OPTIMIZATION OF GLUCOSE PRODUCTION FROM ENZYMATIC HYDROLYSIS OF RICE STRAW USING RESPONSE SURFACE METHODOLOGY

AZNIZAN BINTI SHAARI

UNIVERSITI MALAYSIA PAHANG

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JUDUL: **OPTIMIZATION OF GLUCOSE PRODUCTION FROM ENZYMATIC HYDROLYSIS OF RICE STRAW USING RESPONSE SURFACE METHODOLOGY**

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Date : ……

OPTIMIZATION OF GLUCOSE PRODUCTION FROM ENZYMATIC HYDROLYSIS OF RICE STRAW USING RESPONSE SURFACE METHODOLOGY

AZNIZAN BINTI SHAARI

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

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

APRIL 201

I declare that this thesis entitled "Optimization of Glucose Production from Enzymatic Hydrolysis of Rice Straw Using Response Surface Methodology" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

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Special Dedication to my beloved husband, Mohd Munzir Bin Hamzah,
My parents, Haji Shaari Bin Awang Senik and Hajjah Mek Binti Noh,
Haji Hamzah Bin Md Salleh and Hajjah Rubiah Binti Mustafa,
My family members,
My fellow lecturers,
My friends, my fellow colleague
And all faculty members

For all your care, support and believe in me.

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ABSTRACT

Production cost of cellulase enzyme is very expensive. A research to reduce the cost by optimization of pH, temperature and agitation rate on production of glucose from enzymatic hydrolysis of rice straw by cellulose from *Aspergillus niger* using Response Surface Methodology (RSM) was successfully done. At the first stage of experiment, one factor at a time was employed to screen the best range for pH, temperature and agitation rate. All of the parameters ranges obtained were used in RSM. Response Surface Methodology in Design Expert version 6.0.8 software was used with Central Composite Design (CCD) mode. Seventeen sets of experiments with different parameters values were suggested by the software. The predicted optimum values for pH, temperature, agitation rate and concentration of glucose were 4.23, 43^{o}C, 177 rpm and 10.146 g/L respectively. One set of experiment was run using the optimized parameter and as a result, 9.9835 g/L concentration of glucose was recorded. Before optimization, concentration of glucose was only 5.5622 g/L and the concentration of glucose was increased by 44% after optimization. The optimization also reduces the energy consumption as the temperature was reduced from 45^{o}C to 43^{o}C and agitation rate reduced from 180 to 177 rpm. As conclusion, this research is successful to increase the concentration of glucose production, reduce the energy consumption and also be able to reduce the cost of production.

ABSTRAK

Kos penghasilan enzim sellulase sangat mahal. .Satu kajian untuk merendahkan kos penggunaan enzim dengan mengoptimumkan pH, suhu dan kadar adukan terhadap proses penghasilan glukosa melalui penggunaan enzim sellulase daripada *Aspergillus niger* terhadap jerami padi dengan menggunakan Kaedah Permukaan Tindak balas (RSM) telah berjaya dilakukan. Di awal peringkat eksperimen, kaedah satu faktor pada satu masa telah digunakan untuk menyaring julat pH, suhu dan kadar adukan yang terbaik. Kesemua julat parameter yang diperolehi, digunakan dalam Kaedah Permukaan Tindak balas (RSM). Kaedah Permukaan Tindak balas (RSM) dalam perisian Design Expert versi 6.0.8 telah digunakan dengan mod Rekabentuk Komposit Berpusat (CCD). Tujuh belas set eksperimen berlainan nilai parameter telah dicadangkan oleh perisian ini. Nilai optimum yang diramalkan untuk pH, suhu, kadar adukan dan kepekatan penghasilan glukosa masing-masing 4.23, 43^{o}C, 177 rpm dan 10.1460 g/L. Satu set eksperimen telah dijalankan bagi menguji parameter yang telah dioptimumkan dan sebagai keputusannya, 9.9835 g/L kepekatan glukosa telah direkodkan. Sebelum pengoptimuman, kepekatan glukosa hanyalah 5.5622 g/L dan penghasilan glukosa meningkat 44% selepas pengoptimuman. Pengoptimuman juga menurunkan penggunaan tenaga seperti suhu yang telah direndahkan dari 45^{o}C kepada 43^{o}C dan kadar adukan dikurangkan dari 180 rpm kepada 177 rpm. Sebagai konklusinya, kajian ini telah berjaya meningkatkan penghasilan glukosa daripada jerami padi, mengurangkan penggunaan tenaga dan juga mampu menurunkan kos penghasilan.

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LIST OF SYMBOLS/ABBREVIATIONS

Adj	-	Adjusted
ANOVA	-	Analysis of variance
CCD	-	Central composite design
cm	-	Centimeter
Corr	-	Correlation
DH	-	Degree of hydrolysis
dH_2O	-	Dilution of water
DNS	-	Dinitrosalicylic
GMC	-	Generic model control
g	-	Gram
g/L	-	Gram per liter
h	-	Hour
L	-	Liter
M	-	Molar
mg	-	Milligram
mg/ml	-	Miligram per milliliter
min	-	Minutes
ml	-	Mililiter
NaOH	-	Sodium hydroxide
nm	-	Nanometer
^oC	-	Degree Celcius
OD_{540}	-	Optical density at 540nm
OFAT	-	One factor at a time
Pred	-	Predicted
Prob	-	Probability
R^2	-	Coefficient of design
Rpm	-	Rotation per minutes

RSM	-	Response surface methodology
SEM	-	Scanning electron microscopy
SSF	-	Simultaneous sacharification and fermentation
T	-	Temperature
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Lignocellulosic biomass is consisting of cellulose, hemicellulose and lignin. Biomass comes in many different types, there are wood residues, municipal paper waste, agricultural residues including corn stover and sugarcane bagasse, rice straw and dedicated energy crops. Many of the crops can provide high energy biomass which may be harvested multiple times each year (Sun *et al.*, 2008).

Biomass is a carbon source of energy. It is comes from dead plants, which means that the combustion of ethanol produced from lignocelluloses will produce no net carbon dioxide in the earth's atmosphere. Biomass is also readily available and the fermentation of lignocelluloses provides an attractive way to dispose of many industrial and agricultural waste products (Jin and Chen, 2006).

One of the most abundant lignocellulosic biomass is rice straw. Malaysia is one of the agricultural country and rice straw is the important agricultural corps in Malaysia with a production of 2 362 000 metric ton per year (Sarote and Jowaman, 2005). It was used as feedstock for paper industry, animal feed and organic fertilizer. In order to develop its new uses, the conversion of rice straw into glucose has been studied for fermentation process to produce ethanol.

Rice straw is difficult to convert into the fermentable sugar because the strong crystalline structure of cellulose in rice straw and the presence of the complex structure of lignin and hemicellulose with cellulose, which together limit the accessibility of rice straw to hydrolytic enzymes. Therefore, various pretreatments of rice straw have been developed to remove lignin and hemicelluloses, reduce cellulose crystallinity and increase the porosity thus increases its enzymic hydrolysis. There has been many pretreatment of lignocellulosic material such as acid treatment, alkaline treatment and oxidative delignification ozone (Ma *et al*., 2009).

Enzymatic hydrolysis provides a method to convert cellulose to glucose at high yields without sugar product degradation. Enzymatic hydrolysis of cellulose proceeds in several steps to break glycosidic bonds by the use of cellulase enzymes. Factors effecting hydrolysis of cellulose include type of substrate, cellulase loading and reaction conditions such as temperature, pH and end-product inhibitors. Cellulases are synthesized by fungi, bacteria and plants with most research focused on fungal and bacterial cellulases produced both aerobically and anaerobically. The aerobic mesophilic fungus, *Trichoderma reesei* QM 6a and its mutants have been the most intensely studied sources of cellulases. Cellulase is not a single enzyme but is made up of a family of at least three groups of enzymes: *1,4- β-D-glucan glucanohydrolases* (endoglucanases), 1,*4- β-D-glucan cellobiohydrolases* and *1,4- β-D-glucanglucohydrolases* (exoglucanases) and *β-D-glucoside glucohydrolases* (β-glucosidases) (Silverstein, 2004).

One of the examples from previous research about production of glucose by using enzymatic hydrolysis method is studied by Kunamneni and Singh (2005). This research studied the optimization of enzymatic hydrolysis of maize starch for higher glucose production. Crude amylases were prepared from *Bacillus subtilis* ATCC 23350 and *Thermomyces lanuginosus* ATCC 58160 under solid state fermentation. The effect of various process variables was studied for maximum conversion efficiency of maize starch to glucose using crude amylase preparations. Doses of pre-cooking-amylase, post-cooking-amylase, glucoamylase and saccharification temperature were found to produce maximum conversion efficiency and these were selected for optimization.

In statistics, Response Surface Methodology (RSM) explores the relationships between several explanatory variables and one or more response variables. The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response (Jones, 1996). As an example, Response Surface Methodology (RSM) has been extensively applied in optimization of enzymatic hydrolysis of *Cistus ladanifer* and *Cytisus striatus* for bioethanol production (Ferreira *et al.*, 2009). This reseach studied the optimization of enzymatic hydrolysis using the response surface methodology allowed a study on the influence of the variables (pH, temperature, cellulases concentration, polymer (PEG) concentration and incubation time) and variability due to the type of substrate (*C. ladanifer* and *C. striatus*) used. From the obtained results it can be concluded that the enzymatic hydrolysis was clearly enhanced by temperature, cellulase concentration and incubation time (Ferreira *et al.*, 2009).

1.2 Problem Statement

As crop residue after harvesting time, normally rice straw is discarded as a waste by farmers in Malaysia. This is cause the environmental issue. This research is one of steps to change the waste of rice straw to be valuable thing. Rice straw can be converted to reducing sugars which can be fermented to target product such as ethanol, lactic acid and single cell protein by suitable microorganism.

1.3 Objectives

As a huge potential in producing sugar from biomass, this research is carried out with the objective of optimization of glucose production from rice straw by enzymatic hydrolysis using Response Surface Methodology (RSM).

1.4 Scopes of Research

In order to achieve the above objective, the following scopes have been identified:

i. To study the effect of pH on production of glucose in enzymatic hydrolysis of rice straw.
ii. To study the effect of temperature on production of glucose in enzymatic hydrolysis of rice straw.
iii. To study the effect of agitation rate of shaking on production of glucose in enzymatic hydrolysis of rice straw.
iv. To optimize the pH, temperature and agitation rate on production of glucose using Response Surface Methodology (RSM).

1.5 Rational and Significant

One of the most abundant lignocellulosic biomass is rice straw. Malaysia is one of the agricultural country and rice straw is the important agricultural corps in Malaysia with a production of 2 362 000 metric ton per year (Sarote and Jowaman, 2005).

Rice straw is discarded as waste in Malaysia. This is causing environmental issue as the farmers usually just burn out the rice straw. This research is done as one of the steps to change the waste of rice straw into a valuable thing. The concept of changing waste to wealth is applied in this research.

In this study rice straw has been chosen to be the raw material of producing glucose in this research due to its abundant and low cost rather than using other source as well as it also clean, nontoxic and renewable.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Biorenewable resources are usually classified as either wastes or dedicated as energy crops. Categories of waste materials that qualify as biorenewable resources include agricultural residues, yard waste, municipal solid waste, food processing waste and manure. Agricultural residues such as rice straw, corn stover, rice hulls, wheat straw, cotton stalks and bagasse are the portion of the crop discarded after harvest (Sun *et al.*, 2008).

The straw has traditionally been removed from the field by the practice of open-field burning. This practice clears the field for new plantings and cleans the soil of disease-causing agents. Recently, the impact of open-field burning of rice straw on air quality has led to legislation which will in the future strictly control this practice. In the search for viable alternatives, the rice growers are considering straw as a source of liquid fuels and energy. The carbohydrate portion of the straw, 60% by weight, is being considered as a feedstock for fermentation, in a process that requires both a chemical pretreatment and enzymatic conversion of cell wall polysaccharides to monosaccharides (Yu *et al.*, 1996). Table 2.1 presents the potential supply of crop residues in some selected Southeast Asian countries during 1980/1981(Sarote and Jowaman, 2005).

Table 2.1: Potential supply of crops residues in some selected Southeast Asian countries during 1980/1981

	Indonesia	Malaysia	Thailand	Total
	1 X 10^3 metric ton			
Rice straw	36 300	2 362	20 900	81 881
Maize stover	3 991	8	3 700	11 415
Sweet potato vine	624	11	104	1 789
Cassava leaves	1 098	29	1 432	3 015
Banana stem and leaves	3 423	825	4 446	12 639
Banana fruit wastes	467	112	606	1 723
Pineapple wastes	186	145	1 260	2 676
Sugar-cane tops and leaves	5 268	255	5 580	18 408

Data from Table 2.1 indicate that the main food crops of the region are rice, sugar-cane, cassava, maize and other plantation crops such as banana stem and pineapple wastes. Data shows that Malaysia has the higher potential supply of crop residues for producing of rice straw. Bioconversion of rice straw for bioproduction of ethanol is flourishing as a result of increasing environmental pressure and decreasing fossil-fuel energy supply (Jin and Chen, 2006).

2.2 Rice Straw Composition

Most of the carbohydrate content of plants is structural polysaccharides that provide strength, shape and support for the plant. Lignocelluloses are complex structural material in the cell wall: cellulose, hemicelluloses and lignin are the three main components of lignocellulosic materials with other minor components being ash, protein and extractives (Silverstein, 2004). The distribution of cellulose, hemicelluloses and lignin in a typical plant cell wall are shown below in Figure 2.1.

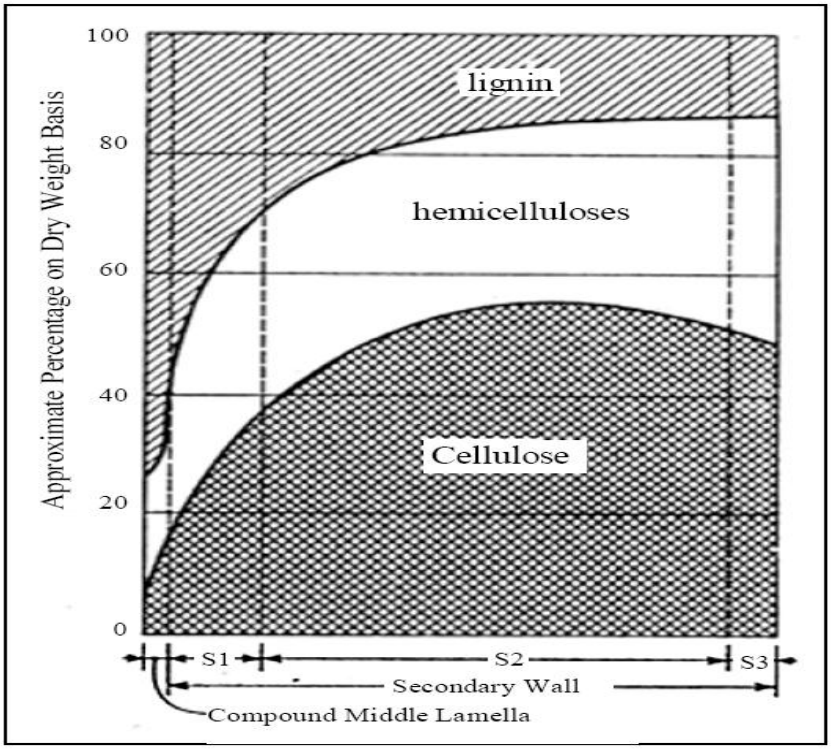

Figure 2.1: Distribution of cellulose, hemicelluloses and lignin in a typical plant cell wall

2.2.1 Lignin

Lignins are the most abundant aromatic plant component in terrestrial ecosystems and represent a significant part of plant litter input (approximately 20%) into soils (Crawford, 1981). In higher plants, lignins are chemically connected to cellulose and hemicellulose in the cellulosic fiber walls, providing strength and rigidity to the plant structures as well as resistance to the biodegradation of carbohydrates (i.e., enzymatic hydrolysis) and to environmental stresses (Brown, 1961).

Lignins are synthesized from L-phenylalanine and cinnamic acids via various metabolic ways to form lignin precursors such as sinapyl and coniferyl alcohols (Higuchi, 1971). The lignin structure consists of aromatic rings with side chains and alkyl alcohol and methoxy groups linked by various strong covalent bonds (alkyl-aryl ether and alkoxyalkane). Lignins are synthesized by oxidative copolymerization of three p-hydroxycinnamyl alcohols (p-coumaryl, coniferyl and sinapyl) which contribute in varying proportions to the macromolecular structure depending upon the morphological parts of plants (Adler, 1977).

Lignin plays a crucial part in conducting water in plant stems. The polysaccharide components of plant cell walls are highly hydrophilic and thus permeable to water, whereas lignin is more hydrophobic. The cross linking of polysaccharides by lignin is an obstacle for water absorption to the cell wall. Thus, lignin makes it possible for the plant's vascular tissue to conduct water efficiently (Silverstein, 2004).

Various chemical or biological treatments have been used to remove or modify lignin and to cleave lignin-matrix cross-links in cell walls, but the specificity of these treatments was poor, making it difficult to attribute changes in degradability to specific changes in cell wall properties. Complex structure of lignin and hemicelluloses with cellulose, which together limit the accessibility of rice straw to hydrolytic enzymes (Thevenot *et al.*, 2010). Therefore, various pretreatments of rice straw have been developed to remove lignin, hemicelluloses, reduce cellulose crystallinity and increase the porosity thus increases its enzymic hydrolysis. There has been many pretreatment of lignocellulosic material such as acid treatment, alkaline treatment and oxidative delignification ozone. The structure of a small section of a lignin polymer is shown below in Figure 2.2 (Silverstein, 2004).

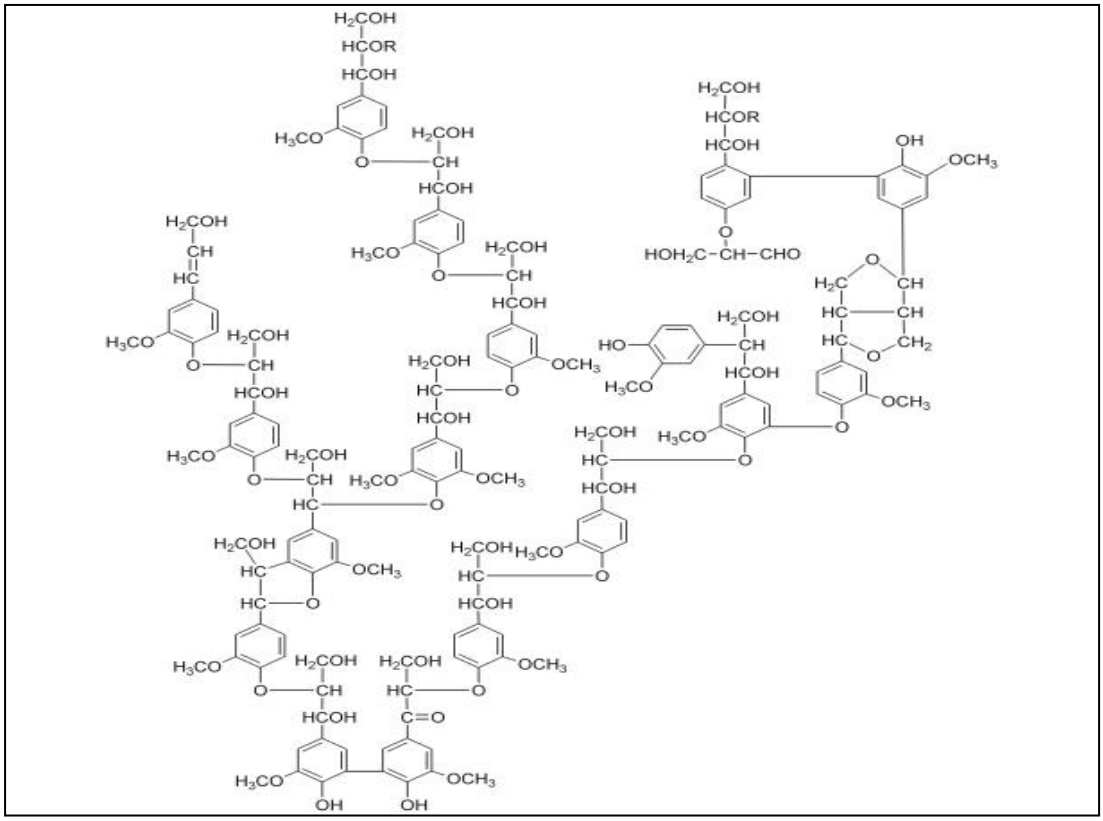

Figure 2.2: Structure of a section of a lignin polymer

2.2.2 Hemicellulose

Hemicelluloses are complex, highly branched polysaccharides that occur in association with cellulose in the cell walls. The monomers that comprise hemicellulose are hexoses (glucose, galactose and mannose) and pentoses (arabinose and xylose). Hemicellulose can be classified into three groups, namely, xylans, mannans and 1,3 galactans based on the polymer backbone that is very often homopolymeric with β-1,4 linkages (Brigham *et al.*, 1996).

In softwoods, the primary hemicellulose components are galactoglucomannans and arabinoglucuronoxylan while the principal hemicelluloses in hardwoods are glucomannans and methylglucoronoxylans (Brigham *et al.*, 1996). Xylan is the most important in terms of the percentage of total hemicellulose found in biomass. Galactoglucomannan consists of β- 1,4-linked mannose and glucose units

in a ratio of 3:1 to which O-acetyl groups and α-1,6- linked galactose side groups are attached (Puls and Schuseil, 1993).

Hemicelluloses are one of the most abundant natural polysaccharides and comprise over 30% of the dry mater of rice straw. They unlike cellulose which is a unique molecule differing only in degree of polymerization and crystalline are inhomogeneous fractions and classically defined as the alkali soluble material after removal of the pectic substances (Sun *et al.*, 2000). Figure 2.3 shows the structure of hemicelluloses.

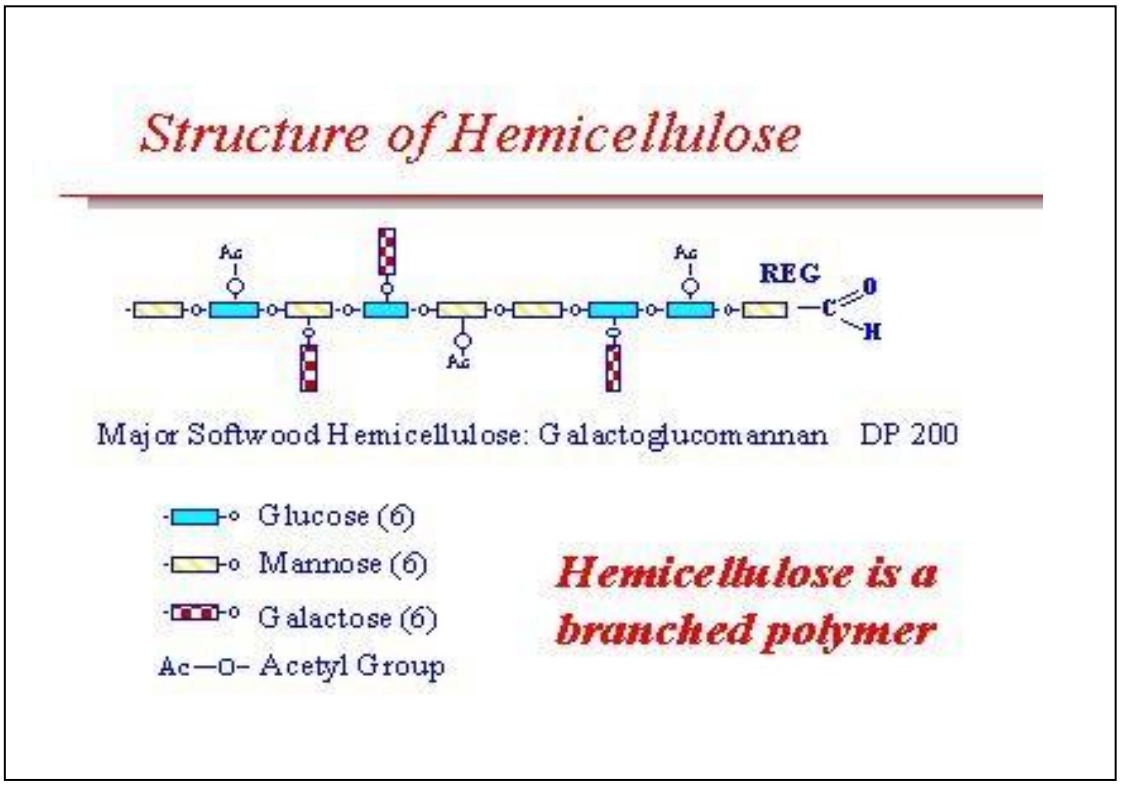

Figure 2.3: Structure of hemicellulose

2.2.3 Cellulose

Cellulose is a linear polymer of anhydro D-glucose units connected by β-1,4 glycosidic bonds as shown below in Figure 2.4. Native cellulose exists in the form of microfibrils which are paracrystalline assemblies of several dozen (1→ 4) β-D glucan chains held together by intermolecular hydrogen bonds. Intramolecular hydrogen bonds also form between two glucose units in the same chain (Carpita and McCann, 2000).

The combined bonding energies of the intermolecular and intramolecular hydrogen bonds increases the rigidity of cellulose and forms the crystalline structure that makes it highly insoluble and recalcitrant to most organic solvents. The cellulose microfibrils are imbedded in a matrix of noncellulosic polysaccharides, mainly hemicellulose and pectic substances (Silverstein, 2004), which complicates hydrolysis of cellulose to glucose even further. The cellulose in lignocellulosic biomass feedstocks provides the main source of glucose used during ethanol fermentation. Figure 2.4 shows the structure of cellulose.

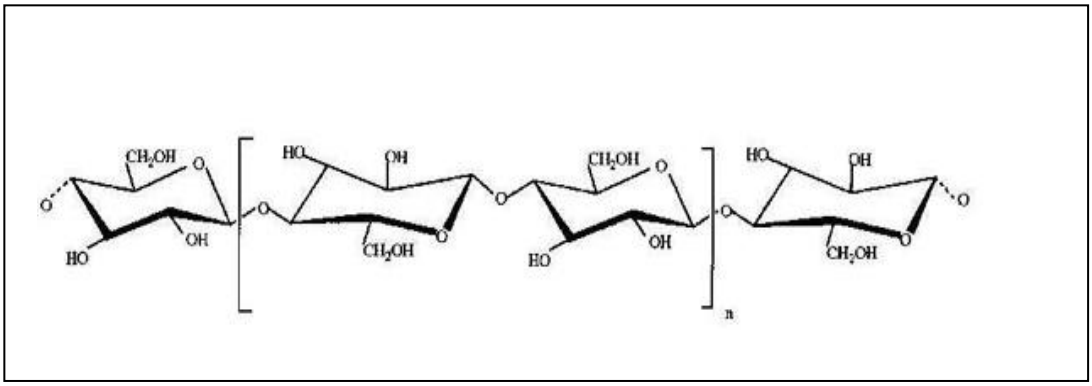

Figure 2.4: The structure of cellulose

2.3 Pretreatment of Lignocellulosic Material

Pretreatment is the first step required to fractionate lignocellulosic materials into its major plant components of lignin, cellulose and hemicellulose. The mechanisms by which pretreatments improve the digestibility of lignocellulose are however not well understood (Brown, 2003). An important goal of pretreatment is to increase the surface area of lignocellulosic material, making the polysaccharides more susceptible to hydrolysis. Along with an increase in surface area, pretreatment effectiveness and hydrolysis improvement has been correlated with removal of hemicellulose and lignin and the reduction of cellulose cyrstallinity (McMillan, 1994). A successful pretreatment must meet the following requirements: (1) improve formation of sugars or the ability to subsequently form sugars by hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes and (4) be cost effective (Sawada *et al.*, 1995).

2.3.1 Alkaline Treatment

Alkaline solutions can be used to pretreat lignocellulosic materials and the effectiveness of pretreatment is dependent upon the lignin content of the material (McMillan, 1994). The mechanism of alkali pretreatment is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components such as lignin and hemicellulose. After alkali pretreatment, the porosity of the material is increased due to the extensive swelling facilitated by removal of the crosslinks (Tarkow and Feist, 1969).

One of the examples for alkaline treatment is studied by Zhang and Chai (2008). Pretreated rice straw by 2% NaOH was analyzed for chemical components (Zhang and Chai, 2008). Cellulose, hemicellulose, lignin and ashes content of rice straw before and after NaOH pretreatment is shown in Table 2.2. As shown in Table 2.2, unpretreated rice straw contains 38.3% cellulose, 28.0% hemicellulose, 14.9% lignin and 18.8% ashes, while pretreated rice straw contains 59.3% cellulose, 10.9%

hemicellulose, 9.5% lignin and 20.3% ashes. Compared with the chemical components in the raw straw, it was clear that NaOH pretreatment increased cellulose by 54.83%, decreased hemicelluloses by 61.07% and lignin by 36.24% respectively. This data shows that pretreatment process is needed to reduce the hemicelluloses and lignin.

Table 2.2: The content of cellulose, hemicelllulose, lignin and ashes in rice straw after 2% NaOH Pretreatment

	Cellulose(%)	**Hemicellulose(%)**	**Lignin(%)**	**Ashes(%)**
Before Pretreatment	38.3	28.0	14.9	18.8
After Pretreatment	59.3	10.9	9.5	20.3

Promotion of cellulose content as well as demotion of hemicelluloses and lignin content can facilitate the process of enzymatic hydrolysis. Since a large quantity of hemicelluloses and lignin content was removed after NaOH pretreatment, it became of interest to examine the morphological changes of the rice straw by scanning electron microscopy (SEM). The SEM micrographs of rice straw before and after pretreatment are shown in Figure 2.5. Figure 2.5 (a) and (b) is the transverse section of rice straw stem before and after NaOH pretreatment, respectively. There were obvious histological changes in the transverse section of rice straw. After NaOH pretreatment, the basic tissue in rice straw severely shrank. The longitudinal section of rice straw stem before and after NaOH (Zhang and Cai, 2008). Pretreatment is shown in Figure 2.5 (c) and (d) respectively.

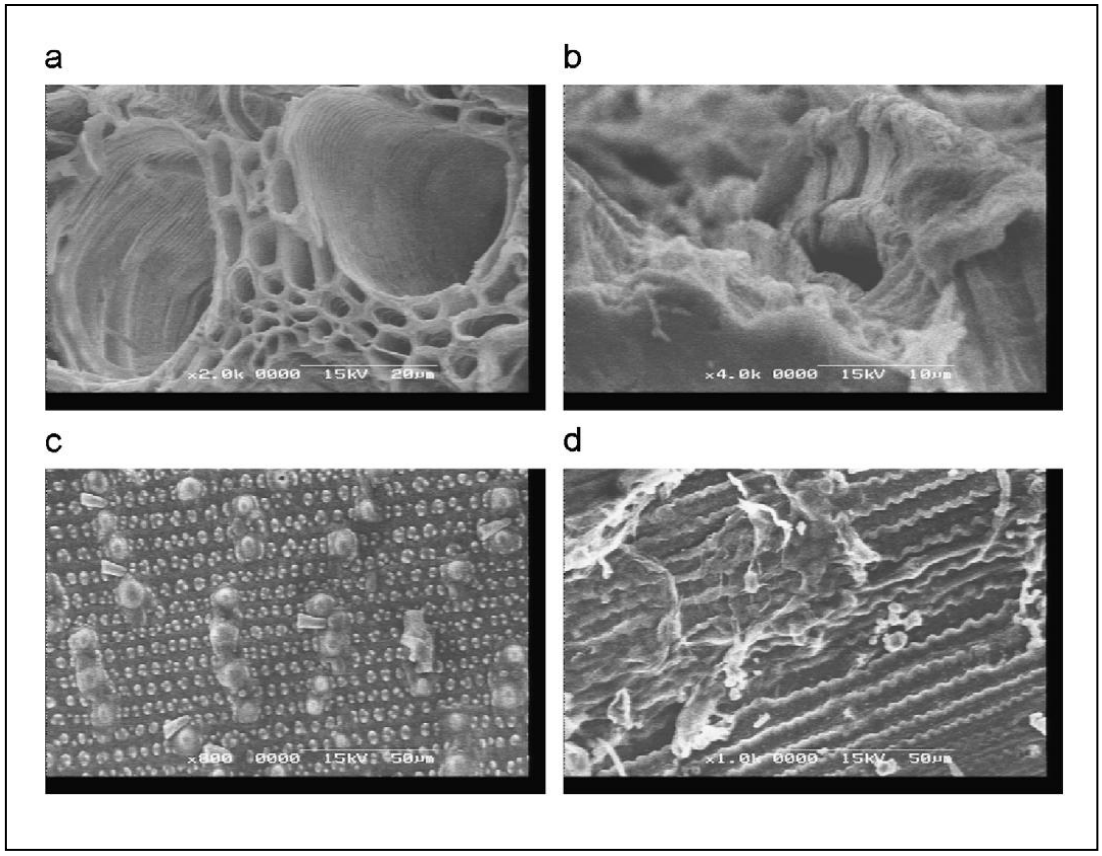

Figure 2.5: SEM micrographs of rice straw before and after 2% NaOH pretreatment. (a)Transverse section of rice straw stem before pretreatment. (b) Transverse section of rice straw stem after NaOH pretreatment. (c) Longitudinal section of rice straw stem before pretreatment. (d) Longitudinal section of rice straw stem after NaOH pretreatment (Zhang and Chai, 2008).

2.3.2 Acid Treatment

Acid pretreatment can utilize either dilute or concentrated acids to improve cellulose hydrolysis. At moderate temperatures, direct saccharification suffers from low yields due to sugar decomposition. However, prehydrolysis with dilute acid at temperatures higher than 121ºC is very effective for increasing the enzymatic digestibility of cellulose. Dilute acid pretreatment (0.2-2.0% sulfuric acid, 121-220ºC) of lignocellulose serves three important functions in the conversion process: 1) hydrolysis of the hemicellulose components to produce a syrup of monomeric sugars; 2) exposure of cellulose for enzymatic digestion by removal of hemicellulose

and part of the lignin; and 3) solubilization of heavy metals which may be contaminating the feedstock (Ingram *et al.*,1997).

As an example for acid treatment is studied by Hsu *et al*. (2009). This study is aim to propose operational conditions for the dilute acid pretreatment of rice straw and to explore the effect of the structural properties of the solid residues on the enzymatic hydrolysis.

2.3.3 Ozone Treatment

Ozone has been used to degrade lignin and hemicellulose in lignocellulosic materials such as cotton stalks ,corn stover ,wheat straw , bagasse and poplar sawdust. Some of the benefits of ozone pretreatment include the fact that no toxic residues are formed since ozone can be easily decomposed to oxygen using a catalytic bed or an increase in temperature thus eliminating the need for extensive downstream processing and ozonation reactions take place at ambient temperature and pressure so energy and investment costs are minimized (Silverstein, 2004).

Ozone is a powerful oxidizing agent and is widely used in various applications which include bleaching of cotton. Its application on the processing of silk is non-existent. Sargunamani and Selvakumar (2006) were studied the effects of ozone treatment on the properties of raw and degummed mulberry silk fabrics. Research studies on degumming and bleaching of silk reveal that almost no work involving ozone has been carried out. Therefore a study was carried out to understand the effects of process parameters namely wet pickup, pH and time in the ozone treatment of raw and degummed mulberry and tassar silk fabrics on their properties.

2.4 Enzymatic Hydrolysis

Cellulose chains can be broken into glucose molecules by cellulase enzymes. This process uses several enzymes at various stages of this conversion. Using a similar enzymatic system, lignocellulosic materials can be enzymatically hydrolyzed at a relatively mild condition (50^{o}C and pH 5), thus enabling effective cellulose breakdown without the formation of byproducts that would otherwise inhibit enzyme activity. All major pretreatment methods including dilute acid pretreatment require an enzymatic hydrolysis step to achieve high sugar yield for ethanol fermentation (Lynd, 1996).

Various enzyme companies have also contributed significant technological breakthroughs in cellulosic ethanol through the mass production of enzymes for hydrolysis at competitive prices. The fungus *Trichoderma reesei* is used by Iogen Corporation to secrete ‘specially engineered enzymes’ for an enzymatic hydrolysis process. The raw material (wood or straw) has to be pre-treated to make it able to hydrolysis (Tengborg *et al.*, 2001).

The first application of enzymes to wood hydrolysis in an ethanol process was to simply replace the cellulose acid hydrolysis step with a cellulase enzyme hydrolysis step. This is called separate hydrolysis and fermentation. An important process modification made for the enzymatic hydrolysis of biomass was the introduction of simultaneous saccharification and fermentation (SSF) which has recently been improved to include the co fermentation of multiple sugar substrates. In the SSF process, cellulase and fermenting microbes are combined. As sugars are produced, the fermentative organisms convert them to ethanol (McMillan, 1994).

Enzymatic hydrolysis provides a method to convert cellulose to glucose at high yields without sugar product degradation. Enzymatic hydrolysis of cellulose proceeds in several steps to break glycosidic bonds by the use of cellulase enzymes. Factors effecting hydrolysis of cellulose include type of substrate, cellulase loading, reaction conditions such as temperature, pH and end-product inhibitors (Silverstein, 2004).

Xu *et al.* (2007) studied about the process enzymatic hydrolysis of pretreated soybean straw. In order to produce lactic acid, from agricultural residues such as soybean straw, which is a raw material for biodegradable plastic production, it is necessary to decompose the soybean straw into soluble sugars. Enzymatic hydrolysis is one of the methods in common use, while pretreatment is the effective way to increase the hydrolysis rate.

2.5 Cellulase

Cellulases are synthesized by fungi with most research focused on fungal and bacterial cellulases produced both aerobically and anaerobically. The aerobic mesophilic fungus, *Trichoderma reesei* QM 6a and its mutants have been the most intensely studied sources of cellulases (Philippidis, 1996). However, there are also cellulases produced by other types of organisms such as plants and animals. Several different kinds of cellulases are known, which differ structurally and mechanistically.

2.5.1 Types of Cellulase

Cellulase is not a single enzyme, but is made up of a family of at least three groups of enzymes: 1,4- β-D-glucan glucanohydrolases (endoglucanases) (EC 3.2.1.21), 1,4- β-D-glucan cellobiohydrolases and 1,4- β-D-glucan glucohydrolases (exoglucanases) (EC 3.2.1.91) and β-D-glucoside glucohydrolases (β- glucosidases) (EC 3.2.1.21) (Silverstein, 2004).

The other names of endoglucanases are endo-1,4-beta-glucanase, carboxymethyl cellulase (CMCase), endo-1,4-beta-D-glucanase, Beta-1,4-glucanase, Beta-1,4-endoglucan hydrolase, Celludextrinase. The other types of cellulase belong to Excocellulases. Bet glucosidases can also be considered as yet another group of cellulases. The expression 'Avicelase' refers to the total cellulase activity of a given sample of the enzyme(s). The cellulase may be the result of the action of more than one type of enzymes (Hoshino *et al.*, 1997)

2.5.2 Action of Cellulase

A collaborative effort among these enzymes is required to break down cellulose. Enzymatic hydrolysis typically involves three steps: adsorption of endoglucanases and exoglucanases onto the surface of cellulose, biodegradation of cellulose to glucose and desorption of cellulases. Native cellulose is hydrolyzed by

the cellobiohydrolases to yield cellodextrins and cellobiose. The cellodextrins are further hydrolyzed to cellobiose, a disaccharide of glucose, by endoglucanases and then β-glucosidase hydrolyzes cellobiose to glucose (Brown, 2003). Cellulases from *T. reesei* have the advantage of having all three groups of enzymes, being more resistant to chemical inhibitors and exhibiting better stability at 50ºC than other fungal cellulases. Unfortunately, they are sensitive to product inhibition and activate slowly even at their optimum temperature (Philippidis, 1994). Increasing the cellulase loading can enhance the yield and rate of hydrolysis, but would increase the cost significantly (Silverstein, 2004).

Figure 2.6 shows the mechanism of cellulolysis. The three types of reaction catalyzed by cellulases: 1. Breakage of the non-covalent interactions present in the crystalline structure of cellulose (endo-cellulase) 2. Hydrolysis of the individual cellulose fibers to break it into smaller sugars (exo-cellulase) 3. Hydrolysis of disaccharides and tetrasaccharides into glucose (beta-glucosidase) (Hoshino *et al.*, 1997).

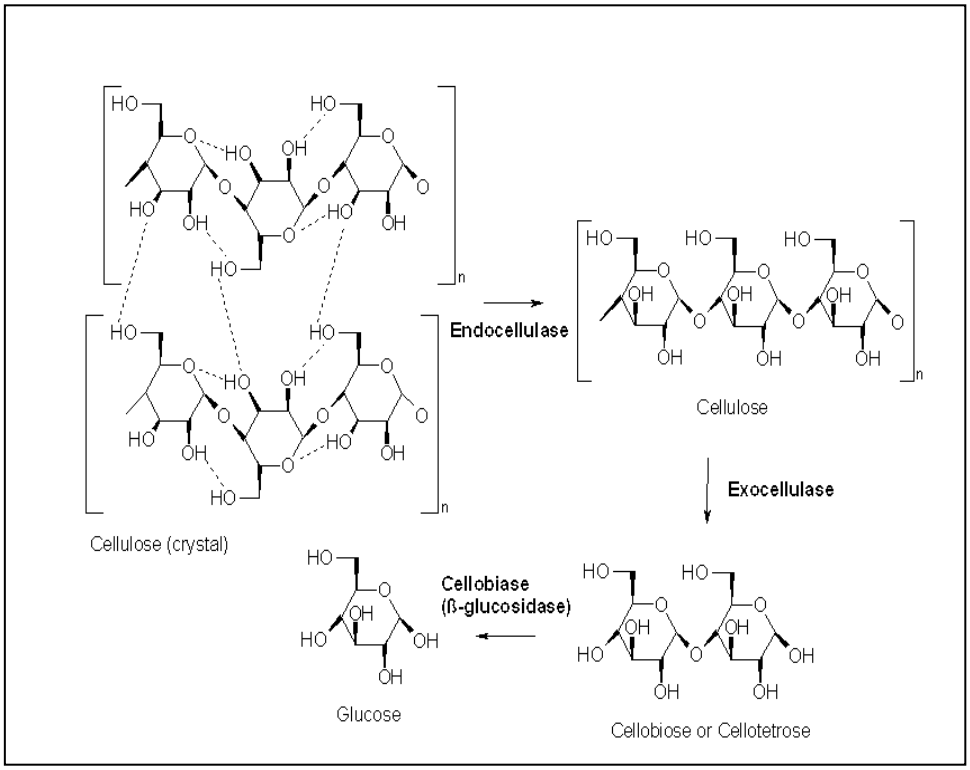

Figure 2.6 : Mechanism of cellulolysis

2.5.3 Applications of Cellulase

Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. Furthermore, cellulases are widely used in textile industry and in laundry detergents. They have also been used in the pulp and paper industry for various purposes, and they are even used for pharmaceutical applications. Cellulase is used in the fermentation of biomass into biofuels, although this process is relatively experimental at present. Cellulase is used as a treatment for Phytobezoars, a form of cellulose bezoar found in the human stomach (Han and He, 2009).

2.6 Production of Glucose

Any biological material that has sugar, starch or cellulose can be used as biomass for producing anhydrous ethanol. In order to maintain the high energy consumption lifestyles that people have grown accustomed to, the prospect of converting renewable biomass resources into biofuels such as ethanol, methanol and biodiesel must be investigated. Starch, which normally constitutes about 70% from the biomass such as corn kernel is easily broken down into glucose that is then fermented to ethanol. Anhydrous ethanol is one of the biofuels produced today and it is a subset of renewable energy. It is considered to be an excellent alternative clean-burning fuel to gasoline. Anhydrous ethanol is commercially produced by either catalytic hydration of ethylene or fermentation of biomass (Kumar *et al.,* 2009).

Many researcher had studied the reseach about glucose production from biomass. Montross and Crofcheck (2004) studied the effect of stover fraction and storage method on glucose production during enzymatic hydrolysis , whileYanez et al. (2004) studied the effect of stover fraction and storage method on glucose production during enzymatic hydrolysis. Furhermore, Kunamneni and Singh (2005) had studied about response surface optimization of enzymatic hydrolysis of maize starch for higher glucose production.

2.7 Factor Affecting the Production of Glucose

There are several factors which can contribute to the high reducing sugar yield in enzymatic hydrolysis.

2.7.1 pH

The productivity and efficiency of cellulase are significant in cellulose hydrolysis. pH for the working condition can give effect on productivity and efficiency of enzyme because it can have an effect of the state of ionization of acidic or basic amino acids. If the state of ionization of amino acids in a protein is altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered.

Others have shown in their research such as combination effect of pH and acetate on enzymatic cellulose hydrolysis (Romsaiyud *et al.,* 2009). The effects of pH and acetate on cellulase produced from *Bacillus coagulans* were studied at various pH. The results showed that the suitable pH range for cellulase production and cellulose hydrolysis (represents eficiency of cellulase) was 2.6–7.5, and 5.3–8.3, respectively. Furthermore, Generic Model Control (GMC)-fuzzy control of pH during enzymatic hydrolysis of cheese whey proteins was studied by Sousa *et al.,* 2004.

2.7.2 Temperature

The enzymatic hydrolysis of cellulose with cellulases takes place fundamentally via two consecutive reactions, firstly the endoglucanases and exoglucanases are responsible for degrading the cellulose into cellobiose and b-1,4-glucosidases then act upon the cellobiose to free glucose molecules. This last stage is of great importance in the efficient hydrolysis of cellulose. In general, one of the

main problems affecting the yields from cellulose hydrolysis is that the cellulases tend to be inhibited by the high temperature (Bravo *et al.*, 2000).

In other work, Bravo *et al*. (2000) studied the influence of temperature upon the hydrolysis of cellobiose by β-1,4-glucosidases from *Aspergillus niger* This research studied into the enzymatic hydrolysis of cellobiose within the temperature range of 40°C to 70°C at pH 4.9, by using β-1,4-glucosidase from *Aspergillus niger*. At 70°C there was significant enzyme deactivation, whereas between 40°C and 60°C noted a substrate inhibition and contributed to glucose production.

2.7.3 Agitation Rate

Agitation rate is one of the indispensable parameter for proper oxygen transfer and homogeneous mixing of the nutrients in fermentation system. Therefore, the effects of different agitation rates on glucose production from enzymatic hydrolysis of rice straw were studied by Ingesson *et al*. (2001). This research studied about the effect of shaking regime on the rate and extent of enzymatic hydrolysis of cellulose. In an attempt to elucidate the effect of mixing on the rate, three shaking regimes were used: continuous at low-speed (25 rpm), continuous at high-speed (150 rpm) and an intermittent regime comprised of high and low-speed shaking intervals. The continuous, high-speed shaking produced the highest conversion yields, whereas the intermittent and low-speed shaking regimes resulted in lower conversions. Thus, it appears that intermittent shaking could be a beneficial process option as it can reduce the mixing energy requirements while producing reasonably high conversion yields.

2.7.4 Hydrolysis Time

Determining the minimum reaction time is important. It is related with to energy consumption that necessary for the shaking and the maintaining of a constant temperature (enzymatic activity depends on the temperature and pH, amongst other factors). Reaction time is also related with the process scale-up.

The influence of hydrolysis time on the percentage of rice straw decomposing is shown in Figure 2.7. It could be seen that in the optimal hydrolysis conditions, *T.reesei ZM4-F3* can decompose 68.21% of rice straw in 120 h (Zhang and Chai, 2008).

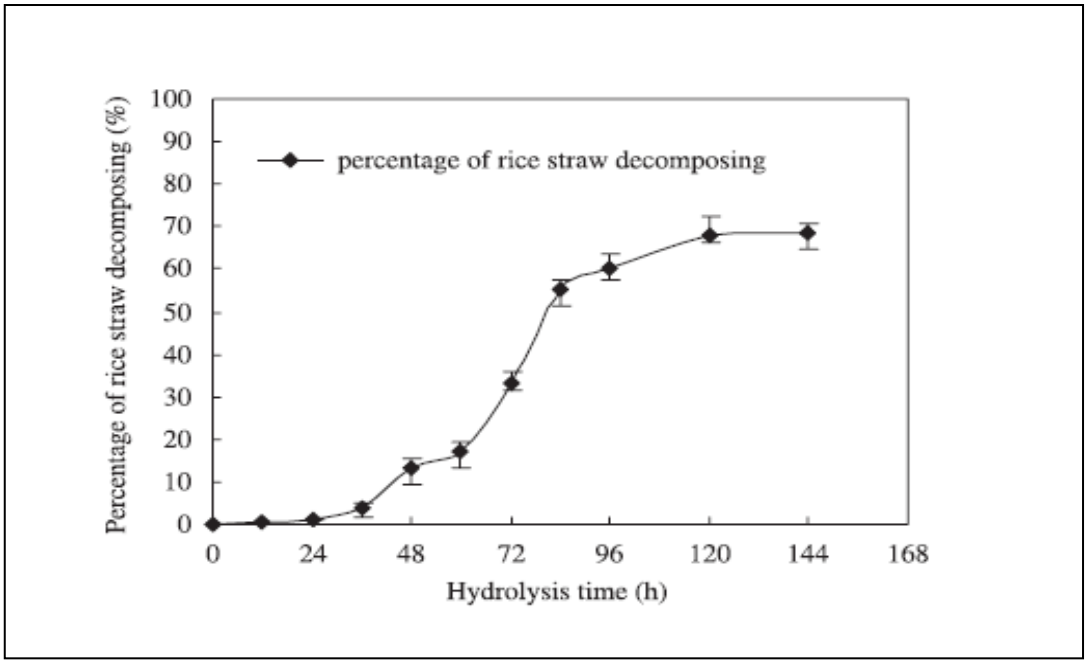

Figure2.7: Influence of hydrolysis time on percentage of rice straw decomposing.

2.8 Optimization of Glucose Production by Using Response Surface Methodology (RSM)

Response Surface Methodology (RSM) can be defines as a statistical method that uses quantitative data from appropriate experiments to determine and simultaneously solve multivarient equations. There are several factors to consider which are critical factors are known system well understood, region of interest where factor levels influencing product is known, factors vary continuously throughout the experimental range tested, a mathematical function relates the factors to the measured response and the response defined by the function is a smooth curve (Cheong *et al.*, 2007).

Response Surface Methodology (RSM) has been extensively applied to optimize culture medium and other process parameters for the production of glucosyltransferase by *Aspergillus niger* (Lee and Chen, 1997), bioconversion of activated sludge (Mannan *et al.*, 2007) and saccharification of rice straw by cellulase from*Trametes hirsuta* (Jeya *et al.*, 2009).

Lee and Chen (1997) studied the optimization of medium composition for the production of glucosyltransferase by *Aspergillus niger* using a statistical and mathematical approach. This research used a central composite design to optimize the medium composition for production of glucosyltransferase by *Aspergillus niger*. Yeast extract was the best nitrogen source after cultivation for 7 days. Addition of minerals to the medium showed no significant increase in the production of glucosyltransferase. A significant decrease in the production of glucosyltransferase was obtained when the initial pH of the medium was adjusted at 3 or 4. The concentration of inoculated spores of 5.8×10^6 or 1×10^7 per ml medium caused the higher production of glucosyltransferase. The central composite experimental design (CCD) and Response Surface Methodology (RSM) were employed to derive a statistical model for the effects of maltose and yeast extract on the production of glucosyltransferase by *Aspergillus niger*. An initial concentration of 4.5% maltose and 6.6% yeast extract have been found optimum to maximize the production of

glucosyltransferase. This concentration of maltose shows that the increment of before and after optimization process is 30%.

One of other examples, Pericin *et al*. (2009) studied about enzymatic hydrolysis of protein isolate from hull-less pumpkin oil cake in application of Response Surface Methodology (RSM). Enzymatic hydrolysis of protein isolate from hull-less pumpkin (*Cucurbita pepo* L) oil cake was studied by response surface methodology, using a central-composite experimental design. The hydrolysis was carried out with an acid protease, at temperature of 30 °C and pH 3.00. Second-order polynomial model was proposed with regard to of effect of time, enzyme and NaCl concentration. The mathematical model showed good fit with the experimental data, since the R^2 of 0.946 indicated that 94.6% of the variability within the range of values studied could be explained by the model. A hydrolysis time of 32.5 h, enzyme concentration of 0.137% (v/v) and NaCl concentration of 0.84% (w/v) were found to be the optimal conditions to achieve the highest value of degree of hydrolysis (DH). An initial degree of hydrolysis was 22.63% and after optimization process was 47.42%. This percentage of degree of hydrolysis shows that the increment of before and after optimization process is 53%.

CHAPTER 3

MATERIALS AND METHODS

3.1 Strategies for Optimization of pH, Temperature and Agitation Rate on Glucose Production from Enzymatic Hydrolysis of Rice Straw

This chapter will elaborate on the materials and methods that have been applied in this experiment. To ensure the flow of experiment goes smooth, it has been divided into 3 stages. The first stage of the experiment was to obtain the standard curve of glucose concentration versus optical density at 540nm (OD_{540}). The second stage of the experiment was carried out to obtain the best range of pH, temperature and agitation rate. One factor at a time method (OFAT) was applied at this stage. For the third stage of this experiment, Response Surface Methodology (RSM) was used to further optimize the data gained from stage 2. Interaction of all parameters was analyzed at this stage. Figure 3.1 show the flow diagram that simplified the strategies of this research and Figure 3.2 shows the flow diagram for process of enzymatic hydrolysis.

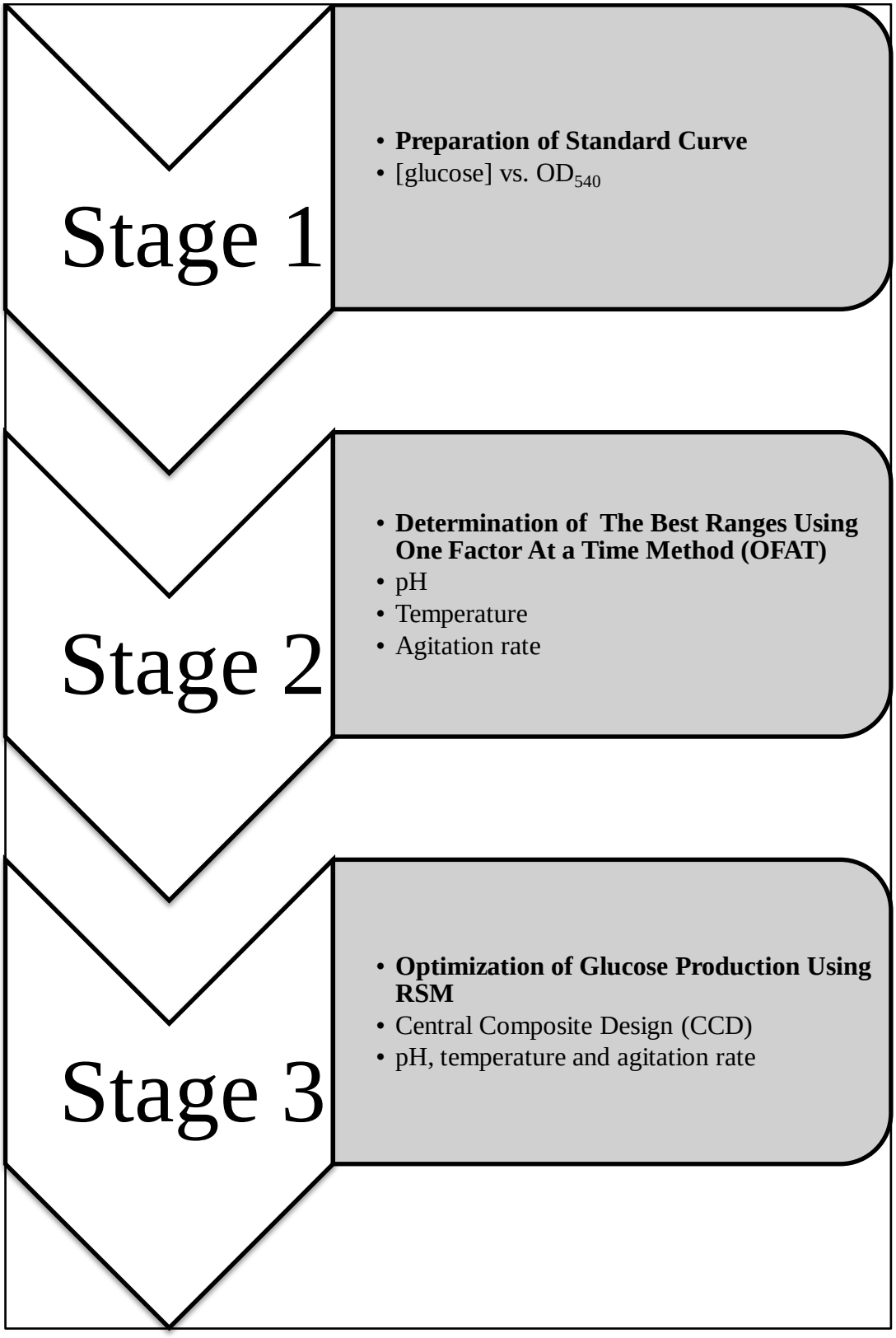

Figure 3.1 : Research design for optimization of glucose production from enzymatic hydrolysis of rice straw.

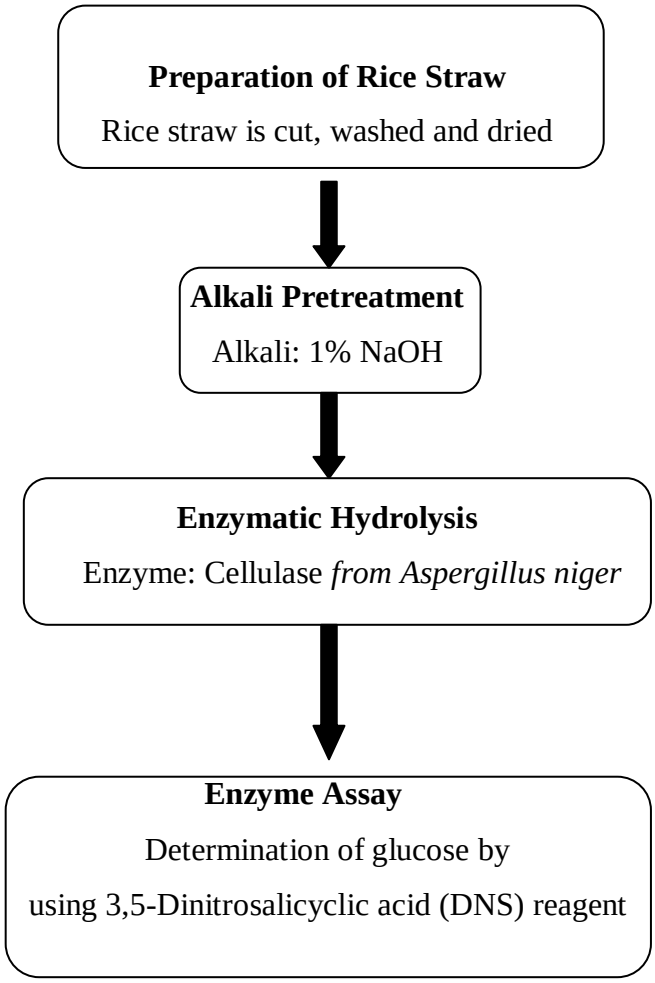

Figure 3.2 : Flow chart of process enzymatic hydrolysis from rice straw

3.2 Preparation of Rice Straw

The rice (*Oryza sativa*) straw was obtained from Kelantan, Malaysia. According to Zhu *et al.* (2005) methods, before any pre-treatment, it was cut to nominally 1–2 cm length and washed thoroughly with tap water until the washings were clean and colourless and then air dried in oven at 70˚C until it kept a constant weight. Then it was stored at room temperature till further treatment.

3.3 Alkali Pretreatment

The alkali pretreatment was carried out as follows: 20 g of rice straw after cutting and washing was suspended in 160 ml of 1% NaOH aqueous solution in a 500 ml beaker and kept boiling in a 500 ml beaker for 2 hour. The residues were collected and washed extensively with tap water until neutral pH, dried at 65˚C until it kept a constant weight.

3.4 Enzymic Hydrolysis

A typical hydrolysis mixture consisted of 1 g treated rice straw, 20 mg of enzyme powder and 20 ml 0.1 M citric acid buffer (pH 4.8), which was supplemented with antibiotics cycloheximide (30 mg/ml) to prevent microbial contamination. The mixture was incubated at 45 ˚C in a rotary shaker at 160 rpm for 48 hour. Then, it was centrifuged for 20 min at 8000 rpm. The supernatant was used for reducing sugar analysis (Zhu *et al.*, 2005).

3.5 Enzyme Assay

Enzyme assay was important to determine the concentration of the glucose which was converted from cellulose catalyzed by cellulase enzyme in a sample. The method used was as described by Bom *et al.* (2001). After two days or the culture had achieved OD_{540} = 1.5 and above, the enzyme can be extract for assay purpose.

The 3,5-Dinitrosalicylic acid (DNS) reagent was prepared in the following way. Firstly, 20 gram 3,5-dinitrosalicylic acid was suspended in 400 ml water. While continuously stirring 300 ml sodium hydroxide solution was added (32 g/300 ml water) and the volume was subsequently adjusted to 1.5 L by the addition of water. Stirring was continued until a clear solution was obtained. Next 600 gram rochelle salt (sodium potassium tartrate) was added and stirring (and, if necessary, heating) was continued until dissolution. The volume was adjusted to 2 L and the solution filtered, if necessary. The solution was kept at room temperature in the dark and protected against carbon dioxide absorption. The reagent was stable for at least one month.

After enzymatic hydrolysis was stopped, 1 ml of sample was put in the test tube and heating the test tube in the boiling water for 3 minutes to stop the enzyme activities,. Then, 1 ml of 3,5-Dinitrosalicylic acid was added into 1 ml of sample. The amounts of reducing sugar groups were determined by heating the test tubes in boiling water for 10 minutes, cooling to room temperature and recording the extinction at 540 nm (Bom *et al.*, 2001).

3.6 Preparation of Standard Curve

Glucose stock solution was prepared by disolved 0.6 g xylose into 100 ml distilled water. The concentration of the stock solution was 6 g/L. The siries of different concentration of glucose solution from 1.0 g/L until 6.0 g/L was prepared in test tube by adding distiled water into stock solution. The volume of each test tube

was adjusted to 10.0 ml. The one tenth dilution was performed by adding 1.0 ml solution into 9.0 ml distiled water. Then put 1 ml of glucose solution in the test tube, 1 ml of 3,5-Dinitrosalicylic acid was added into 1 ml of sample. The amounts of glucose groups were determined by heating the test tubes in boiling water for 10 minutes, cooling to room temperature. The optical density was read using spectrometer with wavelenght 540 nm. Standard curve was ploted by glucose concentrated versus OD_{540}. Table 3.1 shows the concentration of glucose involed in preparation of standard curve.

Table 3.1 : Concentration of glucose involved in preparation of standard curve

Glucose Concentration g/L	0.1	0.2	0.3	0.4	0.5	0.6
dH_2O	0.9	0.8	0.7	0.6	0.5	0.4
Volume, ml	1.0	1.0	1.0	1.0	1.0	1.0
OD_{540}						

3.7 Selection of the Best Value of the Parameters in Glucose Production Using Conventional Method

The hydrolysis mixture was incubated in the incubator shaker. To obtain the best range of temperature, the temperature must be varied while the agitation rate and pH must be set constant. The temperatures of the incubator shaker were set at 25, 35, 45, 50 and 55^{o}C. After the second day, each of the concentration of glucose had reach OD_{540} 1.50 and above. The best temperature was obtained, the value was used in the next step which was to determine best pH.

The second step was to determine the best pH for glucose production. To obtain the best pH, the pH must be varied while the temperature and agitation rate must be set constant. Five different pH were set at 4.0, 4.3, 4.5, 5.0 and 5.5. The temperature and agitation rate for each set was constant at the best temperature obtained in the first step. The best pH was obtained, the value was used in the next step which was to determine best agitation rate.

The next step was to determine the best agitation rate for glucose production. To obtain the best agitation rate, the rate must be varied while the temperature and pH must be set constant. Five different agitation rates were set at 50, 100, 150, 200 and 250 rpm. The temperature and pH for each set was constant at the best temperature and pH obtained in the first and second step. Table 3.2 shows the simplified data in screening the best condition in glucose production using conventional method.

Table 3.2 : The best value of the parameters for screening process in glucose production using conventional method.

Set	**Parameter**	**Manipulated Value**					**Best Value**
1	Temperature,^{o}C	25	35	45	50	55	45
2	pH	4.0	4.3	4.5	5.0	5.5	4.3
3	Agitation rate, rpm	50	100	150	200	250	150

3.8 Optimization of Temperature, pH and Agitation Rate on Glucose Production of Rice Straw Using Response Surface Methodology (RSM)

Based on the previous result of one at a time method (OFAT), the low level and high level between the best value of temperature, pH and agitation rate were used for further study using Response Surface Methodology (RSM). For optimization of glucose production, the design was made up of a full 2^3 factorial with a total of 17 experiments using Central Composite Design (CCD). Among of the 17 experiments, there are 14 star points and 3 replicates at the centre points. The value of alpha was set at 1.68179. The value of alpha determines the location of the star points in a central composite design. Table 3.3 shows the parameters and level involved in optimization of cultural conditions.

Table 3.3 : Initial data used in RSM

Factors	Symbol	Units	Low Level	High Level
pH	A	-	4.0	4.6
Temperature	B	°C	40	50
Agitation Rate	C	rpm	120	180

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Standard Curve of Glucose

A standard curve is a quantitative research tool, a method of plotting assay data that is used to determine the concentration of glucose. The determination on how much of unknown measure will obtain by comparing it to the absorbance of the standard curve. The relationship between absorbance and concentration of glucose was modeled in Equation 1

$$Y = 2.2\,X - 0.32 \qquad \text{Equation (1)}$$

Y = Concentration of glucose (g/L)
X = Absorbance (OD)

The standard curve for measuring the concentration of glucose was showed in Figure 4.1.

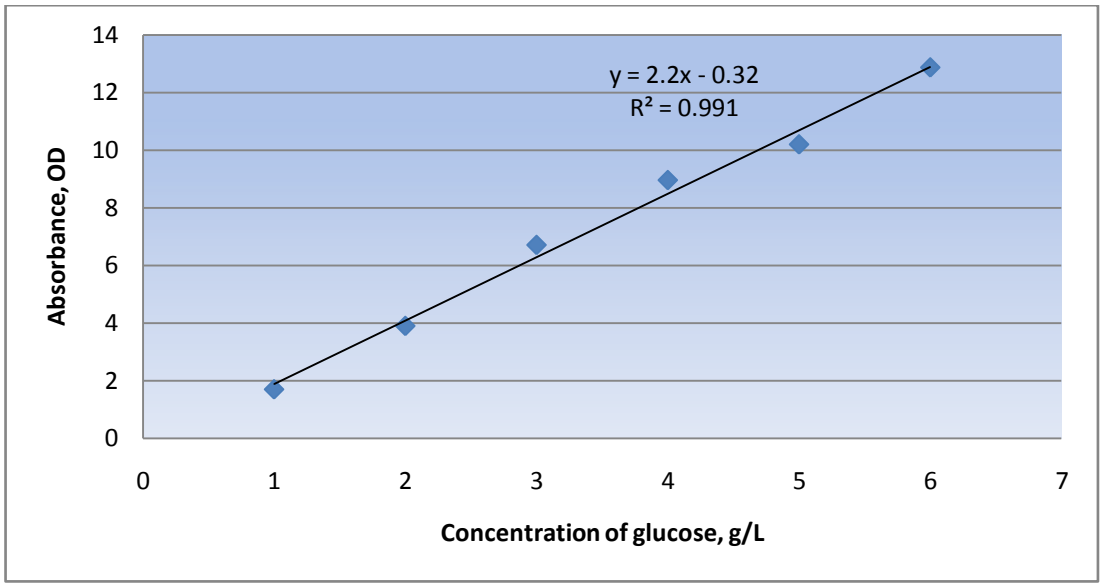

Figure 4.1: Standard curve of glucose

4.2 Screening of the Best Range of Temperature, pH and Agitation Rate on Production of Glucose from Rice Straw Using Conventional Method

Studies on the parameters of enzymatic hydrolysis of rice straw by cellulase from *Aspergillus niger* that affect the production level of glucose were carried out by using the conventional method. The method used was one factor at a time which manipulating one parameter while keeping the other at a certain level. This study was done to determine the best range for all parameters for further optimization process. Basically, the low and high levels of every parameter were obtained before and after peak values respectively in the appropriate study. The parameters involved in this study were temperature, pH and agitation rate.

4.2.1 The Effect of pH on Production of Glucose

The effect of pH was studied to determine the best range of pH on production of glucose from rice straw. The pH were manipulated at 4.0, 4.3, 4.5, 5.0 and 5.5 while agitation rate and temperature were kept constant at 180 rpm and 45 respectively. The data obtained from the experiment was showed in Figure 4.2.

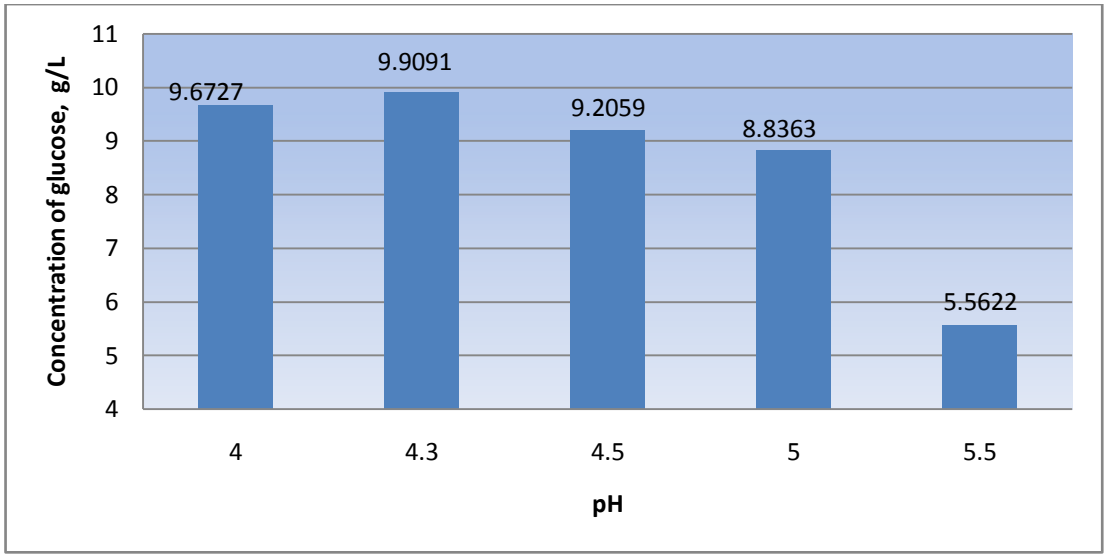

Figure 4.2: The effect of pH on concentration of glucose

From the graph, the concentration of glucose was increased from pH 4 until pH 4.3 and started to decrease after pH 4.3 until pH 5.5. The value of pH 4.3 was the best pH and the concentration of glucose achieved by this pH was 9.9091 g/L. The lowest concentration of glucose was 5.5622 g/L at pH 5.5. According to the results, the optimum pH for glucose production was in the range 4.0 – 4.6. The best pH (4.3) was used for the next step in order to study the effect of temperature on the glucose production.

Zhang and Chai (2008) have reported that 4.5 as an optimal pH for glucose production from rice straw by cellulase from *Trichoderma reesei* ZM4-F3. From this research, the range of pH 4.0 until 5.5 had been used for glucose production from enzymatic hydrolysis of rice straw (Zhang and Chai, 2008).

The optimum pH to get the optimum concentration of reducing sugar was 4.5 (Zhang and Chai, 2008) while the actual value gained from the experiment was 4.3 aimed to obtain optimum concentration of glucose within experiment.

4.2.2 The Effect of Temperature on Production of Glucose

The effect of temperature was studied to determine the best range of temperature on production of glucose from rice straw. The temperature were manipulated at 25, 35, 45, 50 and 55^{o}C while agitation rate and pH were kept constant at 180 rpm and 4.5 respectively. The data obtained from the experiment was showed in Figure 4.3.

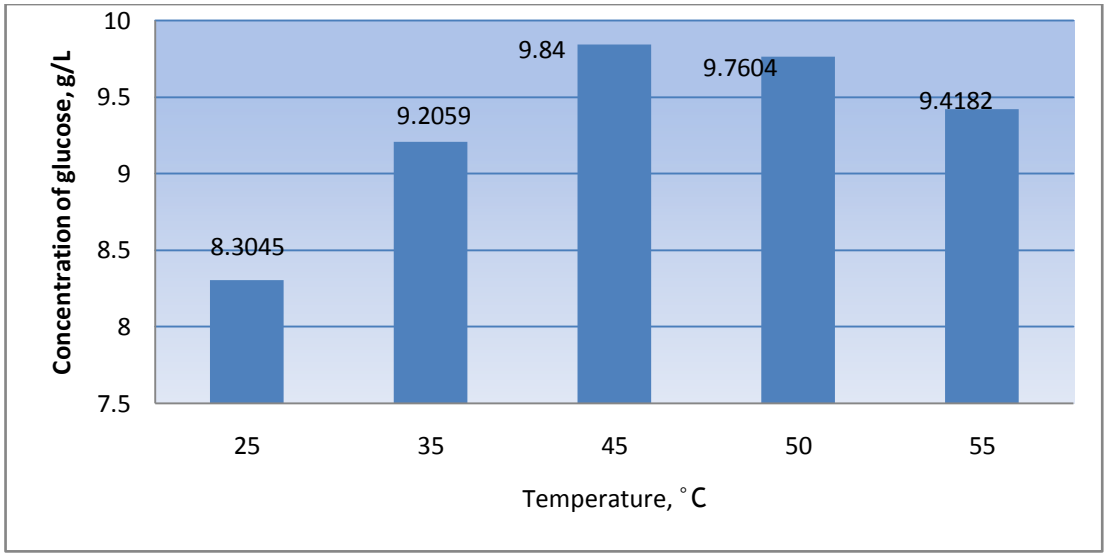

Figure 4.3: The effect of temperature on concentration of glucose

From the graph, the concentration of glucose was increased from temperature 25 ^{o}C until 45^{o}C and started to decrease after temperature 45^{o}C until 55^{o}C. 45^{o}C was the best temperature and concentration of glucose achieved by this temperature was 9.8400 g/L while the lowest concentration of glucose was 8.3045g/L at 25^{o}C. According to the results, the optimum temperature for glucose production was in the range 40^{o}C until 50^{o}C. The best temperature (45 ^{o}C) was used for the next step in order to study the effect of temperature on the glucose production.

Zhu *et al.* (2005) had reported that 45.2^{o}C as an optimal temperature for glucose production by cellulase from *Trichoderma reesei.* The temperature of 40^{o}C until 50^{o}C has been used for glucose production from rice straw (Zhang and Chai, 2008; Kunamneni and Singh, 2005; Lee *et al.*, 2006).

The optimum temperature to get the optimum concentration of reducing sugar was 45.2^{o}C Zhu *et al.* (2005) while the actual value gained from the experiment was 45^{o}C aimed to obtain optimum concentration of glucose within experiment.

4.2.3 The Effect of Agitation Rate on Production of Glucose

The research about effect of agitation rate was done to determine the best range of agitation rate on glucose production using a constant temperature and pH at 45^{o}C and 4.3 respectively which obtained from the previous study. The agitation rate were set at 50, 100, 150, 200 and 250 rpm. The result from the experiment was showed in Figure 4.4.

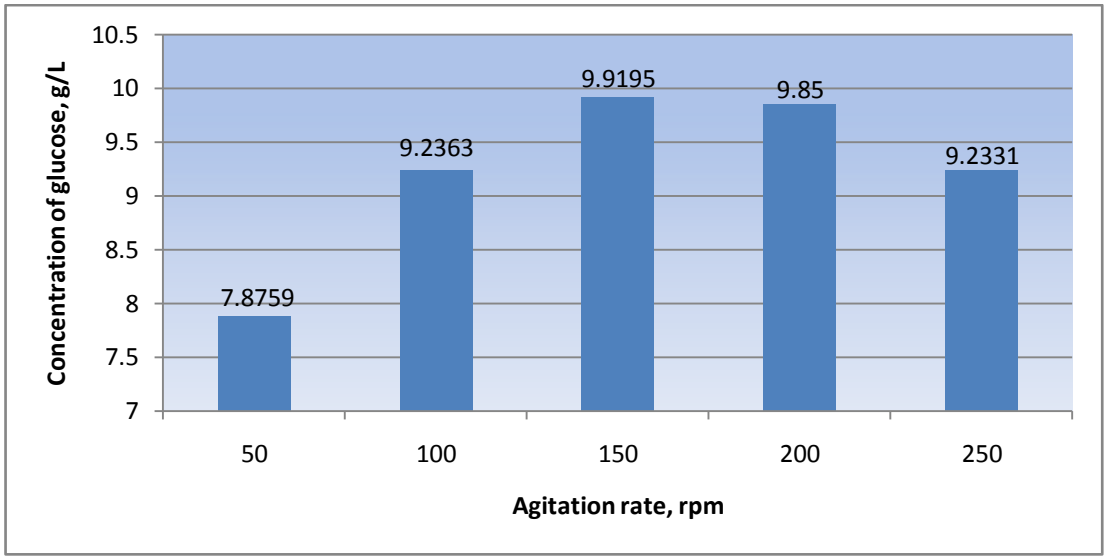

Figure 4.4: The effect of agitation rate on concentration of glucose

From the graph, the concentration of glucose was increased from agitation rate 50 rpm until 150 rpm and started to decrease after 150 rpm until 250 rpm. 150 rpm was the best agitation rate and concentration of glucose achieved by this agitation rate was 9.9195 g/L while the lowest concentration of glucose was 7.8759 g/L at 50 rpm. According to the results, the optimum agitation rate for glucose production was in the range 120 rpm until 180 rpm

Xu *et al.* (2007) reported that the best agitation rate for glucose production from soybean straw was 150 rpm. Furthermore, Zhang and Chai (2008) were using agitation rate of 140 rpm until 200 rpm as initial data in orthogonal experiments for glucose production from rice straw. The best agitation rate for glucose production from rice straw for this research was 180 rpm.

The optimum agitation rate to get the optimum concentration of reducing sugar was 150 rpm Xu *et al.* (2005) while the actual value gained from the experiment was 150 rpm aimed to obtain optimum concentration of glucose within experiment.

As conclusion, the optimum results for glucose production were 4.3, 45^{o}C and 150 rpm for pH, temperature and agitation rate respectively. Those values were used in the Response Surface Methodology (RSM) as centered values. The range of all parameters to be further study in Response Surface Methodology (RSM) for pH, temperature and agitation rate were between 4.0 to 4.6, 40^{o}C to 50^{o}C and 120 rpm to 180 rpm respectively.

4.3 Determination of the Optimum pH, Temperature and Agitation Rate on Glucose Production from Rice Straw Using Response Surface Methodology

The response of glucose concentration from enzymatic hydrolysis, as a function of pH (A), temperature (B) and agitation rate (C) were evaluated in Central Composite Design (CCD). A CCD consisting of three variables was used in this study. The three variables and their levels were pH (A, 4.0-4.6), temperature (B, 40^{o}C -50^{o}C) and agitation rate (C, 120 rpm-180 rpm). This design generated a total of 17 experiments with different settings by using Design Expert 6.0.8 software.

Equation (2) described the relationship between the significant variables and the yield of enzymatic hydrolysis from rice straw was derived by the adjusted model. The equation was shown as follows:

$$\text{Concentration of glucose} = 9.86 - 0.16\,A - 0.23B + 0.42C - 0.59\,A^2 - 0.30B^2 - 0.19C^2 + 0.034\,AB + 0.084A\,C - 0.11\,BC$$

Equation (2)

Concentration of glucose is the predicted response, A is coded value for pH, B is coded value for temperature and C is coded value for agitation rate. A base of inverse transformation was performed with quadratic as original design model which consist of 1 offset, 3 linear and 3 quadratic. The predicted levels of concentration of glucose from enzymatic hydrolysis of rice straw at each experimental point using Equation (1) are given in Table 4.1.

Table 4.1: Central composite design matrix

Standard	pH	Temperature (° C)	Agitation rate (rpm)	Response Concentration of glucose (g/L)	
	A	B	C	Actual Value	Predicted Value
1	4.0	40	120	8.7487	8.7518
2	4.6	40	120	8.1056	8.1936
3	4.0	50	120	8.4138	8.4492
4	4.6	50	120	8.0788	8.0255
5	4.0	40	180	9.6094	9.6448
6	4.6	40	180	9.4765	9.4232
7	4.0	50	180	8.9901	8.8842
8	4.6	50	180	8.8181	8.7971
9	3.8	45	150	8.4486	8.4588
10	4.8	45	150	7.9012	7.9161
11	4.3	37	150	9.4487	9.3964
12	4.3	53	150	8.5381	8.6155
13	4.3	45	100	8.6587	8.6064
14	4.3	45	200	9.9289	9.9806
15	4.3	45	150	9.7069	9.8564
16	4.3	45	150	9.9311	9.8564
17	4.3	45	150	9.9357	9.8564

Based on Table 4.1, the highest concentration of glucose from actual value (experimental value) which produced 9.9357 g/L was read on Standard 17. The parameters of Standard 17 were 4.3 of pH, 45^{o}C of temperature and 150 rpm of agitation rate. The lowest concentration of glucose was recorded at Standard 10 with 7.9012 g/L. The parameters for Standard 10 were 4.8 of pH, 45^{o}C of temperature and 250 rpm of agitation rate.

Using Design Expert 6.0.8, an analysis of variance was conducted for evaluation of the effects of the variables and their probably existed interactions. Coefficients of the full model were analyzed for their significance and the insignificant ones were eliminated from the model by backward elimination. The reduced model was adjusted after all the insignificant variables (p-value > 0.05) were excluded. The results of Analysis of Variance (ANOVA) for the were shown in Table 4.2.

Table 4.2 : ANOVA for response surface quadratic model for production of glucose from enzymatic hydrolysis of rice straw.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	Prob > F	
Model	7.750987	9	0.861221	75.76464	< 0.0001	Significant
Residual	0.079569	7	0.011367			
Lack of Fit	0.045357	5	0.009071	0.530307	0.7547	Not significant
Pure Error	0.034212	2	0.017106			
Corr Total	7.830556	16				

The p-value of the adjusted model was lower than 0.0001 indicating that the model is statistically valid. The p-value of this model was lower than 0.0001 demonstrating that they were the most significant factors than the other ones influencing the response. The Model F-value of 75.76 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant.

Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve this model. The "Lack of Fit F-value" of 0.53 implies the Lack of Fit is not significant relative to the pure error. There is a 75.47% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good. Table 4.3 shows the regression coefficients and p-value calculated from the model.

Table 4.3: Regression coefficients and p-value calculated from the model

Factor	**Coefficient Estimate**	**p-Value Prob > F**
Intercept	9.85646	
A-Ph	-0.16136	0.0008
B-Temp	-0.23218	< 0.0001
C-RPM	0.416158	< 0.0001
A^2	-0.59007	< 0.0001
B^2	-0.30069	< 0.0001
C^2	-0.19448	0.0005
AB	0.033625	0.4020
AC	0.08415	0.0608
BC	-0.1145	0.0189

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2, B^2, C^2, BC are significant model terms Table 4.4 shows the results of determination of coefficient of the design.

Table 4.4: Results of determination of coefficient of the design.

$\boldsymbol{R^2}$	0.989839
Adjusted $\boldsymbol{R^2}$	0.976774
Predicted $\boldsymbol{R^2}$	0.943156
Adeq Precision	25.56112

According to Table 4.4, the R^2 value was 0.9898 in good agreement with the adjusted R^2 value of 0.9768. The vicinity of adjusted R^2 to R^2 means a good adjustment of the theoretical values to the experimental data by the model (Fang *et al* 2010). Meanwhile the lack of fit was insignificant but the R^2-value (0.9898) was high indicating that the model was well adapted to the response. So the adjusted model was suitable to predict the experimental data from enzymatic hydrolysis of rice straw. The Predicted R^2 of 0.9432 is in reasonable agreement with the Adjusted $R^2$of 0.9768. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. This ratio of 25.561 indicates an adequate signal. This model can be used to navigate the design space.

The relationship between the response and variables were visualized by response surface constructed according to the full model (Figure 4.5.4.6 and 4.7). Each 3D response surface plot showed relative effect of two variables on yield of enzymatic hydrolysis while the other ones were staying at level 0.

Response surface plots showing the interaction variables in the conversion of cellulose to glucose. Figure 4.5 shows the effect of pH in glucose production.

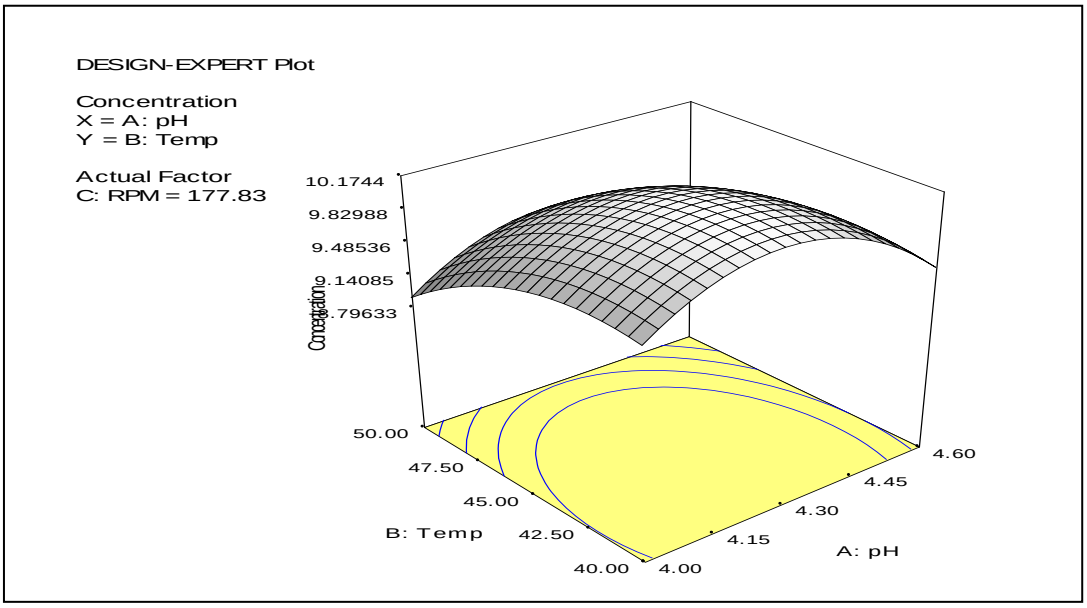

Figure 4.5: 3D response surface displaying relative effect of two variables on yield of enzymatic hydrolysis of rice straw while other at center level: Interaction between temperature and pH.

The effect of pH on the concentration of glucose was shown in Figure 4.5. From that figure, the concentration of glucose increased at pH starting from 4 until 4.23, the optimum concentration of glucose could be obtained at pH 4.23 that was 9.82988 g/L. Then, after the value 4.23 for pH until 4.6, the concentration of glucose was decreased.The lowest concentration of glucose was 8.7963 g/L at pH 4.6.

Lenormand *et al*. (2009) studied the pH effects on the hyaluronan (HA) hydrolysis catalysed by hyaluronidase in the presence of proteins. The results showed the optimum HAase activity could be attained at pH 4.

The optimum pH to get the optimum hyaluronidase activity was 4.0 (Lenormand *et al*., 2009) while the actual value gained from the experiment was 4.23 aimed to obtain optimum concentration of glucose within experiment.

To control the values of pH in this experiment, 0.1 M citric acid mixed with 0.1 sodium hydroxide (NaOH). pH can have an effect of the state of ionization of acidic or basic amino acids. Acidic amino acids have carboxyl functional groups in their side chains. Basic amino acids have amine functional groups in their side chains. If the state of ionization of amino acids in a protein is altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered. This can lead to altered protein recognition or an enzyme might become inactive. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. In general enzymes have a pH optimum. However, the optimum is not the same for each enzyme (Campbell, 2007).

Figure 4.6 shows the effect of temperature in glucose production.

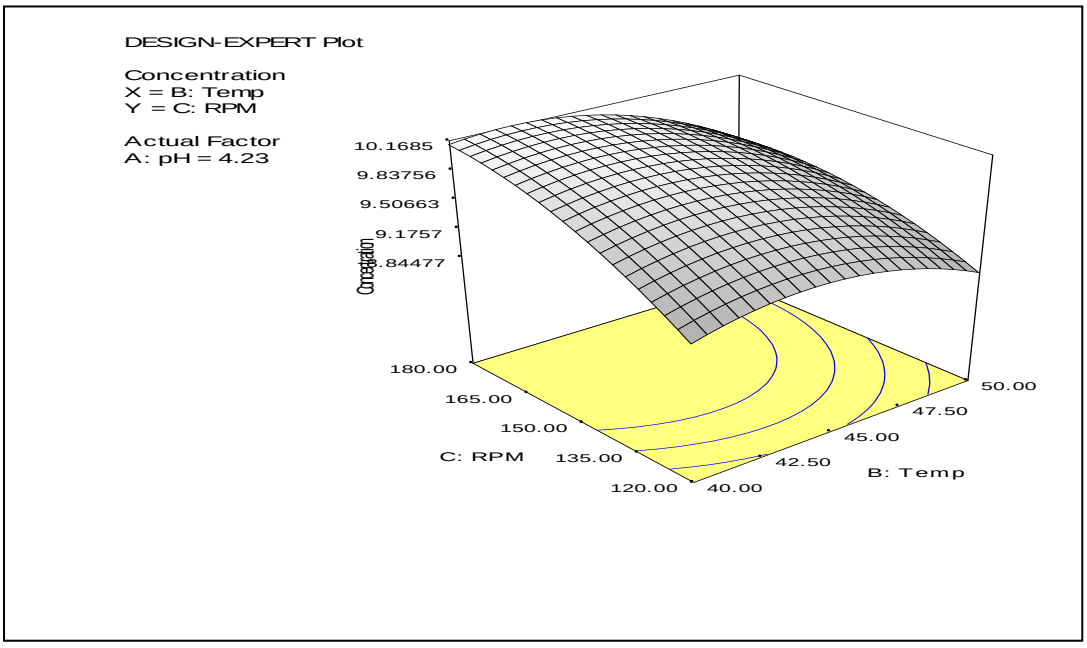

Figure 4.6: 3D response surface displaying relative effect of two variable on yield of enzymatic hydrolysis of rice straw while other at center level: Interaction between agitation rate and temperature.

The effect of temperature on the concentration of glucose was shown in Figure 4.6. From that figure, the concentration of glucose increased at temperature starting from 40°C until 43°C, the optimum concentration of glucose could be obtained at temperature 43°C that was 10.1685 g/L. Then, after the value 43°C until 50°C, the concentration of glucose was decreased. The lowest concentration of glucose was 8.84477 g/L at 50°C.

Eklund *et al*. (2002) studied the optimization of temperature and enzyme concentration in the enzymatic saccharification of steam-pretreated willow. The results showed the highest glucose yield was obtained at 40°C.

The optimum temperature to get highest glucose yield was 40°C (Eklund *et al.*, 2002) while the actual value gained from the experiment was 43°C aimed to obtain optimum concentration of glucose within experiment.

Usually, the reaction rate increases with temperature, but with enzyme reactions, a point is reached when the reaction rate decreases with increasing temperature. At high temperatures the protein part of the enzyme begins to denature, thus inhibiting the reaction. Reaction temperature plays an important role in enzyme activation/deactivation and thus, also affects the glucose yield. A higher reaction temperature allows a higher rate of glucose production. Although an increase in temperature enhances the hydrolytic rate, the cellulase become more susceptible to thermal deactivation (Fareira *et al.*, 2009).

Figure 4.7 shows the effect of agitation rate on glucose production

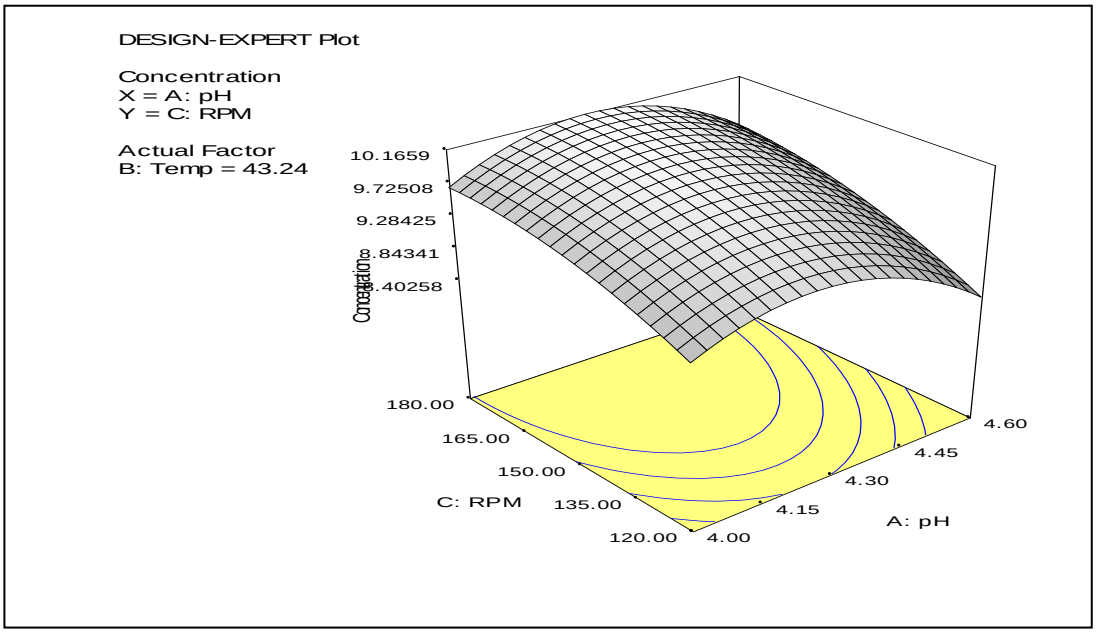

Figure 4.7: 3D response surface displaying relative effect of two variable on yield of enzymatic hydrolysis of rice straw while other at center level: Interaction between agitation rate and pH

The effect of agitation rate on the concentration of glucose was shown in Figure 4.7. From that figure, the concentration of glucose increased at agitation rate starting from 120 rpm until 177 rpm, the optimum concentration of glucose could be obtained at agitation rate 177 rpm that was 10.1659 g/L. Then, after the value 177 rpm until 180 rpm, the concentration of glucose was decreased.The lowest concentration of glucose was 8.40258 g/L at 120 rpm.

Ingesson *et al.* (2001) studied the effect of shaking regime on the rate and extent of enzymatic hydrolysis of cellulose. The results showed the high-speed shaking produced the highest conversion yield that was at 150 rpm.

The optimum agitation rate to get the highest conversion yields was 150 rpm (Ingesson *et al*., 2001) while the actual value gained from the experiment was 177 rpm aimed to obtain optimum concentration of glucose within experiment.

Agitation rate is one of the indispensable parameter for proper oxygen transfer and homogeneous mixing of the nutrients in fermentation system. Therefore, the effects of different agitation rates on glucose production from enzymatic hydrolysis of rice straw were studied. The higher agitation rate (higher than 180 rpm) reduced the glucose production due to sheer stress and heterogeneous mixing effects (Nadeem *et al*, 2009). This effort indicated that proper mixing is crucial for maximum production of glucose.

4.4 Optimization of pH, Temperature and Agitation Rate on Glucose Production Using Response Surface Methodology (RSM)

Table 4.5 shows the summary data before and after optimization for glucose production.

Table 4.5: Summary data before and after optimization for glucose production

Parameters	Before Optimization		After Optimization		
	Parameter Values	Concentration g/L	Parameter Values	Concentration, g/L	
				Predicted	Actual
pH	5.5	5.5622	4.23	10.1460	9.9835
Temperature (°C)	45		43		
Agitation rate (rpm)	180		177		

Based on Table 4.5, concentration of glucose before optimization was determined to be 5.5622 g/L, with pH of 5.5, temperature of 45°C and agitation rate of 180 rpm. To validate the optimum concentration of glucose, an experiment with the specified conditions which were pH of 4.23, temperature of 43°C and agitation rate of 177 rpm yielded a predicted result of 10.1460 g/L. The experimental (actual) result showed 9.9835 g/L compared to predicted value 10.1460 g/L. This result showed that the model was useful for predicting the concentration as well as the optimization of the experimental conditions.

The concentration of glucose before and after optimization was 5.5622 g/L and 9.9835 g/L respectively. This concentration of glucose shows that the increment of before and after optimization process was 44%. Base on Table 4.5, the result indicates that Response Surface Methodology (RSM) was an efficient approach to optimize enzymatic hydrolysis.

Zhang and Chai (2008) studied about enzymatic hydrolysis of alkali-pretreated rice straw by Trichoderma reesei ZM4-F3. Optimization of pH, temperature and agitation rate were implemented on reducing sugar production from enzymatic hydrolysis of rice straw. From this reseach, the results of optimization of pH, temperature and agitation rate were 4.5, 35 ℃ and 180 rpm respectively.

The optimum pH, temperature and agitation rate to get the optimum concentration of reducing sugar were 4.5, 35 ℃ and 180 rpm respectively (Zhang and Chai, 2008) while the actual value gained from the experiment were of 4.23, 43℃ and 177 rpm aimed to obtain optimum concentration of glucose within experiment.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The research on the optimization of pH, temperature and agitation rate on glucose production from enzymatic hydrolysis of rice straw was successfully carried out. By using conventional method, one factor at a time (OFAT), the best range of pH, temperature and agitation rate was able to be determined. The optimum range of pH was within 4.0 to 4.6, temperature range was within 40^{o}C to 50^{o}C and agitation rate range was within 120 rpm to 180 rpm. All of the values are valuable for further optimization using Response Surface Methodology (RSM).

Through the model in Response Surface Methodology (RSM), main effects and interactions between the three reaction parameters (pH, temperature and agitation rate) were successfully elucidated. The optimal reaction conditions of pH, temperature and agitation rate are 4.23, 43^{o}C and 177 rpm respectively. By using the optimized conditions, the concentration of glucose was 9.9835 g/L compared to the predicted value was 10.1460 g/L. The initial concentration of glucose before optimization process was 5.5622 g/L. This concentration of glucose shows that the increment of before and after optimization process is 44%.

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Therefore, the glucose production from enzymatic hydrolysis of rice straw was successfully optimized and improved in term of production of glucose by using Response Surface Methodology (RSM).

5.2 Recommendation

To obtain more significant increment in optimization of glucose production from enzymatic hydrolysis of rice straw, additional of more parameters such as research on incubation time, type of medium culture or concentration of substrate may increase the glucose production. Optimization of many parameters will give the highest concentration of glucose production from enzymatic hydrolysis of rice straw. The production can be multiple folds than initial production.

It is recommended to scale-up the optimal value of parameters to a continuous pilot production of glucose from enzymatic hydrolysis of rice straw. From this, further research could be done to investigate more on the production of glucose from rice straw and the parameters affecting it at larger scale.

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APPENDIX A

Ratio of Glucose and Water to Provide Standard Curve

Glucose (g)	Deionized water (ml)	Concentration of glucose (g/L)
0.6	100	6.0
0.5	100	5.0
0.4	100	4.0
0.3	100	3.0
0.2	100	2.0
0.1	100	1.0
0.0	100	0.0

APPENDIX B

Results for Standard Curve

Concentration of glucose (g/L)	Absorbance (OD_{540})
1.0	1.69
2.0	3.89
3.0	6.70
4.0	8.95
5.0	10.19
6.0	12.86

APPENDIX C

Experiment Data

Table C-1: OFAT on pH

pH	Absorbance (OD_{540}) x10	Concentration of glucose (g/L)
4.0	2.0960	9.6727
4.3	2.1480	9.9091
4.5	1.9933	9.2059
5.0	1.9120	8.8363
5.5	1.1917	5.5622

Table C-2 : OFAT on temperature

Temperature (°C)	Absorbance (OD_{540}) x 10	Concentration of glucose (g/L)
25	1.7950	8.3045
35	1.9933	9.2059
45	2.1328	9.8400
50	2.1153	9.7604
55	2.0400	9.4182

Table C-3 :OFAT on agitation rate

Agitation Rate (rpm)	**Absorbance (OD_{540}) x 10**	**Concentration of glucose (g/L)**
50	1.7007	7.8759
100	2.0000	9.2363
150	2.1503	9.9195
200	2.1350	9.8500
250	1.9993	9.2331

Table C-4 : Experiment data based on RSM suggested parameter

Standard	A: pH	B:Temperature (°C)	C: Agitation rate (rpm)	(OD_{540}) x 10	Concentration of glucose (g/L)
1	4	40	120	1.8927	8.7487
2	4.6	40	120	1.7512	8.1056
3	4	50	120	1.8190	8.4138
4	4.6	50	120	1.7453	8.0788
5	4	40	180	2.0821	9.6094
6	4.6	40	180	2.0528	9.4765
7	4	50	180	1.9458	8.9901
8	4.6	50	180	1.9079	8.8181
9	3.8	45	150	1.8267	8.4486
10	4.8	45	150	1.7063	7.9012
11	4.3	37	150	2.0467	9.4487
12	4.3	53	150	1.8464	8.5381
13	4.3	45	100	1.8729	8.6587
14	4.3	45	200	2.1524	9.9289
15	4.3	45	150	2.1035	9.7069
16	4.3	45	150	2.1528	9.9311
17	4.3	45	150	2.1539	9.9357

APPENDIX D

ANOVA Data

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	7.750987	9	0.861221	75.76464	< 0.0001	
A	0.355566	1	0.355566	31.28039	0.0008	
B	0.736204	1	0.736204	64.76647	< 0.0001	
C	2.3652	1	2.3652	208.075	< 0.0001	significant
A^2	3.925212	1	3.925212	345.3148	< 0.0001	
B^2	1.019259	1	1.019259	89.66783	< 0.0001	
C^2	0.426386	1	0.426386	37.5107	0.0005	
AB	0.009045	1	0.009045	0.795732	0.4020	
AC	0.05665	1	0.05665	4.983682	0.0608	
BC	0.104882	1	0.104882	9.226841	0.0189	
Residual	0.079569	7	0.011367			
Lack of Fit	0.045357	5	0.009071	0.530307	0.7547	not significant
Pure Error	0.034212	2	0.017106			
Cor Total	7.830556	16				

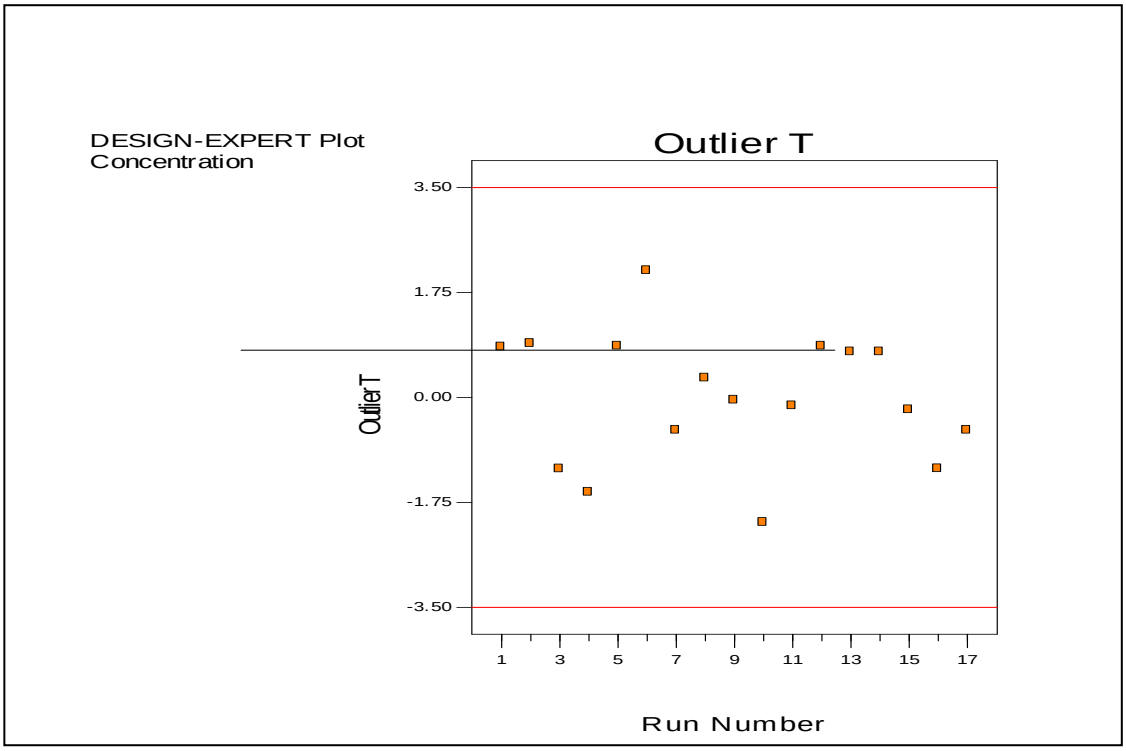
APPENDIX E

Diagnostics Case Statistics

Stan. Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier T	Run
1	8.7487	8.751875	-0.00318	0.669865	-0.05183	0.000545	-0.048	9
2	8.1056	8.193614	-0.08801	0.669865	-1.43675	0.418848	-1.58408	4
3	8.4138	8.449266	-0.03547	0.669865	-0.57896	0.068013	-0.54932	17
4	8.0788	8.025505	0.053295	0.669865	0.870001	0.15358	0.852894	5
5	9.6094	9.644892	-0.03549	0.669865	-0.57937	0.068111	-0.54974	7
6	9.4765	9.42323	0.05327	0.669865	0.869585	0.153434	0.852438	12
7	8.9901	8.884283	0.105817	0.669865	1.727373	0.605436	2.111324	6
8	8.8181	8.797121	0.020979	0.669865	0.34246	0.023797	0.319746	8
9	8.4486	8.458856	-0.01026	0.607498	-0.15355	0.003649	-0.1424	11
10	7.9012	7.916122	-0.01492	0.607498	-0.2234	0.007724	-0.20757	15
11	9.4487	9.396467	0.052233	0.607498	0.781993	0.094647	0.757843	14
12	8.5381	8.615511	-0.07741	0.607498	-1.15894	0.207884	-1.19357	3
13	8.6587	8.606497	0.052203	0.607498	0.78154	0.094538	0.757362	13
14	9.9289	10.00628	-0.07738	0.607498	-1.15848	0.207721	-1.19299	16
15	9.7069	9.85646	-0.14956	0.33203	-1.71638	0.146436	-2.08807	10
16	9.9311	9.85646	0.07464	0.33203	0.856582	0.036472	0.838185	1
17	9.9357	9.85646	0.07924	0.33203	0.909372	0.041106	0.896536	2

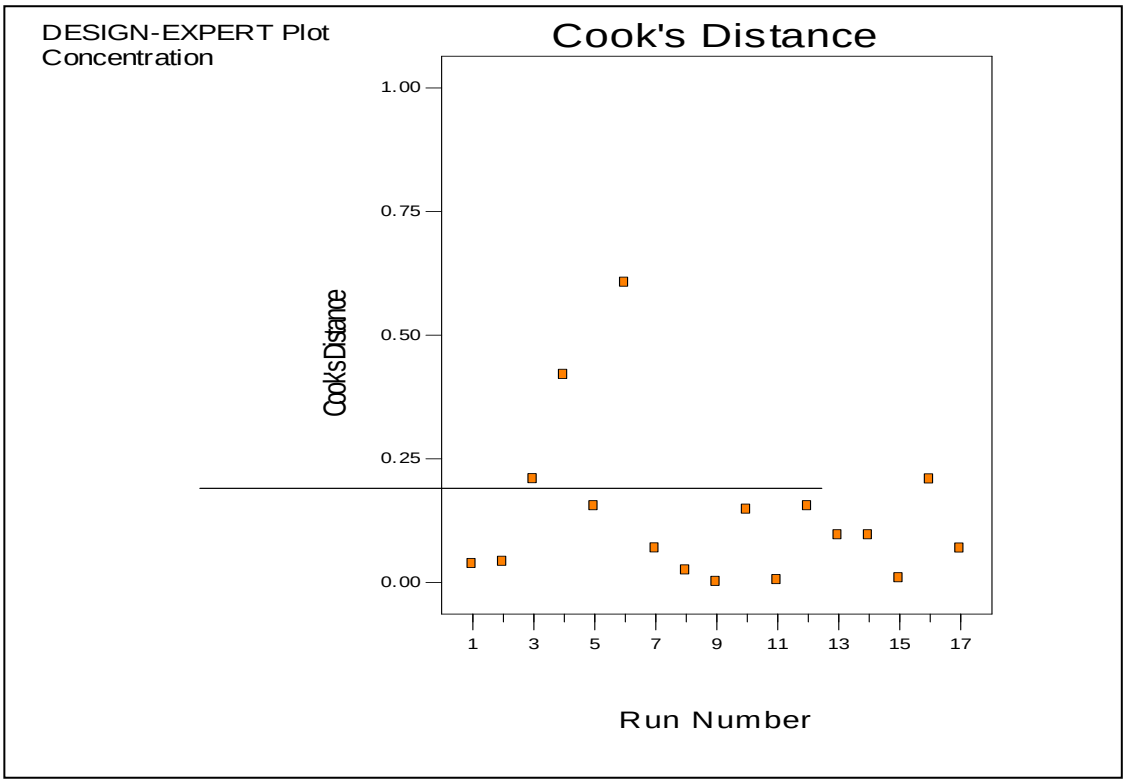
APPENDIX F

Outlier T Analysis



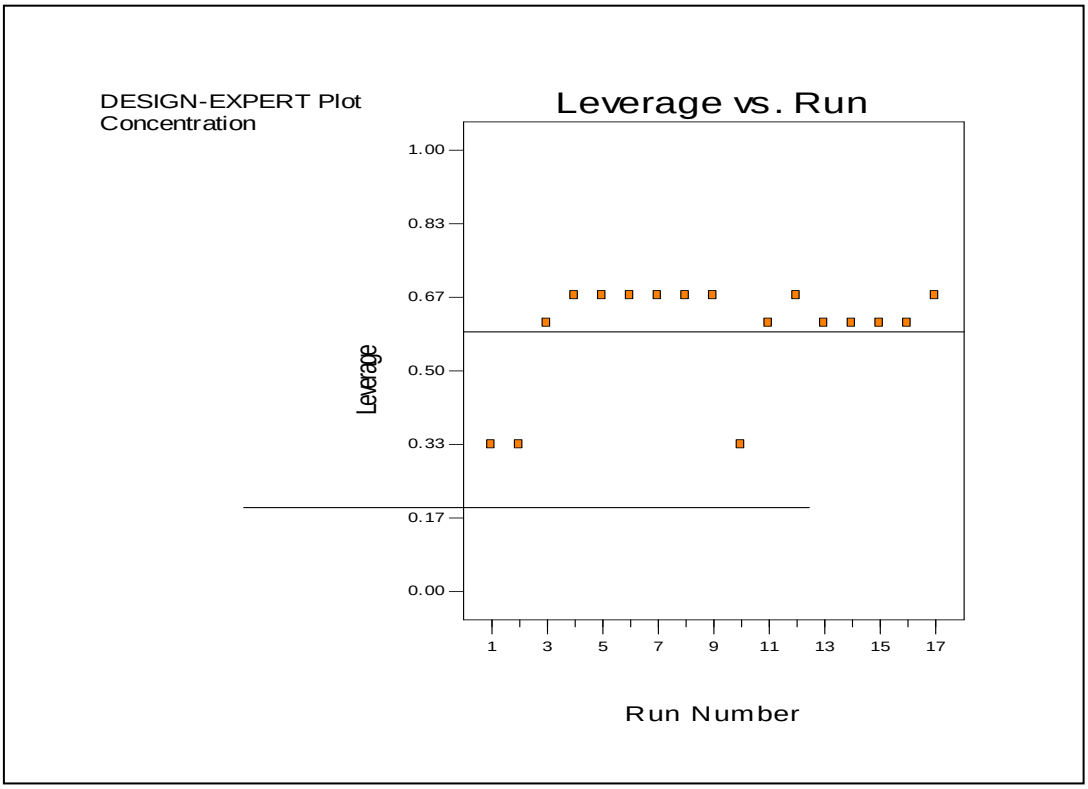
APPENDIX G

Cook's Distance Analysis



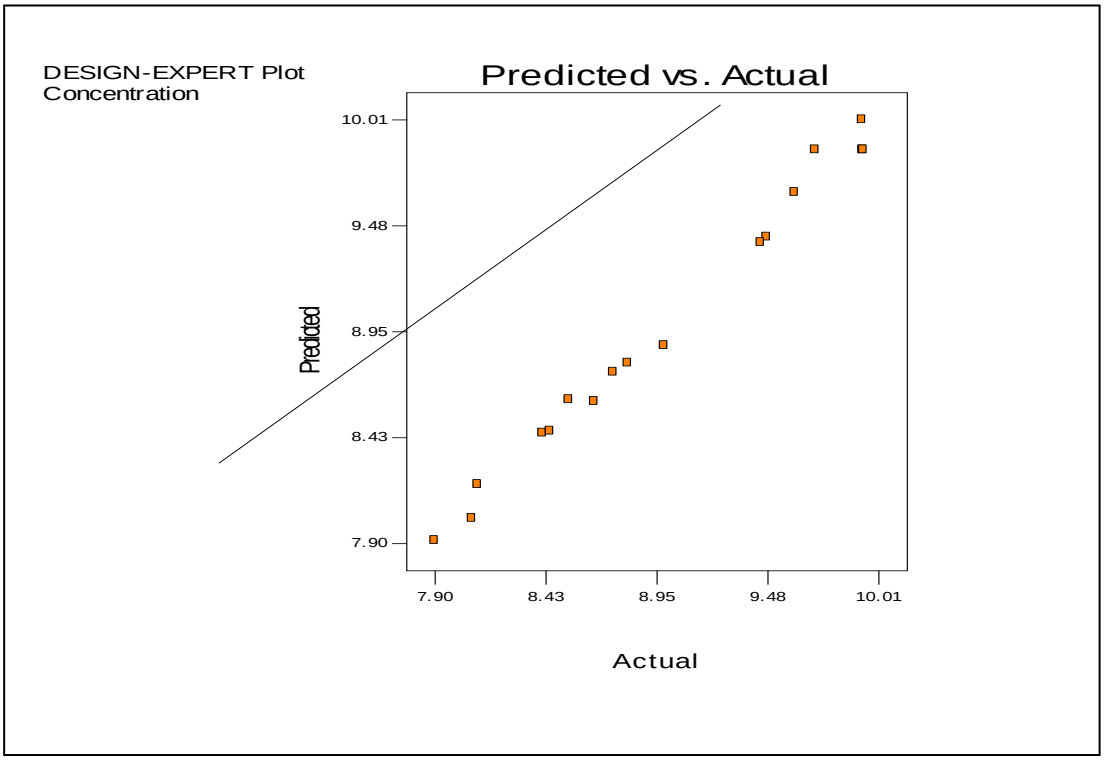
APPENDIX H

Leverage Versus Run



APPENDIX I

Predicted versus Actual value



APPENDIX J

Box Cox Analysis