fermentated to form butanol as petrofuel.

In term of economic view, cost of substrate is a major factor that influences the production cost of biofuels. Renewable resources including molasses, corn, whey and other agricultural by products have been used as substrate to produce butanol but they are expensive and limited sources. Hence, economically viable substrate such as POME should be used other than corn and whey.

Bioethanol is at present the most widely produced biofuel and is used both as an additive to petrol or gasoline and as a fuel in its own right in specially modified vehicles. It also suffers from a number of drawbacks, including low energy density, high vapour pressure and high solubility in water. Hence, butanol may be used as a fuel in an internal combustion engine because it does not suffer from the same drawbacks as ethanol.

1.3 Statement of Objectives

The objective of this research are as follow :

1. To study the effects of substrate concentration (POME) and agitation rate on butanol production from POME by *Clostridium beijerinckii* .

1.4 Scope of The Research

The main scopes of the research are:

1. To study the growth profile of *Clostridium beijerinckii*.
2. To study the effects of substrate concentration (POME) (between 70% to 90 %) on butanol production.
3. To study the effects of agitation rate (between 0 rpm to 250 rpm) on butanol production.
4. To study the glucose consumption in the fermentation broth.

1.5 Rationale and Significance

The rationale and significance of the research are:

1. POME should be used other than corn and whey as substrate in term of of economic view because POME cheaper and better to used waste than food product.
2. Butanol has alternative to replace the ethanol because ethanol corrosive in pipeline and need to modify engine.

CHAPTER 2

LITERATURE REVIEW

2.1 Palm Oil

The history of palm oil can be traced back to the days of the Egyptian paraohs 5000 years B.C. The palm oil however, is a native of West Africa. It was introduced to Malaysia at the start of the 20th century and commercially produced in 1917 (Haris, 2006*)*. Palm oil (*Elaeis guineensis*) is vastly cultivated as a source of oil in West and central Africa where it is originated from and in Malaysia, Indonesia and Thailand as well (Zakaria, 2008). Today Malaysia's palm oil plantations cover 40% of its cultivated land and it has become the world's largest producer and exporter of palm oil (Haris, 2006). Palm oil is one of the most important commercial items and accounts for 20% and 46% of the global oil and fat production and trade respectively. The oil palm planted area increased to 3.5 million hectares by 2001, occupying 60% of the agricultural land in the country and the industry still expanding corresponding to the growing world population (Zakaria, 2008).

Palm oil commercial value lies mainly in the oil that can be obtained from the mesocarp of the fruit palm oil and the kernel of the nut palm kernel oil. Palm oil is used mainly for cooking oil, margarine and shortening and has non-food applications such as soap, detergent and cosmetics (Haris, 2006).

2.2 Palm Oil Mill Effluent (POME)

The production of palm oil results in the generation of large quantities of polluted wastewater, commonly referred to as palm oil mill effluent (POME). At an average about 0.1 tonne of raw POME is generated for every tonne of fresh fruit bunch processed. Typically, 1 tonne of crude palm oil production requires 5-7.5 tonnes of water; over 50 % of which ends up as POME (Lorestani, 2006). Based on palm oil production in 2005 (14.8 million tonnes), an average of about 53 million m^3 POME is being produced per year in Malaysia. POME consists of water soluble components of palm fruits as well as suspended materials like palm fibre and oil. The POME comprises a combination of wastewater from three main sources via clarification (60%), sterilization (36%) and hydrocyclone (4%) units. It contains various suspended components including cell walls, organelles, short fibres, a spectrum of carbohydrates ranging from hemicellulose to simple sugars, a range of nitrogenous compounds from proteins to amino acids, free organic acids and an assembly of minor organic and mineral constituents (Lorestani, 2006).

The palm oil mill industry in Malaysia has thus been identified as the one discharging the largest pollution load into the rivers throughout the country (Wu *et al*., 2008). From environmental perspective, fresh POME is a hot and acidic brownish colloidal suspension, characterized by high amounts of total solids (40,500 mg/l), oil and grease (4000 mg/l), COD (50,000 mg/l) and BOD (25,000 mg/l). The characteristic of a typical POME is shown in Table 2.1. Despite its biodegradability, POME cannot be discharged without first being treated because POME is acidic and has a very high biochemical oxygen demand (BOD). Raw POME is high in BOD and acidic with pH of around 4.0. After treatment, the pH is raised to around 8 and BOD is lowered. In terms of nutrient value, anaerobic sludge of treated POME contains high plant nutrients.

Table 2.1. Typical characteristics of POME (Lorestani, 2006).

Parameter	***Average**	**Metal**	***Average**
pH	4.7	Phosphorous	180
Oil and Grease	4000	Potasium	2270
Biochemical Oxygen Demand (BOD5)	25000	Magnesium	615
Chemical Oxygen Demand(COD)	50000	Calcium	439
Total Solids	40500	Boron	7.6
Suspended Solids	18000	Iron	46.5
Total Volatile Solids	34000	Manganese	2.0
Ammonical Nitrogen	35	Copper	0.89
Total Nitrogen	750	Zinc	2.3

2.3 Butanol

Butanol is a four carbon alcohol. It has double the amount of carbon of ethanol. Butanol is produced by fermentation, from corn, grass, leaves, agricultural waste and other biomass. Butanol can be produced from biomass and from mineral fuel. The butanol from biomass is conveniently denoted as biobutanol despite the fact that it has the same characteristics as the butanol from petroleum. Butanol is a cleaner and superior fuel extender or oxygenate than ethanol with octane numbers 113 and 94 as compared with that of 111 and 94 for ethanol (Qureshi *et al*., 2007).

Industrial producing of butanol began in 1916. Now butanol is produced starting with petroleum via hydrolysis of haloalkanes or hydration of alkenes. Butanol can be produced from biomass and from mineral fuel. The butanol from biomass is conveniently denoted as biobutanol despite the fact that it has the same characteristics as the butanol from petroleum. Butanol which is an excellent biofuel has numerous other applications in the food, plastics and chemical industries (Ezeji *et al*., 2004). World market for this product is estimately 350 million gallons per year, of which 220 million gallon/year is the fraction consumed by USA. Butanol can be used instead of gasoline even in higher degree than ethanol due to its physical properties, economy, safety and because it can be applied without remodeling car engine. Progress in the area of biotechnology allows to use corn and other biomass as economically effective source of biobutanol. The main reason that nobody know butanol as an alternative fuel is the fact that its producing has never been suggested economically reasonable.

The alcohol based fuel including butanol and ethanol is partially oxidized (compared to hydrocarbons) and therefore mixture for engine should be more enriched than in the case of gasoline. As compared with ethanol, butanol can be used as a mixure with gasoline in higher proportion and thus can be used in currently working cars without modification of their system for the formation of air fuel mixture. The alcohol based fuel contains less energy per unit of weight or volume than gasoline and its mixture with air should be more enriched. Per one cycle of engine running, butanol liberates more pure energy than ethanol or methanol and approximately by 10% more than gasoline (Shapovalov and Ashkinazi, 2008). Butanol in particular appears to be a good choice as a fuel additive to reduce exhaust smoke and particulates (Qureshi *et al*., 2000).

Owing to appearance of new highly effective technologies for producing biobutanol, now butanol from corn attracts more attention of specialists than ethanol. There are several advantages of butanol over ethanol which are butanol contains by 25% more energy than ethanol 110 000 Britain heat units per gallon as compared with 84 000 Britain heat units per one gallon of ethanol. Energy content of gasoline is about 115 000 Britain heat units per gallon. Butanol is more safe because is evaporated six times less than ethanol and by factor 13.5 less volatile than gasoline. Its vapor pressure by Reid is 0.33 pounds per squire inch, the same characteristic of gasoline is 4.5 pounds per squire inch, of ethanol 2.0 pounds per squire inch. This makes butanol safe at its application as oxygenate, and need no significant changes in the mixture proportion at summer and winter application. Now it is used as oxygenate in the states Arizona, California and others.

Butanol is much less aggressive substance than ethanol and therefore it can be transported with currently used fuel pipes, while ethanol should be transported by rail way transport. Butanol can be mixed with gasoline. Butanol can be used instead of gasoline, while ethanol can be used as additive to benzene with content of the latter in the mixture not less than 85% and requires significant modernization of engine. Currently predominant are used mixtures with content of ethanol 10%. Producing of butanol can simultaneously solve problems connected with the infrastructure of supplying hydrogen. Butanol provides higher yield of energy (10 Wt-h/g) than ethanol (8 Wt-h/g). At combustion, butanol does not produce sulfur and nitrogen oxides that is advantageous from the ecology viewpoint (Shapovalov and Ashkinazi, 2008).

Butanol is generally produced in concentrations of no greater than 12 g/liter. This limitation is thought to be due to the toxicity of butanol to *Clostridium* (Evans and Wang, 1988). Butanol is an important industrial solvent and potentially a better fuel extender than ethanol. 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.

Table 2.2 Applications of Butanol

Produce	• dibutyl phthalate (as a precursor) • butyl acetate (as a precursor) • butyl acrylate (as a latex) • glycol ethers • amine resins
Other uses	• excellent fuel (it is miscible with gasoline and diesel fuel, has high calorific value, has a lower vapor pressure, and is less miscible with water) • used in plastic industry as a feedstock chemical • food grade extractant • a solvent in the manufacture of oil • pharmaceuticals • perfumes

2.4 Fermentation

2.4.1 Anaerobic Fermentation

Biological treatment processes are cost effective processes that utilize microbial communities of varying degrees of diversity that interact in a multitude of ways to mediate a myriad of biological reactions. Anaerobic digestion has been widely accepted as an effective alternative for wastewater treatment and simultaneous fuel gas production. Its successful application arises from the development of new and innovative reactor designs. Compared to conventional aerobic methods of wastewater treatment, the anaerobic wastewater treatment concept indeed offers fundamental benefits such as low costs, energy production, relatively small space requirement of modern anaerobic wastewater treatment systems, very low sludge production (10-20 % of COD removed) with very high dewaterability, stabilized sludge and high tolerance to unfed conditions. (Lorestani, 2006).

2.4.2 Batch fermentation

Fermentations may be run in one of three modes of operation: batch, continuous or semi-continuous, also referred to as fed-batch. Large volume fermentations for the production of commodities are usually run in the fed-batch or batch modes, while batch operation is used for small-volume processes for the production of fine biologicals. Continuous fermentations are extensively used in research and development and in effluent treatment processes, but are seldom used in industrial fermentations, mainly because of the possibility of contamination and mutation (Simpson *et al*., 2005).

Batch fermentation by *Clostridium* is characterized by two phases. During the first phase or acidogenesis *Clostridium* grows and produces acetate and butyrate from glucose. These acids attain their maximal concentrations and are consumed in the second phase which is known as solventogenesis. The acids are reduced and neutral solvents including butanol, acetone, ethanol and acetoin are produced (Evans and Wang, 1988).

2.4.3 Acetone-butanol-ethanol (ABE) fermentation

The most commonly used metabolically derived liquid bio-energy compounds are ethanol and butanol. Butanol, along with small amounts of acetone and ethanol, is produced biologically from renewable biomass by *Clostridium* under strictly anaerobic condition. This has been called the “acetone–butanol–ethanol (ABE) fermentation” process (Tran *et al*., 2009). The production of butanol by ABE fermentation used to be one of the largest bioprocesses until the 1950s, but later it was replaced by the less expensive petroleum based chemical synthesis. In recent years, interest in bio-based butanol has been revived primarily due to concerns with fossil fuel depletion, and microbial production of butanol is considered to be a potential source of liquid fuels (Liu *et al*., 2004). The ABE fermentation was considered an important industrial process during the first half of the 20th century. This was based on the use of such substrates as corn (Casas *et al*., 2008). The production of ABE by *Clostridium*, which are Gram-positive, obligate anaerobic, spore-forming bacteria, was widely carried out industrially during the first half of the last century (Tashiro *et al*., 2005).

ABE using *Clostridium* could produce acetone, butanol, and ethanol from several biomass types such as palm oil waste, domestic waste and abundant agricultural crops (Shinto *et al*., 2008). There has been a revival of interest in ABE fermentation, since renewable resources as such domestic and agro-industrial wastes have become possible alternative substrates for the production of chemicals and liquid fuels (Kobayashi *et al*., 2005). The ABE fermentation with *Clostridium* was once one of the largest biotechnological processes, second in scale only to ethanol fermentation by yeast. It has a good future potential as a process to convert renewable resources including organic wastes into chemicals. This fermentation has attracted renewed interest as an alternative and environmentally friendly method for the production of solvents (Grube *et al*., 2002).

However, the market for ABE is still tight due to its high production costs. Substrate costs can make up to about 63% of the total cost of ABE production. This is not because of the expense of the substrate itself but mainly because of the low efficiency of *Clostridium* to convert substrate into ABE. This means that the yield of ABE is often low and this together with the formation of by products leads to the high cost for butanol recovery. In addition, the maintenance of strict anaerobic conditions for *Clostridium* to grow requires special conditions such as an addition of costly reducing agents into the medium and flushing with Nitrogen (N_2) gas. These factors additionally increase the costs of the fermentation process.

There are several possible ways to reduce the costs of producing ABE from fermentation. These include using the low cost fermentation substrate or optimizing the fermentation conditions to improve the efficiency of converting substrate to ABE. Another is genetic engineering which could produce highly effective strains able to utilize a variety of substrates so that the need to pretreat substrates will be reduced (Tran *et al*., 2009). Among the cheap and readily available substrates for ABE production is POME, possibly one of the better choices.

2.5 *Clostridium beijerinckii*

Clostridium, a group of Gram-positive,spore-forming, obligate anaerobes, naturally possess pathways that allow the conversion of sugar into solvents,known as acetone–butanol–ethnol (ABE) fermentation (Zheng *et al*., 2009). *Clostridium beijerinckii* is a gram positive, rod shaped, motile bacterium of the genus *Clostridium*. It has been isolated from feces and soil. Its ability to grow in simple, inexpensive media, stability in regard to strain degeneration, good adaptability to continuous processes and sustained production of solvents well into the log phase are other advantages of this bacterium.

The ABE fermentation process is interest for chemical fuel production from renewable resources. The bacterium involved *C. Acetobutylicum* or *C. beijerincki*i can utilize a wide range of substrates, while the fermentation products can be used in combustion engines (Qureshi and Blaschek, 2000).

Different *Clostridium* gender microorganisms have been used for solvents production. They were described by first time in 1880 by Prazmowski (Casas *et al*., 2008). The *Clostridium beijerinckii* (before *Clostridium acetobutylicum*) is a spore forming bacterium, strictly anaerobia condition at 37^0C that produces acetone, butanol and small quantities of ethanol in sugary substrates.

Investigations have been successful in developing a superior culture of *C. beijerinckii* which can produce and tolerate 23 gL^{-1} butanol and 33 gL^{-1} total solvents in batch culture under optimized conditions. This is an increase of 69% over the C. beijerinckii parental strain which produced only 15–20 gL^{-1} total solvents (Qureshi and Blaschek,2000).

CHAPTER 3

METHODOLOGY

3.1 Equipments

3.1.1 Anaerobic Chamber

Figure 3.1 Anaerobic Chamber

An enclosed anaerobic work station, anaerobic chamber was used to provide an oxygen-free environment for cell and tissue homogenization. This chamber uses palladium catalyst wafers and desiccant wafers to maintain strict anaerobiosis to less than 10 ppm oxygen gas (O_2). High purity nitrogen gas (N_2) is used for purging the