BATCH ETHANOL FERMENTATION USING GLUCOSE DESIRED FROM TAPIOCA FLOUR STARCH BY *Saccharomyces cerevisiea*: EFFECT OF INOCULUM AGE AND AGITATION SPEED

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ABSTRACT

The bioethanol can be produced from cellulose and hemicelluloses that originate from various sources of biomass. Besides that, bioethanol can also be produce from starch. This study was focused on the production of ethanol from glucose derive from starch of tapioca flour that containing the starch using *Saccharomyces cerevisiea* as. The objectives of this study are to determine the yield of ethanol from aerobic fermentation at different agitation speed and inoculum ages. Inoculum age is very important in obtaining maximum ethanol production. Enzymatic process is employed to convert the starch in tapioca flour to glucose. In the nut shell, the result showed that the best inoculum age used in ethanol fermentation was 18 hours with 6.45 Vol% of the concentration of ethanol is increased as the agitation speed increased achieved 200 rpm. The cell will contact properly with the fermentation medium at this speed to convert the reducing sugar or glucose to bioethanol.

ABSTRAK

Kini terdapat pelbagai kaedah untuk menghasilkan etanol. Antara kaedah yang digunakan untuk menghasilkan etanol adalah dengan menggunakan hemiselulosa dan juga selulosa yang terdapat pada hasil buangan biojisim. Selain itu, etanol juga boleh dihasilkan menggunakan kanji. Kajian ini dijalankan adalah bertujuan untuk menghasilkan etanol menggunakan tepung ubi kayu yang mengandungi kanji di dalamnya menggunakan *Saccharomyces cerevisiea* ataupun dikenali sebagai kaedah penapaian yis bagi memperluaskan penggunaan tepung ubi kayu . Antara objektif kajian ini adalah untuk mengenalpasti penghasilan etanol menggunakan kaedah penapaian dengan oksigen melalui perbezaan dari segi kelajuan putaran dan jangkamasa inokulum. Jangkamasa inokulum sangat penting dalam menentukan kadar penghasilan etanol. Proses enzimatik digunakan untuk menukarkan kanji yang terdapat dalam tepung ubi kayu kepada bentuk glukosa. Sebagai kesimpulannya, keputusan ujikaji menunjukkan bahawa jangkamasa inokulum pada 18 jam adalah yang terbaik dengan kepekatan etanol sebanyak 6.45 vol% selari dengan peningkatan kelajuan putaran kepada 200 pusingan per minit. Pada kelajuan ini sel dapat bertindak dengan efektif terhadap medium yang digunakan untuk menukarkan gula penurun atau glukosa kepada etanol.

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g	-	gram
ml	-	mililiter
nm	-	nanometer
L	-	liter
kg	-	kilogram
°C	-	degree celcius
%	-	percent
μg/ml	-	microgram per mililiter
mm	-	milimeter
rpm	-	rotation per minute
L/h	-	liter per hour
g/ml	-	gram per mililiter
v/v	-	volume per volume
vol %	-	volume percent
wt %	-	weight percent.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Fermentation is the anaerobic or aerobic conversion of sugar to carbon dioxide and alcohol by microorganisms. A variety of products can be produced by fermentation, such as pharmaceuticals, organic acids, and alcohols. The most widely known fermentation product is ethanol. Ethanol can be produced from a variety of plant-derived raw materials, including agricultural wastes and also from starch, rise husk and sugarcane. Bioethanol is produced using familiar methods, such as fermentation. Two steps are involved on how biomass is converted to bioethanol:

- Hydrolysis of complex polysaccharides in the raw feedstock to simple sugars. The feedstock must first be hydrolyzed into glucose molecules before ethanol production can begin. Biomass-to-bioethanol process, acids and enzymes are used to catalyze this reaction.

- Fermentation is a series of chemical reactions that convert sugars to ethanol. The fermentation reaction is caused by yeast or bacteria, which feed with sugars. Ethanol and carbon dioxide are produced while the sugar is consumed. The reaction for the 6-carbon sugar (glucose) to produce ethanol shown in Equation1.1

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2 \tag{1.1}$$

(Glucose) (Ethanol) (Carbon Dioxide)

One of the greatest challenges for society in the 21st century is to meet the growing demand of energy for transportation, heating and industrial processes and to provide a raw material for those industries (Hahn-Hagerdal, 2006). Increasing concern on the security of the oil supply has been evidenced by increasing oil prices, which is approached US$80 per barrel (Hahn-Hagerdal, 2006). Importantly, the future energy supply must be met with a simultaneous substantial reduction of green house gas emissions.

Ethanol has already been produced on a large scale in Brazil, US and some european countries, and it is expected it to be one of the potential renewable biofuels in the transport sector within next 20 years (Hahn-Hagerdal, 2006). Ethanol can be blended with petrol or used as neat alcohol in dedicated engines, taking advantage of the higher octane number and higher heat of vaporization; furthermore it is an excellent fuel for future advanced flexi-fuel hybrid vehicles (Hahn-Hagerdal, 2006).

Bioethanol is an excellent alternative to fossil fuels, either as a pure fuel with high efficiency and performance or as a gasoline additive. Bioethanol is produced by fermentation. Bioethanol can also be produced from a variety of plant-derived raw material. Ethanol has several attractive features as an alternative fuel. As a liquid it is easily transported and it also can be blended with gasoline to increase the octane rating of the fuel. The huge fluctuations in the price of petroleum within the past twenty years have made commercial production of fermentation ethanol a more attractive, but still risky.

1.2 Problem Statement

The increased concern for the security of the oil supply and the negative impact of fossil fuels on the environment, particularly greenhouse gas emissions, has put pressure on the society to find renewable fuel alternatives. There is a strong reason to produce ethanol as substitutes to fossil fuels in the future. Ethanol usually produces using cheap raw materials such as starch, rise husk and also sugarcane bagasse.

However this raw material base will not be sufficient. Consequently, future large scale use of ethanol will most certainly have to base on production from lignocellulosic materials. This research gives an overview of the new technologies required and the advances achieved in recent years to bring lignocellulosic ethanol towards industrial production.

1.3 Research Objective

Objective of this study is to produce ethanol from batch fermentation of *Saccharomyces Cerevisiae* using tapioca flour as a substrate in a batch system. Studied parameter including:

a) To study the effect of different agitation on batch fermentation process.
b) To study the effect of inoculums ages on fermentation process to produce high yield of ethanol.

1.4 Research Scope

To produce the ethanol from the starch many processes must be done, firstly is how to define agitation speed and also inoculumn age. Besides that, the enzymatic hydrolysis process to convert the starch to glucose before fermentation process also must consider. The research scopes for this experiment are:

a) To determine the age of inoculums that optimize the microbial growth.
b) To produce glucose from tapioca starch via enzymatic hydrolysis.
c) To determine yield of bioethanol that can be produced from starch flour.
d) To optimize inoculum age and agitation speed in fermentation process to produce high yield of ethanol from fermentation of *Saccharomyces Cerevisiae* using tapioca starch as a substrate.

1.5 Research Benefits

a) Ethanol combustion does not cause an increase in atmospheric carbon dioxide (CO_2) concentration.
b) Ethanol is less polluting than gasoline because ethanol produces less toxic substances and less gaseous emissions.
c) Ethanol is a pure substance of known composition, whereas gasoline is a mixture of different compounds.
d) Ethanol is more secure energy source because it is renewable and can be produced anywhere in the world through fermentation.
e) Ethanol has a higher octane number and higher heat of vaporization than gasoline.
f) Ethanol is an excellent fuel for future advanced flexi-fuel hybrid vehicles

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Ethanol plays a key role in policy discussions about energy, agriculture, taxes, and the environment. In the United States it is mostly made from corn; in other countries it is often made from cane sugar. Fuel ethanol is generally blended in gasoline to reduce emissions, increase octane, and extend gasoline stocks. Recent high oil and gasoline prices have led to increased interest in alternatives to petroleum fuels for transportation. Further, concerns over climate change have raised interesting developing fuels with lower fuel-cycle greenhouse-gas emissions. Ethanol is substitutes for transportation fuel when oil crisis arise. It considered as one of alternative way to overcome this problem. Fuel ethanol still taking place at a research institute but in Brazil it already produced on a large scale for the market. From research ethanol performs well as a fuel in cars it also friendly environmental. Another reason to be mentioned the world demand for fuel in this era is predicted to be more than the capacity of global oil production (Johan, 2005)

Ethanol from renewable resources has been of interest in recent decades as an alternative fuel or oxygenate additive to the current fossil fuels. Ethanol made from cellulosic biomass is called bioethanol. In the coming years it is believed that cellulosic biomass will be the largest source of bioethanol. A major challenge is developing biocatalysts capable of fermenting lignocellulosic biomass for efficient industrial application. Lignocellulosic materials are cheap renewable resources, available in large quantities (Caylack, 1998). Rice straw is one of the abundant lignocellulosic waste materials in the world. Cellulose, the major fraction of lignocellulosic biomass, can be hydrolyzed to glucose by cellulase enzymes. This hydrolysis can be affected by porosity of lignocellulosic biomass, cellulose fiber crystallinity and lignin and hemicellulose content. A pretreatment process is essential in order to remove lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity of the materials.

The broad category of biomass for the production of ethanol includes agricultural crops and residues and wood. Biomass resources are abundant and have multiple application potential. Among the various competing processes, bioethanol from lignocellulosic biomass appears to have near-term economic potential. The crops residues such sugarcane bagasse where has are high content of sugar also easy to get in our place will be used as our raw materials, it also used to derive desired economic and environmental benefits and thus they could be important resource bases for bioethanol production (Johan, 2005).

2.1.1 Introduction of starch

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 $C_6H_{10}O_5$ (Raven, 1999). 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.

2.1.2 Composition and structure of starch

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)

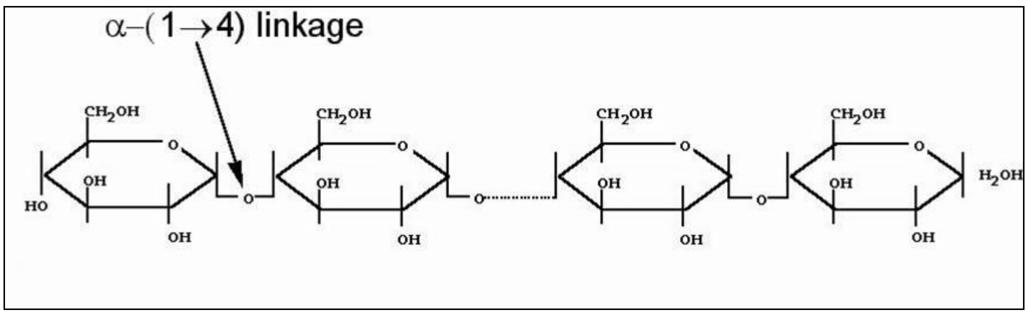

Figure 2.1: Amylose molecule structure (Parker, 2001)

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)

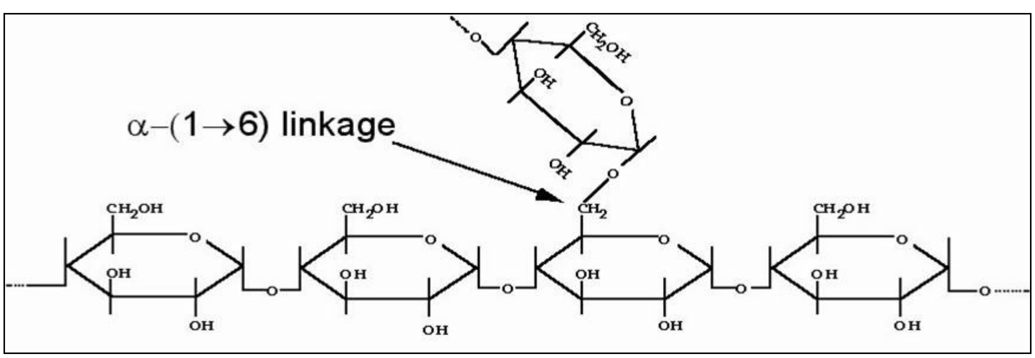

Figure 2.2: Amylopectin molecule structure (Parker, 2001)

Baker's yeast, *Saccharomyces cerevisiae*, is widely used in ethanol production due to its high ethanol yield and productivity, no oxygen requirement, and high ethanol tolerance (Olsson and Hagn-Hägerdal, 1993). These unusual capabilities are the result of adaptation to efficient ethanol production from hexose sugar during thousands of years (Olsson and Hagn-Hägerdal, 1993). However, *S. cerevisiae* cannot transport and use xylose as a substrate, whereas the isomers of xylose (xylulose and ribulose) can be fermented (Jeffries, 2006). Cell metabolic engineering and modification have been extensively carried out to give the ability of xylose assimilation to yeast due to the wide availability of xylose resources. However, the development is still in progress and there is no known recombinant yeast strain that is efficient enough to ferment glucose, xylose and other minor sugars in hydrolysates to ethanol (Jeffries, 2006). Nevertheless, native *S. cerevisiae* is probably still the best choice for softwood hydrolysates, where glucose and mannose are dominant among other sugars. In addition, the native yeasts are inexpensive and widely available.

Saccharomyces cerevisiae is the only yeast that can rapidly grow under aerobic as well as well as anaerobic conditions (Visser, 1990). This unique ability plays a major role in various industrial applications of *S. cerevisiae*, including beer fermentation, wine fermentation and large-scale production of fuel ethanol. Bioethanol is most commonly produced by anaerobic fermentations with *S. cerevisiae.* Many attempts have been made to increase the overall conversion yield from glucose to ethanol.

2.2 Fermentation

The success of fermentation depends upon the existence of defined environmental conditions for biomass and product formation. To achieve this goal it is important to understand what is happening during the fermentation process and how to control it to obtain optimal operating conditions. There are many parameters should be concern like temperature, agitation speed, pH value, dissolved oxygen and nutrient. For the pH, this is very important factor in maintaining a healthy growth for the microorganisms being cultivated. If the pH of the fermenter goes outside an optimum range predefined band, the culture could be lost and produce the side product. For the agitation speed control, the purpose of agitation is two-fold. First, it is to maintain a homogenous mixture within the vessel. Second, it is to help the aeration of the mixture by mixing. In this fermentation process the *S. cerevisiae* will be used. This cell can produce the large amount of ethanol in temperature range around 30-40°C and the agitation speed around 150-200 rpm (Kotter, 1993).

2.3 Ethanol Background

Ethanol (ethyl alcohol, C_2H_5OH) is an alcohol made by fermenting and distilling simple sugars. Ethanol is produced by the fermentation of monosaccharides such as glucose and fructose. The monosaccharides are typically formed through the hydrolysis of any number of biomass options. Ethanol can be manufactured from biomass via the fermentation of sugar derived from grain starches of many crops including wheat wastes and sugar, biomass via the utilisation of the lignocellulosic fraction of crops or petroleum and natural gas. Ethanol is used in the manufacture of alcoholic beverages and it is denatured (made unfit for human consumption) when used for fuel and a variety of other manufactures such as methylated spirits. The biomass source for ethanol production varies depending primarily on local economics and resources. This can be considered an advantage in that regional development of fuel production facilities can optimize for the economics and resources of their own particular surroundings. The two largest producers of Ethanol, Brazil and the United States use biomass sources that are high in carbohydrate content. Other cellulose feed sources are possible as well, such as grass or woodchips.

2.3.1 Processes of ethanol production

Raw materials containing sugars or materials which can be transformed into sugars can be used as fermentation substrates. The fermentable raw materials can be grouped as directly fermentable sugary materials, starchy, lignocellulosic materials and urban/industrial wastes. Direct fermentation of sugarcane, sugar beet and sweet sorghum to produce ethanol has also been reported (Bryan, 1990). Sugar containing materials require the least costly pretreatment, where starchy, lignocellulosic materials and urban/industrial wastes needed costly pretreatment, to convert into fermentable substrates (Sun and Cheng *et al.,* 2002). Sugar containing materials which can be transformed into glucose, can be used as fermentation substrates under anaerobic conditions, glucose is converted to ethanol and carbon dioxide by glycolysis. The phosphorylation of carbohydrates is carried out through the metabolic pathway and the end products are two moles of ethanol and carbon dioxide (Ingram, 1998). Although fungi, bacteria, and yeast microorganisms can be used for fermentation, specific yeast (*S. cerevisiae* also known as Bakers' yeast, since it is commonly used in the baking industry) is frequently used to ferment glucose to ethanol. Theoretically, 100 g of glucose will produce 51.4 gram of ethanol and 48.8 g of carbon dioxide. However, in practice, the microorganisms use some of the glucose for growth and the actual yield is less than 100% (Badger, 2002).

Ethanol production from grain involves milling of grain, hydrolysis of starch to release fermentable sugar, followed by inoculation with yeast. Chemically starch is a polymer of glucose (Peterson, 1995).Yeast cannot use starch directly for ethanol production. Therefore, grain starch has to be wholly broken down to glucose by combination of two enzymes, viz., amylase and amyloglucosidase, before it is fermented by yeast to produce ethanol. Alcohol produced from fermented broth and remaining spillages is processed to produce Distiller's Dried Grain and Soluble (DDGS), which is an excellent ingredient for animal feed (Sheorain, 2000).

Ethanol can be produced by four main types of industrial operations: batch, continuous, fed-batch and semi-continuous. In batch fermentation, substrate and yeast culture are charged into the bioreactor together with nutrients. Most of the ethanol produced today is done by the batch operation since the investment costs are low, do not require much control and can be accomplished with unskilled labour (Caylak and Vardar Sukan *et al.,* 1998). Complete sterilization and management of feedstock are easier than in the other processes. The other advantage of batch operation is the greater flexibility that can be achieved by using a bioreactor for various product specifications. In the continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped continuously into an agitated vessel where the microorganisms are active. The product, which is taken from the top of the bioreactor, contains ethanol, cells, and residual sugar (Maiorella, 1981). The fed-batch operation, which may be regarded as a combination of the batch and continuous operations, is very popular in the ethanol industry. In this operation, the feed solution, which contains substrate, yeast culture and the required minerals and vitamins, are fed at constant intervals while effluent is removed discontinuously. The main advantage of the fed-batch system is that intermittent feeding of the substrate prevents inhibition and catabolite repression. If the substrate has an inhibitory effect, intermittent addition improves the productivity of the fermentation by maintaining a low substrate concentration. It is essential to keep the culture volume constant in continuous operation, whereas there is volume variation in the fed-batch processes. In semi-continuous processes, a portion of the culture is withdrawn at intervals and fresh medium is added to the system. In the continuous processes it is essential to maintain a constant culture volume, whereas there is volume variation in semi-continuous processes. This method has some of the advantages of the continuous and batch operations. There is no need for a separate inoculum vessel, except at the initial startup. Time is also not wasted in non-productive idle time for cleaning and resterilization. Another advantage of this operation is that not much control is required. However, there is a high risk of contamination and mutation due to long cultivation periods and periodic handling. Furthermore, since larger reactor volumes are needed, slightly higher investment costs are required (Caylak, 1998).

2.3.2 Ethanol as fuel

The majority of the energy used today is obtained from the fossil fuels. Due to the continuing increases in the cost of fossil fuels, demands for clean energy have also been increasing. Therefore, alternative fuels sources are sought. Some of the most important fuels are biogas, natural gas, vegetable oil and its esters alcohols and hydrogen. Ethanol which is one of the renewable energy sources and is obtained from biomass has been tested intensively in the internal combustion engines. Some properties of ethanol with comparison to gasoline are given in (Table 1.1). Currently, ethanol for fuel market is produced from sugar or starch at competitive prices. However, this raw material base, which also has to be used for animal feed and human needs, will not be sufficient to meet the increasing demand for fuel ethanol and the reduction of greenhouse gases resulting from use of sugar or starch based ethanol is not as high as desirable (Farrell, 2006).

Table 2.1: Properties of gasoline and ethanol (Das, 1996)

Properties	Gasoline	Ethanol
Chemical formula	C_4–C_{12}	C_2H_5OH
Molecular weight	100–105	46
Oxygen (mass %)	0–4	34.7
Net lower heating value (MJ/kg)	43.5	27
Latent heat (kJ/L)	223.2	725.4
Stoichiometric air/fuel ratio	14.6	9
Vapor pressure at 23.5 °C (kPa)	60–90	17
MON	82–92	92
RON	91–100	111

Due to the high evaporation heat, high octane number and high flammability temperature, ethanol has positive evaporation pressure enable to storage and transportation safely. Since the oxygen contain has positive effect on environment. In spite of its positive effect when used in gasoline engine as alternative fuel, it is necessary to make some modification on the engine. The fuel system requires more fuel. The vehicle takes less distance with ethanol fuel than gasoline. Because of the first cold starting problem of the pure ethanol, the blend called E85 has a widespread usage as alternative fuel. This fuel consists of 15 vol% unleaded gasoline and 85 vol% ethanol. However, the other blend consisting of 90% gasoline and 10% ethanol called as gasohol. In addition, the flame of the ethanol is colorless in the natural burning processes and this is another advantage of ethanol (Das, 1996)

2.3.3 Ethanol economics

Ethanol is made from farm-produced raw products which are usually in surplus. Sugarcane is the primary grain used in ethanol production, and it supplies most of the raw material needed. Ethanol production is the third largest user of sugarcane, behind domestic livestock feed and export uses. The conclusions of the report verify that the federal ethanol program is cost effective. The increase in production and price would raise gross farm income. The increase in farm expenditures and employment opportunities in the ethanol industry is projected to increase annually in relationship to additional ethanol production and use. As the domestic ethanol industry continues to grow, it is witnessing a surge in the construction of farmer-owned ethanol production facilities. Farmers are realizing the added benefits to the ethanol industry through ownership of manufacturing plants. Ethanol’s importance to agriculture is evident like added markets for farmers, stimulating rural economies by increasing sugarcane prices and rural income (Farrell, 2006).

2.3.4 Ethanol and environments

Directly related to fossil energy consumptions the question of greenhouse gas emissions. Proponents of ethanol argue that over the entire fuel cycle it has the potential to reduce greenhouse gas emissions from automobiles relative to gasoline, therefore reducing the risk of possible global warming. Because ethanol contains carbon, combustion of the fuel necessarily results in emissions of carbon dioxide (CO_2), the primary greenhouse gas. Further, greenhouse gases are emitted through the production and use of nitrogen-based fertilizers, as well as the operation of farm equipment and vehicles to transport feedstock and finished products. However, since photosynthesis (the process by which plants convert light into chemical energy) requires absorption of CO_2, the growth cycle of the feedstock crop can serve to some extent as a “sink” to absorb some fuel-cycle greenhouse emissions. Other studies that conclude higher fuel-cycle energy consumption for ethanol production also conclude higher greenhouse gas emissions for the fuel (Brent, 2006).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Ethanol Fermentation Process

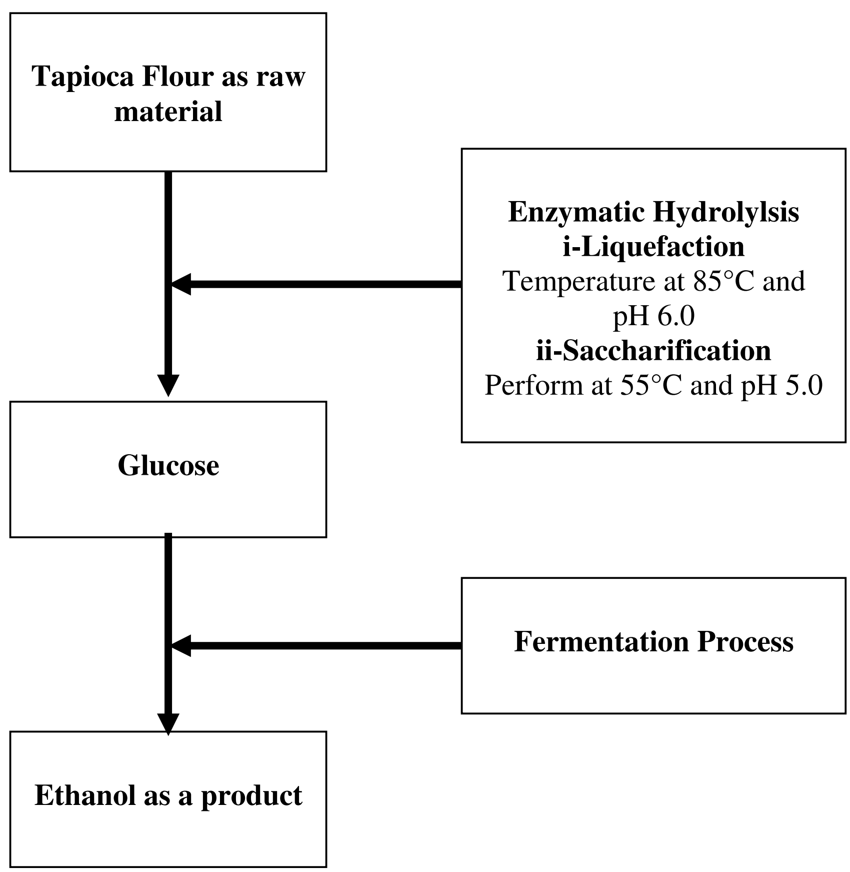

Figure 3.1: Overall flow process of aerobic fermentation process