Valorization of Biomass Waste to Produce Bioethanol

by

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CERTIFICATION OF APPROVAL

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Approved by,

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(Dr Asna Binti Md Zain)

UNIVERSITI TEKNOLOGI PETRONAS

TRONOH, PERAK

May 2013

CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

_____

ROSE AMIRA BINTI KARIM

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ABSTRACT

There are various methods that can be implemented to produce biofuel specifically bioethanol. The bioethanol can be produced from cellulose and hemicelluloses that may be originate from various sources of biomass such as Empty Fruit Bunches (EFB), mesocarp fiber, shell and palm kernel cakes. The studies and research works were focused on the production of bioethanol from oil palm waste, EFB using bacteria, *Saccharomyces Cerevisiae* ATCC 96581 as fermentation aid to expand the usage of oil palm waste and to enhance the production of bioethanol. The purposes of this study are to investigate the effect of FPU loading, pH value and temperatures by using celluloses. Besides, this study also aimed to produce bioethanol from EFB by using Simultaneous Saccharification and Fermentation (SSF) method. Prior fermentation process, enzymatic saccharifications of EFB need to be done to investigate the highest amount of monomeric sugars; glucose and fructose produced from EFB aided by enzyme, *Trichoderma Reesei*. Three sets of experiment were performed; in first set, the sample was hydrolyzed with pretreatment with sodium hydroxide solution then being subjected to sulfuric acid solution. Pretreatment process is necessary to remove lignin from the EFB that could hinder the saccharification of EFB to produce sugars and bioethanol. At the second set of the experiment, the fermentation process which is SSF method was performed aided with *Saccharomyces Cerevisiae* under anaerobic conditions. The result has shown that bioethanol has been produced from the process and the highest amount of bioethanol produced was 0.42 mg/ml at 58 hours. Final sets of experiment were performing to examine the effect of mass loading, pH value and also FPU loading of the celluloses in producing bioethanol. From optimization works of these various fermentation parameters it was found that the highest ethanol production from cellulose (Avicel Ph 101) which ranges from 3.1 mg/mL to 4.6 mg/mL can be achieved at pH 4, 217 FPU, 5.0g of cellulose loading with an agitation rate of 100 rpm for 60 hours incubation.

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CHAPTER 1: PROJECT BACKGROUND

1.1 Background of Study

Since the 20th century, fossil fuels such as oil, coal and natural gas supply a major energy demand to the world. Fossil fuels originate from deceased organisms that lived several million years ago and by time there was shortage and depletion of fossil fuels. Incineration of this fossil remains results in a net increase of today's carbon dioxide level to the atmosphere. Environmental issues such as the increase in temperature caused by the greenhouse effect and the fact that fossil fuels are nonrenewable resources, has increased the interest in producing fuels such as bioethanol from renewable resources such as biomass.

Ethanol or ethyl alcohol is a volatile, flammable, colorless liquid with a boiling point of 78.37^{o}C. Its low melting point of -114^{o}C made it useful as the fluid in thermometers for temperature below -40 ^{o}C, the freezing point of mercury, and for other low temperature purposes, such as antifreeze in automobile radiators. The existence of its hydroxyl group and the shortness of its carbon chain make it capable to participate in hydrogen bonding, rendering it more viscous and less volatile[1]. It is biodegradable, low in toxicity and causes little environmental pollution if spill. Figure 1 shows the structural formula of ethanol where ethanol is the second member of the aliphatic alcohol series.

[1]Lide, D. R., ed. (2000). *CRC Handbook of Chemistry and Physics 81st edition*. CRC press. ISBN 0-8493-0481-4.

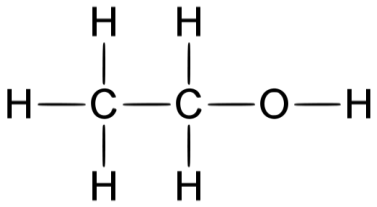

Figure 1: Structural formula of Ethanol

Bioethanol is one form of renewable energy source that is fast gaining position as potential fuel to power automotive engine. Unlike gasoline which is refined through distilling crude oil, ethanol can be synthesized from a wide variety of biological materials such as wheat, corn, barley, wood and sugar cane. In fermentation process, baker's yeast is used to breakdown starch (carbohydrate) into bioethanol and carbon dioxide as a by-product. Fuel ethanol contains 10% of ethanol mixed with 90% gasoline which is commonly known as E10 in United States of America. Because the ethanol is a high-octane fuel with high oxygen content (35% oxygen by weight), it allows the engine to complete the combustion of fuel, resulting in fewer emissions and has replaced lead as an octane enhancer in petrol[2].

Bioethanol is an ethanol synthesized from biomass and it is renewable. Therefore bioethanol has some advantages over petrol as a fuel such as it can help to reduce the amount of carbon monoxide produced by the vehicle thus improving air quality and reduce the emission of greenhouse gases to the atmosphere. Other than that, it gives benefits to Malaysian agriculture such as increase the plantation of the crops such as palm oil, sugar cane and also provides job vacancies to the farmers. These benefits, in turn, could serve to stabilize and improve financial stability for farmers, which would increase the economic well-being of rural and other agriculture-dependent sectors of Malaysia. In addition, it also can give job opportunities whether directly or indirectly in all aspects of ethanol production; from farming to transportation and manufacturing.

[2]Mohammad J. Taherzadeh., Ethanol from Lignocellulose: Physical Effect of Inhibitors. Chalmers University of Technology; 1999.

Bioethanol can be produced from the fermentation process of biomass aided by bacteria to decompose the biomass. There are two key parameters take place on how biomass is transformed to bioethanol:

1. Enzymatic hydrolysis is a chemical process in which acid is used to convert starch (complex sugars) into monomeric sugars such as glucose and fructose. The feedstock must first be hydrolyzed into glucose before proceed with the fermentation process for bioethanol production[3]. In the biomass-to-bioethanol process, acids and enzymes are used to catalyze this reaction.

2. Fermentation is a biological process in which sugars such as glucose, fructose and sucrose are converted into cellular energy and thereby produce ethanol and carbon dioxide as waste products. Fermentation reaction occurs in the presence of yeast or bacteria, which feed on sugars as nutrient. Ethanol and carbon dioxide are being produced as the sugar is consumed. The simplified fermentation reaction of 6-carbon sugar is:

$$C_6H_{12}O_6 \xrightarrow{\text{bacteria}} 2CH_3CH_2OH + 2CO_2$$

Eq. (1)

(Glucose) (Ethanol) (Carbon dioxide)

The sugar formed in the enzymatic hydrolysis reaction is fermented into bioethanol. The common microorganisms use in the fermentation process is *Saccharomyces cerevisiae*, which is known as ordinary baking yeast. It is the critical element in the fermentation process that converts sugar into alcohol[4]. Beside glucose, it also has the ability to ferment mannose as well since soft wood also contains substantial amounts of mannose.

In this study, empty fruit bunches (EFB) of oil palm has been chosen to be the substrate for the fermentation process due to its abundance and low cost of processing. Besides, it also cleans, non-toxic and renewable. EFB is one of the lignocellulosic materials

[3] Kamaruddin,H., H.Mohamad, D.Ariffin and S.Johari. An estimated availability of oil palm biomass in Malaysia. PORIM Occ. Paper Palm Oil Res. Inst. Malaysia 37:1997

[4] Laundry, C.R.,Townsend, J.P., Hartl, D.L. and Cavalieri, D. Ecological and evolutionary genomics of Saccharomyces cerevisiae. Molecular Ecology. 2006. Volume 15.p.575-591.

consists primary of cellulose and hemicellulose component. It was obtained from Sze Tech Engineering Sdn Bhd located in Padang Jawa, Selangor with fiber length of 0.5-1.0 inch.

1.2 Problem Statement

Carbon dioxide is one of the major atmospheric contributors to the greenhouse effect. Greenhouse effect refers to the Earth's trapping of the sun's incoming solar radiation, causing warming of the Earth's atmosphere. Current analysis suggests that the combustion of fossil fuels is a major contributor to the increase in the carbon dioxide concentration, such contributions being 2 to 5 times the effect of deforestation (Kraushaar & Ristinen). Carbon dioxide and other so-called greenhouse gases allow solar energy to enter the earth's atmosphere, but reduce the amount of energy that can radiate back into space, trapping energy and heat causing to global warming[5]. Figure 2 shows the concentration of greenhouse gases emitted such as carbon dioxide, methane and chlorofluorocarbons. It shows that carbon dioxide has the largest concentration followed by methane gas and chlorofluorocarbons. Hence, the opportunity to reduce dependence on fossil fuels, while reducing carbon dioxide is of strategic important today.

[5] Kraushaar & Ristinen, *Energy and problems of a technical society*, 2nd ed (1993)

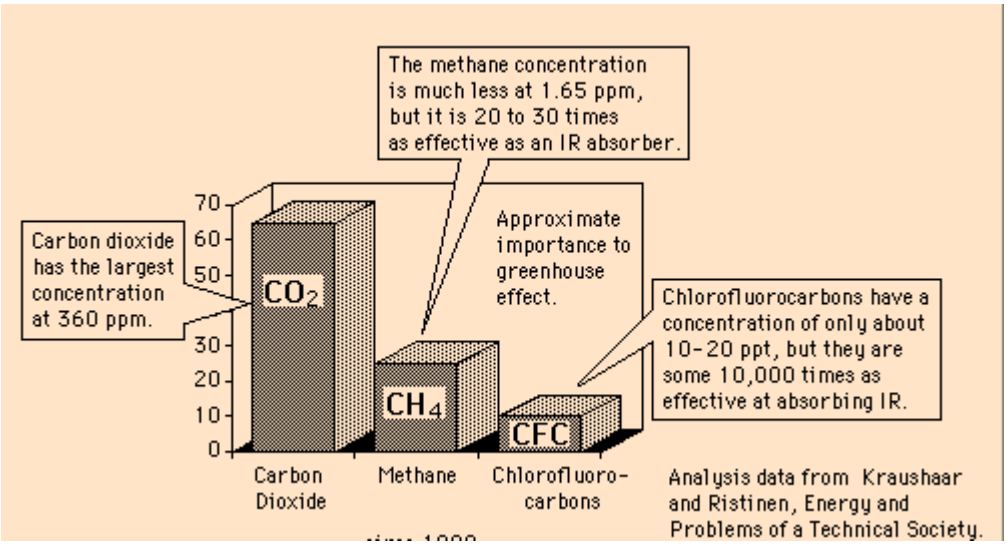

Figure 2: Analysis data for concentration of greenhouse gases emitted to atmosphere (Valentas et al., 2009)

One of the environmental benefits of replacing fossil fuels with biomass-based fuels is that the energy obtained from biomass does not contribute to global warming. All combustion process would produce carbon dioxide as a byproduct, including fuels produced by biomass. Nonetheless, as because plants use carbon dioxide from the atmosphere to grow; for photosynthesis process, carbon dioxide released during combustion is balanced by that absorbed during the annual growth of the plants. Increase the usage of renewable fuels like ethanol will help to counter the pollution and global warming effects of burning gasoline. Use of 10% ethanol-blended fuels results in a 6-10% carbon dioxide reduction and higher level of ethanol can further reduce the net quantity of carbon dioxide emitted into the atmosphere. Ethanol reduces greenhouse gases emissions relative to gasoline by between 40% to 62% depending on agricultural practices and production technologies.[6] Thus, more carbon dioxide will be absorbed by crop growth.

Gasoline and diesel is a liquid mixture distilled from crude oil. They consist of blends of different hydrocarbon chains. During the process of refining, groups of hydrocarbon

[6] Coad.L., Bristow,M.,(2011).Ethanol's Potential Contribution to Canada's Transportation Sector. *The conference board of Canada.*pp: 68

chains with similar molecular size are separated based on the difference in their boiling points. Many of these are toxic and volatile compounds such as benzene, toluene, and xylenes which are responsible for the health hazards and pollution associated with combustion of petroleum – based fuels. The largest single contributor to the rise of man-made greenhouse gases is, of course, the burning of oil and gas to power vehicles, machinery, and produce energy and warmth. Carbon monoxide, nitrogen oxides, sulfur oxides and particulates are the main concerns nowadays. A key environmental benefit of using biofuels as an additive to petroleum-based transportation fuels can give a reduction in these harmful emissions.

Bioethanol is used as fuel oxygenates to improve combustion characteristic. Ethanol reduces pollution through the volumetric displacement of gasoline and by adding oxygen to the combustion process which reduces exhaust emissions to the atmosphere. Hence, the production of bioethanol from empty fruit bunches can spur economic growth because it expands the usage of oil palm and also can reduce the cost in producing fossil fuels.

1.3 Objective of Study

As bioethanol has a huge potential as a substituting agent to gasoline in car fuel and give further advantages to the environment, this research is carried out in order to produce bioethanol from empty fruit bunches (EFB) of oil palm.

1.4 Scope of Study

To achieve the objective, there are three scopes that have been identified:

i. To find the Filter Paper Unit (FPU) value of cellulase derived from *Trichoderma reesei*
ii. To investigate the effect on the production of bioethanol at different temperatures, pH value and FPU loading by using cellulose.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview

Worldwide, biomass is the fourth largest energy resource after coal, petroleum and natural gas. Biomass is biological material from living organism, most often referring to plants or plant-derived materials. Plants use light energy from the sun to convert carbon dioxide and water to sugars through a photosynthesis process. It remains the largest biomass energy source today for example dead trees, branches, tree stumps, wood chips and even municipal solid waste.[7]

Another type of plant matter, called cellulosic biomass, is made up of very complex sugar, and it is not generally used for food. Cellulosic biomass consists of three main components which are lignin, hemicellulose and cellulose. Among these components, the largest portion is cellulose which covers from 38% to 50% followed by hemicellulose (23%-32%) and lignin (15%-25%).[8]

Biomass energy currently contributes 9-13% of the global energy supply accounting for 45±10 EJ per year (Thomas, 2000). Biomass energy includes both traditional uses such as a ring for cooking and heating and modern uses such as producing electricity and steam, and liquid bio-fuels. Use of biomass energy in modern ways is estimated at 7 EJ (exajoule) a year, while the remainder is in traditional uses. Biomass energy is derived from renewable resources. Ethanol derived from biomass, one of the modern forms of biomass energy, has the potential to be a sustainable transportation fuel, as well as a fuel oxygenate that can replace gasoline (Wang, 2000). Shapouri *et al.* (1995, 2002) reported that the energy content of ethanol was higher than the energy required producing ethanol. Kim and Dale (2002) also estimated the total energy requirement for producing ethanol from corn grain at 560 kJ MJ−1 of ethanol, indicating that ethanol used as a

[7] Biomass Energy Centre. http://www.biomassenergycentre.org.uk. Retrieved on December 6, 2012

[8] Valentas.K.,(2009). Biofuel from Cellulosic Biomass: An Overview of Current Technologies & Economic Feasibility. *Biotechnology Institute,University of Minnesota.*pp 1-5.

liquid transportation fuel could reduce domestic consumption of fossil fuels, particularly petroleum. The world ethanol production in 2001 was 31 giga litres (GL) (Berg, 2001). The major producers of ethanol are Brazil and the United States, which account for about 62% of world production.

As a renewable energy source, biomass can either be used directly or indirectly to convert into another type of energy product such as biofuel. The estimated biomass production in the world is 146 billion tons a year.[9] Furthermore, Malaysia is one of the largest producers of palm oil in the region and among the biggest income earners to the country for many years. With the rapid growth of palm oil production in Malaysia, the amount of biomass residues generated also has shown a corresponding increase.

In 2010, the oil palm planted area in the country is 4.8 million hectares. In 2011, Malaysian oil palm accounted for just 1.97% which is about five million hectares of the total 253.9 million hectares. It makes up to71% of agriculture land or 14.3% of total land area.[10] The overall average of 18.03 tones Fresh Fruit Bunches (FFB) per hectare of palm oil plantation has been produced from the oil palm industry (Choo, 2011). Based on this figure, palm oil plantation areas has produced more than 66.63 million tonnes of biomass residues such as Empty Fruit Bunches (EFB), mesocarp fiber, shell, palm kernel cakes, trunks and Palm Oil Mill Effluent (POME) in 2010 (Goh *et al.*, 2009). The EFB represent about 9% of this total. They are the residue left after the fruit bunches has been processed to extract oil at oil mills.

In a country that has significant amount of agricultural activities, biomass can be a very promising alternative source of renewable energy. With increased awareness on reducing greenhouse gas emissions, conversion of biomass residues into renewable such as ethanol, biogas, syngas and bio hydrogen has attracted global responsiveness. The conversion of biomass to this functional compound involve two reaction processes

[9] Schenk,Justin;et al.(2012).Wood Fired Plants Generate Violations. *Wall Street Journal*. Retrieved on December 6,2012.
[10] Palm Oil Facts and Figures 2011. http://www.simedarbyplantation.com/Palm-Oil.pdf

which are biochemical which involve chemicals or enzymes and fermentation and also thermochemical processes; gasification to syngas and pyrolysis.

2.2 Bioethanol as a Fuel

Bioethanol is one form of renewable energy source that is fast gaining foothold as potential fuel to power automotive engine. In comparing to gasoline which is refined through distilling crude fossil fuel, bioethanol can be synthesized from the starchy parts of natural plants. Nowadays, ethanol is one of the most widely used biofuel today. Fuel ethanol has been called 'gasohol'; the most common blends contain 10% ethanol mixed with 90% gasoline. It also can be used in a mixture with gasoline (3-22% ethanol) with no modification of the engine (Taherzadeh, 1999). Because the ethanol is a high-octane fuel with high oxygen content (35% oxygen by weight), it allows the engine to combust the fuel completely, resulting in fewer emissions. Since ethanol is produced from plants that harness the energy from the sun, ethanol is also considered as a renewable fuel. Therefore, ethanol has many advantages as an automotive fuel.[11]

Although ethanol production from corn and sugar bagasse can still expand greatly, its primary used mainly for animal feed, food domestics and beverage industries. Besides, the feedstock may not always be in surplus. Making ethanol from cellulose and hemicellulose dramatically expands different types and amount of available feedstock. This includes many materials now regarded as wastes requiring disposing, as well as corn stalks and wood chips.

Brazil is the world frontrunner in the use of ethanol as an automobile fuel. More than 11 billion litres of ethanol for fuel are produced from sugar cane bagasse each year. About 15% of the vehicles with spark ignition engines (the type normally fueled by gasoline) run on ethanol and the rest use a blend of 20% ethanol in gasoline. Ethanol was introduced to reduce Brazil's dependence on expensive foreign oil, and provides an

[11] http://www.comalc.com/fuel_ethanol.htm

additional market for domestic sugar producers. Beneficial effects on air quality have been an added bonus to the country.

The Clean Air Act Amendments of 1990 authorized the sale of oxygenated fuels in areas of the country with unhealthy levels of carbon monoxide. Since that time, there has been strong demand for ethanol as an oxygenate that blended with gasoline. In United States, ethanol blends make up about 12% of the total gasoline market. In some parts of America, there are projects handled to test the viability of replacing diesel fuel with ethanol. Support for fuel ethanol is a key factor in the current U.S. because of its beneficial effect on air quality. Oxygenated fuel such as ethanol blends, mandated in certain regions to reduce carbon monoxide emissions or ozone.[12] Today, there are more than 55 domestic fuel ethanol production facilities located in 22 states across the country with annual capacity of approximately 1.8 billion gallons.

Ethanol has an octane number of 113 compared with 107 for methanol and 86 to 94 for gasoline, allowing a higher compression ratio in the gasoline engine. Furthermore, it also can be used in reformulated gasoline[13]. The blending octane value of ethanol can actually be much higher than that neat of ethanol, and the blending octane value increases with lower octane-base gasoline. Therefore, ethanol is an excellent additive to prevent engine knock and improve the performance of the engine.

Although bioethanol fuel gives many advantages, but there are also disadvantages contribute from it. One of the most evident disadvantages of ethanol is that the majority of cars in Malaysia are designed to run on petrol. Petrol consists of over one thousand chemical compounds which are mostly petroleum based. Petrol fuels require an extensive range of operating conditions. This includes climate, altitude and driving patterns. This means that the properties of petrol must be balanced to give a satisfactory performance over a range of different driving conditions. This is detrimental in

[12] http://www.comalc.com/fuel_ethanol.htm

[13] Eva-Lena Jakobsson (2002). *Optimization of the pretreatment of wheat straw for production of bioethanol.*Ph.D. Thesis. Department of Chemical, Lund University.

ascertaining the right amount of ethanol that goes in to petrol as it can adversely affect the balance and performance of a vehicle.

Besides, ethanol has lower energy content and suitable for cleansing usage. It cannot be used in two strokes engines as it will clean up the lubricating oil off the cylinder walls and this may leads to overheating. Recent study in United States from Cornell University has shown that 71% more energy is required to produce a litre of ethanol than the energy contained in a litre of ethanol.

2.3 Ethanol Production

2.3.1 Empty fruit bunch (EFB)

Empty Fruit Bunch is composed of 45-50% cellulose and about equal amounts (25-35%) of hemicellulose and lignin (Deraman, 1993). Due to oil palm empty fruit bunch is available in large quantities and contain high amount of cellulose, so empty fruit bunch fiber is appears to be a potential substrate for enzyme and other chemical production (Deraman, 1993). Table 1 below shows the composition of EFB under dry matter basis and fresh matter basis.

Table 1 Composition of EFB under dry matter basis and fresh matter basis

Parameter	Dry matter basis (mean)	Fresh wt. basis (mean)
Ash (%)	6.30	2.52
Oil (%)	8.90	3.56
Carbon (%)	42.80	17.12
Nitrogen (%)	0.80	0.32
Diphosphorous pentoxide (%)	0.22	0.09
Potassium oxide (%)	2.90	1.16
Magnesium oxide (%)	0.30	0.12
Calcium oxide (%)	0.25	0.10
Boron (mg/L)	10	4
Copper (mg/L)	23	9
Zinc (mg/L)	51	20
Ferum (mg/L)	473	189
Manganese (mg/L)	48	19
C/N ratio	54	54

Source: Cheng *et al.* (2007

Figure 3: An empty fruit bunch (top) and its fibrous form (bottom)

In order to obtain the best advantages from the application of EFB in the field, inorganic supplements are also required. They are given for immature and mature plants (Gurmit *et al*. 1999).From the analysis done before, it is stated that one tonne of EFB (fresh weight) would have a fertilizer content equivalent of 3.8 kg urea, 3.9 kg rock phosphate, 18 kg muriate of potash and 9.2 kg kieserite. At current fertilizer prices, this would have a monetary value of RM12.00[14]

2.3.2 Chemical treatment

In order to produce sugars from the biomass, the biomass needs to be pre-treated with acids or enzymes to open up the plant structure and reduce the size of the feedstock. Pretreatment (steam, alkali or acid treatment) may reduce the indigenous microflora particularly required in simultaneous saccharification and fermentation (SSF), where key enzymes must be pre-induced for a quick start of lignocellulose breakdown and fungal growth (Tangerdy and Szakacs, 2003). Rita Rani *et al.* (2006) mentioned that pretreatment of substrate increased the cellulase yields by 33%. The one of the most

[14] Chan, K.W., Chow,M.C., MA,A.N., and Yusof Basiron (2002). The global challenge of GHG emission on carbon reduction:palm oil industry.Paper presented at the 2002 National Seminar on Palm Oil Milling, Refining Technology, Quality & Environment. 19-20 August 2002.12 pp,.

common chemical treatment is by adding sulfuric acid. There are lignin-hemicellulose networks in cellulose fibers. This network interrupts the enzymatic biodegradation of cellulose and hinders the saccharification of EFB to monomeric sugars. To accomplish more effective enzymatic hydrolysis, this network should be removed. In this case, sulfuric acid can resolve hemicelluloses and activate the enzymatic activity to cellulose (Esteghlalian *et al*, 1996).

Alkali is also being used to treat lignocellulosic biomass. To overcome the lignin barrier, lignocelluloses are initially pretreated with alkali to dissolve the lignin caused by the breakdown of ether linkage (Lee 1997). In the case of pretreatment of corn stover by aqueous ammonia, 70 -85% lignin was removed, and 40-60% hemicelluloses were solubilized (Kim *et al*., 2003). Efficient delignifying agent should remove a maximum amount of lignin and minimum of sugars (not more than 5%) (Taherzadeh and Karimi, 2007). Chemical alkali pretreatment at ambient temperatures is simple and time-saving and seems to have strong commercial possibilities (Kim and Holtzapple, 2005).

2.3.3 Cellulose, hemicellulose and lignin

Biomass wastes contain a complex mixture of carbohydrate polymers from the plant cell walls known as cellulose, hemi cellulose and lignin. Typically, this contains 30-50% cellulose, 15-35% hemicellulose and 10-30% of lignin (Lynd *et al.,* 2002). Cellulose, $C_6H_{10}O_5$ is the structural component of the primary cell wall that organized into long, unbranched microfibrils that give support to the cell wall[15].Cellulose from wood pulp has typical chain lengths between 300 and 1700 units; cotton and other plant fibres have chain lengths ranging from 800 to 10,000 units (Klemm *et al.* 2005 and Bailey *et al.,* 1986).

[15] Crawford, R.L. (1981). *Lignin Biodegradation and Transformation*.New York.,John Wiley and Sons.

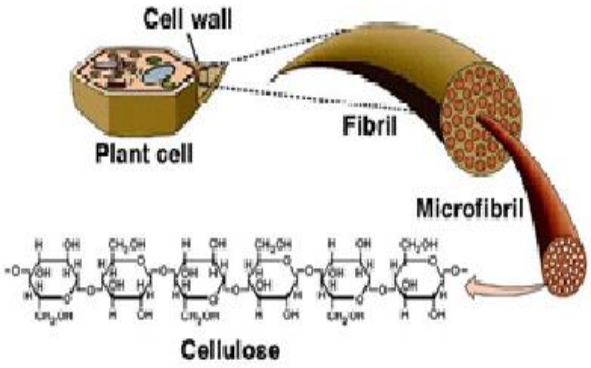

Figure 4: Arrangement of fibrils, microfibrils and cellulose in plant cell walls

Source: (Klemm *et al.* 2005 and Bailey *et al.,* 1986)

Figure 4 shows the arrangement of fibrils, microfibrils and cellulose in plant cell walls. In micro fibrils, the multiple hydroxide groups bonded with each other, holding the chains firmly together and contributing to their high tensile strength. In cell walls, this strength is important as they are meshed into a carbohydrate matrix that helps in keeping the plants rigid and tough.

A hemicellulose is any of several heteropolymers (matrix polysaccharides) such as arabinoxylans that present in almost all plant cell walls. While cellulose is in a form of crystalline, strong and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. Hemicellulose has a molecular weight that is lower than that of cellulose and they have a weak undifferentiated structure compared to crystalline cellulose[16]. It is primarily composed of the 5-carbon sugars and xylose.

[16] Scurlock., Jonathan.,(2004). *Bioenergy Feedstock Characteristics*, Oak Ridge National Laboratory. Retrieved from http://bioenergy.ornl.gov/papers/misc/biochar_factsheet.html

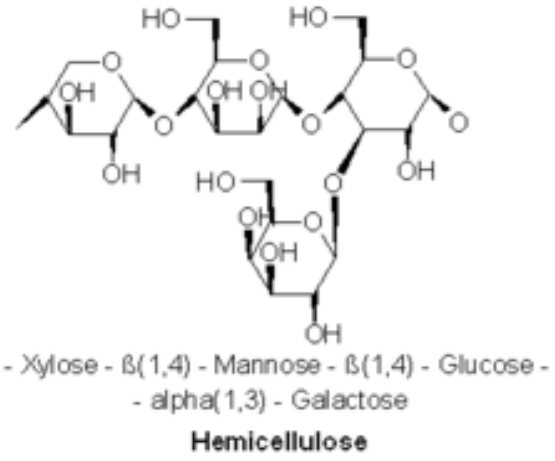

Figure 5: Structure of hemicellulose

Source: (Huber *et al.,* 2006)

Lignin as an irregular polymer forms a network in which cellulose and hemicellulose fibre are embedded and also provides structural integrity in plants (Huber *et al.,* 2006). Due to complex structure of lignocellulose, it is resistant to most chemicals and hydrolysis, which definitely form a barrier for its utilization (Lynd *et al.,* 2002; Zhu *et al.,* 2006). It remains as residual compound after the sugars in the biomass have been converted to ethanol. Figure 6 shows the arrangement of cellulose, lignin and hemicellulose in a plant cell wall that involve in ethanol synthesize.

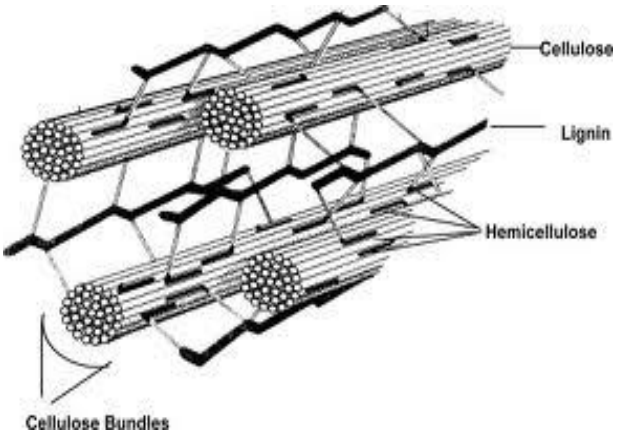

Figure 6: Typical plant cell wall arrangement

Source: (Huber *et al.,* 2006)

2.3.4 Enzymatic hydrolysis

Enzymes are more efficient agents of hydrolysis than are acids (Elwyn *et al.*, 1955). Cellulase enzyme is used to break up cellulose into glucose or other aligosaccharide compounds (Acharya *et al.* 2008). The cellulase system in fungi comprises of three hydrolytic enzymes acting synergistically, endo-1,4-β-D-glucanase (carboxymethyl cellulase), which cleaves β-linkage randomly in the amorphous parts of cellulose; exo-1,4- β-D-glucanase (cellobiohydrolase), which hydrolyzes cellobiose from either non-reducing or reducing end, generally from the crystalline part of cellulose; β-glucosidase (cellobiase), releases glucose from cellobiose and short chain cellooligosaccharides (Wilson *et al.*, 2009). Enzyme complexes for the degradation of lignocelluloses have been produced by solid state fermentation (SSF) on various agricultural residues such as rice straw, wheat bran, corn stover, sugarcane bagasse and pomace (Soccol *et al.*, 2003), using host-specific fungi such as *Saccharomyces cerevisiae* for best results (Nigam and Singh, 1996).

The most essential factor for increasing the rate of hydrolysis is making cellulose accessible to the enzymes. Therefore, chemical pretreatment usually alkaline and acid pretreatment is necessary before enzymatic hydrolysis. Chemical pretreatment not only removes lignin only, but acts as a swelling agent, which will enhances the surface area of the substrate to make it accessible for enzymatic action (Kim *et al.*, 2008).

Usually, enzymatic hydrolysis is conducted at mild conditions; at pH 4.8 with temperature 45-50^{o}C and does not have a corrosion problem (Duff and Murray, 1996). It is possible to obtain hydrolysis of 100% by enzymatic hydrolysis. Nevertheless, there is certain factor that might interfere or inhibit the enzymatic process. Several inhibitory compounds are formed during acid hydrolysis and this problem is not so severe for enzymatic hydrolysis (Lee *et al.*, 1999). The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity and reaction conditions (temperature, pH, etc.). To improve the yield and rate of enzymatic hydrolysis, optimizing the hydrolysis process and enhancing cellulase activity need to be focused (Cantwell *et al.*, 1988).

During the enzymatic hydrolysis, cellulose is depolymerized by the cellulases to monomeric sugars such as hexoses sugars that can be fermented by yeast or bacteria for the production of bioethanol. Figure 7 below is the reaction mechanism for the enzymatic hydrolysis process.

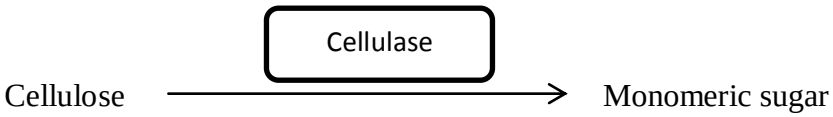

Figure 7: Reaction mechanism for the enzymatic hydrolysis process

2.3.5 Fermentation

Fermentation is a process of converting sugars into alcohols and carbon dioxide. The sugar formed from the enzymatic hydrolysis process will be used by the bacteria or baker's yeast as a nutrient to ferment them to bioethanol. The chemical reaction is shown below in Figure 8:

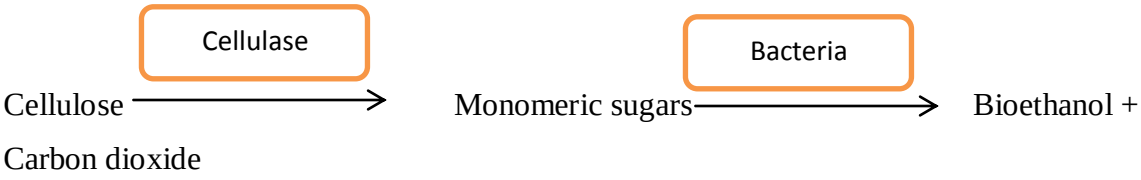

Figure 8: Reaction mechanism for Simultaneous Saccharification and Fermentation (SSF) method

When cellulose is being used as a raw material, the cellulase responsible for enzymatic hydrolysis of pretreated cellulosic biomass strongly inhibited by hydrolysis products: glucose and short cellulose chain. One of the techniques to overcome inhibition of

cellulase is to ferment the glucose to ethanol as soon as it appears in the reaction. Simultaneous saccharification and fermentation (SSF) combines enzymatic hydrolysis with ethanol fermentation to keep the concentration of glucose low. The accumulation of ethanol in the fermenter does not inhibit cellulase as it can remain high concentrations of glucose, so SSF is a good strategy for increasing the overall rate of cellulose to ethanol conversion.In comparison to the process where these two stages are sequential, means that separate saccharification and fermentation, the SSF method enables attainment of higher (up to 40%) yields of ethanol by removing end product inhibition, as well as by eliminating the need for separate reactors for saccharification and fermentation (Stenberg and Bollock *et al.* 2000). Moreover, this approach make a fermentation time shorter and reduced a risk of contamination with external microflora, due to high temperature of the process, the presence of ethanol in the reaction medium, and the anaerobic conditions (Wyman *et al.* 1994).

Ethanol production via fermentation is a complex biochemical process with yeast or bacteria utilizing fermentable sugars as substrate for their growth and converting them to ethanol, carbon dioxide and other metabolic end product. Several factors such as pH condition, temperature and amount of glucose that can affect ethanol fermentation should be considered. During ethanol fermentation, most of the yeast cells used suffers from various stresses, including environmental conditions such as glucose concentration, nutrient deficiency, temperature, rate of agitation and pH value (Graves *et al.*, 2006; Arisra *et al.*, 2008; Yah *et al.*, 2010).

The highest ethanol concentration was 10.29 gL^{-1} obtained when EFB hydrolysate was incubated at 30^{o}C, followed by 9.86 gL^{-1} at 35^{o}C which corresponded to an ethanol yield of 0.51 and 0.54 g ethanol/g glucose, respectively. Kheang reported that the highest ethanol yield was obtained at pH 4 with a maximum ethanol concentration of 10.29 gL^{-1} followed by 8.2 gL^{-1} ethanol at pH 6 at 72 hours of incubation. The highest ethanol fermentation efficiency was obtained at pH 4 with 119% (Kheang and Asyraf et al., 2011).

The focus of this study is to produce bioethanol using empty fruit bunch as raw material. The focus of the study is to determine the effect of the temperature, pH value and the FPU loading of the cellulose to the production of bioethanol. In this study, pure cellulose was used to study the effect of these different parameters to the amount of bioethanol produced. For simultaneous saccharification and fermentation, The EFB must be hydrolyzed first before fermentation process and the best pretreated substrates that can produce high amount of reducing sugars with higher percentage of saccharification will be used as a substrate for the fermentation process. Previous study indicates that simultaneous saccharification and fermentation (SSF) is better than separate saccharification and fermentation. Therefore, the experiment in this research is carried out using SSF method under anaerobic condition.

CHAPTER 3: METHODOLOGY

3.1 Measurement of Cellulase Activity

For this experiment, the method of Union of Pure and Applied Chemistry (IUPAC) guidelines is used[17][18]. The procedure has been designed to measure cellulase activity in terms of "filter paper units" (FPU) per milliliter of original (undiluted enzyme solution).

Filter Paper Unit (FPU) it a unit to measure the activity of a particular enzyme. One international Filter Paper Unit (FPU) was defined as the amount of enzyme that releases 1 μmol glucose per minute during hydrolysis reaction. Activities were reported as FPU/mL. Glucose equivalents (reducing sugars) generated during the assay were estimated by using the 3,5 dinitrosalicylic acid (DNS) method, with glucose as standard.

3.1.1 Preparation of D(+)glucose standard solution

The materials and apparatus used to prepare the standard solution are as followed:

3.1.1.1 Materials and Apparatus

1. D(+)glucose solution
2. Citrate buffer, pH 4.8
3. Filter paper strip Whatman No.1, 1.0 x 6.0 cm (≈50 mg)
4. 2 % DNS reagent
5. Test tubes
6. Hot plate
7. 1000 μL pipette
8. Ice water bath (0^oC- 2^oC)

[17] T.K. Ghose,. 1987.Measurement of Cellulase Activities. *Pure & Applied Chemistry*, 59 (2): 257-268.
[18] B.Adney., J.Baker.,1996. Measurement of Cellulase Activities, Laboratory Analytical Procedure (LAP). *National Renewable Energy Laboratory (NREL)*, pp 1-8

3.1.1.2 Procedure

The procedures for preparing the standard are as followed:

1. Prepare stock solution of 10 mg/mL anhydrous D(+)glucose. Dilute 1.0 g of D(+)glucose in 100 mL of DI water.
2. For dilutions:
   i. 1 ml + 0.5 ml buffer = 1:1.5 = 6.7 mg ml^{-1} (3.35 mg/0.5 ml)
   ii. 1 ml + 1.0 ml buffer = 1:2 = 5.0 mg ml^{-1} (2.5 mg/0.5 ml)
   iii. 1 ml + 2.0 ml buffer = 1:3 = 3.3 mg ml^{-1} (1.65 mg/0.5 ml)
   iv. 1 ml + 4.0 ml buffer = 1:5 = 2.0 mg ml^{-1} (1.0 mg/0.5 ml)
3. Take 0.5 mL of each standard and add 1.0 mL of citrate buffer.
4. Add 3.0 mL of DNS reagent to each tube.
5. Boil the samples at 100^{o}C for 5 minutes.
6. Quench the samples in ice water bath and add 2 mL of DI water into the each test tube.
7. Measure the absorbance of the samples by using UV-vis spectrophotometer at wavelength 540 nm and 575 nm.

3.1.2 FPU value for *T. reesei*

Enzyme was used for the FPU value experiments which are derived cellulase *Trichoderma reesei*. *T. reesei* is a mesophilic and filamentous fungus and capable to secrete large amounts of cellulolytic enzymes (cellulases and hemicellulases)[19].

3.1.2.1 Materials and Apparatus

The materials and apparatus used to prepare the standard solution are as followed:

1. Enzymes; *T. reesei* cellulase (Celluclast 1.5, Novozymes A/S Bagsvaerd, Denmark)
2. Filter paper strips, Whatman No.1 (1.0 cm x 6.0 cm)

[19]Kumar R, Singh S, Singh OV (May 2008). Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *J. Ind. Microbiol. Biotechnol.***35** (5): 377–91

3. Citrate buffer, pH 4.8
4. DNS reagent
5. Ice water bath
6. Test tubes
7. Hot plate with stirrer
8. UV-visible spectrophotometer (Model: UV-1601PC; Shimadzu)

3.1.2.2 Procedures

1. Prepare blank, control and stock solutions at different concentrations.
2. Blank = 1.5 mL citrate buffer
3. Control = 0.5 mL of enzyme solution with 1.0 mL of citrate buffer
4. Preparation of stock solutions at different concentrations (citrate buffer:stock solution).
   i) 5 mL : 5 mL = 0.0025 mg/0.5mL
   ii) 3 mL : 7 mL = 0.0035 mg/0.5mL
   iii) 2 mL : 8 mL = 0.004 mg/0.5mL
   iv) 7 mL : 3 mL = 0.0015 mg/0.5mL
   v) 8 mL : 2 mL = 0.001 mg/0.5mL
5. Add 0.5 mL of stock solutions with different concentrations into the test tubes containing the filter paper strips
6. Incubate all samples at 50^{o}C for one hour
7. Boil the samples for 5 minutes and quench them into the ice water bath
8. Add 2 mL of DI water into the samples
9. Analyze the samples with UV-visible spectrophotometer
10. Prepare the FPU loading for *T. reesei* cellulase at 1.0 mg/ml, 2.5 mg/mL, 5.0 mg/mL, 10 mg/mL and 20 mg/mL. Repeat the same procedures for *A. niger* cellulase.

3.2 High Performance Liquid Chromatography (HPLC)

Determination of oligosaccharides and mono saccharides coming from either acid hydrolysis or enzymatic hydrolysis of cellulose derived from varied means of chemical treatment can be determined using High Performance Liquid Chromatography (HPLC) with refractive index detector (RID). The choice of column for separation of these oligo – and mono – saccharides is of equal importance as retention time, resolution quality and limit of quantification will be highly dependent on it. For the both tasks i.e. enzymatic hydrolysis and separate saccharafication and fermentation (SSF) for ethanol production, several analytes has been identified. Table 2 below listed down all the possible analytes. One or more columns can be used to do such analysis.

Table 2: List of Potential Analytes

Task	Compounds
Enzymatic Hydrolysis	Glucose, fructose, mannose and xylose
Separate Saccharification and Fermentation	Ethanol, glucose, fructose, cellobiose, galactose, mannose, xylose, galactose and arabinose

Selection of column will be evaluated by several criteria: resolution (R_s), separation efficiency (actual vs. theoretical) (N), retention factor (k) and selectivity or separation factor (α). Separation efficiency (N) is the most common method of comparing different column with similar polymeric background (and counter cation). This is best expressed as theoretical plate number. The formula of how to calculate all these parameters are as follows:

$$Efficiency, N = 16\left(\frac{t_r}{w_t}\right)^2$$ Eq. (2)

$$Efficiency, N = 16\left(\frac{t_r}{w_t}\right)^2$$ Eq. (3)

With t_r is the retention time at peak, $w_t$is the peak width at base and $w_{1/2}$ is the width base at half height. The larger the value of N, the better is the separation efficiency. Equation (3) is better if the baseline is noisy, as this can eliminate the influence of noise.

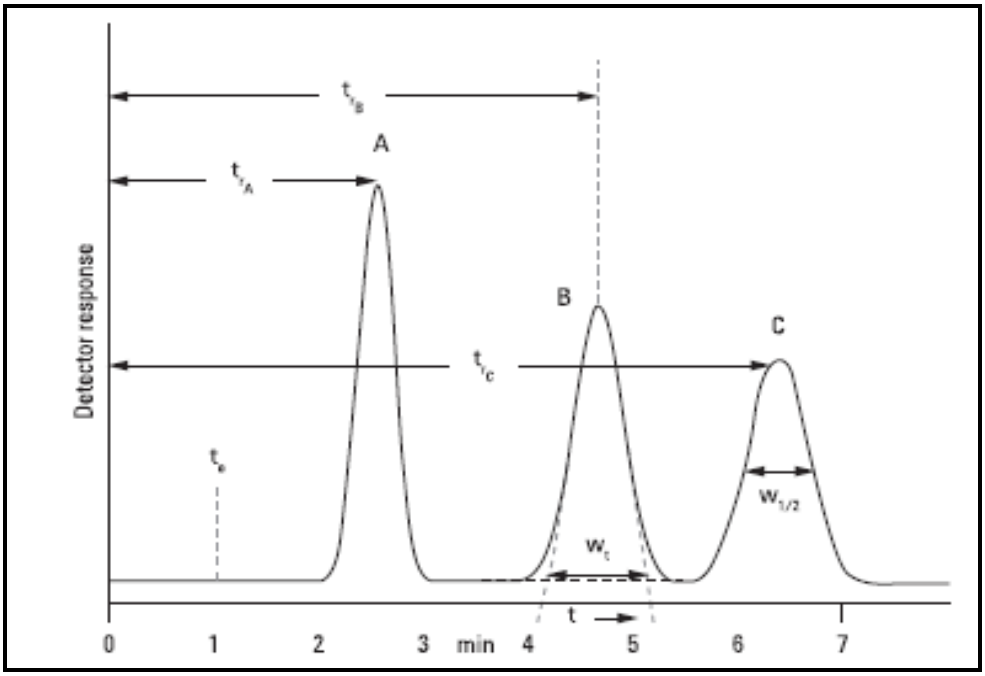

Figure 9: Representation of various processing parameters from an example chromatogram

Retention factor or k is for measuring the residence time for an analyte to reside in the stationary phase relative to its time in the mobile phase.

$$Efficiency, N = 16\left(\frac{t_r}{w_t}\right)^2 \quad \text{Eq. (4)}$$

Where t_0 is the retention time of unretained peak.f

Separation factor or α, is a measure of the time between two maxima of two different peaks. If $\alpha = 1$, the compounds have the same time and will co – elute which in this case is highly undesirable.

$$Efficiency, N = 16\left(\frac{t_r}{w_t}\right)^2 \quad \text{Eq. (5)}$$

Where k_2 is the retention factor of the second peak and k_1 is the retention factor of the first peak.

Resolution (R_s) describes the ability of the column in question to elute compounds of interest with respect to baseline. Resolution will take account of all the parameters above as shown in Equation (6).

$$Efficiency, N = 16\left(\frac{t_r}{w_t}\right)^2 \quad \text{Eq. (6)}$$

3.2.1 Materials and Method

All experiments for calibration curves development are done with Agilent High Performance Liquid Chromatography (HPLC) 1260 series equipped with Refractive

Index (RI) detector. All mobile phase solvent i.e. acetonitrile and deionized water will be filtered with glass fibre 0.2 µm filter and degassed for minimum of 20 minutes and of HPLC grade whenever possible. All external standards i.e. sugars (D – glucose, D – mannose, D – Galactose, D – cellobiose, Sucrose and D – xylose), sugar alcohols (Sorbitol, *meso* – erythritol, mannitol, and xylitol) and polyalcohol (glycerol, ethanol and 1,2 – propanediol)were obtained from Sigma Aldrich. D – arabinose and D – fructose were obtained from Bio Basic Canada Inc. Optical purity of the sugars are of D – variants unless stated otherwise.

Hi – Plex Ca and Hi – Plex Pb column (300 mm x 4.6 mm) were purchased from Agilent Technologies. Shodex Asahipak NH2P – 50 4E were purchased from Showa Denko Inc. Japan.

3.3 Enzymatic Hydrolysis

3.3.1 Substrates

Empty fruit bunches (EFB) of oil palm was used as raw material in this experiment. Processed milled palm oil empty fruit branches (EFB) fibres were obtained from Sze Tech Engineering Sdn Bhd located in Padang Jawa Selangor. The EFB fibres were grounded using grinding equipment in Tenaga Nasional Berhad (TNB) Research Bangi, with fibre length of 0.04 inch.

3.3.2 Delignification

Delignification is a process to remove lignin in the fibre. The presence of the lignin in cellulose substrates hinders the saccharification of them into monomeric sugars. To overcome the lignin barrier, lignocelluloses are usually pretreated initially with alkali to dissolve the lignin caused by the breakdown of ether linkage.

3.3.2.1 Materials and Apparatus

The following are the materials and apparatus used which are:

1. 0.2 M sodium hydroxide solution, NaOH
2. 10 g of EFB fibres
3. Deionized (DI) water
4. 250 mL conical flask
5. pH meter
6. Drying oven (with temperature control and display of 50±2°C)

3.3.2.2 Procedures

These are the procedures for delignification method

1. Soak 10 g of EFB fibres (substrate) into the 0.2 M NaOH solution in the ratio of 1:10.
2. Incubate the substrate at room temperature for 18 hours.
3. Filter the contents and wash the residue repeatedly with DI water until the pH of the residue become neutral (pH 7).
4. Dry the residue at 50^{o}C until the residue is completely dried.
5. Take half of dried alkali treated substrate to subsequently use for acid/enzyme hydrolysis experiments.

3.3.3 Acid Hydrolysis

Acid hydrolysis is a chemical process in which acid is used to convert cellulose or starch to the sugar. 3% of sulfuric acid was used in acid hydrolysis.

3.3.3.1 Materials and Apparatus

1. 3 % sulfuric acid, H_2SO_4
2. 5 g of alkali pretreated sample
3. Deionized (DI) water
4. 250 mL conical flask
5. Drying oven (with temperature control and display of 50±2°C)

3.3.3.2 Procedures

1. Suspend alkali pretreated sample into 3% of H_2SO_4 at temperature 130^{o}C for 30 minutes.
2. Filter the contents and wash the residue repeatedly with DI water until the pH of the residue become neutral.
3. Dry the residue at 50^{o}C
4. until the residue is completely dried.

3.3.4 Enzymatic Hydrolysis of Chemical Treated Empty Fruit Bunches (EFB)

Enzymatic hydrolysis is carried out by using cellulase from *Trichoderma reesei.* Alkali treated, acid hydrolysis and untreated were used as substrates for this experiment.

3.3.4.1 Materials and Apparatus

The following are the materials and apparatus used:

1. Pretreated substrates; alkali treated and acid hydrolysis
2. 0.05 M citrate buffer (pH 4.8)
3. 0.5 g of *Trichoderma reesei* cellulose
4. 0.005 g sodium azide
5. 250 mL conical flask
6. Electronic mass balance
7. Orbital shaker with incubator
8. Centrifuge
9. Test tubes

3.3.4.2 Procedures

1. Soak 1.0 g of each sample substrate with 100 mL of citrate buffer for two hours.
2. Hydrolyze the sample with 5 mg/mL of *T. reesei* with FPU loading at 128 FPU/g.
3. Add 0.005 g of sodium azide into the flask to prevent microbial or fungal contamination.
4. Incubate the sample at 50^{o}C on an orbital shaker at 150 rpm for 84 hours.
5. Take the sample aliquots of 3 mL every 12 hours.
6. Centrifuge the sample aliquots for 20 minutes at 1000 rpm.
7. Take 3 mL supernatant of the sample and run for analysis.
8. Analyze the supernatant by using 3,5- Dinitrosalicylic acid (DNS) method and High Performance Liquid Chromatography (HPLC) to get the amount of reducing sugars in acid hydrolyzate.

3.3 Fermentation Process

The fermentation process has been done using SSF method which is the combination of enzymatic hydrolysis with the fermentation process to produce bioethanol.

3.4.1 Growth media for the microorganism

Culture medium or growth medium is a liquid or gel aimed to support the growth of microorganisms or cells. Media act as a nutrient provider for the cells. There are different types of media for growing different types of cells. In this fermentation process, *Saccharomyces cerevisiae* ATCC 96581 was used as a microorganism to ferment glucose into bioethanol.

3.4.1.1 Broth Yeast extract-peptone-dextrose growth medium (YEPD medium)

The materials and apparatus used for preparing the growth media are:

3.4.1.1.1 Materials and Apparatus:

1. 10 g of Bacto™ Peptone
2. 5 g of yeast extract
3. 10 g of dextrose
4. 500 mL distilled water
5. Analytical balance
6. Autoclave
7. 1 L Schott bottle

3.4.1.1.2 Procedures:

1. Prepare the medium.
2. Dissolve them in 500 mL of distilled water.
3. Retain the medium in Schott bottle
4. Autoclave at 121^{o}C for 15 minutes.

3.4.1.2 Solid agar Yeast extract-peptone-dextrose growth medium (YEPD medium)

3.4.1.2.1 Materials and Apparatus:

1. 10 g of Bacto™ Peptone
2. 5 g of yeast extract
3. 10 g of dextrose
4. 10 g agar
5. 500 mL distilled water
6. Analytical balance
7. Autoclave
8. 1 L Schott bottle

3.4.1.2.2 Procedures:

1. Prepare the medium.
2. Dissolve them in 500 mL of distilled water.
3. Retain the medium in Schott bottle
4. Autoclave at 121^{o}C for 15 minutes.

3.4.2 Bacteria Culturing

Culturing is a method to grow bacteria on media containing nutrients. Bacteria can be grown either in broth (liquid) media or agar (solid) media. Visible colonies will be formed on the agar medium and turbidity of the broth medium can be clearly seen that indicates the growth of the bacteria.

3.4.2.1 YEPD broth medium

3.4.2.1.1 Materials and Apparatus

1. *Saccharomyces cerevisiae* ATCC 96581 ampoules
2. 10 mL YEPD medium
3. 70% ethanol solution
4. Micropipette
5. 250 mL conical flask
6. Bunsen burner
7. Incubator orbital shaker

3.4.2.1.2 Procedures

1. Immediately after thawing frozen ampoules, wipe down ampoule with 70% ethanol. This procedure need to be handled under laminar flow hood to prevent contamination.
2. Transfer 2 mL (or any amount desired) of the strain onto YEPD broth medium.
3. Incubate the strain at 30^{o}C with agitation speed of 190 rpm.
4. Prepare multiple amounts of flasks for culturing the strain.

3.4.2.2 YEPD solid agar medium

3.4.2.2.1 Materials and Apparatus:

1. *Saccharomyces cerevisiae* ATCC 96581 ampoules
2. YEPD agar medium
3. 70% ethanol solution
4. Micropipette
5. Hockey stick
6. Streak stick
7. 250 mL conical flask
8. Bunsen burner
9. Incubator orbital shaker

3.4.2.2.2 Procedures:

1. Pour 10 mL of YEPD agar medium into the petri plates. This procedure need to be handled under laminar flow hood to prevent contamination.
2. Apply ultraviolet rays onto the petri plates for 10 minutes.
3. Transfer 10 µL (or any amount desired) of the strain onto petri plates containing YEPD gar medium.
4. Spread the strains by using hockey stick or streak onto agar.
5. Incubate the strain at 30^{o}C with agitation speed of 190 rpm.

3.4.3 Growth profile of *Saccharomyces Cerevisiae* ATCC 96581

Bacterial growth is the division of one bacterium into two daughter cells in a process called binary fission. Bacterial growth over time can be graphed as cell number versus time. This is called a growth profile.

3.4.3.1 Materials and Apparatus:

1. 5 mL *Saccharomyces cerevisiae* ATCC 96581
2. 100 mL YEPD broth medium
3. 70% ethanol solution
4. 1 mL pipette

5. Bunsen burner
6. 250 mL conical flask
7. UV-Visible Spectrophotometer (Model: UV-1601PC; Shimadzu)

3.4.3.2 Procedures:

1. Pour 5 mL of inoculum strains into 100 mL of YEPD broth medium.
2. Incubate the strain at 30^{o}C with agitation speed of 190 rpm.
3. After 24 hours, take 1 mL of the sample for every two hours retention times.
4. Analyze the sample with UV-visible spectrophotometer to get the optical density or cell concentration of saccharomyces at wavelength 600 nm and 620 nm.

3.4.4 Inoculum Preparation for Fermentation

3.4.4.1 Materials and Apparatus:

1. *Saccharomyces cerevisiae* ATCC 96581
2. YEPD broth medium
3. 8.5 g/L saline solution
4. 1 mL pipette
5. Incubator orbital shaker
6. Centrifuge

3.4.4.2 Procedures:

1. Grow *Saccharomyces cerevisiae* ATCC 96581 on YEPD broth medium.
2. Incubate the inoculum at 30^{o}C with agitation speed of 190 rpm for 24 hours.
3. Centrifuge the inoculum at 3000 rpm for 15 minutes.
4. Rinse the pellet twice with sterilized saline solution before being re-suspended in sterilized saline solution to yield desired Optical Density (OD) value.
5. Take 1 mL of dilute inoculum and analyze using UV-visible spectrophotometer at wavelength 600 nm or 620 nm.
6. Use standardized S.*cerevisiae* for fermentation process.

3.4.4 Shaker Flask Preparation

The following are the steps and procedure for preparing shaker flask for fermentation.

1. Make cotton- gauze plugs to fit the mouth of 250 mL shaker flasks.
2. Plug the flask and cover the plug with a piece of aluminum foil before autoclaving. The aluminum foil will prevent dust from directly settling on the cotton plug while standing on the shelf waiting to be used. This is generally the case where many flasks are simultaneously autoclaved for later use.
3. After autoclaving the flasks, cool them to room temperature.

3.4.6 Separate Saccharification and Fermentation

3.4.6.1 Materials and Apparatus:

1. Avicel PH-101
2. Deionized water
3. *Trichoderma reesei*cellulose
4. *Saccharomyces* Cerevisiae ATCC 96581
5. Sodium hydroxide(NaOH) solution
6. 250 mL heavy-wall filtering flask
7. Gas-washing bottle, fritted cylinder
8. Retort stand with clamp
9. Orbital shaker
10. Thermometer
11. Electronic mass balance

3.4.6.1.1 Testing On Mass Loading of Cellulose to the Production of Bioethanol

3.4.6.1.1.1 Procedures:

1. Pour 100 mL of deionized water (pH 4) into the flask containing 1.0 g of acid hydrolyzate pretreated substrates.
2. Autoclave the samples at 121^{o}C for 15 minutes.

3. Cool down the flasks at room temperature.
4. Add 0.5 g of *T.reesei* cellulase with 128 FPU/mL.
5. Incubate the samples at 30^{o}C with rate of agitation of 100 rpm for 60 hours.
6. Add 3 mL of *S. cerevisiae* with OD 0.5.
7. Take the sample aliquots for every 12 hours retention times.
8. Centrifuge the sample aliquots at 3000 rpm for 15 minutes.
9. Take 1 mL of supernatant and analyze using High Performance Liquid Chromatography (HPLC)
10. Record the area of the compounds exist and calculate the amount of ethanol and reducing sugars produced.
11. Repeat step 1 to 10 by varying the mass loading of cellulose which are 0.5g, 1.0g, 2.0g and 5.0g.

3.4.6.1.2 Testing on pH Value to The Production of Bioethanol

3.4.6.1.2.1 Procedures:

1. Pour 100 mL of deionized water (pH 4) into the flask containing 1.0 g of acid hydrolyzate pretreated substrates.
2. Autoclave the samples at 121^{o}C for 15 minutes.
3. Cool down the flasks at room temperature.
4. Add 0.5 g of T.reesei cellulase with 128 FPU/mL.
5. Incubate the samples at 30^{o}C with rate of agitation of 100 rpm for 72 hours.
6. Add 3 mL of *S. cerevisiae* with OD 0.5.
7. Take the sample aliquots for every six hours retention times.
8. Centrifuge the sample aliquots at 3000 rpm for 15 minutes.
9. Take 1 mL of supernatant and analyze using High Performance Liquid Chromatography (HPLC)
10. Record the area of the compounds exist and calculate the amount of ethanol and reducing sugars produced.
11. Repeat step 1 to 10 by varying the pH value of the medium, 6, 7 and 8

3.4.6.1.3 Effect of FPU Value to the Production of Bioethanol

3.4.6.1.3.1 Procedures

1. Pour 100 mL of deionized water (pH 4) into the flask containing 1.0 g of acid hydrolyzate pretreated substrates.
2. Autoclave the samples at 121^{o}C for 15 minutes.
3. Cool down the flasks at room temperature.
4. Add 0.5 g of *T.reesei* cellulase with 128 FPU/mL.
5. Incubate the samples at 30^{o}C with rate of agitation of 100 rpm for 60 hours.
6. Add 3 mL of *S. cerevisiae* with OD 1.0
7. Take the sample aliquots for every twelve hours retention times.
8. Centrifuge the sample aliquots at 3000 rpm for 15 minutes.
9. Take 1 mL of supernatant and analyze using High Performance Liquid Chromatography (HPLC)
10. Record the area of the compounds exist and calculate the amount of ethanol and reducing sugars produced.
11. Repeat step 1 to 10 by varying the FPU value, 77 FPU/ml, 154 FPU/ml, 218 FPU/ml.

3.4.7 Simultaneous Saccharification and Fermentation

3.4.7.1 Materials and Apparatus

1. Acid hydrolyzate pretreated EFB
2. Deionized water (pH 3.5)
3. 0.5 g *Trichoderma reesei*cellulose
4. Saccharomyces Cerevisiae ATCC 96581
5. Sodium hydroxide, NaOH
6. 250 mL heavy-wall filtering flask
7. Gas-washing bottle, fritted cylinder
8. Retort stand with clamp
9. Orbital shaker
10. Thermometer

3.4.7.2 Procedures

12. Pour 100 mL of deionized water (pH 3.5) into the flask containing 1.0 g of acid hydrolyzate pretreated substrates.
13. Autoclave the samples at 121^{o}C for 15 minutes.
14. Cool down the flasks at room temperature.
15. Add 0.5 g of T.reesei cellulase with 128 FPU/mL and 3 mL of *S. cerevisiae* with OD 0.5.
16. Incubate the samples at 30^{o}C with rate of agitation of 100 rpm for 72 hours.
17. Take the sample aliquots for every six hours retention times.
18. Centrifuge the sample aliquots at 3000 rpm for 15 minutes.
19. Take 1 mL of supernatant and analyze using High Performance Liquid Chromatography (HPLC)
20. Record the area of the compounds exist and calculate the amount of ethanol and reducing sugars produced.

3.4.8 Analysis Method

There are two methods used in this experiment to qualify and quantify the amount of reducing sugars produced for enzymatic hydrolysis process. The analysis method used are by using High Performance Liquid Chromatography (HPLC) and 3, 5 Dinitrosalicyclic acid (DNS) method.

3.4.8.1 Calibration curves of Total Reducing Sugars using HPLC Method

All experiments for calibration curves development are done with Agilent High Performance Liquid Chromatography (HPLC) 1260 series equipped with Refractive Index (RI) detector. All mobile phase solvent i.e. acetonitrile and deionized water will be filtered with glass fibre 0.2 µm filter and degassed for minimum of 20 minutes and of HPLC grade whenever possible.

3.4.8.1.1 Materials and apparatus:

1. D(+) glucose and D(+) fructose
2. Analytical balance
3. Test tubes
4. Micropipette – 100-1000µL

3.4.8.1.2 Procedures:

1. Prepare D(+) glucose solution with concentration of 0.02 wt% up to 5.0 wt%. Prepare double standard solution for each concentration.
2. For each concentration, take 1.0mL of glucose solution and put into sample bottle.
3. Run the sample with HPLC method under LC conditions (refer table 2)
4. Record the area of the sample at various concentrations and draw calibration curve.
5. Repeat step 1 to 4 using D(+) fructose.
6.

3.4.8.2 Analysis of Enzymatic Hydrolysis using HPLC Method

The supernatant of the samples; alkali treated, untreated and acid hydrolysis were analyzed by using HPLC method to determine the amount of reducing sugars formed. Table 3 shows the liquid conditions for HPLC

Table 3: Liquid Chromatography (LC) conditions of HPLC

Parameter	Value
Instrument	High performance liquid chromatography, HPLC
Mobile phase	75% acetonitrile and 25% water
Column	Shodex Asahipack, NP2-50
Column temperature	50^{o}C
Flow rate	1.0 mL/min

Detectors	Refractive index at 30^{o}C
Injection volume	6.0 µL
System pressure	65 bars (maximum: 100 bar)

3.4.8.3 Analysis of Enzymatic Hydrolysis Using 3, 5 - Dinitrosalycylic acid (DNS) Method

3.4.8.3.1 Preparation of 2% DNS Reagent

3.4.8.3.1.1 Materials and Apparatus:

1. 3, 5 - Dinitrosalicyclic acid powder (CAS No. 609-99-4)
2. Deionized (DI) water
3. Sodium hydroxide (NaOH) pellets
4. Potassium sodium tartarate (Rochelle salt) (Na-K tartrate)
5. Analytical balance
6. Volumetric flask, 1000mL
7. Aluminum foil
8. Hot plate with stirrer
9. 1 L Schott bottle

3.4.8.3.1.2 Procedures:

1. Prepare 10.67 w/v % sodium hydroxide solutions. Weigh 16 g of NaOH pellets to 150 mL of deionized water.
2. Add 10.0 g of DNS acid powder into the 500 mL of DI water in the volumetric flask.
3. Mix the solution with 100 mL of 10.67 w/v% sodium hydroxide solution.
4. Place the solution into the 45^{o}C water bath until the contents are fully dissolved.
5. Add 300 g of potassium sodium tartarate with continuous mixing.

6. Add DI water to bring the total volume of reagent up to 1.0 L

7. Transfer the reagent to the Schott bottle and wrap the Schott bottle with aluminum foil as DNS reagent is very sensitive to light.

3.4.8.3.2 Calibration curves of Total Reducing Sugars by using DNS method

UV- Visible spectrophotometer was used to determine the calibration curves of total reducing sugars using D(+)glucose as model compound by using DNS method.

3.4.8.3.2.1 Materials and Apparatus:

1. D(+)glucose
2. Deionized (DI) water
3. DNS reagent
4. Oil bath
5. UV-Visible Spectrophotometer (Model: UV-1601PC; Shimadzu)
6. Analytical balance
7. Test tubes
8. Micropipette (100-1000 µL)
9. Hot plate with stirrer

3.4.8.3.2.2 Procedures:

1. Prepare D(+) glucose with different concentration ranging from 0.1 mg/ml to 1.5 mg/ml (a minimum of 5 concentrations)
2. Prepare duplicate solution for each concentration.
3. For each concentration, take 1.0 mL of D(+) glucose solution and add with 3.0mL of DNS reagent.
4. Boil for 5 minutes in the oil bath at 100^{o}C.
5. Quench the samples in ice water bath and add 2 mL of DI water.
6. Measure the absorbance for each concentration using UV-visible spectrophotometer at the wavelength 540 nm and 575 nm.

3.4.8.3.4 Analysis of Enzymatic Hydrolysis using DNS method

The supernatant from each enzymatic hydrolysis sample will be analyzed using the DNS method.

3.4.8.3.4.1 Procedures

1. Take 1 mL of supernatant from the four samples which are untreated, alkali treated, acid hydrolysis and ionic liquid treated (washed and unwashed)
2. Add 3 mL of DNS reagent into the each test tube.
3. Boil the samples for 5 minutes at 100^{o}C.
4. Quench the samples in the ice water bath and add 2 mL of DI water.
5. Measure the absorbance of the samples by using UV-vis spectrophotometer at wavelength 540 nm and 575 nm.

CHAPTER 4: RESULT AND DISCUSSION

4.1 Enzymatic Hydrolysis Using Different Pretreated Samples

For enzymatic hydrolysis, derived *Trichoderma reesei* cellulase was used throughout the process.

4.1.1 Calibration Curves of Total Reducing Sugars using DNS Method

3,5-dinitrosalicyclic acid (DNS) method test the presence of free carbonyl group (C =O), so called reducing sugars such as glucose and fructose. This involves the oxidation of aldehyde functional group present in ketone. Simultaneously, DNS is reduced to 3-amino, 5-nitrosalicyclic acid under alkaline condition since pH value of DNS reagent is 13.2.

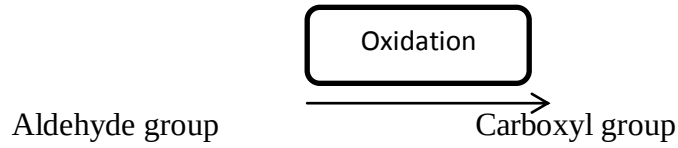

Figure 10: Oxidation reaction of aldehyde group to form carboxyl group

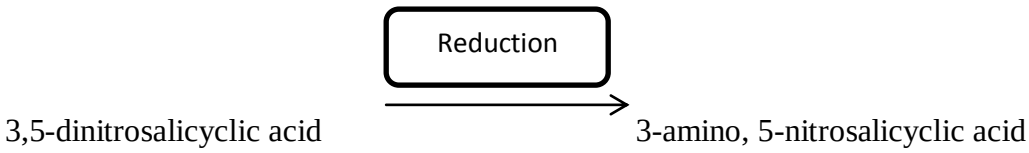

Figure 11: Reduction of 3,5-dinitrosalicyclic acid to form 3-amino,5-nitrosalicyclic acid

Monosaccharide group converts the oxidized form of DNS to reduced form which absorbs at 540 nm and 575 nm. The color conversion reaction is changed from yellow to red brown. UV-visible spectrophotometer will detect the chromophore of 3-amino, 5-nitrosalicyclic acid. The absorbance value determined from the spectrophotometer

shows the amount of reduced DNS which directly related to the amount of reducing sugars.

Figure 11 shows the reduction of glucose (sugars) by DNS to yield 3-amino-5-nitrosalicyclic acid which is in red-brown colored. Aromatic amino acid has a strong absorbance of light at wavelength 540 nm and 575 nm.

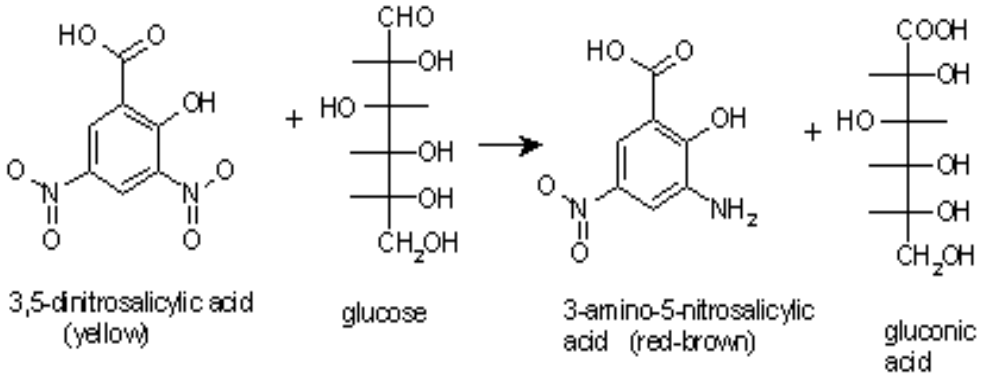

Figure 12: Reaction mechanism for reducing sugars

Figure 13 shows the calibration curves of D(+)glucose at two different wavelengths. The calibration curve will be used to determine the concentration of reducing sugars from the unknown sample by extrapolating the absorbance of the unknown sample with the calibration curve. Based on the graph, both wavelengths at 540 nm and 575 nm can be used with good confidence; RSD < 0.01.

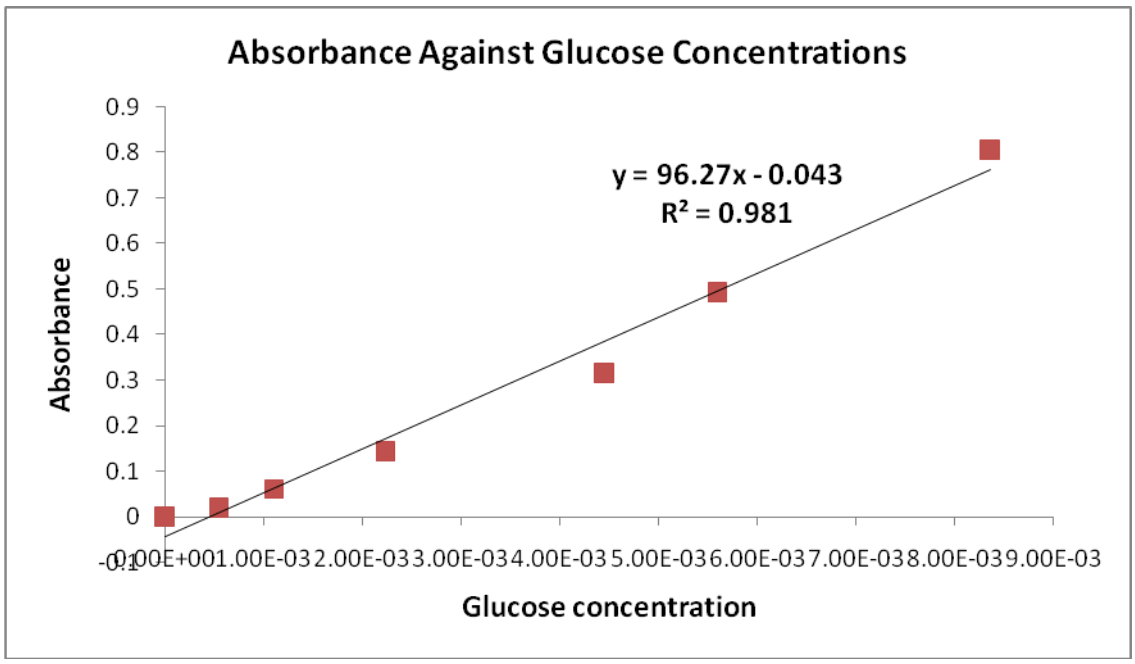

Figure 13: Calibration curves for total reducing sugars using glucose as model compound at 575 nm using UV-Vis Spectrophotometer

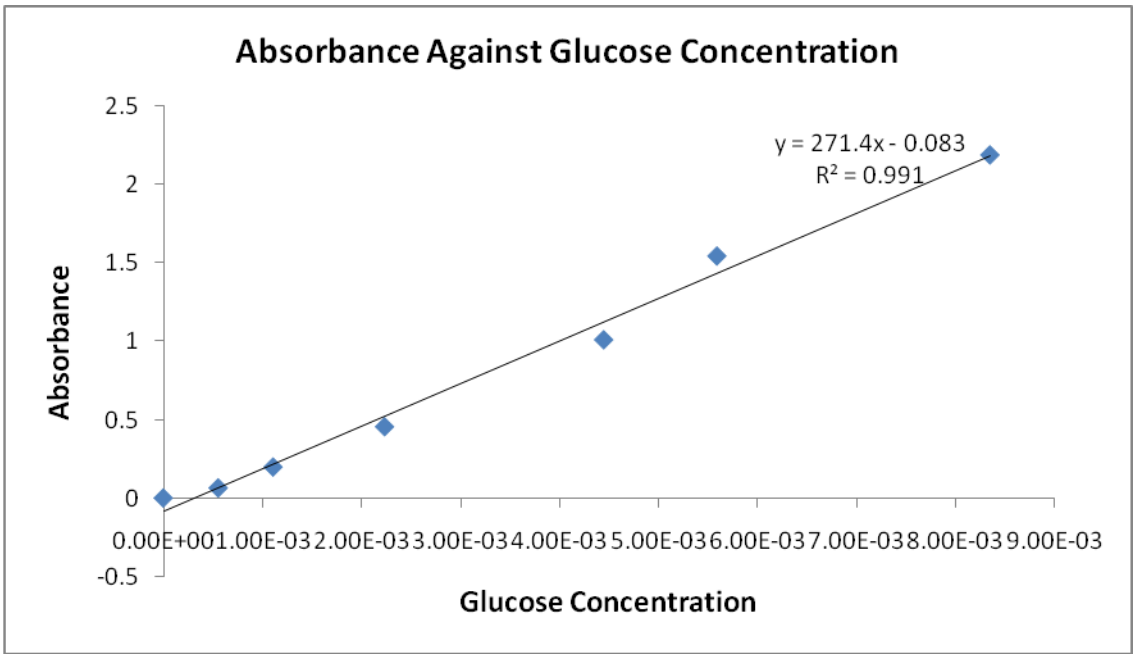

Figure 14: Calibration curves for total reducing sugars using glucose as model compound at 540 nm using UV-Vis Spectrophotometer

4.1.2 Enzymatic Hydrolysis using DNS Method

Figure 15 shows the color of each pretreated samples before reaction while figure 16 shows the color of the pretreated samples after the reaction. There are color changes after the reaction for acid hydrolysis, alkali treated and untreated pretreatment samples. The color intensity would depend on the amount of reducing sugars produced. Acid hydrolysis and alkali pretreated samples show high intensity of red brown color compared to untreated sample.

The following graph (Figure 17) shows the results of reducing sugars in acid hydrolyzate by using DNS method from enzymatic hydrolysis of untreated, alkali treated and acid hydrolysis. Based on the graph, the maximum sugar yield per substrate for each pretreatment sample were acid hydrolysis with 5.26 mmol/mL, alkali treated with 4.35 mmol/mL and finally the untreated substrate with 1.73 mmol/mL. Acid hydrolysis pretreatment with 3% of sulfuric acid shows the good trend and had increased the total reducing sugar in acid hydrolyzate by time followed by alkali treated.

Effective delignifying agent should remove a maximum of lignin and minimum of sugars[20].However, for ionic liquid treated; washed samples gave the lower value of reducing sugar compared to the unwashed samples. Furthermore, washed sample also gave lower value compared to untreated samples. It shows that THPC was may not suitable chemical for delignification of EFB. For unwashed sample, the higher value obtained might be instigated by the interaction of DNS reagent with the cation in THPC. In addition, there would be presence of inhibitors in the reaction that consequently slow down or stop the analysis. Hence, further analysis to investigate the inhibitors will be conducted to identify the exact inhibitor.

The percentage of saccharification or percentage of hydrolysis can be calculated by dividing the reducing sugars in acid hydrolyzate with total holocellulose in pretreated

[20] Taherzadeh MJ, Karimi K (2007) Acid based hydrolysis process for bioethanol production from lignocellulosic material: a review. Bioresources 2: 472-499.

samples. Different pretreated sample will have different holocellulose contents including the cellulose, hemicellulose, lignin and ash. Therefore, total holocellulose for each pretreatment samples need to be determined before the percentage of saccharification can be obtained. In the other way, the percentage of saccharification (percentage of hydrolysis) can also be calculated by using equation provided (Ghose, 1987).

$$\%\ Saccharification = \frac{reducing\ sugar\ \left(\frac{mg}{mL}\right)\ x\ 0.9\ x\ 100}{initial\ substrate\ \left(\frac{mg}{mL}\right)}$$

Based on Figure 18, the highest percentage of saccharification for each pretreatment sample were obtained from acid hydrolysis with 47.37% followed by alkali treated with 39.11% and finally untreated substrates with 15.59%.

Kinetic rate of reaction of enzyme in acid hydrolysis is higher compared to other substrates. The amount of reducing sugars produced for acid hydrolysis increases with time. At 72 hours to 84 hours, there was only small increment for every substrate as time proceeds. It shows that there was a limitation of substrates for the reaction to proceed. For the improvement of enzymatic hydrolysis, it is necessary to optimize the critical process parameters such as optimum cellulase loading, temperature, saccharification time and substrate to liquid ratio.

Making cellulose accessible to the enzymes is an important factor to increase the rate of hydrolysis[21]. Therefore, chemical pretreatment, usually alkaline and acid hydrolysis pretreatment are necessary before enzymatic hydrolysis. Chemical pretreatment not only removes lignin but also acts as a swelling agent, which will enhance surface area of the substrate accessible for enzymatic action[22].

[21] Phillippids, G. P. and Smith, T. K. (1995) Limiting factors in the simultaneous saccharification and fermentation process for conversion of cellulosic biomass to fuel ethanol. *Applied Biochemistry Biotechnology*. **51(52)**:117-124.

[2222] Kim, H. T., Kim, J. S., Sunwoo C. and Lee, Y. Y. (2008) Pre-treatment of corn stover by aqueous ammonia. *Bioresource Technology*. **90**:39-47.

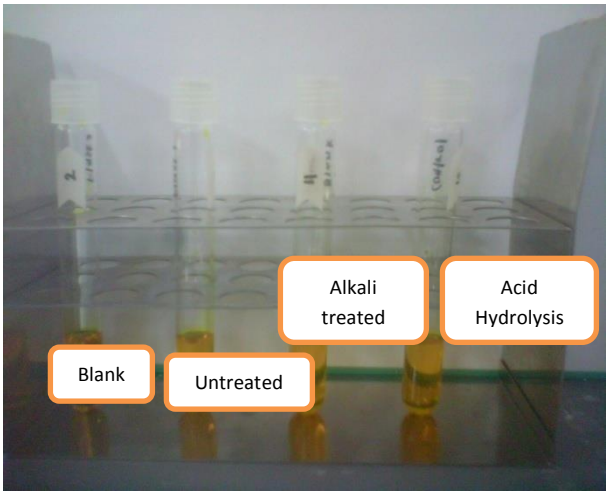

Figure 15: Pretreated samples before reaction

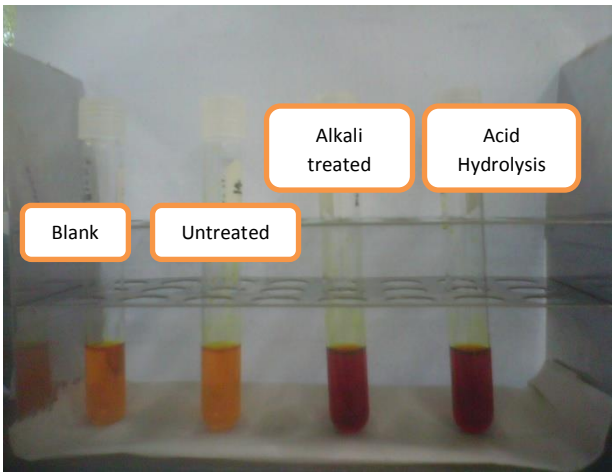

Figure 16: Pretreated samples after reaction

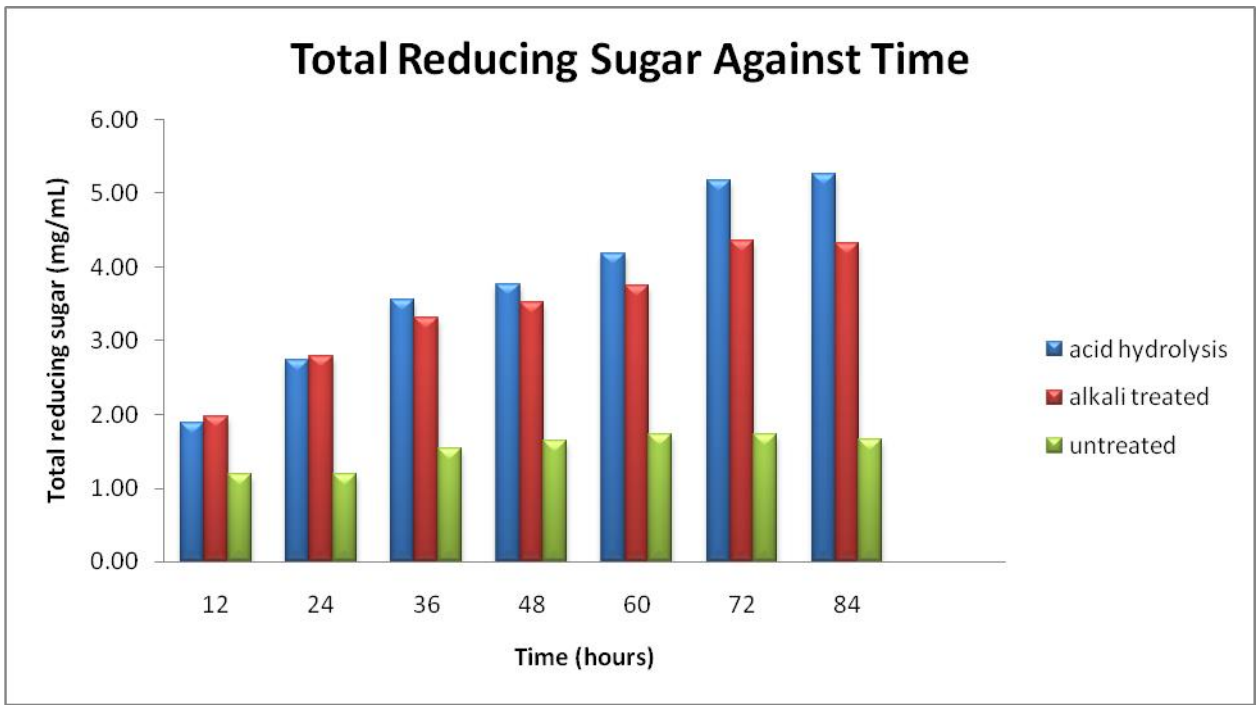

Figure 17: Total amount of reducing sugars in acid hydrolyzate using DNS method for every treatment from 12 hours to 84 hours

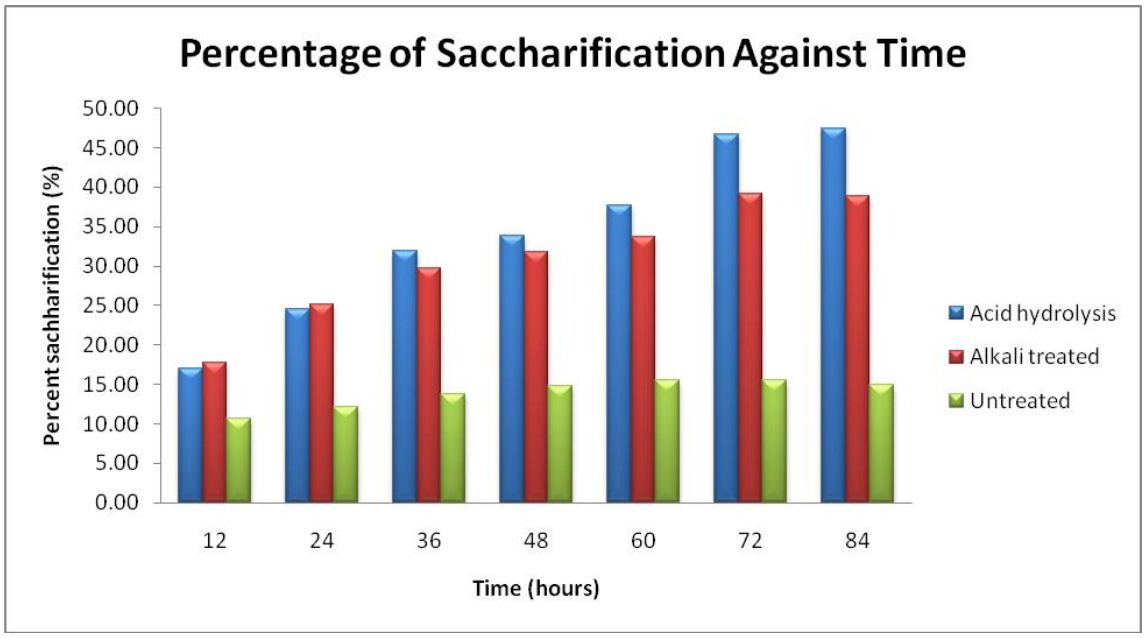

Figure 18: Percentage of saccharification in acid hydrolyzate using DNS method for every treatment from 12 hours to 84 hours

4.1.3 High Performance Liquid Chromatography (HPLC) Method

Determination of oligosaccharides and mono saccharides coming from either acid hydrolysis or enzymatic hydrolysis of cellulose derived from different types of chemical treatment can be determined using High Performance Liquid Chromatography (HPLC) with refractive index detector (RID). The choice of column for separation of these oligo – and mono – saccharides is of equal significance as retention time, resolution quality and limit of quantification will be highly dependent on it.

4.1.3.1 Calibration Curves of Total Reducing Sugars Using HPLC Method

Calibration curve is the general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of a known concentration. Figure 19 and figure 20 shows the calibration curve of D(+) glucose and D(+) fructose. Highest regression analyses (R^2) were found for both calibration curves. Therefore, the calculation for the total amount of reducing sugars for HPLC method is based on the specified calibration curve.

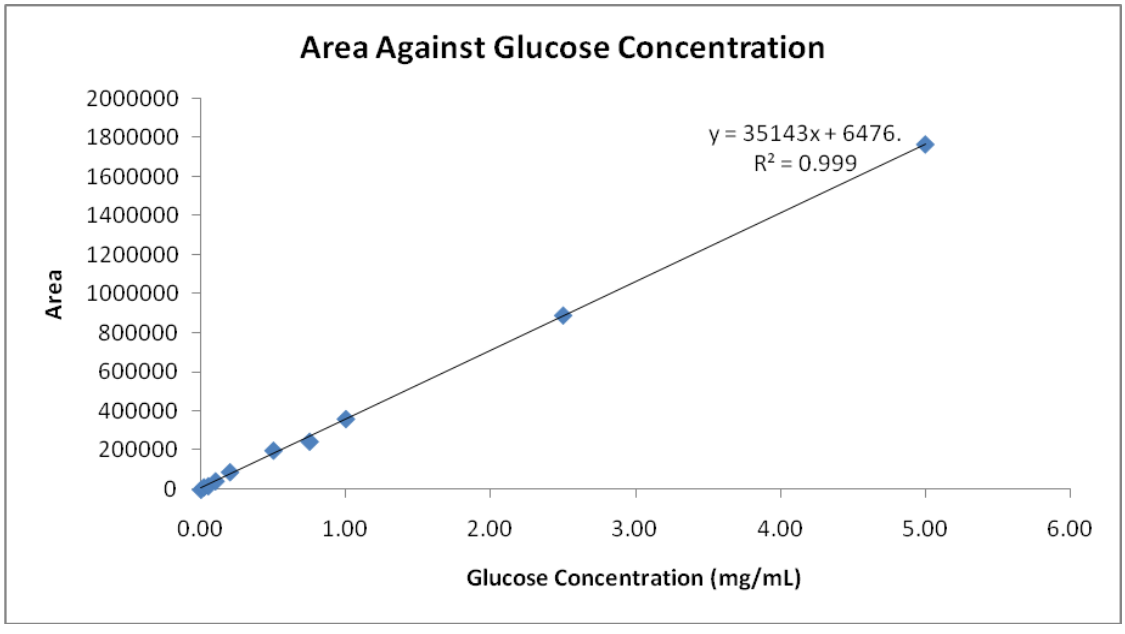

Figure 19: Calibration curve of D(+)glucose using HPLC

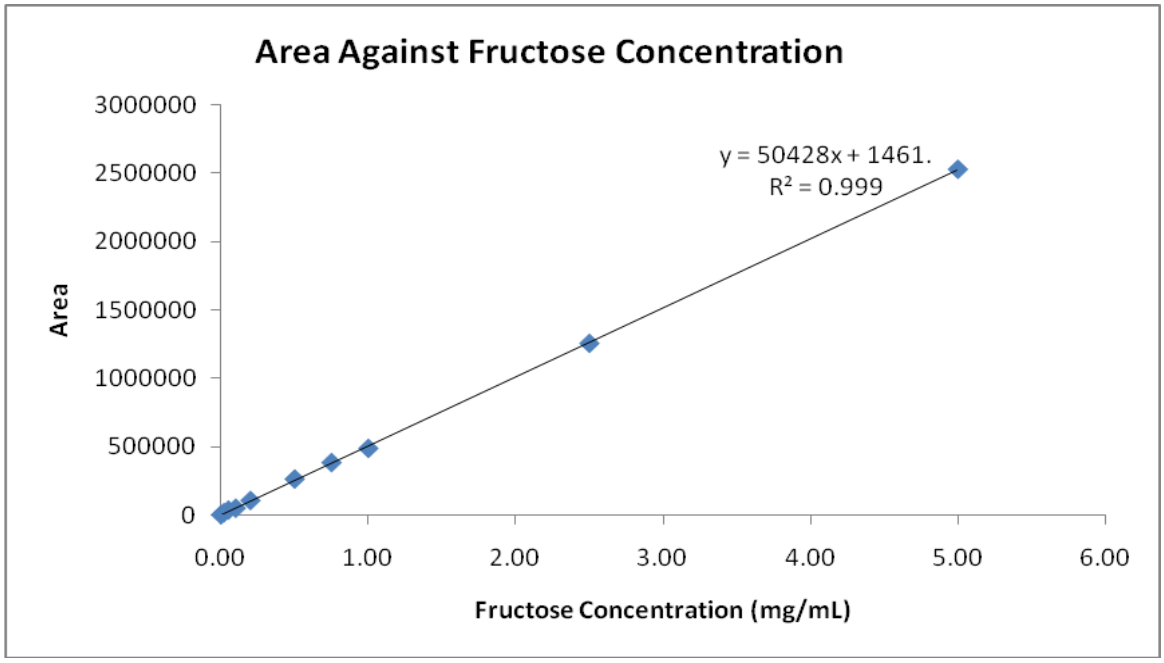

Figure 20: Calibration curve of D(+)fructose using HPLC

4.1.3.2 Analysis of Enzymatic Hydrolysis using HPLC Method

The graph (Figure 21) show the results of total reducing sugars in acid hydrolyzate by using HPLC method from enzymatic hydrolysis of untreated, alkali treated and acid hydrolysis. Based on this method, the results for all pretreated samples were not correlated with the results from DNS method. This is due to the improper keeping of the samples for analysis. Hence, the experiments need to be repeated to find the accurate results.

The common sugars obtained from HPLC quantification were glucose and fructose. These two compounds were detected at different retention time, the time it takes for that specific compound to elute from the column after injection. Glucose was detected at 7.4 minutes while fructose was detected at 5.8 minutes. Monomeric sugars are quantified by HPLC with refractive index detection. Enzymatic hydrolysis will produce reducing sugars which is hexoses sugars; glucose and fructose. Therefore, by using the standard calibration curve of glucose and fructose in HPLC, determination of reducing sugars

concentration from unknown samples can be obtained. In this method the area of the unknown sample will be extrapolated from the calibration curve.

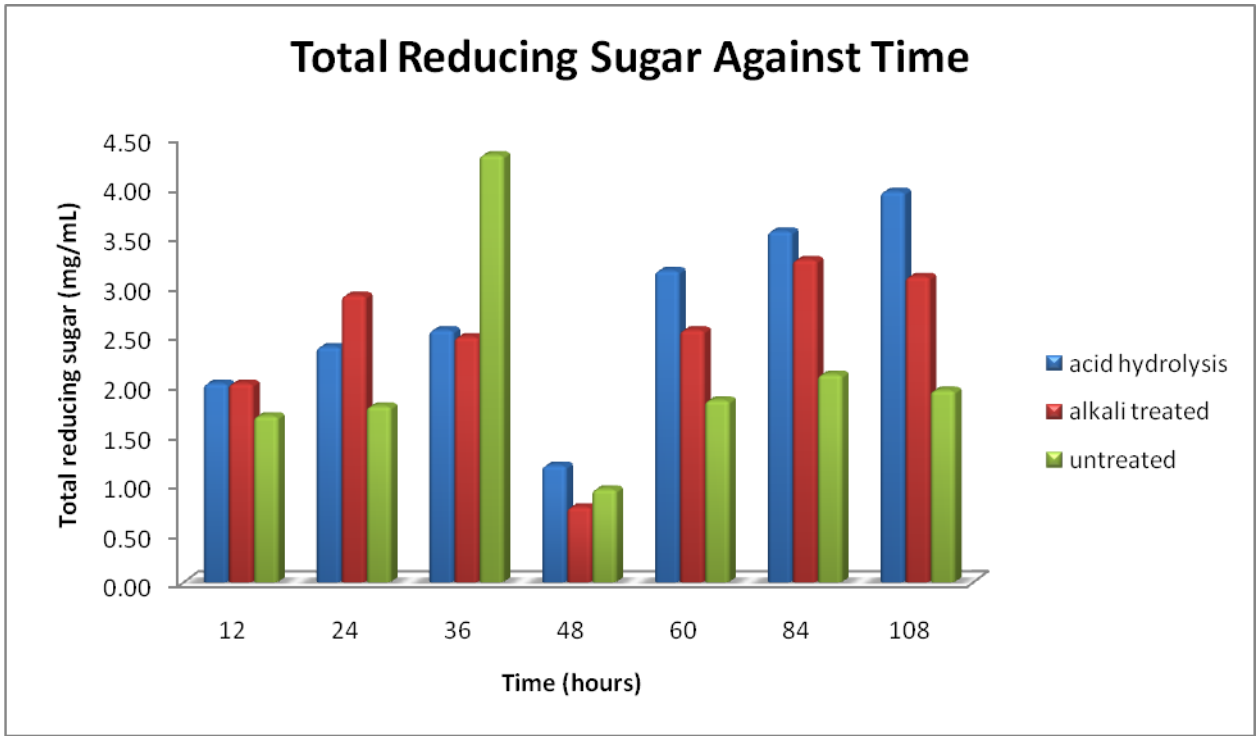

Figure 21: Total amount of reducing sugars in acid hydrolyzate using HPLC method for every treatment from 12 hours to 84 hours

Figure 22 shows the percentage of saccharification for each pretreated samples by using HPLC method. Based on inaccurate results obtained from total reducing sugar, the percentage of saccharification will be also affected. In conjunction, to obtain the percentage of saccharification, the experimentation for enzymatic hydrolysis using HPLC method need to be repeated.

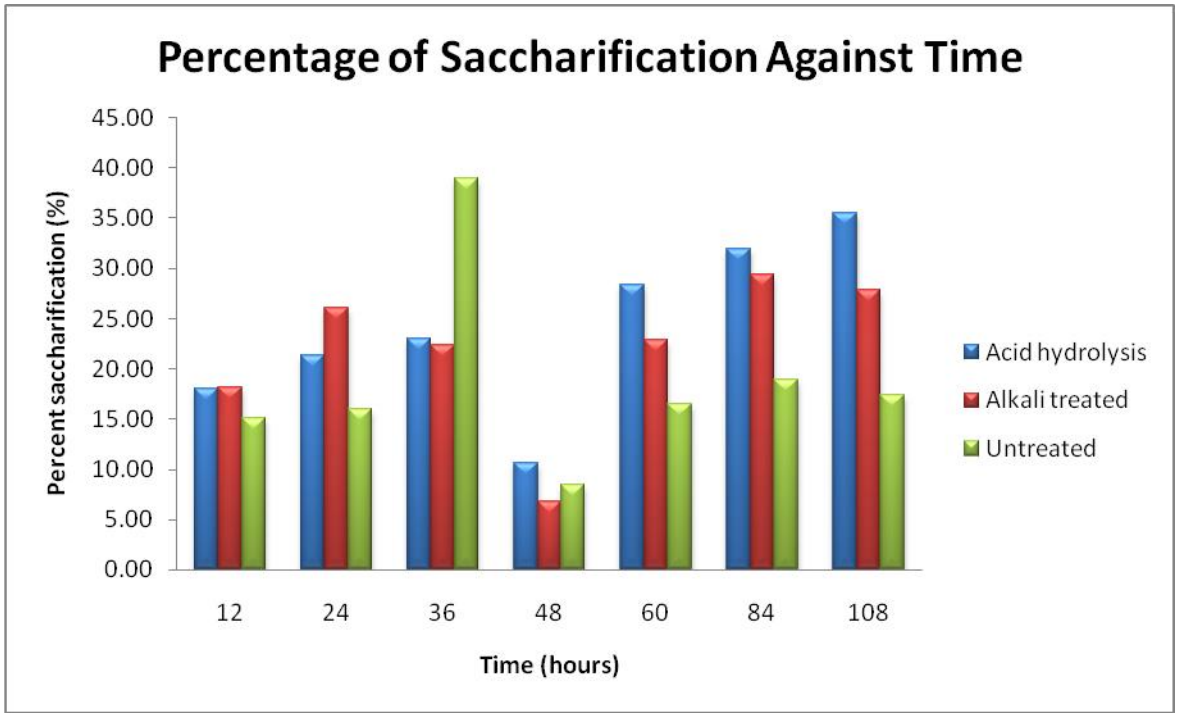

Figure 22: Percentage of saccharification in acid hydrolyzate using HPLC method for every treatment from 12 hours to 84 hours

The results for enzymatic hydrolysis were different by using DNS method and HPLC method. DNS method could measure any reducing compounds based on its reaction mechanism and widely used with its simplicity and inexpensive reagents. Based on the both graphs, DNS method shows the good trend of results with most of the pretreatment samples gave the increasing total reducing sugars by time. For HPLC method, the results were not accurate because of the unsuitable condition for samples storage before analysis. The sample aliquots need to be analyzed immediately or else the aliquots need to be frozen to retard the enzymatic reaction. Frozen supernatants need to be thawed and vortex before the HPLC analysis.

Theoretically, HPLC allows analysis to be done in a shorter time and achieves a higher degree of resolution, that is, the separation of constituents is more complete and the results of analysis are more highly reproducible. For DNS method, it is plagued by long assay times, exacting dilutions and many manual manipulations such as different mass of filter paper strips. It is valid only at low levels of hydrolysis and generally

requires several iterations to ensure the valid activities measurement. Furthermore, for DNS method, at lower concentration of reducing sugars, it could be limit of quantification (LOQ) in which the analyte cannot only reliably detected but at which some predefined areas for bias and imprecision are met.

4.2 Measurement of Cellulase Activity by using UV-Visible Spectrophotometer

4.2.1 Calibration Data

Figure 23 and figure 24 show the FPU assay value for glucose at different concentrations. **T**he regression analysis (R^2) in the graph gave the good confidence and calibration. If the value of R^2 is closer to 1, the variables in this study can be deduced to be strongly correlated. The calibration curve of FPU assay at wavelength 575 nm show high confidence value compared to wavelength 540 nm. Therefore,the calculation for the FPU value of *T. reesei* cellulase and *A. niger* cellulase were based on the wavelength at 575 nm

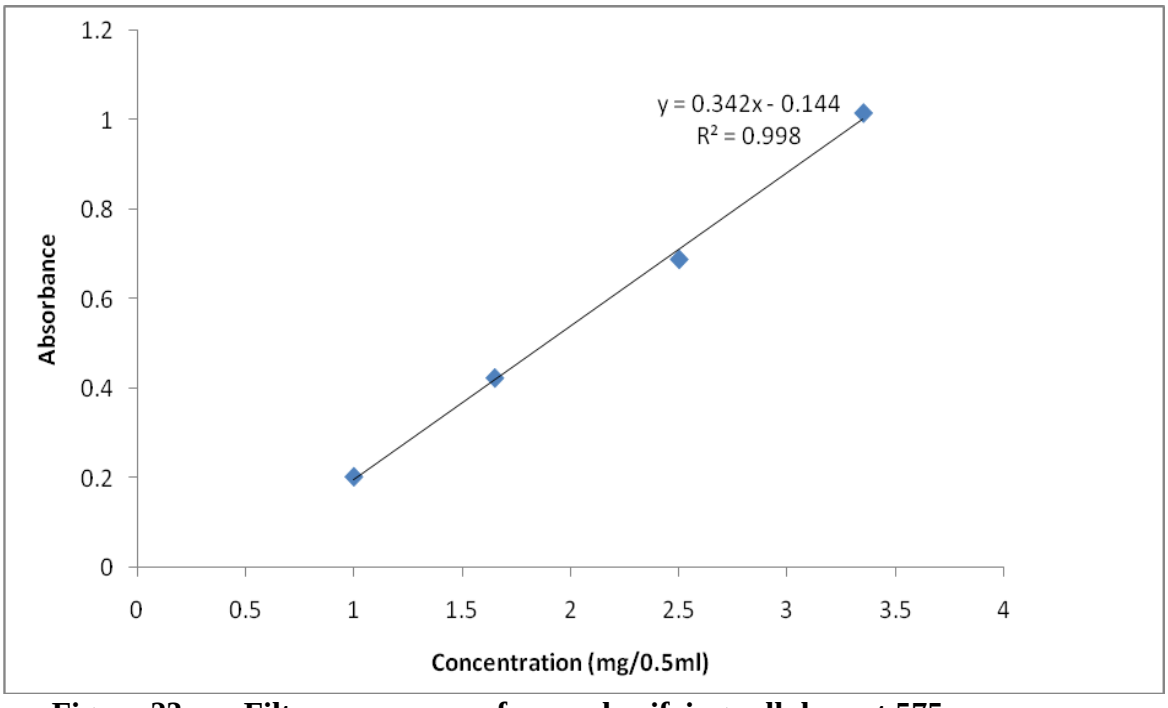

Figure 23: Filter paper assay for saccharifying cellulose at 575 nm

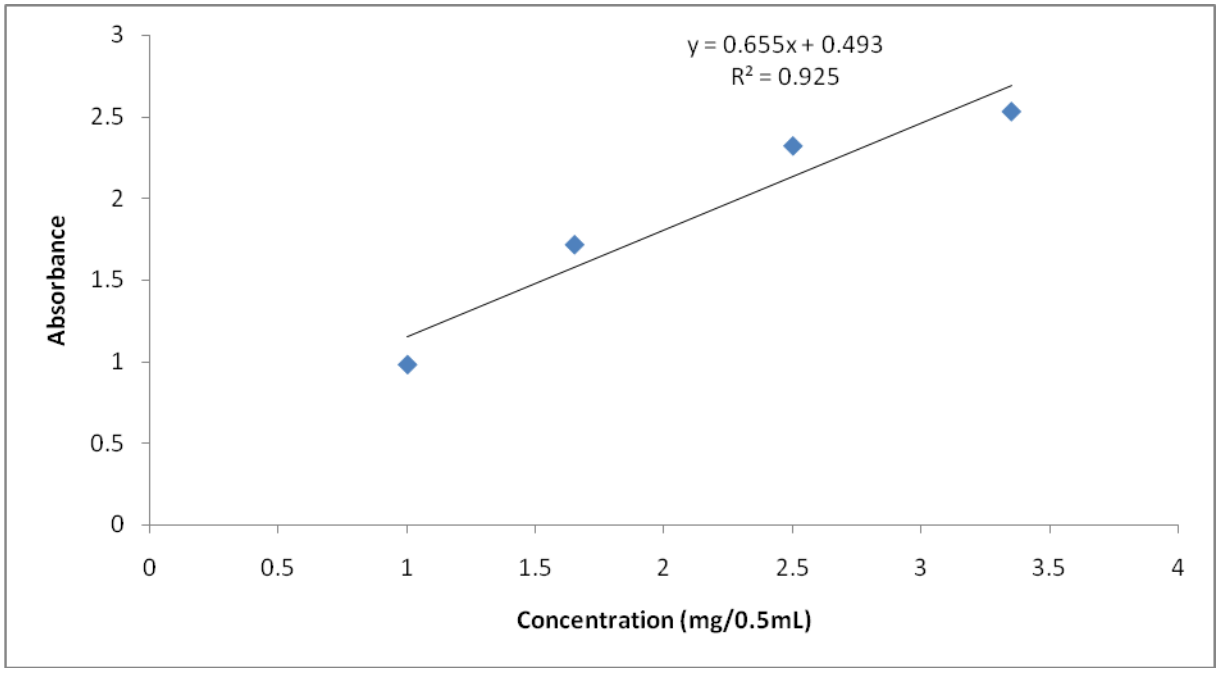

Figure 24: Filter paper assay for saccharifying cellulose at 540 nm

4.2.2 FPU Value of derived *Trichoderma reesei* celullase loading

For FPU value of *T. reesei*, various concentrations of enzyme loading has been completed which were at 1.0 mg/mL to 20 mg/mL. Based on Figure 25, the highest FPU value of the cellulose was at concentrations of cellulase loading at 20 mg/mL. High concentration of cellulase loading should be continued to obtain the optimum loading (highest FPU value). It is necessary to optimize the process parameter such as optimum cellulase loading for the improvement of enzymatic hydrolysis. An optimum enzyme concentration is required to hydrolyze the cellulose into glucose and the sugar yield would be increased. Therefore, the effect of enzyme loading on the enzymatic hydrolysis of pretreated samples must be studied. However, FPU value is not a linear function of the quantity of enzyme loading in the assay mixture; twice the amount of enzyme loading would not be expected to yield twice the FPU value.

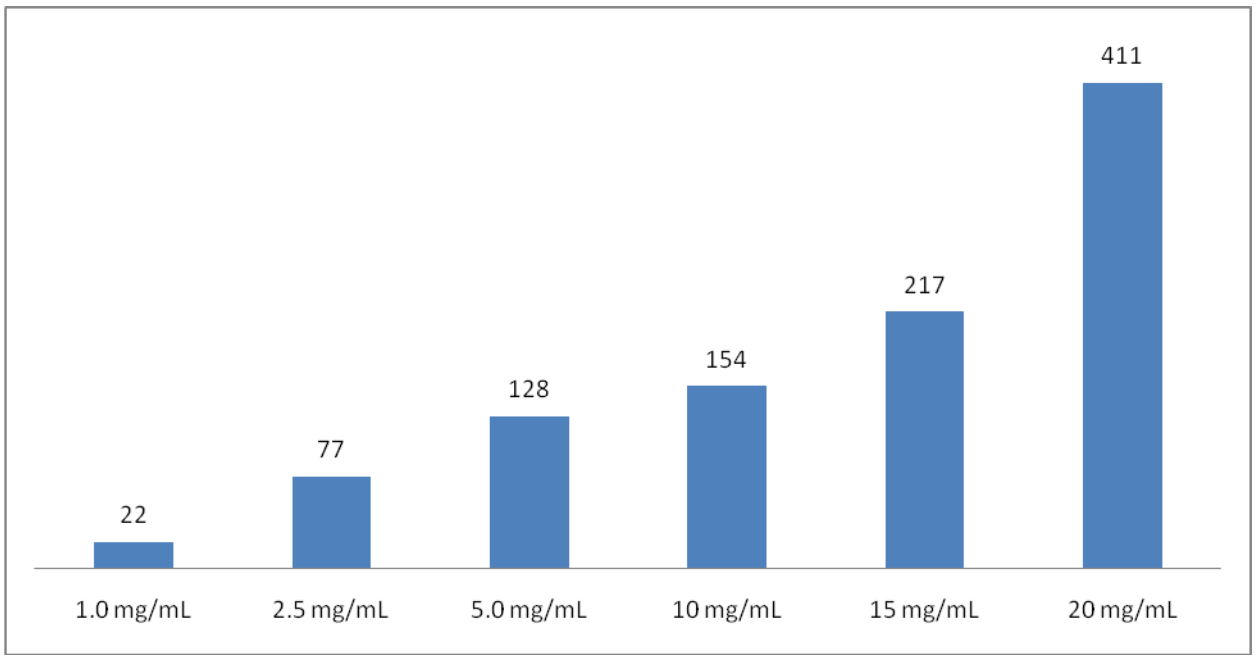

Figure 25: FPU value (FPU/mL) for each concentration of *T.reesei* cellulase loading

4.2.3 Growth Profile of *Saccharomyces Cerevisiae* ATCC 96581

Prior to fermentation process of cellulose using *Saccharomyces cerevisiae*, it is important to determine the population growth dynamics of bacterial culture by drawing the growth profile/ curve of the bacteria. The bacterial growth curve shows the rate of reproducibility and a lifespan of bacteria in a certain amount of media. A bacterium undergoes division by simple binary fission. This means that one cell grows to about double its original size and then splits into two genetically identical cells. Since DNA replication occurs before division of the cells, each new cell, called a daughter cell gets a complete genome (full sets of genes). Figure 26 clearly shows the mitosis process of *Saccharomyces cerevisiae* ATCC 96581 under electron microscope, OLYMPUS BH2. The size of the whole cell is 6µ with 6 scale division.

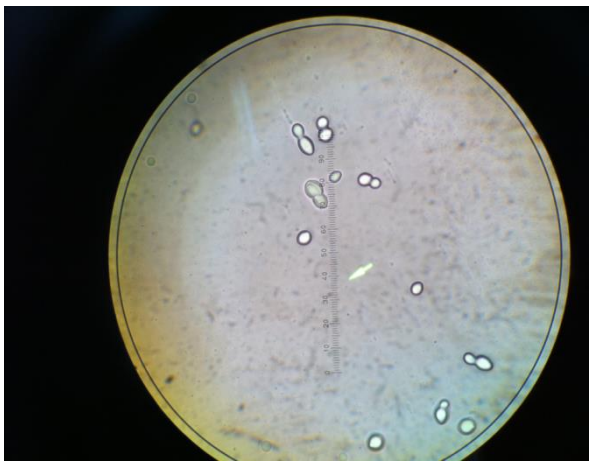

Figure 26: Mitosis of Saccharomyces Cerevisiae ATCC 96581

Figure 27 shows the growth profile of Saccharomyces cerevisiae ATCC 96581. The optical density of the strain was analyzed using UV-visible spectrophotometer at wavelength 600 nm. Bacterial growth in batch culture can be modeled with four different phases; lag phase (A), exponential phase (B), stationary phase (C) and death phase (D).

At 0 hours to 2 hours, *S.cerevisiae* undergoes lag phase. During lag phase, the bacteria adapt themselves to growth condition. It is the period where the individual bacteria are maturing and not yet prepared to divide. Besides, during lag phase of the bacterial growth cycle, synthesis of ribonucleic acid (RNA), enzymes and other molecules occur. Exponential phase is a period where the bacteria grow rapidly and mitosis process occurred. Based on the graph obtained, from 3 hours to 8 hours, it can be categorized under exponential or logarithmic phase. The number of new bacteria formed per unit time is proportional to the present population. However, exponential growth cannot continue indefinitely since the nutrients slowly depleted and enriched with wastes. At 10 hours to 30 hours, due to growth-limiting factor, cell viability of *S. cerevisiae* begins to decline. There are no cell growths during that stationary phase. After 32 hours, *S.cerevisiae* run out of nutrients and died.

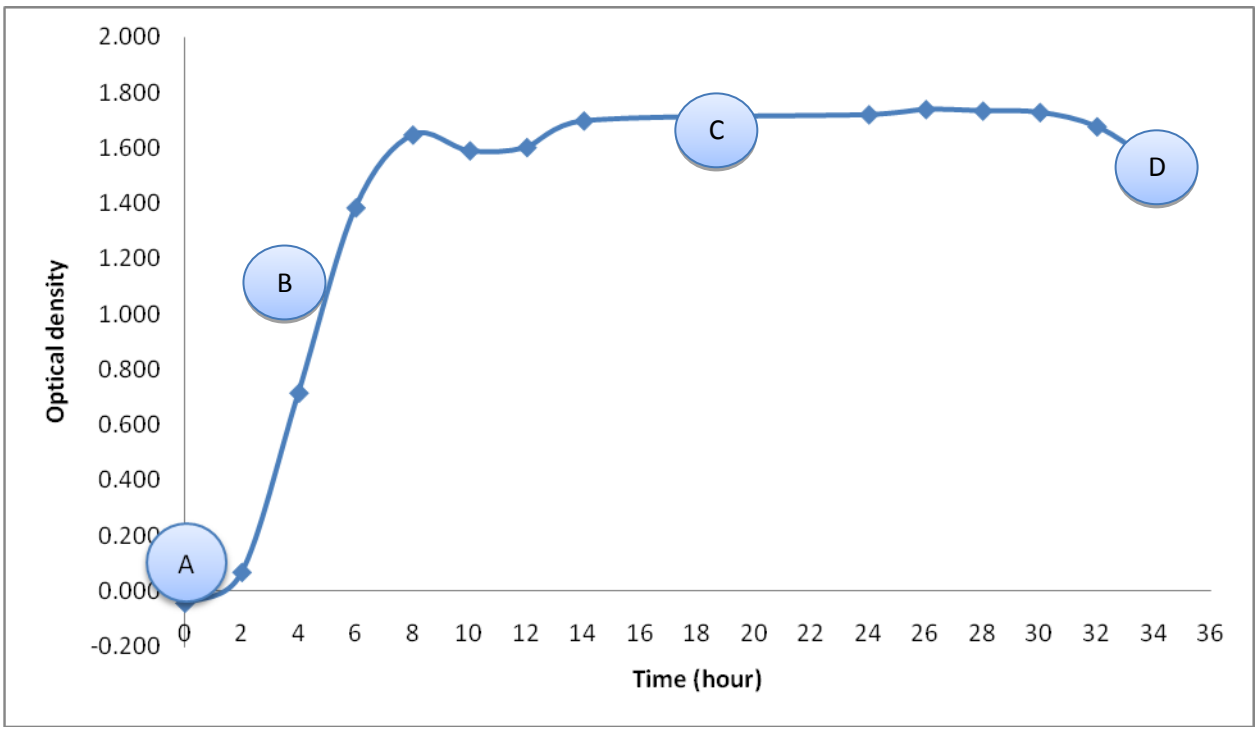

Figure 27 Growth profile of Saccharomyces Cerevisiae ATCC 96581 at wavelength 600 nm

4.3 Spectroscopic Elemental Analysis

4.3.1 Fourier Transform Infrared Spectroscopy (FT-IR)

The FTIR analysis for cellulose samples including reference sample of Avicel PH101, Alkali Lignin and microcrystalline Cellulose were done using Perkin Elmer Spectrum 100 with Horizontal Attenuated Total Reflectance (HATR) in UTP. IR spectra (4000 cm^{-1} – 650 cm^{-1}) were recorded with a resolution of 4cm^{-1} and 16 scans per sample. The samples are needed to be grinded before the analysis.

The crystallinity index (CI) of the sample was measured by two methods:

1. The absorbance ratio from 1385 cm^{-1} (A≈1385) and 2900 cm^{-1} (A≈2900) bands as shown in Equation 7.

$$(Crystallinity\ Index)Cr.I._1 . = \frac{A_{\approx 1385}}{A_{\approx 1385} + A_{\approx 2900}} \qquad \text{Eq. (7)}$$

2. The absorbance ratio from 1430 cm^{-1} (A≈1430) and 890 cm^{-1} (A≈890) bands as shown in Equation 8.

$$(Crystallinity\ Index)Cr.I._2 . = \frac{A_{\approx 1430}}{A_{\approx 1430} + A_{\approx 890}} \qquad \text{Eq. (8)}$$

Absorbance (quantity of light that a sample neither transmits nor reflects) can be determined using the following formula equation:

$$Absorbance(A) = -\log\left(\frac{transmittance}{100}\right) \qquad \text{Eq. (9)}$$

Determination of CI using FT-IR is the simplest method but it can only give relative values, as the spectrum always contains contribution from both crystalline and amorphous regions23.

4.3.2 Solid State ^{13}C Nuclear Magnetic Resonance (NMR)

Solid-state ^{13}C NMR spectra were collected at 4.7 T with cross-polarization and magic angle spinning (MAS) using 200MHz Bruker ASX200. Variable amplitude cross-polarization was used to minimize intensity variations of the non-protonated aromatic carbons that are sensitive to Hartmann-Hahn mismatch between protons and dilute spins that can cause intermolecular magnetization transfer between the low-gamma nuclei

23Sunkyu Park, J. O. (2010). Cellulose Crystallinity Index : Measurement Techniques and Their Impact on Interpreting Cellulase Performance. *Biotechnology for Biofuels*.

over long distances at higher MAS rotation rates. The ^{1}H and ^{13}C fields were matched at 125.7 kHz. MAS were performed at 8000 Hz. The number of scans was 1000 with a relaxation delay of 5 seconds.

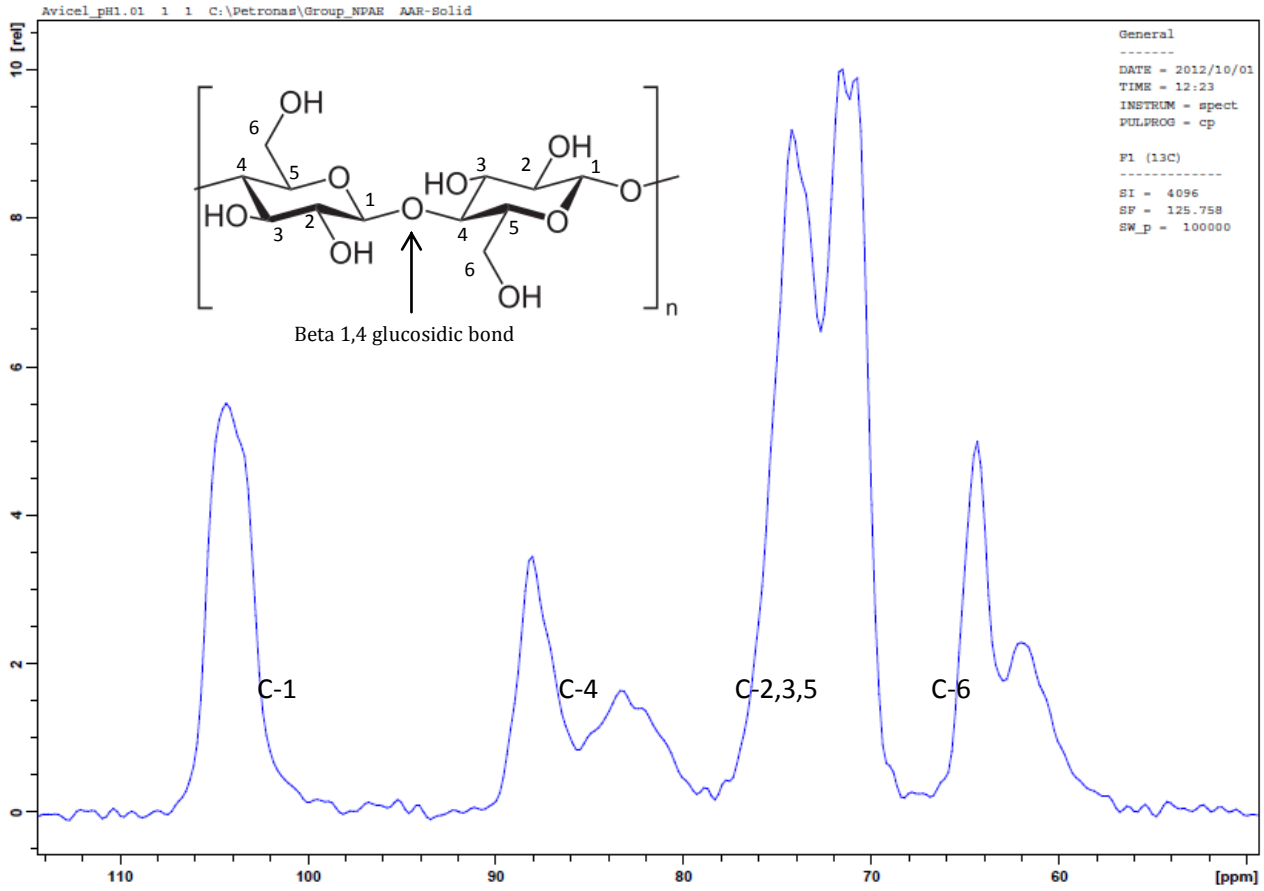

Figure 28: Solid State ^{13}CNMR spectrum of Avicel PH-101. (Spectrum showing peaks assignment to the carbons in cellulose)

The CI of the sample was determined by separating C4 region of the spectrum into crystalline and amorphous peaks, and calculated by dividing the area of crystalline peak (87 to 90 ppm) by the total of C4 peak area (78 to 90 ppm).

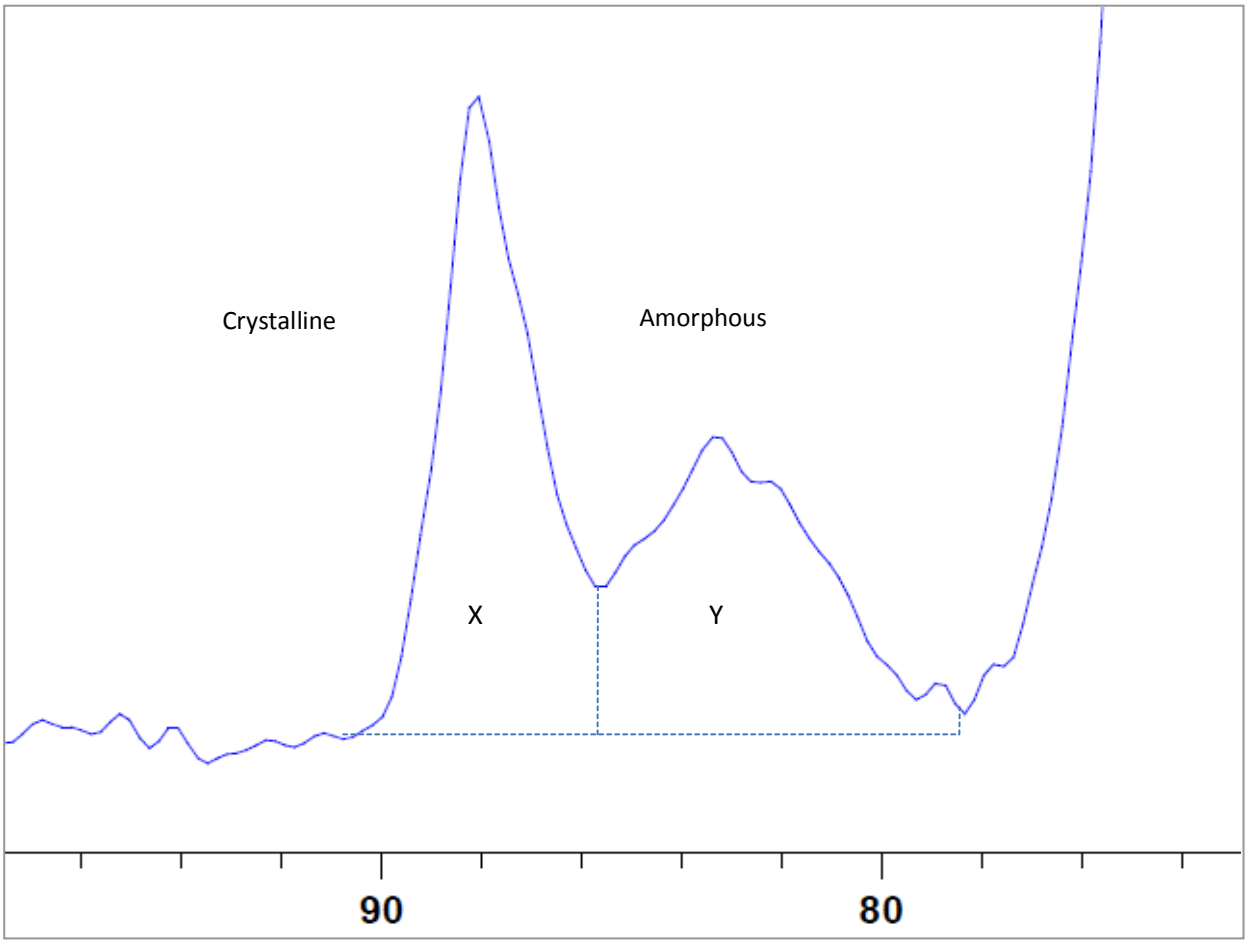

Figure 29: Sub- spectrum of C-4 peaks in cellulose. (X represents crystalline peaks and represents amorphous peaks)

The calculation for the CI is:

$$Crystallinity\ Index\ (CI) = \frac{A_{87-90\ ppm}}{A_{87-90\ ppm} + A_{78-87\ ppm}} \times 100\%$$

Eq. (10)

The CI of cellulose is crucial in interpreting changes in the cellulose structure after physicochemical and biological treatments24.

24Sunkyu Park, J. O. (2010). Cellulose Crystallinity Index : Measurement Techniques and Their Impact on Interpreting Cellulase Performance. *Biotechnology for Biofuels*.

4.4 Effect of FPU Value to the Production of Bioethanol

The experiment is carried out with different Filter Paper Unit (FPU) value of derived cellulase, *Trichoderma reesei* as stated in the experiment before. The FPU value used in the experiment was FPU 77, FPU 128, FPU 154 and FPU 217.This experiment aimed to study the effect of FPU value to the production of ethanol. The sample analytes were analyzed by using High Performance Liquid Chromatography (HPLC) with the following LC conditions.

Table 3: LC conditions for HPLC

Column	**Hi- Plex Ca**
Mobile phase	DI – water
Flowrate (ml/min)	0.6
Injection (µl)	20
Column temperature (°C)	80
RID temperature	35
Column Pressure (bar)	35.5
Analysis time (mins)	45
Separation, N	26361

The common sugars obtained from HPLC quantification were glucose and fructose. Glucose was detected at 14.32 minutes while fructose was detected at 20.51 minutes. Monomeric sugars are quantified by HPLC with refractive index detection. However, ethanol eluent compound was detected at 23.59 minutes. SSF will produce reducing sugars which is hexoses sugars; glucose and fructose and ethanol itself. Therefore, by using the standard calibration curve of glucose, fructose and ethanol in HPLC, determination of reducing sugars and ethanol concentration from unknown samples can be obtained. In this method the area of the unknown sample will be determined from the calibration curve with good regression (R^2) andconfidence; RSD < 0.01. Figure 17 to figure 19 show the calibration curve for glucose, fructose and ethanol.

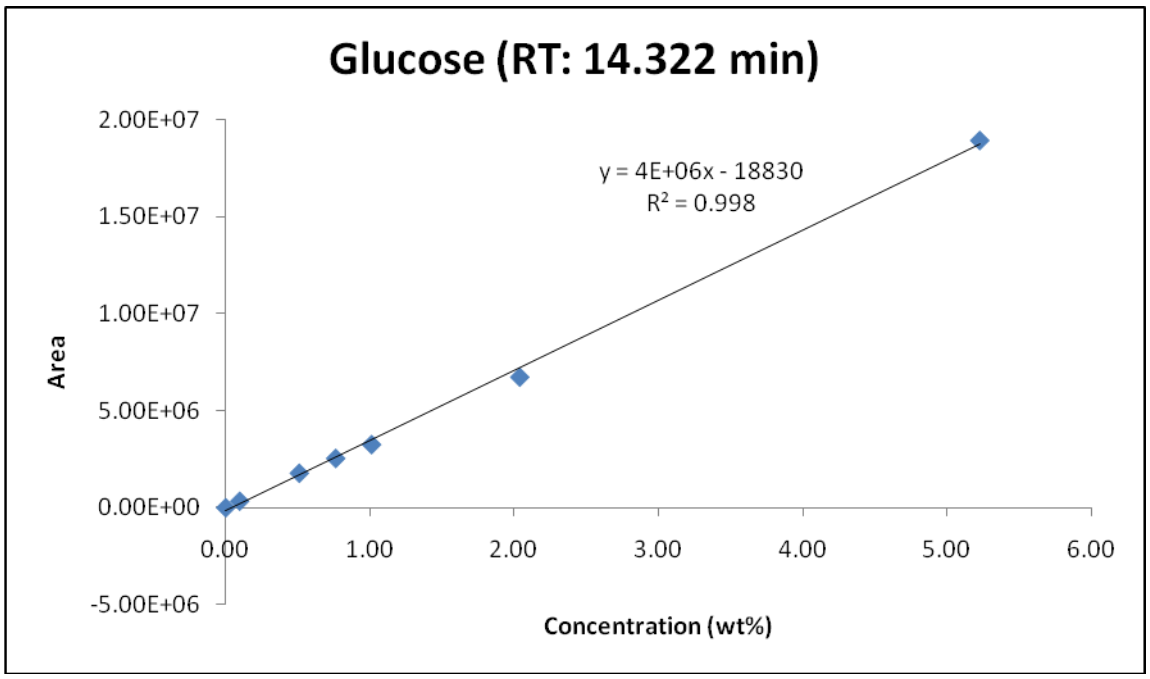

Figure 30: Calibration curve of D(+) glucose

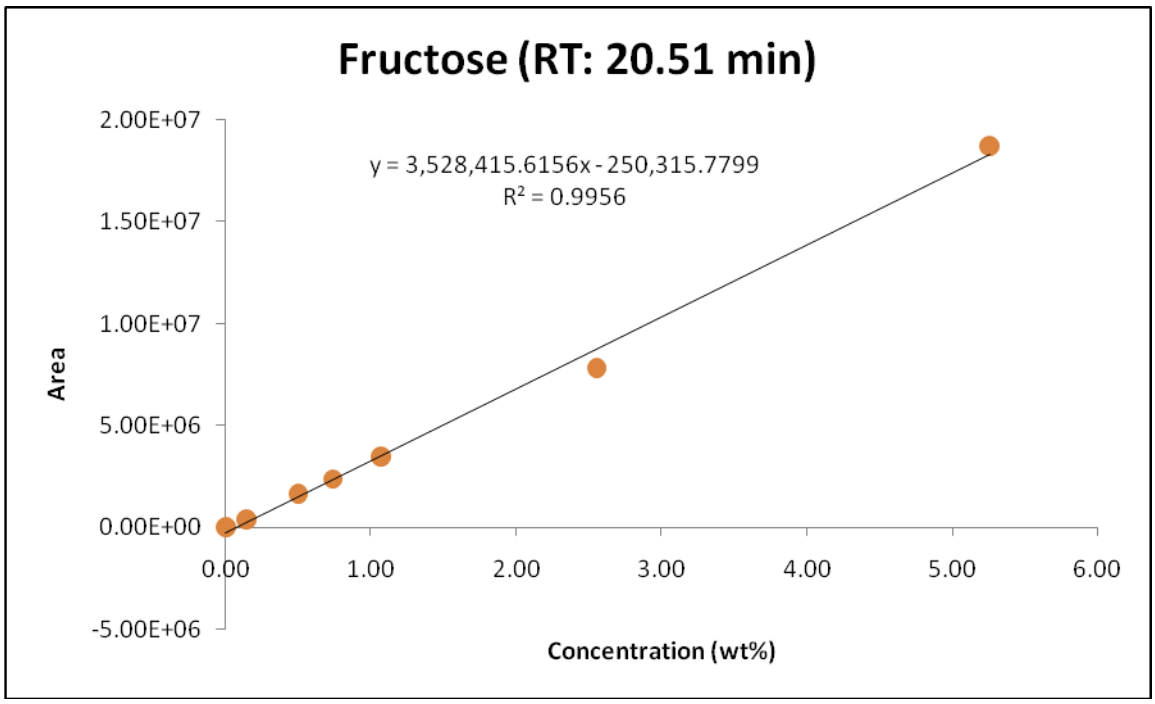

Figure 31: Calibration curve of D(+) fructose

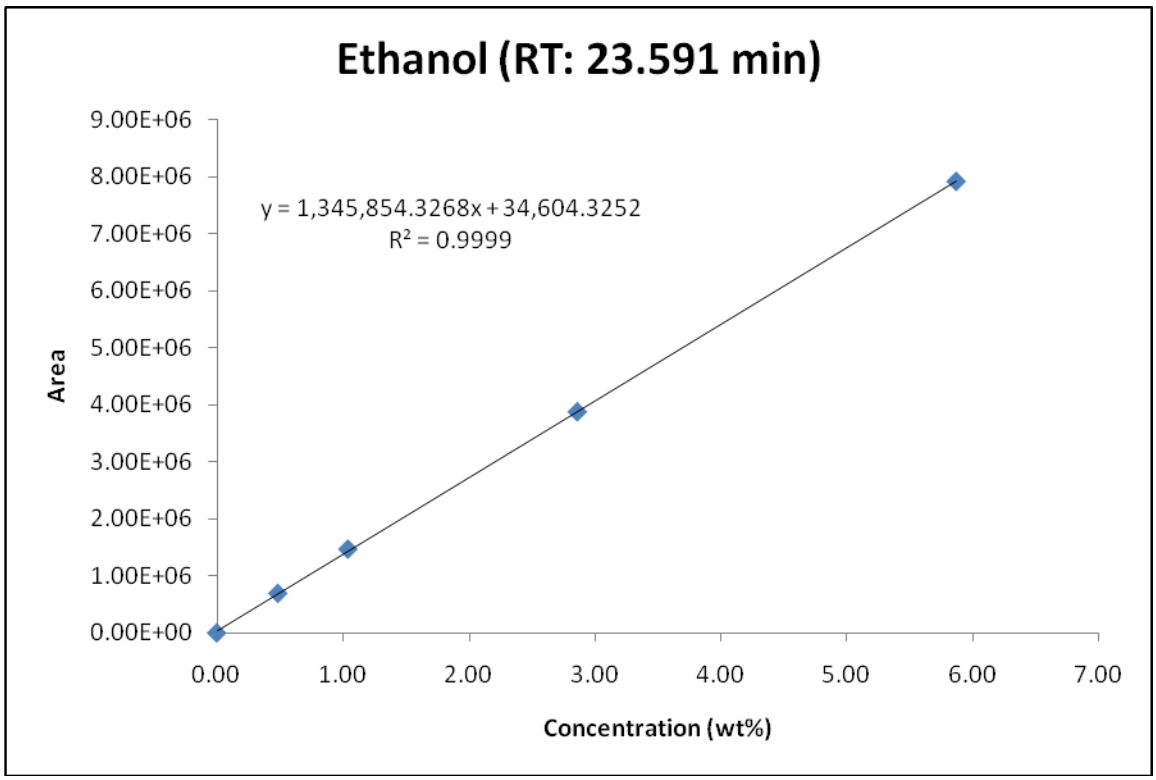

Figure 32: Calibration curve of ethanol

Table 4: Effect of different FPU values on ethanol production from cellulose hydrolysate at optimum condition

FPU	Glucose consumption (mg/hr)	Ethanol formation (mg/hr)	Glucose concentration (mg/mL)	Ethanol concentration (mg/mL)	Fermentation efficiency (%)
77	0.08	0.01	4.73	0.52	25.94
128	0.08	0.02	4.61	1.14	58.66
154	0.05	0.04	3.25	2.36	192.28
217	0.03	0.05	1.52	3.10	947.12

Figure 33 shows the graph of total glucose produced from the separate saccharification and fermentation (SSF) process from 24 hours to 60 hours. Generally, as the FPU value increased, the amount of glucose was reduced as the times were increasing. The highest FPU value gives the highest reduction of glucose from time to time compared to the sample with the lowest FPU value. For example, at 217 FPU value, the lowest amount of glucose is at 60 hours with 1.52 mg/mL followed by 2.80 mg/mL at 48 hours, 3.95

mg/mL at 36 hours and 5.64 mg/mL at 24 hours. Higher FPU value gives the higher amount of derived cellulase, *trichoderma reesei* and thus it will produce large amount glucose since the cellulose is changed into glucose with the help of cellulase.

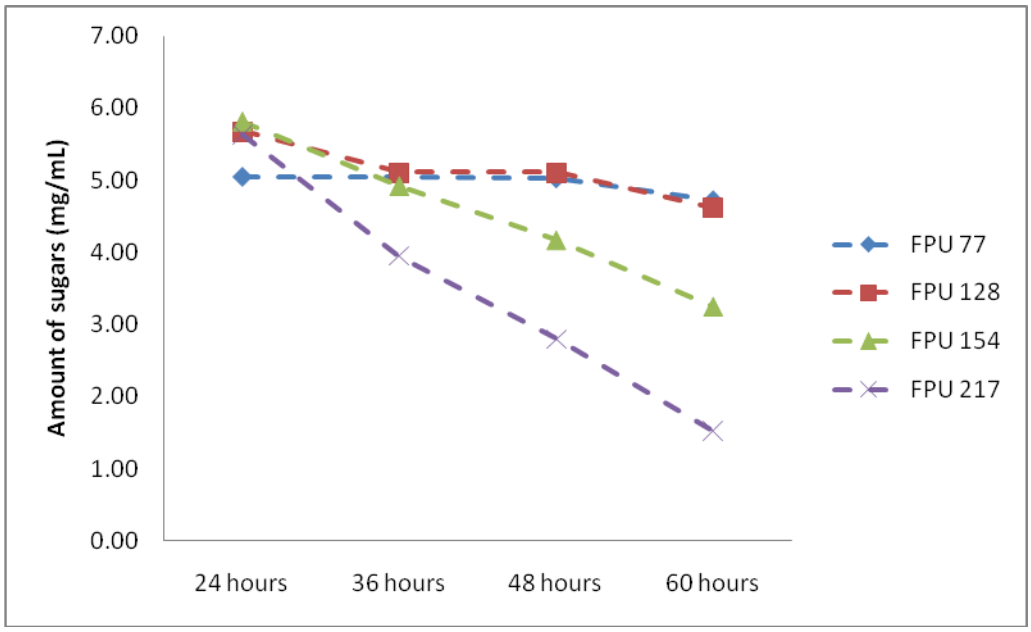

Figure 33: Glucose consumption by *S. cerevisiae* at different FPU values incubated at pH 4 and rate of agitation 100 rpm

Figure 34 shows the amount of ethanol production from the fermentation process. From the graph, it is clearly shown that high FPU value gives massive amount of ethanol production compared to lower FPU value. The highest amount of ethanol produce is 3.10 mg/mL which is at 217 FPU value followed by 2.41 mg/mL at 154 FPU value and 2.36 mg/mL at 217 FPU value. Furthermore, figure 22 shows the graph of ethanol yield against time. The ethanol yield was increased as the FPU value increased with time. The highest amount of ethanol yield was at 217 FPU (60 hours) with 947.12 followed with 242.91 at 217 FPU (48 hours) and 192.28 at 154 FPU (60 hours).

From these two graphs, it has been investigated that the amount of FPU value would affect the production of ethanol. High FPU value will give high amount of glucose produced as the glucose will be used by bacteria, *Saccharomyces cerevisiae*as nutrients for fermentation process to produce ethanol. Initially, the optical density used for each sample is 1.0 and the value was increased to 1.72 (24 hours), 2.04 (36 hours), 2.47 (48

hours) and 2.89 (60 hours). Hence, as time increases, the amount of glucose produced decreased by time to time as glucose will be used up by the bacteria to produce ethanol and give high amount of ethanol from time to time. In a nutshell, it has been proved that the FPU value will affect the amount of ethanol produced as the highest FPU value gives the highest amount of ethanol yield.

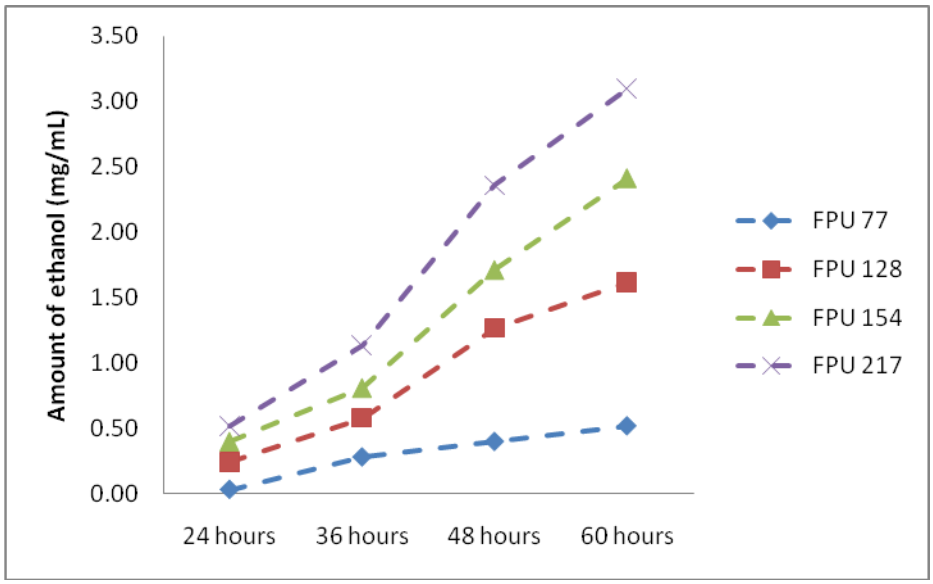

Figure 34: Ethanol production from cellulose hydrolysate at different FPU values incubated at pH 4 and rate of agitation 100 rpm

4.5.1 Effect of pH Value to the Production of Bioethanol

The effect of different initial pH on glucose consumption by *S. cerevisiae* ATCC 96581 and ethanol yield using Avicel pH -101 are shown in Figure 23 and 24. From the graph, the highest ethanol yield was obtained at pH 4 with maximum ethanol concentration of 3.76 mg/mL followed by 3.17 mg/mL at pH 6 at 60 hours incubation. The respective ethanol formation for both these pH were 0.06 (pH 4) and 0.05 (pH 6) as showed in figure 24. Fermentation of cellulose hydrolysate at initial pH 7 and pH 8 showed the lower ethanol concentration corresponding to ethanol formation of 0.04 mg/hr for both pH respectively. It was happen because the bacteria became inactive or less active as the alkilinity of the medium is high.

Table 5: Effect of different pH values on ethanol production from cellulose hydrolysate at optimim condition

pH	Glucose consumption (mg/hr)	Ethanol formation (mg/hr)	Glucose concentration (mg/mL)	Ethanol concentration (mg/mL)	Fermentation efficiency (%)
4	0.07	0.06	1.23	3.76	599.32
6	0.06	0.05	0.55	3.17	439.59
7	0.11	0.04	0.56	3.11	406.66
8	0.11	0.04	0.55	3.08	341.48

Figure 35 also showed that glucose consumption rate was the highest at initial pH 4 in which it showed highest glucose utilization over 60 hours of incubation. Besides, Figure 36 has showed that at initial pH 4, the ethanol concentration was the highest which is 3.76 mg/mL followed with pH 6 and 7 which were 3.17 mg/mL and 3.11 mg/mL respectively. The fermentation efficiency was calculated based on the ratio of ethanol yield obtained against theoretical maximum ethanol yield. The highest ethanol fermentation efficiency was obtained at pH 4 with 599%. The high fermentation efficiency value may be due to the presence of other simple sugars such as mannose and arabinose in the hydrolysate, thus contribute to higher concentration of ethanol obtained in the process. As fermentation of cellulose hydrolysate at pH 4 showed the highest ethanol production, hence, this pH value was used in all the following experiments.

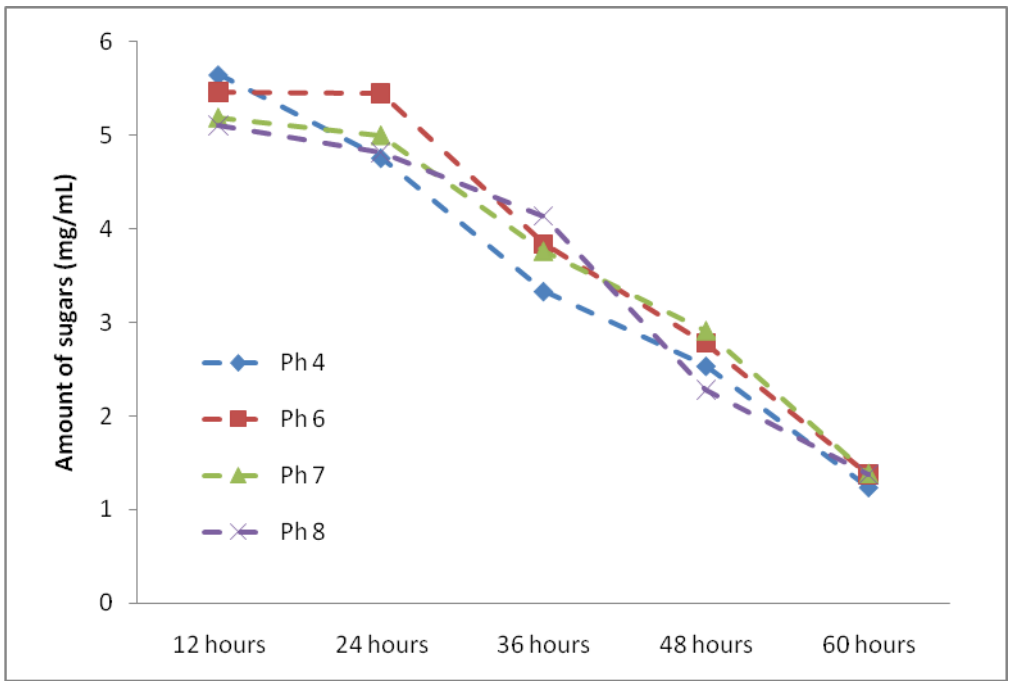

Figure 35: Glucose consumption by S.cerevisiae at different initial pH incubated at 30°C and agitated at 100 rpm

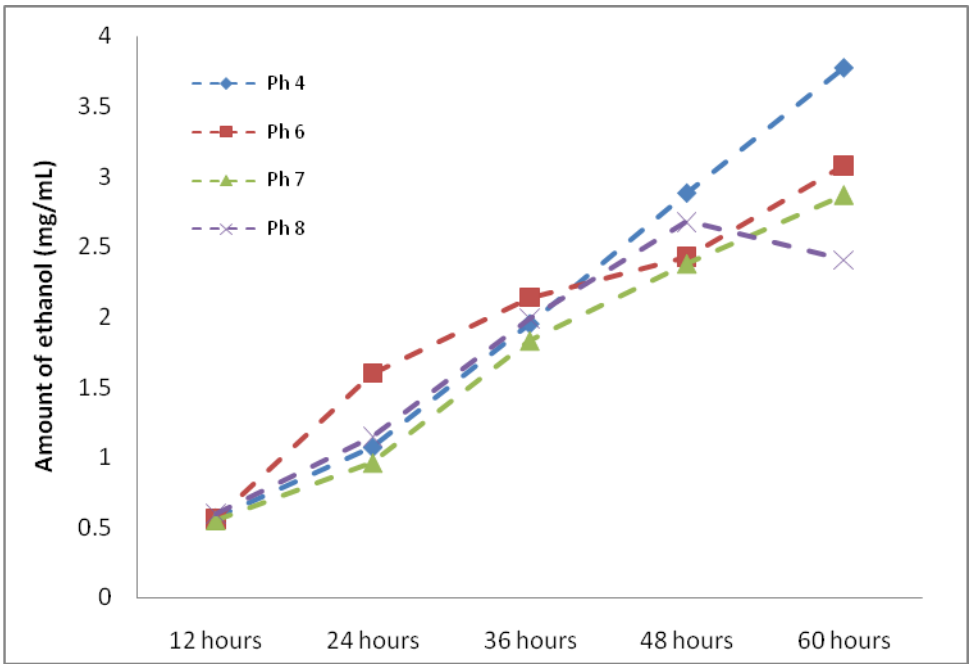

Figure 36: Ethanol production from cellulose hydrolysate at different initial pH incubated at 30°C and agitated at 100 rpm

The effect of initial pH has been reported to show a significant influence on fermentation, mainly on yeast growth, fermentation rate and by-product formation (Chaudary and Qazi, 2006; Sheela *et al.,* 2008; Manikandan *et al.,* 2008). The results obtained from this study shows that the most suitable initial pH value for ethanol production from cellulose was pH 4. It was found that increment of pH value was able to reduce ethanol production rate and glucose consumtion rate. This current study is in agreement with other studies reported that the growth of yeast and fermentation process performs best in natural or slightly acidic environment (Noor *et al.,* 2005; Manikandan *et al.,* 2008).

This study indicates that ethanol production at higher pH value was lower. The lower ethanol productivity may be due to lower metabolic rate of yeast cell used (Mariam et *al.,* 2009). Increment of pH value will increase the permeability of the cell membrane resulted reduction of the rate of sugar fermented enzyme production. The lower ethanol yield and sugar conversion obtained at higher pH value were also probably due to the formation of undesired product suchas glycerol and organic acid during the fermentation process.

4.6 Effect of Mass of Substrate to the Production of Bioethanol

The effect of different mass loading of cellulose on glucose consumption by *S. cerevisiae* and ethanol production from cellulose hydrolysate is shown in Figure 37 and Figure 38. As referred to Figure 38, the highest ethanol concentration was 4.63 mg/mL obtained when the mass loading of cellulose is 5.0 g followed by 3.76 mg/mL at mass loading of 2.0 g which corresponded to an ethanol formation of 0.07 mg/hr and 0.06 mg/hr respectively. Ethanol production is lower at lower mass loading of cellulose.

Figure 37 shows the amount of glucose consumed by the bacteria in producing ethanol. From the graph, it was clearly shown that at higher loading of cellulose, more glucose has been consumed compared to lower loading of cellulose. The decrement for amount of glucose also higher at higher mass loading of cellulose from time to time which is from 14.86 mg/mL (24 hours) to 11.21 mg/mL (60 hours).

Table 6: Effect of different mass loading of cellulose on ethanol production from cellulose hydrolysate at optimum condition

Mass loading of cellulose (g)	Glucose consumption (mg/hr)	Ethanol formation (mg/hr)	Glucose concentration (mg/mL)	Ethanol concentration (mg/mL)	Fermentation efficiency (%)
0.5	0.11	0.02	1.14	1.33	148.53
1.0	0.14	0.05	1.73	2.89	182.89
2.0	0.41	0.06	4.95	3.76	228.13
5.0	0.53	0.07	11.21	4.63	326.65

Based on the calculation, the fermentation efficiency is higher at 5.0 g of cellulose loading followed by 2.0 g of cellulose loading with 326% and 228% respectively. Lowest cellulose loading (o.5g) gave the lowest fermentation efficiency which was 148.53%.

During hydrolysis, the released sugars inhibit the cellulase activity which needs cellulose loading to ensure the higher production of ethanol. The higher the cellulose loading, the higher ethanol production as high amount of cellulose can be converted to sugars and directly fermented by bacteria to produce ethanol.

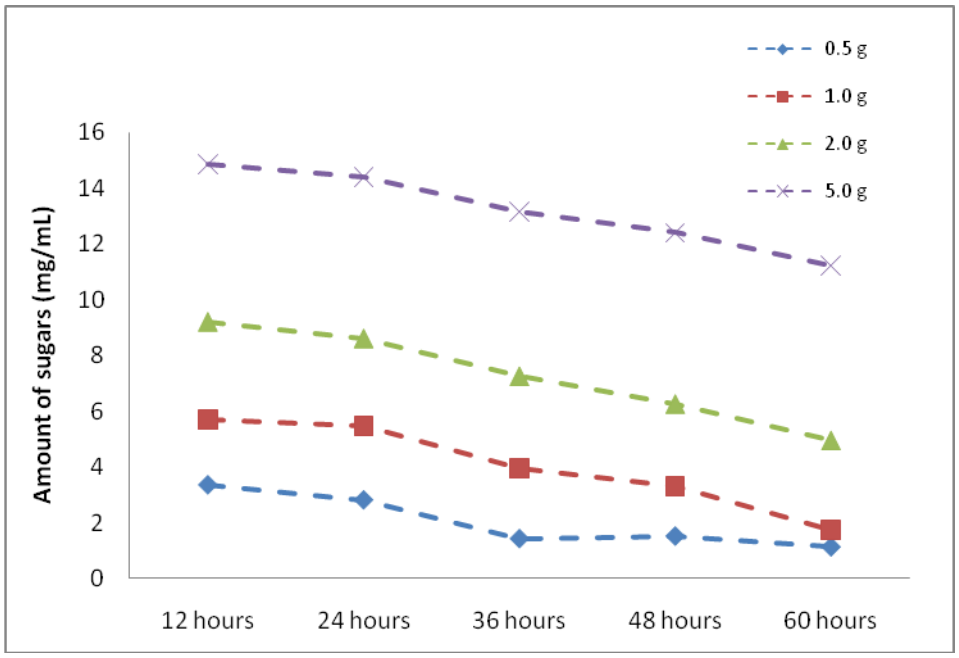

Figure 37: Glucose consumption by S. cerevisiae at different mass loading of cellulose incubated at pH 4 and rate agitation 100 rpm

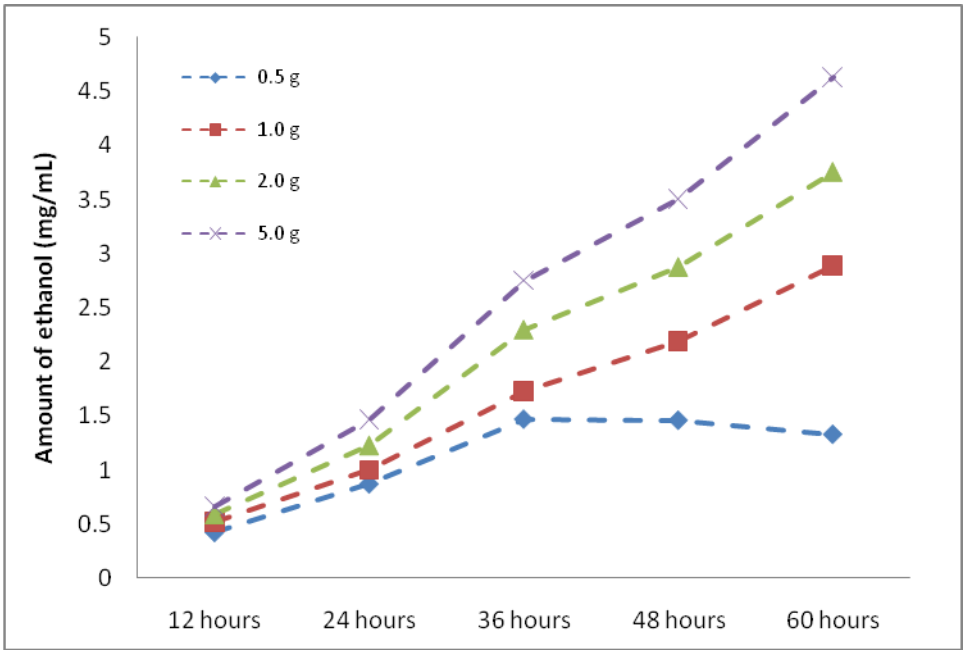

Figure 38: Ethanol production from cellulose hydrolysate at different mass loading of cellulose incubated at pH 4 and rate of agitation 100 rpm

4.7 Calculation for Ethanol Production

Example of ethanol production of 217 FPU value at 24 hours

Step 1

Determine glucose concentration:

Area of glucose (flask 1) = 1598230

Area of glucose (flask 2) = 1536329.6

From calibration curve of glucose;

Y = 3622122.2118 X -188302.7885

Average concentration, X = 4.85 mg/mL

Step 2

Determine ethanol concentration:

Area of ethanol (flask 1) = 121414.3

Area of ethanol (flask 2) = 148162.9

From calibration curve of ethanol;

Y = 1345854.3268 X – 34604.3252

Average concentration, X = 0.74 mg/Ml

Step 3

Assume 100% of ethanol conversion:

Molecular weight of glucose:180.16 g/mol

$$\frac{0.485\ g}{L}\ X\ \frac{mol}{180.16\ g} = \frac{0.00269\ mol}{L}$$

2 (0.00269 mol/L) = 0.005384 mol/L

Step 4

Find amount of ethanol produced:

Molecular weight of ethanol: 46.06844 g/mol

$$\frac{0.74\ g}{L}\ X\ \frac{mol}{46.06844\ g} = 0.016\ \frac{mol}{L}$$

Step 5

Ethanol production rate:

$$Ethanol\ yield = \frac{Actual\ amount}{Theoretical\ amount}\ x\ 100$$

Eq. (11)

$$Ethanol\ yield = \frac{0.016\ mol/L}{0.005384\ mol/L}\ x\ 100 = 29.834$$

CHAPTER 5: CONCLUSION AND RECOMMENDATION

Extensive research during the past decade has shown that simultaneous Saccharification and fermentation (SSF) is a promising way to biochemically convert cellulose to ethanol. The process combines cellulose hydrolysis and fermentation in one step. Because glucose consumed by the hydrolysis process is immediately consumed by the ethanologenic microorganism, only very low levels of fructose and glucose are observed in the system. This reduces cellulase inhibition, which in turn increases sugar production rates, concentrations, and yields, and decreases enzyme loading requirements. The number of vessels required for SSF is reduced compared to separate hydrolysis and fermentation (SHF), because hydrolysis and fermentation and are performed in the same bioreactor, resulting in capital cost savings. Furthermore, the presence of ethanol during SSF reduces the likelihood of contamination, especially in continuous operations of commercial interest. The delignified EFB fibres after acid hydrolysis has the highest reducing sugar in acid hydrolyzate compared to the others pretreated samples by using DNS method. The results for enzymatic hydrolysis using HPLC method were inaccurate because of unsuitable storage condition. Trichoderma Reesei cellulase gave highest FPU value for highest concentrations of cellulase loading. Suitable fermentation conditions are crucial in producing optimum ethanol yield from cellulose hydrolyzate. In this study, the optimum ethanol yield from cellulose (Avicel Ph 101) which ranges from 3.1 mg/mL to 4.6 mg/mL can be achieved at pH 4, 217 FPU, 5.0g of cellulose loading with an agitation rate of 100 rpm for 60 hours incubation. Further study on other parameters such as different temperatures, agitation rate and types of feedstock in Simultaneous Scarification and Fermentation (SSF) will be carried out to make ethanol production from palm oil biomass is produce in economical viable and in sustainable way.

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APPENDICES

Enzymatic Saccharification Process

Figure 39: Preparation of DI water at pH 4.0

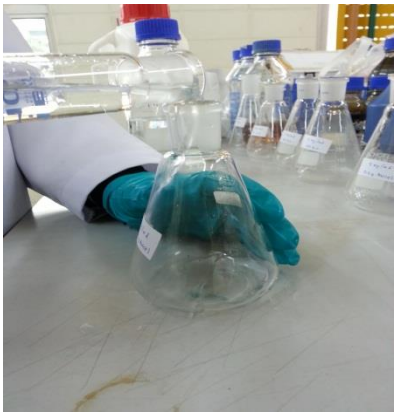

Figure 40: Pour DI water into the flasks

Figure 41: Autoclave the samples

Figure 42: Add *Trichoderma reesei* into the flask

Figure 43: Incubate and shake the flasks

Figure 44: Take out the sample and centrifuge

Figure 45: Analyze the sample with HPLC

Fermentation Process

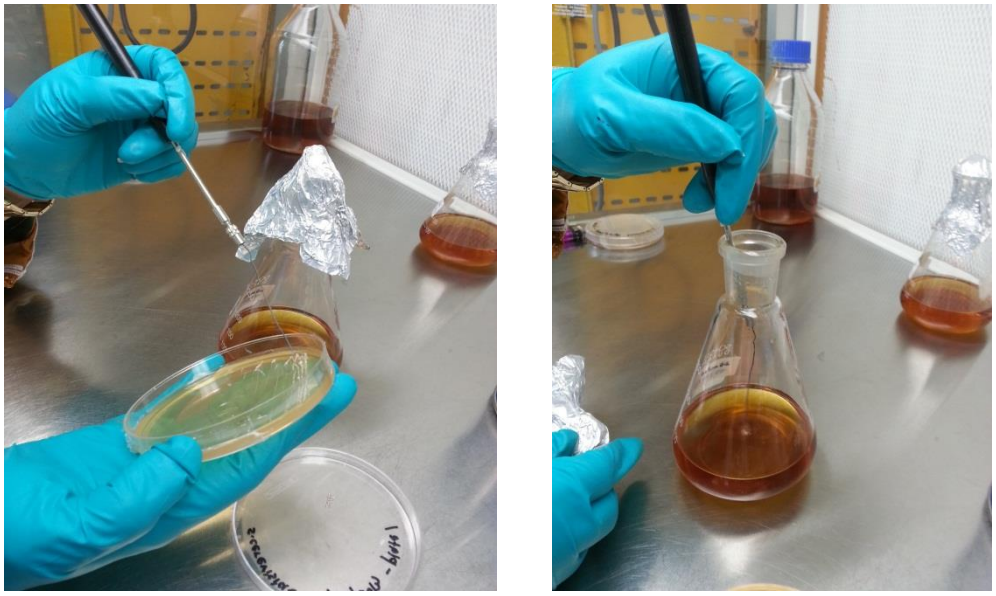

Figure 46: Preparation of Inoculum (fresh culture)

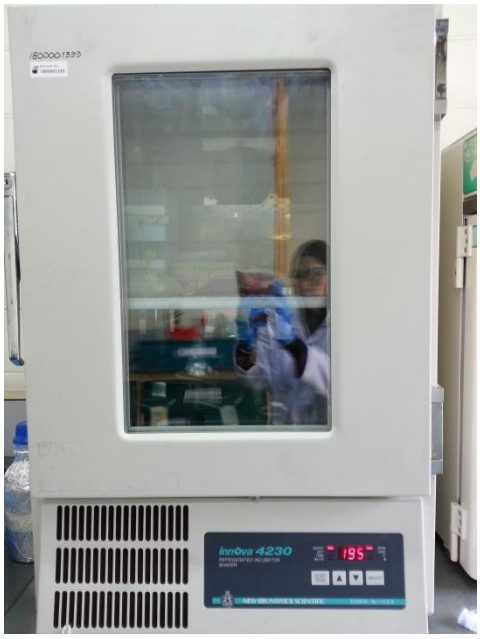

Figure 47: Incubate and shake the culture for 24 hours

Figure 48: Add Avicel pH-105 into the flask contains autoclaved DI water

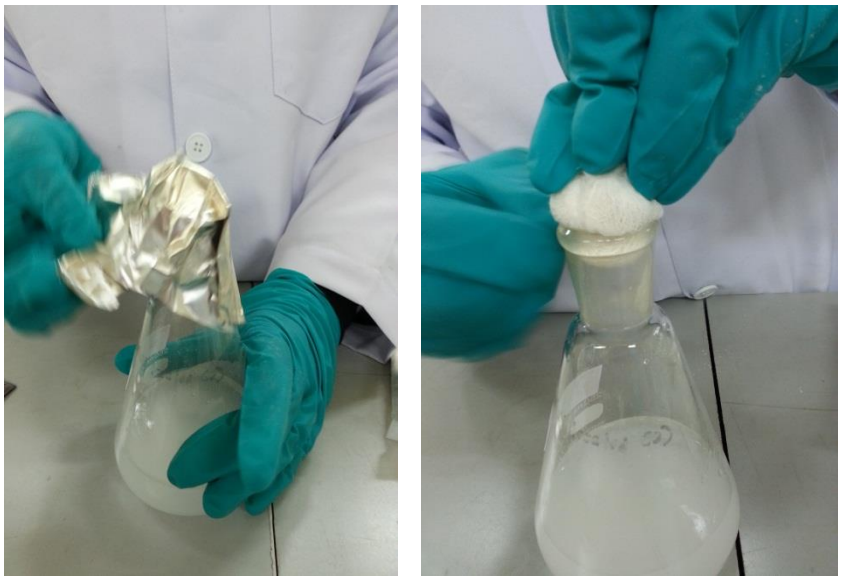

Figure 49: Close the flask tightly with cotton wool and aluminum foil

Figure 50: Autoclave the samples

Figure 51: Add *Saccharomyces cerevisiae* into the flasks

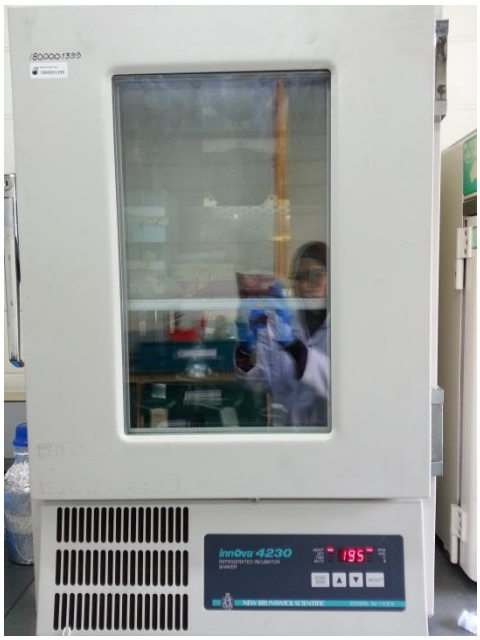

Figure 52: Incubate and shake the samples for 72 hours

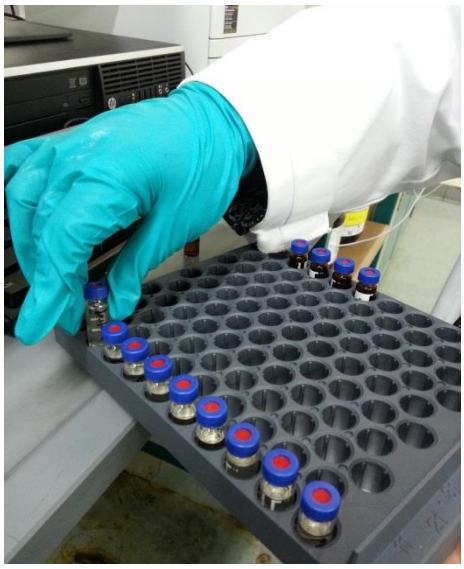

Figure 53: Put the samples into the vials and analyses using HPLC