BATCH ETHANOL FERMENTATION USING GLUCOSE DERIVED FROM TAPIOCA FLOUR STARCH BY *Saccharomyces Cerevisiae* FOR EFFECT OF TEMPERATURE AND AGITATION RATE

MOHD AZIMIE BIN AHMAD

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

BORANG PENGESAHAN STATUS TESIS

JUDUL **BATCH ETHANOL FERMENTATION USING GLUCOSE DERIVED FROM TAPIOCA FLOUR STARCH BY *Saccharomyces Cerevisiae* FOR EFFECT OF TEMPERATURE AND AGITATION RATE**

SESI PENGAJIAN: **2007/2008**

Saya **MOHD AZIMIE BIN AHMAD**
(HURUF BESAR)

mengaku membenarkan tesis (PSM/Sarjana/Doktor Falsafah)* ini disimpan di Perpustakaan Universiti Malaysia Pahang dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Pahang
2. Perpustakaan Universiti Malaysia Pahang dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. **Sila tandakan (✓)

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)
☐	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
✓	TIDAK TERHAD	

Disahkan oleh

_____ (TANDATANGAN PENULIS)

Alamat Tetap: **NO 167, JALAN WIRA 3,**
TAMAN WIRA EMAS 3
06200 KEDAH DARUL AMAN

Tarikh: May 2008

_____ (TANDATANGAN PENYELIA)

SUPERVISOR
Nasratun Binti Masngut

Tarikh: May 2008

CATATAN:
* Potong yang tidak berkenaan.
** Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT atau TERHAD.
◆ Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau disertai bagi pengajian secara kerja kursus dan penyelidikan, atau Laporan Projek Sarjans Muda (PSM).

"I hereby declare that I have read this thesis and in
my opinion this thesis is sufficient in terms of scope and
quality for the award of the degree of Bachelor of Chemical Engineering
(Biotechnology) "

Signature : .....

Supervisor : Miss Nasratun Binti Masngut

Date : May 2008

BATCH ETHANOL FERMENTATION USING GLUCOSE DERIVED FROM TAPIOCA FLOUR STARCH BY *Saccharomyces Cerevisiae FOR* EFFECT OF TEMPERATURE AND AGITATION RATE

MOHD AZIMIE BIN AHMAD

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
Universiti Malaysia Pahang

May, 2008

DECLARATION

I declare that this thesis entitled "Batch Ethanol Fermentation Using Glucose Derived from Tapioca Flour Starch by *Saccharomyces cerevisiae* for Effect of Temperature and Agitation 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."

Signature :…….

Name of Candidate : Mohd Azimie Bin Ahmad

Date : May , 2008

DEDICATION

Special Dedication to my Mother and Father,
My family members that always love me,
My friends, my fellow colleague, Kak Zai & Abang Solihon
and all faculty members.

For all your Care, Support and Believe in me.

Sincerely
Mohd Azimie Bin Ahmad

ACKNOWLEDGEMENT

Bismillahirrahmanirahim,

I am so thankful to Allah S.W.T for giving me patient and spirit throughout this project and the research is successfully complete.

To my beloved father and mother, Ahmad Bin Mat Esa and Faridah Binti Man. I am grateful to have both of you in my life and giving me full of support to through this life. I pray and wish to both of you are always in a good health and in Allah mercy. You are the precious gift from Allah to me.

I am indebted to my supervisor, Miss Nasratun Binti Masngut, lecturer from the Faculty of Chemical Engineering and Natural Resources for her advice, insightful comments and generous support. Thank for your guide and without your guide this research will not complete and well organized. And not forgetting for my panels, Miss Asmida Binti Ideris and Madam Norashikin Binti Mat Zain for your brilliant ideas that you gave to me, thank you.

I would like to dedicate my appreciation to all the lecturers that involve in this subject/project for their invaluable time, guidance and advice. Special thanks also dedicated to staff at Clean Room Laboratory for their guidance and help in doing my study. Without your cooperation and sacrifices this research will not able to complete and published.

I would also like to thank my beloved fiance Siti Khadijah Binti Abdul Halim, and especially who have accompanied me throughout this project. Thank you very much.

ABSTRACT

Bioethanol can be produced from amylose and amylopectin that originate from various sources of biomass. This study was focusing on the production of ethanol from glucose derived from tapioca flour starch using *Saccharomyces cerevisiae*. The objectives of this study are to determine the yield of ethanol produced from certain amount of tapioca flour, the effects of the fermentation temperature and agitation speed in fermentation process. Enzymatic process is purposely done to convert the tapioca flour starch to its glucose. Two types of enzyme were employed for these processes which were α-amylase and amyloglucosidase. This study is done in batch mode of aerobic fermentation process. The temperature and agitation used in this experiment are 30, 35, 37°C and 100, 200, and 300 rpm. Determination of cell growth concentration in inoculation process plays an important role to ensure the microbial activity and determination of microbe growth. Results show that yield of ethanol production will increase as the temperature and agitation increase until it reaches the optimum point. From this study, it was observed that optimum condition for ethanol fermentation by *Saccharomyces cerevisiae* was at 35°C with agitation speed of 200 rpm

ABSTRAK

Bioetanol boleh dihasilkan daripada amilose dan amilopektin yang boleh diperolehi dari pelbagai sumber biojisim. Kajian ini dijalankan untuk menghasilkan bioethanol daripada glukosa yang diperolehi daripada kanji tepung ubi kayu dengan menggunakan mikroorganisma dikenali sebagai *Saccharomyces cerevisiae*. Objektif kajian ini adalah untuk menentukan kadar penghasilan etanol dari kuantiti tertentu tepung ubi kayu yang digunakan, juga mengkaji kesan fermentasi etanol akibat perubahan suhu dan perubahan halaju adukan. Proses penukaran kanji tepung ubi kayu kepada glukosa melibatkan penggunaan dua jenis enzim iaitu α-amylase dan amyloglucosidase. Fermentasi etanol dijalankan dalam mod sekumpul secara aerobik. Perubahan suhu yang dikaji adalah pada 30°C, 35°C dan 37°C dan kadar halaju pengadukan pula adalah 100, 200 dan 300 rpm. Keputusan eksperimen menunjukkan penghasilan etanol akan meningkat dengan peningkatan suhu fermentasi dan kadar kelajuan pengadukan. Daripada kajian ini, keadaan optimum untuk fermentasi etanol oleh *Saccharomyces cerevisiae* adalah pada 35°C dengan kelajuan adukan pada 200 rpm.

TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURES

LIST OF SYMBOLS

%	-	percent
°C	-	°Celcius
µg/ml	-	microgram per mililiter
g	-	gram
g/ml	-	gram per mililiter
kg	-	kilogram
L	-	liter
L/h	-	liter per hour
ml	-	mililiter
mm	-	milimeter
rpm	-	rotation per minute
v/v	-	volume per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Fermentation may be thought of as chemical reactions catalyzed by living cell. Fermentation is the anaerobic or aerobic conversion of sugar to carbon dioxide and alcohol by microorganisms. Variety of products can be produced by fermentation, such as pharmaceuticals, organic acids and alcohols. However, to be commercially viable, the bioprocesses must be economically competitive with alternative processes, such as petrochemical manufacturing. Advances in recombinant-DNA technology allow fermentation to mass produce chiral and complex biomolecules (such as human insulin) more economically than by other means. Most widely known fermentation product is ethanol. Batch yeast fermentations have been used for hundreds of years to produce alcoholic beverages. Ethanol can be produced from a variety of plant-derived raw materials, including agricultural wastes. Currently, there is a small market for ethanol as a fuel additive, but for ethanol to successfully compete with petroleum, additional improvements in the process economics are needed. The use of baker's yeast, such as *Saccharomyces cerevisae*, that grow faster and give higher ethanol yields than the conventionally used yeast could provide such an improvement. Objectives of this study are to carry out an ethanol fermentation using *S. cerevisiae* utilizing cheap carbon sources and to study optimum condition (agitation speed and temperature) of the fermentation.

1.2 Problem Statement

S. cerevisiae is facultative anaerobic yeast which ferments hexose sugars under aerobic and anaerobic conditions (Hohmann, 1997). Yeast will be grown in glucose-rich medium where it will utilize the sugar at different modes to produce energy depending upon the specific growth conditions.

Organisms employ cellular respiration to harvest energy in the form of ATP in the presence of oxygen (Campbell, 1996). Enzymatic activity is the driving force behind the three main stages of cellular respiration – glycolysis, the Krebs cycle and the electron transport chain. Without oxygen, organisms undergo fermentation which only involves the first stage of cellular respiration, glycolysis, and subsequently requires less enzymes (Campbell, 1996). Cellular respiration yields 38 ATP molecules while fermentation only produces 2 ATP molecules (Campbell, 1996). Organisms rely on ATP to drive their cellular processes and promote growth. Very little growth occurs during lag phase where the yeast acclimate themselves to their environment and synthesize enzymes necessary to catabolize nutrients (Hohmann, 1997). The yeast will synthesize fewer enzymes in anaerobic environment than in an aerobic environment since fermentation requires fewer enzymes than cellular respiration (Mills, 1967). It is possible that yeast growth will be inhibited under anaerobic conditions since fermentation produces alcohol, which also decreases pH over time and kill the yeast (Miller, 1900). Consequently, the partial pressure of oxygen is a key factor affecting yeast growth in addition to temperature and pH. The optimum temperature growth range of yeast is between 25°C and 37°C (Miller 1900). The optimum agitation speed range of yeast is between 100 to 200 rpm (Kotter, 1993).

1.3 Research Objectives

1) To determine ethanol yield from fermentation of *Saccharomyces cerevisiae* utilizing starch from tapioca flour.
2) To study the effect of different fermentation temperature and agitation speed on ethanol fermentation in flask.

1.4 Research Scope

~~1)~~ Glucose conversion from starch derived from tapioca flour by enzymatic hydrolysis and two types of enzyme was employed for these processes which were α-amylase and amyloglucosidase.
2) Variation of process condition for temperature 30°C, 35°C, 37°C and for agitation speed is 100 rpm, 200 rpm, and 3000 rpm and its effect on bioethanol production in fermentation process.

1.5 Research Benefits

1) Provide an optimum operation condition (temperature & agitation speed) for continuous ethanol production.
2) Ethanol fermentation using cheap carbon source (sugar derived from tapioca flour starch).

CHAPTER 2

LITERATURE REVIEW

2.1 Bioethanol

Ethanol or ethyl alcohol (CH_3CH_2OH) is an important organic chemical because of its unique properties, and therefore can be used widely for various purposes. Under ordinary conditions, ethanol is a volatile, flammable, clear, colorless liquid, miscible in both water and non-polar solvents.

The production of ethanol has two routes: synthetic and biological. The synthetic ethanol production is commonly carried out by a catalytic hydration of ethylene in vapor phase and often as a by-product of certain industrial operations (Logsdon, 2006). Ethanol produced from this process is mostly used as a solvent (60%) and chemical intermediate (40%). Fermentation ethanol production accounts for 93% of the total ethanol production in the world. The ethanol is produced from fermentation of sugar extracted mostly from crops. *S. cerevisiae* is most popular microorganism used for ethanol production due to its high ethanol yield and high tolerance to rather high ethanol concentration. Ethanol is mostly used as fuels (92%), industrial solvents and chemicals (4%) and beverages (4%) (Logsdon, 2006).

Crops were main feedstock used for ethanol fermentation nowadays, Brazil is the largest ethanol producer and uses sugar cane as feedstock, while the USA in second place by using corn as feedstock (Rosillo-Calle and Walter, 2006). However these crops are also food for human and animals, thus the expansion of production capacity, especially as ethanol becomes a worldwide alternative fuel, is limited by supply of the feedstock. In contrast, cheap starch materials are available as alternative feedstock for ethanol production. Therefore, the use of starch materials for ethanol production is very promising.

In this study, ethanol as fuel and starch derived from tapioca flour as feedstock for ethanol production is in focus. The first part discusses the advantages of ethanol as fuel in regard to combustion as well as to its environmental impact, while the second part discusses the structure of starch materials, followed by the enzymatic hydrolysis process which breaks down of starch to obtain fermentable sugars (glucose).

2.1.1 Ethanol as Fuel

The use of ethanol as fuel goes back to origin of the use of vehicles itself. For example, Henry Ford's Model T. built in 1908, ran on ethanol. It was continued until the availability of cheap petrol effectively killed off ethanol as a major transport fuel in the early part of the 20th century. The energy crisis of the 1970s renewed interest in ethanol production for fuels and chemicals (Marrs, 1975). Although the interest study in the following decade due to oil price, the environmental issue of reducing greenhouse gas, rising vehicle fuel demand, and the security of energy supply sustain the development of ethanol production from renewable resources.

Ethanol is used in vehicle either as a sole fuel or blended with gasoline. As an oxygenated compound, ethanol provides additional oxygen in combustion, and hence obtains better combustion efficiency. The physicochemical properties of some oxygenated high-octane additives are shown in Table 2.1. Since the completeness of combustion is increased by the present of oxygenated fuels, the emission of carbon monoxide is reduced by 32.5% while the emission of hydrocarbon is decreased by 14.5% (Rasskazchikova *et al*., 2004). In addition, the emission of nitrogen oxides is reduced by using ethanol as additive.

Table 2.1: The physicochemical properties of some oxygenated high-octane additives to gasoline. Source: (Rasskazchikova *et al*., 2004)

Properties	Gasoline	Oxygenates		
		Methanol	Ethanol	MTBE
Density at 15.56, kg/m^3	719-779	794	792	742
Heat, kJ/kg				
Combustion (lower)	41,800-44200	19,934	26,749	35,123
Evaporation	~349	1104	839	326
Flash point, °C	-42.8	6.5	12	-28
Octane number				
Research (RON)	90-100	107	108	116
Motor (MON)	81-90	92	92	101
Reid vapor pressure, kPa	55-103	32	16	54

Methyl-tertiary-butyl-ether (MTBE) has properties similar to those of gasoline, but a higher octane number, and therefore is very suitable for high-octane additive. However, MTBE is reported responsible for groundwater pollution as a result, for example is the leaking of the underground tanks. Low levels of MTBE can make drinking-water supplies undrinkable due to its offensive taste and odor. MTBE has higher water solubility compare with other gasoline constituents, thus is rather difficult to purge from ground water (Rong, 2001). Moreover, biodegradation of MTBE needs lots of oxygen which is almost impossible to carry out naturally in ground water. The use of methanol as oxygenated is limited, or in many countries prohibited, due to its high toxicity, volatility and hygroscopic behavior. Ethanol has become more competitive as an oxygenated fuel especially because ethanol is produced from renewable resources by fermentation, resulting in less dependency on fossil fuel. Moreover, ethanol is less hygroscopic, contains a reasonable heat of combustion, has lower evaporation heat and, most importantly, is not toxic like methanol. In addition, acetaldehyde as a product of partial oxidation of ethanol in the exhaust gas of vehicles is much less toxic than formaldehyde, which is formed when using methanol.

As a high-octane additive, ethanol has drawbacks: emitting acetaldehyde of 2-4 times as much as does gasoline that highly corrosive, which is a function of water content which can bring a negative effect on rubber and plastic, and the blend with gasoline tends to separate in the presence of traces of water (Rasskazchikova *et al.,* 2004). Fortunately, these drawbacks have been overcome. An additional 5% of water in a blend of ethanol and gasoline can reduce the emission of acetaldehyde. Stabilizers like higher alcohols, aromatic amine, ethers or ketones are useful to prevent separation. For example, 2.5-3% of isobutanol stabilized the gasoline-ethanol blend in the presence of 5% water at low temperature of -20°C. Some corrosion inhibitor such as hydroxyethylated alkylphenols and alkyl imidazolines can attain essential anticorrosion resistance. Additionally, polymer industries have developed special material that are resistant to penetration of alcohols (Rasskazchikova *et al*., 2004).

2.1.2 Environmental Impact

The main environmental advantages of fuel ethanol are its sustainability in using a renewable resource as a feedstock, thus promoting independence of fossil fuel, and maintaining the level of greenhouse gas (CO_2). While crops are useful as energy sources for human and animals, some crops like starch or oil-containing crops can be converted to fuels or chemicals. Combustion of these fuels produces CO_2 gas which would be assimilated again by plants. How effectively ethanol reduces greenhouse gas emission has been widely discussed. The issues are mainly related to the net energy content in ethanol, and depend on the assumption of ethanol production routes. A number of life-cycle assessments have been studied, and show that a change from fossil fuel to biofuels could reduce CO_2 emission by factor of 1/2 to 1/5, depending on how significant the use of renewable fuels is at all stages in the process (Bernesson *et al*., 2006; Hu *et al*., 2004; Kadam, 2002; Kim and Dale, 2005; Rosillo-Calle and Walter, 2006; von Blottnitz and Curran, 2006). Ethanol is harmless to the environment. In ground water and soil mixtures, ethanol can be rapidly degraded both aerobically (100 ml/L in 7 days) and anaerobically (100 mg/L in 3-25 days) (Armstrong, 1999). Ethanol in surface water is also rapidly degraded and thus not harmful as long as it is not present in concentrations directly toxic to microorganisms. The half-time of ethanol in surface water is 6.5 to 26 hours. While ethanol releases volatile organic compounds (VOC) due to its low vapor pressure, degradation of ethanol in the atmosphere is also predicted to be rapid.

Exposure of humans to ethanol is harmless. The exposure may be carried out mostly by inhalation of ethanol vapor as VOC, and by body contact or, rarely, ingestion from either blended fuel or denatured fuel. Biological exposures and responses to ethanol are typically evaluated in terms of blood ethanol concentrations (BEC). The endogenous level is 0.02-0.15 mg/dL while the legal limit for vehicle drivers is 80- 100 mg/dL (Armstrong, 1999). In addition, (von Blottnitz and Curran, 2006) studied the potential health effects of gasoline and ethanol engine exhaust fumes. He concluded that the acute toxicity of the exhaust gas of a gasoline-fueled engine is significantly higher than that of an ethanol-fueled engine.

2.2 Structure of Starch Materials

Starch is a complex carbohydrate which is soluble in water. It is used by plants as a way to store excess glucose and can be used as a thickening agent when dissolved and heated. The word is derived from Middle English *sterchen*, meaning to stiffen. The formula for starch is $C_6H_{10}O_5$ (Hedley, 2002). In terms of human nutrition, starch is by far the most important of the polysaccharides. It constitutes more than half the carbohydrates even in affluent diets, and much more in poorer diets. It is supplied by traditional staple foods such as cereals, roots and tubers. Starch contains a mixture of two molecules: amylose and amylopectin. Usually these are found in a ratio with amylopectin found in larger amounts than amylose. Starch is often found in the fruit, seeds, rhizomes or tubers of plants. The major resources for starch production and consumption worldwide are rice, wheat, corn, and potatoes.

2.2.1 Composition and Structure of Starch

Starches are found in a large number of plants as the major carbohydrates reserve and provide an essential source of energy to us. The largest and most important source of starch comes from corn (maize). The other common source of starch comes from wheat, potatoes, tapioca and rice (Marrs, 1975).

Starches have wide commercial use and it extends beyond the food and drinks industries due to its inexpensive and abundant supplies. Starches are used as thickening agents in baby food formulation and semi-solids food such as sauces, custards and pie fillings. They are also used as binding agents in products such as sausages and processed meat. For non food purpose, starches are used mainly in paper, packaging and textiles industries. They are also used as fillers in the pharmaceutical industry for pill manufacture (Marrs, 1975; Galliard, 1987).

Starch are predominantly composed of two polysaccharides macromolecules, amylose (20-30%) and amylopectin (70-80%), which are packed in a form of partially crystalline granules (Barsby *et al*, 2003). Starch is produced as granules in most plants cells and is referred to native when in this particular granular state. Native starches from different botanical sources vary widely in structure and composition, but all granules consist of two major molecular components, amylose and amylopectin, both of which are polymers of α-D-glucose units in the $^4C_1$ conformation. Molecule structure of amylose shown in Figure 2.1, these are linked (1 → 4), with the ring oxygen atoms all on the same side, whereas in amylopectin about one residue in every twenty is also linked (1 → 6) forming branch-points as shown in FIGURE 2.2.

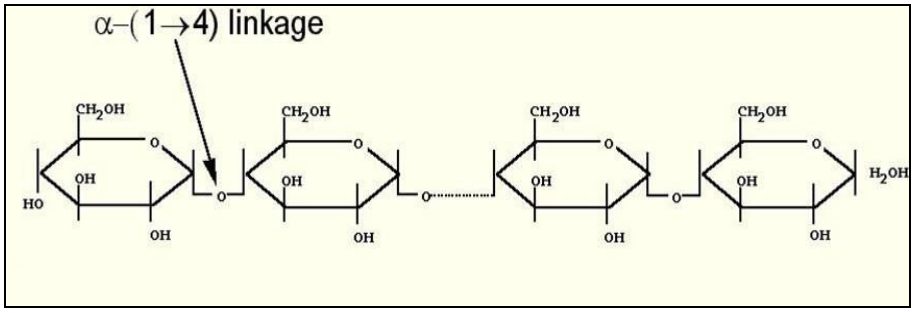


Figure 2.1 *Amylose* molecule structure

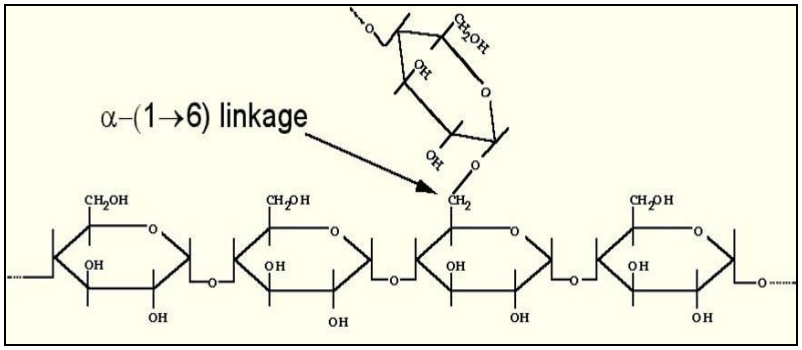


Figure 2.2 Amylopectin molecule structure (Hedley, 2002)

Amylose consist of anhydroglucose units that are linked by α-D-1,4 glucoside bonds to form linear chains. Amylose molecules are typically made up from 200-2000 anhydroglucose units. The level of amylose and its molecular weight vary between different starches types. Aqueous solutions of amylose are unstable due to intermolecular attraction and association of neighboring amylose molecules. This leads to viscosity increase, retrogradation and, under specific conditions, precipitation of amylose particles. Amylose forms a helical complex with iodine giving a characteristic blue colour (Hedley, 2002).

Amylopectin have a polymeric, branched structure. Its consist of anhydroglucose units that are linked by α-D-1,6 bonds that occur every 20-30 anhydroglucose units, in addition it also have α-D-1,4 bonds that are present in amylose. The level of amylopectin varies between different starches types, waxy starches are almost 100% amylopectin. Aqueous solutions of amylopectin are characterized by high viscosity, clarity, stability and resistance to gelling. Amylopectin binds weakly with iodine and the complex usually gives a red/brown colour (Hedley, 2002). Of the two components of starch, amylose has the most useful functions as a hydrocolloid. Its extended conformation causes the high viscosity of water-soluble starch and varies relatively little with temperature. The extended loosely helical chains possess a relatively hydrophobic inner surface that is not able to hold water well and more hydrophobic molecules such as lipids and aroma compounds can easily replace this. Amylose forms useful gels and films. Its association and crystallization (retrogradation) on cooling and storage decreases storage stability causing shrinkage and the release of water (syneresis). Increasing amylose concentration decreases gel stickiness but increases gel firmness. Amylopectin interferes with the interaction between amylose chains (and retrogradation) and its solution can lead to an initial loss in viscosity and followed by a more slimy consistency. At high concentrations, starch gels are both pseudo plastic and thixotropic with greater storage stability. Their water binding ability (high but relatively weak) can provide body and texture to foodstuffs and is encouraging its use as a fat replacement (Chaplin, 2004b).

2.3 Enzymatic Hydrolysis

Starchy substances constitute the major part of the human diet for most of the people in the world, as well as many other animals. They are synthesized naturally in a variety of plants. Some plant examples with high starch content are corn, potato, rice, sorghum, wheat, and cassava. It is no surprise that all of these are part of what we consume to derive carbohydrates. Similar to cellulose, starch molecules are glucose polymers linked together by the alpha-1,4 and alpha-1,6 glucosidic bonds, as opposed to the beta-1,4 glucosidic bonds for cellulose. In order to make use of the carbon and energy stored in starch, the fermentation process to produce ethanol, with the help of the enzyme amylases, must first break down the polymer to smaller assimilable sugars, which is eventually converted to the individual basic glucose units.

Starch is generally insoluble in water at room temperature. Because of this, starch in nature is stored in cells as small granules which can be seen under a microscope. Starch granules are quite resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the same molecule and with other neighboring molecules. However, these inter- and intra-hydrogen bonds can become weak as the temperature of the suspension is raised. When an aqueous suspension of starch is heated, the hydrogen bonds weaken, water is absorbed, and the starch granules swell. This process is commonly called *gelatinization* because the solution formed has a gelatinous, highly viscous consistency. The same process has long been employed to thicken broth in food preparation (Barsby *et al*., 2003).

Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose, maltose, and glucose, etc. Dextrins are shorter, broken starch segments that form as the result of the random hydrolysis of internal glucosidic bonds. A molecule of maltotriose is formed if the third bond from the end of a starch molecule is cleaved; a molecule of maltose is formed if the point of attack is the second bond; a molecule of glucose results if the bond being cleaved is the terminal one; and so on. The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called *liquefaction* because of the thinning of the solution. The final stages of depolymerization are mainly the formation of mono-, di-, and tri-saccharides. This process is called *saccharification*, due to the formation of saccharides (Barsby *et al*., 2003).

Since a wide variety of organisms, including humans, can digest starch, alpha-amylase is obviously widely synthesized in nature, as opposed to cellulase. For example, human saliva and pancreatic secretion contain a large amount of alpha-amylase for starch digestion. The specificity of the bond attacked by alpha-amylases depends on the sources of the enzymes. Currently, two major classes of alpha-amylases are commercially produced through microbial fermentation. Based on the points of attack in the glucose polymer chain, they can be classified into two categories, liquefying and saccharifying (Azrul, 2006).

Because the bacterial alpha-amylase to be used in this experiment randomly attacks only the alpha-1,4 bonds, it belongs to the liquefying category. The hydrolysis reaction catalyzed by this class of enzymes is usually carried out only to the extent that, for example, the starch is rendered soluble enough to allow easy removal from starch-sized fabrics in the textile industry. The paper industry also uses liquefying amylases on the starch used in paper coating where breakage into the smallest glucose subunits is actually undesirable (Galliard, 1987).

On the other hand, the fungal alpha-amylase belongs to the saccharifying category and attacks the second linkage from the nonreducing terminals (for example C4 end) of the straight segment, resulting in the splitting off of two glucose units at a time. Of course, the product is a disaccharide called maltose. The bond breakage is thus more extensive in saccharifying enzymes than in liquefying enzymes. The starch chains are literally chopped into small bits and pieces. Finally, the amyloglucosidase (also called glucoamylase) component of an amylase preparation selectively attacks the last bond on the nonreducing terminals. The type to be used in this experiment can act on both the alpha-1,4 and the alpha-1,6 glucosidic linkages at a relative rate of 1:20, resulting in the splitting off of simple glucose units into the solution. Fungal amylase and amyloglucosidase may be used together to convert starch to simple sugars (Azrul, 2006).

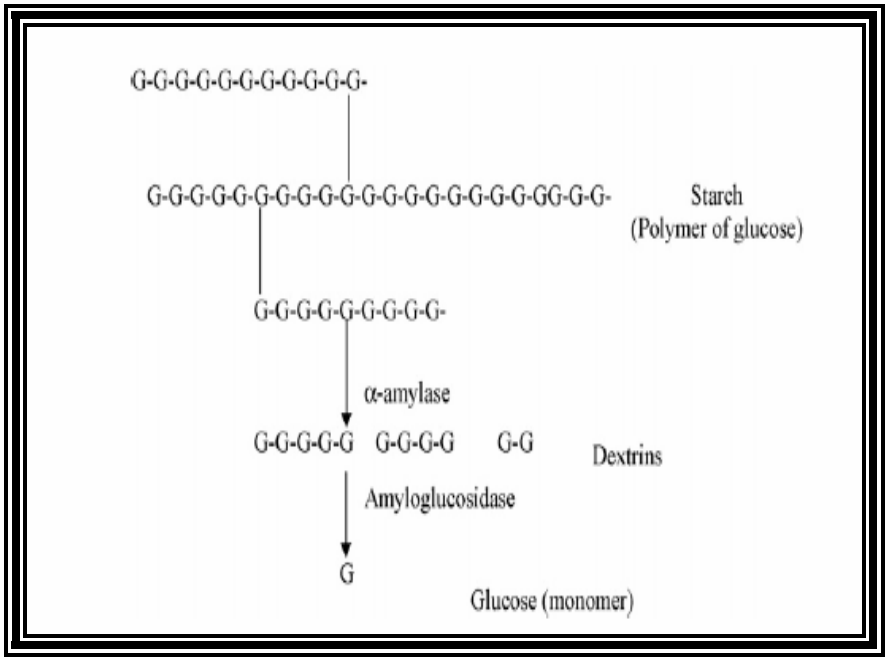


Figure 2.3 Enzymatic Hydrolysis (Barsby *et al*., 2003).

2.4 Metabolism of Saccharomyces Cerevisiae

S. cerevisiae exhibits different modes of metabolism to gain energy when glucose is used as a carbon source (Table 2.2). In general, the route of metabolic flux starting from pyruvate, the end product of glycolysis, can be alcoholic fermentation (fermentative metabolism) or respiration (respiratory metabolism) through tricarboxylic acid (TCA) cycle and respiratory chain. However, both types of metabolism can also occur at the same time (respirofermentative metabolism) in *S. cerevisiae*. There are two important regulatory phenomena depending on the availability of oxygen and the concentration of glucose in the medium, referred to as Pasteur Effect and Crabtree Effect (for details see Walker, 1998). Pasteur Effect can be defined as the suppression of fermentation by oxygen. However, this phenomenon is only observable when glucose concentrations are low for example 5 mM in *S. cerevisiae*) or under certain nutrient limited conditions (Lagunas, 1979).

Crabtree Effect, also referred to as the "glucose effect" or "contre-effect Pasteur" was defined as the suppression of respiration by high glucose. As other Crabtree-positive yeasts, *S. cerevisiae* has a strong tendency towards alcoholic fermentation. It shows a respirofermentative metabolism when the sugar concentration in the cultivation medium exceeds a threshold value of about 5 mM even under fully aerobic conditions (Verduyn *et al*., 1984) or when the specific growth rate is higher than two third of the maximal growth rate (Postma *et al*., 1989). Respiration is the sole route of glucose catabolism when the concentration of glucose is very low. Fermentative metabolism alone occurs only under completely anaerobic conditions. Glycerol is formed as the quantitatively most important by-product of fermentative metabolism and is therefore also mentioned in Table 2.2. By using glucose-limited chemostat, the mode of metabolism can be adjusted.

Table 2.2: Metabolism of *S. cerevisiae*.Source :(Huyen *et al*., 2004)

Glucoce concentration	Oxygen supply	
	Aerobic conditions	Anaerobic conditions
2%- Batch culture	Respirofermentative	Fermentative
Low dilution rate - chemostat	Respiratory	
High dilution rate - chemostat	Respirofermentative	

Respirofermentative: Glucose + O_2 = biomass + ethanol + glycerol + H_2O

Repiratory: Glucose + O_2 = biomass + CO_2 + H_2O

Fermentative: Glucose = biomass + ethanol + CO_2 + glycerol

CHAPTER 3

MATERIALS & METHOD

3.1 Introduction

This chapter will discuss about process ethanol fermentation, step by step from agar preparation, liquid medium preparation, culture maintenance, enzymatic hydrolysis to fermentation process until analytical analysis. In this study, aerobic condition will be used in fermentation process. The conical flask will be used for fermentation process. Tapioca flour is the raw material for this study. Finally the reducing sugar analysis, turbidity analysis to measure the cell growth, cell concentration and also ethanol determination will be showed as analysis procedures in this study.

3.2 Framework of Study

These frameworks give a view of the research methodology applied to obtain fermentation result from the glucose derived from tapioca flour by using *S. cerevisiae.*

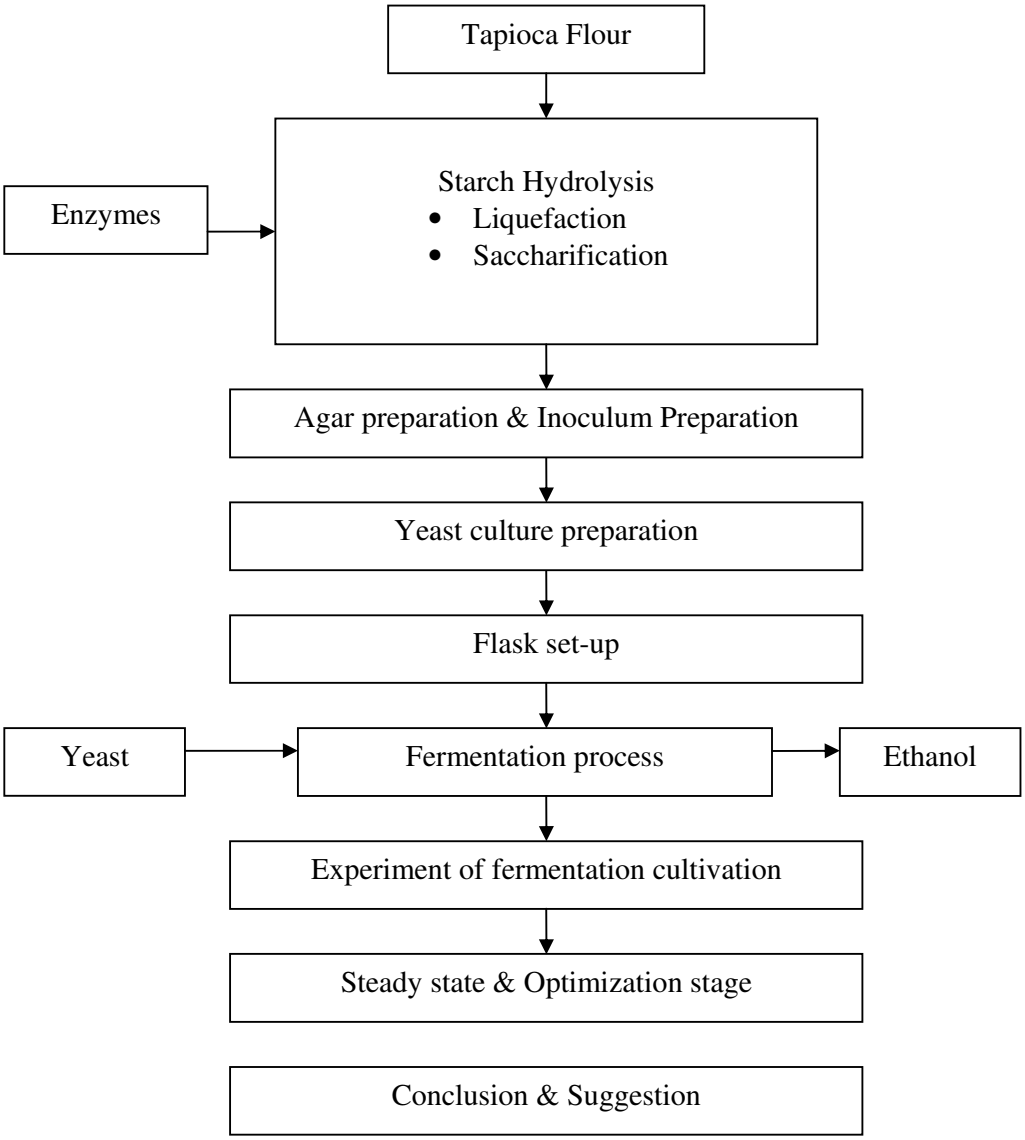


Figure 3.1: Framework of study

3.3 Methodology

3.3.1 Enzymatic Hydrolysis

3.3.1.1 Enzymes

Heat-stable α-amylase (Termamyl 120L) from *Bacillus licheniformis* will be used for tapioca flour liquefaction. This enzyme activity is 120KNU/g (KNU, kilo novo units α-amylases the amount of enzyme which breakdown 5.26 g of starch per hour). Glucoamylase (Supersan 240L) from *Aspergillus niger*, activity 240AGU/g (AGU is the amount of enzyme which hydrolyses 1 μmol of maltose per minute under specified conditions) will be used for tapioca flour saccharification (Mojović *et al*., 2006).

3.3.1.2 Hydrolysis experiments

Tapioca flour, 2g was mixed into 100 ml of distilled water with 60 ppm of Ca^{2+} (as $CaCl_2$) ions are added. The mixture was treated with enzymes in two steps. The first step, liquefaction, was performed at 85°C and pH 6.0 with various concentration of α-amylases, and the second step, saccharification, was perform at 55°C and pH 5.0 with various concentration of glucoamylase. The liquefaction with α-amylases was usually done in 1 hour, while the saccharification with glucoamylase was usually operated in 4hours. The hydrolysis is performed in flasks in water bath with shaking at 150 rpm (Mojović *et al*., 2006).

3.3.2 Agar Preparation

Agar plate containing (per liter): 50g of glucose, 5g of yeast extract, 2g of KH_2PO_4 ,1g of $MgCl_2$, 1g of $NH_4Cl_2.H_2O$, 15g of nutrient agar and maintaining the pH at 5.5. A 100 ml of agar solution is prepared in a 500 ml Schott bottle. The Schott bottle is filled with distilled water until 100 ml. The solution of agar plate is poured into the Schott bottle. Next, by using magnetic stirrer the solution is stirred and boiled. The solution is mixed well. The solution in the bottle was sterilized for 15 minute at 121^{o}C (Noranisah *et al*., 2005). Agar solution is poured into petri dishs. Each of the petri dishs were capped then held at slanted position until it hardens. After that the petri dish are kept in refrigerator at 6˚C-8 ˚C (Noranisah *et al*., 2005).

3.3.3 Liquid Medium of Fermentation

A seed culture of *Saccharomyces cerevisiae* is grown in liquid medium of continuous fermentation containing (per liter): 20g of glucose (derived from enzymatic hydrolysis), 10g of yeast extract, 4g of KH_2PO_4 ,2g of $MgCl_2$, 2g of $NH_4Cl_2.H_2O$ and maintaining the pH at 5.5. Mix ingredient in a beaker; A 1000 ml of liquid medium solution is prepared in 2L beaker. Then, use magnetic stirrers to mix the solution and boiled. The cooked media is transferred into 2 Schott bottle, with quantity of each bottle is 500ml. Sterilized for 15 minute at 121^{o}C (Noranisah *et al*., 2005). After that the bottles are kept in refrigerator at 6˚C-8 ˚C (Noranisah *et al*., 2005) before been filled in the flasks.

3.3.4 Culture Maintenance

Culture maintenance of *Saccharomyces cerevisiae* is done in two different ways, which is in single colony unit form on agar slant and as spore suspension and kept in refrigerator at 4 ^{o}C as a stock culture. 10 % (v/v) of stock culture is grown up in oxygen free liquid medium and kept it at 35 ^{o}C for 72 hours. Single colony unit of *S. cerevisiae* is done by streaking the yeast from the solution on to agar slant using the inoculating loop in a laminar flow cabinet to prevent any contamination. All petri dishs were incubated at 35 ^{o}C for 72 hours in aerobic condition. Agar slant which has good single colony unit on agar surface will be keep as a stock.

3.4 Fermentation Procedures

3.4.1 Inoculum Preparation

There are two stages of inoculum preparation. First is yeast activation stage and secondly is inoculum producing stage. The activation stages is done by transfer the single colony unit of yeast from the agar plate into the fresh liquid medium of batch fermentation and keep in anaerobic condition at 35^{o}C for 30 hours. Then followed by the second stage, inoculum producing stage, 10% (v/v) from activation culture is inoculating into fresh liquid medium of batch fermentation. All transferring process was done in sterilized condition. One of the serum bottles is used as controller. The bottles then are incubated in Double Stake Shaking Incubator Infors at 30^{o}C and 150 rpm for 18 hours (Noranisah *et al.*, 2005). This inoculum will be used as seed culture in fermentation using flasks.

3.4.2 Fermentation in shake flask 250 mL

Batch fermentations were done in the 250ml flasks containing glucose from enzymatic hydrolysis were inoculated with 10% of inoculums size from working volume of 100 ml in fermentation medium. Flasks were incubating at 35 °C (optimum) at different agitation speeds of 100, 200 and 300 rpm using optimum inoculum age of 18 hr. Samples of fermented were taken at different time interval and centrifuged at 3,000 rpm for 10 minutes with temperature 30°C to remove the cells (Azrul *et al*., 2006). Each sample was analyzed to monitor ethanol production.

3.5 Analytical Procedures

3.5.1 Di-Nitro Salicylic Acid (DNS) Reagent

DNS reagent was prepared by adding 10-gram of Di-Nitro Salicylic Acid (DNS), 2-gram of phenol and 0.5-gram of sodium sulphate into a 500ml 2 % NaOH solution. The solution was added with distilled water until the volume is 1 liter. The reagent has to be stored in a dark area where purposed to prevent from exposed to light (Noranisah *et al*. 2005).

3.5.2 Concentration of Reducing Sugar Determination

3 ml of sample is taken out and put in a lightly capped test tube. The test tube covered with a piece of prafilm (to avoid loss of liquid due to evaporation for plain tube). Then 3 ml of DNS reagent is added to each of test tube before heated at 90^{o}C for 5-15 min to develop red-brown colour (Noranisah *et al*., 2005). After that 1 ml of 40% potassium sodium tartrate (Rochelle salt) solution is added to stabilize the color. The sample is cooled at room temperature in a cold water bath. The absorbance

reading at 575 nm is recorded using UV-Visible Single Beam Spectrophotometer (Model U-1800) (Noranisah *et al*., 2005).

3.5.3 Ethanol Determination

The amount of ethanol was determined by measuring the measuring the weight decrease of the system at certain intervals and by using refractometer (METTLER TOLEDO Refractor 30PX and 30GS) in order to determine the weight percent and volume percent of ethanol in each sample. This phenomenon is reflected as a weight decrease, which can be correlated to the amount of the ethanol produced (Noranisah *et al*., 2005).

In terms of weight, every gram of glucose can theoretically yield 0.51 g of ethanol. It is assumed that 50% of glucose was used to produce ethanol and 50% of it to produce CO_2; thus there is a weight decrease due to the amount of CO_2 removed from the system and the amount of the ethanol that was produced (Noranisah *et al*., 2005).

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

In order to produce bioethanol from tapioca flour there were many processes need to be done. In order to get sugar from tapioca flour, enzymatic hydrolysis should be done. After sugar can be provided, the sugar will be used as the substrate in the fermentation medium. In this study of bioethanol production from tapioca, it is important to define the optimum temperature and agitation speed in fermentation process, the enzymatic hydrolysis should be standardized. Optimum conditions during the fermentation process are important to get high yield of bioethanol during the process. Method that used to ascertain this condition with constant all the parameter that can affect the yield of ethanol such as:

i) The concentration of the glucose derived from 10% wt/vol of flour in water by using enzymatic hydrolysis.

ii) The pH condition in fermentation experiment.

(High concentration of acid will lead to the destruction of the glucose structure and reduced the amount of bioethanol produced)

iii) Temperature and agitation speed in enzymatic process.

The results were divided into two parts, which consist the results of enzymatic hydrolysis and fermentation process. In this study the results for fermentation process were discussed in order to find the best temperature and agitation speed of fermentation process to produce ethanol from tapioca flour.

4.2 Enzymatic Hydrolysis

During enzymatic hydrolysis, the operating weight of tapioca flour per volume of deionized water is 2%. The operating temperature and time for the liquefaction process is set at 85°C for one hour and for the saccharification process at 55°C for 4 hours. The agitation speed for both processes is maintained at 150 rpm. In this process, the best operating weight of tapioca flour / volume (w/v) of deionized water are 2%. Yield of the glucose is measured using the standard calibration curve of glucose. The yield of glucose is important to determine the ethanol that can be produced. From the standard curve line (Figure 4.1), the reducing sugars that have been produce during enzymatic hydrolysis are about 1.6714 gram. The calculations for sugar concentration are shown in Appendix D.

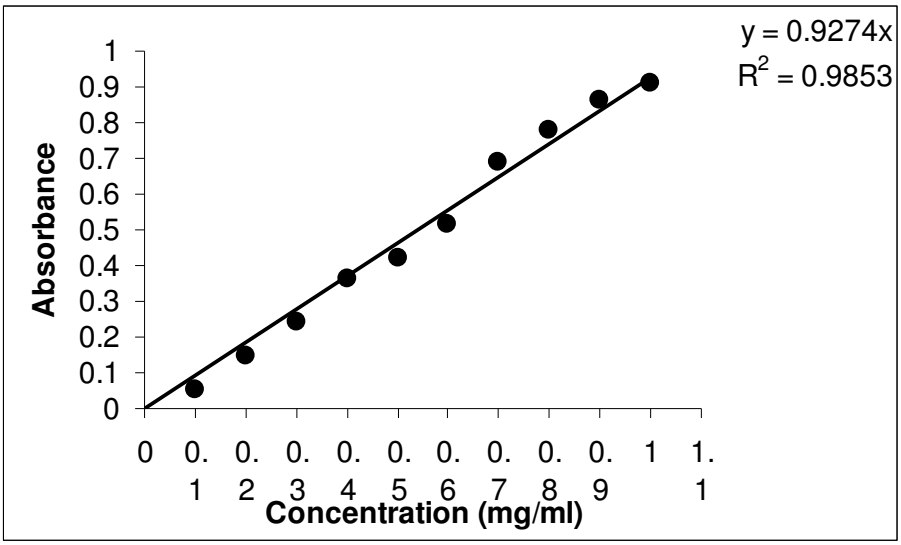


Figure 4.1: Standard Calibration Curve of Glucose

4.3 Fermentation Process

In this research, the fermentation process of sugar derived from tapioca flour by *S. cerevisae* was done with different parameter of agitation speed and temperature. Three different agitation speed were used, 100, 200 and 300 rpm with three different temperature; 30, 35 and 37°C.

4.3.1 Effect of temperature

The temperature has a marked influence on the production of biomass and ethanol. Usually, the rate of alcoholic fermentation increases with temperature to an optimum between 30°C and 40°C using conventional yeast. However, both optimum and temperature tolerance for growth and fermentation are strongly strain dependent (Rousseau *et al*., 1992). Therefore, fermentation experiments were conducted under varying temperature in the range 30-38°C to see the effect of the newly developed strain towards ethanol production. The results are shown in Figure. 4.2, Figure 4.3 and Figure 4.4.

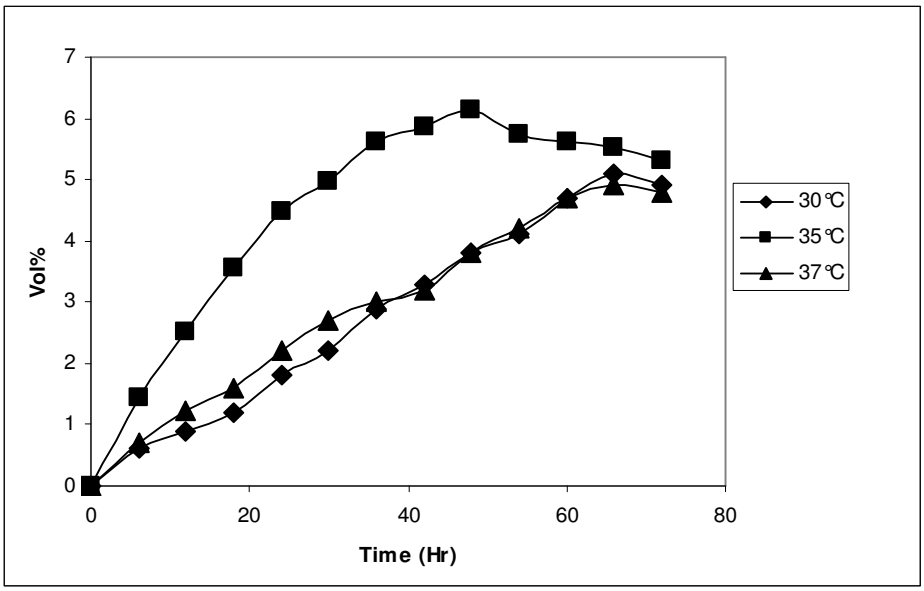


Figure 4.2: Fermentation profile in conical flask at agitation speed of 100 rpm

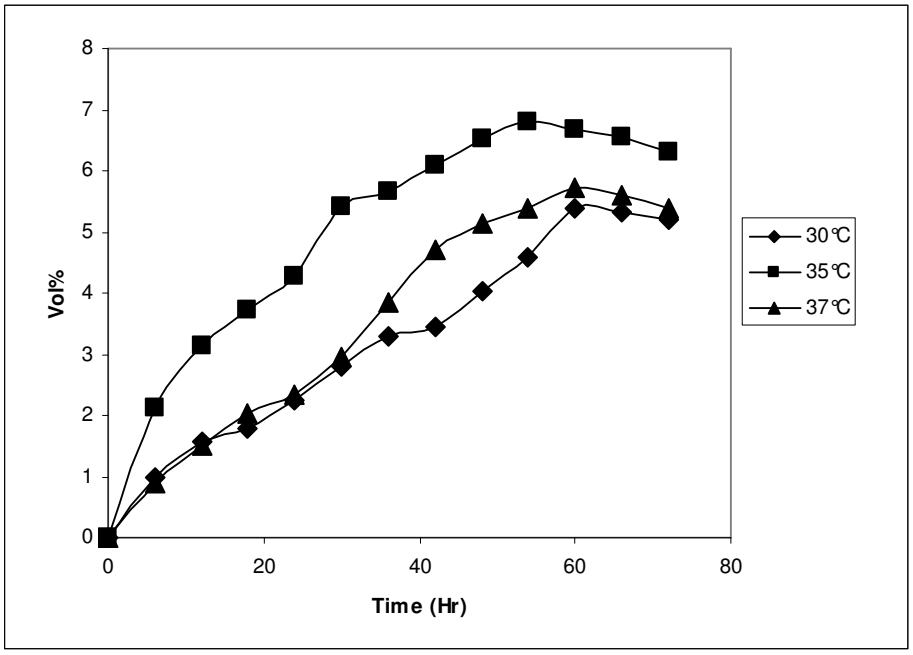


Figure 4.3: Fermentation profile in conical flask at agitation speed of 200 rpm

Figure 4.2 showed the fermentation profile in conical flask at agitation speed of 100 rpm and using various temperatures which are 35°C, 37°C and 37°C. From this agitation speed, the highest volume percent of ethanol was 5.3 Vol%. The fermentation profile at temperature 35°C shows significant increase of ethanol from 0 hr fermentation until 48hr. But the ethanol production starts to decrease after that due to vaporization.

Figure 4.3 shows that the ethanol concentration increased steadily with time for all experimental temperature. Though, the rate of production was initially found to be higher at the high temperatures of 37°C but the highest ethanol concentration was achieved at 35°C. The lower efficiency of the yeast towards ethanol formation may be attributed to the loss of enzyme activity at higher temperatures. So, temperature of 35°C was found to be optimum temperature compared with the other experimental temperature with respect to the highest ethanol concentration in volume percent almost reached 7% from total fermentation broth. Since the result was obtain at temperature range between 30-40°C, this result is agreed with the finding by Rousseau *et al*., (1992) which stated that at temperature range of 30-40°C, the ethanol fermentation will be produced at highest concentration.

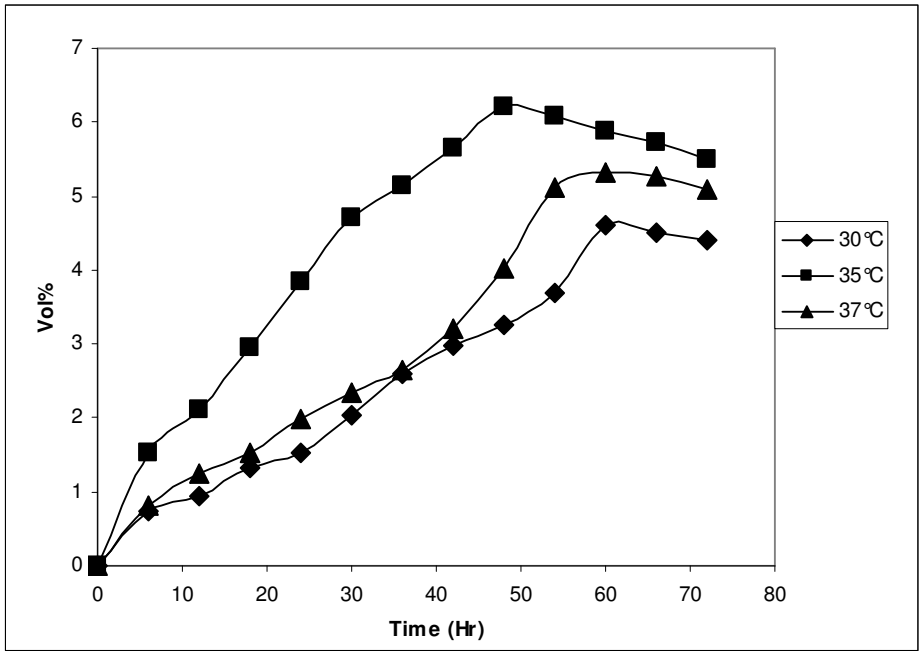


Figure 4.4: Fermentation profile in conical flask at agitation speed of 300 rpm

Figure 4.4 shows that the ethanol concentration increased steadily with fermentation time. Though, the rate of production was initially found to be the highest at temperatures of 35°C. From the fermentation profile, the highest ethanol concentration was observed at agitation speed of 300 rpm and temperature 35°C. The ethanol productivity is high due to sufficient heat during fermentation. After 48 hours of fermentation, the ethanol production was decreasing due to too much heat which was generated from the agitation and accumulated. Increasing in fermentation temperature will reduce the enzyme ability leading to decreasing in ethanol production by the yeast. High agitation speed also, reduces the yeast contact with fermentation broth which lead to unsuitable condition to the fermentation process (Johan, 2005).

4.3.2 Effect of agitation speed

The fermentation was performed with the various agitation speeds of 100, 200 and 300 rpm. At the end of the fermentation process the ethanol composition is determine by using refractometer (METTLER TOLEDO Refracto 30 PX/GS). Result for ethanol fermentation at various agitation speeds at 30°C was showed in Figure 4.5.

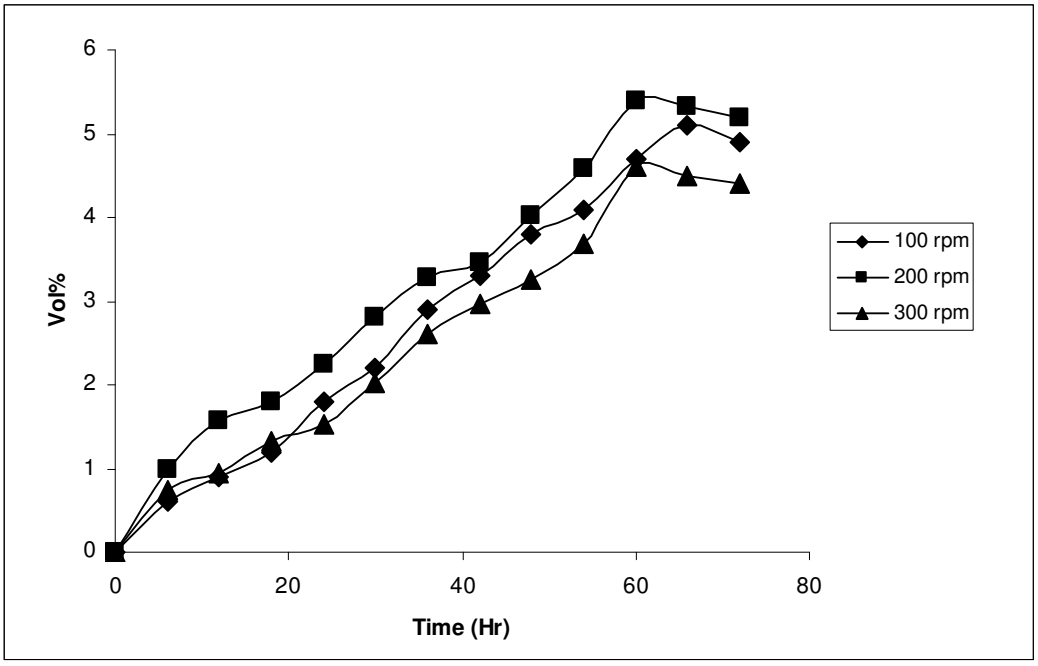


Figure 4.5: Fermentation profile in conical flask at temperature of 30°C

Figure 4.5 showed the fermentation profile in conical flask at various agitation speeds of 100 rpm 200 rpm, and 300 rpm and operating temperature of 30°C. The maximum volume percent of 5.2 Vol% of ethanol was achieved at 200 rpm of agitation speed. At 300 rpm, it was observed to give the lowest of ethanol concentration of 4.4 Vol% due to sheer stress factor that occur during the fermentation because of vigorous mixing intensity. This sheer stress factor will destroy the cell wall of the yeast which resulted in low concentration of ethanol production.

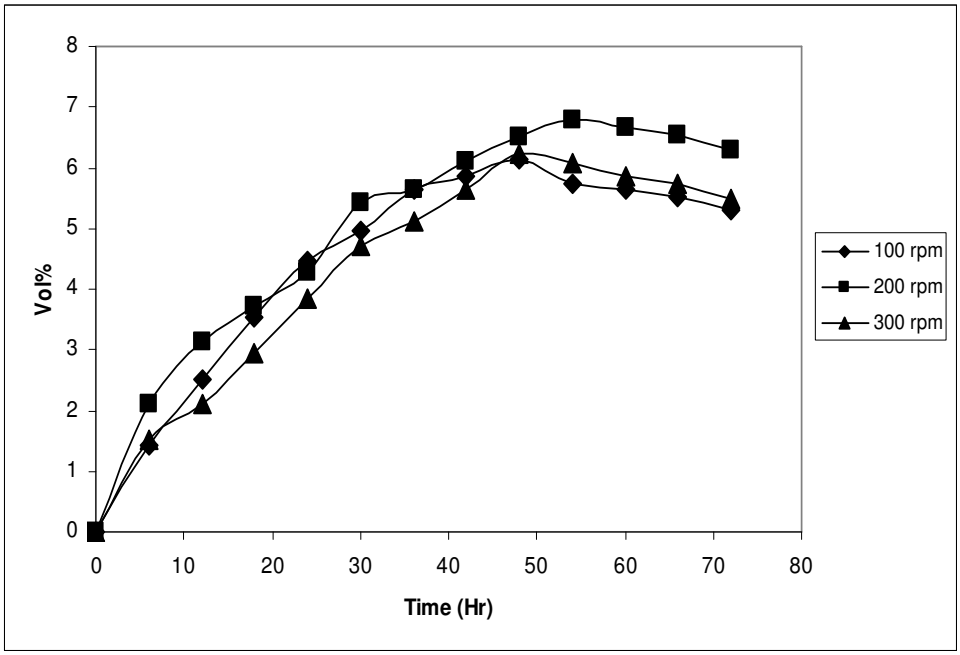


Figure 4.6: Fermentation profile in conical flask at temperature of 35°C

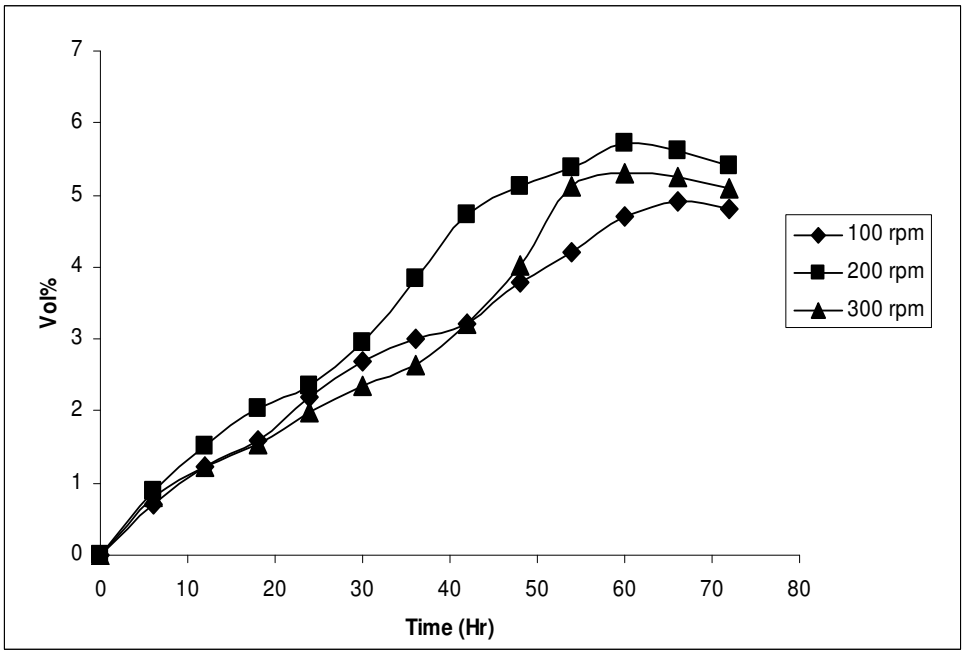


Figure 4.7: Fermentation profile in conical flask at temperature of 37°C

Figure 4.6 showed the fermentation profile in conical flask at various agitation speeds of 100 rpm, 200 rpm, and 300 rpm and at operating temperature of 35°C. From this agitation speed, the highest volume percent of ethanol was 6.3 Vol % which produced at 200 rpm agitation speed. .

Figure 4.7 showed the fermentation profile in conical flask at various agitation speeds and at 37°C. From the agitation speed, the highest of volume percent of ethanol was 5.4 Vol% is recorded at 200 rpm.

The composition of ethanol is increased when the agitation speed achieved 200 rpm. During this rate, the cell will contact properly with the fermentation medium to convert the glucose to bioethanol. The best agitation speed in fermentation process is around 150-200 rpm as stated by Kotter (1993) in his study. The result for ethanol production at agitation speed of 100 rpm showed that, at this rate of agitation, low concentration of ethanol will be produce due to low contact between the yeast and the fermentation broth. However, if the rate of agitation was increased from 200 rpm to 300 rpm, it also will reduce the ethanol concentration produced. It can be consider that, at the high speed of agitation, for example 300 rpm, this will bring extra heat that then accumulated inside the broth and will denatured the enzyme slowly resulted in low ethanol production.

4.3.3 Conclusion

The ethanol fermentation was performed at various temperature and agitation speed. -Summary of the data was presented in Table 4.1.

Table 4.1: Data of Ethanol Composition

Temperature, ° C	**Agitation Speed, rpm**	**Ethanol Composition**
		Vol%
30	100	4.9
	200	5.2
	300	4.4
35	100	5.3
	200	**6.3**
	300	5.5
37	100	4.8
	200	5.4
	300	5.1

In this study the highest ethanol composition comes from fermentation with temperature 35°C and agitation speed 200 rpm with the ethanol composition value is 6.3 % in volume percent. This is because, in order to produce bioethanol, the important things that must be consider was the fermentation condition of *S. cerevisae* where the optimum operating temperature is between 30-40°C (Johan, 2005). At temperature 30°C and the agitation speed at 100, 200 and 300 rpm was not good enough for fermentation due to insufficient of heat supply. The concentration of ethanol increased when the agitation speed increased from 100 rpm to 200 rpm (Kotter, 1993). It can be consider that the higher speed of agitation will bring extra heat that can be negligible and it also increase the cell and medium contact to accelerate fermentation process when the solution was homogenous. However in case of fermentation process at 37°C the ethanol produce at higher rate at the beginning, but at the end of fermentation period which is three days, the operating set up of 35°C and 200 rpm produced higher yield of bioethanol in term of vol %. The cell will contact properly with the fermentation medium at this speed to convert the reducing sugar to bioethanol.

The reason for the ethanol composition is lowest compared to the theoretically is because there are some error occurred during the experiment. It was important to ensure all parameters were standardized. Besides that the glucose that produced after the enzymatic hydrolysis is not 100% purity. The reducing sugar determination shows only for the total for reducing sugar using DNS method. In reducing sugar, there are maltose, fructose and glucose. Bioethanol only can be derived from fermentation of glucose and fructose (Hedley, 2002). Due to long operating time, there also will be vaporization of bioethanol that have been produced. So, the concentration of ethanol produced is decreasing.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

Ethanol fermentation was successfully carried out in flask fermentation using glucose derived from tapioca flour by solvent production yeast *S. cerevisae*. The highest ethanol concentration of 6.3% vol/vol was determined from temperature of 35°C and agitation speed of 200 rpm. Ethanol concentration from this fermentation still can improve not only by manipulating agitation speed and fermentation temperature but other parameter because there are many factors that should be concern such as mode of fermentation, concentration of inoculums and so on. Previous study showed that enzymatic hydrolysis of starch material was important process in order to achieve higher yield of glucose. Theoretically, the bond in starch material which is amylose and amylopectin molecules has to be break down to smaller sugar. The exact concentration of enzyme will lead to the perfect hydrolysis of the amylose and amylopectin structure and increased the amount of glucose produced. Temperature also plays an important role in order to obtain the optimum condition of the hydrolysis.

Enzymatic hydrolysis is an important part to convert the amylose and amylopectin into glucose. The combination α-amylase from *Bacillus lichenformis* and glucoamylase from *Aspergillus niger* has been achieved to convert the amylose and amylopectin structure into glucose. In this process the parameters like temperature, time and agitation speed can be considered in order to optimize the conversion of glucose from tapioca flour. In this study, the temperature was set according to process. The liquefaction with α-amylase, temperature is 85°C was done in one hour with agitation speed at 150 rpm. The saccharification with glucoamylase was done in 4 hours at agitation speed of 150 rpm and temperature 55°C.

5.2 Recommendations

There are several recommendations proposed in order to produce a large amount of bioethanol using biomass sources:

- Another types of biomass waste should be used in order to find which biomass wastes can yield a higher amount of glucose This becomes interesting because biomass wastes are abundant and can get with lower cost in Malaysia
- Commercialization of bioethanol production in Malaysia so that production will meet the demand in environmental friendly way. This will help saving the earth, our health and also as a precautions steps to face of with the depletion of crude oil in the world
- Further research must be conduct to study another critical factor in bioethanol production into details:
    - substrate concentration,
    - pH of medium,
    - Impeller speed if using bioreactor.

REFERENCES

Armstrong, S. M., (1999), Ethanol: Brief Report on its Use in Gasoline (544-etoh),EPA, http://www.epa.gov

Ausbel, F. M., (1998). Current Protocols in Molecular Biology. Vol 2. New York: Wiley Insterscience.

Azrul A. B. Y., (2006). Production of Bioethanol from biomass waste (sugarcane baggase).Thesis of degree of Bachelor of Chemical Engineering.Kolej Universiti Kejuruteraan Dan Teknologi Malaysia.

Barsby, T. L., Donald, A. M., and Frazier, P. J. (2003). Starch advanced in structure and function. *The Royal Society of Chemistry, United Kingdom*. 27(16):26

Bernesson, S., (2006). A limited LCA comparing large and small-scale production of ethanol for heavy engines under Swedish conditions. *Biomass and Bioenergy* 30(1):46

Bro, C., Regenberg, B., (2005). In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. Metabolic Engineering.

Campbell, Neil A., (1996). Biology. 5th Edition. Menlo Park: Addison Wesley.

Castellan, Stephan, (1983). Physical Chemistry. 3rd Edition. New York: Addison Wesley.

Chaplin, M. (2004b). Water structure and behaviour 'Starch'. London South Bank University, Viewed 20th July 2007, URL: http://www.lsbu.ac.uk/water/hysta.html

Galliard, T. (1987). Starch properties and potential, Critical reports on applied chemistry Volume 13. John Wiley & Sons, Britain. 28(36):10-11.

Hedley, C. (2002). Starch Structure. Viewed 20th July 2007, URL: http://www.jic.bbsrc.ac.uk/staff/cliff-hedley/Starch.htm.

Hohmann, Stefan, 1997. Yeast Stress Response. Austin: R.G Landes Company.

Hu, Z., et al., (2004). Economics, environment, and energy life cycle assessment of automobiles fueled by bio-ethanol blends in China. *Renewable Energy* 29(14):2183.

Johan Sendelius, (2005). Steam Pretreatment Optimization for Sugarcane Bagasse In Bioethanol Production, Master of Science Thesis. Deparment of Chemical Engineering, Lund University, Sweden.

Kadam, K. L., (2002). Environmental benefits on life cycle basis of using bagasse-derived ethanol as a gasoline oxygenate in India. *Energy Policy* 30(5):371.

Kim, S. and Dale, B. E., (2005). Environmental aspects of ethanol derived from no-tilled corn grain: nonrenewable energy consumption and greenhouse gas emissions. *Biomass and Bioenergy* 28(5):475.

Kotter, P. and Ciriacy, M. M. (1993), Xylose Fermentation by *Saccharomyces cerevisiae*. *Applied Microbiol Biotechnology*. 43(2):31

Logsdon, J. E., (2006). Ethanol, in: R. E. KIRK, D. F. Othmer, et al. (EDS.). Encyclopedia of Chemical Technology. John Wiley & Sons. Inc., New York.

Marrs, W. M. (1975). The properties and uses of natural and modified starches, Scientific & Technical Surveys No. 85. *Leatherhead Food R.A. Publication*, pp. 1.

Miller, Alex K., (1900). Micro-organisms and fermentation. 3rd Edition. London: Macmillan.

Mills, A.K., (1967). Aspects of Yeast Metabolism. Oxford: Blackwell Science Publications.

Noranisah H., (2005).Production of Bioethanol from biomass waste (rice husk).Thesis of degree of Bachelor of Chemical Engineering.Kolej Universiti Kejuruteraan Dan Teknologi Malaysia

Nur Suriani M. I., (2006). Optimization of Ethanol Production in Yeast Fermentation Process Based on Mixing Time http://eprints.utm.my/3172/1/NUR_SURIANI_MOHD._ISMAIL.pdf cited 28th Ogos 2007.

Olsson, L. and Hagn-Hägerdal, B., (1993). Fermentative performance of bacteria and yeast in lignocellulosic hydrolysates. *Process Biochemistry*. 42(1):13-20

Rasskazchikova, T. V., et al., (2004). Ethanol as high-octane additive to automotive gasoline: production and use in Russia and abroad. *Chemistry and Technology of Fuels and Oils*. 120(6):146

Rong, Y., (2001). The MTBE Paradox of Groundwater Pollution. Environmental Forensics.

Rosillo-Calle, F. and Walter, A., (2006). Global market for bioethanol: historical trends and future prospects. Energy for Sustainable Development.

von Blottnitz, H. and Curran, M. A., (2006). A review of assessments conducted on bio-ethanol as a transportation fuel from a net energy, greenhouse gas, and environmental life cycle perspective. *Journal of Cleaner Production In Press, Corrected Proof*. 120(5):96

APPENDIX A (Equipment)

Appendix A.1.: Laminar air Flow Cabinet (Model AHC-4A1)

Appendix A.2: UV-Visible Single Beam Spectrophotometer (Model U-1800)

Appendix A.3: Autoclave H + P E500 Varioklav

Appendix A.4: Double Stack Shaking Incubator Infors

Appendix A.5: Shaking Water Bath (Model BS-21)

Appendix A.6: Oven Heraeus

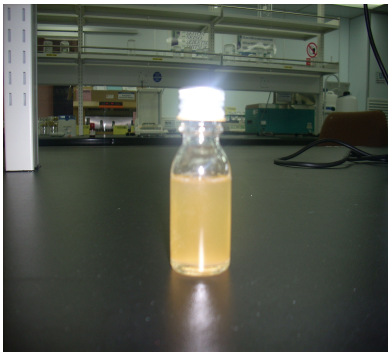
Appendix A.7: High Speed Centrifuging (SORVALL)

Appendix A.8: Inoculating Loop

APPENDIX B

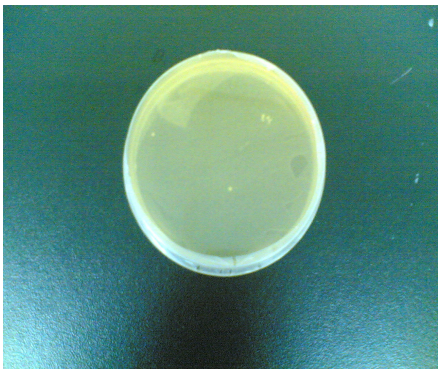
Appendix B.1: Enzymatic Hydrolysis

Appendix B.2: Tapioca Flour



Appendix B.3: Saccharomyces Cerevisiae.

Appendix B.4: Glucose from Tapioca Flour



Appendix B.5: Single Colony of Yeast at Agar Plate

Appendix B.6: α-amylase and Amyloglucosidase

Appendix B.7: METTLER TOLEDO Refracto 30 PX/GS

Appendix B.8: DNS Reagent Test

APPENDIX C

Appendix C.1: Data from Standard Calibration Curve for Glucose

Concentration of Glucose (mg/ml)	**Absorbance @ 575nm**
0.1	0.053
0.2	0.146
0.3	0.241
0.4	0.362
0.5	0.422
0.6	0.516
0.7	0.691
0.8	0.781
0.9	0.863
1	0.913

APPENDIX D

Appendix D.1 Reducing Sugar Yield on Tapioca Flour Weight Basis

Sample Concentration, C_G*=1.6714 mg/ml

(This sample concentration get after the dilution has been done)

The absolute $C_G = C_G$* x 10

C_G = 1.6714mg/ml x 10

C_G = 16.714mg/ml

The amount of reducing sugar, $m_g = C_G$ x V

Volume of buffer used, V= 100ml

$m_g = C_G$ x V

= 16.714mg/ml x 100ml

= 1671.4mg

=1.6714g

From 2g substrate (tapioca flour) will produce 1.6714g of reducing sugar. The efficiency in the enzymatic hydrolysis is 83.57%