EFFECTS OF pH AND TEMPERATURE ON GLUCOSE PRODUCTION FROM TAPIOCA STARCH USING ENZYMATIC HYDROLYSIS: A STATISTICAL APPROACH

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SITI NOR SHADILA BINTI ALIAS

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
University Malaysia Pahang

May 2009

I declare that this thesis entitled **"Effects of pH and Temperature on Glucose Production from Tapioca Starch using Enzymatic Hydrolysis: A Statistical Approach"** 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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Date :

Special Dedication to my family members,
my supervisor, my lecturers, my love, my friends, my fellow colleagues
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

The aim of this study was to maximize the glucose concentration produced from enzymatic hydrolysis of tapioca starch. The tapioca starch was enzymatically hydrolyzed using α-amylase from *Bacillus lichenformis* followed by amyloglucosidase action from *Aspergillus niger*. Effects of liquefaction temperature (X_1), saccharification temperature (X_2), liquefaction pH (X_3) and saccharification pH (X_4) were evaluated. 2^4 full factorial design with 1 replicate and 3 centered point was applied to determine the significant parameters affecting the production of glucose concentration. The range of the factors employed were 60 -90˚C (liquefaction temperature), 40-60˚C (saccharification temperature), 5-7 (liquefaction pH) and 4-6 (saccharification pH). The maximum glucose concentration obtained experimentally was 329.10 g/L. The ANOVA shows that the effects of liquefaction temperature and saccharification pH on glucose production were very significant. The saccharification temperature and liquefaction pH, on the other hand did not influence the glucose production**.** The optimum liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH suggested by the design of experiment were 90˚C, 60˚C, 7 and 6 respectively. From that optimum condition, the maximum glucose concentration of 331.8 g/L was estimated.

ABSTRAK

Tujuan kajian ini adalah untuk memaksimumkan kepekatan glukosa yang terhasil daripada kanji ubi kayu oleh hidrolisis enzim. Kanji ubi kayu ditindakbalaskan oleh enzim α-amylase daripada *Bacillus lichenformis,* diikuti oleh tindakan daripada enzim amyloglucosidase berasal daripada *Aspergillus niger*. Kesan suhu *liquefaction* (X_1), suhu *saccharification* (X_2), pH *liquefaction* (X_3) and pH *saccharification* (X_4) telah dikaji. '2^4 full factorial design' dengan 1 'replicate' dan 3 'centered point' diaplikasikan untuk menentukan parameter yang memberi kesan kepada penghasilan kepekatan glukosa. Julat yang ditetapkan oleh setiap parameter adalah seperti berikut: 60-90˚C (suhu *liquefaction*), 40-60˚C (suhu *saccharification*), 5-7 (pH *liquefaction*) dan 4-6 (pH *saccharification*). Kepekatan glukosa maksimum yang didapati daripada eksperimen adalah 329.10 g/L. Analisis ANOVA telah menunjukkan bahawa suhu *liquefaction* dan pH *saccharification* memberi kesan yang signifikan terhadap penghasilan glukosa. Sebaliknya, suhu *saccharification* dan pH *liquefaction* tidak mempengaruhi penghasilan glukosa. Keadaan optimum bagi suhu *liquefaction*, suhu *saccharification*, pH *liquefaction* dan pH *saccharification* yang dicadangkan oleh 'design of experiment' masing-masing adalah 90˚C, 60˚C, 7 dan 6. Daripada keadaan optimum yang dicadangkan, kepekatan glukosa maksimum adalah dianggarkan sebanyak 331.824 g/L.

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LIST OF SYMBOLS/ ABBREVIATIONS

%	-	percentage
˚C	-	degree Celsius
ABS	-	Absorbance
ANOVA	-	Analysis of Variance
DP	-	degree of polymerization
DNS	-	Di-Nitro Salicylic Acid
EPA	-	Environment Protection Agency
g	-	gram
g/L	-	gram per liter
gcm^{-3}	-	gram per centimeter cubic
$gmol^{-1}$	-	gram per mol
h	-	hour
H_2SO_4	-	Sulfuric Acid
HCl	-	Hydrochloric Acid
mg protein/ml	-	milligram protein per milliliter
mg	-	miligram
mL	-	mililiter
NaOH	-	Sodium Hydroxide
nm	-	nanometer
rpm	-	revolution per minute
RSM	-	Response Surface Methodology
U/mg	-	Unit per miligram
w/w	-	weight per weight
µL	-	microliter

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ethanol has been produced commercially and acts as an alternative energy for crude oil. The American Petroleum Institute has estimated that the world's oil supply would be depleted between 2062 and 2094. Ethanol has a potential to be used as a promise fuel as ethanol is more volatile than water, flammable, burns with a light blue flame, and has good fuel properties for spark ignition internal combustion engines. Ethanol can be produce via two paths which are: (1) fermentation of simple sugar (fermentable sugar) degraded from starch or cellulosic materials and (2) reaction of ethylene with water. The most preferable path is fermentation process since ethylene is too expensive. Utilizing enzymatic and fermentation technology ethanol can be produced from low cost raw material such as agricultural waste, agro-industrial waste, woody crops, corn, sorghum, potato and sago (Patle and Lal, 2008; Wyman, 2004; Mojović *et al.,* 2006; Suriani, 2002). The new route will reduce the consumption of petroleum and less pollution to environment.

Fermentable sugar mainly being produced from two major feedstocks which are lignocellulosic (wood, straw and grasses) and starchy materials (wheat, corn, barley and tapioca) by enzymatic hydrolysis (Balat *et al.,* 2008).

Lignocellulosic materials build up from three basic formulas which are cellulose $(C_6H_{10}O_5)_x$, hemicellulose such as xylan $(C_5H_8O_4)_m$ and lignin $[C_9H_{10}O_3.(OCH_3)_{0.9\text{-}1.7}]_n$ (Balat *et al.,* 2008). Due to their continuously abundant

availability, the cost for lignocellulosic materials is very low. However, the lignin component is highly branched, substituted, and composed of mono-nuclear aromatic polymers cell wall which caused hydrolysis of lignocellulosic to be non-effective process. Pretreatment process is required to alter or remove the complex structure of lignocellulosic material to enhance the rate of enzyme action and increase yield of fermentable sugar. Yu and his coworkers (2008) have reported that hot-compressed water (HCW) pretreatment is one of the most cost-effective pretreatment processes for enzymatic hydrolysis. Water under pressure penetrates the cell structure of biomass, hydrates cellulose, and degrades hemicellulose and lignin. In addition, the acidity of water at high temperature and the organic acids produced from hemicellulose facilitate the disruption of lignocellulosic structure during pretreatment. Peiris and Silva (1986) have noticed that without pretreatment, 5.6 g/L of reducing sugar was produced in five days from hydrolysis of wheat straw compared to 29.5 g/L of reducing sugar produced after a pretreatment using NaOH.

Starch is one type of complex sugar which known as polysaccharide. Similar to cellulose, starch molecule are glucose polymers linked with α-1, 4 and α-1, 6 glucosidic bonds. To produce glucose syrup from starch, it is necessary to break down the chain of this carbohydrate. Differed to lignocellulosic material, starch can be directly converted to fermentable sugar via either enzymatic or acid hydrolysis without performing the pretreatment process. Hydrolysis is a reaction of starch with water, which is normally used to cleave the starch to fermentable sugar (glucose). Enzymes are act as the catalysts for the reaction (Balat *et al.,* 2008).

Tapioca (*Manihot esculenta*) is the example of starchy material (Figure 1.1). Tapioca also known as cassava in Africa while in South America, it is called as manioc or yucca. The taste is bitter and sweet varieties. Tapioca is traditionally used as dessert or breakfast meal. As the development of technology become more sophisticated, tapioca starch is useful in textile industry, paper industry and for miscellaneous uses (Vandamme *et al.,* 2002). Tapioca starch is cheap and easy to find in the tropical and subtropical areas like Asia and Southern Africa. Tapioca is believed as the cheapest sources of starch compared to the cereals, tubers and root crops (Patle and Lal, 2008). Tapioca contains almost 70-75% of starch. Therefore it

is suitable in renewal source of bio-ethanol and would be alternatively replaces the petroleum demand.

Figure 1.1: Tapioca Block

1.2 Objective

The aim of this study was to maximize glucose concentration produced from enzymatic hydrolysis of tapioca starch. Hence, the objectives of this study are:

- To determine the effects of pH and temperature on the amount of glucose produced from enzymatic hydrolysis of tapioca starch.
- To determine the optimum temperature and pH for liquefaction and saccharification steps that maximize glucose concentration produced.

1.3 Scope of Study

Glucose has been produced by enzymatic hydrolysis that comprised of two main processes which are liquefaction and saccharification. The entire work was conducted is to determine the yield of glucose that can be produced from locally available tapioca starch via enzymatic hydrolysis process. Various liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH were investigated. Agitation speed, reaction time and enzyme loading were fixed during the experiment and glucose produced was analyzed by using DNS method.

All experiments were assisted by 2^4 full factorial design from Design Expert Software Version 7.1.6 with 1 replicate and 3 centered points.

1.4 Problem Statement

Bioethanol is an alternative fuel as many researchers and manufacturers are very interested to involve with. Bioethanol can be produced from fermentation of fermentable sugar. Fermentable sugar can be derived from starch or lignocellulosic material. The lignocellulosic materials such as biomass waste, crop and grasses are abundantly available compared to the starchy material. However, the cost of the materials was elevated due to the pretreatment employed for the lignocelluloses. Hence starchy material was chosen to be the raw material for this study.

Tapioca contains almost 70-75% of starch and available in Malaysia and in Asian countries such as Indonesia, Thailand and Vietnam. Tapioca is believed as the cheapest sources of starch compared to the cereals, tubers and root crops. (Patle and Lal, 2008).

In order to obtain fermentable sugar (glucose syrup), starch can be treated either via enzymatic or acid hydrolysis Enzymatic hydrolysis was preferred compared to acid hydrolysis because acid is very corrosive and difficult to handle. Besides, acid hydrolysis tends to produce other undesired by product like 5-hydroxymethylfurfural and make the product change the color to brownish. Enzymatic hydrolysis is typically chosen because it produce better yield, less byproduct and easy to handle due to not have a corrosive problem. Additionally, utility cost of enzymatic hydrolysis is recently found to be lower compared to acid hydrolysis (Balat *et al.,* 2008) as enzymatic hydrolysis is usually conducted at mild condition. Hence, enzymatic hydrolysis is more preferable in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of Starch

Starch is the main carbohydrate storage in many plants. Starch from all plant sources occurs in the form of water soluble granules which differ in size and physical characteristics from species to species (Madihah *et al.*, 2001). Starch is one type of complex sugar which is polysaccharide. Starch polysaccharides are macromolecules that consist of a large number of glucose units. They are sometime known as glycans.

Starch is a mixture of two polysaccharides that built from glucose units which are amylose (a linear chain molecule) and amylopectin (a branched polymer molecule of glucose) (Sun *et al.,* 2006; Madihah *et al.*, 2001; Nigam and Singh, 1995). The structures of both polymers are illustrated in Figures 2.1 and 2.2. The relative amount of amylose and amylopectin depends on the sources of the starch. However, the major component is usually amylopectin (73 - 86%) and the minor component would be amylose (14 - 27%) (Aehle, 2007). Corn starch from waxy maize for example consist only 2% amylose and almost 80% is amylopectin. Meanwhile, cereal typically contains of 70% amylopectin and 30% amylase (Nigam and Singh, 1995). Suraini (2002) has reported that sago contains 65% of starch while rye, wheat and corn contain 59.6, 61.2 and 71.5% or starch respectively (Czarnecki and Grajek, 1990). In separate analysis Mojović *et. al* (2006) reported corn meal contained 70.82% (w/w) of starch.

Alpha- amylose is a linear polymer that consist of several thousands of glucose units, alpha-(1, 4)-glycosidically linked. Amylopectin on the other hand carries α-(1, 6)-connected branches every 24 to 30 glucose units of the alpha-(1, 4)-linked chain and become tree or brush-like structure (Sun *et al.,* 2006). Starch is susceptible to enzyme attack and influenced by several factors such as amylose and amylopectin content, particle size, crystalline structure and the presence of enzyme inhibitor (Shariffa *et al.*, 2008).

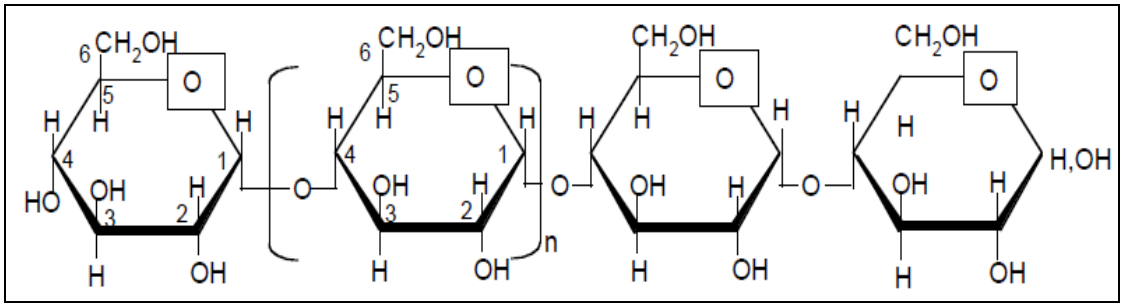

Figure 2.1: Amylose Structure (Tester *et al.,* 2004)

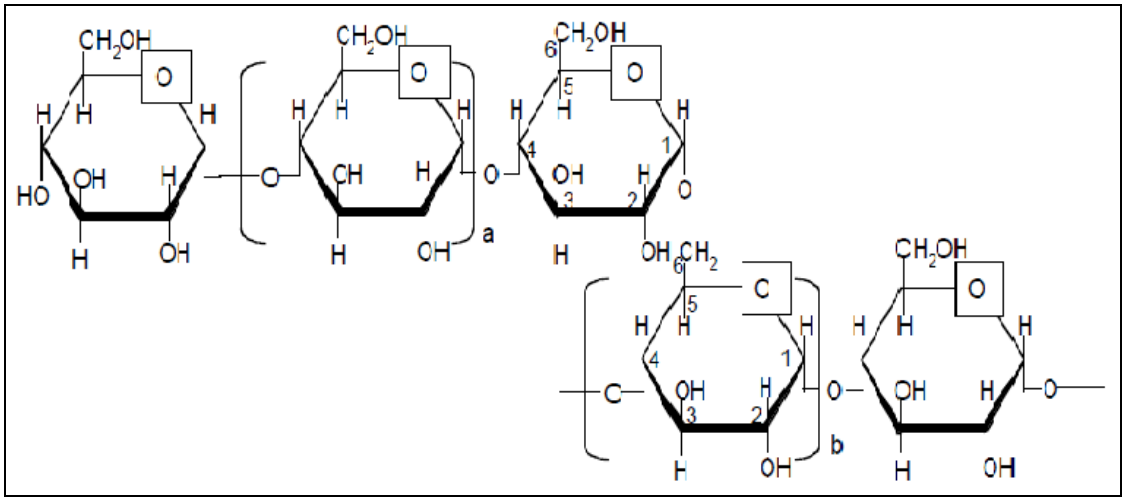

Figure 2.2: Amylopectin Structure (Tester *et al.,* 2004)

Starch is widely used in textile industry to hold colors in the desired area of the fabric and to avoid spreading and mixing of the color. In addition, starch is used to enhance the strength of the sheet in paper industry. It is also used as an adhesive for paper bag (Vandamme *et al.,* 2002).

2.2 Background of Glucose

Glucose ($C_6H_{12}O_6$) contains six carbon atoms, one of which is part of an aldehyde group, and therefore known as an aldohexose (Figure 2.3). Glucose commonly presents in a form of white substance or as a solid crystal. Glucose also known as confectioners' syrup and can be dissolved in water as an aqueous solution (Vandamme *et al.,* 2002). The molar mass and density of glucose is 180, 16 $gmol^{-1}$ and 1.54 gcm^{-3}, respectively. The melting point of α-D-glucose and β-D-glucose is 146˚C and 150˚C, respectively.

In Brazil, glucose from cane was widely used to produce fuel bioethanol (Wyman, 2004). In UK, glucose syrup and high maltose syrup is used in brewing as fermentable carbohydrate. Liquid glucose is used as substrate for the production of stabilizer xanthan gum from *Xanthomonas campestris* and for the growth of mycoprotein from *Fusarium graminearum* (Vandamme *et al.,* 2002). Glucose also is important in food sweetener industries as well as in the production of antibiotics (Vandamme *et al.*, 2002; Chowdary *et al.*, 2000; Nigam and Singh, 1995). In addition, organic acid like lactic, citric, and ascorbic (vitamin C) acids are also being produced from glucose (Vandamme *et al.*, 2002; Madihah *et al.*, 2001).

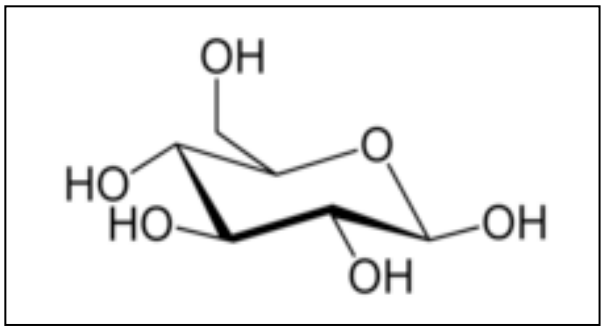

Figure 2.3: Glucose structure (Vandamme *et al*., 2002)

2.3 Enzymatic Hydrolysis

Starch degradation process catalyzed by enzyme is known as enzymatic hydrolysis. Liquefaction and saccharification are the main steps of this process (Sun *et al.,* 2006; Suraini, 2002; Linko and Wu, 1993).

Starch is degraded by enzymes called α-amylase which is derived from bacteria (Nigam and Singh, 1995). α-amylase is an endo-acting enzyme which randomly hydrolyze α-(1, 4)-glycoside bonds inside the starch structure and quickly destroy the whole starch structure (Sun *et al.,* 2006; Kaur and Satyanarayana, 2004; Nigam and singh, 1995). The degradation products would be oligosaccharide fragments such as glucose, maltotetrose, maltose, maltoriose as well as oligosaccharide containing α-(1, 6)-branches. All the components are known as dextrin mixture (Sun *et al.,* 2006). However the percentage of glucose is very low and need further enzyme treatment. The oligosaccharides formed from amylase activity are further hydrolyzed by exo-acting enzyme, glucoamylase which can cleave both α-(1, 4) and α-(1, 6)-branches from the non-reducing ends of the starch polymers and forms exclusively glucose (Sun *et al.,* 2006; Kaur and Satyanarayana, 2004; Nigam and Singh, 1995; Linko and Wu, 1993).

2.3.1 Liquefaction and Saccharification Steps

In liquefaction step, gelatinization is required to increase the rate of hydrolysis as the native starch is slowly degraded by α-amylase (Shariffa *et al.*, 2008). Therefore, gelatinization and swelling are needed to make the starch easy to breakdown by enzyme (Aehle, 2007). Gelatinization is achieved by heating starch with water which occurs automatically when starchy materials are cooked. At this condition, the pores of the starch become larger than usual and the enzyme easily can penetrate into the starch polymer and interrupt the hydrogen bond between the polymer chains to become weak (Shariffa *et al.*, 2008).

Liquefaction process is employed to loosen the structure of starch polymer and reduce the viscosity of the gelatinized starch and ease the next hydrolysis processing. α-amylase enzyme which is thermostable enzyme (Liu *et al.,* 2008; Vandamme *et al.,* 2002; Nigam and Singh, 1995) is being used in liquefaction step where it will initially attack the interior bonds of starch granules composed of long chain of glucosyl residues linked by α-(1,4)glycosidic bond. It branches was comprised of amylopectin fraction linked by α-(1,6)glycosidic bonds. α-amylase is employed due to its active actions (1) degrade the long starch chains so that starch will not form a gel at lower temperature and, (2) produce more chain ends, as glucoamylase, the enzyme used in the sacchatification step, will cleave glucose molecules only from the non reducing ends of the chains. In liquefaction, pH is not allowed to drop below 4.5 otherwise the α-amylase will be denatured (Nigam and Singh, 1995). Liu *et al.* (2008) has reported that α-amylase is sensitive to acidic circumstances, and this could result in the loss of its hydrolysis activity. Additionally, α-amylase operates optimally at 90˚C and pH 6 (Liu et al., 2008). Previously, liquefaction step in corn starch hydrolysis was performed at 85˚C and pH 6.0 (Mojović *et al.*, 2006). Again, the optimum liquefaction pH was reported to be 6 by Vandamme *et al.* (2002). The optimum α-amylase action and reducing sugar production in continuous enzymatic hydrolysis was obtained at pH 6 and 30˚C. The amount of reducing sugar produces from sago starch was 0.464 g/Lh.

Saccharification step is important to further hydrolyze the liquefied starch. Glucoamylase also known as amyloglucosidase is being used in the saccharification step. The glucoamylase breaks the α-(1,6)glycosidic bonds in the liquefied starch chains. Saccharification leads to about 96% yield of glucose, and about 4% byproduct. The overall saccharification reaction occurs in the hydrolysis process is as follows:

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

$C_6H_{12}O_6$ is glucose unit that produced when the alpha bonds linking *n* unit of $C_6H_{10}O_5$ in starch polymer are cleaved and as its hydrolyzed by *n* molecules of water, H_2O. Saccharification of corn starch has been reported to be performed at 55˚C and

pH 5 (Mojovic *et al.*, 2006) while according to Vandamme *et al.* (2002), the optimum saccharification has been conducted at 60˚C and pH 4.5. In separate study, Aggarwal *et al.* (2001) has found that at high temperature, the rate of saccharification reduced substantially at the optimum condition for saccharification were at 45˚C and pH 5.

Figure 2.4 describes the action of hydrolytic enzymes on amylose and amylopectin. β-amylase is an exo-acting enzyme cleaving β-maltose molecules from the non-reducing end of amylose or from the outer branches of amylopectin. Meanwhile, α-amylase is an endo-acting enzyme hydrolyzing α-(1-4) bonds at random, producing the malto oligosaccharides (linear or branched). Debranching enzymes (e.g. isoamylase or pullulanase) on the other hand, hydrolyse α-(1-6) bonds at the branching points of amylopectin. Meanwhile, amyloglucosidase is an exo-acting hydrolase which releases single glucose molecules from the non-reducing end of α-(1-4) oligo or polysaccharides. This enzyme is unique because it can hydrolyse α-(1-6) branching points, and completing the hydrolysis of starch (Tester *et al.,* 2004).

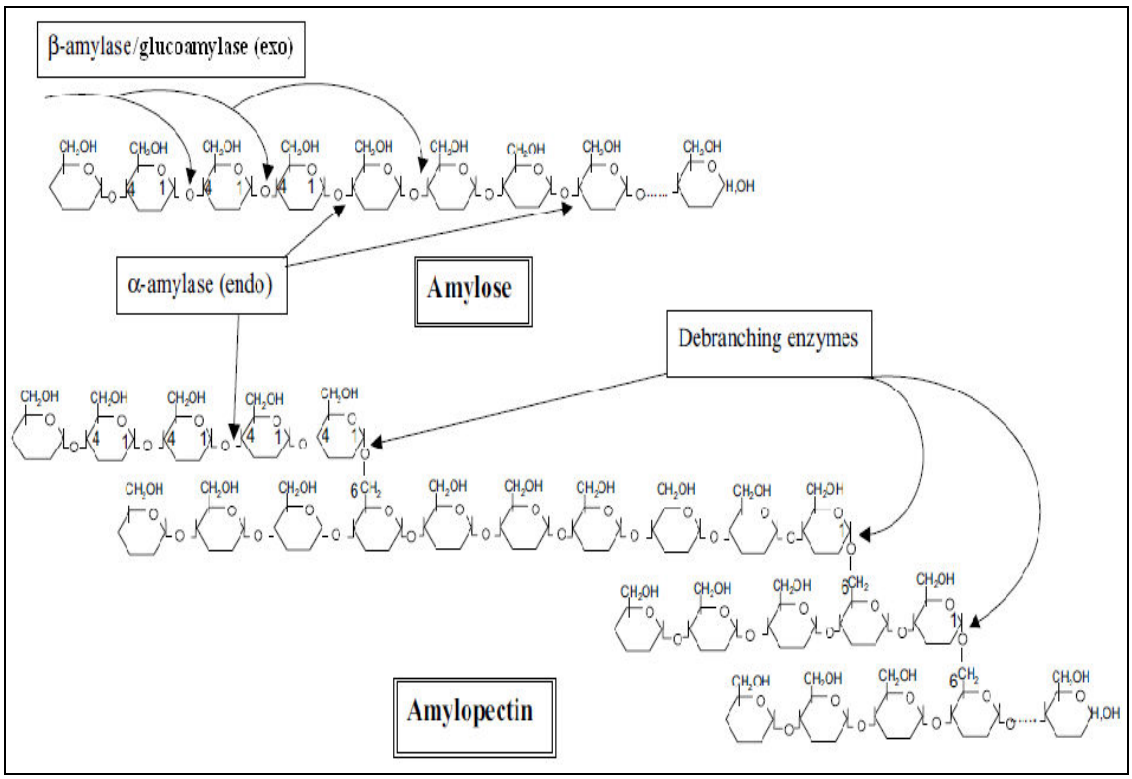

Figure 2.4: Enzymatic Hydrolysis Mechanism (Tester *et al.,* 2004).

2.3.2 Factors Effecting Hydrolysis Yield

Both liquefaction and saccharification steps have different optimum temperature and pH values for the maximum reaction rate and product yield purpose. According to Kunamneni and Singh (2005), the glucose product will slightly increases with the increase of pH value. Same goes to temperature where there is slight increment of glucose amount with increasing the temperature (Kunamneni and Singh, 2005).

Hydrolysis yield also depends on substrate concentration, type of starch, enzyme dose, time taken, and speed of agitation, granule size and viscosity of the raw starch (Balat *et al.*, 2008; Zulfikri *et al.*, 2008; Madihah *et al.*, 2001; Marlida *et al.*, 2000; Czarnecki and Grajek, 1991). Lower substrate concentrations are more suitable in order to avoid substrate inhibition. For example, when a 16% suspension of corn flour is hydrolyzed, the glucose yield is 76%, while when a 40% suspension is hydrolyzed the yield is only 50.2 % (Mojović *et al.*, 2006). Moreover, hydrolysis rate is influenced by duration of the hydrolysis process (Sun *et al.,* 2006). Figure 2.5 depicts the effect of time toward hydrolysis rate for different type of starch. Figure 2.6 shows that glucose yield increases with rising temperature (Yu *et al.,* 2008). Longer hydrolysis time and high enzyme dose showed the highest increment in percentage of glucose yield as temperature rise (Yu *et al*., 2008).

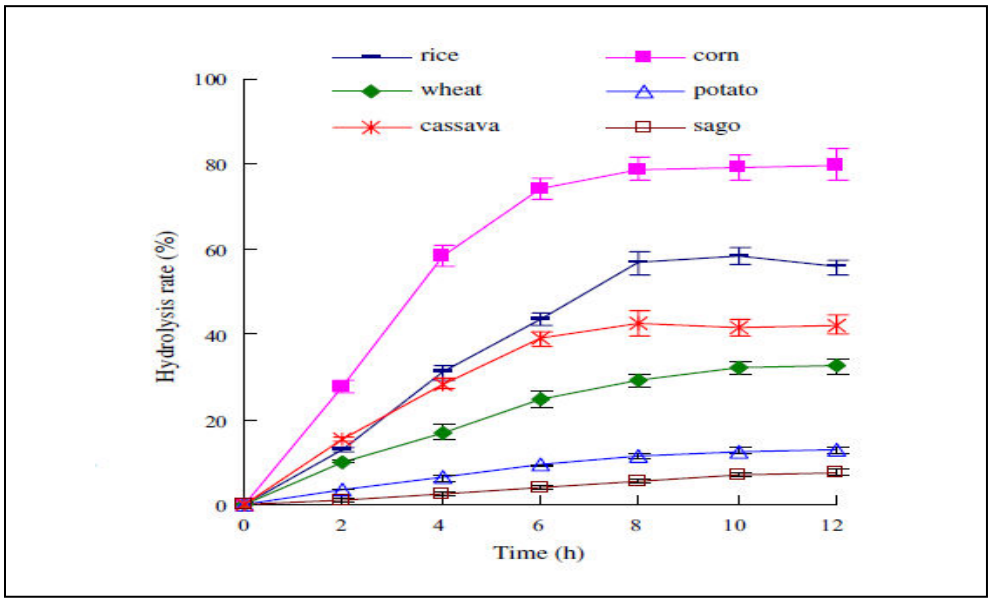

Figure 2.5: The Effect of Incubation Time on the Hydrolysis of Different Raw Starches (Sun *et al*., 2006)

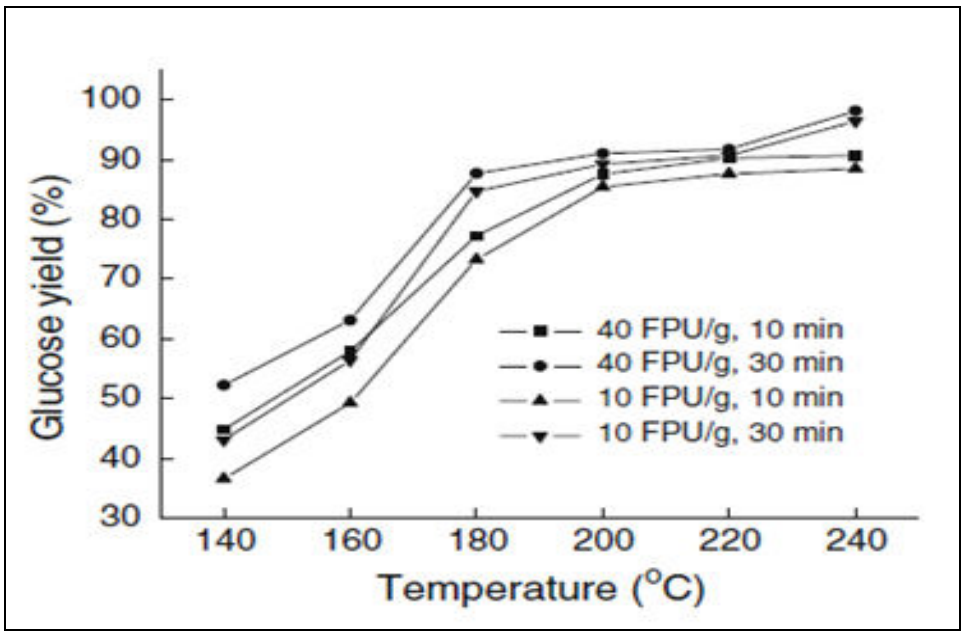

Figure 2.6: Glucose Yield by Enzymatic Hydrolysis (Yu *et al*., 2008)

2.4 Acid hydrolysis

Before enzymatic hydrolysis is being introduced, acid hydrolysis was widespread use in the past to degrade starch. Acid hydrolysis was introduced by German chemist, Kirchoff in 1811 (Vandamme *et al.,* 2002). Normally, starch that hydrolyzed by acid will result in contamination to the glucose due to undesirably coloured and flavoured breakdown products (Vandamme *et al.,* 2002).

There are two types of acid hydrolysis which is dilute acid hydrolysis and concentrated acid hydrolysis. Application of high concentrations of H_2SO_4 of course makes more solubilization of raw materials but no significant increase was observed in reducing sugar (Patle and Lal, 2008). This process also makes the product turn browning or charring (Vandamme *et al.,* 2002). Dilute concentration of H_2SO_4 also does not give any advantage where this process has a tendency to yield more undesired by-products from its high temperature application. They are furfural and 5-dihydroxymethyl furfural (Patle and Lal, 2008; Vandamme *et al.,* 2002; Aggarwal *et al.,* 2001) that capable to inhibit the fermentation process. Although these compounds are reported to be produced in small concentration but they may be toxic to fermentation and reduce the sugar yield (Patle and Lal, 2008). Moreover, acid is corrosive solution and difficult to handle. Acid hydrolysis mainly is performed at high temperature around 150˚C-180˚C and may be lead to sugar degradation, reducing the carbon source and lowered the production of ethanol. Evidently, acid hydrolysis has several drawbacks and enzymatic hydrolysis was optimized for various materials. Patle and Lal (2008) have found that reducing sugar yield from thippi was 395.2 gKg^{-1} from acid hydrolysis while 535.33 gKg^{-1} was resulted from enzymatic hydrolysis.

2.5 Factorial Design

The design is utilized to estimate the main effects and interaction effects in a process. Full factorial design usually used to screening the factors that significant towards the target production before further the process is further optimized. (Zulfikri *et al.,* 2008; Kunamneni and Singh, 2005; Chowdary *et al.,* 2000). Kunamneni and Singh (2005) have used full factorial composite experimental design and response surface in design experiment to evaluate the optimum condition of enzymatic hydrolysis of maize starch to get higher glucose production.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter will discuss on the process of producing glucose through enzymatic hydrolysis. All the experiments condition was designed by 2^4 factorial from Design Expert Version 7.1.6 sofware with 3 centered points. There are 19 sets of experiments utilized for evaluating the significant factors. The temperature and pH value of liquefaction and saccharification step were varied. Glucose produced was analyzed by DNS method (Miller, 1959). Figure 3.1 shows the overall process of this study.

Experimental Design

2^4 full factorial was applied to design the experiment. A total of 19 sets of experiment with 1 replicate and with 3 centered points were employed.

Enzymatic Hydrolysis

Sample preparation

10g tapioca starch + 30g distilled water

(1:3) w/w

Liquefaction step

80µL α-amylase (144 KNU/mL) from *Bacillus lichenformis*

1 hour

150 rpm

Saccharification step

5.75mg amyloglucosidase (24 AGU/g) from *Aspergillus niger*

4 hours

150 rpm

Centrifuge at 10000 rpm 15 minutes

Determination of glucose by using DNS reagent

Determination of absorbance by using spectrophotometer (UV-Vis) at 540nm

Analysis

ANOVA analysis

Figure 3.1: Overall Study Flow

3.2 Experiment Design and ANOVA Analysis

Experimental design was carried out using Design Expert Software Version 7.1.6. The factors involved and range selected in this research were given in Table 3.1. The selections of range of each parameter were based on previous studies (Liu et al., 2008; Mojović *et al.*, 2006; Vandamme *et al.,* 2002; Nigam and Singh, 1995). A 2^4 full factorial was applied to determine the effects of pH and temperature of liquefaction and saccharification steps on the production of glucose through enzymatic hydrolysis of tapioca starch statistically. A total of 19 sets of experiment with 1 replicate and with 3 centered points were employed. The experiment designed was shown in **Appendix A.8**.

Table 3.1: Selected Parameters and Range in Factorial Design

Factors	Low level	High level
Liquefaction Temperature, ˚C	60	90
Saccharafication Temperature, ˚C	40	60
Liquefaction pH	5	7
Saccharification pH	4	6

3.3 Raw Material

Tapioca starch was used as the starchy substrate for the production of glucose.

3.3.1 Sample preparation

Ten grams of tapioca starch was mixed with distilled water in weight ratio of 1:3. The mixture was stirred until homogeneous. The final mixture is 25% (w/w).

3.4 Enzyme

Enzyme α-amylase solution, type Xll-A from *Bacillus licheniformis* 24mg protein/ml (75 KNU/mg) and Amyloglucosidase from *Aspergillus niger* (138 U/mg) were employed for liquefaction and saccharification steps, respectively.

3.4 Hydrolysis Experiment

3.5.1 Liquefaction Step

In liquefaction step, the sample was treated with 80 μL of α-amylase (144 KNU/mL) from *Bacillus licheniformis* (Mojović *et al.,* 2006). The temperature and pH value were controlled at specific values as suggested by the factorial design. The mixture was continuously mixed in a shaking water bath at 150 rpm for 1 hour. 0.1NHCl and 0.1 NaOH were used for pH adjustment.

3.5.2 Saccharification Step

The liquefied mixture was then treated by 5.75 mg amyloglucosidase (24 AGU/g) from *Aspergillus niger* (Mojović *et al.,* 2006). The temperature and pH value were set at specific value as suggested by the factorial design. The mixture was hydrolyzed in a shaking water bath at 150 rpm for 4 hours. 0.1NHCl and 0.1NaOH were used for pH adjustment.

3.6 Analysis Method

3.6.1 Preparation of Dinitrosalicylic Acid (DNS) Reagent

A 2.5 g of 3, 5-dinitrosalicylic acid (Acros) was dissolved into 50mL of NaOH 2M resulting the orange colour mixture. The mixture was heated and stirred on hot plate until homogeneous. In separate beaker, 75 g of sodium potassium tartarate (Merck) was added to 125mL of distilled water. Then, the solution was heated and stirred on hot plate to obtain hot salt solution. After that, both DNS and salt solution were mixed together and stirred continuously until homogeneous. The solution was then cooled at ambient temperature. The solution was then transferred into 250mL standard volumetric flask. Finally, distilled water was added to the solution until the marked level.

3.6.2 Determination of Reducing Sugar by DNS Method

The hydrolysate was centrifuged at 10000 rpm for 15 minutes to obtain the supernatant (sample). One mL of DNS reagent was added to 1 mL of the sample. Then, the mixture was soaked in water bath of 90˚C for 10 minutes. After that, the mixture is cooled under running tap water to the ambient temperature. Eight mL of distilled water was then added to the sample and shaked vigorously. Then, the solution was pipette into cuvette and analyzed by spectrophotometer at 540 nm. The absorbance reading was recorded in **Appendix B.1** and compared with standard curve (absorbance versus concentration of glucose, g/L) in **Appendix A.7**.

3.6.3 Preparation of Standard Calibration Curve for Glucose

Glucose stock solution, 1 g/L was prepared by adding 0.1 g of D (+)-glucose anhydrous to 100mL of distilled water. The solution was stirred until homogeneous. Eleven different glucose concentrations were prepared from previous glucose stock

ranging from 0g/L to 1.0g/L. Glucose sample preparation is shown in **Appendix A.5**. One mL of the solution was pipetted into eleven different test tubes. Then, 1mL of DNS was added to each test tube. All the test tubes were soaked into the boiling water bath for 10 minutes. After 10 minutes, the test tubes were removed from the water bath and cooled under running tab water to room temperature. Next, 8mL of distilled water was added to the test tubes and vigorously shacked. Finally, the mixed solutions were analyzed by spectrophotometer (UV-Vis) at 540nm. The readings were recorded in **Appendix A.6** and the graph absorbance versus glucose concentration was plotted. The curve is shown in **Appendix A.7**.

3.6.4 Preparation of Hydrochloric Acid (HCl) 0.1M for pH Adjustment

Hydrochloric acid was used to lower the pH of the sample to the desired value. Hydrocloric acid 0.1M was prepared by adding 2.07mL of 37% HCl into the 250mL of distilled water. The solution was mixed until homogeneous.

3.6.5 Preparation of Sodium Hydroxide (NaOH) 0.1M for pH adjustment

Sodium hydroxide 0.1M was used to increase the pH of the sample to the desired value. One g of NaOH pellet was measured and dissolved in 250mL of distilled water until being homogeneous.

3.7 Material and Equipment

3.7.1 Raw Material

1. Tapioca starch
2. Alpha- amylase solution from *Bacillus licheniformis*
3. Amyloglucosidase from *Aspergillus niger.*
4. DNS reagent

3.7.2 Experimental Equipment/Apparatus

1. Shaking Water Bath (Model BS-21)
2. Refrigerated centrifuge (5810 R)
3. Hot plate and magnetic stirrer
4. Analytical balance
5. pH meter
6. Conical flask, 100mL
7. UV-Visible Single Beam Spectrophotometer (Model U-1800)

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

In the study of enzymatic hydrolysis of tapioca starch, the effects of temperature and pH for both liquefaction and saccharification processes on the glucose concentration were evaluated. The mixture for both steps was continuously shaked in shaking water bath at 150 rpm and reaction time for liquefaction and saccharification were fixed at 1 and 4 hours respectively (Mojovic *et al.*, 2006). For liquefaction process, the amount of α-amylase was fixed at 80μL (144 KNU) while 5.75mg (24 AGU) of amyloglucosidase was used for saccharification process. Glucose produced from the enzymatic hydrolysis was analyzed using DNS method (Miller, 1959).

4.2 Glucose Concentration Result

Total of 19 sets of experiment were employed and the result were shown in Table 4.1. The highest amount of glucose produced was 329.10 g/L as the liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH were at 90˚C, 40˚C, 7 and 4 respectively. On the other hand, the lowest glucose concentration obtained was 152.10 g/L. The production of glucose is depending upon the best combination factors of liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH. All these parameters directly influence the enzyme action. α-amylase carried two main

characteristics which are thermostable and sensitive to acidic environment. The α-amylase will be operated optimally at high temperature (85-90˚C). Besides, it will be denatured if exposed to extremely acidic circumstance (pH below 4.5). Therefore, this situation will directly influence the glucose productivity. Meanwhile amyloglucosidase is active at mild condition and lost its stability when exposed to too high temperature. Maximum glucose will obtained if both processes are conducted at optimum condition.

Table 4.1: Glucose Production Evaluated from Enzymatic Hydrolysis of Tapioca Starch

Run	Liquefaction Temperature (˚C)	Saccharification Temperature (˚C)	Liquefaction pH	Saccharification pH	Glucose Concentration (g/L)
1	75	50.00	6.00	5.00	312.00
2	90	60.00	5.00	4.00	300.60
3	90	60.00	5.00	6.00	300.60
4	75	50.00	6.00	5.00	172.50
5	75	50.00	6.00	5.00	280.80
6	60	60.00	7.00	4.00	195.60
7	60	40.00	7.00	6.00	301.20
8	90	40.00	7.00	6.00	289.80
9	90	40.00	7.00	4.00	329.10
10	60	40.00	7.00	4.00	173.40
11	90	40.00	5.00	6.00	294.00
12	90	60.00	7.00	4.00	245.40
13	90	40.00	5.00	4.00	208.50
14	60	60.00	5.00	6.00	327.90
15	60	40.00	5.00	4.00	152.10
16	90	60.00	7.00	6.00	325.50
17	60	60.00	7.00	6.00	228.30
18	60	60.00	5.00	4.00	186.00
19	60	40.00	5.00	6.00	243.00

4.3 Analysis of Variance (ANOVA)

The effects of temperature and pH of liquefaction and saccharification were observed using 2^4 full factorial design. The analysis of variance (ANOVA) was employed to evaluate the effects of parameters statistically (Table 4.2). The p-value of less than 0.05 indicates that model term is significant. In enzymatic hydrolysis of tapioca starch, model term was significant where the p-value was 0.0407. F-value of the model was 3.41 implies that the model is significant. There is only 4.07% that a 'Model F-value' this large could occur due to noise.

The glucose concentration produced from enzymatic hydrolysis was significantly influenced by liquefaction temperature and saccharification pH where the p-values for both factors are 0.0278 and 0.0201, respectively. Whereas, sachharification temperature and liquefaction pH seemed to be not significant in influencing the glucose concentration. Their p-values are 0.5557 and 0.7057, respectively. The R^2=0.5123 shows that there was an agreement between the experimental and predicted data. For lack of fit, the p-value is 0.9006 which means the model fits well to the data.

Table 4.2: Analysis of Variance, ANOVA Table

Source	Sum of Square	df	Mean Square	F- Value	p-value Prob>F
Model	32855.90	4	8213.98	3.41	0.0407
Liquefaction T	14753.14	1	14753.14	6.13	0.0278
Saccharafication T	879.86	1	879.86	0.37	0.5557
Liquefaction pH	358.63	1	358.63	0.15	0.7057
Saccharification pH	16864.27	1	16864.27	7.01	0.0201
Residual	31272.43	13	2405.57		
Lack of Fit	20551.57	11	1868.32	0.35	0.9006
Pure Error	10720.86	2	5360.43		
Cor Total	64131.99	18			

R^2=0.5123

The mathematical relationship of the process variables and the glucose concentration were calculated by the first order polynomial equation. Equation 4.1 describes the concentration of total glucose concentration as a function of the

independent factors analyzed (liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH) in term of coded factors. Meanwhile, Equation 4.2 presents the suggested model in term of actual factors.

Model Equation in Term of Coded Factors

$$Y = 256.30 + 30.37X_1 + 7.42X_2 + 4.73X_3 + 32.47X_4 \quad \text{(Equation 4.1)}$$

Model Equation in Term of Actual Factors

$$Y = -123.33750 + 2.02437X_1 + 0.74156X_2 + 4.73437X_3 + 32.46562X_4 \quad \text{(Equation 4.2)}$$

Where,

Y=Glucose Concentration; X_1=Liquefaction Temperature;

X_2=Saccharification Temperature; X_3=Liquefaction pH; X_4=Saccharification pH

4.4 Effect of Liquefaction Temperature on Glucose Concentration

Mukhopadhyay (1992) has reported that the increase of liquefaction temperature will increase the glucose yield. Figure 4.1 shows the relationship between liquefaction temperature and glucose concentration. The plot describes that glucose concentration boosts with the increment of the liquefaction temperature. At 60˚C glucose concentration was around 227g/L while at 90˚C glucose produced was increased to 285 g/L. This is because at higher temperature, dextrinizing activity of α-amylase will be increased, resulting in high glucose yield (Mukhopadhyay, 1992). Tester *et al.* (2004) has reported that at high temperature starch crystallinity has been lost where the accessibility of enzyme to degrade the starch structure is greater. Liu and his coworkers (2008) also reported that α-amylase is thermostable enzyme and operate optimally at 90˚C. In separate sources, Aehle (2007) stated that optimum temperature for α-amylase from *B. lichenformis* was 75-90˚C. However at the temperature above 90˚C, the α-amylase activity will rapidly decrease due to its denaturation (Mukhopadhyay, 1992).

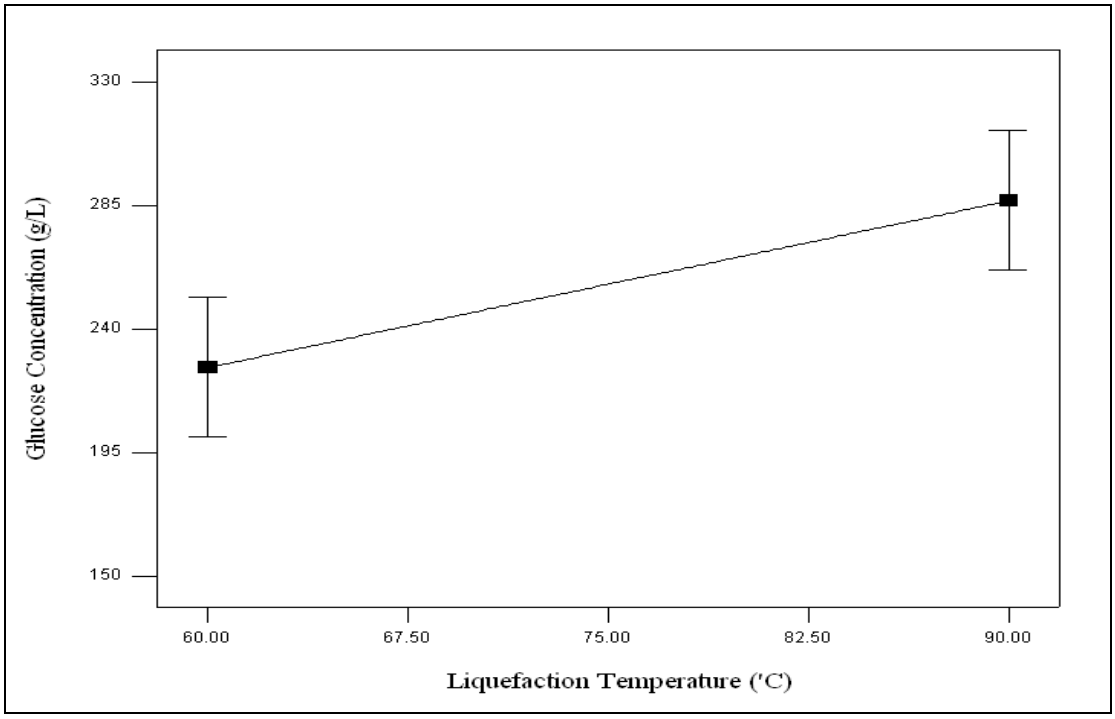

Figure 4.1: Effect of Liquefaction Temperature on Glucose Concentration.

4.5 Effect of Saccharification Temperature on Glucose Concentration

Saccharification is the process where starch is completely converted into glucose. In this study, saccharification temperatures only give slight effect on the glucose concentration. A similar finding was reported by Aggarwal *et al.* (2001) where the increase of temperature has resulted in slight increment in saccharification percentage causing a slight increment in glucose concentration. Saccharification rate is dependent upon the liquefaction yielded. As the starch structure was decrystallized and liquefied, saccharification enzyme will easily attacked the end branched of the starch (Aehle, 2007). Figure 4.2 clearly shows that as the saccharification temperature was raised up from 40˚C to 60˚C, their glucose concentration were increased from 250 g/L to 264 g/L. From previous research, it have found that the optimum temperature for saccharification process were 45˚C (Aggarwal *et al.,* 2001), 55˚C (Mojovic *et al.*, 2006) and 60˚C (Vandamme *et al.,* 2002). Amyloglucosidase actives at this temperature range. Therefore, there is no significant effect to the glucose concentration although the temperature was increased up to 60˚C.

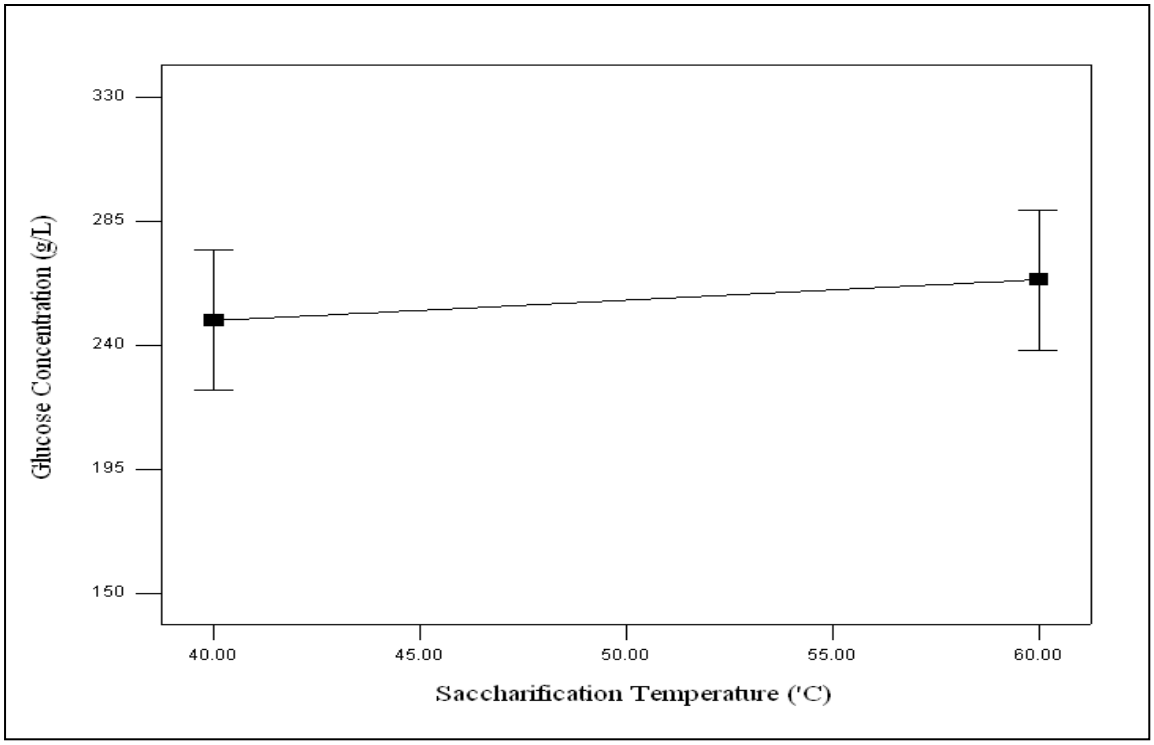

Figure 4.2: Effect of Saccharification Temperature on Glucose Concentration.

4.6 Effect of Liquefaction pH on Glucose Concentration

From Figure 4.3, there is a slight increment of glucose concentration as the liquefaction pH is increased from 5 to 7. The glucose concentration was around 251 to 262g/L. Liu and his coworkers (2008) have found that α-amylase is sensitive to acidic circumstances. In addition, liquefaction pH of lower than 4.5 will cause the enzyme to be denatured (Nigam and Singh, 1995). Several researchers have reported that α-amylase operates optimally at pH 6 (Liu *et al.,* 2008; Mojović *et al.*, 2006; Vandamme *et al.,* 2002). Aehle (2007) also reported that α-amylase from *B. lichenformis* active at pH in range of 6 - 9. However, if the pH value is greater than 9 the enzyme tends to lost its stability (Aehle, 2007).Therefore, the enzyme will operate almost at the same rate although the pH value was increased. This situation did not give any significant effect to the glucose production.

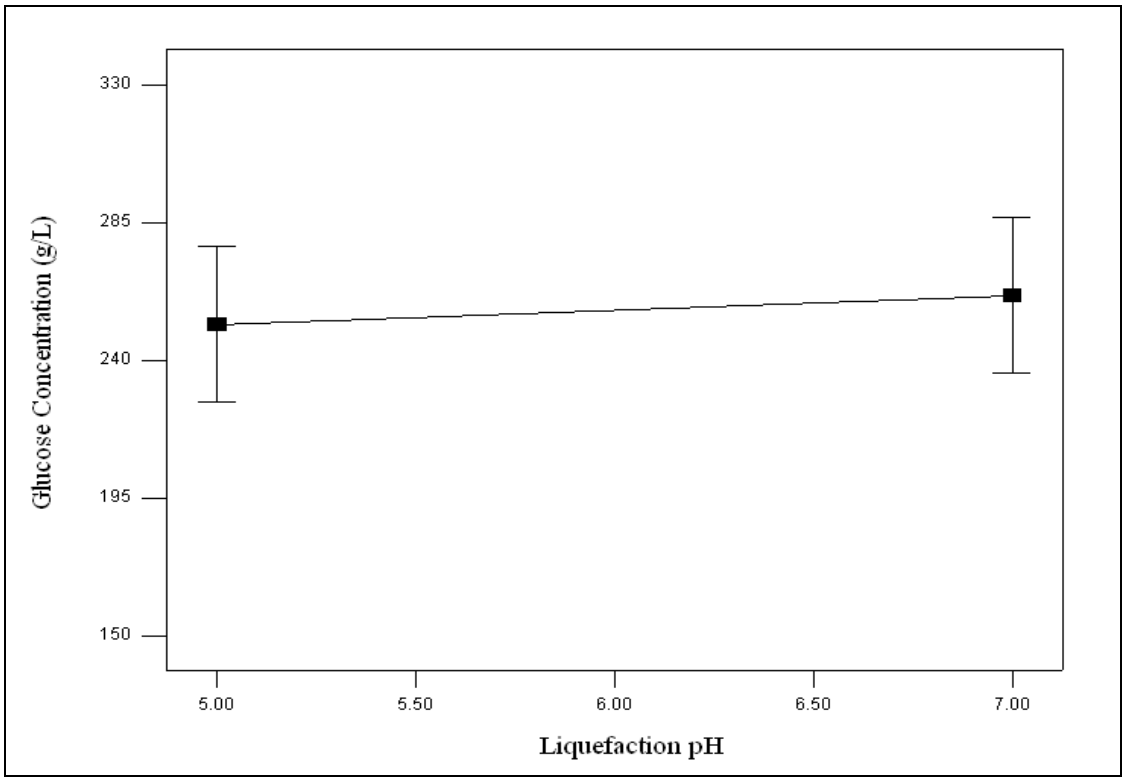

Figure 4.3: Effect of Liquefaction pH on Glucose Concentration

4.7 Effect of Saccharification pH on Glucose Concentration

From previous study Aggarwal *et al.* (2001) has reported that, saccharification percentage was improved with the increase of pH value. From Figure 4.4, glucose concentration was drastically increased when the saccharification pH increased from 4 to 6 where the glucose concentration was improved from 217.5 to 285 g/L. Earlier researchers noticed that optimum condition of saccharification pH was 4.5 (Vandamme *et al.,* 2002) and 5 (Mojovic *et al.*, 2006; Aggarwal *et al.*, 2001). Hence, amyloclocosidase enzyme operates optimally at this condition and resulted in high glucose concentration.

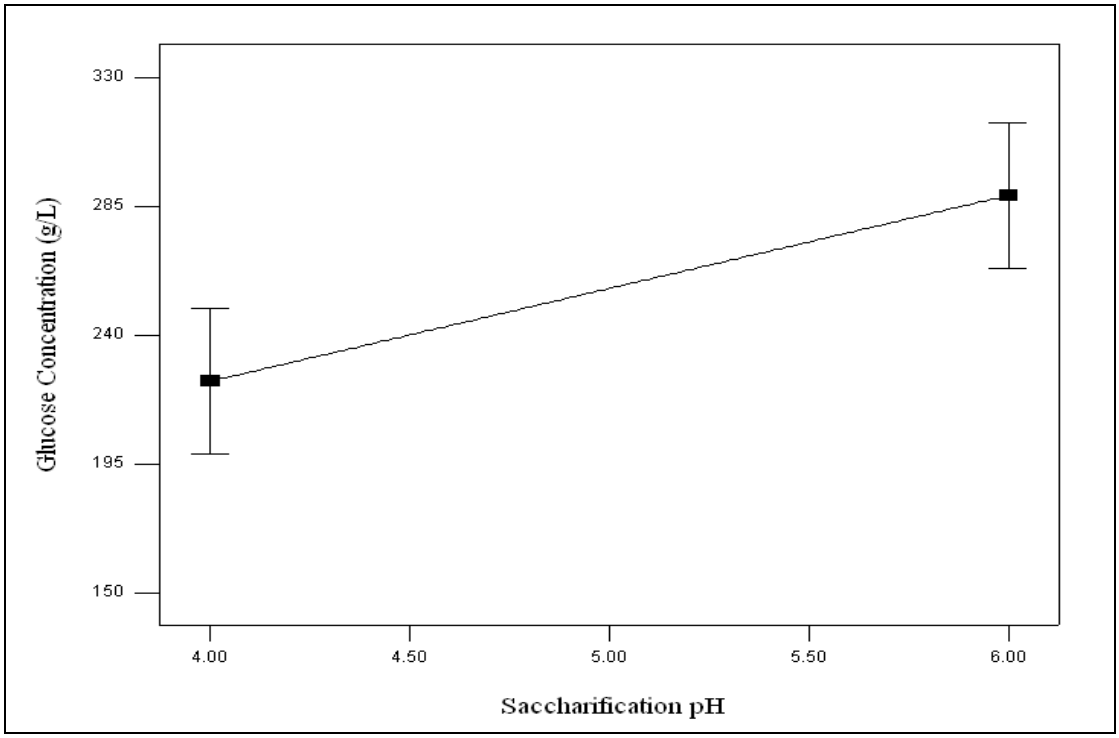

Figure 4.4: Effect of Saccharification pH on Glucose Concentration

4.8 Suggestion Optimization

There are 5 sets of optimum conditions of liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH for maximizing the glucose concentration suggested by the design experiment. From Table 4.3, the highest glucose concentration of 331.824g/L was estimated where the suggested conditions for liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH were 90.00˚C, 60.00˚C, 7.00 and 6.00 respectively.

Table 4.3: Optimum Conditions Suggested by Design of Experiment

No	Liq. T	Saccha.T	Liq. pH	Saccha. pH	[Glucose, (g/L)]	Desirability
1	90.00	60.00	7.00	6.00	331.284	1.0000
2	89.91	59.65	6.96	5.98	330.097	1.0000
3	89.99	59.73	6.91	5.99	330.229	1.0000
4	89.45	59.73	6.95	5.99	329.559	1.0000
5	89.72	59.86	6.96	5.97	329.484	1.0000

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study has focused on the effects of liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH on the enzymatic hydrolysis of tapioca starch. The maximum glucose production was aimed. Enzymatic hydrolysis of tapioca starch was strongly influenced by liquefaction temperature and saccharification pH. However, saccharification temperature and liquefaction pH were only resulted in slight variation in glucose concentration produced. From the experiment, the optimum conditions of liquefaction temperature, saccharification temperature, liquefaction pH and saccharification pH were 90˚C, 40˚C, 7.0 and 4.0, respectively where the maximum glucose concentration produced was 329.10 g/L. The optimized conditions suggested by the experiment design were 90˚C, 60˚C, 7.0 and 6.0 for liquefaction temperature, saccharification temperature, liquefaction pH and saccharification, respectively with glucose concentration up to 331.82 g/L was estimated.

5.2 Recommendation

In order to enhance the glucose concentration from enzymatic hydrolysis of tapioca starch, α-amylase treatment in liquefaction steps can be replaced with thermostable amylopullanase of *G. thermoleovorans* NP33 enzyme. According to Kaur and Satyanarayana (2004), this enzyme will liquefy the starch by specifically attacking the 1, 6-linkage of the starch structure. Moreover, amylopullanase will increase starch saccharification up to 91% compared to α-amylase only 70% starch saccharification (Kaur and Satyanarayana, 2004). Additionally, in order to improve the result, further optimized by Response Surface Methodology (RSM) is required and factor range can be wider.

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APPENDIX A

MATERIAL AND METHODOLOGY

Appendix A.1: Shaking Water Bath (Model BS-21)

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

Appendix A.3: Refrigerated Centrifuge (Model 5810 R)

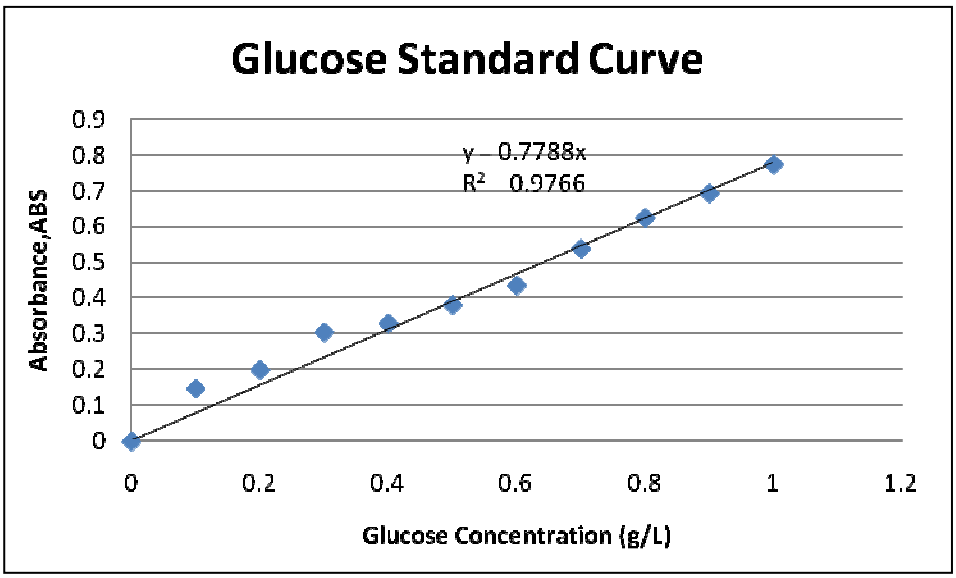
Appendix A.4: Hot Plate & Magnetic Stirrer

Appendix A.5: Glucose Standard Preparation

Test Tube	Volume of 1 g/L Glucose Stock (mL)	Volume of Distilled Water (mL)	Glucose Concentration (g/L)
1	0.0	1.0	0.0
2	0.1	0.9	0.1
3	0.2	0.8	0.2
4	0.3	0.7	0.3
5	0.4	0.6	0.4
6	0.5	0.5	0.5
7	0.6	0.4	0.6
8	0.7	0.3	0.7
9	0.8	0.2	0.8
10	0.9	0.1	0.9
11	1.0	0.0	1.0

Appendix A.6: Data for Standard Calibration Curve of Glucose

Glucose Concentration (g/L)	Absorbance, ABS at 540 nm
0.00	0.0000
0.10	0.1475
0.20	0.1995
0.30	0.3045
0.40	0.3290
0.50	0.3805
0.60	0.4350
0.70	0.5370
0.80	0.6230
0.90	0.6920
1.00	0.7725



Appendix A.7: Glucose Standard Curve

Appendix A.8: Experiment Designed by 2^4 Full Factorial

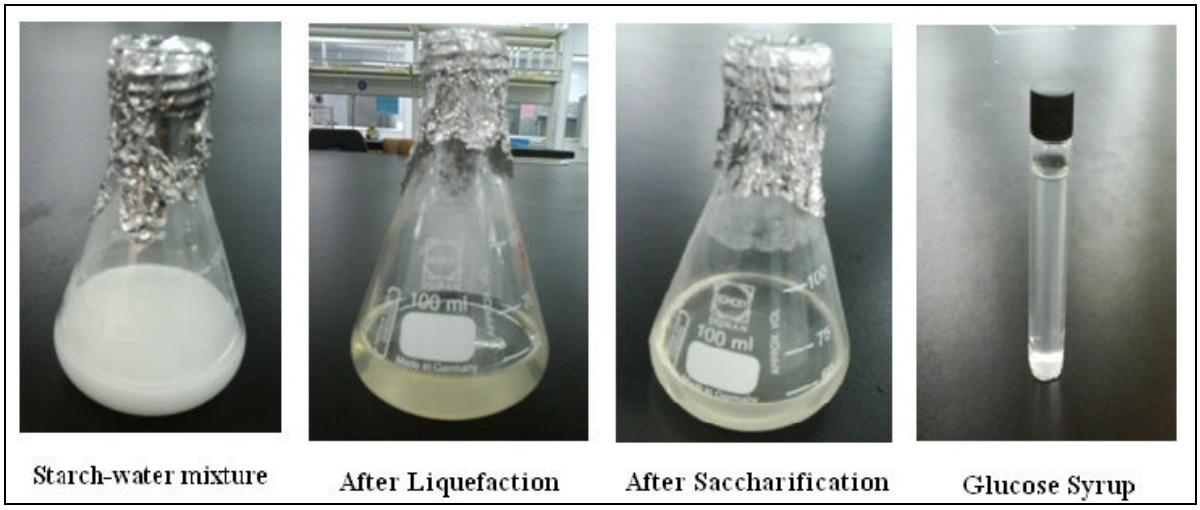
Run	Liquefaction Temperature ('C)	Saccharification Temperature ('C)	Liquefaction pH	Saccharification pH
1	75	50.00	6.00	5.00
2	90	60.00	5.00	4.00
3	90	60.00	5.00	6.00
4	75	50.00	6.00	5.00
5	75	50.00	6.00	5.00
6	60	60.00	7.00	4.00
7	60	40.00	7.00	6.00
8	90	40.00	7.00	6.00
9	90	40.00	7.00	4.00
10	60	40.00	7.00	4.00
11	90	40.00	5.00	6.00
12	90	60.00	7.00	4.00
13	90	40.00	5.00	4.00
14	60	60.00	5.00	6.00
15	60	40.00	5.00	4.00
16	90	60.00	7.00	6.00
17	60	60.00	7.00	6.00
18	60	60.00	5.00	4.00
19	60	40.00	5.00	6.00

APPENDIX B

RESULT

Appendix B.1: ABS and Glucose Concentration Result

Experiment	ABS at 540nm	Glucose concentration (g/L) (300X dilution)	Actual concentration (g/L)
1	0.81	1.040	312.00
2	0.78	1.002	300.60
3	0.78	1.002	300.60
4	0.448	0.575	172.50
5	0.729	0.936	280.80
6	0.508	0.652	195.60
7	0.782	1.004	301.20
8	0.752	0.966	289.80
9	0.854	1.097	329.10
10	0.45	0.578	173.40
11	*0.763*	0.980	245.40
12	0.637	0.818	294.00
13	*0.541*	0.695	208.50
14	0.851	1.093	327.90
15	0.395	0.507	152.10
16	0.845	1.085	325.50
17	*0.593*	0.761	228.30
18	0.483	0.620	186.00
19	*0.631*	0.810	243.00



Appendix B. 2: Enzymatic Hydrolysis Observation