PRODUCTION OF BIOETHANOL FROM TAPIOCA STARCH USING *Saccharomyces cerevisiae*: EFFECTS OF pH AND AIR FLOW RATE

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PRODUCTION OF BIOETHANOL FROM TAPIOCA STARCH USING *Saccharomyces serevisiae*: EFFECTS OF pH AND AIR FLOW RATE

MUNA SUHAILI BINTI ZAKARIA

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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April 2008

I declare that this thesis entitled “Production of Bioethanol from Tapioca Starch Using *Saccharomyces cerevisiae*: Effects of pH and Air Flow Rate” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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Special Dedication to 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

The effects of pH and air flow rate on fermentation process for bioethanol production in 2 L bioreactor using *Saccharomyces cerevisiae* were studied. Other conditions such as temperature, agitation and inoculum concentration were fixed at specific values. Two-step enzymatic hydrolysis of tapioca starch by commercially available α-amylase and glucoamylase were employed at the beginning of the process for glucose production. The cell growth study was conducted in order to observe the cell growth profile in the resulted sugar. The fermentation study was conducted in aerobic condition using 2 L bioreactor with various pHs (4, 5, and 6) and air flow rates (1.0, 1.5 and 2.0 L/min). Based on the results, the optimum pH and air flow rate were pH 5 and 1.0 L/min respectively. The highest yield of ethanol was 3.64 g/L which produced at pH and air flow rate at 1.0 L/min.

ABSTRAK

Kesan pH dan kadar aliran udara terhadap proses penghasilan etanol menggunakan *Saccharomyces cerevisiae* di dalam bioreaktor 2 L telah dikaji. Keadaan lain seperti suhu, pengadukan dan kepekatan inokulum telah ditetapkan pada nilai-nilai tertentu. Proses hidrolisis enzim oleh α-amilase dan glucoamilase digunakan terhadap kanji ubi kayu bagi penghasilan gula. Kajian pertumbuhan sel telah dijalankan bagi mengenal pasti profil pertumbuhan sel pada gula yang dihasilkan. Proses fermentasi telah dijalankan di dalam bioreaktor 2 L pada keadaan aerobik dengan menggunakan pelbagai pH (4, 5, and 6) dan kadar aliran udara (1.0, 1.5 and 2.0 L/min) dijalankan. Berdasarkan data yang diperolehi, keadaan pH dan aliran udara yang optimum adalah masing-masing pH 5 dan 1.0 L/min. Kadar etanol yang tertinggi adalah sebanyak 3.64 g/L yang dihasilkan pada pH 5 menggunakan aliran udara 1.0 L/min.

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LIST OF SYMBOLS/ABBREVIATIONS

Ca^{2+}	-	ion calcium
CO_2	-	carbon dioxide
DNS	-	Di-Nitro Salicylic Acid
Glu.	-	Glucose
g	-	gram
h	-	hour
KNU	-	kilo
mg/L	-	milligram per liter
Mg^{2+}	-	ion magnesium
min	-	minutes
mL	-	mililiter
Mm^3	-	megameter
v/v	-	volume per volume
v/w	-	volume per weight
w/v	-	weight per volume
w/w	-	weight per weight
µg/mL	-	microgram per mililiter
%	-	percentage
°C	-	degree Celsius
°F	-	degree Fahrenheit
µmol	-	micromole

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ethanol (ethyl alcohol, grain alcohol) is a colourless, clear liquid and completely miscible with water. In dilute aqueous solution, ethanol has sweet flavor, but in concentrated form, it has a burning taste, and an ether-like odor. Ethanol is also known as ethyl alcohol (CH_3CH_2OH) or fermentation alcohol. It is referred to a group of chemical compounds, whose molecules contain a hydroxyl group, –OH, bonded to a carbon atom. Ethanol has been proposed as a valuable liquid fuel and an alternative to crude oil (Wyman, 2004).

Bioethanol is mainly produced by sugar fermentation process. Industrial ethanol production has utilized using various starchy materials such as corn, wheat, rice or waste straw, potatoes and sago starch as its raw materials. One of the greatest challenges for the 21st century is to meet the growing demand of bioethanol production as energy in sustainable way (Wyman, 2004).

Bioethanol has the potential to provide partial solution to the world's energy whereby it has high octane fuel and able to replace lead as an octane enhancer in petrol. Bioethanol may significantly reduce the amount of imported oil and also allowing large savings in imports costs or increased revenues from export countries (Suraini, 2002).

Blending ethanol with gasoline will oxygenate the fuel mixture caused the fuel to burn completely and reduces polluting emissions (Alexender, 2008).

An agricultural raw material such as rice, wheat and starch is the major carbohydrates suppliers in Malaysia. Tapioca starch has a great potential as an alternative cheap carbon source for fermentation which attract both economic and geographical considerations. In 2003, the world's bioethanol production was 23 Megameter^3 (Berg, 2004). The major world producers, Brazil and United States are together account for about 80% of the world production. The main feedstock for bioethanol production in Brazil is sugarcane while USA utilizes corn grain (Mojović *et al*., 2006).

Production of bioethanol from starch requires the conversion of polymer starch into glucose at the first place. A fermentable sugar is normally recovered by an enzymatic process that comprises two reaction steps which are liquefaction and saccharafication. Afterward, the process was followed by fermentation process for bioethanol production (Suraini, 2002). α-amylase and glucoamylase are used for enzymatic hydrolysis of starchy materials while *Saccharomyces cerevisiae* yeast is employed in fermentation process (Mojović *et al*., 2006).

1.2 Objective

The aim of this research is to determine the optimum conditions of few parameters in fermentation process for the production of bioethanol from tapioca starch. Hence, the objectives of this research are:

i. To determine the effect of pH on the production of bioethanol from tapioca starch.
ii. To determine the effect of air flow rate on the production of bioethanol from tapioca starch.

1.3 Scope of Study

Bioethanol production has been conducted by two-step process; enzymatic hydrolysis followed by fermentation process. Various air flow rate and pH in fermentation process were investigated. The bioethanol concentrations produced and glucose consumption were analyzed during the entire study. Other parameters such as enzymatic hydrolysis parameters, fermentation temperature, agitation speed and inoculums concentration were fixed at specific conditions.

1.4 Problems Statement

In 2000, the United States consumed almost 10^{17} BTUs of energy, with almost 40% coming from imported petroleum. High dependence on imported oil would expose to price vulnerability and availability disruption as occurred during "energy crises" on 1970s. Additionally, vehicles miles and total number of vehicles are continued to rise, thus, increased the petroleum demand. Petroleum presents as a finite resource and cannot be sustained indefinitely, and its price will increase over time. Hence, abundant alternative domestic energy sources for transportation are vital (Wyman, 2004).

According to *Oil & Gas Journal* (Malaysian Energy Data, Statistics & Analysis - Oil, Gas, Electricity, 2007), Malaysia held oil reserves of 3.0 billion barrels as of January 2007, down from a peak of 4.6 billion barrels in 1996. Average production for 2006 stood at 798,000 barrels/day decreased by 7 % from 2005's. During 2006, Malaysia consumed an estimated of 515,000 barrels/day of oil while the net exports was about 283,000 barrels/day (Malaysian Energy Data, Statistics & Analysis - Oil, Gas, Electricity, 2007). Malaysia's oil production and consumption during 1998-2008 is given by Figure 1.1.

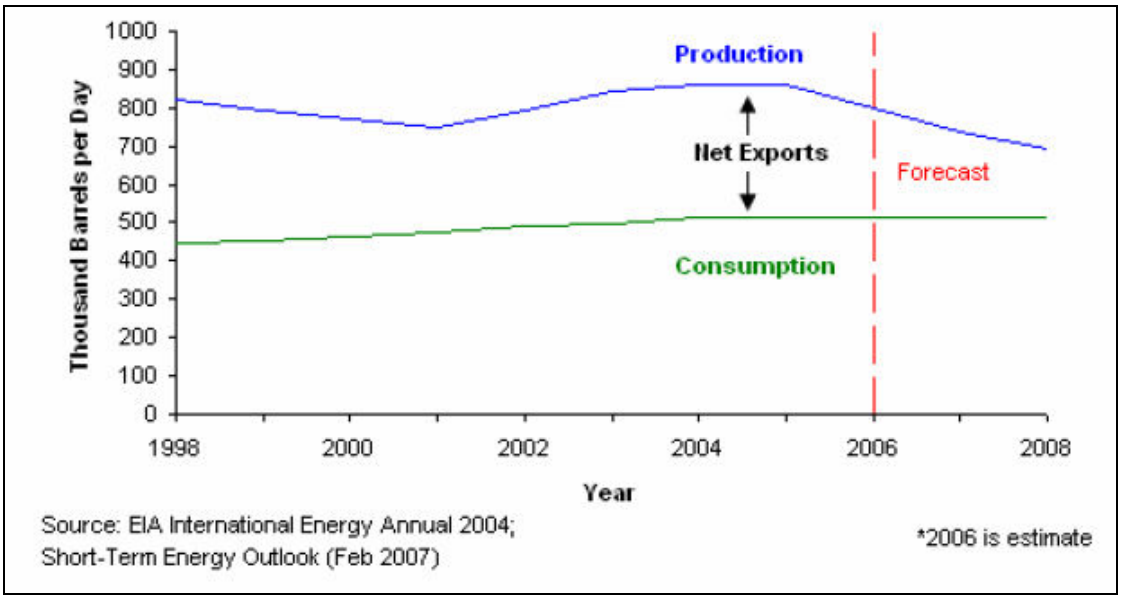

Figure 1.1: Malaysia's Oil Production and Consumption, 1990-2008* (Malaysian Energy Data, Statistics & Analysis - Oil, Gas, Electricity, 2007)

An alternative source for fuel and others petroleum-based products is critically needed. The global demand and unstable conditions in worldwide prices has forced developing country such as Malaysia to re-evaluate their ability to meet their future energy replacements (Malaysian Energy Data, Statistics & Analysis - Oil, Gas, Electricity, 2007).

Bioethanol posed as a valuable liquid fuel alternative to transportation sector. It is one of the most important renewable fuels that contribute to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels. The United States for example has become the largest contributor of greenhouse gases where burning petroleum has caused about 43% of carbon dioxide release into the atmosphere each year. Bioethanol performs as a better transportation fuel as it substantially improves urban air quality and reduces environmental effects. Thus, bioethanol has a great potential and high efficiency for reducing petroleum consumption and practically environmental friendly compared to the occasional fuel from gasoline (Wyman, 2004).

1.5 Rationale and Significance

In Brazil, transportation sector uses over two-thirds of the petroleum consumed; with over 96% of transportation energy comes from this ultimate source. Thus, it will continue on increasing the world petroleum demand. Many other countries also suffer from this similar oil-related strategic which resulted in significant economic difficulties (Wyman, 2004). Therefore, abundant alternative domestic energy sources such as bioethanol would dramatically reduce the total dependent on petroleum. Bioethanol also present as cheap energy source that would be a better alternative fuel compared to occasional fuel. The blending bioethanol with petrol would help to prevent the diminishing of oil supplies and ensure greater fuel security, avoiding heavy reliance on oil producing nations (Prasad *et. al*., 2007).

Bioethanol has a favourable fuel, resource, and environmental attributes as a transportation fuel. In quantities up to 5%, bioethanol can be blended with conventional fuel without the need of engine modifications. Bioethanol is also biodegradable and far less toxic that fossil fuels. Ethanol contains 35% oxygen that helps complete combustion of fuel and thus reduces particulate emission that pose health hazard to living beings. In addition, using bioethanol in older engines can help reduce the amount of carbon monoxide produced by the vehicle thus improving air quality. Thus, bioethanol would give better urban air quality and reduce environment effects of consuming fuels (Chandel *et. al*., 2009).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In the past, fossil fuels were used for transportation and it is totally relied upon the petroleum supply. During the last few decades, the petroleum supply was continuously diminished. There has been a considerable interest in the development of fuels generated from renewable resources, that is to say bio-fuels (Marcos *et. al*, 2007). Ethanol is a favourable fuel and environmental friendly transportation fuel. Ethanol can be made from starch, sugar, abundant, sustainable sources of low-cost cellulosic biomass such as agricultural and forestry residues, portions of municipal waste, herbaceous and woody crops. The transformation of the energy-rich crops such as sugar cane, corn and starch or lignocellulosic biomass requires the pretreatment of the feedstock for fermenting organisms to convert them into ethanol (Cardona, 2007).

The development of cost-effective technologies for fuel ethanol production is a priority for many research centers, universities, private firms, and even for governments. Process engineering applied into the production of bioethanol includes the design of new innovative process configurations, aimed at reducing ethanol production costs. On the other hand, the development of environmental friendly technologies for bioethanol production can be carried out by utilizing the different design approaches. Particularly, the production of bioethanol gives lower impact on global climate change of the

greenhouse gas emissions. Ethanol has been trusted as an alternate fuel for the future and is already produced on a fair scale (about 14-26 million tons) worldwide. The bulk of the production is located in Brazil (16 billion liters produced in 2005) and the USA (10.6 billion liters in 2003) (Hamelinck *et al*. 2005). Bioethanol is expected to be one of the dominating renewable biofuels in the transportation sector within the next 20 years (Hägerdal *et al*. 2006).

2.2 Background of Ethanol

Ethanol is known as ethyl alcohol or fermentation alcohol. Ethanol is a member of the alcohol family which has a chemical formula C_2H_5OH. It is a monohydric primary alcohol. Ethanol melts at -117.3°C and boils at 78.5°C. It is miscible with water in all proportions and is separated from water only with difficulty. Other than chemically produced from ethylene, ethanol can be produced from starch, sugar crops such as sugar-cane, maize, sorghum, and wheat and other grains, or even cornstalks, fruit and vegetable waste. Ethanol also can be made from cellulosic biomass such as agricultural residues, industrial waste, herbaceous plants and portions of municipal waste. Ethanol is used extensively as a solvent in the manufacturing of varnishes and perfumes, essences and flavorings, medicines and drugs, and as a fuel and gasoline additives (Wyman, 2004).

2.3 Ethanol Production from Sugar Fermentation

Ethanol can be produced by two routes; (1) fermentation of sugars derived from sugar, starch, or cellulosic materials or (2) reaction of ethylene with water. Production of ethanol from sugar required microbial fermentation. The fermentable raw materials can be categorized as fermentable sugary materials, starchy or lignocellulosic

materials. Starch and cellulose components in those raw material can be transformed into sugars specially glucose that be used as fermentation substrates (Equation 2.1). In sugar fermentation, the phosphorylation of carbohydrates is carried out through the metabolic pathway and the end products are two moles of ethanol and carbon dioxide (Prasad *et. al*, 2007). Two moles of ethanol and CO_2 were produced for every mole of glucose consumed (Equation 2.2).

$(C_6H_{10}O_5)n + nH_2O \rightarrow nC_6H_{12}O_6$ (Equation 2.1)

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$ +energy (stored as ATP) (Equation 2.2)

Since the yeast cannot use starch directly for ethanol production, starch has to be wholly convert into glucose where two known enzymes, which are α-amylase and glucoamylase are basically used. Hence, ethanol production from starch involves enzymatic hydrolysis to release fermentable sugar, followed by fermentation with yeast (Prasad *et. al*, 2007).

2.4 Raw Materials or Feedstock for Ethanol Fermentation

2.4.1 Sugar

Direct sugar feedstock for bioethanol is essentially comprised of sugar cane and sugar beet. Direct fermentation of sugarcane, sugar beet and sweet sorghum to produce ethanol has been widely reported. Sugar containing materials required the least costly pretreatment, whereas starchy, lignocellulosic materials and industrial wastes needed prior pretreatment to convert into fermentable substrates (Prasad *et. al*, 2007).

2.4.2 Starch

Another type of feedstock which can be used for bioethanol production is starch-based materials. Starch is a biopolymer consisting only one monomer, D-glucose. To produce bioethanol from starch, it is necessary to break down the chains of this carbohydrate for obtaining glucose, which can be converted into bioethanol by yeasts (Figure 2.1). This is the most utilized feedstock for bioethanol production in North America and Europe where corn and wheat are mainly employed (Balat *et.al*, 2008).

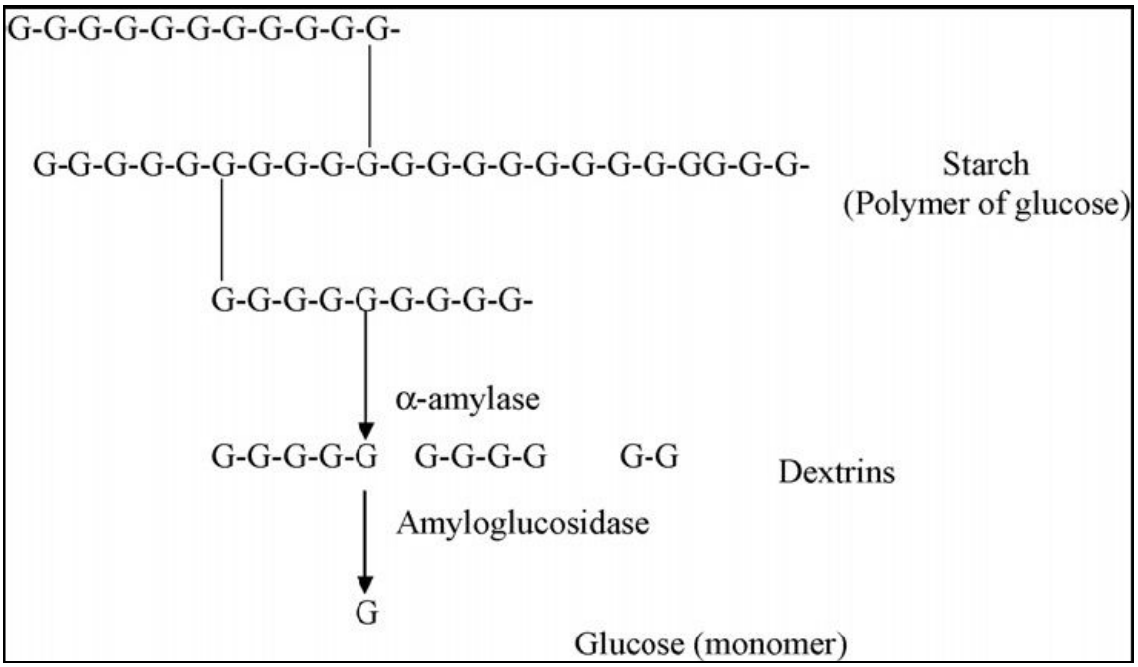

Figure 2.1: Scheme of an enzymatic hydrolysis of starch to glucose (Prasad *et.al*, 2007).

Starch is the major dietary source of carbohydrates, and the most abundant storage polysaccharide in plants, occurring as granules of size 1 to 100 μm (Phillips and Williams, 2000). Starch is composed of a mixture of two kinds of polyglucans, namely amylose and amylopectin. The structure of amylose and amylopectin is shown in Figure 2.2. Starch is the most essential component in most of the food preparation and also the major source of calories in the tropics.

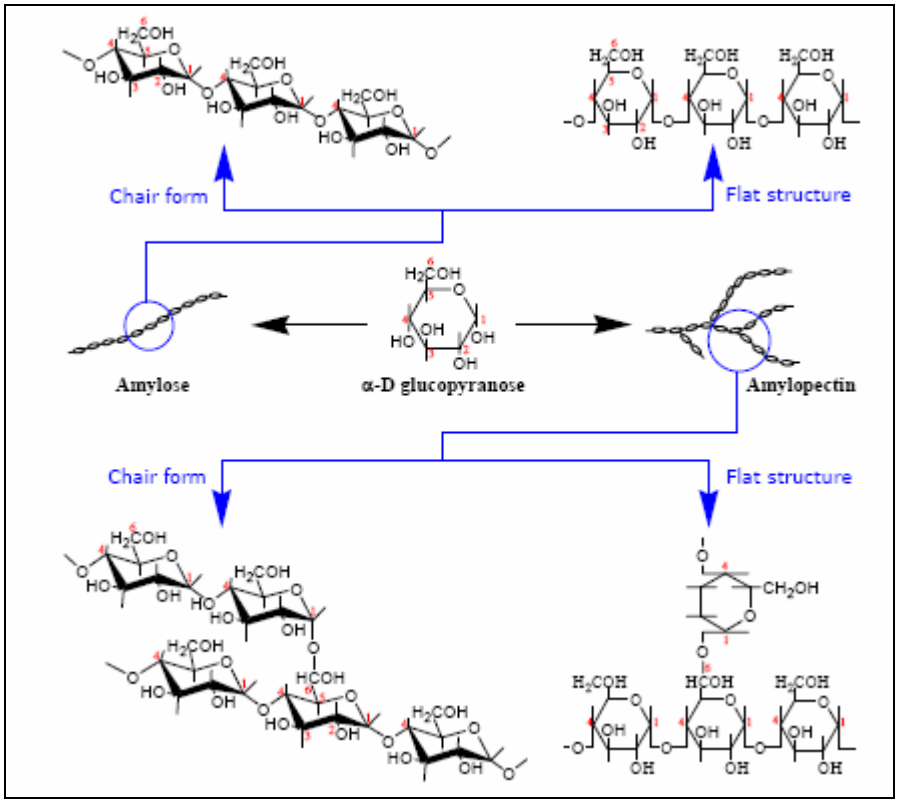

Figure 2.2: Scheme representing the linear and branched starch building polymers. (Marcos *et. al.*, 2007).

Industrial ethanol production has been reported using various starchy materials such as starch, corn, wheat, potatoes, and cassava root. Cassava (*Manihot esculenta*) is a perennial woody shrub with up to 32% of starch content (Nigam and Singh, 1995). Tapioca starch is a potential raw materials which rich in fermentable carbohydrates. It is abundantly found in Malaysia and the prices are cheaper compared to the starchy-based materials. Nutritional value of the cassava tubers is given by Table 2.1.

Table 2.1: Nutritional composition of cassava tubers per 100 g of edible portion (Bajaj, 1999).

Nutrients	Tubers
Calories	149 %
Calcium	68 mg
Water	62 mL
Carbohydrates	35 g
Vitamin C	31 mg
Vitamin A	30 mg
Iron	1.9 mg
Protein	1.2 g
Fiber	1.1 g
Fat	0.2 g

2.4.3 Ligno-Cellulosic Material

Another feedstock for ethanol production is rougher and woodier parts of plants so-called “lingo-cellulosic material”. This field has gained attention in the latest decades, as lignocellulosic biomass is a potential source for ethanol that is not directly linked to food production (Chum and Overend, 2001). The conversion of lignocellulosic material to ethanol is generally more complex, compared to sugar and starch. In case of lingo-cellulose, more drastic hydrolysis steps are necessary for achievement of high conversion yields (Marcos *et. al*, 2007).

The used of lingo-cellulosic biomass feedstock to produce ethanol would face several problems. The lignin sheath, present in all lingo-cellulosic materials would prevent the hydrolysis restriction. Therefore, in order to produce ethanol from lingo-cellulosic biomass feedstock by fermentation, thermo-mechanical, mechanical, chemical or a combination of these pretreatments are necessary before the feedstock is ready for fermentation (Regulation of Fuels and Fuel Additives: Renewable Fuel Standard Program; Final Rule, 2007).

2. 5 Enzymes and Microorgasnisms

Endo-1, 4-α-D-glucan glucohydrolase (α-amylase) is currently used in a broad array of industrial applications. Since 1980, the most widely used enzyme for these applications would be the α-amylase isolated from the ubiquitous mesophilic soil bacterium *Bacillus licheniformis*. Liquefaction involved with a process of semi-purifying starch to glucose oligomers by the *B. licheniformis* α-amylase, ideally conducted at a pH 6 and a temperature of 90 °C (Richardson *et. al,* 2002)**.** The major end products from the action of α-amylase on starch are glucose, maltose, malotriose, maltotetraose, maltopentose, and maltohexaose (Nigam and Singh, 1995).

Enzymes amyloglucosidase is also known as glucoamylase. Crude glucoamylase enzyme isolated from *Aspergillus niger* was normally used for the saccharification of liquefied starch. The optimum of glucoamylase activity was at pH 5 with a temperature of 60°C. Prior to this final step, the calcium added in the first step and the salts generated from the second step must be removed. Removal of these compounds is an expensive part of the overall process and could be avoided if the liquefaction step capable without the addition of calcium (Richardson *et. al,* 2002).

Saccharomyces cerevisiae is a traditional yeast and its characteristic make it easy to be transposable for industrial applications. *S. cerevisiae* mostly used for the leavening of dough and for the production of ale and distilled beverages. The choice of *S. cerevisiae* was conditioned by ethanol industry due to their competitiveness to several properties such as fast growth, efficient glucose repression, good ability to produce and consume glucose, and a tolerance for several environmental stresses (Piskur *et. al*, 2006).

2.6 Acid Hydrolysis

Dilute acid hydrolysis is the oldest technology for converting cellulose biomass to sugar production. The dilute acid process normally uses a 1% sulfuric acid in a continuous flow reactor at about 215°C. The process involves two reactions with a sugar conversion efficiency of about 50 %. One way to decrease sugar degradation is to use a two-stage process which the first stage is conducted under mild process conditions to recover the 5-carbon sugars, while the second stage is conducted under harsher conditions to recover the 6-carbon sugars. Both hydrolyzed solutions are then fermented to ethanol. Lime is used to neutralize the residual acid before the fermentation stage. The residual cellulose and lignin are used as boiler fuel for electricity or steam production (Regulation of Fuels and Fuel Additives, 2007).

Concentrated acid hydrolysis, on the other hand uses a 70% sulfuric acid solution followed by water hydrolysis. The reaction times are typically slower than those of the dilute acid process. The critical factors needed to make this process economically viable are to optimize sugar recovery as well as acid for recycling. The concentrated acid process is somewhat more complicated and requires more time, but it has the primary advantage of yielding up to about 90% of sugars. In addition, a significant advantage of the concentrated acid process is that it is carried out at relatively low temperatures and low pressure (Regulation of Fuels and Fuel Additives, 2007).

However, in this acid hydrolysis process, high capital cost is unavoidable because of expensive corrosion resistant equipment, acid recovery plants and higher operation costs. Moreover, one of the major problems with hydrolyzates produced by acid hydrolysis is the poor fermentability caused by the presence of inhibitors in the hydrolyzates. Furfural is known to be one of the most important of these inhibitors whereby it is a degradation product from pentoses and which formed in a browning reaction during hydrolysis in the presence of strong acids. It is impossible to completely

avoid furfural formation in a chemical hydrolysis process designed to give a high sugar yield (Taherzadeh *et. al*., 1999).

2.7 Enzymatic Hydrolysis

There are two processes involve in enzymatic hydrolysis; liquefaction and saccharification. The main role of enzymatic hydrolysis is to effectively convert the starch polymers (amylose and amylopectin) into fermentable sugar that could subsequently be converted to ethanol by yeast. Starch molecules are glucose polymers linked together by the α-1, 4 and α -1, 6 glucosidic bonds. The initial step in the depolymerization is the splitting of large chains into various smaller sized segments. The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called *liquefaction*. The α-amylase attacks the second linkage from the non-reducing terminals of the straight segment, resulting in the splitting off of two glucose units at a time. The product is called maltose. The final stage of depolymerization is called *saccharification* (Kim and Park, 2004). The bond is breakage more extensive by saccharifying enzymes than by liquefying enzymes. The starch chains are literally chopped into small bits and pieces. Finally, the amyloglucosidase attacks the last bond on the nonreducing terminals to form mono-, di-, and tri-saccharides (Wang, 2007).

2.7.1 Liquefaction

The starch content of tapioca powder was estimated to be 95% and moisture content 2% (Aggarwal *et al*., 2001). Starch hydrolysis can be carried out by two basic ways – acidic and enzymatic. The traditional method is acidic hydrolysis which requires highly acidic medium (pH 1-2) obtained through mineral acids, high temperatures (150-

230 ºC) and also high pressure (Kolusheva and Marinova, 2006). Thermomechanical liquefaction of starch under elevated temperature, pressure and shear was demonstrated in the early 1970s (Oliveira *et. al*, 1996). As a result of thermal processing, acidic hydrolysis produces unnecessary byproducts which contaminate the end products-hydrolysate. Acid-catalyzed liquefaction causes furfural or substituted furfuraldehydes, and also gives poor yields of the desired sugar, thus, there has been an increasing trend to use bacterial α-amylase for liquefaction (Nehete *et. al*, 1992). The bacterial α-amylase enzymes attack the polysaccharide molecules in the inner part of the chain. They act on the amylase of starch so they destroy the spiral of the polysaccharide chain. The viscosity of the starch solutions is quickly lowered. In the beginning dextrin are obtained and if the enzymes act continuously, maltose accumulates (Kolusheva and Marinova, 2006).

Liquefaction under pressurized steam (104ºC) was found to be more effective than that of using water bath at 95ºC since the slurry of tapioca powder was liquefied in a significantly shorter time. 30% slurry of tapioca powder which was liquefied in 150 min under pressurized steam, stained blue even after 210 min at 95ºC. The slurry of 25% consistency was found to be more appropriate for this process as the liquefaction took only 45 min at 104 ºC and 120 min at 95 ºC. Since the pressurized heat yielded better results, the liquefaction using 25% slurry tapioca powder under this condition was carried out (Aggarwal *et al*., 2001). Starch slurry at 25% (w/w) consistency was found to be more appropriate for liquefaction process as liquefied starch with 3 to 5% reducing sugars resulted in maximum maltose yields and minimum glucose contamination after the hydrolytic step (Nehete *et. al*, 1992).

In order to determine the optimum temperature for liquefaction, tapioca slurry was prepared with temperature values 60, 70, 80, 90, and 100ºC. Temperatures higher than 100 ºC were not examined due to the intensive boiling and formation of foam of the suspension at atmospheric pressure. Optimal temperature was at 90 ºC as the foam almost does not form if the suspension is continuously stirred (Kolusheva and Marinova, 2006).

In order to determine the optimum pH for liquefaction, tapioca slurry was prepared with pH values of 5.0, 5.5, 6.0, 6.5 and 7.0 and liquefied using 0.15% enzyme dose. The results of starch–iodine reaction showed that efficient liquefaction of tapioca was achieved in a pH range of 6.5–7.0. To examine the effect of divalent ions on the process of liquefaction, various concentrations of calcium chloride and magnesium sulphate providing Ca^{2+} and Mg^{2+} ranging from 25 to 250 mg/l were added to the slurry. The results obtained suggested a requirement of Ca^{2+} was required for liquefaction process of similar duration in presence of Ca^{2+}. Therefore to reduce the enzyme dose from 0.15 to 0.10% (w/v), the calcium chloride supplementation was optimized and found to be 120 mg/l of Ca^{2+}. At concentrations of calcium lower than 120 mg/l, liquefaction required 0.15% enzyme concentration i.e. 50% more. Supplementation of divalent ions Mg^{2+} and Zn^{2+} does not show any effect on liquefaction (Aggarwal *et al*., 2001).

2.7.2 Saccharification

The second step of enzymatic hydrolysis process is saccharification of starch by using *Aspergillus niger* glucoamylase. The addition of buffer during the preparation of extruded cassava flour had no influence on enzyme susceptibility with glucoamylase. The processing conditions used for the extrusion produced a cassava flour extrudate expansion of 2.75% +/- 0.03. In terms of enzymatic activity, the extrusion procedure was shown to be an effective gelatinization process. Glucoamylase activity with extruded cassava flour (ECF) was higher than 99% of that using boiled cassava flour as substrate and 94.6% of that of the standard substrate (soluble cassava starch). Glucoamylase showed a very good stability at optimum conditions (pH 4.5, 55°C) with an estimated half-life of about 80 days. However, stability of glucoamylase was greatly affected at 60°C or higher suggesting a strict control of this parameter in the reactor (Lopez-Ulibarri and Hall, 1997).

The saccharification improved with the increasing enzyme units within the range of 10–30 U/ml. The enzyme was required at the concentration of 30 U/ml, which was close to the expected value to achieve 92% saccharification. Higher units did not prove effective. Addition of calcium chloride, magnesium and zinc sulphate will provide an indicated of irrespective of type and the concentration saccharification was similar in all cases as in control showing no effect of divalent ions for the level of saccharification in presence of these ions. Moreover, the efficiency of an enzymatic starch saccharification process depends on the activity of the glucoamylase and also on the purity of enzyme. The saccharification process also effective when the tapioca was liquefied under pressurized steam in autoclave. This is due to the decrease in the crystallinity of the granules as intermolecular hydrogen and hydrophobic bonds between double helices of the starch chains are broken during the melting process of gelatinization (Aggarwal *et al*., 2001).

2.8 Ethanol Fermentation

Ethanol fermentation by *S. cerevisiae* under anaerobic conditions is shown by Equation 2.2 in subtopic 2.3.

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2\ CO_2 \quad \text{(Equation 2.2)}$$

The fundamental physiological characteristic of beer and wine-brewing yeast, *Saccharomyces,* it's ability to degrade carbohydrates, usually six-carbon (C_6) molecules such as glucose, to two-carbon (C_2) components, in particular ethanol, without completely oxidizing them to CO_2, even in the presence of oxygen (Alexander and Jeffries, 1990). Moreover, *Saccharomyces cerevisiae* owe it's competitiveness to several properties such as fast growth, efficient glucose repression, good ability to produce and consume glucose, and a tolerance for several environmental stresses (Piskur *et. al*, 2006).

Before proceed to the fermentation process, enzymatic hydrolysis was basically performed for glucose production. During the fermentation process, the resulted sugar was consumed by *S.cerevisae* for the formation of ethanol. In order to attain a higher yield of ethanol and fermentation rate, main parameters of ethanol fermentation including temperature, pH, agitation rate, and initial substrate concentration were mainly investigated. Mojović *et al*. (2006) reported that simultaneous process of the second hydrolysis step and fermentation can be applied in order to reduce the time to complete the process and make beneficial energy savings. According to Liu and Shen (2007) findings, the optimal fermentation of ethanol production for 5 L bioreactor was achieved at 37 °C, 200 rpm, and pH 5.0.

Ethanol produced can be determined by Gas Chromatography (GC) with a FID detector. The internal standard method was applied. Chromatogram column and samples injection were stainless steel column and auto headspace samples injection. Ethanol and normal propyl alcohol were used for the standard curve and internal standard substance respectively. The column temperature was controlled at 200 °C. N_2 was used as a carrier gas (40 mL/min). The flow rates of H_2 and O_2 were 40 mL/min and 500 mL/min respectively. Samples of 0.3 μL were injected directly into the column. All determinations were done by means of standard curves, and results were the mean of two repetitions (Liu and Shen, 2007). Liu and Shen (2007) has conducted ethanol fermentation sugar resulted from stalk juice of sweet sorghum in 5L bioreactor. Glucose was depleted during the fermentation as the glucose was continuously consumed by the yeast and converted to ethanol. The result of ethanol accumulation and glucose consumption is illustrated as in Figure 2.3.

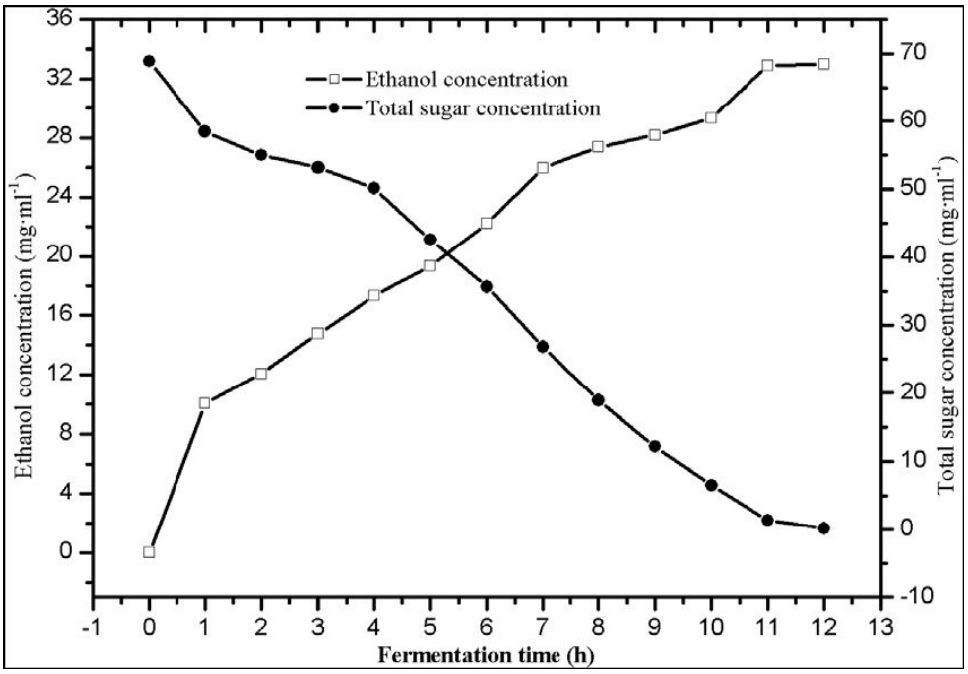

Figure 2.3: The result of ethanol accumulation and glucose consumption using 5L bioreactor (Liu and Shen, 2007).

CHAPTER 3

METHODOLOGY

This chapter will discuss about the process of producing ethanol using enzymatic hydrolysis and fermentation process. Initially, the growth profile has been conducted shake flask 250 mL in aerobic condition. The effects of pH and air flow rate on production of bioethanol were evaluated in 2 L bioreactor. The reducing sugar, cell optical density and ethanol concentration were analyzed.

3.1 Starch

Tapioca flour was purchased from the local market and used as the starchy substrate for the sugar production.

3.2 Yeast

The yeast for fermentation of tapioca starch is *Saccharomyces cerevisiae*.

3.3 Enzymes

A heat-stable α-amylase from *Bacillus licheniformis* was used for tapioca flour liquefaction. This enzyme activity is 144 KNU/mL (KNU, kilo novo units α-amylases-the amount of enzyme which breakdown 5.26 g of starch per hour). Meanwhile, glucoamylase, *Aspergillus niger* glucoamylase with the enzyme activity 240 AGU/g was utilized for tapioca flour saccharification (AGU is the amount of enzyme which hydrolyses 1 μmol of maltose per minute for 100 g of starch under specified conditions).

3.4 Hydrolysis Experiment

Tapioca starch, 100 g was mixed with water in 1:3 of tapioca to water weight ratio and 120 mg/L of Ca^{2+} (as $CaCl_2$) ions was added. The mixture was treated with enzymes in two steps. In the liquefaction step, 80 μL (11.52 KNU/mL) of α-amylase was uitlized at 85ºC, pH 6.0 and 150 rpm for 1 hour in water bath. For the saccharification step, 57.5 μg (38 AGU/g) of glucoamylase was used at 55 ºC, pH 5.0 and 150 rpm for the next 4 hours.

3.5 Fermentation Procedures

3.5.1 Preparation of Medium Culture

The medium in 250 mL shake flask containied 1 g/L of glucose, 1 g/L of yeast extract, and 1 g/L of the peptone. A 100 mL of liquid medium solution is prepared from 50 mL glucose and a mixture of 50 mL peptone and yeast extract. Both solutions were then placed in an autoclave for 15 minutes at temperature 121 ºC. The glucose and peptone were autoclaved separately to avoid Maillard reaction. In 2 L bioreactor, the

larger amount of liquid medium was prepared. A 2000 mL of liquid medium solution is prepared consisted of 1000 mL glucose and 1000 mL peptone and yeast extract.

3.5.2 Seed Culture Preparation

Eighteen milliliters of solution from medium culture was poured into a 100 mL conical flask. Then 0.18 g of *Saccharomyces cerevisiae* were put inside the seed flask and incubated for about 6 hours at 25 ºC and 150 rpm.

3.5.3 The Study of Cell Growth Profile

To determine the optimum time for cell growth during fermentation, the fermentation process in 250 mL shake flasks was performed. Nine shake flasks were required for preparation of cell growth profile corresponding with the time needed (6-48 hours). All the samples were carried out in duplicate. 18 mL of seed culture and 162 mL of medium was prepared for all flasks. Next, the seed culture solutions were transfered to the conical flask contained 162 mL media. An aseptic technique was applied to avoid the probability of contamination on the fermentation process. The mixtures are fermented at 25 ºC and 150 rpm. After all the samples were analyzed to determine the Optical Density (OD) of cell pellet and the total reducing sugars.

3.5.4 Fermentation Preparation

From seed culture solution, 18 mL of the solution was transferred into a 250 mL conical flask which contained 162 mL of media. An aseptic technique (laminar air flow, steriled pipette, Bunsen burner, 70% ethanol) was employed to avoid the contamination.

The mixture was incubated for 24 hours at temperature at 25 ºC and 150 rpm. After that, 180 mL of seed culture were transferred into 2 L bioreactor which is contained 1620 mL of media. An aseptic technique was employed to reduce the risk of contamination during fermentation at 2 L bioreactor. The fermentation was carried out 48 hours and the sample was taken at the end of experiment.

3.6 Methods of Analysis

3.6.1 Preparation of Di-Nitro Salicylic Acid (DNS) Reagent

To determine the glucose concentration in each sample of hydrolysate**,** DNS method was performed by measuring the absorbance at 540 nm (Miller, 1959). In order to prepare the DNS reagent, a 50 mL of 2 M NaOH solution was initially prepared and 2.5 g of 3, 5-dinitrosalicylic acid was added into the solution of sodium hydroxide. The mixture was heated and stirred continuously on a hot plate stirrer. In a separated beaker, a 75 g of potassium-sodium tartarate was dissolved in 125 mL of distilled water. The solution also heated and stirred continuously to obtain a hot salt solution and well-mixed solution. Then, both mixtures were transferred to 250 ml Schott bottle and covered with aluminum foil to avoid light penetrate into the reagent. The mixed solution was then cooled down to ambient temperature.

3.6.2 OD Analysis for Cell Pellet

Ten milliliter sample was taken from every 1800 mL of fermentation broth for optical density (OD) analysis. The sample was centrifuged to separate the supernatant and cell pellet with 6000 rpm for 10 minutes and temperature at 4 ºC. Then, the cell pellet was dissolved with distilled water while the supernatant was stored for further

analysis of total reducing sugar and ethanol. Vortex was used to ensure the well-mixing the samples were analyzed using UV-visible Spectrophotometer.

3.6.3 Total Reducing Sugar Determination by DNS Method

One milliliter of supernatant sample was taken and put in a test tube. Then 1 ml of DNS reagent was added and the solution was vigorously shaken and later was heated at 90^{o}C for 10 minutes (Noranisah, 2005). After that, the sample is cooled at room temperature in a cold water bath for another 10 min. Then, the solution was later diluted with additional 8 mL of distilled water and analyzed for total reducing sugar concentration. The analysis of absorbance was carried out using UV-visible spectrophotometer at 540 nm.

3.6.4 Preparation of Standard Calibration Curve for Glucose

The standard calibration curve for glucose was constructed based on the data obtained after the standard glucose was tested by using DNS method. This graph was important in order to find the concentration of glucose in every sample being tested. A 1000 µg/mL standard glucose solution is prepared by adding 180.16 mg of D (+) - glucose anhydrous into 100 mL of distilled water. The solution is stirred until all glucose dissolved in the water. Next, 1 mL glucose solution of 10 different concentrations is prepared from 1000 µg/mL glucose solution. The data for the required concentrations and standard calibration curve can be found at Appendix B.1. After that, 1 mL of glucose with different concentration was put it in a test tube which containing 1 mL DNS reagent. Then, all the solution is being test with DNS method and analysis of absorbance from the sample can be observed by UV-visible spectrophotometer at 540 nm. The graph of standard calibration curve for glucose is given in Appendix B.

3.6.5 Ethanol Determination

The amount of ethanol was determined using flame ionization detector gas chromatography (FID-GC). The column employed was a capillary column, HP-INNOWax 19091 N-133 (30 m x 0.25 mm x 0.25 μm). The internal standard method was applied. The column temperature was controlled at 280 °C. Helium was used as a carrier gas (1.5 mL/min) at constant flow. Samples of 1 μL were injected directly into the column. All determinations were done by means of standard curves, and results were the mean of two repetitions. The graph of standard calibration curve for ethanol and the example for peak of bioethanol graph are shown at APPENDIX B in Figure B.5 and Figure B.10 respectively.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

In this study, the effects of pH and air flow rate on fermentation process for bioethanol production were investigated. The agitation speed of 150 rpm and 25°C temperature were employed for the entire study. This study were conducted in aerobic condition using various range of pH (4.0, 5.0 and 6.0) and air flow rate (1.0, 1.5 and 2.0 L/min). The cell optical density, glucose consumption and ethanol concentration were subsequently analyzed.

4.1.1 Cell Growth Profile

The growth profile of *Saccharomyces cerevisae* in the fermentation broth using glucose from tapioca starch was observed. From Figure 4.1, the cell was initially dropped at the first 6 hour. The trend so-called *lag phase* and it was occurred immediately after inoculation and it is a period of adaptation of cells to a new environment. The phase was strongly influenced by the age of inoculums where the age should be sufficient enough for the adaptation of cell to the growth medium (Shuler and Kargi, 2002).

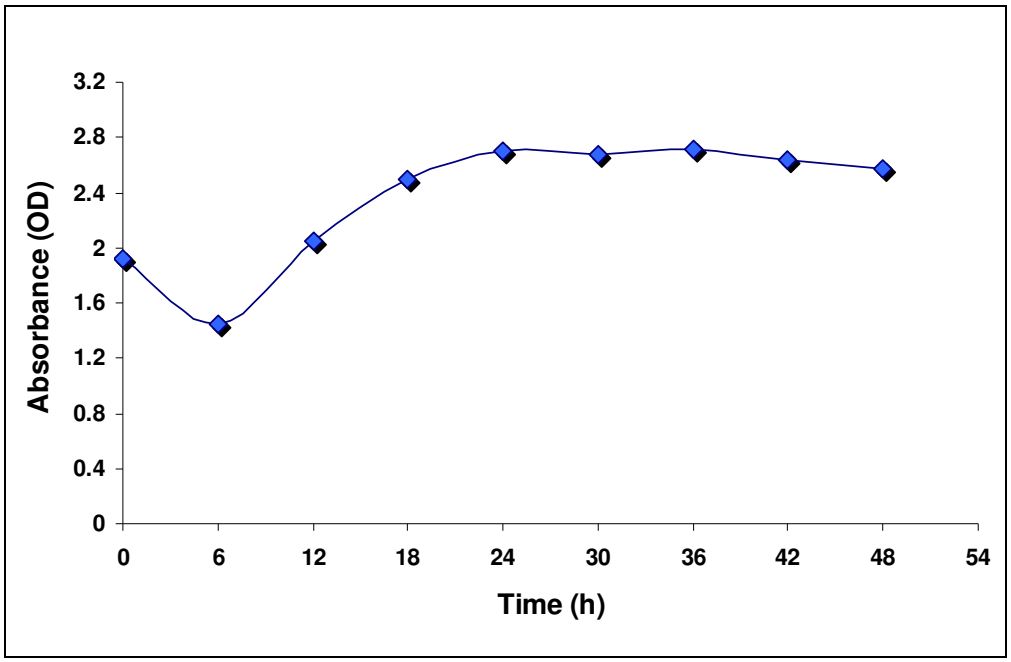

Figure 4.1: Cell growth profile from fermentation broth.

At time 6 hours until 24 hours, the number of cell was increased and trend was identified as *exponential growth phase.* It is believe that the cells were multiply rapidly and cell density increased exponentially with time. From the graph, at time within 24 hours to 26 hours, the growth was decelerates. This might be due to the depletion of one or more essential nutrients or the accumulation of toxic by-products of growth. At time at 26 hours until 30 hours, the results remain constant due to net growth rate is zero (no cell division) and this trend classified as *stationary phase*. At the end of fermentation, the trend of the graph was declined due to some of the cell death. This trend was called as *death phase* where the higher depletion of nutrients or toxic product accumulation occurred. It is concluded that, the optimum time for cell growth for the fermentation in ethanol production was at 24 hours (refer Figure 4.1) which the cell growth rate were at the maximize value (Shuler and Kargi, 2002).

4.1.2 Effect of Different pH on Ethanol Yield

Several values of pH were employed (pH 4, 5 and 6) to study the effect of pH on production of bioethanol from cassava starch. Fermentation process was conducted in 2 L bioreactor and other parameters such as temperature and agitation rate were fixed at specific values. The temperature used for this study was 25°C and the agitation speed was at 150 rpm.

The fermentation process was conducted for 24 hours. At pH 5, the lowest glucose concentration was occurred presenting the highest consumption of glucose. From a finding by Mojović *et al*. (2006), the highest productions of ethanol were at pH 5 and the concentration of glucose remained was the lowest at this pH. The concentration of glucose was reduced during the fermentation as the glucose was continuously consumed by the yeast and converted into ethanol (Mojović *et al*., 2006). The concentration of ethanol was increased as the pH increased from pH 4 to pH 5. However, the concentration was dropped as the pH of 6 was employed. Based on the results, the highest yield of ethanol produced was 3.6391 g/L at pH 5 compared to 1.10827 g/L at pH 4 and 2.26402 g/L at pH 6. The results of ethanol accumulation and glucose consumption with various pH are illustrated as in Figure 4.2.

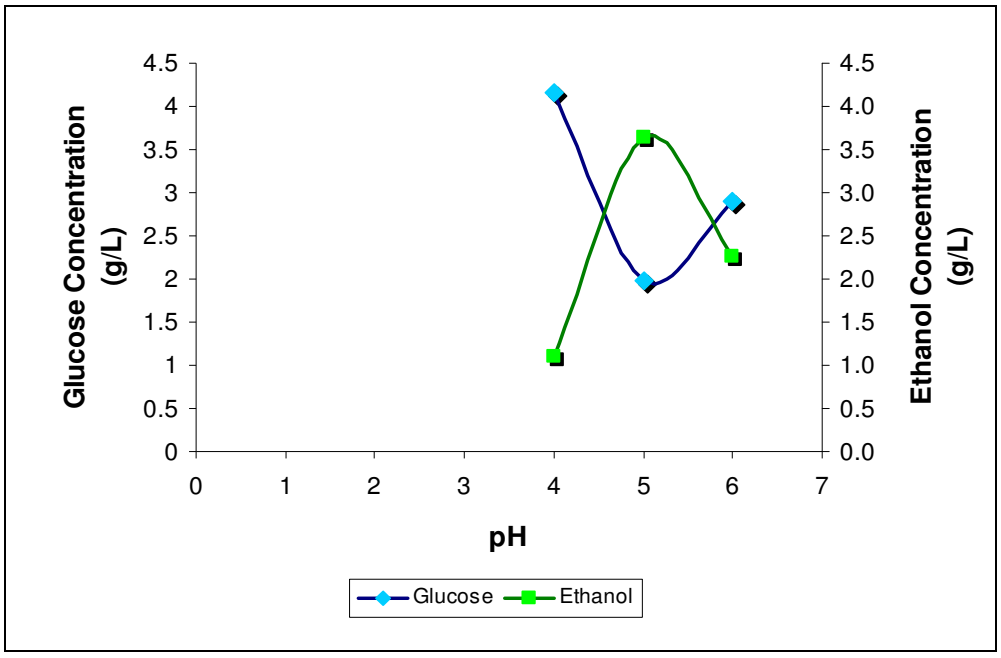

Figure 4.2: The effect of pH on glucose consumption and ethanol production.

Kunamneni (2005) also found that the value of pH on fermentation process influenced the production of ethanol significantly. Figure 4.3 shows the profile of glucose consumption and ethanol accumulation at optimum pH (pH 5). It is found that after 24 hours, the glucose concentration reduction was observed and it is presenting the increasing of glucose consumption. This is due to the highest cell growth activity during the exponential growth phase. The production of bioethanol was increased as the time increased from 0 to 24 hours. Beyond 24 hours, the glucose consumption and ethanol concentration was reduced. The results of glucose consumption and ethanol accumulation at pH 5 are illustrated as in Figure 4.3.

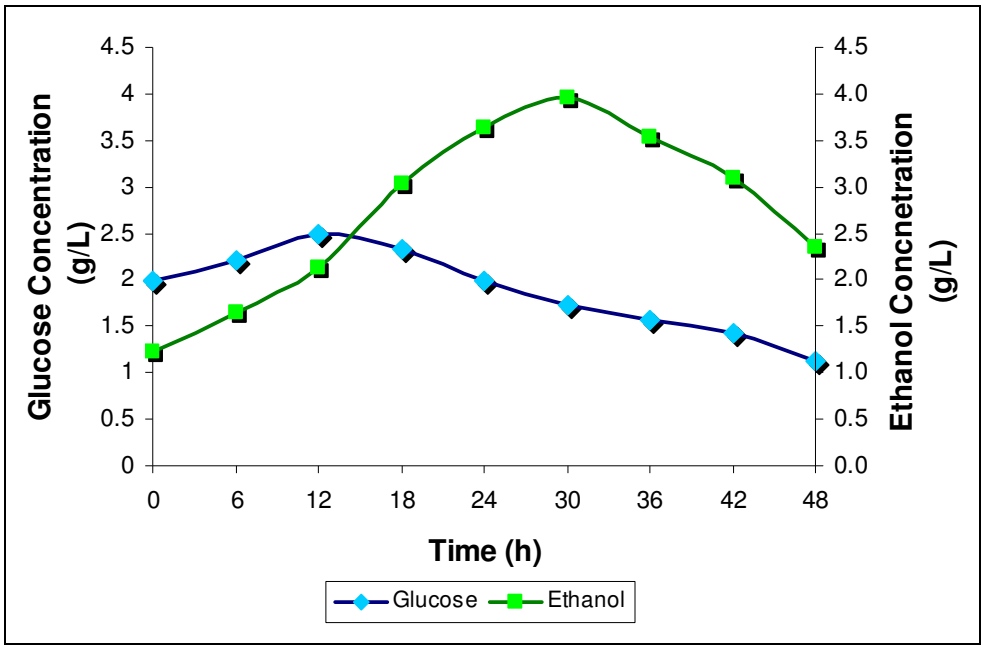

Figure 4.3: The glucose consumption and ethanol production at optimum pH 5.0.

4.1.3 Effect of Air Flow Rate on Ethanol Yield

Several value of air flow rate was employed (1.0, 1.5 and 2.0 L/min) to study the effect of air flow rate on ethanol production. Fermentation process was conducted in 2 L bioreactor and the conditions of fermentation such as temperature, agitation rate and pH were fixed at 25°C, 150 rpm and pH 5 respectively.

Based on Figure 4.5, as the air flow rate supplied to the bioreactor was increased, the glucose consumption was reduced. Mojović *et al.* (2006) also reported that the consumption of glucose by yeast decreased as the air flow rate supplied to the bioreactor was increased. From the graph, it is clear that air flow rate at 1.0 L/min has demonstrated the highest consumption of glucose whereby air flow rate at 1.5 L/min and 2.0 L/min showed lower glucose consumptions. The high yield of ethanol was accumulated when the glucose consumption by yeast increased. From the result obtained, the highest yield of ethanol was 3.46 g/L at 1.0 L/min.

Piskur et.al, (2006) has stated that *Saccharomyces cerevisiae* can accumulate ethanol in the presence of oxygen. The yeast is group in Crabtree-positive-type yeasts. It was also found that ethanol produced may become a substrate and is degraded if oxygen is present. This change in metabolism is known as the 'diauxic shift'. Therefore, the presence of oxygen at some extents during fermentation may interfere the accumulation of ethanol (Piskur *et. al*, 2006). The results of ethanol accumulation and glucose consumption with various air flow rate are illustrated as in Figure 4.4.

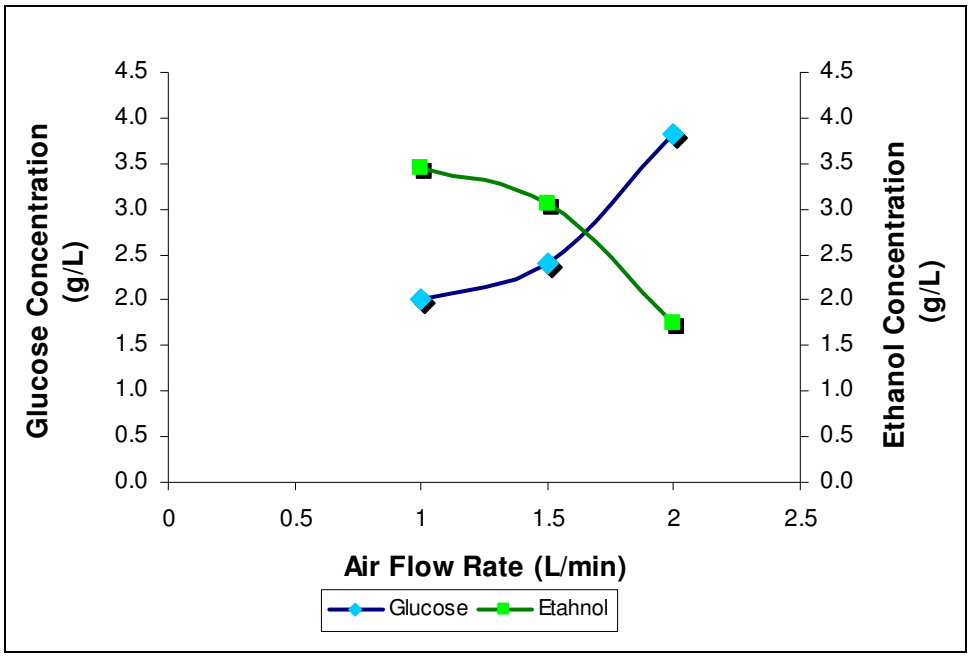

Figure 4.4: The effect of air flow rate on glucose consumption and ethanol production.

From the Figure 4.5, it is found that the glucose concentration continuously decreased as the time of fermentation is increased. This is due to the number of cells which multiply rapidly and increased exponentially with time. From the graph below, it shows the profile of glucose consumption and ethanol accumulation at optimum air flow rate 1.0 L/min. The ethanol concentration was increased within the first 24 hours of fermentation time. The concentration was continued to drop as the fermentation time as carried out longer than 24 hours.

From the findings by Yuguo *et.al,* (1999), the effects of various air flow rate or aeration rate on fermentation process were previously investigated using the air flow rate in the range of 0 – 2 L/min. An air flow rate of 2 L/min was found to be the critical rate where above this air flow rate, the production of ethanol or glucose consumption would remain constant. This condition is due to the forces generated between molecules of air inside the bioreactor (Yuguo *et.al,* 1999*)*. In addition, it is clear that gas holdup increased as the air flow rate increased. During the fermentation, the microorganism grows slowly when dissolved oxygen is insufficiently supplied. However, when dissolved oxygen concentration was excessive, the ethanol accumulation or glucose consumption was also limited. The results by Yuguo *et.al* (1999) also detemined that 1.0 L/min was an optimum air flow rate.

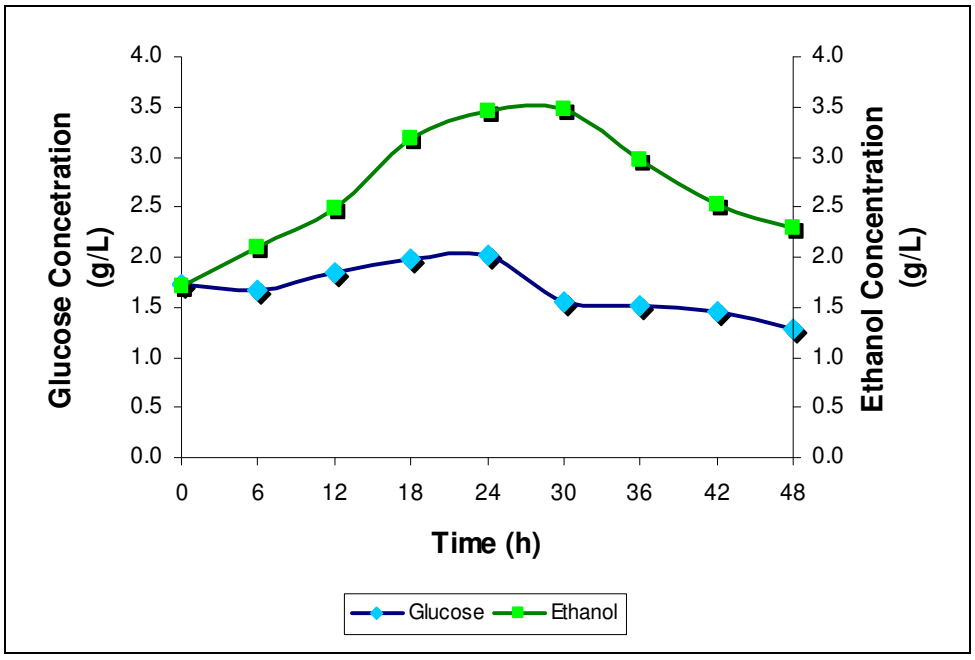

Figure 4.5: The glucose consumption and ethanol production at optimum air flow 1.0 L/min.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Bioethanol production from tapioca starch has provided a valuable liquid fuel. In order to attain a higher yield of ethanol in ethanol fermentation, main parameters of ethanol fermentation by *S. cerevisiae*; air flow rate and pH have been investigated. From various air flow rate employed in 2 L fermentation, it was found that the ethanol concentration was lower when the air flow rate supplied to the bioreactor was increased. From various pH employed in 2 L fermentation, it was observed that the ethanol concentration was increased when the pH inside the bioreactor became higher. The optimum fermentation conditions were obtained: pH 5.0 and 1.0L/min air flow rate. Based on the result, the highest yield of ethanol produced was 3.64 g/L at pH 5.0 and 1.0L/min air flow rate.

5.2 Recommendations

In order to improve this research, there are several things should be stress out in the future. Firstly, to improve the enzymatic hydrolysis, α-amylase can be replaced with thermostable amylopullanase of *G. thermoleovorans* NP33 enzyme in liquefaction step. This due to its ability to increase the saccharification to 91% compared to α-amylase only 70% starch saccharification (Kaur and Satyanarayana, 2004).

Furthermore, in order to reduce the time to complete the process and make beneficial energy savings, simultaneous process of the second hydrolysis step and fermentation can be applied. The mixture of liquefied starch can be added to the glucoamylase and the saccharafication and fermentation can be simultaneously carried out.

Lastly, fermentation can be performed using fed-batch as the process will be able to increase maximum viable cell concentration, prolong culture lifetime, and allow product accumulation to a higher concentration.

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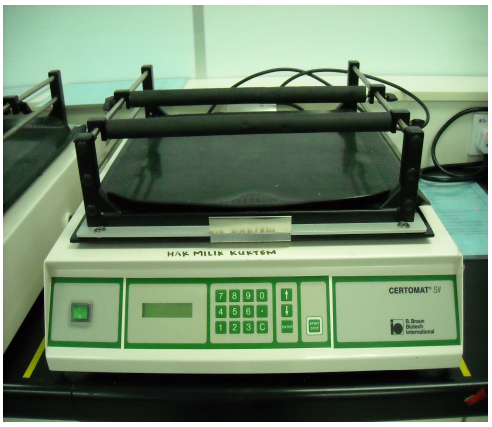
APPENDIX A



Appendix A.1: Orbital Shaker CERTOMAT S-II

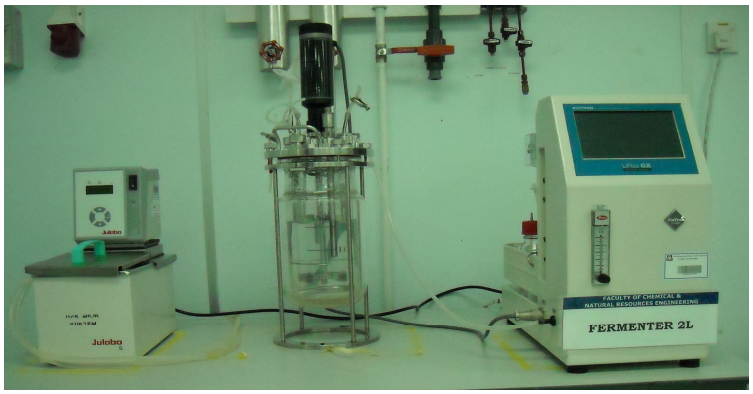
Appendix A.2: Shaking Water Bath (Model BS-21)

Appendix A.3: UV-Visible Single Beam Spectrophotometer (Model U-1800)

Appendix A.4: Autoclave 50L (Model HICLAV HVC-50)

Appendix A.5: Laminar air Flow Cabinet (Model AHC-4A1)

Appendix A.6: Double Stack Shaking Incubator Infors



Appendix A.7: Fermenter 2L (Lflus-GX)

Appendix A.8: Refrigerated Centrifuged (Model 5810 R)

APPENDIX B

Appendix B.1: Data for preparation of concentration required of glucose standard calibration curve.

Concentration of glucose concentration/ Stock solution = 1000µg/ml

Test Tube Number	**0 (Blank)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Glucose Stock Solution (ml)	-	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Distilled Water (ml)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	-
DNS Reagent (ml)	1	1	1	1	1	1	1	1	1	1	1

Appendix B.2: Data for standard calibration curve of glucose.

Concentration of Glucose (mg/mL)	**Absorbance at 540 nm**
0.0 (Blank)	0
0.1	0.149
0.2	0.297
0.3	0.344
0.4	0.405
0.5	0.516
0.6	0.597
0.7	0.664
0.8	0.773
0.9	0.868
1.0	0.961

The equation obtained: $y = 0.9821\ x$

$R^2 = 0.9808$

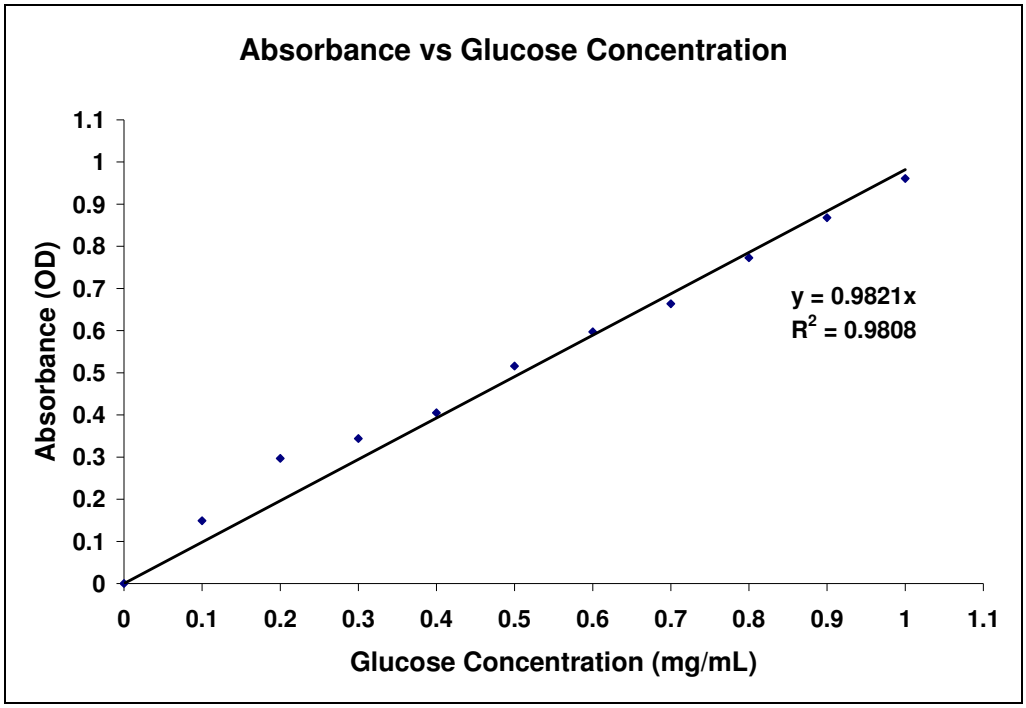


Figure B.1: Graph of Standard Calibration Curve for Glucose.

Appendix B.3: Data for the effect of pH on glucose concentration.

Time (h)	pH = 4	pH = 5	pH = 6
	Glucose Conc. (g/L)		
0	10.53	2.58	6.49
6	9.51	2.21	6.12
12	11.49	2.5	6.71
18	11.55	3.34	7.62
24	11.69	3.48	7.51
30	10.87	3.22	7.66
36	9.72	2.57	7.22
42	9.65	2.43	6.82
48	9.27	2.12	5.99

Appendix B.4: Data for the effect of air flow rate on glucose concentration.

Time	1.0 L/min	1.5 L/min	2.0 L/min
	Glucose Conc. (g/L)		
0	1.73	2.19	3.79
6	1.67	2.18	3.71
12	1.85	2.29	3.86
18	1.99	2.26	3.76
24	2.02	2.41	3.83
30	1.55	2.02	3.41
36	1.51	1.99	2.99
42	1.5	1.88	2.91
48	1.38	1.59	2.77

Appendix B.5: Data for preparation of concentration required of ethanol standard calibration curve.

Concentration of Ethanol (g/L)	Peak Area (pA*s)
0	0
0.2	94.1
0.4	218.759
0.6	237.6
0.8	327.745
1.0	456.7
1.2	556.192
1.4	640.876
1.6	692.778
1.8	701.172
2.0	908.3

The equation obtained: $y = 436.34\ x$

$R^2 = 0.9832$

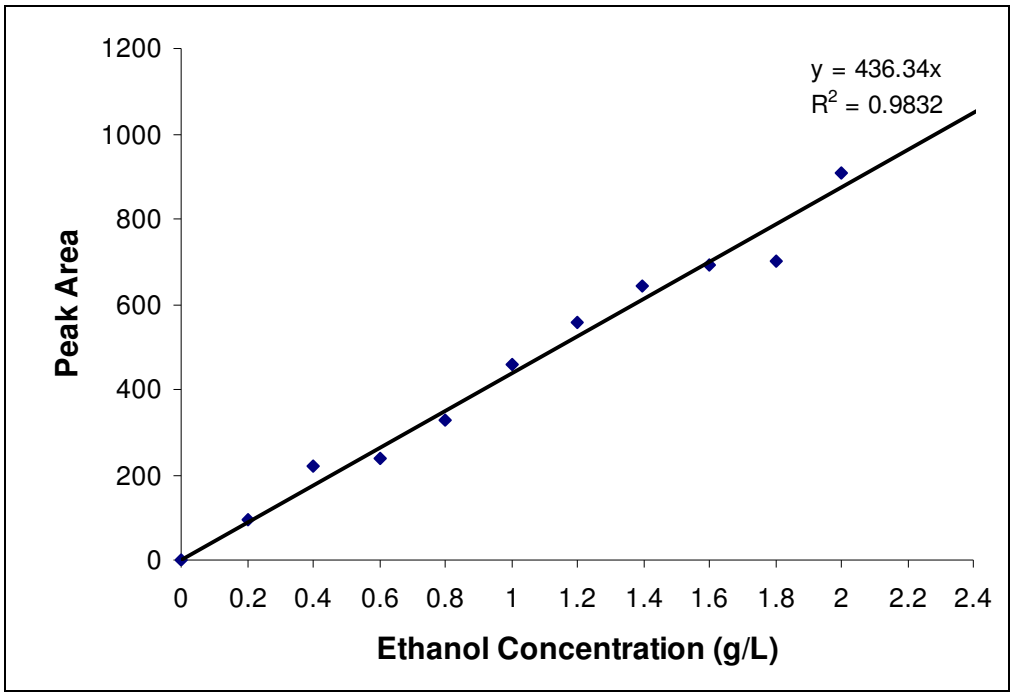


Figure B.2: Graph of Standard Calibration Curve for Ethanol.

Appendix B.6: Data for the effect on glucose and ethanol concentration at pH 5.

Time (h)	Glucose Concentration (g/L)	Peak Area (pA*s)	Ethanol (g/L)
0	1.98	538.96637	1.23520
6	2.21	718.56559	1.64680
12	2.5	925.34712	2.12070
18	2.34	1323.0859	3.03224
24	1.98	1587.88281	3.63910
30	1.72	1731.03972	3.96718
36	1.57	1542.21503	3.53443
42	1.43	1352.02526	3.09856
48	1.12	1025.97268	2.35131

Appendix B.7: Data for the effects of pH on glucose and ethanol concentration.

pH	Glucose Concentration (g/L)	Peak Area (pA*s)	Ethanol (g/L)
4	4.17	483.58200	1.10827
5	1.98	1587.88281	3.63910
6	2.91	987.88281	2.26402

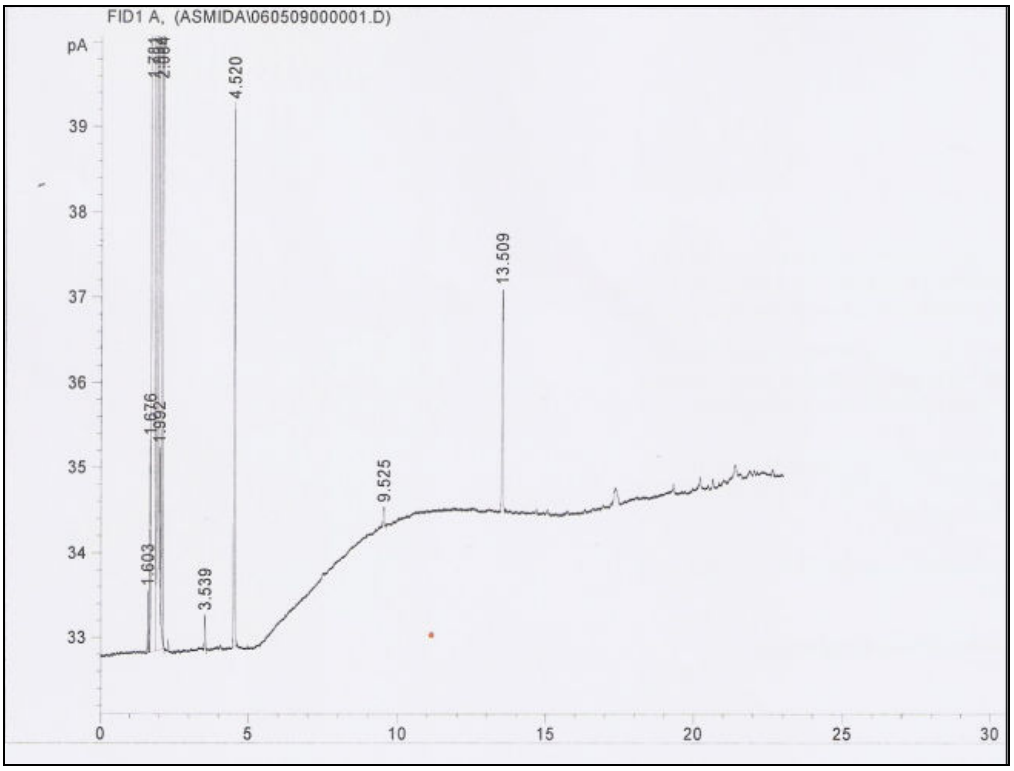
Appendix B.8: Data for the effect of air flow rate on glucose and ethanol concentration at 1.0 L/min.

Time (h)	Glucose Concentration (g/L)	Peak Area (pA*s)	Ethanol (g/L)
0	1.73	746.44141	1.71069
6	1.67	916.36792	2.10012
12	1.85	1083.13421	2.48232
18	1.99	1391.63679	3.18934
24	2.02	1507.86389	3.45571
30	1.55	1512.8418	3.46712
36	1.51	1298.04707	2.97485
42	1.45	1098.04712	2.51649
48	1.28	995.70879	2.28196

Appendix B.9: Data for the effects of air flow rate on glucose and ethanol concentration.

pH	Glucose Concentration (g/L)	Peak Area (pA*s)	Ethanol (g/L)
1	2.02	1507.86389	3.45571
1.5	2.41	1330.38879	3.04897
2	3.83	766.78461	1.75731

Appendix B.10: Graph of peak area and data of ethanol for the effects of pH 6 at time 24 hours.



Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	1.603	BB	0.0198	9.35507e-1	7.35110e-1	0.00015
2	1.676	BV	0.0151	2.37155	2.49589	0.00039
3	1.781	VV S	0.0397	5.39657e5	2.27388e5	88.54187
4	1.934	VB S	0.0266	6.93447e4	4.36112e4	11.37742
5	1.992	BV X	0.0114	7.82312e-1	1.14174	0.00013
6	2.084	VB X	0.0209	464.33862	362.86057	0.07618
7	3.539	BB	0.0308	7.85174e-1	4.02437e-1	0.00013
8	4.520	BB	0.0365	15.00581	6.37464	0.00246
9	9.525	BB	0.0375	5.87882e-1	2.18047e-1	9.645e-5
10	13.509	BB	0.0429	7.09302	2.59395	0.00116