Effects of Enzyme Loading and Time on Production of Sugar from Tapioca Starch Using Enzymatic Hydrolysis: A Statistical Approach

AHMAD NAHRI BIN ABDUL AZIZ

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

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AHMAD NAHRI BIN ABDUL AZIZ

A dissertation 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

April 2009

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Special Dedication to my family members,
my friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

Two-step enzymatic hydrolysis of tapioca starch by commercially available α-amylase and glucoamylase was carried out in this study. Effects of enzyme loading and hydrolysis time for liquefaction and saccharification process of enzymatic hydrolysis on production of sugar from tapioca starch were investigated. A statistical approach was employed to study the effect of selected parameters where two level factorial design was applied for the experimental design. From the result, it is shown that the highest glucose concentration achieved was 277.80g/L. From the analysis of variance (ANOVA), it is found that the liquefaction time and the interaction effect between α-amylase loading and saccharification time show significant effects on the enzymatic hydrolysis. The optimum conditions suggested by the design of experiment were: 80μL α-amylase loading, 5.75mg glucoamylase loading 5.75mg, liquefaction time 1 hour and saccharification time 4 hours. From this condition, an amount of high glucose concentration was estimated.

ABSTRAK

Kaedah dua langkah proses hidrolisis enzim bagi kanji ubi kayu menggunakan enzim-enzim komersil, α-amylase dan glucoamylase telah digunakan dalam kajian ini. Kesan kandungan/amaun enzim dan masa hidrolisis bagi proses likuefaksi dan sakarafikasi dalam proses hidrolisis enzim terhadap penghasilan gula daripada kanji ubi kayu telah. Pendekatan statistik telah digunakan untuk mengkaji kesan terhadap parameter yang telah dipilih dan rekabentuk 2-level factorial. Kaedah dua tahap rekabentuk faktor telah dipilih dalam merekabentuk eksperimen. Kepekatan gula yang paling tinggi diperolehi daripada keputusan eksperimen ialah sebanyak 277.80g/L. Berdasarkan analisa varians (ANOVA), didapati masa bagi proses likuefaksi memainkan kesan yang paling ketara terhadap proses hidrolisis enzim. Selain itu, kesan gabungan kandungan enzim α-amylase dan masa proses saccharification juga memberi kesan yang signifikan terhadap penghasilan gula. Keadaan optimum yang dicadangkan daripada rekabentuk eksperimen adalah: kandungan α-amylase: 80μL, kandungan glucoamylase: 5.75mg, masa proses likuefaksi: 1 jam dan tempoh masa proses sakarafikasi: 4 jam. Daripada kondisi ini, kepekatan gula sebanyak 271.8g/L adalah dianggarkan.

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LIST OF SYMBOLS/ABBREVIATIONS

DE	-	dextrose equivalent
DNS	-	Di-Nitro Salicylic Acid
g	-	gram
h	-	hour
KNU	-	kilo novo units α-amylases
AGU	-	amount of enzyme which hydrolyses 1 mmol of maltose per minute under specified conditions
g/L	-	gram per liter
mL	-	mililiter
w/v	-	weight per volume
w/w	-	weight per weight
%	-	percentage
°C	-	degree Celsius
μL	-	microliter
CO_2	-	carbon dioxide

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

1.1.1 Oil Depletion

After the invention of steam engine in 1860's during Industrial Revolution, it has brought a bright future for humankind since then. Engine became widely applied, initially from the utilization of wood and coal, later oil and natural gas.

Fossil fuels which a successful helper for amazing economic growth were the medicine to cure deprivation. As human keep consuming the fossil fuels, two important predicaments started to emerge:

(1) the fossil fuels would be depleted in a foreseeable future since it was first being drilled in 1859.

(2) the fossil fuels is causing global environmental problem (Veziroglu *et al.*, 2008).

1.1.2 Renewable Energy

Due to the depletion of oil in oil reservoir, energy researchers are trying to find any possible alternative source of energy to replace the fossil fuels. There is numerous numbers of possible alternative primary energy sources such as thermonuclear energy, nuclear breeders, solar energy, wind energy, hydropower, geothermal energy, ocean currents, tides and waves.

Ironically to fossil fuel, none of the mentioned can be directly used as a fuel in transportation. For choosing best fuel to replace fossil fuel, the choice must satisfy the following conditions:

i. It must be convenient fuel for transportation.
ii. It must be versatile or convert with ease to other energy
iii. forms at the user end
iv. It must have high utilization efficiency
v. It must be safe to use (Veziroglu *et al*., 2008).

1.1.3 Ethanol as a Renewable Energy

A transportation sector is dependent on one finite energy source which is petroleum. Recently, ethanol has been found to be a valuable fuel, acts as alternative for the transportation sector (Wyman *et al*., 2004). Based on the statistic, most of transportation fuel comes from hydrocarbon fuel mostly in developed country. As a new transportation fuel, ethanol is favorable on resource, and environmental attributes. Ethanol is a liquid fuel that widely accepted for blending with gasoline, and can be used in core form (Wyman *et al*., 2004).

Today, there are two technically alternative methods in reducing CO_2 emissions which are increase thermal efficiency of engines and the use of liquid biorenewable fuels (Cataluña *et al*., 2008). Thus, ethanol would be the most efficient candidate.

1.1.4 Production of ethanol

Ethanol is manufactured from two major paths which are fermentation of sugars derived from sugar, starch or cellulosic material and reaction of ethylene with water. The former is favored for production of fuel. Most researchers are focusing on fermentation route since it can potentially meet fuel cost and volume need for large-scale fuel production, while ethylene is too expensive and supplies would likely be streached (Wyman *et al*., 2004).

1.1.4.1 Raw Material

In ethanol production, a critical issue has been considered is whether the process is economical. Research efforts to produce a sustainable transportation fuel are focused on design and process improvement. This can be done by using low cost feedstock in establishing a cost effective technology. In order to analyze the cost effectiveness of bioethanol, several researches had found that ethanol can be economically derived from starch (Mojovic *et al*., 2006).

High yielding ethanol resource is offered by starch. Industrial ethanol production has been reported using various starchy materials. The carbohydrates in the starchy must be pretreated in order to hydrolyze the starch to simple sugar

before it is further fermented by yeasts to ethanol. Starch is hydrated and gelatinized, and particular enzyme will break down the starch polymer chain to fermentable sugar for ethanol production (Nigam *et al*., 1995).

Another potential raw material for bio-ethanol production would be lignocellulose. The principal carbohydrates contained in lignocellulose resources are the structural carbohydrates. These carbohydrates, along with proteins and lignin, form the complex matrix of plant cell walls that give plants structural stability and protection from the environment (Graf and Koehler, 2000)

Cellulose is a straight chained (linear) polymer of glucose molecules joined by ß (1-4) glycosidic bonds. The multiple hydroxyl groups (OH) situated along a cellulose chain bond with the hydroxyl groups of other cellulose chains to form tight crystalline structures (microfibrils). Microfibrils have high tensile strength and are the major structural components of all plant cell walls (Bioweb, 2008).

Compared to starch, cellulose is far more abundant in nature than is starch. However the high tensile strength and chemical stability of cellulose make it much more difficult to break down into glucose molecules. Therefore the process of converting cellulose to ethanol is more complex than that for starch. The recalcintrance feature of cellulose requires pretreatment, processing steps preceding hydrolysis of cellulose and hemicellulose into fermentable sugars. The purpose of pretreatment is to alter or remove structural and compositional factors present in plant that prevent the breakdown (hydrolysis) of cell wall polysaccharides into the fermentable simple sugars (Bioweb, 2008).

The cellulose stability make hydrolysis by using cellulose as a raw material in ethanol production become more complex than starch as an additional pretreatment process, is required. Hence, starch is a feasible raw material for bioethanol production.

1.1.5 Overview of glucose production from starch

Production of ethanol from starch required two steps which are hydrolysis of starch materials, followed by fermentation of the hydrolysis products. Hydrolysis of starch materials is a crucial step as it revealed the effectiveness of the process and raw material selected. Industrial production of starch hydrolysis products such as glucose syrup can be achieved either by using acid or enzyme catalyst. For enzymatic hydrolysis process, two enzymes basically being used are α-Amylase and glucoamylase. Conventional processes for glucose production involved liquefaction and saccharification process of starch. Liquefaction and saccharification in the process require the starch granule to be extensively gelanatinized at high temperature (Lim, 2002, Shariffa *et al.*, 2008).

1.2 Objective

The aim of this study is to obtain the optimum conditions of enzymatic hydrolysis for the production of glucose from tapioca starch. Hence the objectives of this research are:

I. To determine the effect of enzyme loading on the production of glucose from tapioca starch using enzymatic hydrolysis.
II. To determine the effect of hydrolysis time on the production of glucose from tapioca starch using enzymatic hydrolysis.

1.3 Scope of Study

The scope of the study is to determine the concentration of glucose that can be produced locally available tapioca starch via enzymatic hydrolysis. The investigation has been conducted to investigate the effects of two parameters, enzyme loading and hydrolysis time period on the glucose produced. Two-level factorial design was employed to statistically investigate the effects of enzyme loading and hydrolysis time. DNS method has been used to determine the resulted glucose from the experiment.

1.4 Problem Statement

Carbon dioxide (CO_2) gas is the main contributor for greenhouse effect. The continuation usage of fossil (hydrocarbon) fuels to meet world energy's demand is a reason for the increasing of CO_2. Almost 73 % of the CO_2 production

is comes from combustion of fossil fuel. Global warming issue has made high awareness on reducing the usage of fossil fuel.

Bioethanol has been recognized as a potential alternative to hydrocarbon fuels for reduction of CO_2 emissions (Balat *et al*., 2007). Bioethanol has higher oxygen content that makes it implies less amount required additive. It also allows better oxidation of the gasoline hydrocarbon and reduce the emissions of CO_2. Greater octane booster properties make ethanol become non-toxic and do not contaminate water sources (Sánchez *et al*., 2007).

In conventional method of ethanol production, there are two processes involve which are hydrolysis and fermentation. The purpose of hydrolysis process is to breakdown raw material into while fermentation process is to convert glucose into ethanol. Acid hydrolysis and enzymatic hydrolysis is two main routes in hydrolysis process.

In acid hydrolysis, browning or charring occurred as acid is employed as catalyst. Dilute acid hydrolysis tends to produce some undesirable by-products. They are furfural and 5-dihydroxymethyl furfural, which are known to inhibit fermentation. These compound may reproduces in a small amount but they be very toxic to fermentation (Graf and Koehler, 2008). Concentrated acid hydrolysis formed fewer by-products but in order the process to be economical, the acid has to be recycled. Sulphuric acid (H_2SO_4) recycling process involving separation and reconcentration of the acid make this method becomes more complex. Neutralization of hydrolysate is required before the fermentation, which leads to sludge formation at the bottom. This is condition the required for solid–liquid separation. High temperature (150-180°C) for acid hydrolysis can degrade the sugars, reducing the carbon source and ultimately lowering the ethanol yield (Patle *et al.*, 2008).

Enzymatic hydrolysis offers major advantages over acid hydrolysis. Enzymatic hydrolysis give such as higher yields, minimal byproduct formation, low energy requirements, mild operating conditions, and low chemical disposal costs (Wyk, 2001).

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Starch

In human diet, starch is the most important energy source. Besides being an energy source, starch is widely used as a functional texturizer in food industries. Starch found naturally in granules forming most plants. It is also found in cereal seeds in maize, wheat, rice, barley and in tubers/roots from potato, tapioca) are especially rich in starch. Starch is a composition of two different types of a-glucan polymers: Generally, starch is a mixture of α-amylose (20-30%; water soluble linear polymer) and amylopectin (70-80%; water insoluble branched polymer). α-amylose, a linear molecule consisting almost exclusively of α-1, 4-linked glucose residues (Figure 2.1). While amylopectin is linear chains of α-1,4-linked glucose, also contains α-1,6-linked branch points (Figure 2.2) (Hansen *et al*., 2007).

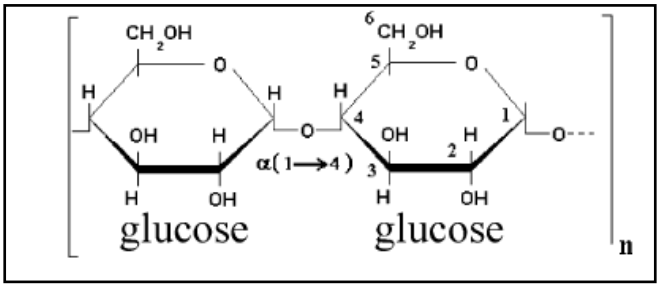

Figure 2.1: Chemical structures of α-amylase macromolecule (Reis et al, 2002)

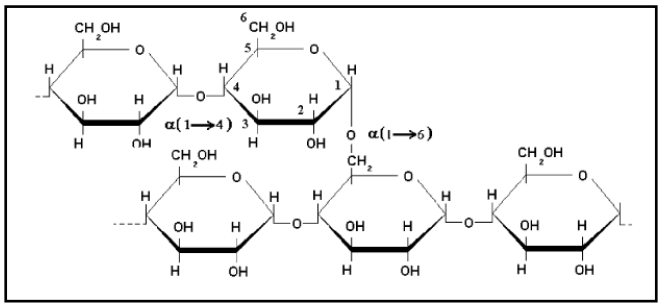

Figure 2.2: Chemical structure of amylopectin macromolecule (Reis *et al*., 2002)

The way of starch granules being stored suits to its role. Although it is insoluble in water and densely packed but it still allows for plant's metabolic enzymes. Starch technologists who are interested in the hydrolysis of the component macromolecules noted that once the granule has been destroyed, it will be useful. Effect of hydrolysis enzyme on native starch granules has been prevailed by closer examination by many researchers (Oates, 1997).

2.2 Overview of glucose

Glucose, a monosaccharide also known as grape sugar, is an important carbohydrate in biology. The living cells use it as a source of energy and

metabolic intermediate. Glucose is one of the main products of photosynthesis in both prokaryotes and eukaryotes.

From two stereoisomers of the aldohexose sugars, only one of its form which D-glucose is biologically active. This form (D-glucose) is often referred to as dextrose monohydrate, or, especially in the food industry, simply dextrose (from *dextrorotatory glucose*).

Glucose is produced commercially via enzymatic hydrolysis of starch. Many crops can be used as the source of starch. Maize, rice, wheat, potato, cassava, arrowroot, and sago are all used in various parts of the world. In the United States, cornstarch (from maize) is used almost exclusively (Wikipedia, 2009)

2.3 Hydrolysis

The carbohydrates in the starchy materials must be pretreated in order to hydrolyze the starch to simple sugar before it being pretreated by most of yeasts. Hydrolysis process for fermentable sugar involved two methods which are enzymatic and acid hydrolysis (Figure 2.3). Both two methods can be used either one. Acid hydrolysis uses acid as a catalyst for the reaction while enzymatic hydrolysis uses enzyme as its catalyst.

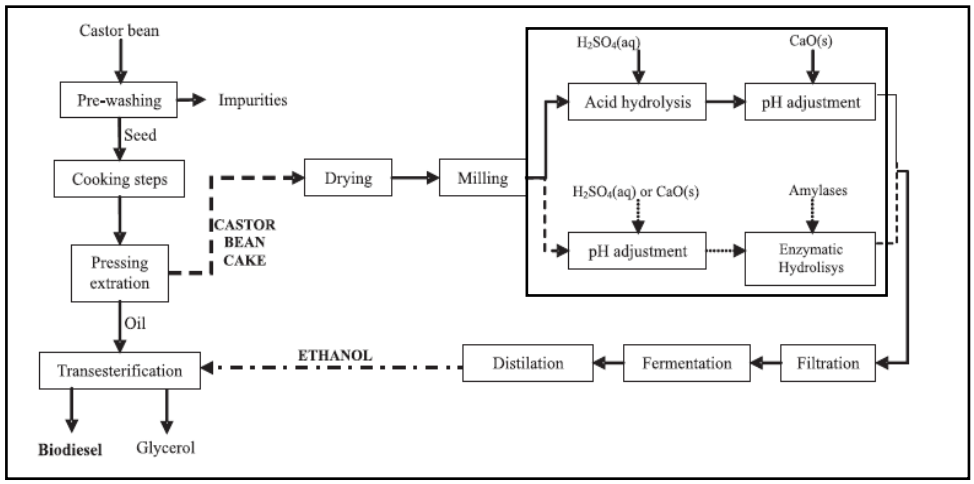

Figure 2.3: Two different path route in hydrolysis stage (Melo *et al*., 2008).

2.3.1 Enzymatic Hydrolysis

In enzymatic hydrolysis, it has two steps which are liquefaction and saccharafication. Enzyme used for liquefaction is α-amylase while for saccharification, glucoamylase is employed.

In liquefaction, starch is treated using heat-stable bacterial α-amylase (Nigam *et al*., 1994 & Mojovic *et al*., 2006). During this period, starch grains become dissolved to form a viscous suspension (gelatinisation) due to the cooking effect of heat. At the same time, α-amylase digests partially some of the starch molecules; this reduces the viscosity of the suspension. The suspension contains solubilized starch molecules, and dextrins (Indsutrial Alcohol Production, 2008).

Cereals like maize, wheat and sorghum, etc. contain 60-75% w/w starch. Conventional enzymatic hydrolysis for liquefaction involves temperature above 90°C. At the end of the liquefaction process, maltodextrin is obtained which

contains mainly different oligosaccharides and dextrins. Maltodextrins are only slightly sweet and they usually undergo further conversion (Mapsenzymes, 2007).

The later process of enzymeatic hydrolysis is called saccharafication. The liquefied starch is saccharified into low-molecular-weight saccharides by a specific enzyme. A glucoamylase is used to break down the maltodextrins. The glucoamylase can hydrolyse starch completely into glucose. Besides, a pullulanase is a de-branching enzyme that can also be used along to aid saccharification (Mapsenzymes, 2007). Saccharification was mainly performed at 55°C and pH 5.0 with various concentrations of glucoamylase and in range of 1-5 hours time (Nigam *et al*., 1994 , Mojovic *et al*., 2006).

2.4 **Enzymes used**

2.4.1 α-Amylases

The enzyme is basically obtained from *Aspergillus oryzae, Bacillus amyloliquefaciens*, and *Bacillus licheniformis* and used in starch hydrolysis for sugar syrups and bewing. Glucose, maltose, maltotriose, maltotetraose, maltopentose and maltohexaose are major end products from the activity of α-amylase.

2.4.2 Glucoamylases

The enzyme is usually obtained from *Aspergillus niger* or *Rhizopus* species and used in glucose syrup production from liquefied starch. The starch is initially liquefied with a heat-stable bacterial α-amylase prior to sterilization (Nigam *et al*., 1994).

2.5 Effect of enzyme loading

α-amylase and glucoamylase are basically employed in enzymatic hydrolysis. Like any other enzyme both have optimum activity so do the loading in obtaining highest glucose concentration. For α-amylase, higher enzyme activity may lead to hydrolysis of internal α-D-(1–4)-glucosidic linkages in starch thus resulted to more sugar being produced in the process. The concentration of reducing sugar was directly proportional to the activity of the enzyme in a given time of enzyme action (Mojovic *et a*l., 2006).

In saccharification of starch, excess glucoamylase is needed to obtain high rates of hydrolysis process since the enzyme hydrolyzes α-1,4 and α -1,6 linkages. On the other hand, excess of glucoamylase also leads to formation of 'reversion products' due to slow hydroxylation process which lower final dextrose equivalent (DE) value (Roy and Gupta., 2003).

2.6 Effect of hydrolysis time

From previous work, on appropriate for the liquefaction was for 120 min (2h) at 95°C (Aggarwal and Singh., 2001). Meanwhile, according to Mojovic *et al* (2006)., time required to optimize saccharication time is 4 hours.

2.7 Experimental Design with 2-Level Factorial

Two-level factorial designs normally employed to study the effects of parameters estimated the optimum conditions of a process. To determine optimum values of the selected variables, regression equation solving was mainly used. The results of the second order response surface model in the form of analysis of variance (ANOVA)

In determining the significance of each effect, p-test normally been done evaluated. The larger the magnitude of the F-value and smaller the p-value (< 0.05), the more significant is the corresponding coefficient. Hence the significance of p-value means that the effect or the interaction effect is significance toward the process (Kunamneni and Singh., 2005).

The effects of selected parameters is presented by a regression equation :

$$Y = b_0 + \sum b_i x_i + \sum\sum b_{ij} x_i x_j + \sum b_{ii} x^2_i + e \qquad \text{(Equation 2.1)}$$

where Y is the measured response, b_0, intercept term, b_i, b_{ij}, and b_{ii} are constant, respectively, the measures of the effects of variables of X_i, X_iX_j, and X^2_i, respectively (Kunamneni and Singh, 2005).

CHAPTER 3

METHODOLOGY

This chapter will discuss on the process of producing glucose through enzymatic hydrolysis. In this study, tapioca starch was hydrolyzed in two-steps enzymatic hydrolysis which comprised of liquefaction and saccharification processes. Finally glucose produced will be analyzed by using DNS reagent.

3.1 Starch

Starch from tapioca was applied as the raw material (starchy substance) glucose production.

3.2 Enzymes

α-Amylase (Termamyl Type L,) from *Bacillus licheniformis* was used for liquefaction process. The activity of these is commercially available enzyme was 24mg protein/mL x 75 KNU/mg protein which equals to 1800KNU/mL^{-1} (KNU, kilo novo units α-amylases— the amount of enzyme which breaks down 5.26 g of

starch per hour according to Novozyme's standard method for the determination of α-amylase). Meanwhile, for the glucoamylase employed, its initial activity is 138 AGU/mg (AGU is the amount of enzyme which hydrolyses 1 mmol of maltose per minute under specified conditions). Enzyme activity which gave optimum condition reported by Mojovic *et al*., 2006 and Melo *et al*., 2008 is for α-amylase loading is 144KNU/mL while for glucoamylase loading is 240AGU. For the whole experiment, the enzyme activity used was based on what been reported by Mojovic *et al*., 2006 and Melo *et al*., 2008.

3.3 Hydrolysis experiments (Liquefactions & Saccharification)

100 g of tapioca flour was mixed with water to get 25% w/v concentration. The mixture was treated with enzymes (α-Amylase and glucoamylase) in two steps. The tapioca starch was initially liquefied with various loadings of α-amylase at 85°C and pH 6.0 in water bath. Then, the saccharyfying enzyme, glucoamylase was added in various amount and hydrolysis was conducted at 55°C and pH 5.0.

3.4 Determination of selected variables using factorial design

A 2-level factorial with 2^4 factor was used to determine the effect of four variables α-amylase dose, glucoamylase dose, liquefaction time, and saccharification time). The lowest and the highest levels of selected variables are shown in Table 3.1.

Table 3.1: Range for parameters

Variables	Low level	High level
α-amylase loading	60μL	80μL
Glucoamylase loading	4.75mg	6.75mg
Liquefaction time	0.5 hours	1.5 hours
Saccharafication time	3 hours	5 hours

These selected variables ranges were used in Expert Design software by using 2^4 factorial designs. From the factorial design, 19 independent runs are generated, three of them as center points.

3.5 Analytical methods

3.5.1 Preparation of DNS reagent

The determination of glucose concentration in each sample of tapioca was conducted using DNS method (Miller, 1959). Fifty mL of 2M NaOH was prepared and 2.5 g of 3-5-dinitrosalicylic acid was added to the alkaline solution. The mixture was heated and stirred on hot plate stirrer. In separated beaker, 75 g of sodium-potassium tartarate was added into 125 mL of distilled water and was heated. Both DNS and salt solutions were mixed and stirred continuously. The mixture was let to cool down to ambient temperature. The mixture was then poured into 250 mL volumetric flask and diluted with distilled water.

3.5.2 Total Reducing Sugar Determination by DNS Method

One millimeter of sample was put into a test tube. One millimeter of DNS reagent was added to each tube and the solution was vigorously shaken and heated at 90°C for 10 minutes until the solution turn until red-brown color. The sample was cooled to room temperature in cold water bath for 10 minutes. The sample was diluted by adding 8 mL distilled water and subsequently analyzed for total reducing sugar concentration. The analysis was carried out using UV-visible spectrophotometer at 540 nm. Concentration of glucose in the sample was being determined with standard curve of glucose concentration.

3.5.3 Preparation of Standard Calibration Curve for glucose

Glucose solutions of 10 different concentrations were prepared from 1000 µg/ml standard glucose solution. DNS method was used to determine the glucose concentration. The absorbance of each sample was determined at the standard curve was plotted in Figure 3.1. Data for standard glucose calibration curve can be referred at Appendix B.1.

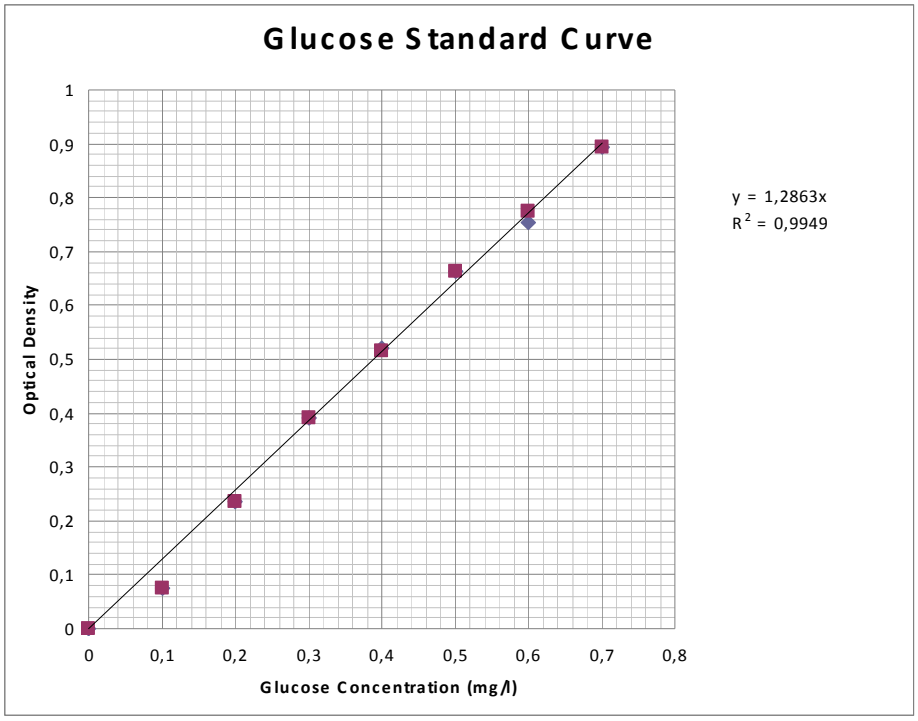

Figure 3.1: Standard calibration curve for glucose

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

In this study, the effects of enzyme loading and time towards enzymatic hydrolysis of tapioca starch were studied. Enzymes that used in this study were α-amylase and glucoamylase. The enzymes for each loading were in range of 60-100μL for α-Amylase and 4.75-6.75mg for glucoamylase. The hydrolysis time employed for liquefaction was 0.5-1.5 hours and that for saccharification were 3-5 hours. The optimum conditions for enzymatic hydrolysis were also determined. The Design Expert software was employed for analyzing data from the 2^4 factorial experimental design.

4.2 Optimization using 2-level factorial desigm

The concentration of glucose resulted from 19 experiments are shown in Table 4.1.

Table 4.1: Result for design experiment with 2-level factorial

Run	Factor 1 A:glucoamylase loading (mg)	Factor 2 B:α-amylase loading (µL)	Factor 3 C:Liquefaction time (hour)	Factor 4 C:Saccharification time (hour)	Response R1 (g/L)
1	4.75	100	0.5	5	217.20
2	4.75	60	1.5	5	245.40
3	5.75	80	1.0	4	271.80
4	6.75	100	1.5	5	277.20
5	6.75	100	1.5	3	258.00
6	4.75	60	1.5	3	212.40
7	6.75	100	0.5	3	252.60
8	5.75	80	1.0	4	255.60
9	4.75	60	0.5	3	256.02
10	4.75	100	1.5	5	191.40
11	4.75	100	0.5	3	192.60
12	4.75	100	1.5	3	250.80
13	6.75	60	0.5	5	201.00
14	6.75	100	0.5	3	235.80
15	6.75	60	0.5	3	259.80
16	6.75	60	1.5	3	213.66
17	6.75	60	1.5	5	239.76
18	4.75	60	0.5	5	232.80
19	5.75	80	1.0	4	277.80

From Table 4.1, range for concentration of glucose obtained is from 191.4 to 277.8 g/L. The effects of the parameters, enzyme loading and time were observed based on the concentration of glucose obtained at the end of the hydrolysis process. Analysis of variance (ANOVA) which used to predict the effects of enzyme loading and reaction time on enzymatic hydrolysis of tapioca starch is tabulated in Table 4.2.

From Table 4.2, p-value for the model (p = 0.0342) indicates that the model is significant throughout the research For lack of fit, the p-value is 0.7244 which means the model fit the data very well.

The liquefaction time and the interaction effect between α-amylase loading and saccharification time were found significant throughout the process, with the p-values of 0.0078 and 0.0473 respectively. By referring to Table 4.2, it implies that the first order main effects of liquefaction time is highly significant as is evident from it respective *p*-values is 0.0128. Interaction between α-amylase loading and saccharification time is significant as devoted from its *p*-value. These suggest that the liquefaction time and interaction between α-amylase loading and saccharification time have a direct relationship with the concentration of glucose. Meanwhile other parameters were found to be not significant.

Table 4.2: ANOVA for selected effect

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	6464.60	6	1077.43	4.417	0.0162	significant
A-glucoamylase loading	223.77	1	223.77	0.917	0.3587	
B-alpha-amylase loading	317.89	1	317.89	1.303	0.2779	
C-Liquefaction time	2574.11	1	2574.11	10.553	0.0078	
D-Saccharification time	388.90	1	388.90	1.594	0.2328	
BD	1216.23	1	1216.23	4.986	0.0473	
Lack of Fit	2238.79	8	279.85	1.900	0.3253	not significant
Pure Error	444.267	3	148.10			
Cor Total	*14032.84*	18				
R2 = 0.8990; R = 0.948; Adj. R2 = 0.97139						

The adequacy of the model was checked by the determination coefficient (R^2). Referring to Table 4.2, the value of the determination coefficient (R^2 = 0.8990) indicates that only 10.1 % of the total variations are not explained by the model. The R^2 shows that there good agreement between the experimental data and the predicted values from the model

The application of 2 level of factorial yielded the following equation which is an empirical relationship between the values of glucose yields and the variables in coded unit:

$$Y = 243.29 - 4.48X_1 + 3.86X_2 - 12.24X_3 - 5.50X_4 - 7.26X_1X_2 - 4.17X_1X_4$$
$$-0.62X_2X_3 - 9.46X_2X_4 + 2.84X_3X_4 - 5.69X_1X_2X_4 - 7.89X_2X_3X_4 \quad \text{(Equation 4.1)}$$

where Y is the response, that is, the concentration of glucose (g/L) and X_1, X_2, X_3 and X_4 are the coded values of glucoamylase loading, α-amylase loading, liquefaction time and saccharification time, respectively. Whereas the variables X_1X_2, X_3X_4, X_2X_3, X_2X_4, $X_3X_2X_4$ and $X_2X_3X_4$ represent interaction effects of parameters involved.

4.3 Effect of α-Amylase loading

α-Amylase has been used during liquefaction stage to catalyze the hydrolysis of internal α-D-(1–4)-glucosidic linkages in starch in a random manner. To study the effect of α-amylase loading during liquefaction, a range of the enzyme loading was being chosen which is from 60 to 100μL based on enzyme activity selected by Mojovic *et al*., (2006).

Figure 4.1 shows the effect of α-amylase loading on glucose concentration produced. From the figure, it is shown that as the enzyme loading increased from the concentration of glucose was directly increased (239.43 – 248.508 g/L). The highest concentration of sugar is 248.508 g/L at 100µL of α-amylase loading. Higher enzyme is corresponded to higher enzyme activity. Therefore, more internal a-D-(1–4)-glucosidic linkages in starch will be hydrolyzed which lead to more sugar being produced in the process (Mojovic *et al*., 2006). This observation also reported by Melo *et al*. (2008) where the concentration of reducing sugar was directly proportional to the activity of the enzyme in a given time of enzyme action.

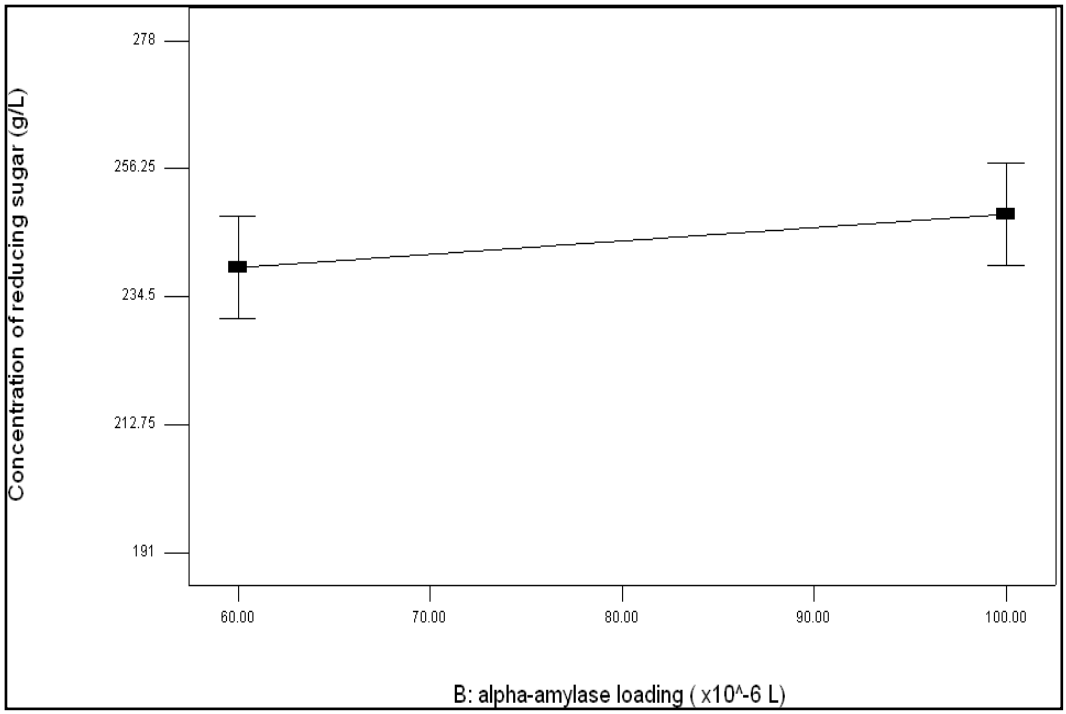

Figure 4.1: Effect of α-amylase loading on concentration of glucose

4.4 Effect of glucoamylase loading

In enzymatic hydrolysis, glucoamylase was being used during sacharafication stage. The range of enzyme loading was being employed is from 4.75 to 6.75mg and the effect of glucoamylase loading is shown in Figure 4.2. From the graph, as glucoamylase loading increased, the concentration glucose decreased from 2447.778 – 238.81 g/L. The highest concentration of glucose was obtained at the lowest range of the enzyme loading which is 247.778 g/L. In saccharification of starch, excess glucoamylase is needed to obtain high rates of process. However, the excess of glucoamylase also leads to formation of 'reversion products' (Roy and Gupta, 2003).

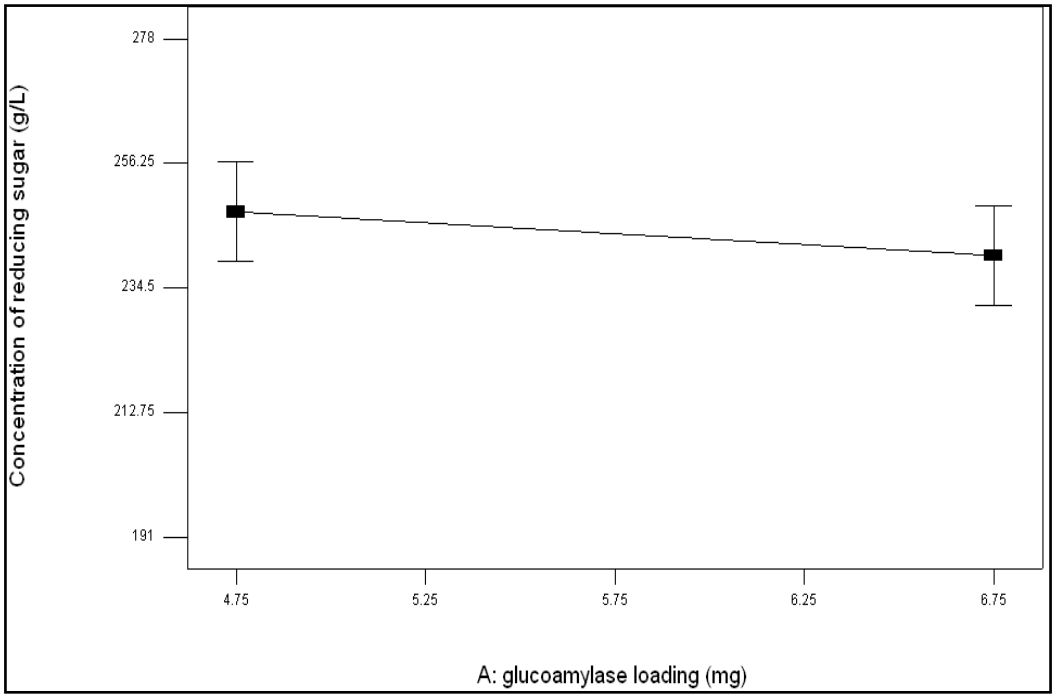

Figure 4.2: Effect of glucoamylase loading on concentration of glucose

4.5 Effect of liquefaction time

Liquefaction time period indicates time fixed for α-amylase activity in the liquefaction process. Range for the time was from 0.5 to 1.5 hour. Figure 4.3 shows the effect of liquefaction time on glucose concentration produced from tapioca starch. Concentration of glucose is found to be at the highest (256.886 g/L) at the lowest range employed (0.5 hour). As liquefaction time being increased from 0.5-1.5 hour, the concentration of sugar also decreased from 256.886 to 231.053 g/L. In other words, the concentration of sugar is diversely proportional to liquefaction time. It is believed that the longer time for the enzyme being exposed to high temperature could lead to the slight enzyme deactivation (Mojovic *et al*., 2006). Aggarwal and Singh (2001) also reported that at ≈85°C temperature for 30% slurry, starch cannot be further liquefied if the time is exceeded 120 minutes.

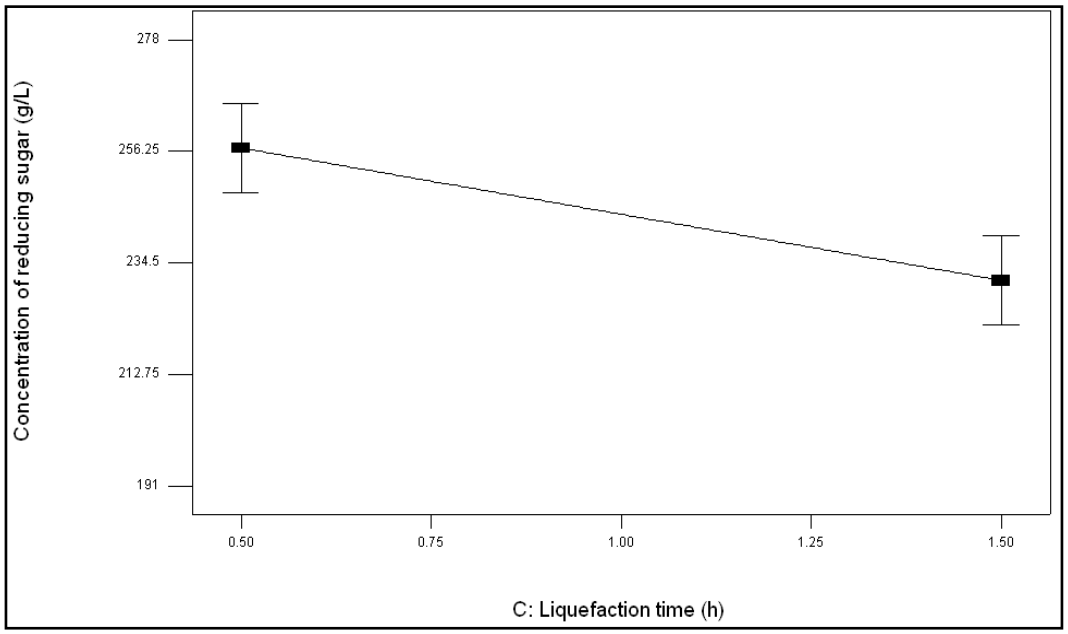

Figure 4.3: Effect of liquefaction time on concentration of glucose

4.6 Effect of saccharafication time

Sacharafication time period means the retention time for glucoamylase to catalyze the hydrolysis of α-D-(1–4) and α-D-(1–6)-glucosidic bonds of starch (Mojovic *et al*., 2006). Range for the time selected was from 3 to 5 hours. Figure 4.4 is shows the effect of saccharification time on glucose concentration. As range the saccharification time increased, concentration of glucose decreases from 249.143 to 237.795 g/L. Concentration of glucose is at the value highest (249.143 g/L) at the lowest range of saccharification time (3 hours). Roy and Gupta (2003) reported that as saccharification time being increased, the glucoamylase activity slightly decreased. This due to decrease of the enzyme action will resulted in the low concentration of glucose.

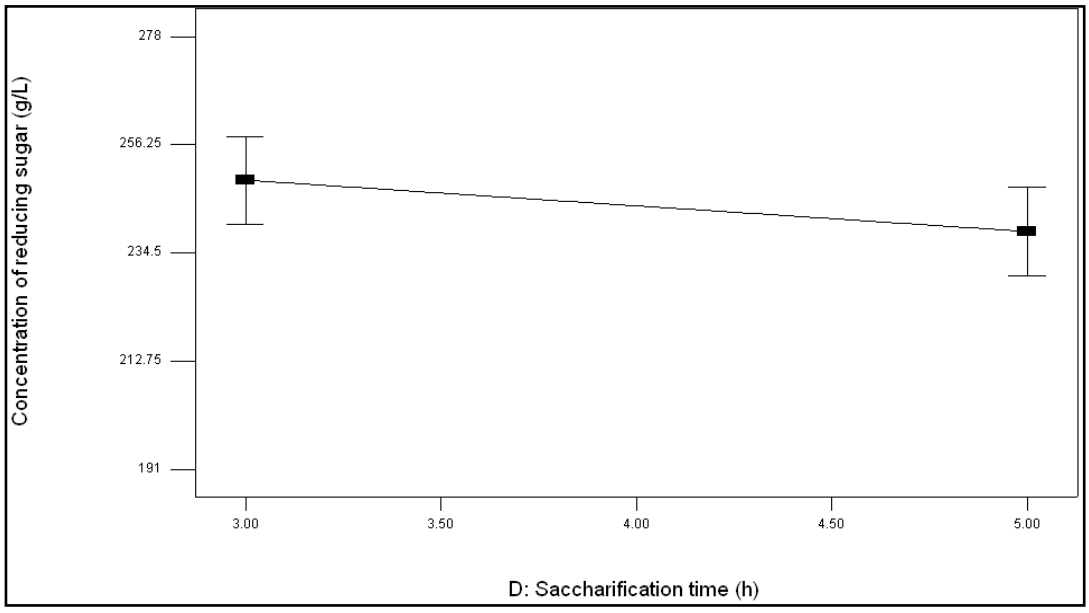

Figure 4.4: Effect of saccharification time on concentration of glucose

4.7 Interaction of α-amylase loading and saccharification time

From Anova in Table 4.2, interaction between α-amylase loading and saccharification time is a significant effect towards the hydrolysis process. These means that whenever action taking by changing the α-amylase loading, determination of saccharifiaction time plays critical action in obtaining the concentration of glucose.

From Figure 4.5, line D+ indicates high range of saccharification time while line for D- indicates low range of saccharification time. At low range of sacchrification time (D-), with the increment of α-amylase loading, the concentration of glucose increased. While at high range of saccharification time (D+), α-amylase loading increased, the concentration of glucose decreased. The highest glucose concentration is 257.129 g/L was obtained at 100 μL of enzyme loading and 3 hours of saccharification time period According to Apar *et al., 2004* and Roy and Gupta (2003), increasing the amount of enzyme will resulted in increment of hydrolysis degree value. Meanwhile, short time for saccharification process will prevent the product from being converted to substrate (Roy and Gupta, 2003).

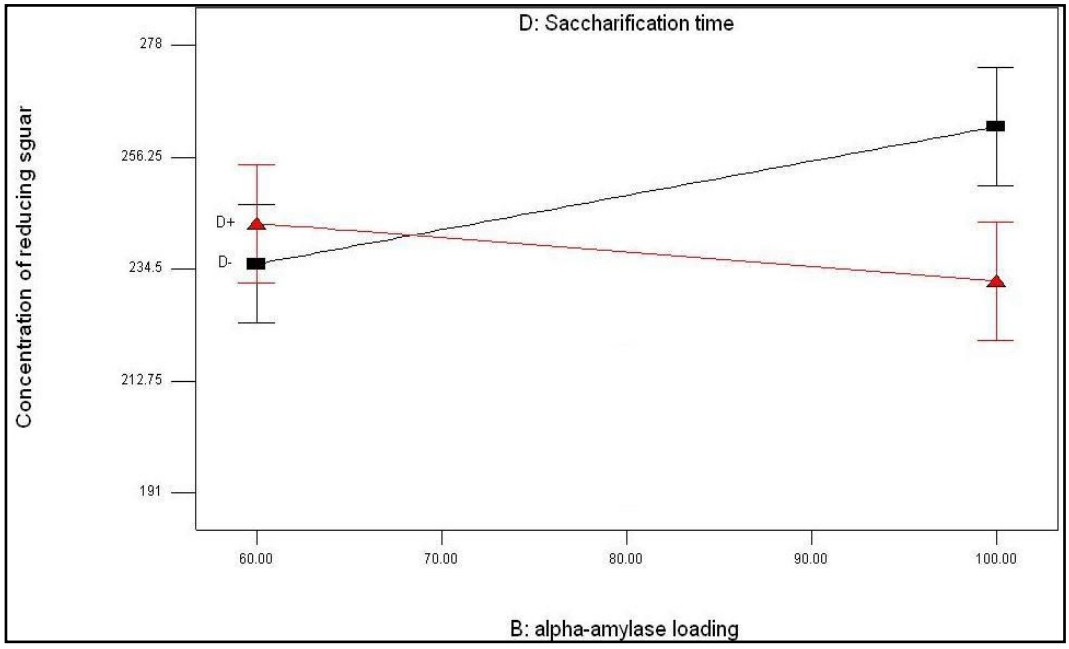

Figure 4.5: Effect of α-amylase loading and saccharification time on concentration of glucose

4.8 Determination of optimum condition

In determining the optimum condition, maximum concentration of glucose was aimed. The optimum conditions suggested by the software, is given in Table 4.3. It is found that the optimum conditions for enzymatic hydrolysis of tapioca starch are α-amylase loading: 80, glucoamylase loading: 5.75mg, liquefaction time: 1 hour and saccharification time: 4 hours. The predicted amount of glucose at the suggested condition is 271.80 g/L. The same optimum conditions also reported by Mojovic *et al*., (2006). and Melo *et al*.,(2008).

Table 4.3: Selection of optimum condition for maximum concentration of glucose

No.	Glucoamylase Loading (mg)	α-amylase Loading (μL)	Liquefaction Time (h)	Saccharification Time (h)	Glucose Concentration (g/L)	Desirability	
1	5,75	80	1	4	271.80	0.931	Selected
2	6	80	0,999995901	4	271.79	0.930	
3	6	80	1,000000021	4	271.78	0.930	

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Enzymatic hydrolysis for glucose production has been conducted using tapioca starch as the raw material. Based on 19 experiment runs the glucose concentrations were from 191.4 to 277.8g/L, which prove that tapioca is feasible as raw material for glucose production. The enzymatic hydrolysis was carried out to study the effects of α-amylase loading, glucoamylase loading, liquefaction time and saccharification time. The optimum conditions for the process were also determined. Among all effects studied, liquefaction time and the interaction between α-amylase loading and saccharification time show the most significant effects on concentration of glucose produced. Optimum conditions suggested for enzymatic hydrolysis of tapioca starch were α-amylase loading: 80μL, glucoamylase loading: 5.75mg, liquefaction time: 1 hour and saccharification time: 4 hours. From this condition, as high 277.80 g/L of glucose concentration was estimated.

5.2 Recommendations

In order to improve the research, there are several recommendations:

- Otimization should continue with central composite design (CCD) for better determination of optimum conditions.
- Usage of glucoamylase alone during saccharification will not give high conversion of final product due to product-end inhibition. This process should be improving with addition of pullulanase.
- Since use of food as a raw material would be an issue in most countries, raw material from waste or any non-food source should be considered.

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APPENDIX A

Appendix A.1: UV-Visible Single Beam Spectrophotometer (Model U-1800)

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

APPENDIX B

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

Concentration of Glucose (mg/mL)	Absorbance at 540 nm
0.1	0.074
0.2	0.237
0.3	0.392
0.4	0.52
0.5	0.663
0.6	0.755
0.7	0.894
0.8	1.026
0.9	1.154
1.0	1.231

Appendix B.2: Full factorial 2^4 design consisting of 19 experiments for the study of four experimental factors in coded units.

Run	X1	X2	X3	X4	Coefficient assessed by
1	-1	1	-1	1	Fractional 2^4 factorial design
2	-1	-1	1	1	
3	0	0	0	0	Central points
4	1	1	1	1	Fractional 2^4 factorial design
5	1	1	1	-1	
6	-1	-1	1	-1	
7	1	1	-1	-1	
8	0	0	0	0	Central points
9	-1	-1	-1	-1	Fractional 2^4 factorial design
10	-1	1	1	1	
11	-1	1	-1	-1	
12	-1	1	1	-1	
13	1	-1	-1	1	
14	1	1	-1	-1	
15	1	-1	-1	-1	
16	1	-1	1	-1	
17	1	-1	1	1	
18	-1	-1	-1	1	
19	0	0	0	0	Central points

Appendix B.3: Combinations of variables in determination of optimum condition

No.	Number of Starting Points: 47			
	glucoamylase loading	α-amylase loading	Liquefaction time	Saccharification time
1	4.750	60.000	2.000	3.000
2	5.750	80.000	1.000	4.000
3	4.750	100.000	1.000	5.000
4	6.750	100.000	1.000	3.000
5	4.750	100.000	2.000	5.000
6	6.750	100.000	2.000	3.000
7	4.750	100.000	2.000	3.000
8	6.750	60.000	1.000	3.000
9	6.750	60.000	2.000	5.000
10	4.750	60.000	1.000	3.000
11	6.750	100.000	2.000	5.000
12	6.750	60.000	1.000	5.000
13	4.750	100.000	1.000	3.000
14	6.750	100.000	1.000	3.000
15	6.750	60.000	1.500	3.000
16	4.750	60.000	0.500	5.000
17	4.750	60.000	1.500	5.000
18	5.049	71.892	0.871	4.805
19	6,054	62.808	0.706	4.903
20	6.322	99.172	0.567	3.047
21	5.990	96.604	0.667	3.675
22	5.872	85.764	1.251	3.012
23	4.913	65.264	0.761	3.397
24	5.561	69.388	0.877	4.247
25	6.080	71.760	0.866	3.223
26	6.692	86.384	1.132	4.181
27	6.001	87.092	1.379	3.435
28	6.424	66.840	1.292	4.939
29	4.931	97.996	1.140	3.895
30	6.429	74.816	1.214	3.415
31	6.069	79.592	1.490	3.736
32	5.817	93.424	1.450	4.595
33	5.594	73.052	1.438	4.309
34	6.288	75.024	1.482	4.121
35	4.960	88.240	0.968	3.380
36	5.557	96.396	1.088	4.117
37	5.432	71.356	1.460	4.5734
38	5.009	98.856	0.829	4.2552
39	4.872	97.632	1.247	4.065
40	5.620	93.432	0.662	4.0792
41	4.782	68.940	1.285	4.5328
42	6.117	71.032	0.680	4.5734

43	5.594	76.504	0.955	4.348
44	5.393	97.596	0.973	4.983
45	6.205	68.496	0.991	4.022
46	5.172	74.200	0.620	4.884
47	5.767	62.744	0.530	3.901

Appendix B.4: Calculation for determining enzyme loading

1. α-amylase loading

Enzyme activity available = 24mg protein/mL : 75 KNU/mg protein
= 11.52 KNU/mL

Enzyme activity from journal (Melo et al, 2008) = 144KNU/mL

$$\frac{1800\text{KNU}}{?\text{mL}^*} = \frac{144\text{KNU}}{1\text{mL}}$$

* in other words, how much amount needed for available enzyme activity to achieve target enzyme acitivity

Solving the equation, 0.08mL needed (80μL)

2. Glucoamylase loading

Enzyme activity available = 138 AGU/mg

Enzyme activity from journal (Mojovic et al, 2006) = 240 AGU/g for 100g of starch

= 24 AGU/g for 10 g of starch

$$\frac{138\text{AGU}}{?\text{mg}^*} = \frac{24\text{AGU}}{1\text{g}}$$

* in other words, how much amount needed for available enzyme activity to achieve target enzyme acitivity

Solving the equation, 0.00575g needed (5.75mg)