EFFECT OF TEMPERATURE AND pH ON GLUCOSE PRODUCTION USING ENZYMATIC HYDROLYSIS

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SITI SOLEHAH BINTI AHMAD

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

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

APRIL 2010

I declare that this thesis entitled "*Effect of Temperature and pH on Glucose Production Using Enzymatic Hydrolysis*" 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 Solehah Binti Ahmad
Date	:	30 APRIL 2010

Special dedication to my whole family members, my supervisor, my lecturers, staffs of FKKSA laboratory and all my colleagues at Universiti Malaysia Pahang.

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ABSTRACT

Enzymatic hydrolysis is one of the effective methods in glucose production due to enzyme properties which are highly specific and very sensitive. Therefore, this paper was designed to study the effect of temperature and pH on glucose production using enzymatic hydrolysis. By using enzymatic hydrolysis, cellulase from *Trichoderma reesei* was used and supplemented with cellobiase from *Aspergillus niger* for increasing the glucose production. Cellulose as a substrate was hydrolyzed by these enzymes to produce glucose and the amount of glucose production was determined by high-performance liquid chromatography (HPLC). The result showed that the highest glucose concentration was produced at the temperature of 50°C and pH 5.0 while the lowest glucose production was produced at 30°C and pH 6.5 which were 21.09 mg/ml and 3.55 mg/ml, respectively. By changes the temperature and pH above or below 50°C and pH 5.0 results in low glucose production because the enzyme was lose their three-dimensional functional shape due to disruption from unstable condition of the environment system. As a conclusion, all the requirement for complementary in the configuration of cellulose and enzyme must be considered seriously for the highest glucose production.

ABSTRAK

Hidrolisis enzim merupakan salah satu kaedah berkesan dalam penghasilan glukosa disebabkan oleh sifat enzim yang sangat khusus dan sensitif. Oleh itu, tujuan kajian ini dilaksanakan untuk mengetahui kesan suhu dan pH kepada penghasilan glukosa dengan menggunakan kaedah hidrolisis enzim. Dengan menggunakan kaedah ini, enzim selulase daripada *Trichoderma reesei* dan dilengkapi dengan selobiase daripada *Aspergillus niger* telah digunakan untuk meningkatkan penghasilan glukosa. Selulosa yang digunakan sebagai bahan mentah dihidrolisis oleh enzim untuk menghasilkan glukosa dan jumlah penghasilan glukosa dianalisis menggunakan sistem kromatografi (HPLC). Keputusan kajian menunjukkan bahawa penghasilan glukosa tertinggi adalah pada suhu 50°C dan pH 5.0 manakala penghasilan glukosa terendah adalah pada suhu 30°C dan pH 6.5. Jumlah kepekatan tertinggi yang dihasilkan ialah 21.09 mg/ml dan jumlah kepekatan terendah yang dihasilkan pula ialah 3.55 mg/ml. Perubahan suhu dan pH di atas atau di bawah nilai maksimum akan menyebabkan penghasilan glukosa rendah kerana enzim kehilangan fungsinya disebabkan oleh gangguan dari sistem persekitaran yang tidak stabil. Sebagai kesimpulan, semua keperluan dalam konfigurasi enzim dan selulosa, harus dipertimbangkan secara serius untuk menghasilkan glukosa yang tertinggi.

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LIST OF ABBREVIATIONS/SYMBOLS

%	-	Percent
mg/ml	-	Milligram per milliter
°C	-	Degree celsius
µm	-	Micro meter
α	-	Alpha
β	-	Beta
MPa	-	Mega Pascal
FPU	-	Filter paper unit
CBU	-	One Cellobiase Unit
HPLC	-	High-performance Liquid Chromatography
ml	-	Milliliter
w/v	-	Weight per volume
rpm	-	Rotation per minute
M	-	Molarity
cm	-	Centimeter
µL	-	Microliter
ml/min	-	Milliliter per minute
nRIU*s	-	Unit area of chromatogram peak

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Glucose is a commercially important product widely used by the food and pharmaceutical industries (Johnson *et al*., 2009). In general, glucose is used in the food industry as a partial or complete substitute for sucrose. Glucose is the common name for the syrup which is used in large quantities in fruit canning, confectioneries, jams, jellies, preserves, ice cream, bakery products, pharmaceuticals, beverages and alcoholic fermentation. The functional purpose of glucose in the confectionery industry is to prevent crystallization of the sucrose while in the bakery products industry it is to supply fermentable carbohydrates. In the ice-cream and fruit-preserves, it used to increase the solids without causing an undue increase in the total sweetness. In pharmaceutical industry, glucose is used as a precursor to make vitamin C in the Reichstein process, to make citric acid, gluconic acid, polylactic acid and sorbitol. Currently, glucose is utilized as an intermediate raw material for bio-ethanol production.

Commonly, glucose is prepared commercially via the enzymatic hydrolysis of starch instead of acid hydrolysis. Many crops can be used as the source of the initial starch. Maize, rice, wheat, potato, cassava, arrowroot and sago are all used in various parts of the world. Nevertheless, using the starch needs to compete with their primary use as food crops. Due to the abundant of non-food energy crops like cellulosic material, they are use to reduce the utilization of starch as raw material for

production of glucose. Cellulosic materials including agricultural, agro-industrial and forestry lignocellulosic residues have potential as cheap and renewable feedstocks for large scale production of fuels and chemicals. Currently, bio-processing of lignocellulosics is focused on enzymatic hydrolysis of the cellulose fraction to glucose, followed by fermentation to fuel-grade ethanol. However, enzymatic hydrolysis of cellulosic materials to produce fermentable sugars has also enormous potential in meeting global food and energy demand via biological route (Gan *et al*., 1994).

In lignocellulosic materials cellulose is physically associated with hemicellulose, and physically and chemically associated with lignin. The presence of these two fractions is reported to make the access of cellulase enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis (Mussatto *et al*., 2008). There are several kinds of pretreatment able to disrupt the lignocellulosic structure for increasing the efficiency of the hydrolysis which have been investigated but are not within the scope of this study. However, the effect of temperature and pH are also significant in cellulose hydrolysis which will be studying in this study. The temperature and pH influence the efficiency of cellulase to degrade the cellulose for producing glucose.

1.2 Problem Statement

In order to produce glucose, cellulose is essential to break it down first. By using acid hydrolysis, conversion of cellulose to glucose only produces low glucose concentration because acid is no selectivity. Furthermore, by using acid causes the cost production of glucose is high due to demand of neutralization after hydrolysis which can contribute to corrosion problem if there is no neutralization process. Other that, the need of high temperature during acid hydrolysis process also contributes to the cost of production because high energy is consumed. Waste from acid hydrolysis also gives the bad effect to the environment which is using high concentration of acid can cause harmful to the environment. Therefore, the

investigation attempted to determine the glucose production by using enzymatic hydrolysis process in order to replace acid hydrolysis process.

Nevertheless, by using enzymatic hydrolysis need highly specific and very sensitive. Their environmental condition such temperature and pH influence the activity of the enzymes in the system. Hence, the effect of temperature and pH is investigated to determine the maximum conditions of enzymatic hydrolysis process in glucose production.

1.3 Research Objective

The main purpose of this study is to study the effect of temperature and pH on glucose production using enzymatic hydrolysis.

1.4 Scope of Study

The scope of this study includes studying the effect of temperature at 30°C, 40°C, 50°C, 60°C and 70°C which control by incubator. For the effect of pH at 4.5, 5.0, 5.5, 6.0 and 6.5 are control by citrate buffer. This study also includes analyzing the glucose concentration by using high performance liquid chromatography (HPLC).

1.5 Significant of Study

The significant of this study is to increase glucose yield from enzymatic hydrolysis by the maximum temperature and pH. Furthermore, by using enzymatic hydrolysis can reduce the cost production of glucose due to less energy consumption because the temperature consume in this process is low. Besides that, this process is green technology because it will not produce harmful waste.

CHAPTER 2

LITERATURE REVIEW

2.1 Glucose Overview

Glucose is a simple monosaccharide sugar also known as grape sugar, blood sugar or corn sugar which is a very important carbohydrate in biology. The living cell uses it as a source of energy and metabolic intermediate. Glucose is one of the main products of photosynthesis and starts cellular respiration in both prokaryotes and eukaryotes. Glucose ($C_6H_{12}O_6$) contains six carbon atoms, one of which is part of an aldehyde group (Figure 2.1). Therefore, glucose is an aldohexose. Glucose is commonly available in the form of a white powder or as a solid crystal, called dextrose. It can also be dissolved in water as an aqueous solution, glucose syrups. Its solubility level is very high (McMurry, 1988).

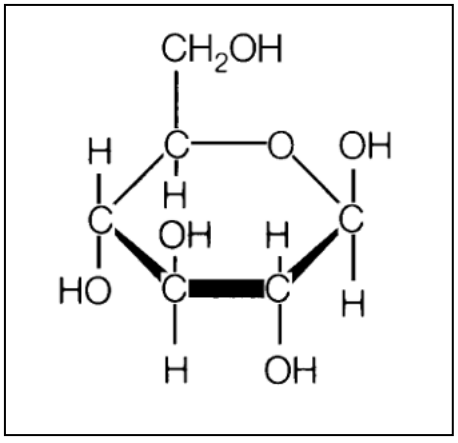

Figure 2.1 Structure of Glucose

Glucose can be forms disaccharide when two of monosaccharide are linked together such sucrose, the combination of glucose with fructose (Figure 2.2). Sucrose is the most common sweetener in the modern, industrialized world, although it has been displaced in industrial food production by some other sweeteners such as glucose syrups or combinations of functional ingredients and high intensity sweeteners.

In lactose, another important disaccharide, glucose is joined to galactose (Figure 2.3). It used as the predominant sugar in milk. For maltose, a product of starch digestion is glucose-glucose disaccharide (Figure 2.4). Glucose also can be forms polysaccharides when the units (either mono- or di-saccharides) are repeated and joined together by glycosidic bonds like cellulose. Cellulose is yet a third polymer of the monosaccharide glucose (Carpi, 2003).

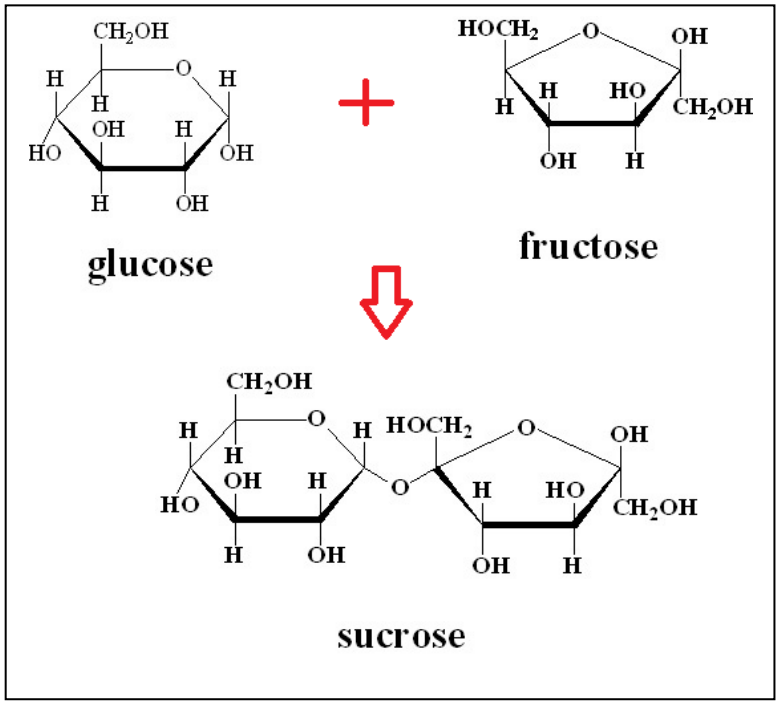

Figure 2.2 Structure of Sucrose

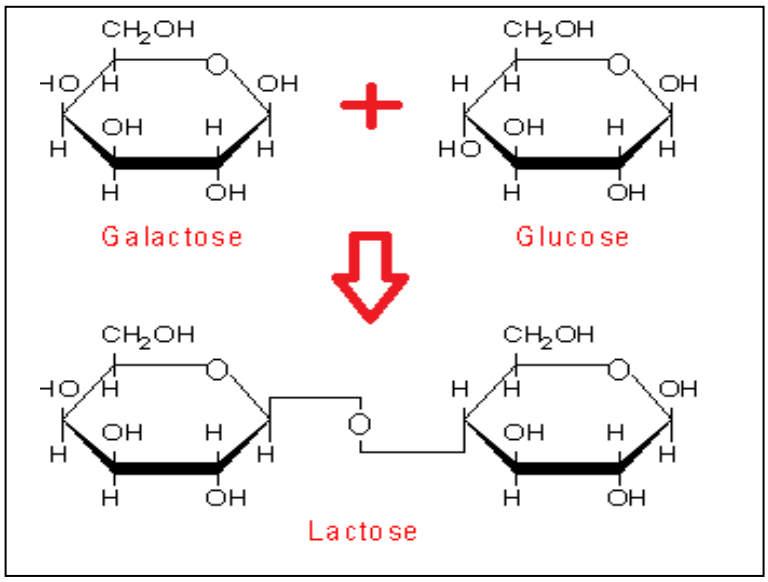

Figure 2.3 Structure of Lactose

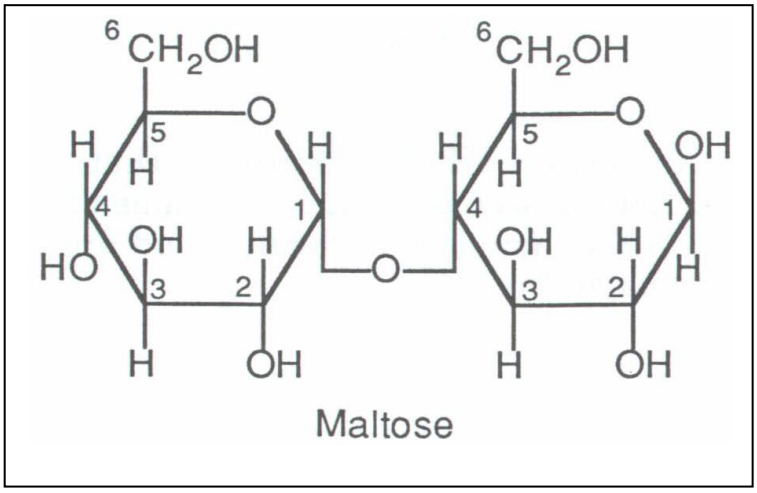

Figure 2.4 Structure of Maltose

2.1.1 Glucose as Energy Source in Living Cell

Glucose used as an energy source in most organisms from bacteria to human. In human, glucose is the main source of energy for the body, especially for the brain and the red blood cells. It is utilized by the cells to generate energy that is needed to carry out important cellular functions. If the glucose level becomes very low (hypoglycemia), the cells cannot function normally and it results in headache, confusion, nervousness, convulsions and coma in extreme cases (Gailliot *et al*., 2007). Since the brain is so sensitive to the glucose level in the blood, a number of mechanisms are in place to ensure that the blood glucose remains more or less constant. If the blood glucose level increases, it is promptly converted into glycogen in the liver and stored. On the other hand, if the glucose level falls, the stored glycogen is readily converted back to glucose and released in the blood stream.

Glucose may come directly from dietary carbohydrates or from glycogen stores in the liver and the muscles. Glucose is the results of the breakdown of glycogen. Several hormones, including insulin, work rapidly to regulate the flow of glucose to and from the blood to keep it at a steady level. While in animal, glucose is synthesized in the liver and kidneys from non-carbohydrate intermediates, such pyruvate and glycerol, by a process of gluconeogenesis. For plant, glucose is produced from photosynthesis process which is used as an energy source in cells via aerobic or anaerobic respiration.

2.1.2 Commercial Production of Glucose

Nowadays, glucose is valued in almost all industrial countries for its unique properties. It is used in the manufacture of a number of products in food industries, pharmaceuticals and industrial fermentations. In food industries, glucose is extremely popular in the sweet manufacturing business (Figure 2.5). It is extensively used in confectionery as a doctoring agent to prevent crystallization. Being a non-crystallizing substance, it helps produce homogenous confectionery like chewing gums and chocolates. It provides a smooth texture, possesses good preservative qualities for a longer shelf life and has several desirable organoleptic properties. In processed foods like jams and jellies, glucose syrup is used to prevent crystallization of sugar. It acts as a good preservative and prevents spoilage of the product without unduly increasing its sweetness. It is used in the preparation of common syrups as it is easily digestible and provides an instant source of energy. Baby foods and baby syrups also favour glucose syrup as it serves as a rich source of carbohydrates.

Furthermore, glucose syrup adds body, bulk and optimum sweetness to bakery products. This is why it is so often used by bakery houses in pie and cream fillings. It also prevents crystallization, enhances shelf-life and its non-crystallizing and hygroscopic properties keep the preparations fresh and longer ice creams. Nobody likes ice creams that crystallize, melt soon or are rough to the tongue. This is exactly what glucose syrup prevents. It prevents crystallization, gives a smooth texture and ensures that ice creams do not melt soon. It prevents sucrose crystallization and lends a creamy, soft mouthful to the ice cream, lending it homogenous sweetness. There is no undesirable taste and it can even replace expensive ingredients like non-fat milk solids.

Figure 2.5 Usage of Glucose in Food Industries

Otherwise, in pharmaceuticals, glucose is a valuable vehicle for cough syrups and vitamin-based tonics and may be used as a granulating agent for tablet coating. Cough lozenges also use glucose syrup as one of the principal ingredients. It provides body, consistency, a good mouth feel and balanced sweetness when used with other carbohydrate sweeteners like sucrose and sorbitol. Glucose syrup adds flavour to tobacco and lends it a smooth texture. When used in the preparation of chewing tobacco, it enhances the shelf-life. It also helps in the dressing of cigarette tobacco. Others usage, glucose syrup also finds use as a preservative in pan masalas, besides helping in the brewing and fermentation industries. It is also used in the traditional oil extraction industries for its gumming properties. It is used to improve stability in adhesives, as a setting retardant in concrete, as humectants in air fresheners and for evaporation control in cologne and perfumes.

2.1.3 Substrate for Glucose Production

Glucose is produced from many plant sugars. Cellulose and starch are just two examples but in no matter what sources, glucose production process takes polysaccharides or complex sugars from the plant and breaks them into single sugar, glucose. Nevertheless, there have some difference process between cellulose and starch because the structure of cellulose and starch is different. By using the starch, the structure is easy to breakdown because it joined together by α- (1-4) acetal linkage and the enzyme that used in the breakdown process is α-amylase. While for cellulose, the structure is difficult to breakdown because it joined together by β- (1-4) acetal linkage and the enzyme that used in the breakdown process is cellulose (Figure 2.6). Therefore, it makes sense that starch is easier to convert to glucose and production process is faster and not complicated compare to cellulose which the production process is slower and more complicated.

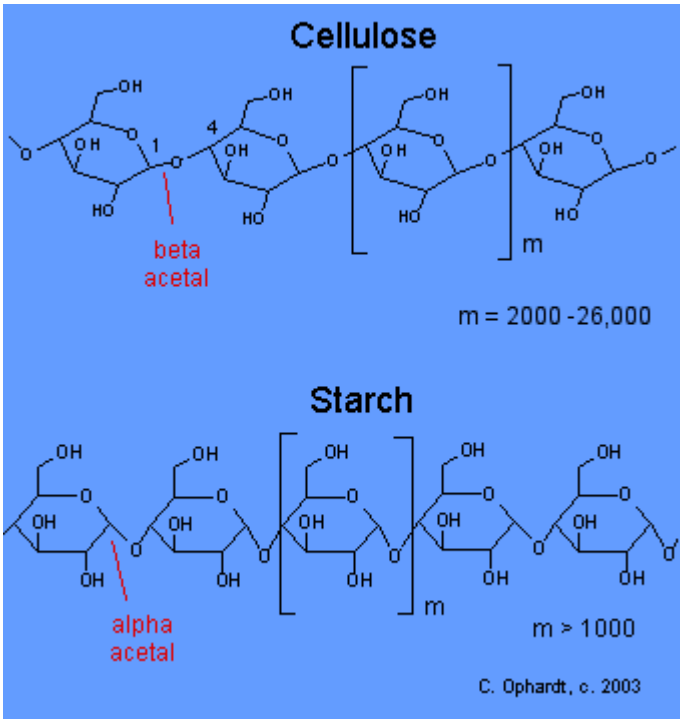

Figure 2.6 The Comparison Structure of Starch and Cellulose

Due to the starch advantages, glucose is prepared commercially via the enzymatic hydrolysis of starch. Starch, the ubiquitous storage carbohydrate of plants like corn, potato, rice, sorghum, wheat and cassava are becomes the primary raw material for the production of glucose (Aschengreen *et al*., 1979). But it creates a problem when starch becomes the primary raw material because starch needs to compete with their primary use as food crops. Therefore, due to the abundant of non-food energy crops like cellulosic material, cellulose is use to reduce the utilization of starch as raw material for production of glucose. Cellulosic materials including agricultural, agro-industrial and forestry lignocellulosic residues have potential as cheap and renewable feedstocks for large scale production of fuels and chemicals. Currently, bioprocessing of lignocellulosics is focused on enzymatic hydrolysis of the cellulose fraction to glucose, followed by fermentation to fuel-grade ethanol. However, enzymatic hydrolysis of cellulosic materials to produce fermentable sugars has also enormous potential in meeting global food and energy demand via biological route (Gan *et al*., 1994).

2.2 Cellulose

The major component in the rigid cell walls in plants is cellulose. Cellulose is an organic compound with the formula $(C_6H_{10}O_5)n$, which is a linear polysaccharide polymer with many glucose monosaccharide units are linked together by β-(1→4)-glycosidic bonds (Figure 2.7). A linear chain of (1-4) linked β-D-glucopyranose units aggregated to form a highly ordered structure due to its chemical constitution and spatial conformation. The highly order structure and crystallinity of cellulose makes it recalcitrant to hydrolysis which it should be disrupted in a pretreatment step in order to hydrolyze cellulose efficiently (Kua and Lee, 2009).

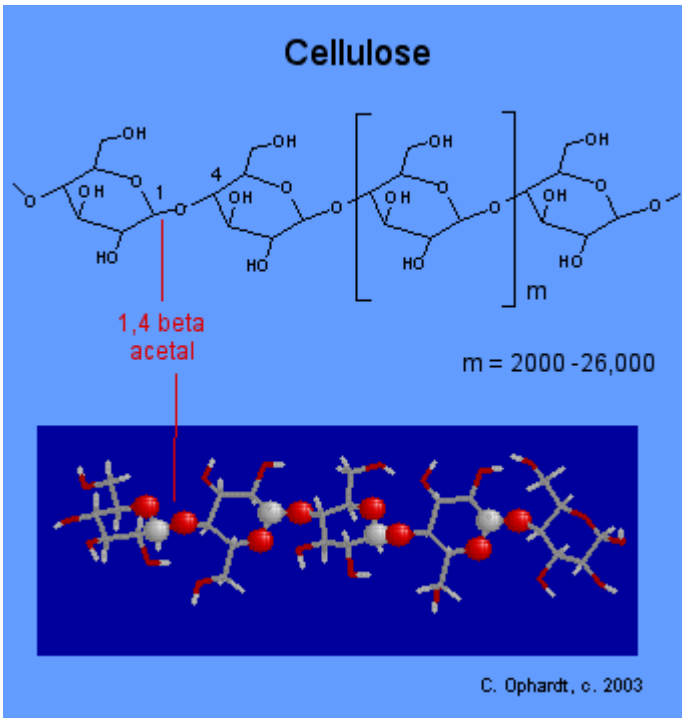

Figure 2.7 Structure of Cellulose

On the other hand, the beta acetal linkage makes cellulose different from starch, glycogen and other carbohydrates linkages. This peculiar difference in acetal linkages results in a major difference in digestibility in humans. Humans are unable to digest cellulose because the appropriate enzymes to breakdown the beta acetal linkages are lacking. Animals such as cows, horses, sheep, goats, and termites have symbiotic bacteria in the intestinal tract. These symbiotic bacteria possess the necessary enzymes to digest cellulose in the GI tract. They have the required enzymes for the breakdown or hydrolysis of the cellulose, the animals do not, not even termites, and have the correct enzymes. No vertebrate can digest cellulose directly.

Cellulose has no taste, is odourless, is hydrophilic in water and most organic solvents, is chiral and is biodegradable. Commercially, it can be broken down chemically into its glucose units by treating it with concentrated acids at high temperature. Due to the highly order structure and crystallinity, cellulose requires a temperature of 320°C and pressure of 25 MPa to become amorphous in water (Deguchi *et al*., 2006). Many properties of cellulose depend on its chain length or degree of polymerization, the number of glucose units that make up one polymer molecule.

2.2.1 Sources of Cellulose

Cellulose is the main component of higher plant cell walls and one of the most abundant organic compounds on earth. It can be derived from a number of sources using a number of techniques that are considered synthetic, and some that might be considered non-synthetic (natural). Cellulose from such major land plants as forest trees and cotton is assembled from glucose which is produced in the living plant cell from photosynthesis. These are macroscopic, multi-cellular photosynthetic plants with which we are all familiar.

In the oceans, however, most cellulose is produced by unicellular plankton or algae using the same type of carbon dioxide fixation found in photosynthesis of land plants. In fact, it is believed that these organisms, the first in the vast food chain, represent Nature's largest resource for cellulose production. Without photosynthetic microbes, all animal life in the oceans would cease to exist. Several animals, fungi, and bacteria can assemble cellulose; however, these organisms are devoid of photosynthetic capacity and usually require glucose or some organic substrate synthesized by a photosynthetic organism to assemble their cellulose. Some bacteria can utilize methane or sulfur substrates to produce glucose and other organic substrates for cellulose (Brown, 1979).

Nowadays, due to the abundant of biomass wastes, it becomes major resources to obtain the cellulose. Biomass wastes are including agricultural residues such as straws, corn stalks and cobs, bagasse, cotton gin trash and palm oil wastes. Paper such recycled newspaper, paper mill sludge's and sorted municipal solid waste. For wood wastes are prunings, wood chips and sawdust while for green wastes are leaves, grass clippings, vegetable and fruits wastes. By utilizing the biomass wastes for the production of value-added product, it becomes environmental-friendly alternative to the disposal of solid waste.

Figure 2.8 Types of Biomass Wastes

2.2.2 Usages of Cellulose

Cellulose is one of the most widely used natural substances and has become one of the most important commercial raw materials. The major sources of cellulose are cotton (about 91 percent cellulose) and wood (about 42 percent cellulose). Since cellulose is insoluble in water, it is easily separated from the other constituents of a plant. Generally, cotton and wood are the major resources for all cellulose products such as paper, textiles, construction materials, cardboard, as well as such cellulose derivatives as cellophane, rayon, and cellulose acetate. Cellulose has been used to make paper since the Chinese first invented the process around A.D. 100. Cellulose is separated from wood by a pulping process that grinds woodchips under flowing water. The pulp that remains is then washed, bleached, and poured over a vibrating mesh. When the water finally drains from the pulp, what remains is an interlocking web of fibers that, when dried, pressed, and smoothed, becomes a sheet of paper.

Otherwise, raw cotton and its fiber cells are found on the surface of the cotton seed. There are thousands of fibers on each seed, and as the cotton pod ripens and bursts open, these fiber cells die. Because these fiber cells are primarily cellulose, they can be twisted to form thread or yarn that is then woven to make cloth. Since cellulose reacts easily to both strong bases and acids, a chemical process is often used to make other products. For example, the fabric known as rayon and the transparent sheet of film called cellophane are made using a many-step process that involves an acid bath. In mixtures if nitric and sulfuric acids, cellulose can form what is called guncotton or cellulose nitrates that are used for explosives. However, when mixed with camphor, cellulose produces a plastic known as celluloid, which was used for early motion-picture film. However, because it was highly flammable (meaning it could easily catch fire), it was eventually replaced by newer and more stable plastic materials. Although cellulose is still an important natural resource, many of the products that were made from it are being produced easier and cheaper using other materials.

2.2.3 Cellulose as a Raw Material of Bio-ethanol Production

Bio-ethanol has some advantages over petrol as fuel. Bio-ethanol is made from biomass and it is renewable. It is an alcohol and is fermented from sugars, starches or lignocellulosic biomass. Lignocellulosic biomass is considered a future alternative for the agricultural products that are currently used as feedstock for bio-ethanol production, because it is more abundant and less expensive than food crops, especially when waste streams are used. Furthermore, the use of lignocellulosic biomass is more attractive in terms of energy balances and emissions. Production of bio-ethanol from lignocellulosic biomass is one way to reduce both the consumption of crude oil and environmental pollution. As a result, by using bio-ethanol instead of petrol the emission of green house gases decreases. It is due to as the biomass grows it consumes as much carbon dioxide as it form during the combustion of bio-ethanol which makes the net contribution to the green house effect zero (Galbe and Zacchi, 2002). Besides that, by using bio-ethanol can enhance energy security, improve the economy and improve air quality.

There are mainly two processes involved in the conversion of raw material to bio-ethanol, first process is hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars and second process is fermentation of the sugars to ethanol (Arthe *et al*., 2008). Lignocellulosic biomass consists of three main components are carbohydrate polymers called cellulose and hemicellulose that can be converted to sugars, and a non-fermentable fraction called lignin that can be utilized for the production of electricity or heat (Figure 2.9). Cellulose is the major component in lignocellulosic biomass which is about 44% while hemicellulose is about 30% and the minor component is lignin which contains 26%.

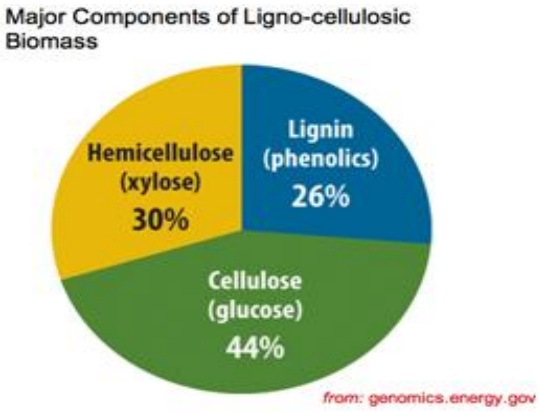

Figure 2.9 Major Components of Lignocellulosic Biomass

In order to produce bio-ethanol from cellulosic biomass, a pretreatment process is used to reduce the sample size, breakdown the hemicelluloses to sugars and open up the structure of the cellulose component (Figure 2.10). The cellulose portion is hydrolyzed by acids or enzymes into glucose sugar that is fermented to bio-ethanol.

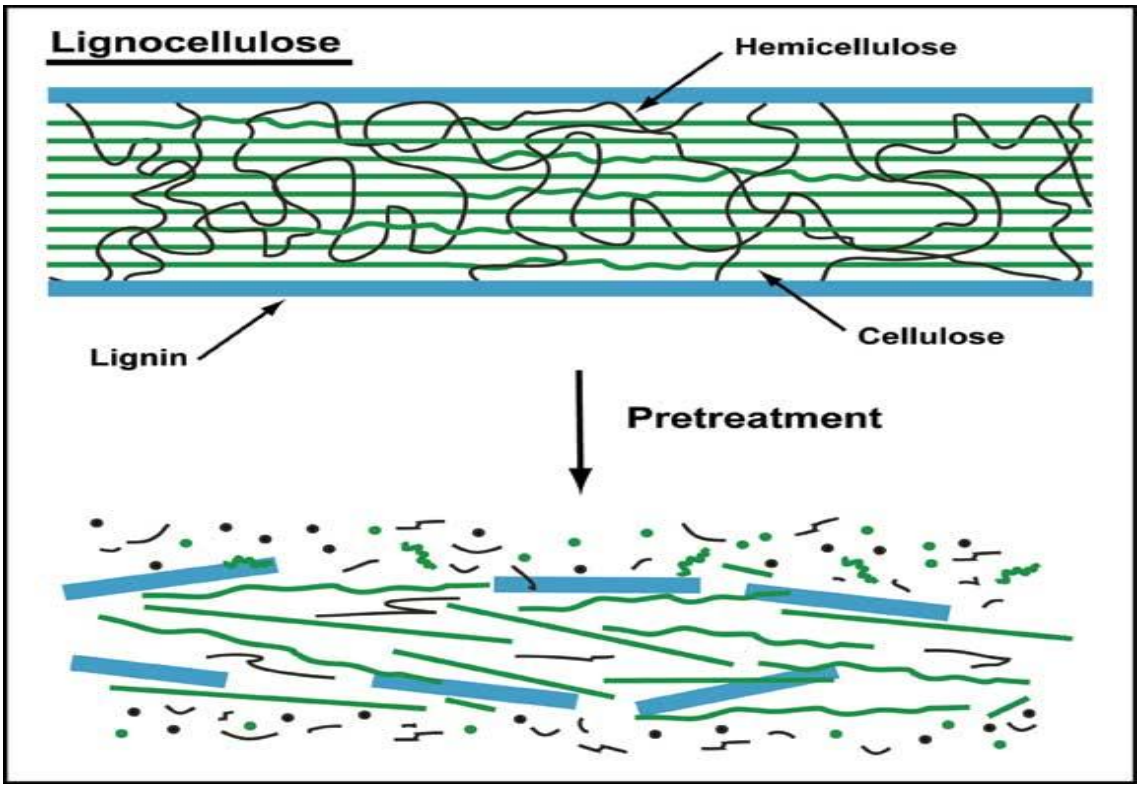

Figure 2.10 Lignocellulose Model Showing Lignin, Cellulose and Hemicelluloses

2.3 Hydrolysis Process

Hydrolysis is a chemical process in which a molecule is cleaved into two parts by the addition of a molecule of water. One fragment of the parent molecule gains a hydrogen ion (H+) from the additional water molecule. The other group collects the remaining hydroxyl group (OH−). However, under normal conditions, only a few reactions between water and organic compounds occur. In general, strong acids or bases must be added in order to achieve hydrolysis where water has no effect. The acid or base is considered a catalyst. They are meant to speed up the reaction, but are recovered at the end of it.

In this study, hydrolysis is defined as the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units. Because cellulose molecules bind strongly to each other, hydrolysis is relatively difficult compared to the breakdown of other polysaccharides. Cellulose degradation to glucose accomplished by the addition of water molecule for each glucose molecule produced (Imai *et al.*, 2004). Currently, there are two major ways of converting cellulose to glucose whereby chemical (acid) hydrolysis versus enzymatic hydrolysis. The research on both methods has for decades occupied the attention of many investigators worldwide.

2.3.1 Acid Hydrolysis

Acid hydrolysis is performed by attacking cellulose with an acid. By using dilute acid, it may be used under high heat and high pressure while for concentrated acid can be used at lower temperature and pressure. As evidence of this, the reaction rates measured at the high temperature region (above 200°C) exhibited a sudden change in apparent activation energy at a certain temperature, deviating from Arrhenius Law (Xiang *et al*., 2003).

Acid-catalyzed of cellulose hydrolysis is a complex heterogeneous reaction. As such the reaction is influenced of by physical factors as well as the hydrolytic chemical reaction. Therefore, the reaction not only controlled by acid concentration and temperature but also physical state of the cellulose. The sudden shift of physical structure and reaction pattern in response to acid concentration and temperature indicates that the main factor causing the change in cellulose structure is disruption of hydrogen bonding (Xiang *et al*., 2003).

2.3.2 Enzymatic Hydrolysis

Enzymatic hydrolysis is performed by attacking cellulose with an enzyme. This reaction is carried out by cellulase enzyme, which are highly specific (Begiun and Aubert, 1994). Cellulase is most often employed to hydrolyze cellulose to glucose because it mild reaction condition and specific action in hydrolysis, producing virtually no glucose degradation products. Cellulase is usually a mixture of several enzymes at various stages of this conversion. Among its which at least three major groups are involved in the hydrolysis process of cellulose which are β-1-4-endoglucanase, attacks regions of low crystallinity in the cellulose fiber creating free chain ends, β-1-4-exoglucanase or cellobiohydrolase, which degrades the molecule further by removing cellobiose units from the free chain ends and lastly, β-glucosidase or cellobiase, which hydrolyzes cellobiose to produce glucose (Prasad *et al*., 2007; Sun and Cheng, 2002; Cao and Tan, 2002).

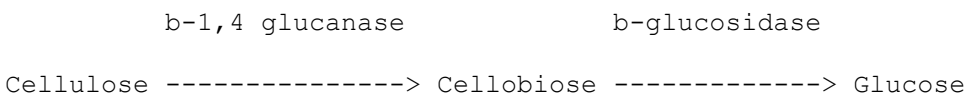

Figure 2.11 Mechanism of enzymatic hydrolysis

Because enzymatic hydrolysis is a heterogeneous reaction and requires direct physical contact between enzyme and substrate, cellulose hydrolysis by cellulase involves three steps, firstly cellulase adsorption onto cellulose surface, secondly subsequent breakdown of cellulose through the synergistic action of the endo- and exo-cellulase, and lastly cellulase desorption from cellulose residue into supernatant (Kuo and Lee, 2009). The accessibility of cellulase to the limited adsorption sites on crystalline cellulose structure is generally believed to play an important role in determining the cellulose hydrolysis rate.

Pan *et al*. (2005) claimed that the hydrolysis of polysaccharides is usually catalyzed by hydrolytic enzymes, because enzymatic hydrolysis produces better yields than acid-catalyzed hydrolysis. Furthermore, by using acid hydrolysis have a lot of disadvantages such no selectivity, a relatively low yield, by-product formation, need to high temperature, demand of neutralization after the hydrolysis and can cause corrosion problem. Otherwise, enzyme hydrolysis is selective, results in a relatively high yield and the hydrolysis is done at lower temperature (Galbe and Zacchi, 2002).

Nevertheless, the enzymatic hydrolysis is a very complex system with multiple factors generated in the processes, rendering it difficult for operation and control (Gan *et al*., 2003). The multiple factors are including size of cellulose particles, substrate concentration, enzyme concentration, agitation intensity, pH of the buffer and the temperature of the process. The optimization of these factors will require more detailed understanding based on enzymes regulation and activity which highly controlled.

2.3.2.1 Effect of pH on Enzymatic Hydrolysis

The pH is plays an important role in enzymatic hydrolysis because it influences enzyme activity. In general, enzymes have a pH optimum which the optimum is not the same for each enzyme. A change in pH above or below the pH optimum reduces the rate of enzyme reaction considerably. It because changes in pH lead to the breaking of the ionic bonds that hold the tertiary structure of the enzyme in place which gives effect to the total net charge of enzymes. Therefore, enzyme begins to lose its functional shape, particularly the shape of the active site, such that the substrate will no longer fit into it, causes denatured and unable to catalyze chemical reactions. As a result, the production rate of product will decrease by the decreasing of hydrolysis rate. Furthermore, changes in pH may not only effect the shape of enzyme but it may also change the shape or change properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis.

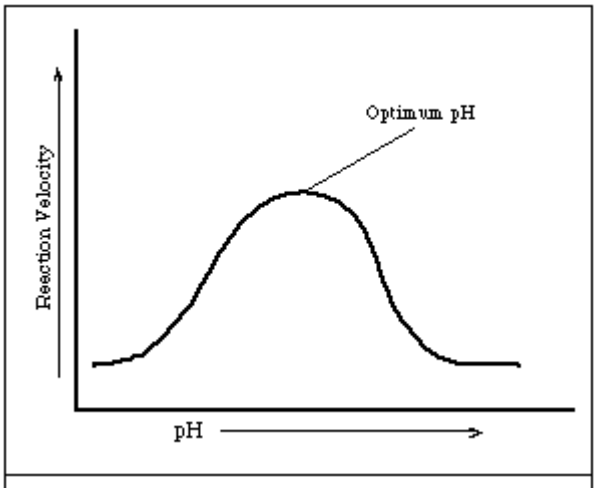

Figure 2.12 Effect of pH on Reaction Rate

2.3.2.2 Effect of Temperature on Enzymatic Hydrolysis

The other factor that affects the enzyme activity is temperature. Enzyme action is greatly affected by temperature. In order to convert substrate into product, enzyme must collide with and bind to the substrate active site. Therefore, the enough energy is necessary to make the good orientation of collision by increasing the temperature. As a result, the number of substrate and enzyme molecule collides will increase and produce higher conversion of product. Nevertheless, too much heat can cause the rate of enzyme catalyzed reaction to decrease because the enzyme or substrate becomes denatured and inactive. Generally, when the temperature is increased by 10^0C the most chemical reaction is doubled. However, above optimum temperature of 50^0C, catalytic activities were severely diminished because denaturation occurs at that temperature.

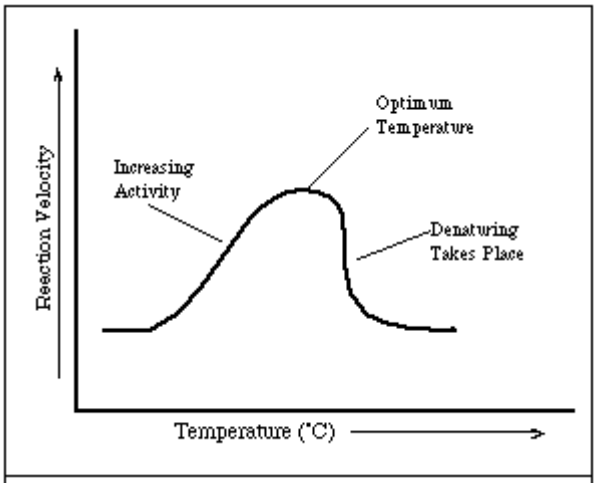

Figure 2.13 Effect of Temperature on Reaction Rate

2.4 Cellulase Enzyme

Aspergillus and *Trichoderma* are the more potent producers of cellulase (Elad, 2000; Kang, 1994). The enzymatic degradation of waste cellulose by fungal cellulases has been suggested as a feasible alternate for the conversion of lignocellulosics into fermentable sugars and fuel ethanol (Oksanen *et al*., 2000; Chin *et al*., 2000). These funguses are widely used in the food and feed industries and recently also used in the textile, pulp and paper industries (Wang *et al*., 2004).

The high cost of cellulase enzymes often restricts the large scale application of these enzymes in the bioconversion of lignocellulosic biomass. In previous publication, Xia and Cen (1999) have reported low-cost cellulose production by *Trichoderma reesei* ZU-02 using solid-state fermentation. However, the most widely used cellulase from *T. reesei* is poor in cellobiase, and thus restricts the conversion of cellobiose to glucose (Shen and Xia, 2004). The accumulation of cellobiose will cause severe feedback inhibition to the cellulase reaction. Therefore, improving the activity of cellobiase in the cellulase system is crucial to raise the enzymatic hydrolysis yield.

Hence, enzymatic hydrolysis with cellulase from *T. reesei* ZU-02 and supplemented by cellobiase from *Aspergillus niger* ZU-07 are used to improve the activity of cellobiase for increasing the hydrolysis yield. The ratio of *Trichoderma reesei* cellulase and *Aspergillus niger* cellobiase is 2FPU:1CBU (Chen *et al*., 2008). One FPU is defined as the amount of enzyme that releases 1μmol of glucose equivalents from Whatman No. 1 filter paper per min. One CBU is the amount of enzyme that converts 1μmol of cellobiase to 2μmol of glucose per min (Bailey *et al*., 1992).

2.5 High Performance Liquid Chromatography (HPLC)

Analysis of glucose and others sugars are among the most widespread chemical analysis that are performed within the industries of food, forage, biomass, pulp and paper (Slimested and Vagen, 2006). The analysis is often performed by use a normal phase high-performance liquid chromatography (HPLC). High-performance liquid chromatography is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds (Figure 2.14).

The HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. By using HPLC, the column temperature plays an important role with respect to chromatography performance and detection limits of glucose when using a specific carbohydrate column. The most widely column used for sugar analysis is cyano- or amino-bonded silica column and the detector is refractive index (RI) detector.

The normal phase chromatographic methods require an organic solvent, often acetonitrile, as a major eluent in combination with water and other solvents for modification of the elution profile (e.g methanol). The mobile phase flow rate was another important factor for chromatography which at fast, the response current reduced. There are reason was that the samples were diluted much more at high flow speed (Sato *et al*., 2008). Furthermore, by using HPLC, different compounds have different retention times. For a particular compound, the retention time will vary depending on the pressure used because that affects the flow rate of the solvent and the nature of the stationary phase which not only what material it is made of, but also particle size.

Figure 2.14 High-performance Liquid Chromatography

2.6 Summary of Hydrolysis Process

Table 2.1 Summary of Previous Research Using Hydrolysis Process

Author	Title	Description
Chen *et al.* (2008)	Enzymatic hydrolysis of maize straw polysaccharides for the production of reducing sugars	• Hydrolysis process is run at 50°C and pH 4.8. • Using mixed of cellulase (cellulase + cellobiase) to raise the enzymatic hydrolysis yield.
Andreaus *et al.* (1999)	Effect of temperature on the cellulose binding ability of cellulase enzymes	• Above optimum temperature of 50°C, catalytic activities were severely diminished.
Angsana *et al*. (2009)	Combination effect of pH and acetate on enzymatic cellulose hydrolysis	• The shift of pH values above or below optimum value was followed by the rapid decrease of hydrolyzed cellulose. • pH will affect the total net charge of enzyme.
Imai *et al*. (2004)	High-performance hydrolysis of cellulose using mixed cellulose species and ultrasonication pretreatment	• The hydrolysis reaction was carried out at pH 4.8 and 50°C. • A mixed-enzyme system of cellulases gave improved hydrolytic reactivity over single-enzyme systems.
Mussatto *et al*. (2008)	Effect of hemicelluloses and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain	• The hydrolysis reaction was carried out at pH 4.8 and 45°C. • The lower hemicelluloses and lignin contents in lignocellulosic biomass, results in the better performance of cellulose enzymatic hydrolysis into glucose.
Liao *et al*. (2006)	Acid hydrolysis of fibers from dairy manure	• By using concentrated acid hydrolysis has been proved ineffective and contribute to the environmental issues.

		• By using dilute acid condition did not convert much cellulose at low temperature. • Therefore, the optimum temperature for dilute acid hydrolysis is 135°C.
Jakobsson (2002)	Optimization of the pretreatment of wheat straw for production of bio-ethanol	• In hydrolysis step, enzymatic is preferable due to it selectivity, results in a relatively high yield and the hydrolysis is done at low temperature. • Most cellulose and β-glucosidase have an optimum at 50±5 ºC and pH 4.0-5.0.
Iranmahboob *et al*. (2002)	Optimizing acid-hydrolysis: a critical step for production of ethanol from mixed wood chips.	• Acid concentration and heating period were two main factors affecting dextrose yields. • For low concentrated acid process, the reaction carried out at high temperature (160°C) but the glucose yields still poor. • Therefore, moderate concentration about 26% by weight able to produce high glucose.
Nidetzky *et al.* (1994)	Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction	• Hydrolysis was carried out at 50°C and pH 4.8. • Combination of cellulase system showed the most powerful synergism.
Karimi *et al.* (2006)	Conversion of rice straw to sugars by dilute-acid hydrolysis	• Hydrolysis of rice straw by dilute sulfuric acid at high temperature and pressure and need several step to achieve high yield of sugars. • Dilute-acid hydrolysis of lignocelluloses results in sugars and other by-product.

The summary of hydrolysis process in previous studies is shown in Table 2.1. From all these previous studies, enzymatic hydrolysis is commonly used due to it mild condition. Actually, different enzymes have different optimum condition based on its properties. Therefore, the cellulases enzyme from *Trichoderma reesei* achieved their optimum values at temperature and pH range of 45°C-55°C and 4.0-5.0, respectively. However, some researcher claimed that enzymatic hydrolysis requires feedstock pretreatment, enzyme production and enzyme recovery which may make this option economically unfeasible. Hence, further research is needed to make this process economically feasible.

CHAPTER 3

RESEARCH METHODOLOGY

3.0 Introduction

In chapter 3, the research methodology is discussed clearly with a specific focus on the enzymatic hydrolysis of cellulose. By using enzymatic hydrolysis, this process is green technology because it will not produce harmful waste and results in environmental friendly. Therefore, the method that has been used in this study is based on the study of which the title is "Enzymatic hydrolysis of maize straw polysaccharides for the production of reducing sugars" by Chen *et al*. (2008).

3.1 Apparatus

During this research, a few apparatus and equipments had been used. The apparatus and equipments would be are :

- Erlenmeyer Flask
- Measuring Cylinder
- Micropipette
- Analytical Balance
- Bunsen Burner
- Double Stack Shaking Incubator (Brand : INFFORS/MULTITRON 11)
- Refrigerated Centrifuge (Brand : EPPENDORF – 5810 R)

- Shaking Water Bath (Brand : JEIO TECH)
- pH Meter (Brand : Methrom)

3.2 Raw Material

The commercial microcrystalline cellulose powder was supplied from Sigma-Aldrich (M) Sdn. Bhd (Figure 3.1). The celullose concentration of 3% (w/v) were prepared in the 100 ml of citrate buffer.

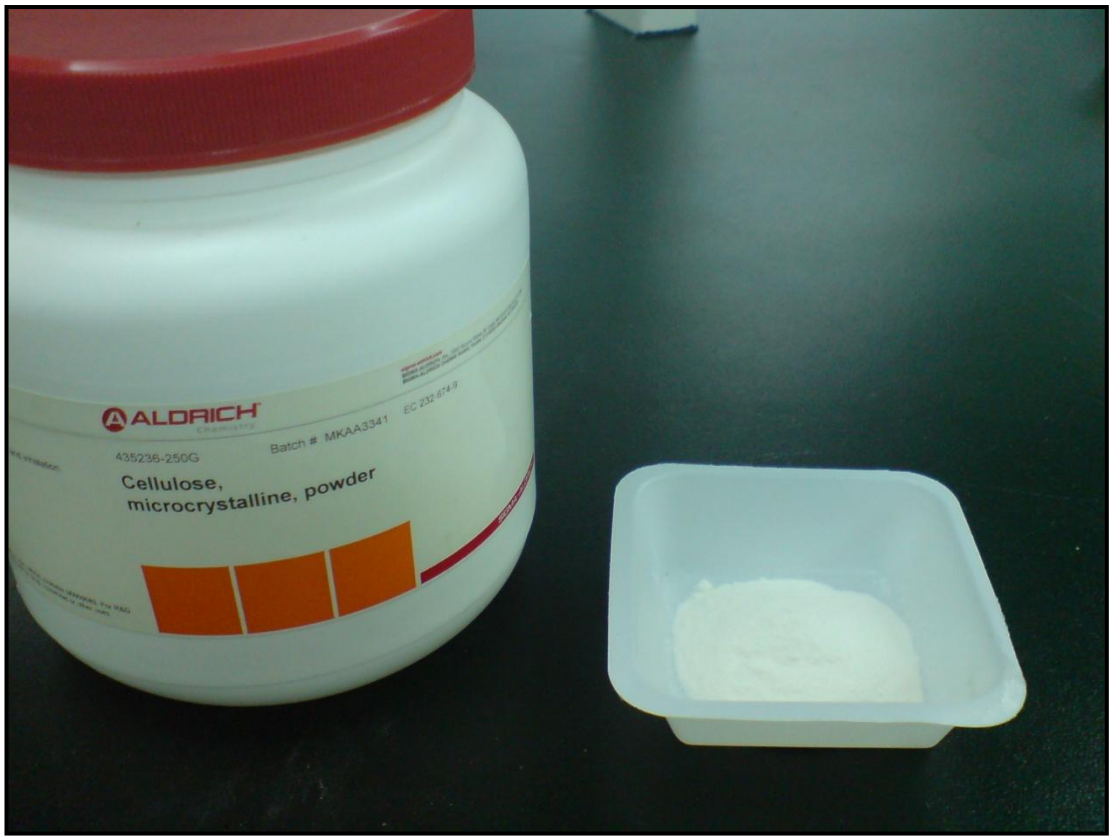

Figure 3.1 Microcrystalline Cellulose Powder

3.3 Enzymes

Cellulase from *Trichoderma reesei* ATCC 26291 were used and supplemented with cellobiase from *Aspergillus niger* (Novozymes 188) by the ratio of 2 FPU/g substrate : 1 CBU/g substrate. These entire enzymes were supplied from Sigma-Aldrich (M) Sdn. Bhd (Figure 3.2).

Figure 3.2 Cellulase and Cellobiase

3.4 Flow Chart of Methodology

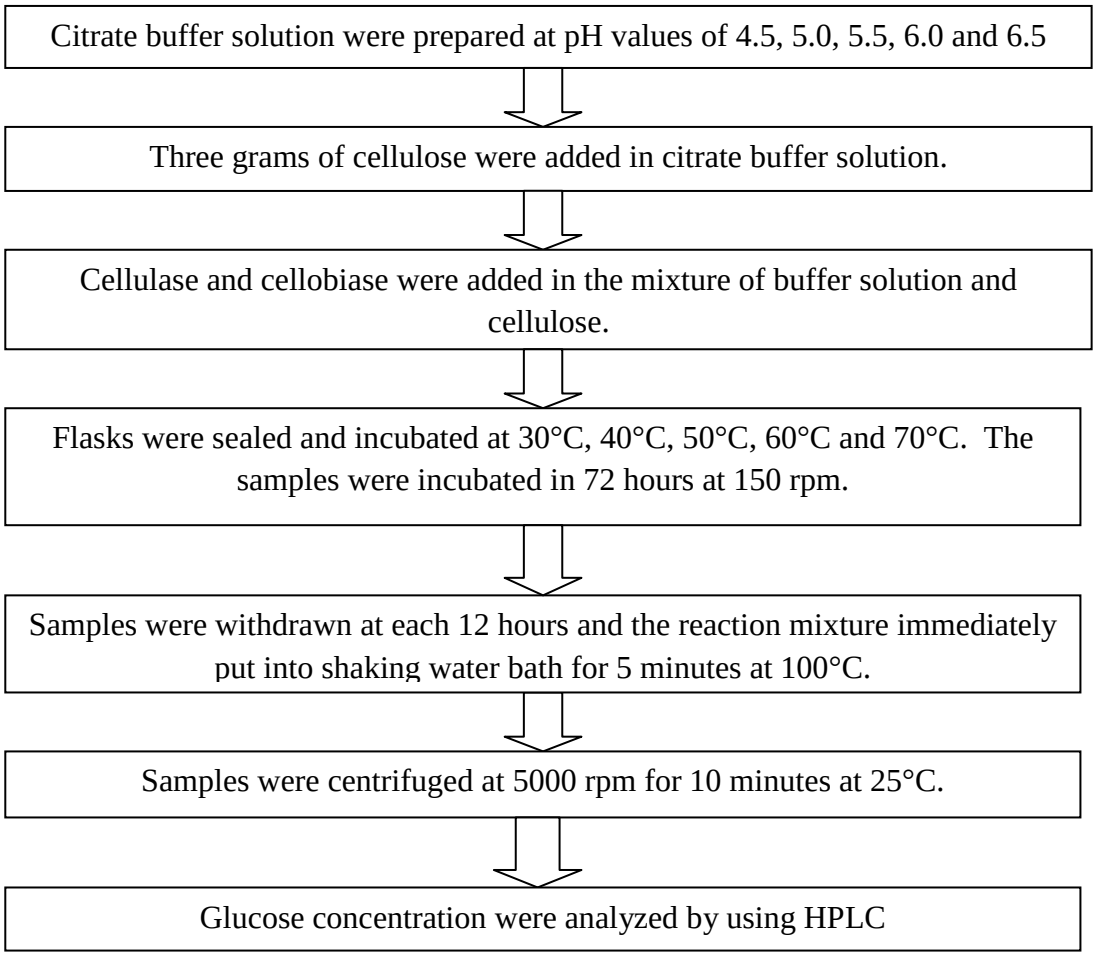

Figure 3.3 The Flow Chart of the Enzymatic Hydrolysis Method

Figure 3.3 shown the flow chart of the enzymatic hydrolysis process to produce glucose. The process was began with the sample preparation stage and end up with analyzing glucose concentration. This flow chart were used as a guideline for the experiment.

3.5 Preparation of Citrate Buffer Solution

Citrate buffer solution was prepared to control the pH values of enzymatic hydrolysis condition. The citrate buffer was prepared by adding 0.05 M of citric acid solution and 0.05 M of *tri*-sodium citrate solution. Before that, the stock solution of both solution was prepared in 1000 ml of volume.

Firstly, a 1 liter of volumetric flask was filled up with deionized water to 1 liter and then, 14.705 grams of *tri*-sodium citrate was weighed and poured it inside the flask. The solution was stirred until a powder dissolved in deionized water. For citric acid stock solution, 9.622 grams of citric acid was weighed and poured it with 1 liter of deionized water in 1 liter of volumetric flask. The solution was stirred until mixed well.

Next, in order to prepare the citrate buffer solution at the desired pH, both stock solution was mixed in 100 ml of volumetric flask and filled up the flask with deionized water to 100 ml. Thus, the desired volume for certain pH values can be conclude in the Table 3.1 .

Table 3.1 Buffer Tables for Citrate Buffer Solution (Source : Ruzin, 1999)

Desired pH	0.05 M Citric Acid	0.05 M *tri*-sodium Citrate
4.5	26.8	23.3
5.0	20.5	29.5
5.5	14.9	35.2
6.0	9.5	41.5
6.5	5.2	44.8

3.6 Enzymatic Hydrolysis Process

The microcrystalline cellulose powder was hydrolyzed or degraded to glucose by the action of enzyme that attacks the cellulose structure. Three grams of microcrystalline cellulose powder was weighed and poured it into the 250 ml Erlenmeyer flask that already contains 100 ml of citrate buffer solution. Due to the Substrate-Enzyme concentration which is in the ratio of 0.5 : 0.2 (g: ml), thus 0.8 ml of cellulase and 0.4 ml of cellobiase were pipetted into Erlenmeyer flask by using micropipette (Rajendran *et al*., 2008).

Next, the flask were sealed with aluminium foils and put it inside the incubator shaker at desired temperature (30°C, 40°C, 50°C, 60°C and 70°C) for 72 hours. The speed was controlled at 150 rpm. At the time interval of 12 hours, 5 ml of samples were withdrawn from the flask and put it into shaker water bath at 100°C for 5 minutes to prevent futher hydrolysis which any enzymes that present within the samples will deactivated (Chen *et al*., 2008).

After 5 minutes, the samples were centrifuged by refrigerated centrifuge at speed of 5000 rpm and the temperature at 25°C for 10 minutes to separate the unreacted substrate from the solution. The pellets were removed and the supernatants were collected for analyzing step to determine the amount of glucose that produced by the hydrolysis process.

3.6.1 Preparation of Sample

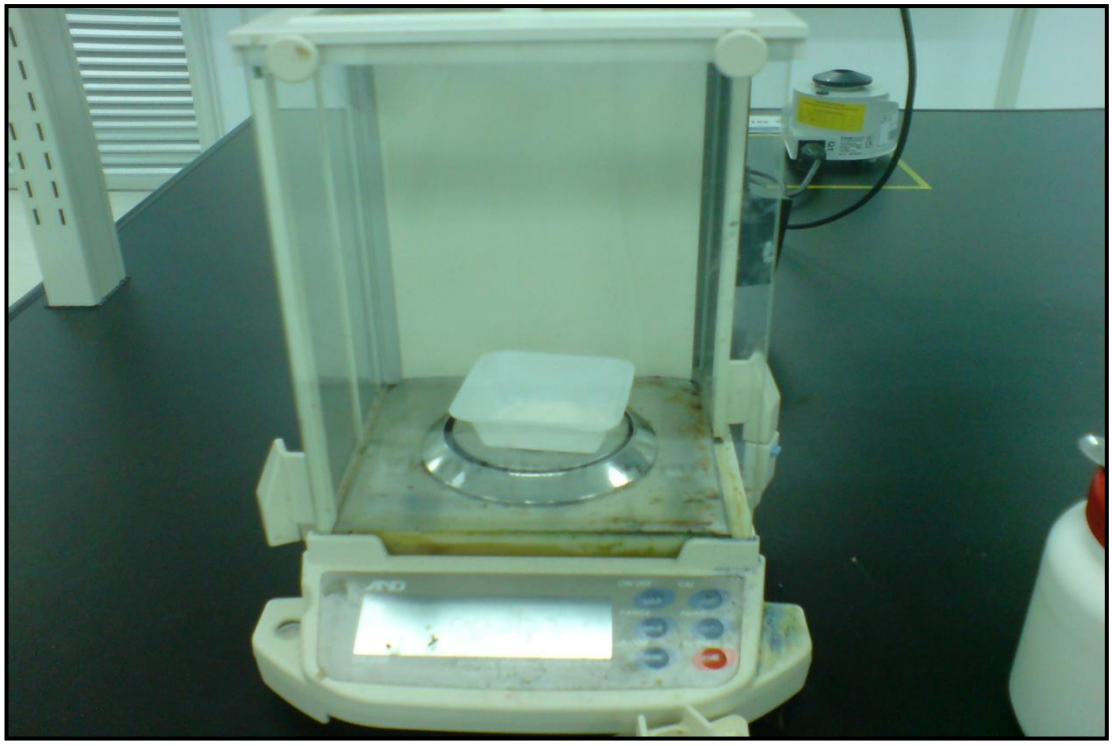

Figure 3.4 Weighing Cellulose by Using Analytical Balance

Figure 3.5 Cellulose in Five Different pH Values

Concentration of sample about 3% (w/v) was prepared by weighing three grams of cellulose powder using analytical balance in FKKSA laboratory (Figure 3.4). After that, three grams of cellulose were poured into 250 ml Erlenmeyer flask that contains 100 ml citrate buffer solution in various pH values which were illustrated in Figure 3.5. There were five Erlenmeyer flasks that contain five different pH values which are 4.5, 5.0, 5.5, 6.0 and 6.5.

3.6.2 Preparation of Enzymes

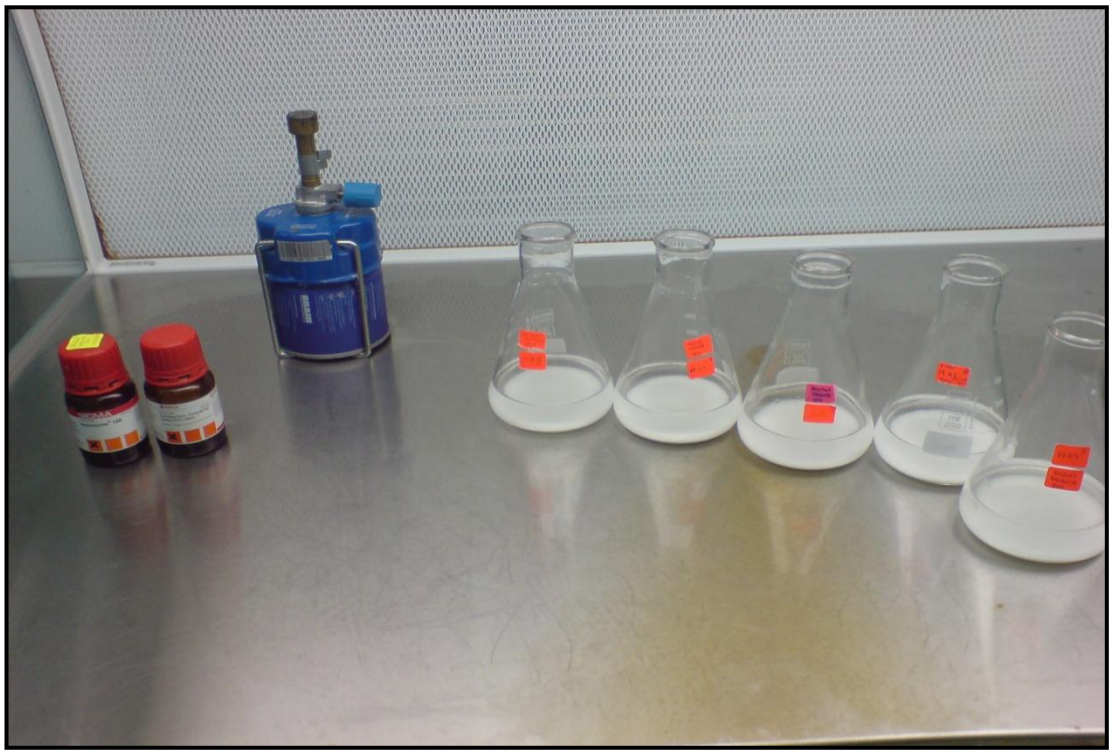

Figure 3.6 Preparation of Enzymes in Laminar Flow

Figure 3.7 The Mixture of Cellulose and Enzymes

Preparation of enzymes were made in the laminar flow to minimize contamination from the environment such dust. Furthermore, aseptic technique also used to make sure the flasks free from any contaminant which the mouth of flasks were steriled by using bunsen burner (Figure 3.6). The amount of enzymes that used in this research was based on the cellulose concentration. By the ratio of Substrate-Enzyme concentration which was in the ratio of 0.5 : 0.2 (g: ml), thus 0.8 ml of cellulase and 0.4 ml of cellobiase were pipetted into Erlenmeyer flasks by using micropipette. The mixture of cellulose and enzyme was showed in Figure 3.7. After that, as illustrated in Figure 3.8, pH values were measured by using pH meter to ensure the pH was in the desired values before incubate all these samples.

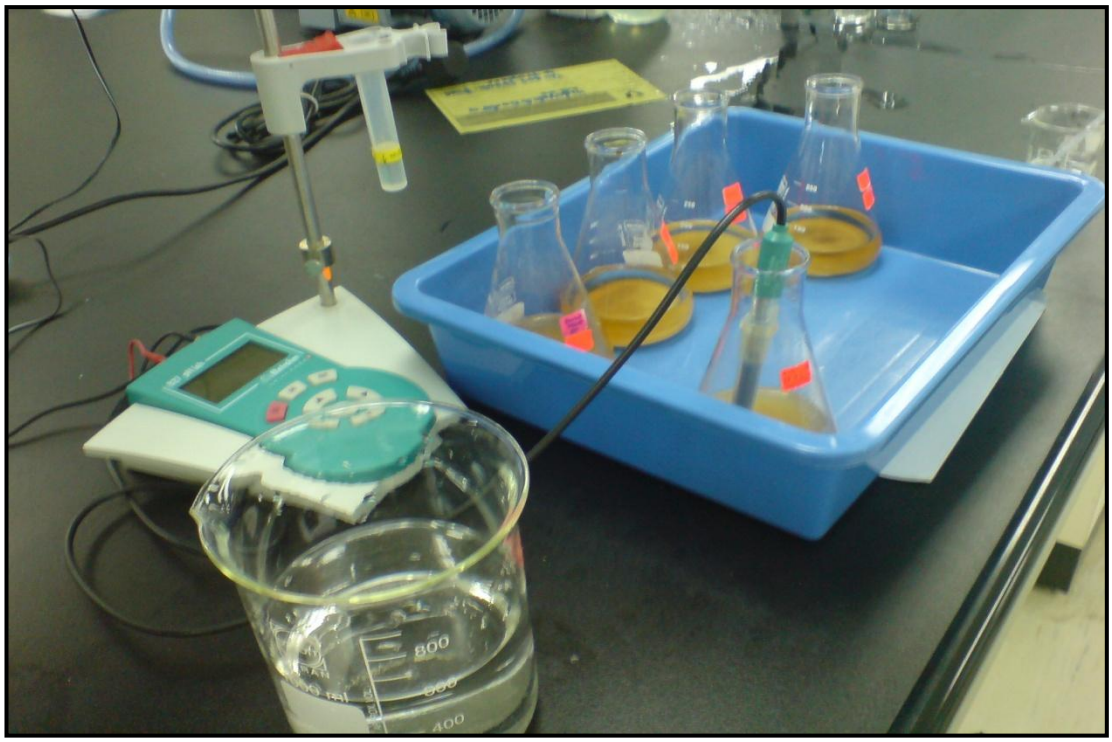

Figure 3.8 Measuring pH Values by Using pH Meter

3.6.3 Hydrolysis Process

Figure 3.9 Stackable Incubator Shaker

Hydrolysis process was ran in the incubator shaker for 72 hours which illustrated in Figure 3.9. In this incubator shaker, temperature were controlled at desired values which are 30°C, 40°C, 50°C, 60°C and 70°C. For the procedure, firstly flasks were sealed with aluminum foils then placed it in the incubator shaker. Then, speed of rotation was adjusted at 150 rpm. During hydrolysis process, 5 ml of samples were taken from the reaction mixture periodically which at interval time of 12 hours for glucose analysis. Next, the 5 ml of samples were put into shaking water bath immediately at 100°C for 5 minutes to prevent further hydrolysis which any enzymes that present within the samples will deactivated (Figure 3.10).

Figure 3.10 Shaking Water Bath

After that, the pH values of other samples that remained in the flasks were measured by using pH meter to ensure the pH is constant at desired values before the samples were placed again into the incubator shaker for continue the hydrolysis process. Figure 3.11 and Figure 3.12 shown the changes of samples color before and after hydrolysis process.

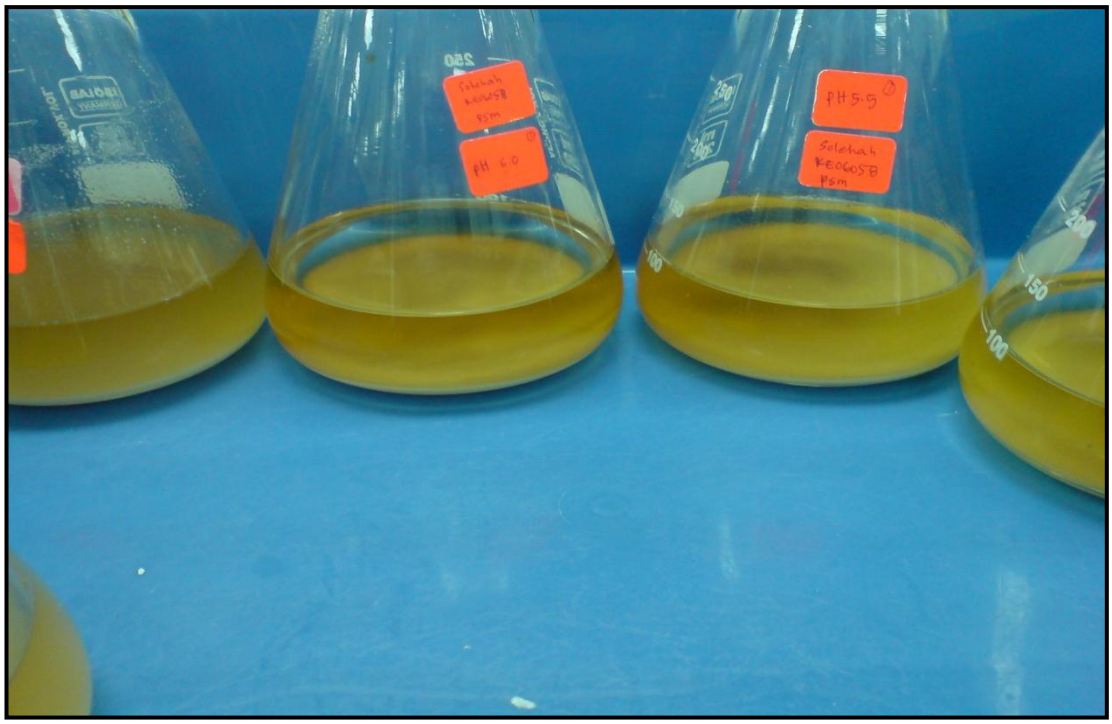

Figure 3.11 Samples Before Hydrolysis Process

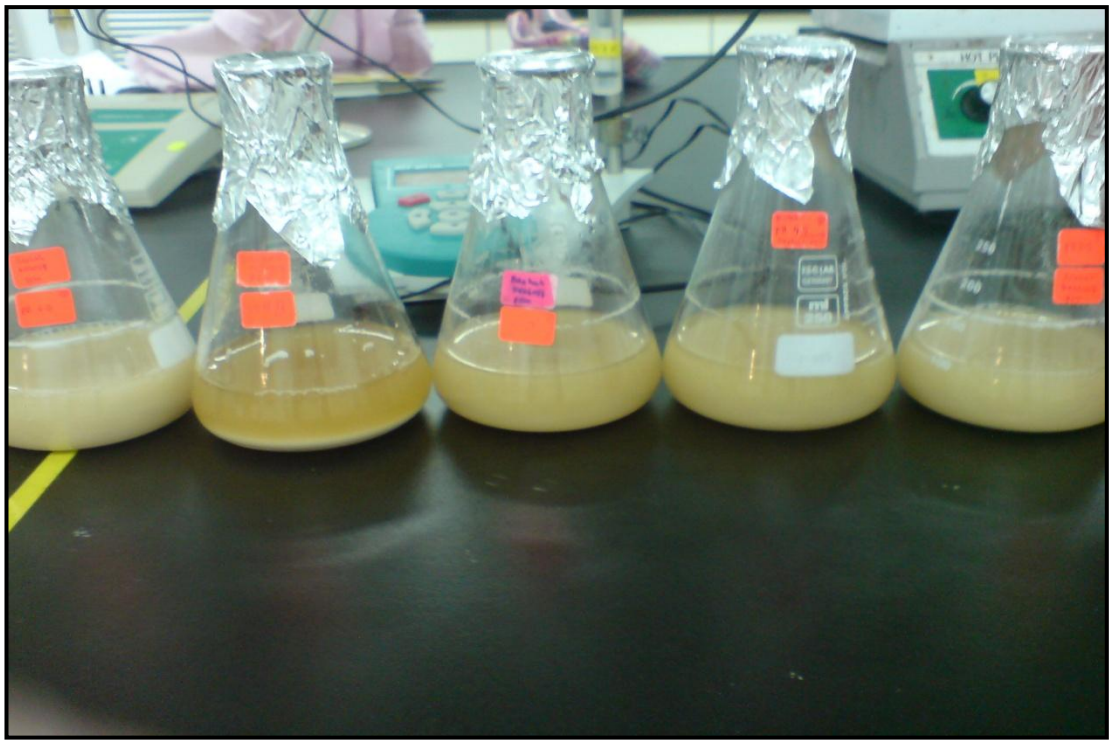

Figure 3.12 Samples After Hydrolysis Process

3.6.4 Centrifugation

Figure 3.13 Refrigerated Centrifuge

After hydrolysis process was stopped by heated the samples in boiling water bath, the 5 ml samples were then brought to be centrifuged at speed of 5000 rpm for 10 minutes at the temperature of 25°C. Figure 3.13 shown the refrigerated centrifuge that used in this research. Before that, the samples have to be filled in the 15 ml centrifuge tube and tightly closed of the lid. During placing the tube in the centrifuge, the placement of the tube must be balance because the imbalance of the placement in the centrifuge would affect the spinning of the machine. Figure 3.14 shown the samples before being centrifuged while Figure 3.15 shown the samples after centrifuged which the samples were separated well and the supernatants were clearly seen.

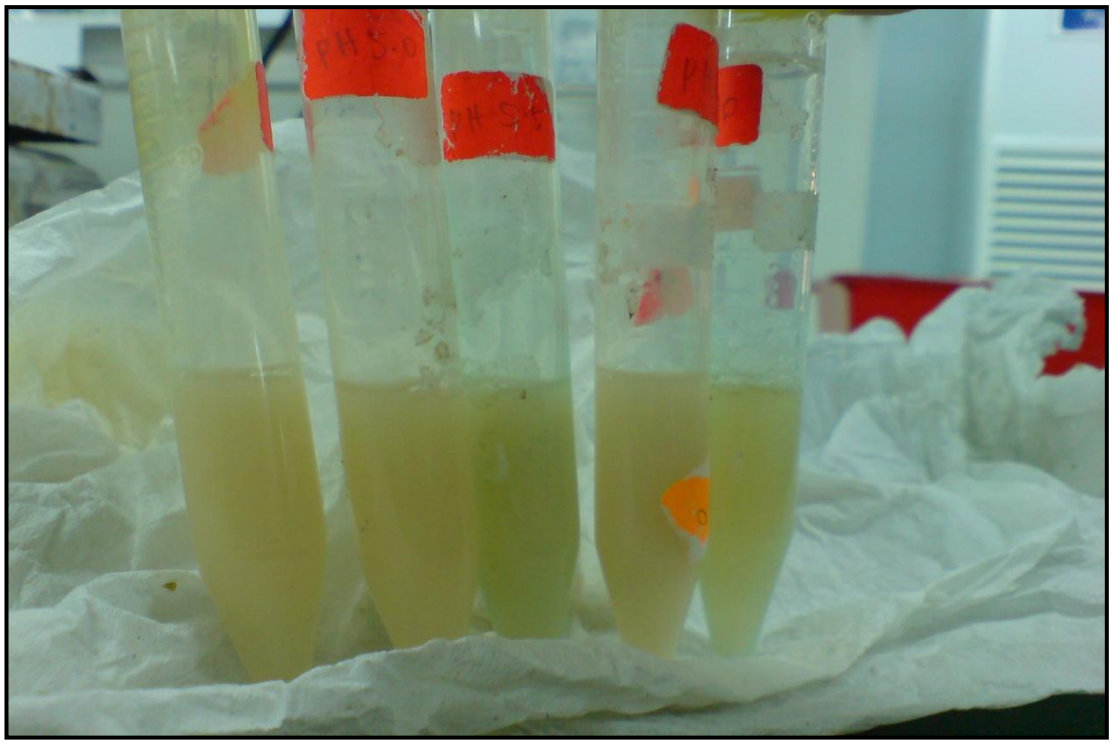

Figure 3.14 Samples Before Being Centrifuged

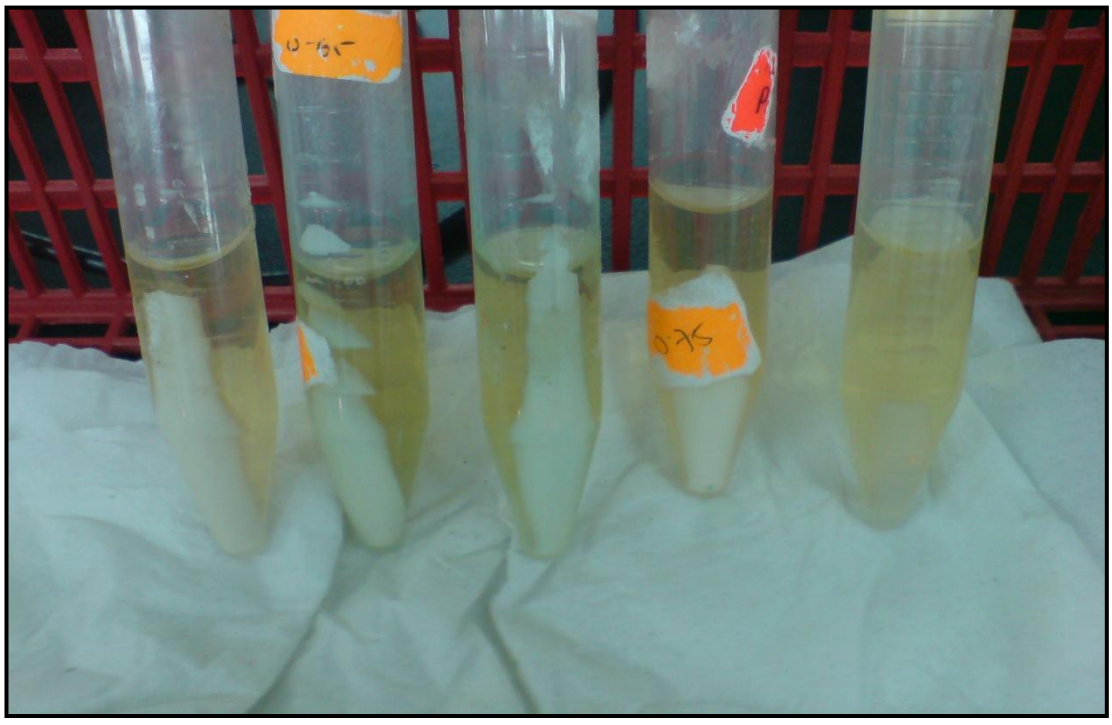

Figure 3.15 Samples After Being Centrifuged

3.7 Preparation of Standard Calibration Curve for Glucose

A standard calibration curve were prepared to determine the unknown concentration of glucose in a sample solution by comparing the values of unknown concentration to a values of standard sample of known glucose concentration. By using high performance liquid chromatography (HPLC), the unknown concentration of glucose was measured by the linear equation of standard sample of known glucose concentration which the area of the peak versus glucose concentration.

Therefore, 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml of standard glucose concentration were prepared by adding D (+)-Glucose in different amount which 0.2 grams, 0.4 grams, 0.6 grams, 0.8 grams and 1 grams, respectively and dissolved in 10 ml of distilled water. The solution was stirred until all glucose dissolved in water.

After all of the standard glucose solution were ready, 1.5 ml of each concentration was syringed out by syringe and filtered it by using 0.45µm of syringe filter before put it in a lightly capped vials. Next, all the solution is being analyze with HPLC.

3.8 Glucose Analysis by Using HPLC

Figure 3.16 High-performance Liquid Chromatography in FKKSA Lab, UMP

The HPLC system in this study was an Agilent Technologies with refractive index (RI) detector as shown in Figure 3.16. The column used is SUPELCOSIL LC-NH_2 (25cm X 4.5cm, 5µm) with the column temperature of 30^0C. Acetonitrile and water were used as a mobile phase with the ratio of 75% to 25%. The flow rate was set to 2 ml/min and injections of 10µL were made. The monitor screen will show about the retention time and the area of peak. By the sharp peak, the analysis will produce the best results.

Before start the analyzing process, the glucose was filtered by using 0.45 µm of syringe filter into 1.5 ml lightly capped vials (Figure 3.17). The acetonitrile for HPLC grade and water that used also must be filtered. This filtration was very important to avoid any large molecule enter the column due to the sensitivity of column.

Figure 3.17 Filtration of Glucose Before Being Analyzed

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Standard Calibration Curve of Glucose

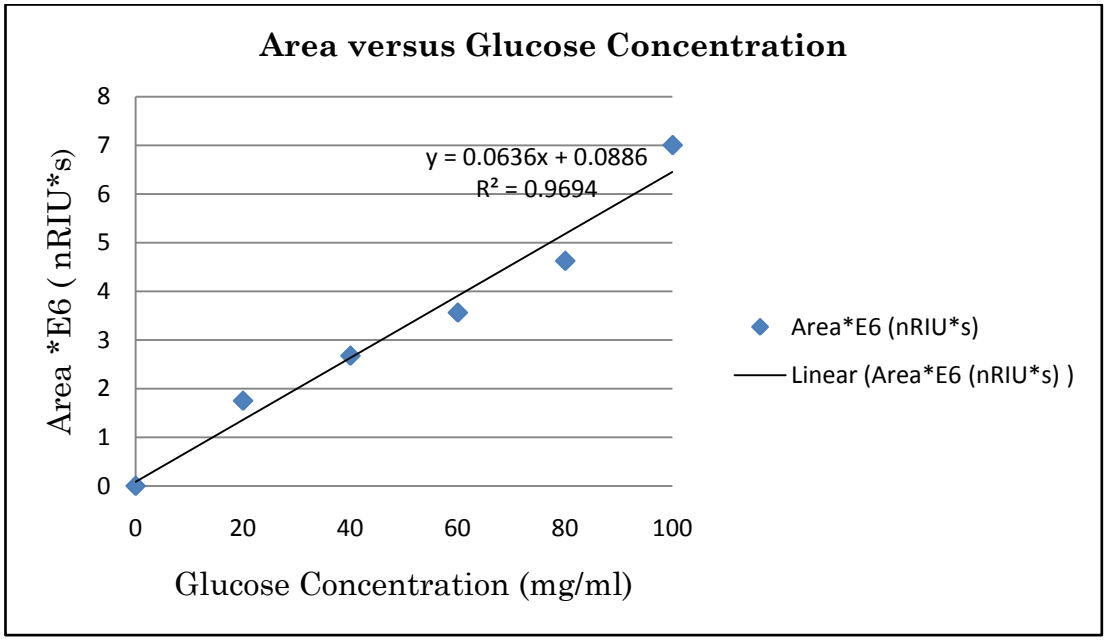

Figure 4.1 Standard Calibration Curve of Glucose

Figure 4.1 above shown the area of the chromatogram peak versus glucose concentration. This graph as a standard reference to determine glucose concentration in the sample by using the linear equation of this graph. Based on this graph, increasing the glucose concentration results in large area of chromatogram peak. Therefore, the results of area of chromatogram peak is shown in Table 4.1.

Table 4.1 Area of Standard Glucose Concentration

Glucose Concentration (mg/ml)	**Area*E6** (nRIU*s)
0	0
20	1.75099
40	2.67647
60	3.56295
80	4.62626
100	7.00709

Based on table above, at 20 mg/ml, 40 mg/ml, 60mg/ml, 80mg/ml and 100mg/ml of standard glucose concentration, the area of chromatogram peaks were 1.75099, 2.67647, 3.56295, 4.62626 and 7.00709, respectively.

4.2 Effect of pH on Enzymatic Hydrolysis

4.2.1 Effect of pH at 30°C

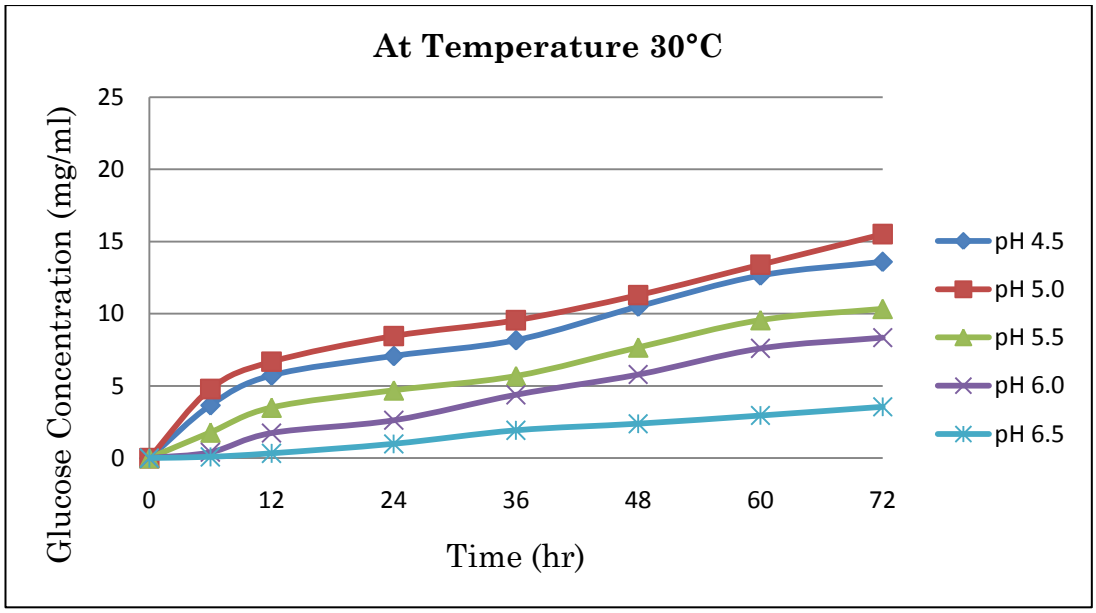

Figure 4.2 Effect of pH at 30°C

Figure 4.2 shown the effect of various pH on enzymatic hydrolysis that influences the glucose production at temperature constant 30°C. Based on this graph about glucose concentration versus time, the glucose concentration increase smoothly when time of the enzymatic hydrolysis increase. The pattern of this graph is same in all five different initial pH values that have been investigated. Therefore, the results that obtained after 72 hours shown pH 5.0 produced high glucose concentration then followed by pH 4.5, pH 5.5, pH 6.0 and pH 6.5. The amount of glucose concentration produced were 15.51 mg/ml, 13.60 mg/ml, 10.34 mg/ml, 8.35 mg/ml and 3.56 mg/ml, respectively.

Based on this results, the rate of glucose production was represented by the gradient percentage of graph for each of pH values. For pH values of 5.0, 4.5, 5.5, 6.0 and 6.5, the percent gradient of each lines were 18.09 %, 16.96 %, 13.74 %, 12.10 % and 5.21 %, respectively. It was clearly seen that the rate of glucose production at pH 5.0 was higher than others pH values which resulting in high

glucose concentration at 72 hours. Therefore, it can concluded that as the gradient percentage increase, the glucose production also increase.

4.2.2 Effect of pH at 40°C

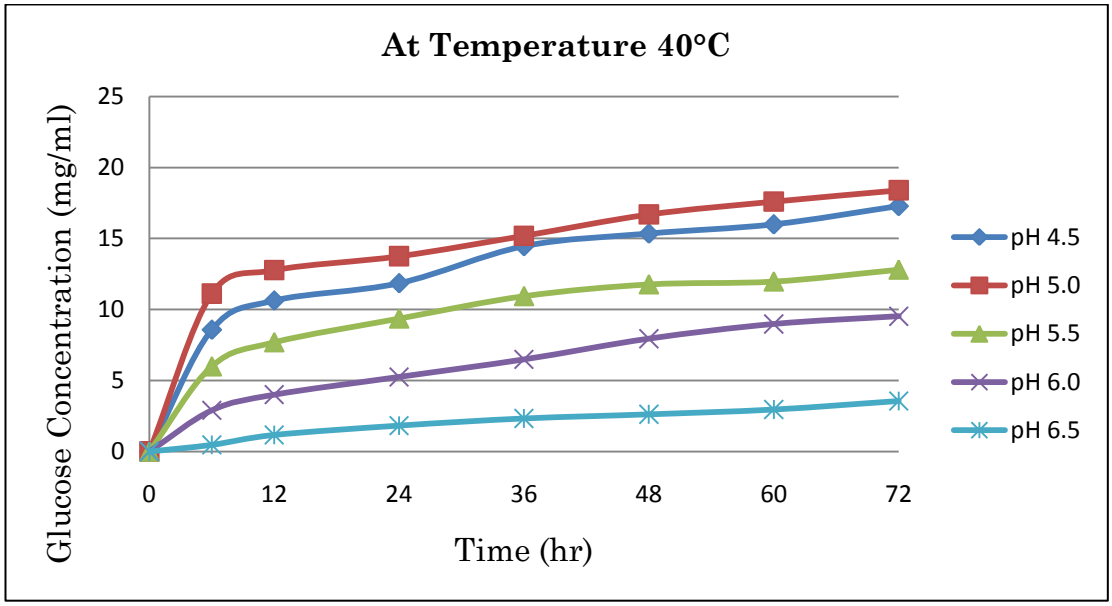

Figure 4.3 Effect of pH at 40°C

The results for the effect of pH on enzymatic hydrolysis at temperature constant 40°C was illustrated as Figure 4.3. Based on the results, glucose concentration increase rapidly at 6 hours then continuous increase smoothly when the time of enzymatic hydrolysis increase. Same goes like the pattern at temperature constant 30°C, pH 5.0 produced the highest glucose concentration and followed by other pH values. Nevertheless, the amount of glucose concentration after 72 hours at pH 5.0, 4.5, 5.5, 6.0 and 6.5 in this graph was higher than previous graph which were 18.39 mg/ml, 17.28 mg/ml, 12.81 mg/ml, 9.52 mg/ml and 3.55 mg/ml, respectively.

In this graph, the gradient percentage of graph for pH 5.0 was less than pH 4.5 which are 18.17 % less than 18.44 %. It was shown that rate of glucose production at pH 4.5 was higher than pH 5.0. However, the amount of glucose concentration at pH 5.0 still higher than pH 4.5 because the rate of reaction at the beginning stage gives the effect to the glucose production at 72 hours. For pH 4.5,

the rate of reaction starts to increase at 36 hours and by the end of 72 hours, the amount of glucose production was quite closed with the amount of glucose at pH 5.0. For the pH values of 5.5, 6.0 and 6.5, the gradient percentage were 14.05 %, 11.96 % and 4.61 %, respectively.

4.2.3 Effect of pH at 50°C

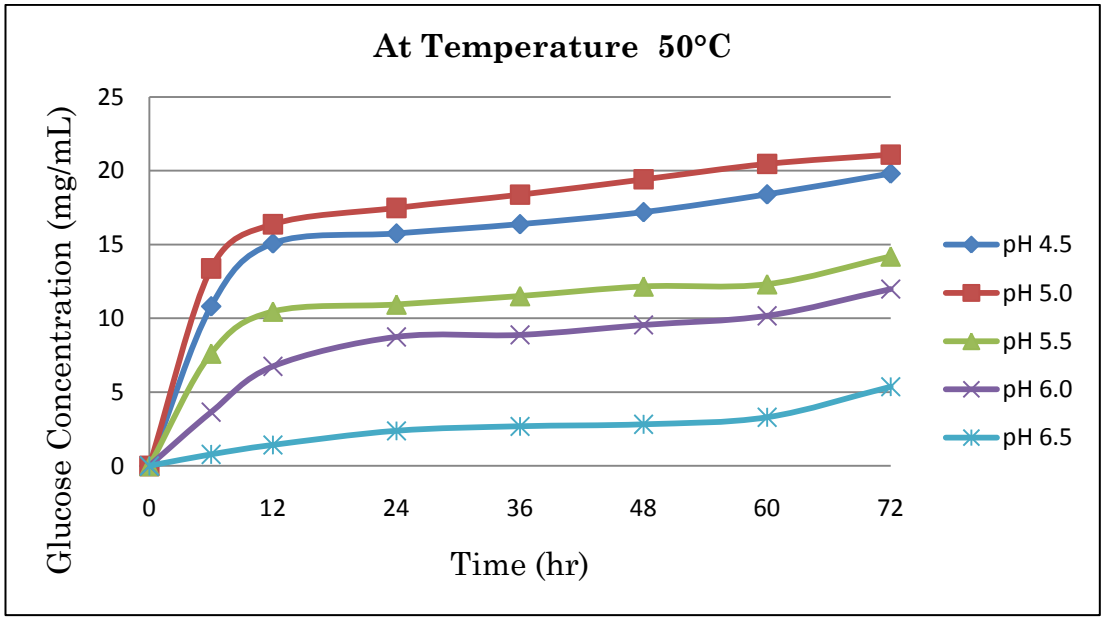

Figure 4.4 Effect of pH at 50°C

Figure 4.4 above shown the effect of pH on enzymatic hydrolysis at temperature constant 50°C. It clearly seen that glucose concentration increase sharply at 6 hours and continuous increase smoothly when the time of enzymatic hydrolysis increase. The value of pH 5.0 shown the highest glucose concentration which was 21.09 mg/ml. It was followed by pH 4.5, 5.5, 6.0 and 6.5 which the amount of glucose concentration were 19.79 mg/ml, 14.20 mg/ml, 11.98 mg/ml and 5.36 mg/ml, respectively.

Due to pH 5.0 was the highest glucose concentration, it means that its gradient percentage also was the highest compare the other pH values. Therefore, the gradient percentage of pH 5.0, 4.5, 5.5, 6.0 and 6.5 were 19.79 %, 18.90 %, 13.25 %, 13.24 % and 6.02 %, respectively.

4.2.4 Effect of pH at 60°C

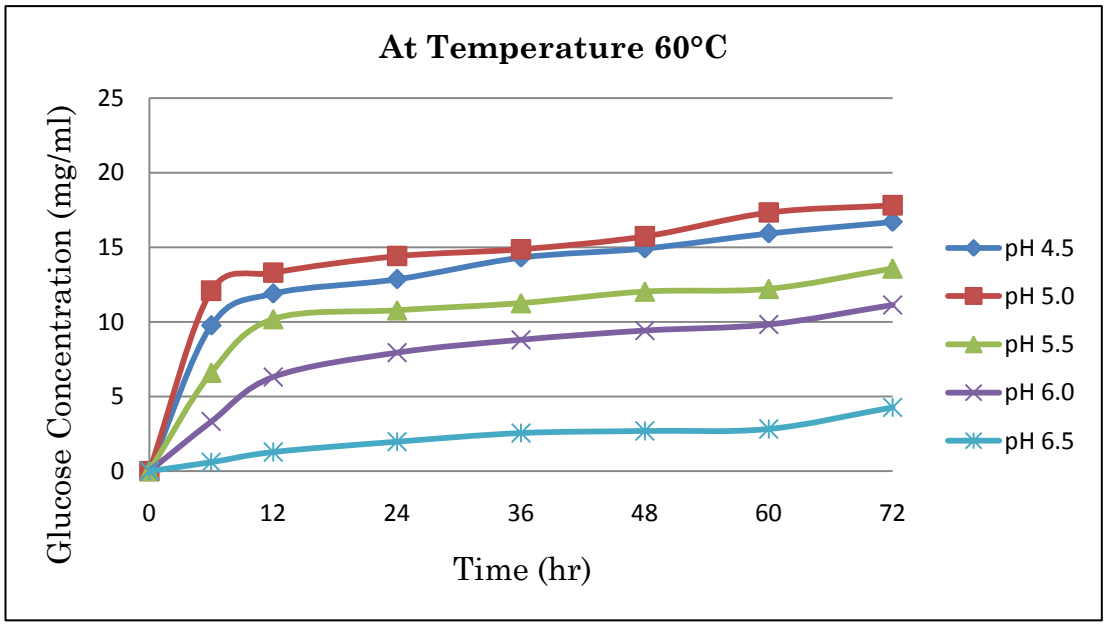

Figure 4.5 Effect of pH at 60°C

Figure 4.5 shown the effect of pH on enzymatic hydrolysis at temperature constant 60°C. Based on this graph about glucose concentration versus time, the glucose concentration increase smoothly when time of the enzymatic hydrolysis increase. The maximum activity of enzyme were constant at pH 5.0 which same goes like the other results but the amount of glucose concentration was 17.81 mg/ml. While the amount of glucose concentration at pH 4.5, 5.5, 6.0 and 6.5 were 16.69 mg/ml, 13.57 mg/ml, 11.12 mg/ml and 4.26 mg/ml, respectively.

In this graph, the highest gradient percentage at pH 4.5 which 16.42 % followed by pH 5.0, 5.5, 6.0 and 6.5 which the percentage were 16.34 %, 13.32 %, 12.78 % and 5.02 %, respectively. This case was same goes like the effect of pH at temperature constant of 40°C which pH 4.5 was the highest gradient percentage but its amount of glucose production is low than pH 5.0. It because at 36 hours, the rate of reaction started to increase and by the end of 72 hours, the amount of glucose production was slightly different with the amount of glucose at pH 5.0.

4.2.5 Effect of pH at 70°C

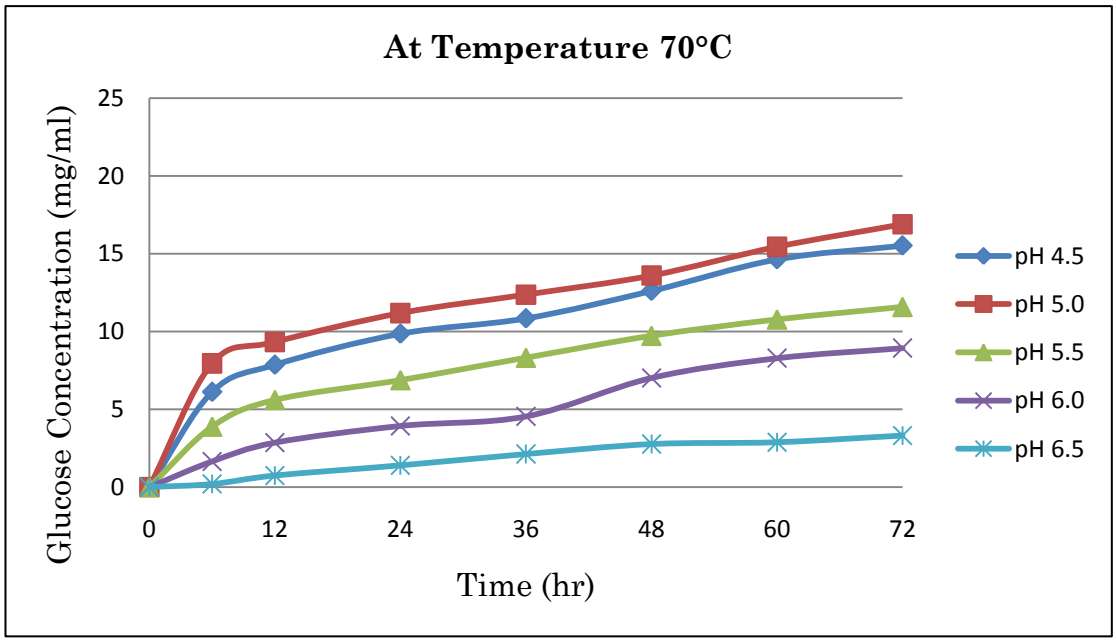

Figure 4.6 Effect of pH at 70°C

The effect of pH on enzymatic hydrolysis at temperature constant 70°C is illustrated as Figure 4.6. Based on that figure, glucose concentration increase smoothly when the time of enzymatic hydrolysis increase. The amount of glucose concentration at pH 5.0, 4.5, 5.5, 6.0 and 6.5 were 16.88 mg/ml, 15.52 mg/ml, 11.57 mg/ml, 8.93 mg/ml and 3.31 mg/ml, respectively. The value of pH 5.0 produced the highest glucose concentration again same goes like in the other graphs.

Based on this graph, pH 5.0 was the highest rate of glucose production due to it high gradient percentage which about 18.06 % followed by pH 4.5, 5.5, 6.0 and 6.5 and the percentage were 17.87 %, 13.92 %, 12.01 % and 4.8 %, respectively.

4.2.6 Combination Effect of pH on Enzymatic Hydrolysis

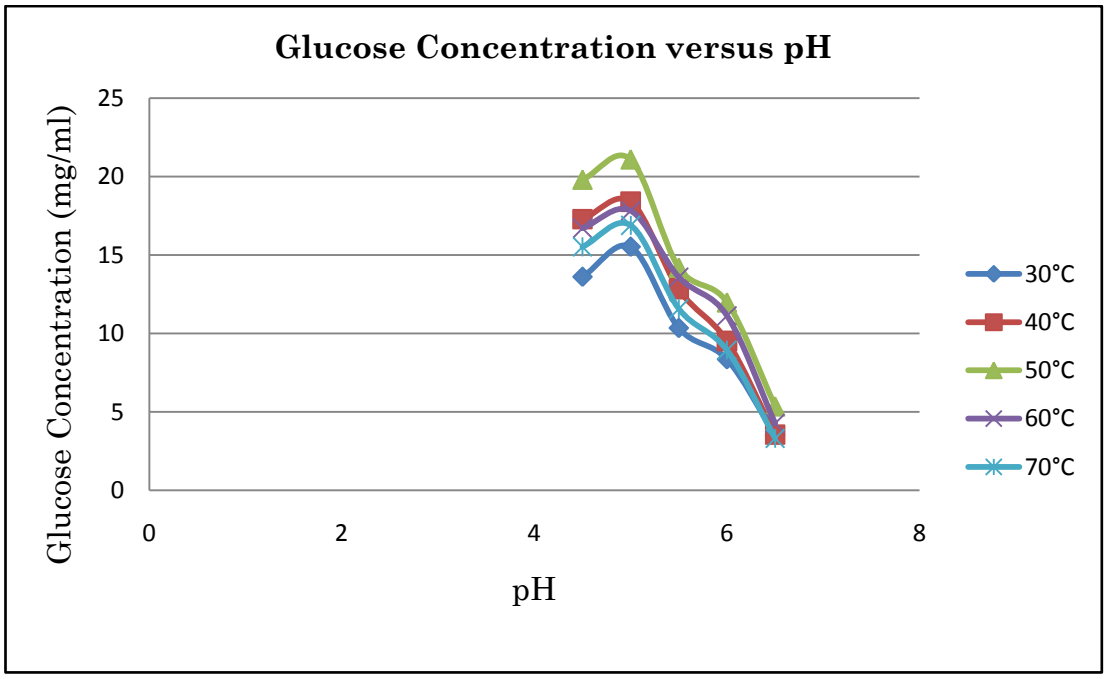

Figure 4.7 Graph of Glucose Concentration Versus pH

Figure 4.7 above shown the effect of various pH on enzymatic hydrolysis that influences glucose production. Based on this graph about glucose concentration versus pH value, the glucose concentration increase slightly from pH 4.5 to pH 5.0 then decrease slightly from pH 5.0 to pH 6.5. At constant temperature, it was clearly seen that pH 5.0 produced the highest glucose concentration compare to other pH values that have been investigated.

Therefore, it can be concluded that enzyme was achieved their maximum activity to hydrolyze cellulose at pH 5.0 and results in high glucose production. During this condition, the three-dimensional shape of enzyme was maintain because the total net charge of the enzyme that contribute to its functional shape was not altered by the charge ion of pH. Therefore, the enzyme can bind to the cellulose at it's active site exactly. As a results, the rate of hydrolysis reaction of enzyme to hydrolyze the cellulose to glucose was increased thus glucose production also increase.

The shift of pH values above or below pH 5.0 was followed by the decreasing of glucose production. From this results, it can be considered that the total net charge of enzyme is disrupted resulting in changes the three-dimensional shape of enzyme. Hence, the enzyme becomes denatured and unable to catalyze chemical reaction or lack of their action. Therefore, the enzyme cannot bind to the cellulose at it's active site causes the hydrolysis process to hydrolyze cellulose to glucose becomes slow and the process might become terminate.

4.3 Effect of Temperature on Enzymatic Hydrolysis

4.3.1 Effect of Temperature at pH 4.5

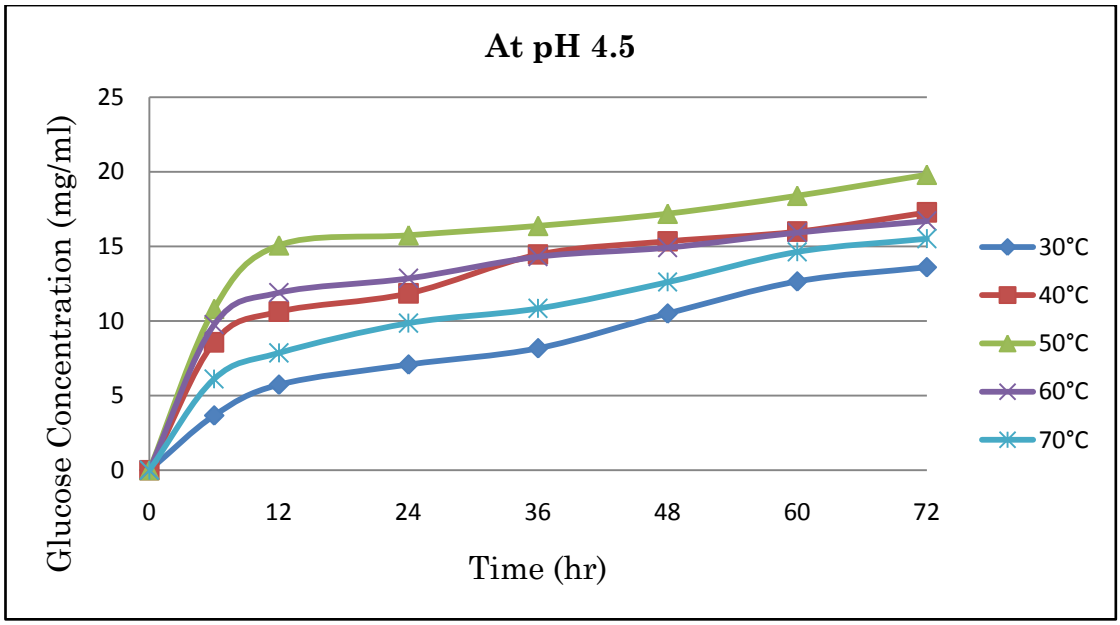

Figure 4.8 Effect of Temperature at pH 4.5

Figure 4.8 shown the effect of various temperature on enzymatic hydrolysis that influences the glucose production at pH constant of 4.5. Based on this graph about glucose concentration versus time, the glucose concentration slightly increase when time of the enzymatic hydrolysis increase. Therefore, the results that obtained after 72 hours shown temperature of 50°C produced high glucose concentration then followed by temperature of 40°C, 60°C, 70°C and 30°C. The amount of glucose concentration produced were 19.79 mg/ml, 17.28 mg/ml, 16.69 mg/ml, 15.52 mg/ml and 13.60 mg/ml, respectively. For the 40°C and 60°C, the amount of glucose was slightly different.

4.3.2 Effect of Temperature at pH 5.0

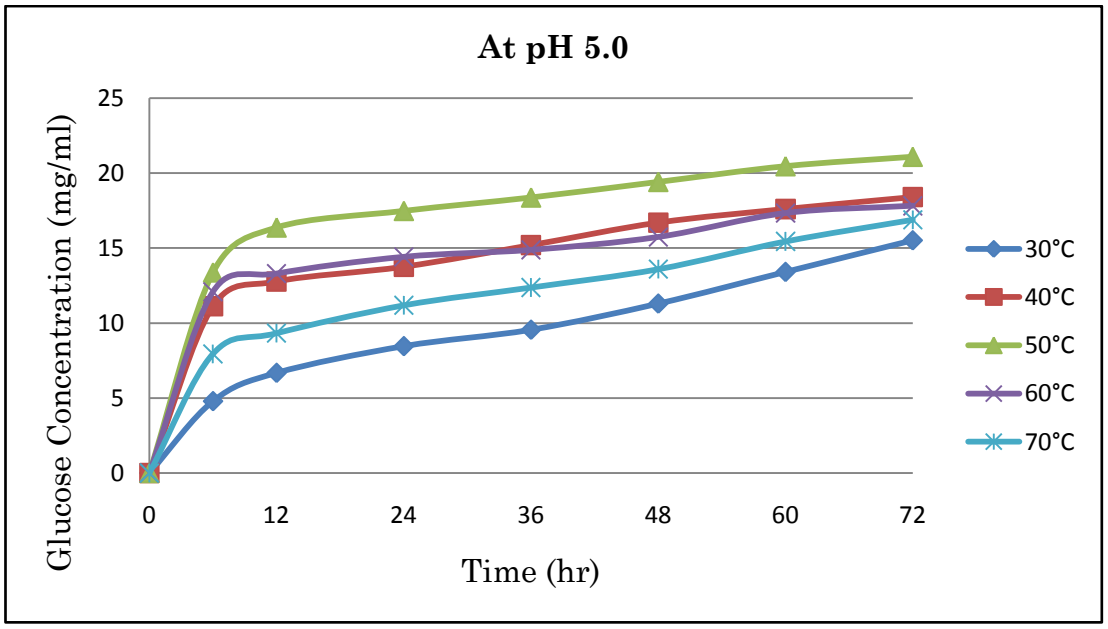

Figure 4.9 Effect of Temperature at pH 5.0

Figure 4.9 shown the effect of various temperature on enzymatic hydrolysis that influences the glucose production at pH constant of 5.0. Based on this graph about glucose concentration versus time, the glucose concentration slightly increase when time of the enzymatic hydrolysis increase. Therefore, the results that obtained after 72 hours shown temperature of 50°C produced high glucose concentration then followed by temperature of 40°C, 60°C, 70°C and 30°C. The amount of glucose concentration produced were 21.09 mg/ml, 18.39 mg/ml, 17.81 mg/ml, 16.88 mg/ml and 15.51 mg/ml, respectively. Same goes like previous graph, the amount of glucose at 40°C and 60°C were slightly different.

4.3.3 Effect of Temperature at pH 5.5

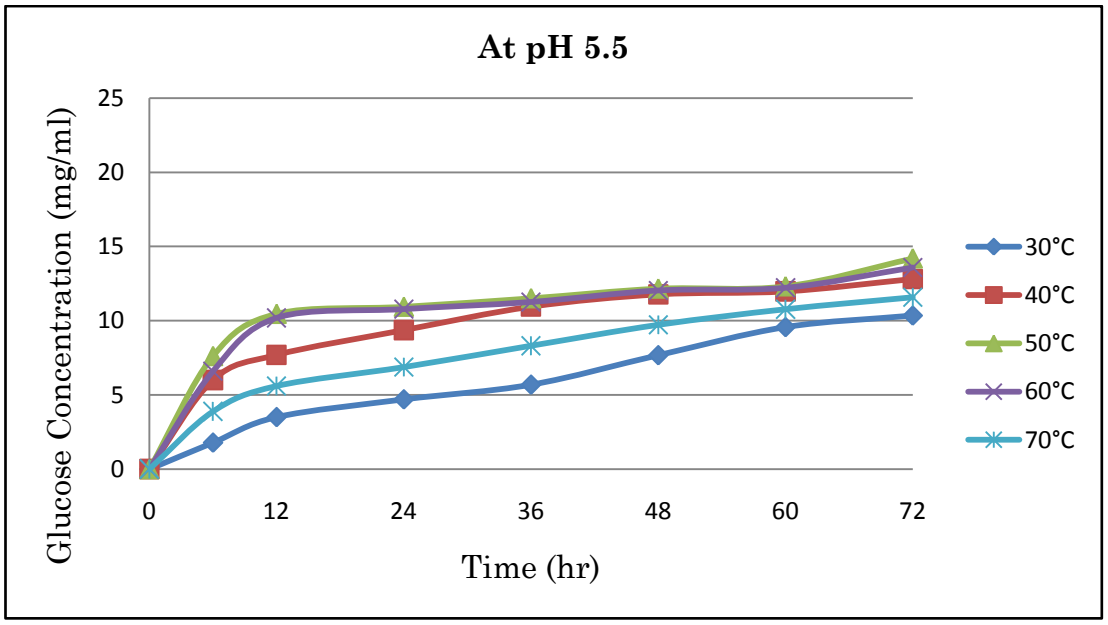

Figure 4.10 Effect of Temperature at pH 5.5

The effect of temperature on enzymatic hydrolysis at pH constant 5.5 was illustrated as Figure 4.10. Based on that figure, glucose concentration slightly increase when the time of enzymatic hydrolysis increase. However, at temperature 50°C and 60°C, the rate of reaction began to constant at 12 hours due to its constant line of the graph. The amount of glucose concentration at 30°C, 40°C, 50°C, 60°C and 70°C were 10.34 mg/ml, 12.80 mg/ml, 14.19 mg/ml, 13.57 mg/ml and 11.57 mg/ml, respectively. In this graph, the glucose concentration at 40°C, 50°C and 60°C were slightly different at 72 hours.

4.3.4 Effect of Temperature at pH 6.0

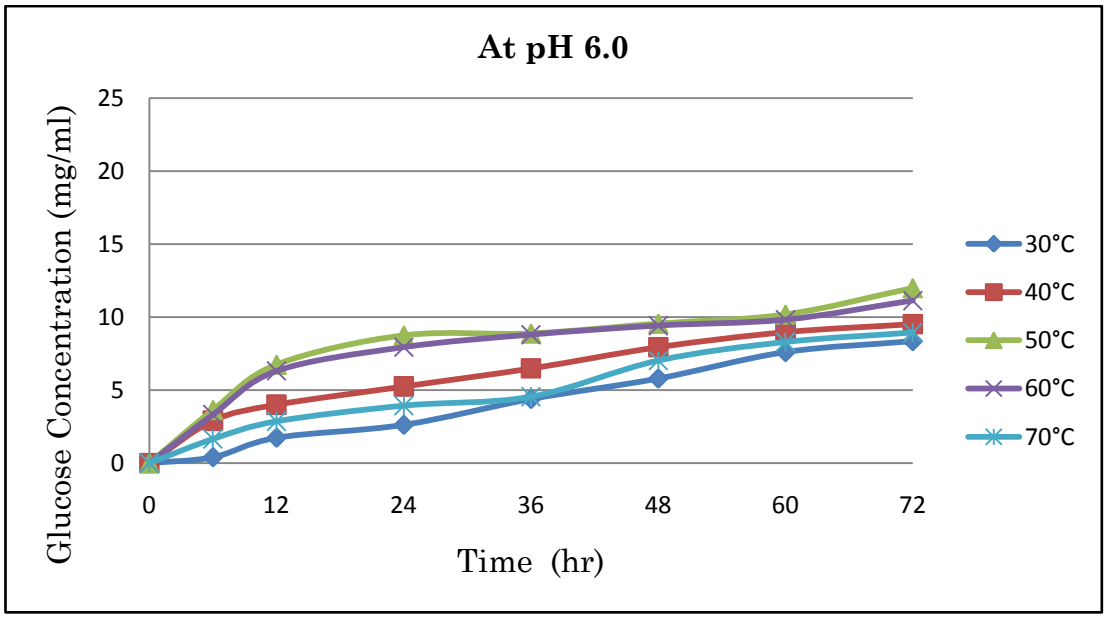

Figure 4.11 Effect of Temperature at pH 6.0

Figure 4.11 above shown the effect of temperature on enzymatic hydrolysis at pH constant 6.0. It clearly seen that glucose concentration slightly increase when the time of enzymatic hydrolysis increase. Based on this graph, the glucose concentration at all the various temperature that had been investigated were slightly different because the enzyme was began to lose of its function due to the effect of temperature and pH on its structure. Therefore, the amount of glucose concentration at 30°C, 40°C, 50°C,60°C and 70°C were 8.35 mg/ml, 9.52 mg/ml, 11.98 mg/ml, 11.12 mg/ml and 8.93 mg/ml, respectively.

4.3.5 Effect of Temperature at pH 6.5

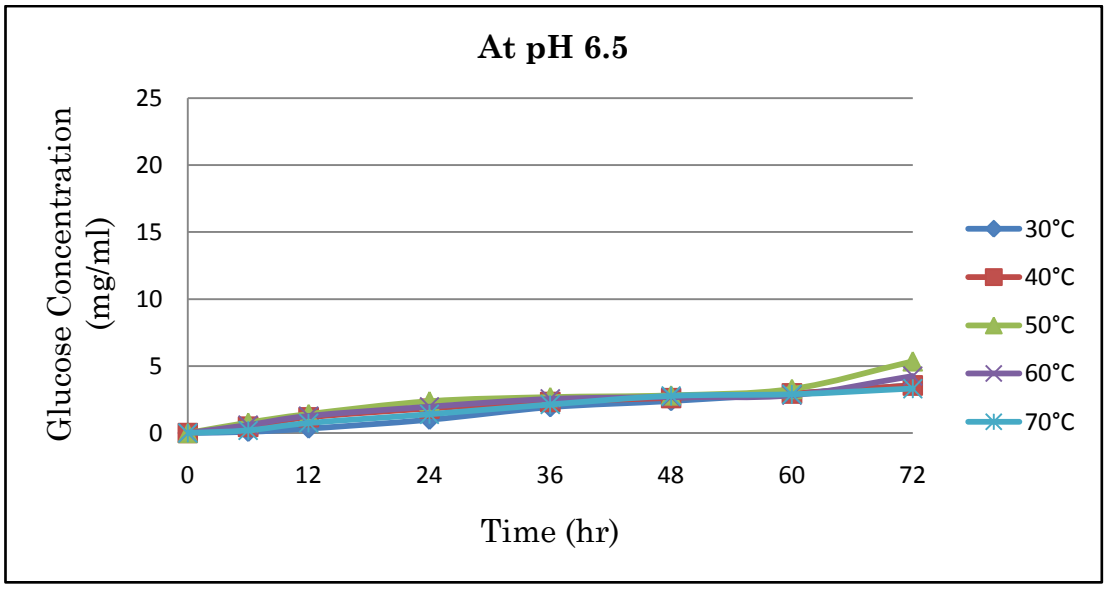

Figure 4.12 Effect of Temperature at pH 6.5

Figure 4.12 shown the effect of various temperature on enzymatic hydrolysis that influences the glucose production at pH constant of 6.5. Based on this graph, the glucose concentration at all various temperature that had been investigated was very closed each other. During this condition, some enzyme might be denatured and results in low glucose production. Therefore, the amount of glucose concentration at 30°C, 40°C, 50°C,60°C and 70°C were 3.56 mg/ml, 3.55 mg/ml, 5.35 mg/ml, 4.26 mg/ml and 3.31 mg/ml, respectively.

4.3.6 Combination Effect of Temperature on Enzymatic Hydrolysis

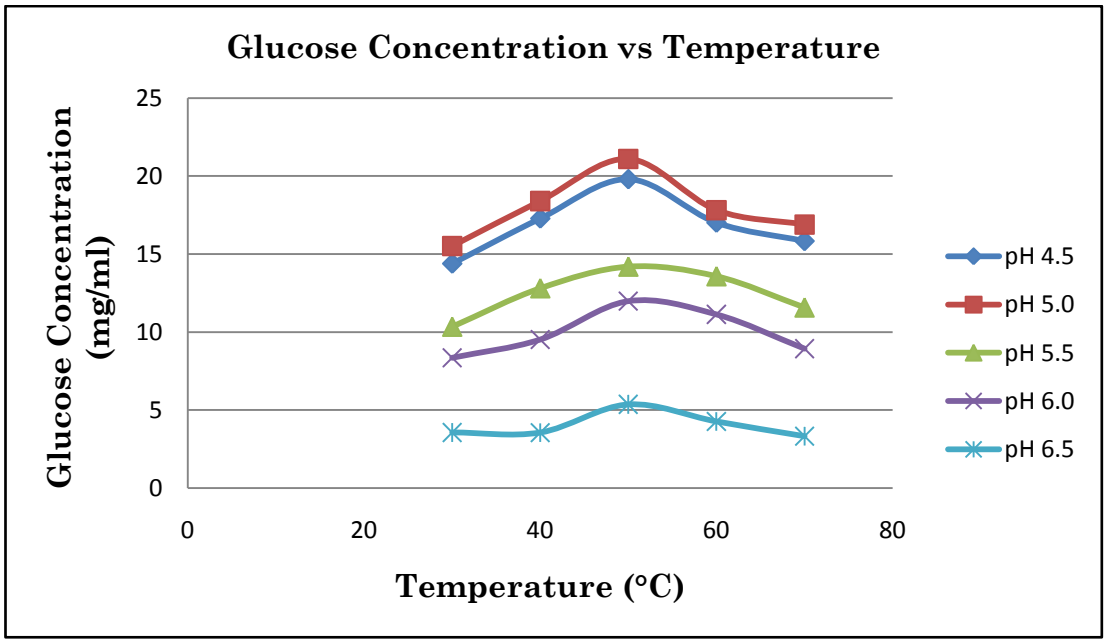

Figure 4.13 Graph of Glucose Concentration Versus Temperature

Figure 4.13 above shown the effect of various temperature on enzymatic hydrolysis that influences glucose production. Based on this graph about glucose concentration versus temperature, the glucose concentration increase slightly from 30°C to 50°C then decrease slightly from 50°C to 70°C. At constant pH values, it was clearly seen that temperature of 50°C produced the highest glucose concentration compare to other temperature that have been investigated.

Therefore, 50°C was the maximum activity of enzyme which it works best. A higher temperature generally results in an increase in enzyme activity because as temperature increases, molecular motion increases resulting in more molecular collisions between enzyme and cellulose. As a results, more cellulose was hydrolyzed to produce glucose. However, when the temperature rises above their maximum value, the heat will denature the enzyme which causing it to lose its three-dimensional functional shape by denaturing its hydrogen bonds. On the other hand, for temperature below their maximum value or cold temperature results in slow down enzyme activity by decreasing molecular motion. As a results, the hydrolysis reaction of cellulose to glucose was slowed causes low glucose production.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

As a conclusion, the maximum glucose production at temperature and pH were 50°C and 5.0, respectively which the amount of glucose produced was 21.09 mg/ml. While the minimum glucose production at temperature and pH were 30°C and 6.5, respectively which the amount of glucose produced was 3.55 mg/ml. Therefore, it can be concluded that at 50°C and pH 5.0, enzyme was in the most stable condition results in high reaction rate because the system consists of enough energy to collide between enzyme and cellulose besides the active site of enzyme in the best shape thus enzyme can bind to the cellulose at active site exactly.

By increasing the temperature above the maximum value can cause the enzyme denature resulting in termination of glucose production while by decreasing the temperature below the maximum value causing the enzyme have not enough energy to collide with the celloluse. As a results, glucose production becomes decrease. On the other hand, by increasing and decreasing pH values from maximum value can cause the total net charge of enzyme is disrupted results in lose of its three-dimensional functioanl shape. Therefore, enzyme cannot bind at it's active site.

Based on that results, it can be concluded that temperature and pH influenced the enzymatic hydrolysis which by changes temperature above or below the maximum value can reduce the glucose production. It is because enzyme is very

sensitive with their environment condition and due to it's specificty properties, all the requirement for complementary in the configuration of cellulose and enzyme must be considered seriously.

5.2 Recommendations

As a recommendation for future research, Design Expert Software should be use for modelling and analyzing problems in which a response of interest is influenced by several variables and the objective is to optimize this response by collection of mathematical and statistical techniques. Therefore, the optimum value of temperature and pH for the hydrolysis process can determine clearly by the figure shown from the software.

Furthermore, enzyme activity should be calculated to determine the amount of cellulose that has been hydrolyzed per unit time by using Michaelis-Menten Equation. Based on this equation, it will show the rate of reaction of enzyme to hydrolyze the cellulose for glucose production which the substrate and enzyme concentration is plays an important role. Actually, substrate and enzyme concentration also becomes factor that affect the enzymatic hydrolysis process. For further interesting research, continue this research by fermentation process for bio-ethanol production because bio-ethanol becomes alternative fuel source which is emission less results in reduce environmental pollution.

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APPENDICES

Appendix A

Calculation for 0.05 M Citrate Buffer Preparation :

$$M = \frac{m \times 1000}{V \times MW}$$

M = Molarity (mol/L)
m = Mass (gram)
V = Volume (mL)
MW = Molecular weight (g/mol)

For citric acid stock solution :

$$m = \frac{(0.05 \text{ mol/L})\ (1000 \text{ mL})(192.43 \text{ g/mol})}{1000 \text{ mL/L}}$$

m = 9.6215 grams of citric acid.

For *tri*-sodium citrate stock solution :

$$m = \frac{(0.05 \text{ mol/L})(1000 \text{ mL})(294.10 \text{ g/mol})}{1000 \text{ mL/L}}$$

m = 14.705 grams of *tri*-sodium citrate.

Therefore, 9.6215 grams of citric acid was needed for 1000 ml of citric acid stock solution while 14.705 grams of *tri*-sodium citrate was needed for 1000 ml of *tri*-sodium citrate stock solution.

Appendix B

Data for glucose concentration from HPLC at constant temperature :

a) For temperature constant 30°C :

pH Values	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
4.5	0	0	0
	6	3.663836478	0.32162
	12	5.728380503	0.452925
	24	7.07922956	0.538839
	36	8.171792453	0.608326
	48	10.49971698	0.756382
	60	12.65150943	0.893236
	72	13.60253145	0.953721
5.0	0	0	0
	6	4.787861635	0.393108
	12	6.675345912	0.513152
	24	8.456619497	0.626441
	36	9.550613208	0.696019
	48	11.29331761	0.806855
	60	13.40606918	0.941226
	72	15.51455975	1.075326
5.5	0	0	0
	6	1.782845912	0.201989
	12	3.502783019	0.311377
	24	4.696352201	0.387288
	36	5.68672956	0.450276
	48	7.669528302	0.576382
	60	9.5625	0.696775
	72	10.34207547	0.746356
6.0	0	0	0
	6	0.400660377	0.114082

	12	1.735172956	0.198957
	24	2.618569182	0.255141
	36	4.388742138	0.367724
	48	5.790172956	0.456855
	60	7.601745283	0.572071
	72	8.351289308	0.619742
6.5	0	0	0
	6	0.086194969	0.094082
	12	0.328160377	0.109471
	24	0.989072327	0.151505
	36	1.925141509	0.211039
	48	2.385518868	0.240319
	60	2.950959119	0.276281
	72	3.557059748	0.314829

b) For temperature constant 40°C :

pH Values	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
4.5	0	0	0
	6	8.572169811	0.63379
	12	10.62798742	0.76454
	24	11.85110063	0.84233
	36	14.45628931	1.00802
	48	15.34496855	1.06454
	60	15.99355346	1.10579
	72	17.28050314	1.18764
5.0	0	0	0
	6	11.11507862	0.795519
	12	12.76907233	0.900713
	24	13.75207547	0.963232
	36	15.19342767	1.054902
	48	16.69303459	1.150277
	60	17.60092767	1.208019
	72	18.39643082	1.258613
5.5	0	0	0
	6	6.001179245	0.470275
	12	7.697421384	0.578156
	24	9.365833333	0.684267
	36	10.94413522	0.784647
	48	11.77234277	0.837321
	60	11.97451258	0.850179
	72	12.80803459	0.903191
6.0	0	0	0
	6	2.900158805	0.2730501
	12	3.998268868	0.3428899
	24	5.24908805	0.422442
	36	6.482311321	0.500875

	48	7.94163522	0.593688
	60	8.972641509	0.65926
	72	9.520283019	0.69409
6.5	0	0	0
	6	0.471509434	0.118588
	12	1.166855346	0.162812
	24	1.82495283	0.204667
	36	2.324261006	0.236423
	48	2.614292453	0.254869
	60	2.952751572	0.276395
	72	3.551666667	0.314486

c) For temperature constant 50°C:

pH Values	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
4.5	0	0	0
	6	10.81053459	0.77615
	12	15.0677673	1.04691
	24	15.74245283	1.08982
	36	16.37201258	1.12986
	48	17.18726415	1.18171
	60	18.38977987	1.25819
	72	19.79984277	1.34787
5.0	0	0	0
	6	13.37798742	0.93944
	12	16.37342767	1.12995
	24	17.47940252	1.20029
	36	18.37468553	1.25723
	48	19.41289308	1.32326
	60	20.46084906	1.38991
	72	21.09040881	1.42995
5.5	0	0	0
	6	7.618553459	0.57314
	12	10.46084906	0.75391
	24	10.93962264	0.78436
	36	11.51163522	0.82074
	48	12.16273585	0.86215
	60	12.31493711	0.87183
	72	14.19528302	0.99142
6.0	0	0	0
	6	3.638050314	0.31998
	12	6.743238994	0.51747
	24	8.740251572	0.64448
	36	8.872798742	0.65291

	48	9.547484277	0.69582
	60	10.17248428	0.73557
	72	11.98097484	0.85059
6.5	0	0	0
	6	0.775314465	0.13791
	12	1.416352201	0.17868
	24	2.375628931	0.23969
	36	2.680974843	0.25911
	48	2.81336478	0.26753
	60	3.291037736	0.29791
	72	5.359433962	0.42946

d) For temperature constant 60°C:

pH Values	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
4.5	0	0	0
	6	9.757232704	0.70916
	12	11.89921384	0.84539
	24	12.85880503	0.90642
	36	14.31179245	0.99883
	48	14.92122642	1.03759
	60	15.92735849	1.10158
	72	16.6995283	1.15069
5.0	0	0	0
	6	12.08506289	0.85721
	12	13.29339623	0.93406
	24	14.40927673	1.00503
	36	14.87374214	1.03457
	48	15.73459119	1.08932
	60	17.32861635	1.1907
	72	17.81477987	1.22162
5.5	0	0	0
	6	6.593238994	0.50793
	12	10.18632075	0.73645
	24	10.77767296	0.77406
	36	11.25660377	0.80452
	48	12.03050314	0.85374
	60	12.21446541	0.86544

	72	13.57751572	0.95213
6.0	0	0	0
	6	3.316666667	0.29954
	12	6.305503145	0.48963
	24	7.934591195	0.59324
	36	8.794496855	0.64793
	48	9.41336478	0.68729
	60	9.819339623	0.71311
	72	11.12657233	0.79625
6.5	0	0	0
	6	0.59418239	0.12639
	12	1.275314465	0.16971
	24	1.968553459	0.2138
	36	2.544968553	0.25046
	48	2.68663522	0.25947
	60	2.81918239	0.2679
	72	4.262578616	0.3597

e) For temperature constant 70°C:

pH Values	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
4.5	0	0	0
	6	6.118081761	0.47771
	12	7.863726415	0.588733
	24	9.858176101	0.71558
	36	10.8422956	0.77817
	48	12.60786164	0.89046
	60	14.63694969	1.01951
	72	15.52012579	1.07568
5.0	0	0	0
	6	7.947327044	0.59405
	12	9.323427673	0.68157
	24	11.18113208	0.79972
	36	12.36823899	0.87522
	48	13.5913522	0.95301
	60	15.44040881	1.07061
	72	16.88773585	1.16266
5.5	0	0	0
	6	3.883490566	0.33559
	12	5.598742138	0.44468
	24	6.871698113	0.52564
	36	8.310220126	0.61713
	48	9.718396226	0.70669
	60	10.76698113	0.77338
	72	11.57342767	0.82467

6.0	0	0	0
	6	1.65	0.19354
	12	2.859591195	0.27047
	24	3.930345912	0.33857
	36	4.543553459	0.37757
	48	7.017295597	0.5349
	60	8.285062893	0.61553
	72	8.934748428	0.65685
6.5	0	0	0
	6	0.199528302	0.10129
	12	0.743710692	0.1359
	24	1.397798742	0.1775
	36	2.124842767	0.22374
	48	2.765566038	0.26449
	60	2.883647799	0.272
	72	3.313050314	0.29931

Appendix C

Data for glucose concentration from HPLC at constant pH:

a) For pH constant at 4.5:

Temperature (°C)	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
30°C	0	0	0
	6	3.663836478	0.32162
	12	5.728380503	0.452925
	24	7.07922956	0.538839
	36	8.171792453	0.608326
	48	10.49971698	0.756382
	60	12.65150943	0.893236
	72	13.60253145	0.953721
40°C	0	0	0
	6	8.572169811	0.63379
	12	10.62798742	0.76454
	24	11.85110063	0.84233
	36	14.45628931	1.00802
	48	15.34496855	1.06454
	60	15.99355346	1.10579
	72	17.28050314	1.18764
50°C	0	0	0
	6	10.81053459	0.77615
	12	15.0677673	1.04691
	24	15.74245283	1.08982
	36	16.37201258	1.12986

	48	17.18726415	1.18171
	60	18.38977987	1.25819
	72	19.79984277	1.34787
60°C	0	0	0
	6	9.757232704	0.70916
	12	11.89921384	0.84539
	24	12.85880503	0.90642
	36	14.31179245	0.99883
	48	14.92122642	1.03759
	60	15.92735849	1.10158
	72	16.6995283	1.15069
70°C	0	0	0
	6	6.118081761	0.47771
	12	7.863726415	0.588733
	24	9.858176101	0.71558
	36	10.8422956	0.77817
	48	12.60786164	0.89046
	60	14.63694969	1.01951
	72	15.52012579	1.07568

b) For pH constant at 5.0:

Temperature (°C)	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
30°C	0	0	0
	6	4.787861635	0.393108
	12	6.675345912	0.513152
	24	8.456619497	0.626441
	36	9.550613208	0.696019
	48	11.29331761	0.806855
	60	13.40606918	0.941226
	72	15.51455975	1.075326
40°C	0	0	0
	6	11.11507862	0.795519
	12	12.76907233	0.900713
	24	13.75207547	0.963232
	36	15.19342767	1.054902
	48	16.69303459	1.150277
	60	17.60092767	1.208019
	72	18.39643082	1.258613
50°C	0	0	0
	6	13.37798742	0.93944
	12	16.37342767	1.12995
	24	17.47940252	1.20029
	36	18.37468553	1.25723
	48	19.41289308	1.32326
	60	20.46084906	1.38991
	72	21.09040881	1.42995

60°C	0	0	0
	6	12.08506289	0.85721
	12	13.29339623	0.93406
	24	14.40927673	1.00503
	36	14.87374214	1.03457
	48	15.73459119	1.08932
	60	17.32861635	1.1907
	72	17.81477987	1.22162
70°C	0	0	0
	6	7.947327044	0.59405
	12	9.323427673	0.68157
	24	11.18113208	0.79972
	36	12.36823899	0.87522
	48	13.5913522	0.95301
	60	15.44040881	1.07061
	72	16.88773585	1.16266

c) For pH constant at 5.5:

Temperature (°C)	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
30°C	0	0	0
	6	1.782845912	0.201989
	12	3.502783019	0.311377
	24	4.696352201	0.387288
	36	5.68672956	0.450276
	48	7.669528302	0.576382
	60	9.5625	0.696775
	72	10.34207547	0.746356
40°C	0	0	0
	6	6.001179245	0.470275
	12	7.697421384	0.578156
	24	9.365833333	0.684267
	36	10.94413522	0.784647
	48	11.77234277	0.837321
	60	11.97451258	0.850179
	72	12.80803459	0.903191
50°C	0	0	0
	6	7.618553459	0.57314
	12	10.46084906	0.75391
	24	10.93962264	0.78436
	36	11.51163522	0.82074
	48	12.16273585	0.86215
	60	12.31493711	0.87183
	72	14.19528302	0.99142

60°C	0	0	0
	6	6.593238994	0.50793
	12	10.18632075	0.73645
	24	10.77767296	0.77406
	36	11.25660377	0.80452
	48	12.03050314	0.85374
	60	12.21446541	0.86544
	72	13.57751572	0.95213
70°C	0	0	0
	6	3.883490566	0.33559
	12	5.598742138	0.44468
	24	6.871698113	0.52564
	36	8.310220126	0.61713
	48	9.718396226	0.70669
	60	10.76698113	0.77338
	72	11.57342767	0.82467

d) For pH constant at 6.0 :

Temperature (°C)	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
30°C	0	0	0
	6	0.400660377	0.114082
	12	1.735172956	0.198957
	24	2.618569182	0.255141
	36	4.388742138	0.367724
	48	5.790172956	0.456855
	60	7.601745283	0.572071
	72	8.351289308	0.619742
40°C	0	0	0
	6	2.900158805	0.2730501
	12	3.998268868	0.3428899
	24	5.24908805	0.422442
	36	6.482311321	0.500875
	48	7.94163522	0.593688
	60	8.972641509	0.65926
	72	9.520283019	0.69409
50°C	0	0	0
	6	3.638050314	0.31998
	12	6.743238994	0.51747
	24	8.740251572	0.64448
	36	8.872798742	0.65291
	48	9.547484277	0.69582
	60	10.17248428	0.73557
	72	11.98097484	0.85059

60°C	0	0	0
	6	3.316666667	0.29954
	12	6.305503145	0.48963
	24	7.934591195	0.59324
	36	8.794496855	0.64793
	48	9.41336478	0.68729
	60	9.819339623	0.71311
	72	11.12657233	0.79625
70°C	0	0	0
	6	1.65	0.19354
	12	2.859591195	0.27047
	24	3.930345912	0.33857
	36	4.543553459	0.37757
	48	7.017295597	0.5349
	60	8.285062893	0.61553
	72	8.934748428	0.65685

e) For pH constant at 6.5 :

Temperature (°C)	Time (hr)	Glucose Concentration (mg/mL)	Area*E6 (nRIU*s)
30°C	0	0	0
	6	0.086194969	0.094082
	12	0.328160377	0.109471
	24	0.989072327	0.151505
	36	1.925141509	0.211039
	48	2.385518868	0.240319
	60	2.950959119	0.276281
	72	3.557059748	0.314829
40°C	0	0	0
	6	0.471509434	0.118588
	12	1.166855346	0.162812
	24	1.82495283	0.204667
	36	2.324261006	0.236423
	48	2.614292453	0.254869
	60	2.952751572	0.276395
	72	3.551666667	0.314486
50°C	0	0	0
	6	0.775314465	0.13791
	12	1.416352201	0.17868
	24	2.375628931	0.23969
	36	2.680974843	0.25911
	48	2.81336478	0.26753
	60	3.291037736	0.29791
	72	5.359433962	0.42946

60°C	0	0	0
	6	0.59418239	0.12639
	12	1.275314465	0.16971
	24	1.968553459	0.2138
	36	2.544968553	0.25046
	48	2.68663522	0.25947
	60	2.81918239	0.2679
	72	4.262578616	0.3597
70°C	0	0	0
	6	0.199528302	0.10129
	12	0.743710692	0.1359
	24	1.397798742	0.1775
	36	2.124842767	0.22374
	48	2.765566038	0.26449
	60	2.883647799	0.272
	72	3.313050314	0.29931

Calibration Table

CALIBRATION CURVE FOR GLUCOSE

Calib. Data Modified : 2/19/2010 3:10:14 PM

Rel. Reference Window : 5.000 %
Abs. Reference Window : 0.000 min
Rel. Non-ref. Window : 5.000 %
Abs. Non-ref. Window : 0.000 min
Uncalibrated Peaks : not reported
Partial Calibration : Yes, identified peaks are recalibrated
Correct All Ret. Times: No, only for identified peaks

Curve Type : Linear
Origin : Included
Weight : Equal

Recalibration Settings:
Average Response : Average all calibrations
Average Retention Time: Floating Average New 75%

Calibration Report Options :
Printout of recalibrations within a sequence:
Calibration Table after Recalibration
Normal Report after Recalibration
If the sequence is done with bracketing:
Results of first cycle (ending previous bracket)

Signal 1: DAD1 A, Sig=191,2 Ref=360,100
Signal 2: RID1 A, Refractive Index Signal

RetTime [min]	Sig	Lvl	Amount [mg/ml]	Area	Amt/Area	Ref Grp	Name
3.956	2	1	20.00000	1.75099e6	1.14221e-5		Glucose
		2	40.00000	2.67647e6	1.49450e-5		
		3	60.00000	3.56295e6	1.68400e-5		
		4	80.00000	4.62626e6	1.72926e-5		
		5	100.00000	7.00709e6	1.42713e-5		

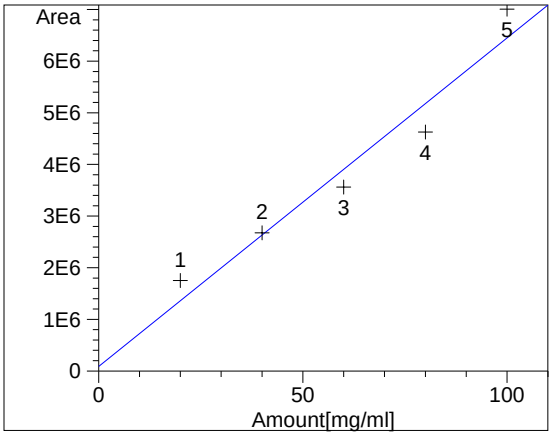
Peak Sum Table

No Entries in table

==========

Calibration Curves

==========



Glucose at exp. RT: 3.956
RID1 A, Refractive Index Signal
Correlation: 0.98460
Residual Std. Dev.: 472666.38884
Formula: y = mx + b
m: 63639.58161
b: 88648.02381
x: Amount
y: Area

==========

Data File C:\CHEM32\1\DATA\GLUCOSE RI\GLUCOSE RI 2010-02-17 12-30-17\SOLEHAH000041.D
Sample Name: SAMPLE 36

```
==========================================================================
Acq. Operator   : IDAYU/SOLEHAH (PSM)          Seq. Line :  41
Acq. Instrument : Instrument 1                  Location : Vial 41
Injection Date  : 2/17/2010 7:55:33 PM               Inj :   1
                                              Inj Volume : 10 µl
Acq. Method     : C:\CHEM32\1\DATA\GLUCOSE RI 2010-02-17 12-30-17\GLUCOSE RI.M
Last changed    : 2/17/2010 12:29:44 PM by IDAYU/SOLEHAH (PSM)
Analysis Method : C:\CHEM32\1\METHODS\DEF_LC.M
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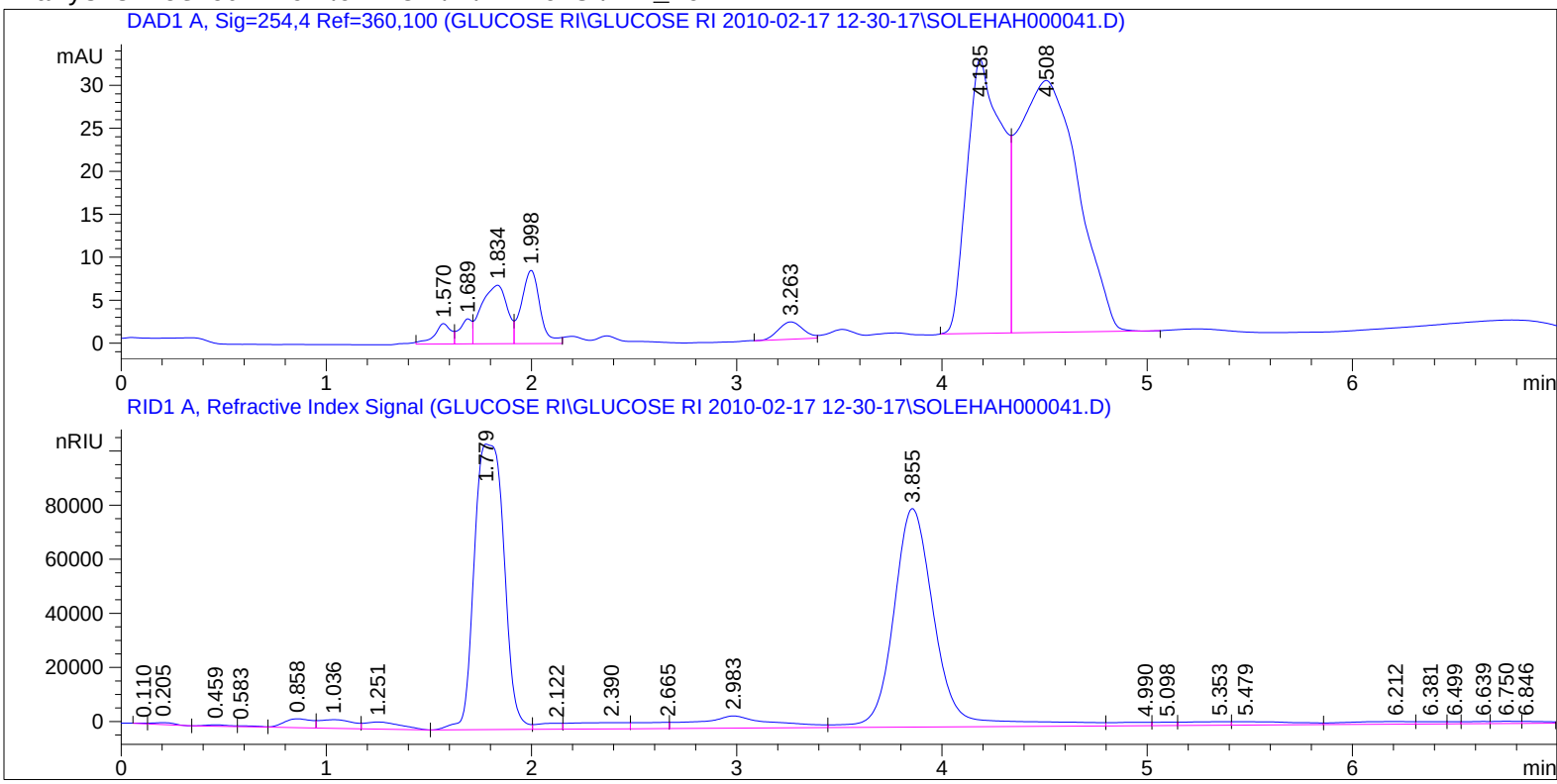


```
==========================================================================
                        Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   1.570 BV    0.0796   13.36825    2.37662   1.1866
   2   1.689 VV    0.0584   12.12446    2.92513   1.0762
   3   1.834 VV    0.1419   59.54492    6.81760   5.2854
   4   1.998 VB    0.0954   53.69053    8.53212   4.7657
   5   3.263 BV    0.1316   16.92180    2.02307   1.5020
   6   4.185 BV    0.1698  382.92941   31.95987  33.9898
   7   4.508 VB    0.2800  588.02081   29.31163  52.1943

Totals :                   1126.60019   83.94603
```

Data File C:\CHEM32\1\DATA\GLUCOSE RI\GLUCOSE RI 2010-02-17 12-30-17\SOLEHAH000041.D
Sample Name: SAMPLE 36

Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.110	BV	0.0457	732.72748	244.61140	0.0277
2	0.205	VV	0.1081	5023.18652	732.74792	0.1900
3	0.459	VV	0.1214	4254.10352	531.69818	0.1609
4	0.583	VV	0.1224	2428.00269	322.45779	0.0918
5	0.858	VV	0.1418	3.08072e4	3286.50854	1.1652
6	1.036	VV	0.1701	3.64547e4	3188.62866	1.3788
7	1.251	VV	0.1798	3.16677e4	2622.53760	1.1978
8	1.779	VV	0.1392	1.06419e6	1.05618e5	40.2511
9	2.122	VV	0.1279	1.85468e4	2216.54175	0.7015
10	2.390	VV	0.2614	4.50399e4	2316.95972	1.7035
11	2.665	VV	0.1491	2.57680e4	2283.19604	0.9746
12	2.983	VV	0.3289	1.13871e5	4514.20898	4.3070
13	3.855	VV	0.2140	1.13820e6	8.08233e4	43.0500
14	4.990	VV	0.1713	1.67228e4	1249.28101	0.6325
15	5.098	VV	0.1038	9153.61133	1234.87903	0.3462
16	5.353	VV	0.2024	2.00330e4	1308.22705	0.7577
17	5.479	VV	0.2751	2.66220e4	1246.67297	1.0069
18	6.212	VV	0.3028	2.32555e4	972.21692	0.8796
19	6.381	VV	0.1297	7642.82471	859.38727	0.2891
20	6.499	VV	0.0589	3186.03687	772.31396	0.1205
21	6.639	VV	0.1152	6602.60791	804.23364	0.2497
22	6.750	VV	0.1219	7339.50928	835.73236	0.2776
23	6.846	VBA	0.1374	6349.75195	720.17236	0.2402
Totals :				2.64389e6	2.18705e5	

*** End of Report ***

Data File C:\CHEM32\1\DATA\GLUCOSE RI\GLUCOSE RI 2010-02-17 12-30-17\SOLEHAH000042.D
Sample Name: SAMPLE 37

```
==================================================================
Acq. Operator   : IDAYU/SOLEHAH (PSM)          Seq. Line :  42
Acq. Instrument : Instrument 1                  Location : Vial 42
Injection Date  : 2/17/2010 8:06:39 PM             Inj :   1
                                               Inj Volume : 10 µl
Acq. Method     : C:\CHEM32\1\DATA\GLUCOSE RI 2010-02-17 12-30-17\GLUCOSE RI.M
Last changed    : 2/17/2010 12:29:44 PM by IDAYU/SOLEHAH (PSM)
Analysis Method : C:\CHEM32\1\METHODS\DEF_LC.M
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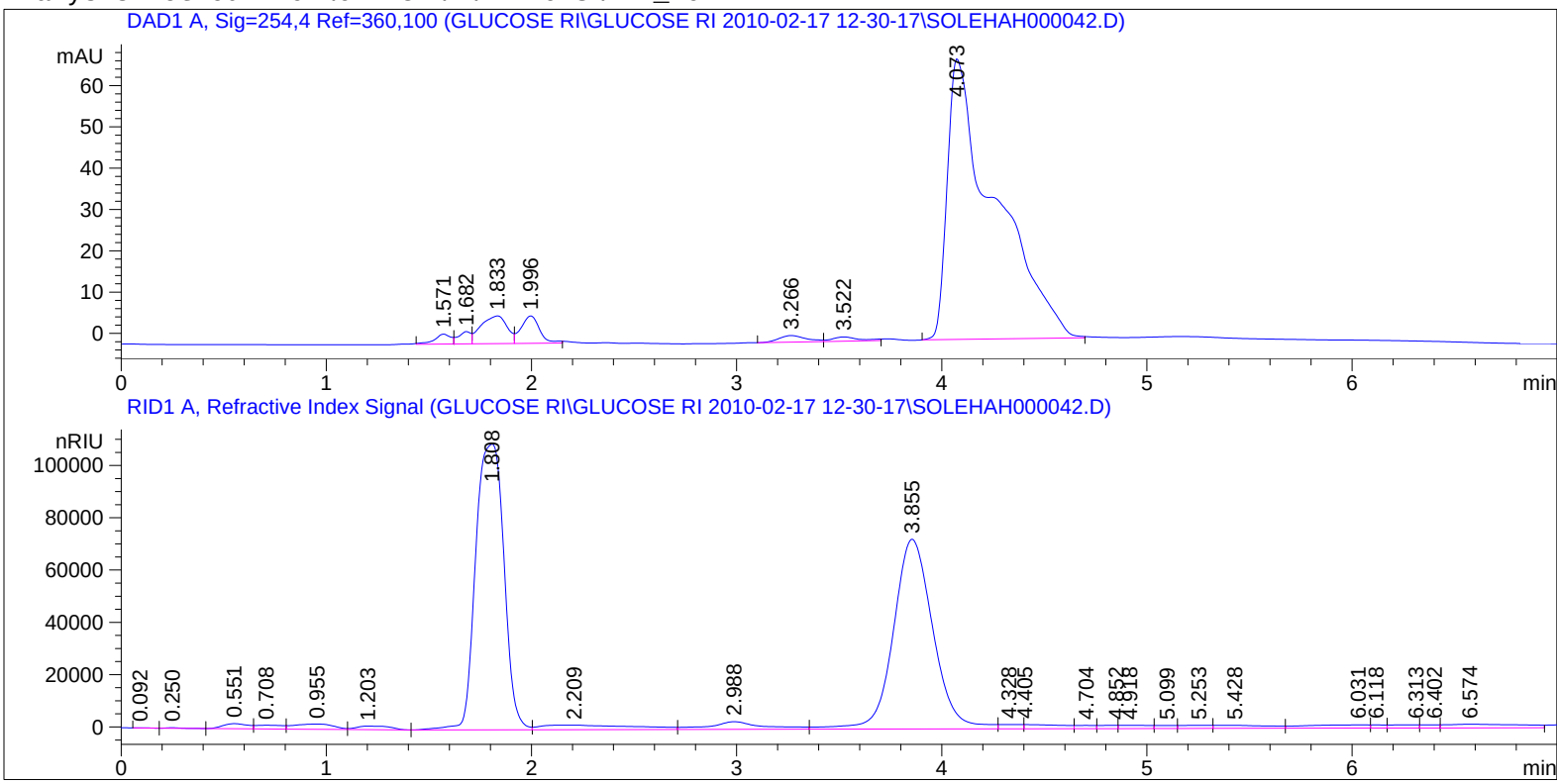


```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   1.571 BV    0.0763   13.31850    2.41476   1.0676
   2   1.682 VV    0.0589   12.43176    2.96621   0.9965
   3   1.833 VV    0.1228   59.10179    6.70975   4.7376
   4   1.996 VB    0.0945   41.28526    6.64606   3.3094
   5   3.266 BV    0.1393   14.61159    1.58824   1.1713
   6   3.522 VB    0.1312    9.26911    1.02777   0.7430
   7   4.073 BB    0.2204 1097.49915   67.97745  87.9747

Totals :                   1247.51715   89.33022
```

Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.092	BV	0.0789	284.26666	57.87621	0.0119
2	0.250	VV	0.0756	990.68060	205.63927	0.0413
3	0.551	VV	0.1283	1.63912e4	1949.24036	0.6839
4	0.708	VV	0.1397	1.30372e4	1416.97119	0.5440
5	0.955	VV	0.2134	2.67962e4	2016.41260	1.1181
6	1.203	VV	0.1563	1.58077e4	1368.79834	0.6596
7	1.808	VV	0.1570	1.04635e6	1.09305e5	43.6593
8	2.209	VV	0.3967	5.62476e4	1739.43213	2.3469
9	2.988	VV	0.2454	5.32716e4	2947.25195	2.2228
10	3.855	VV	0.2063	9.74330e5	7.25953e4	40.6541
11	4.328	VV	0.1021	1.22037e4	1636.17261	0.5092
12	4.405	VV	0.2173	2.07701e4	1592.74609	0.8666
13	4.704	VV	0.0913	8137.52637	1245.76782	0.3395
14	4.852	VV	0.0915	7689.82129	1241.80151	0.3209
15	4.918	VV	0.1528	1.27993e4	1240.16138	0.5341
16	5.099	VV	0.0960	7878.22119	1167.36841	0.3287
17	5.253	VV	0.1423	1.22336e4	1204.42444	0.5105
18	5.428	VV	0.2436	2.16403e4	1171.26990	0.9029
19	6.031	VV	0.2773	2.74233e4	1240.86218	1.1442
20	6.118	VV	0.0694	5850.27930	1208.95081	0.2441
21	6.313	VV	0.1223	1.13887e4	1215.68811	0.4752
22	6.402	VV	0.0873	7345.13965	1223.53015	0.3065
23	6.574	VV	0.3435	3.77637e4	1416.04736	1.5757
Totals :				2.39663e6	2.10406e5	

*** End of Report ***