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

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A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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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.”

Signature :........................................
Name : Siti Nor Shadila binti Alias
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.
ACKNOWLEDGEMENT

I would like to forward my appreciation to my thesis supervisor, Miss Asmida binti Ideris and my panels Miss Nurul Aini binti Mohd Azman and Miss Shalyda binti Md Shaarani @ Md Nawi for their guidance and support. I would also very thankful to my academic advisor, Mr. Rozaimi bin Abu Samah for his support and believe in me during my studies. Again, very thanks to all laboratory’s staffs for their guidance and support.

I’m very grateful to Universiti Malaysia Pahang (UMP) for providing good facilities in the laboratory and in campus. To all the staff in Faculty of Chemical & Natural Resources Engineering, a very big thanks you to all.

My sincere appreciation also extends to all my fellow colleagues and others who have provided assistance at various occasions. Their views and tips are useful indeed. Thank you for the time sacrificed to accompany me. And last but not least, I am grateful to all my family members.
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 memaksimurnkan 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*. Kesah suhu *liquefaction* \( (X_1) \), suhu *saccharification* \( (X_2) \), pH *liquefaction* \( (X_3) \) and pH *saccharification* \( (X_4) \) telah dikaji. ‘2⁴ 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$_2$SO$_4$ - 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 \((\text{C}_6\text{H}_{10}\text{O}_5)_x\), hemicellulose such as xylan \((\text{C}_5\text{H}_8\text{O}_4)_m\) and lignin \([\text{C}_9\text{H}_{10}\text{O}_5;\text{(OCH}_3)_{0.9-1.7}]_n\) (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](image)

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 consists 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$_6$H$_{12}$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, maltotriose 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 amylpectin 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 saccharification 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 breaks 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 amyllopectin. β-amylase is an exo-acting enzyme cleaving β-maltose molecules from the non-reducing end of amylose or from the outer branches of amyllopectin. 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 amyllopectin. 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).
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).