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LAPORAN AKHIR PENYELIDIKAN

TAJUK PROJEK : THE KINETIC OF DILUTE-ACID HYDROLYSIS OF AGROWASTE FOR THE PRODUCTION OF REDUCING SUGAR

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THE KINETIC OF DILUTE-ACID HYDROLYSIS OF AGROWASTE FOR THE PRODUCTION OF REDUCING SUGAR

(Kinetik Hydrolisis Acid Lemah ke atas Sisa Pertanian bagi Penghasilan Gula Ringkas)

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ABSTRACT

There is a growing incentive for us to identify alternative and ideally renewable energy sources. Among the sources, grains, plant matters (biomass) and their wastes are commonly been used for biofuel production due to their sustainability. Oil palm empty fruit bunch (EFB) is a type of lignocellulosic waste from palm oil mills. Fermentable sugars mainly the glucose from EFB can be further fermented for the production of bioethanol. This study aims to investigate the effect of the key parameters during dilute acid pretreatment and enzymatic treatment for the hydrolysis of EFB to produce optimised yield of glucose. Three parameters for the dilute acid pretreatment, namely the reaction temperature, acid concentration and reaction time and two parameters for the enzymatic treatment, namely the substrate concentration and treatment time were investigated to optimise the yield of glucose. Batch reactions were carried out under different combination of operational conditions as proposed by the experimental design produced by the RSM (response surface methodology). RSM was used to optimise both the pretreatment and enzymatic processes in order to obtain the highest glucose yield. An optimised glucose yield of 53.96 % was obtained at the operating condition of 130 °C pretreatment temperature, 6 %w/w sulphuric acid concentration, 37 min of pretreatment time and 96 hours of enzymatic treatment using 6 %w/v of substrate concentration. The optimised yield has also been validated through experiment work.

ABSTRAK

Terdapat insentif yang semakin meningkat untuk mengenalpasti sumber tenaga alternatif yang sebaiknya dapat diperbaharui dari semasa ke semasa (*renewable*). Di antara sumber tersebut, bijirin, bahan tumbuhan (biomas) dan sisanya adalah biasa digunakan untuk penghasilan *biofuel* (bahan api berasaskan biologi) kerana kepertanggungannya. Tandan kosong kelapa sawit (EFB) merupakan sejenis sisa lignoselulosa dari kilang minyak kelapa sawit. Penapaian gula terutamanya glukosa daripada EFB dapat ditapai untuk penghasilan bioetanol. Tujuan kajian ini adalah untuk menyelidik kesan pra-rawatan asid cair dan juga rawatan enzim untuk menghidrolisiskan EFB bagi memberikan hasilan glukosa yang optimum. Tiga parameter untuk pra-rawatan asid cair iaitu suhu reaksi, kepekatan asid dan masa reaksi dan juga dua parameter untuk rawatan enzim iaitu kepekatan substrak serta masa rawatan telah diselidik untuk mengoptiumkan hasilan glukosa. Tindakbalas-tindakbalas berkelompok telah dilakukan di bawah pelbagai keadaan operasi yang dicadangkan oleh rekabentuk eksperimen yang dihasilkan oleh Kaedah Permukaan Respons (RSM). RSM telah digunakan untuk mengoptimumkan kombinasi proses pra-rawatan dan juga proses enzim untuk mencapai hasilan glukosa yang tertinggi. Hasilan glukosa optimum yang setinggi 53.96 % telah dicapai pada suhu pra-rawatan 130 °C, kepekatan asid 6 %w/w, 37 min masa pra-rawatan dan 96 jam proses enzim pada kepekatan subtrak 6 %w/v. Hasilan glukosa yang optimum juga disahkan melalui kerja eksperimen.

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LIST OF ABBREVIATIONS

9MP	-	Ninth Malaysia Plan
ANOVA	-	Analysis of Variance
C	-	Carbon
C. V.	-	Coefficient of Variance
$Ca(OH)_2$	-	Calcium Hydroxide
$CaSO_4.2H_2O$	-	Hydrated gypsum
CCD	-	Central Composite Design
CO	-	Carbon Monoxide
CO_2	-	Carbon Dioxide
df	-	Degree of Freedom
DOE	-	Design of Experiment
EFB	-	Empty Fruit Bunch
FFB	-	Fresh Fruit Bunches
FPU	-	Filter Paper Units
HMF	-	5-hydroxymethyl furfural
HPLC	-	High Performance Liquid Chromatography
LAP	-	Laboratory Analytical Procedure
MS	-	Mean Square
MS_A	-	Mean Square of Factors
MS_E	-	Mean Square of Error
MS_T	-	Total Mean Square
NO_x	-	Nitrogen Oxide
O	-	Oxygen
PKC	-	Palm Kernel Cake

PKS	-	Palm Kernel Shell
POME	-	Palm Oil Mill Effluent
PPF	-	Palm Press Fiber
PRESS	-	Predicted Residual Error Sum of Squares
R	-	Correlation Coefficient
R^2	-	*R*-squared
R^2_{adj}	-	Adjusted R^2
$R^2_{prediction}$	-	Predicted R-squared
RI	-	Refractive Index
rpm	-	Revolutions per minute
RSM	-	Response Surface Methodology
SC	-	Sludge Cake
SO_2	-	Sulphur Dioxide
SS	-	Sum of Square
SS_{Error}	-	Sum of Square of Error
SSF	-	Simultaneous Saccharification and Fermentation
$SS_{Residual}$	-	Sum of Square of Residual
SS_T	-	Total Sum of Squares
Std. Dev.	-	Standard Deviation
TS	-	Total Solids content
VIF	-	Variance Inflation Factor
WIS	-	Water-Insoluble Solids

LIST OF SYMBOLS

%	-	percents
%w/v	-	percentage of weight per volume
%w/w	-	percentage of weight per weight
°C	-	degree Celsius
µL	-	microLitre
µm	-	micrometer
g	-	gram
g/L	-	gram per Litre
hrs	-	hours
L	-	Litre
M	-	Molar
$mg.dm^{-3}$	-	milligram per cubic decimetre
min	-	minutes
mL	-	milliLitre
$mL.min^{-1}$	-	miliLitre per minute
MPa	-	Megapascal
α	-	alpha
β	-	beta
□	-	lambda

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Since the beginning of 1970, palm oil industry has emerged as one of the top industries in Malaysia. In order to process the fruits from oil palms, many factories and palm oil mills had been set up. Enormous amount of wastes derived from oil palm industries are generated every year and this already created a great concern in environmental safety. In Malaysia, about 50 million tonnes of palm oil mill effluents and 40 million tonnes of oil palm biomass, in forms of empty fruit bunch (EFB), oil palm trunks and oil palm fronds are generated from palm oil industries, every year (Kabbashi *et al.*, 2007).

Lignocellulose is the major structural component of woody and herbaceous plants such as oil palm tree. It represents a major source of renewable organic matter. Lignocellulose consists of lignin, hemicellulose, and cellulosic material. The chemical properties of the components of lignocellulose make them a substrate of enormous biotechnological value. Much of the lignocellulosic wastes are disposed by biomass

burning, which is not restricted to developing countries alone, but it is considered a global phenomenon. In addition, the problem arises when all of this biomass is not being treated and left to rot in the plantations to provide some nutrient. Unfortunately, these wastes may create environmental problems due to accumulation of high organic content. Therefore, environmental management is placing greatest emphasis in waste minimisation at source or recycling. Moreover, a growing awareness of the "need not to pollute" has forced this industry to look more closely at the milling operation. It is recommended to treat and manipulate the waste to produce useful product (Kabbashi *et al.*, 2007).

The need for alternative sources of bioenergy is expected to increase sharply in the coming years with the rising prices of crude oil due to increase in fuel demands. The principle fuel used as a petrol substitute for road transport vehicles is bioethanol. Bioethanol is mainly produced by the sugar fermentation process. The main sources of sugar required to produce ethanol come from fuel or energy crops. These crops are grown specifically for energy use and include corn, maize, wheat crops, waste straw, sugarcane and sorghum plants. There is also ongoing Research and Development into the use of municipal solid wastes and agrowastes to produce ethanol in order to reduce the demand of energy crop for biofuel production.

Among the potential alternative of bioenergy resources, lignocellulosic biomass has been identified as the prime source of biofuels and other value-added products. Lignocelluloses, as agricultural, industrial and forest residuals, account for the majority of the total biomass present in the world. Therefore, the bioconversion of large amounts of lignocellulosic biomass into fermentable sugars has potential application in the area of bioenergy generation.

EFB have been chosen in this study for the monomeric sugars production. Ethanol can be produced from the biomass by the hydrolysis and sugar fermentation processes. Biomass wastes contain a complex mixture of carbohydrate polymers from the plant cell walls known as cellulose, hemicellulose and lignin. In order to produce sugars from the biomass in this research, the lignocellulosic biomass is milled and pre-treated in order to reduce the size of the feedstock and to open up the plant structure. The cellulosic and the hemicellulosic portions are then hydrolysed, by enzymes or dilute acids, into monomeric sugar which is then fermented into ethanol. There are three principle methods of extracting sugars from biomass. These are concentrated acid hydrolysis, dilute acid hydrolysis and enzymatic hydrolysis.

Dilute acid hydrolysis is among the oldest method for sugar extraction process. This process dates back to 1800 that the first commercial plant was set up in year 1898 (Chiaramonti, 2007). The dilute acid hydrolysis process first hydrolyses the hemicellulose in mild process conditions to recover the 5-carbon sugars. The reaction has to be controlled under mild conditions in order to avoid sugar degradation during the hydrolysis process. This not only reduces yield but also causes the formation of inhibitors such as furfural and other by-products of the fermentation process. Cellulose in the remaining solids is then hydrolysed in a more strict condition at temperature more than 200 °C. The liquid hydrolates are then neutralised and recovered from the process. Dilute acid hydrolysis is a simple process and no acid recovery is needed after this process. Unfortunately, the yields of fermentable sugar are low and it has high potential for the production of degradation product.

Apart of using acid to hydrolyse the biomass into monomeric sugar, enzymes can be used to break down the biomass in a similar way. Enzymatic processes use selected cellulase and hemicellulose degrading enzymes to break the polymeric chain of the cellulose and hemicellulose, leaving the monosaccharide available for fermentation. It performs a higher hydrolysis yields than the chemical hydrolysis process. However, a

barrier for these processes is represented by its costs that accounts for approximately 40 % of the total costs. As only 20 % of the biomass's pore volume can be reached by the large cellulase enzymes molecules, biomass pretreatment becomes an essential step in the enzymatic hydrolysis processes and thus increasing the cost (Chiaramonti, 2007). Enzymatic treatment however can be considered as a mild hydrolysis process. A clean and clear sugar solution can be produced which consists higher amount of fermentable sugars. In this study, combined dilute acid hydrolysis pretreatment and enzymatic hydrolysis treatment were used. Various variables among the combined hydrolysis processes were study to obtain the miximised yield of glucose.

To prepare a successful experimental design for this research, response surface methodology (RSM) was chosen. Among the RSM, there are several types of models that can be used. Central composite design was chosen due to the usefulness of this model without the need of using a complete three-level factorial experiment. Through the RSM, the experimental runs were proposed. The analysis of the data after the proposed condition of experiment can also be evaluated accordingly. The optimised condition for the highest yield can be obtained after the analysis of experiments.

1.2 Research Objective

Due to the cost but the good efficiency of the enzymatic treatment, it is desirable to incorporate enzymatic hydrolysis treatment with the existing acid hydrolysis pretreatment to further enhance the conversion of oil palm empty fruit bunch into the sugar products. The objective of this research is to optimise the parameters or the operating conditions for both the acidic pretreatment and the enzymatic treatment to maximise the yield of glucose.

1.3 Research Scopes

i. To investigate the effect of the key parameters for dilute sulphuric acid pretreatment, these parameters include:
   - reaction temperature
   - acid concentration
   - reaction time

ii. To investigate the effect of substrate concentration and reaction time for the enzymatic hydrolysis of pretreated oil palm empty fruit bunch at a specific enzyme concentration.

iii. To optimise the combined process of acid hydrolysis pretreatment and enzymatic treatment in order to obtain highest glucose yield using the statistical model of RSM.

1.4 Organisation of Report

This report consists of five chapters. Chapter one introduce the research background, problem statement, objective and scopes of the study. Besides, the organisation of the report is also included in this chapter.

Chapter two presents the detailed literature survey concerning the main elements involved in this study as well as researches involved in this area. It reviews the related

studies on biofuel, oil palm and its waste, lignocellulosic biomass and the statistical optimisation model.

Chapter three describes the materials and the methodology involved in this study. The analytical methods including characterisation and testing procedures for the determination of sample composition, the design and analysis of experiments and the experiments of hydrolysis procedures are discussed in this chapter.

Results and Discussion are presented in chapter four. The results on sample characterisation are firstly presented and discussed. The total solids content and main composition of EFB is reported. The effectiveness of the hydrolysis treatments is compared and the highest yield of glucose is identified and discussed statistically. The optimum values for the variables are obtained respectively.

Chapter five concludes the study. It presents the conclusion for the objective and the scopes of this study. Recommendation and suggestions are presented for further improvement of this work in the future.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

A number of indicators suggest that petroleum supplies will begin to dwindle during the 21st century. Identifying new resources of fuel supply is the worldwide trend now. Grains, plant matter and sometimes their waste are commonly used in biofuel production and are generally considered to be renewable. Sugarcane and corn were used to produce bioethanol in Brazil and United States of America, respectively. Using these feedstocks to produce biofuel has raised many social issues especially the rising of food price and eventually causing the overall implication for food security. Therefore, the use of non food crops or inedible waste products would have less impact on food and has become the trend of bioethanol production recently.

Palm oil is produced primarily in South East Asia where Malaysia is the largest producer of palm oil at the world. Palm oil which is the primary product of oil palm trees are now the most traded vegetable oil in the world. Therefore, it generates a large

amount of waste in the mill after the processing of palm oil from the fresh fruit bunch of oil palm tree yearly. These wastes especially the empty fruit bunch is useless and sometimes cause disposal problem. Hence, it can be chosen as the substrate for the production of fermentable sugars and subsequently to bioethanol.

Oil palm empty fruit bunch (EFB), an agrowaste from oil palm industry is one type of lignocellulosic biomass in nature. Lignocellulose is the "woody" structural material of plants. This feedstock is abundant and diverse, and in some cases it is a significant industry-specific disposal problem or waste. It comprises of lignin, cellulose and hemicellulose. Each of the components has their own usage. The polymeric sugar part, cellulose and hemicellulose can be treated or hydrolysed into monomeric sugar. The lignin can be used as fuel for boiler in the ethanol production plant.

In order to convert the lignocellulosic biomass to useful chemical, three main stages are involved. The first stage is the pretreatment process. Then, it is followed by the hydrolysis of the cellulose part to release the monomeric sugar. Finally, the last stage is the fermentation of the monomeric sugar.

The pretreatment process aims to dissociate the cell components of lignocellulose. Hemicellulose can sometime be broken down into its subunits after the pretreatment process. For the cellulose part, two types of hydrolysis processes are commonly employed to degrade it into the monomeric sugar which requires a fermentation step to convert it into the ethanol. The most common hydrolysis methods used are acid (dilute and concentrated) and enzymatic methods. In this research, dilute acid pretreatment followed by enzymatic hydrolysis was used to convert the EFB into glucose which are ready for the further fermentation process. Fermentation is the last stage in the bioethanol production. However, this stage was not the scope of this study.

This chapter also reviews the statistical approaches used to optimise the operating condition of the hydrolysis process so that the yield of glucose is optimised.

2.2 Biofuel

With the increase in awareness and importance relevance to environmental issues such as global warming, renewable and more environment-friendly fuels are being developed as alternatives to the fossil fuel. One such fuel, which has gained prominence in recent years, is biofuel. Clean and renewable, biofuel has been touted as the solution to the issue of the diminishing of energy reserves.

Biofuel, which can be broadly defined as solid, liquid, or gas fuel consisting of or derived from biological material or biomass are now being used globally to overcome the depletion of crude oil worldwide. Biofuel can be produced from any carbon source that can be replenished rapidly such as plants. Many different plants and plant-derived materials are used for the production of biofuel. Among the potential alternative bioenergy resources, lignocellulosic biomasses have been identified as the prime source of biofuels and other value-added products. The most common use for biofuels is as liquid fuels for automotive transport. The use of renewable biofuels provides increased independence from petroleum and enhances energy security.

Biofuel consist of two major categories of fuels which are bioethanol and biodiesel. Therefore, there are at least two different procedures of producing biofuel from biomass. The methods followed have a strong impact on the end results. There are

two key reactions that are involved in the production of bioethanol, one is hydrolysis and the other one is fermentation which will be discussed in Section 2.7 and 2.8.

2.3 Agriculture Sector and Oil Palm Industry in Malaysia

Malaysia is well known for the production and plantation of oil palm trees. The first oil palm plantation in Malaysia was set up in the year of 1917. Since then, the palm oil industry has grown to become the largest producer and exporter of palm oil in the world, accounting for 52 % of world production and 64 % of world exports in 1997. The Malaysian oil palm industry is sustaining its performance in 2003 after staging a significant recovery in 2002 despite a weak global economy. Palm oil and palm oil-based products is Malaysia's second largest export revenue, with a total value of RM 20.8 billion (US$ 5.47 billion) during the first nine months of 2003, accounting for 7.6 % of total exports of RM 271.5 billion (POIC, 2007). Crude palm oil production increased by 6.8 % per annum, from 7.8 million tonnes in 1995 to 10.8 million tonnes in 2000 due to improvement in yield and expansion in hectarage of matured trees (The 8th Malaysia Plan, 2001).

During the Seventh Plan period, the agriculture sector remained as one of the major sectors of the economy after manufacturing and services, contributing to national income and export earnings. In line with the Third National Agricultural Policy, the sector contributed not only as a supplier of raw materials to the resource-based industries, but also in terms of food production. The increase in earnings of major commodities, particularly palm oil and pepper as well as food commodities enabled the sector to retain its workforce and withstand the economic downturn of year 1997 to 1998 (The 8th Malaysia Plan, 2001).

In many important ways, agriculture was accorded a very different treatment in the Ninth Malaysia Plan (9MP), starting with the revitalizing of the sector as one of the key aims of the Plan, and the sector itself featured strongly in each of the five key thrusts of the National Mission. Table 2.1 below shows the agriculture and agro-based manufactured export from year 2000 to year 2010 which is provided by Department of Statistics and Economic Planning Unit of Malaysia.

Table 2.1: Agriculture and agro-based manufactured export (2000 – 2010) (Wong, 2007)

Commodity	*RM million*			*% of Total*			*Average Annual Growth Rate (%)*	
	2000	*2005*	*2010*	*2000*	*2005*	*2010*	*8MP Achieved*	*9MP Target*
Agriculture Exports	**22,892**	**37,421**	**54,992**	**48.1**	**50.0**	**47.5**	**10.3**	**8.0**
% to Total Exports	*6.1*	*7.0*	*6.8*					
Industrial Commodities	18,428	31,509	37,244	38.7	42.1	32.2	11.3	3.4
Palm Oil	9,948	19,036	26,735	20.9	25.4	23.1	13.9	7.0
Rubber	2,571	5,787	5,156	5.4	7.7	4.5	17.6	-2.3
Sawlogs	2,489	2,465	2,100	5.2	3.3	1.8	-0.2	-3.2
Sawntimber	3,020	4,051	2,995	6.3	5.4	2.6	6.0	-5.9
Cocoa	33	50	128	0.1	0.1	0.1	8.8	20.5
Pepper	367	120	130	0.8	0.2	0.1	-20.0	1.6
Food Commodities	4,464	5,913	17,748	9.4	7.9	15.3	5.8	24.6
Agro-Based Manufactured Exports	**24,686**	**37,442**	**60,660**	**51.9**	**50.0**	**52.5**	**8.7**	**10.0**
% to Total Exports	6.6	7.0	7.6					
Food	4,509	8,627	15,803	9.5	11.5	13.7	13.9	12.9
Beverages and Tobacco	1,207	1,755	2,446	2.5	2.3	2.1	7.8	6.9
Wood Product	6,801	9,665	13,909	14.3	12.9	12.0	7.3	7.6
Furniture and Parts	6,077	8,454	14,335	12.8	11.3	12.4	6.8	11.1
Paper and Paper Product	1,397	2,018	2,799	2.9	2.7	2.4	7.6	6.8
Rubber Product	4,695	6,923	11,368	9.9	9.3	9.8	8.1	10.4
Total Agriculture and Agro-Based Exports	**47,578**	**74,863**	**115,652**	**100.0**	**100.0**	**100.0**	**9.5**	**9.1**
% to Total Exports	*12.7*	*14.0*	*14.4*					
Total Exports	**373,270**	**533,790**	**803,163**				**7.4**	**8.5**

Table 2.2 provides an indication of land use over the period of 2000 to 2010. Again the dominance of oil palm over other tree crops is quite obvious (Source: Ministry

of Agriculture and Agro-Based Industry and Ministry of Plantation Industries and Commodities).

Table 2.2: Agricultural land use over the period of 2000 to 2010 (Wong, 2007)

Crop	*Hectares ('000)*			*Average Annual Growth Rate (%)*		
				8MP		*9MP Target*
	2000	*2005*	*2010*	*Target*	*Achieved*	
Oil Palm	3,377	4,049	4,555	3.2	3.7	2.4
Rubber	1,431	1,250	1,179	-2.7	-2.7	-1.2
Padi[10]	478	452	450	-0.5	-1.1	-0.1
Fruits	304	330	375	5.1	1.7	2.6
Coconut	159	180	180	-0.6	2.5	0.0
Cocoa	76	33	45	-2.4	-15.2	6.2
Vegetables	40	64	86	4.2	9.9	6.1
Tobacco	15	11	7	2.5	-6.0	-7.4
Pepper	13	13	14	2.1	0.0	0.6
Total [11]	**5,893**	**6,383**	**6,891**	**1.5**	**1.6**	**1.5**

2.4 Oil Palm and Mill Wastes

Palm oil is produced primarily in South East Asia where Malaysia is the largest producer of palm oil followed by Indonesia. As the climatic conditions in the south are suitable for palm trees, the oil palm plantation area has expanded ever since in Malaysia.

The oil palms (*Elaeis*) actually comprise two of species in the order Arecales of the family Aracaceae. They are planted commercially for the production of palm oil.

One of the oil palm is African Oil Palm, *Elaeis guineensis* which is originated from West Africa and another is American Oil Palm, *Elaeis oleifera* is native to tropical Central and South America. *Elaeis guineensis* produces palm oil and palm kernel oil. It is a native of tropical Africa that is extensively planted commercially in many other tropical countries which include Malaysia (Rival, 2007).

Recently, Sabah's Oil Palm Sector is mainly confined to the primary processing of oil palm in Malaysia. Presently, there are 58 palm oil mills in Sabah producing crude palm oil, palm kernel oil and palm kernel cake. Only a small number of refineries producing higher value added oil palm products. There is roughly one oil palm mill for every 10,000 ha of oil palm planted (POIC, 2007). Raw material supplied to the mills consists of fresh fruit bunches (FFB). There are various forms of solid and liquid wastes from the mills after processing. These include empty fruit bunch (EFB), palm press fiber (PPF), palm kernel cake (PKC), palm kernel shell (PKS), sludge cake (SC) and palm oil mill effluent (POME). Only EFB, PPF, PKS and POME appear in large quantities and are considered as wastes. The others can be sold for animal feed or fertilizer. The quantity of the wastes depends on the quality of the raw material which is the fresh fruit bunches (Prasertsan and Prasertsan, 1996).

Figure 2.1 shows the process of palm oil milling and the waste products of each process. Straight line indicates the process whereby the dotted line indicates production of wastes (Prasertsan and Prasertsan, 1996).

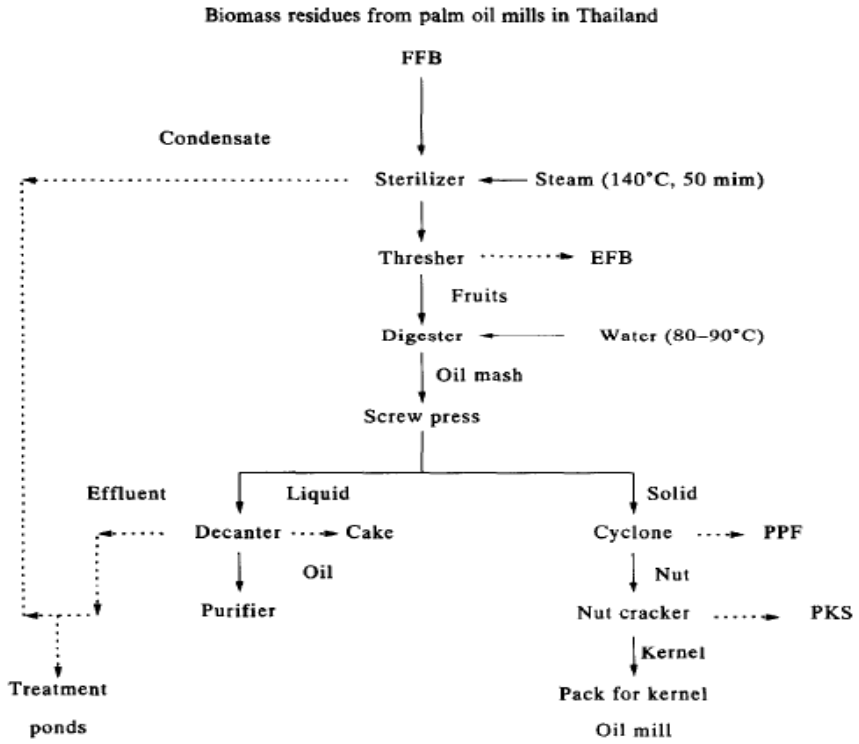

Figure 2.1: Palm oil milling process. (-) Process; (- - -) waste (Prasertsan and Prasertsan, 1996)

In a well run palm oil mill, it is expected that each 100 tonnes of fresh fruit bunches (FFB) processes yields 20 to 24 tonnes of crude palm oil and about 4 tonnes of palm kernels. Thus between 72 to 76 % of the FFB comes out at various stages of the process as waste (Poku, 2002). Empty fruit bunch (EFB) is the major component of all solid wastes from the palm oil mills. The steam from the sterilisation process makes the moisture content of EFB as high as 60 %. Thus, the EFB cannot be used directly as fuel. It was reported that the EFB has 42 % Carbon, 0.8 % Nitrogen, 0.06 % Phosphorus, 2.4 % Kalium and 0.2 % Magnesium. The bulky nature of the EFB causes a high land-fill disposal cost. The mills, therefore, burn the EFB down to ashes after the drying process. Burning a ton of EFB produces 4 kg of ash. Unfortunately, particulates and gases (SO_2,

CO_2, CO and NO_x) emitted from the furnaces of burning cause air pollution to the nearby communities and results in public protests (Prasertsan and Prasertsan, 1996).

Palm kernel shell (PKS) is the waste most difficult to be decomposed. The shell size is uniform and is not as bulky as the EFB. They are usually left unused in the factory or disposed of by the land-fill method (Prasertsan and Prasertsan, 1996). The PKS is also used as a source of fuel for the boilers. However, the shell of palm kernel contains silicates that form a scale in the boilers if too much shell is fed to the furnace, thus limiting the amount of shell that can be utilised in the boilers. The shell residual is disposed of as gravel for roads maintenance within the plantation (Poku, 2002).

POME is the wastewater produced from the palm oil industry. It is a colloidal suspension which consists of 95 - 96 % water, 0.6 - 0.7 % oil and 4 - 5 % total solids including 2 - 4 % suspended solids. There are three major sources of waste water, namely steriliser condensate (17 %), decanter or separator sludge (75 %) and hydrocyclone water (8 %). POME contains 4,000 $mg.dm^{-3}$ of oil and grease, which is relatively high compared to the limit of only 50 $mg.dm^{-3}$ set by the Malaysian Department of Environment. Therefore, this effluent must be treated before being discharged to avoid serious environmental pollution. Normally, waste water is treated anaerobically in a series of ponds. Over half of the land has to be spared for the waste water treatment pond (Prasertsan and Prasertsan, 1996). Sometimes, the liquid waste treatment involves anaerobic fermentation followed by aerobic fermentation in large ponds until the effluent quality is suitable for discharge (Poku, 2002).

Figure 2.2 shows the composition of fresh fruit bunch (FFB). Figures in the brackets are the percentage of FFB. Round brackets, ( ) indicates high-quality FFB and square brackets, [ ] indicates the low-quality FFB (Prasertsan and Prasertsan, 1996).

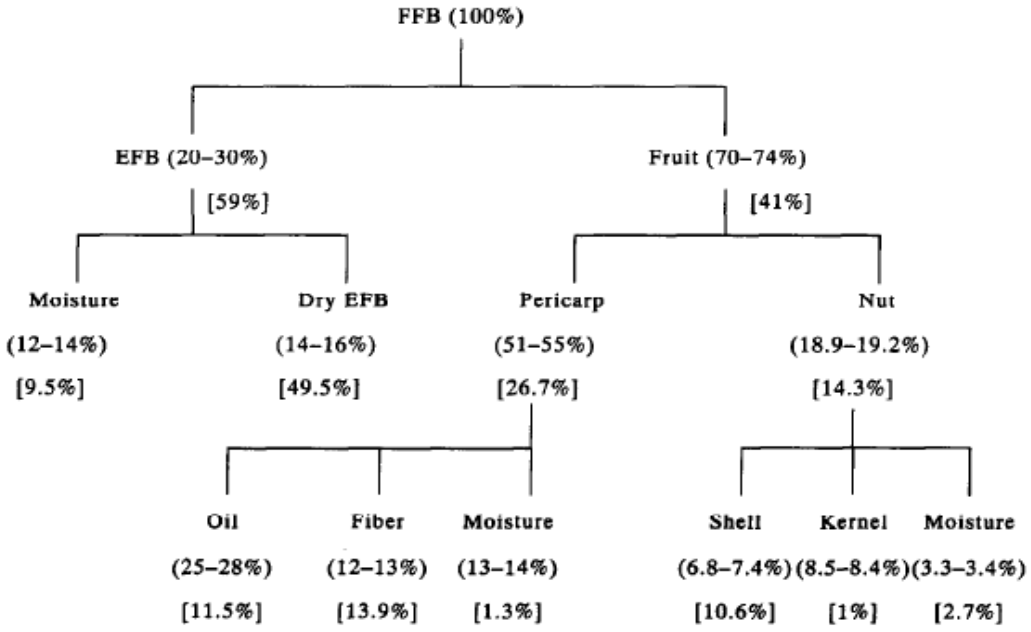

Figure 2.2: Composition of fresh fruit bunch (FFB) (Prasertsan and Prasertsan, 1996)

2.5 Lignocellulosic Biomass

Lignocellulosic biomass in nature is by far the most abundant raw material. It may be grouped into four main categories which are wood residues, municipal paper waste, agricultural residues such as EFB and dedicated energy crops. Lignocellulose is a substrate with its structure more complex than starch. It composes of lignin and a mixture of carbohydrate polymers, cellulose and hemicellulose (Lee, 1997). Whereby, starch is monopolymer. It is a mixture of amylose and amylopectin. Both amylose and amylopectin are complex carbohydrate polymers of glucose.

The carbohydrate polymers of lignocellulose are tightly bound to lignin mainly by hydrogen bonds but also by some covalent bonds (Lee, 1997). Therefore, it is difficult to degrade and hydrolyse lignocellulosic biomass. Recently, conversion of lignocellulose to bioethanol becomes attractive and dominant in all research due to the depleting stores of crude fuels and the abundance of lignocellulosic biomass in nature. Lignocellulose is a carbon source of energy, since it is primarily from dead plants. It means that the combustion of ethanol produced from lignocellulose will produce no net carbon dioxide and thus reduce the pollution in the earth's atmosphere. The abundance amounts of lignocellulosic materials are disposed by many industrial and agricultural sectors as waste byproducts that can be used as renewable resources. Many dedicated energy crops can provide high energy biomass and be harvested on multiple times each year such as switchgrass planted in United State of America.

The biological process for converting the lignocellulose to biofuel of ethanol involves three stages. Firstly, it involves the liberation of cellulose and hemicellulose from their lignin complex by the process of delignification. Then, the carbohydrate polymers will be depolymerised to produce free monomeric sugars. Finally, the fermentation process that converts the mixed hexose and pentose sugars to ethanol (Lee, 1997).

2.5.1 Lignin

Lignin was derived from a Latin word, *lignum* which means wood by F. Schulze at year 1865 (Sjostrom, 1981). Lignin is a complex, variable, hydrophobic, cross-linked, three-dimensional aromatic non-sugar polymer of *p*-hydroxyphenylpropanoid units connected by C–C and C–O–C link with molecular masses in excess of 10,000 units

(Lee, 1997). The degree of polymerisation in nature is difficult to measure, since it is fragmented during extraction and the molecule consists of various types of substructures which appear to repeat in a haphazard manner. Lignin is most commonly derived from wood and an integral part of the cell walls of plants. It can be isolated from extractive-free wood as an insoluble residue after hydrolytic removal of polysaccharides. Sometimes, Klason lignin is used to define as lignin, but Klason lignin is obtained after removing of polysaccharides from extracted wood by hydrolysis with 72 %w/w sulphuric acid (Sjostrom, 1981).

Lignin is the most abundant organic polymer on earth after cellulose. It employs 30 % of non-fossil organic carbon. Lignin accounts 10 to 30 % of wood's dry weight (Chiaramonti, 2007). It consists of complex phenolic cell wall that endows the xylem and other tissues of plants with compression and decay resistance. It is largely responsible for preservation of plants as fossil. It gives wood strength and confers resistance to microbial attack. The compound has several unusual properties as a biopolymer. It is heterogeneity which lacks a defined primary structure (Graham *et al.*, 2006). Lignin is particularly abundant in compression wood, but scarce in tension wood. It is fairly resistant to chemical and enzymatic degradation.

2.5.2 Cellulose

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$. It is a polysaccharide consisting of a linear chain of several hundred to over ten thousand $\beta(1\rightarrow4)$ linked *D*-glucose units, $C_6H_{10}O_5$. Cellulose is the structural component of the primary cell wall of green plants, many forms of algae, some protist and bacterial. It is also produced by various bacteria for use as an attachment material (Graham *et al.*,

2006). It is the most common organic compound on Earth. Many properties of cellulose are depending on its degree of polymerisation or chain length, the number of glucose units that make up one polymer molecule. Fiber walls mainly consist of cellulose have made up 30 to 60 % of wood (lignocellulosic) dry weight (Chiaramonti, 2007).

Cellulose is built up from a linear chain of *D*-glucose units, which is held together by β-(1-4)-glycosidic bonds. This linkage is in contrast with α-(1-4)-glycosidic bonds which is present in starch, glycogen, and other carbohydrates. Cellulose is a straight chain polymer. No branches are found in the polymer of cellulose. The multiple hydroxyl groups on the glucose residues from one chain form hydrogen bonds with oxygen molecules on another chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength. This strength is important in cell walls, where they are meshed into a carbohydrate matrix, conferring rigidity to plant cells. Plants will use this glucose chain as building material, linking the subunits together in different orientations not recognised by most enzymes (Johnson, 2006).

Compared to starch, cellulose is much more crystalline. Starch is the storage polysaccharide whereby cellulose is the structural polysaccharide. Starch undergoes a crystalline to amorphous transition when heated below 100 °C in water (as in cooking condition), but cellulose requires a temperature of 320 °C and pressure of 25 MPa to become amorphous in water. Chemically, cellulose can be broken down into its glucose units at extreme condition. It can be treated with concentrated acids at high temperature to break down into subunits. Biologically, this polymer can be hydrolysed by the action of cellulases into glucose units. The cellulose molecule is complex in nature, and therefore a group of enzymes acting synergistically with different binding sites is desired which including endoglucanase, exoglucanase, and β-glucosidase (Lee, 1997).

2.5.3 Hemicellulose

Hemicellulose is a polysaccharide related to cellulose. It is found in cell wall that is similar to cellulose but it is more soluble. It corresponds to 10 to 40 % of wood dry weight (Chiaramonti, 2007). In addition to cellulose, plant cell walls contain other polysaccharide, such as hemicellulose, pectin and protein. A hemicellulose can be any of several heteropolymer (matrix polysaccharides), a polymer of glucose and other sugars that is important in binding cellulose fibrils together. In contrast to cellulose, which contains only anhydrous glucose, hemicellulose contains many different sugar monomers. It is derived from several sugars in addition to glucose, mainly including xylose but also mannose, galactose, rhamnose, and arabinose. Hemicellulose contains most of the *D*-pentose sugars, and occasionally small amounts of *L*-sugars as well. Xyloglucan is a type of hemicellulose which is present mostly in cell walls of plants in land. It contains mainly of xylose and glucose sugars (Graham *et al.*, 2006). Hemicellulose in hardwood mainly contains xylans, while in softwood glucomannans are most common (Kumar *et al*., 2008).

Hemicellulose consists of shorter chains of around 200 sugar units as compared to 7,000 to 15,000 glucose molecules in the average of cellulose polymer. Furthermore, hemicellulose is branched, whereas cellulose is linear and unbranched. While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolysed by dilute acid or base as well as myriad enzymes, hemicellulase. There are various enzymes responsible for the degradation of hemicellulose. In xylan degradation, for instance, endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterase all act on the different heteropolymers available in nature. In glucomannan degradation, β-mannanase and β-mannosidase cleave the polymer backbone. Like cellulose, hemicellulose is also an important source of fermentable sugars for biorefining applications (Kumar *et al*., 2008).

Figure 2.3 shows the chemical structure of cellulose, hemicellulose and lignin. The lignin structure is hypothesised by Chiaramonti (2007).

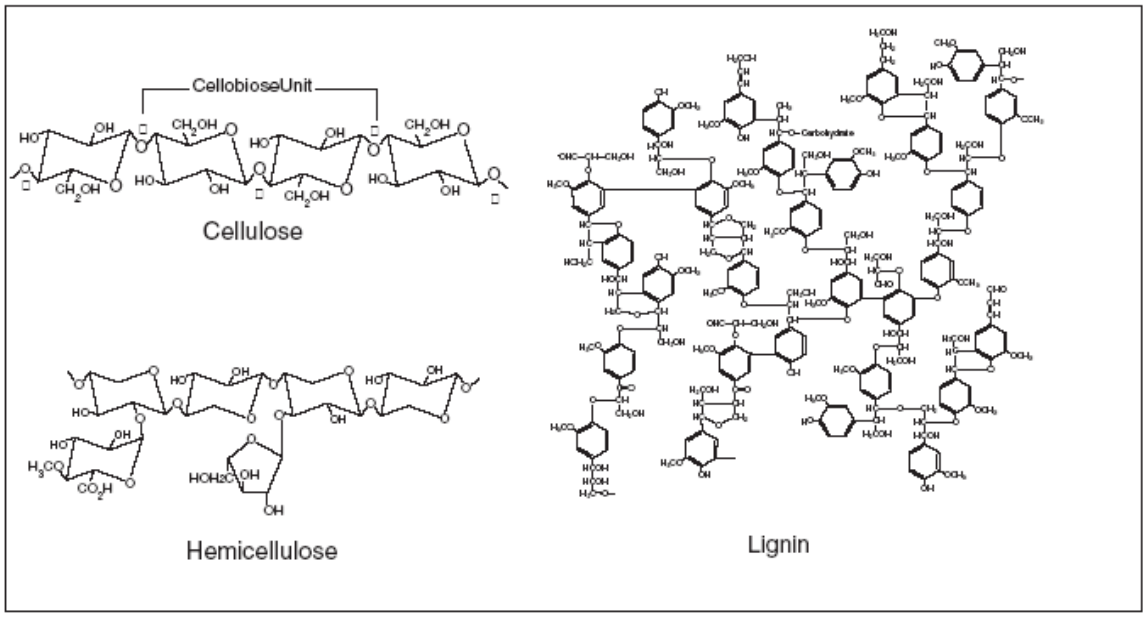

Figure 2.3: Cellulose, hemicelluloses and hypothesised lignin chemical structure (Chiaramonti, 2007)

2.6 Conversion of Lignocellulosic Biomass to Chemical

Basically, three main stages are involved in the conversion of lignocellulosic biomass or woody material to useful chemical like ethanol. The first stage is the dissociation of the cell components or pretreatment process. Secondly, is the hydrolysis of the cellulose to release the monomeric sugars. The last stage is the sugars fermentation process.

Biomass wastes contain a complex mixture of carbohydrate polymers from the plant cell walls known as cellulose, hemicellulose and lignin. In order to produce sugars from the biomass, the biomass is pretreated with acids and/or enzymes in order to reduce the size of the feedstock to dissociate the plant structure. The cellulose and the hemicellulose portions are broken down or hydrolysed by enzymes or dilute acids into monomeric sugars that are then fermented into ethanol. The lignin which is also present in the biomass is normally used as a fuel for the boilers in ethanol production plants. There are three principle methods of extracting sugars from biomass. These are concentrated acid hydrolysis, dilute acid hydrolysis and enzymatic hydrolysis.

2.7 Pretreatment Processes

Among the three key processes to convert the lignocellulosic biomass to chemical, the delignification of lignocellulosic raw materials to release the monomeric sugars is the key rate-limiting step. The potential method for removing lignin and releasing fermentable sugars is pretreatment followed by enzymatic or acidic hydrolysis. In the past decade, most research has focused on the pretreatment process and significant progress was achieved in thermal, mechanical, and chemical pretreatments and enzymatic hydrolysis. The method of pretreatment or hydrolysis and conditions employed will consequently affect the hydrolysis rate and the composition of the resulting sugars in the hydrolysate. The final constituents in enzyme hydrolysates are mainly glucose and xylose which are released from cellulose and hemicellulose, respectively (Lee, 1997).

The major obstacle in effective utilisation of the lignocellulose is its crystalline unreactivity and in particular its resistance to hydrolysis. Pretreatment is required to

alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes so that the carbohydrate polymers can be converted into fermentable monomeric sugars. This will alter the macroscopic and microscopic size of the biomass as well as its submicroscopic chemical composition and structure so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly with greater yields. Pretreatment affects the structure of biomass by solubilising hemicellulose, reducing crystallinity and increase the available surface area and pore volume of the substrate. Hemicellulose is broken down to its subunits. Pretreatment has been considered as one of the most expensive processing steps in converting biomass to fermentable monomeric sugar. Native lignocellulosic biomass is extremely recalcitrant to enzymatic digestion. Pretreatment methods are either physical or chemical and sometimes both are used. Physical pretreatment methods include comminution (mechanical reduction in biomass particulate size), steam explosion, and hydrothermolysis. Chemical pretreatment will use acids or bases that promote hydrolysis and improve the yield of glucose recovery from cellulose by removing hemicellulose or lignin during pretreatment (Moiser *et al.*, 2005).

2.8 Hydrolysis of Cellulose

After the pretreatment process as stated above, there are normally two types of hydrolysis processes to degrade the lignocellulosic material into the monomeric sugar. The most common hydrolysis methods used are acid (dilute and concentrated) and enzymes. To improve the enzymatic hydrolytic efficiency, the lignin-hemicellulose network has to be loosened for the better amenability of cellulases to residual carbohydrate fraction for sugar recovery. Dilute acid treatment is employed for the degradation of hemicellulose leaving lignin and cellulose network in the substrate (Chandel *et al.*, 2007).

Hydrolysis is a chemical decomposition reaction or process in which chemical bonds of compound is broken down or split by reaction with water. Hydrolysis of cellulose refers to the process of cellulolysis which is relating to or causes the hydrolysis of cellulose. The hydrolysis of cellulose or starch into glucose is called saccharification.

During the hydrolysis of lignocellulosic materials especially the process involving high temperature under acidic condition, a wide range of compounds which are inhibitory to microorganisms will be formed or liberated. Based on their origin, the inhibitors are usually divided into three major groups which are weak acids, furan derivatives, and phenolic compounds. At high temperature, xylose from hemicellulose will further be degraded to furfural. Similarly, 5-hydroxymethyl furfural (HMF) is formed from the degradation of hexose sugar. Formic acid will be formed when furfural and HMF are broken down. Whereby, levulinic acid is the product of HMF degradation. Phenolic compounds are generated from partial breakdown of lignin (Palmqvist and Hahn-Hägerdal, 2000). These degraded byproducts would reduce the yield and productivity of ethanol during the fermentation process by inhibiting on the growth of microorganism. As such, in order to enhance the productivity of fermentation process, the process prior to the fermentation has to be optimised to give reduced byproducts.

2.8.1 Acid Hydrolysis

Two types of acid hydrolysis processes are commonly used for the treatment of lignocellulosic material, namely the dilute acid hydrolysis and concentrated acid hydrolysis. The dilute acid hydrolysis treatment is conducted under high temperature and pressure and has reaction time in the range within minutes. The concentrated acid hydrolysis uses relatively mild temperatures, but conducted at very high concentration of

sulfuric acid and at a minimum pressure. The process involves pumping the materials from one vessel to another vessel to minimise the corrosive of strong acid on the vessel. Reaction times for concentrated acid hydrolysis are typically much longer than for dilute acid process (Chandel *et al.*, 2007).

2.8.1.1 Concentrated Acid Hydrolysis

Concentrated acid hydrolysis process is accomplished by adding 70 - 77 %w/w sulfuric acid to the oven-dried biomass with a moisture content of 10 %. This process will be followed by a dilution with water to dissolve and hydrolyse the substrate of lignocellulose into fermentable monomeric sugar. Firstly, the operation temperature is controlled in the range of 40 to 50 °C with reaction time of 2 to 4 hours in a reactor for components dissociation and hemicellulose hydrolysis processes. In the next step, the cellulose part in the lignocellulosic biomass will be hydrolysed. Water is then added to the solid residue and the acid will be diluted to 30 to 40 %w/w. The mixture is then heated to 100 °C for 50 minutes. The gel produced from this mixture is then pressed to release an acid-sugar mixture. A chromatographic column is used to separate the acid and sugar mixture. Both the sugar streams from two concentrated hydrolysis steps are combined and will be used as substrate for subsequent fermentation process for ethanol production (Chandel *et al.*, 2007).

This process provides complete and rapid conversion of cellulose to glucose and hemicellulose to xylose with low degree of degradation. High sugar recovery efficiency is the primary advantage of the concentrated acid hydrolysis process. Up to 90 % of cellulose and hemicellulose degrade to their subunits after the treatment process. The low temperatures and pressure of the concentrated acid hydrolysis will lead to minimised sugar degradation (Chandel *et al.*, 2007). Sugars derived from this hydrolysis process are easily fermented by microorganism.

The disadvantage of the concentrated hydrolysis will be the difficulty of single sugar recovery after the acid hydrolysis and neutralisation processes. The hydrolysate which contains 10 % acid and 10 % glucose after the hydrolysis of cellulose, need to be neutralised by adding lots of calcium hydroxide, $Ca(OH)_2$. Hydrated gypsum ($CaSO_4.2H_2O$) is precipitated after the neutralisation process and need to be removed before proceeding to the subsequent fermentation process. Lignin is a solid residue of the process, which remains available for various uses, as heat and power generation (Chiaramonti, 2007).

2.8.1.2 Dilute Acid Hydrolysis

The dilute acid hydrolysis process is one of the simple, oldest and most efficient methods of producing monomeric sugars from biomass. Dilute acid is used to hydrolyse the biomass to monomeric sugars. The liquid hydrolates will then be neutralised and recovered from the process of hydrolysis. In dilute acid hydrolysis, the hemicellulose fraction is depolymerized at lower temperature than the cellulosic fraction. Dilute sulphuric acid is mixed with biomass to hydrolyse hemicellulose to xylose and other sugars. Dilute acid interacts with the biomass and the slurry is held at temperature ranging from 120 to 220 °C for a short period of time. Thus, the hemicellulosic fraction of plant cell wall is depolymerised and will lead to the enhancement of cellulose digestibility in the residual solids. Dilute acid hydrolysis has some limitations. If higher temperatures (or longer residence time) are applied, the hemicellulose derived monomeric sugars will degrade and give rise to fermentation inhibitors like furan compounds, weak carboxylic acids and phenolic compounds. These fermentation inhibitors are known to affect the ethanol producing performance of fermenting microorganisms. Therefore, dilute acid hydrolysis is carried out in two stages to avoid inhibitor production (Chandel *et al.*, 2007).

The first stage of the dilute acid hydrolysis process uses 0.7 %w/w sulfuric acid at 190 °C to hydrolyse the hemicellulose present in the biomass. The second stage aims to yield the more resistant cellulose fraction. This is achieved by using 0.4 %w/w sulphuric acid at 215 °C. The liquid hydrolysate is then neutralised and recovered from the process. Dilute acid hydrolysis is a simple process and no acid recovery is needed, but the sugars yielded in this hydrolysis process are lower when compared to concentrated acid hydrolysis.

First-stage dilute acid hydrolysis

At the first-stage, hemicellulose will be hydrolysed under mild process condition. Typically, 0.5 to 0.7 %w/w of dilute sulphuric acid, H_2SO_4 interacts with the lignocellulosic material under the temperatures of 160 to 190 °C to recover the five carbon sugars from hemicellulose (Chiaramonti, 2007). About 80 % of the hemicellulose and 29 % of cellulose are hydrolysed in the first reactor. The hydrolysate is further incubated at a lower temperature for a residence time of 2 hours to hydrolyse most of the oligosaccharides into monosaccharides followed by the separation of solid and liquid fractions (Chandel *et al.*, 2007). The first-stage dilute acid hydrolysis must be controlled at mild conditions to avoid the degradation of sugars to the byproducts which will act as inhibitors in the fermentation process.

The solid material is washed with lots of water to maximise sugar recovery. The separated solid material is then sent to the next stage of acid hydrolysis reactor (Chandel *et al.*, 2007).

Second-stage dilute acid hydrolysis

In contrast to the first-stage dilute acid hydrolysis which treats the biomass at relatively mild conditions during which the hemicellulose fraction is hydrolysed, the second-stage is normally carried out at higher temperature for degradation of cellulose into glucose (Chandel *et al.*, 2007).

After the first-stage of the hydrolysis process, the hydrolysate containing the monomeric sugars is removed to avoid the further degradation of monosaccharides and inhibitors formation. The cellulose in the remaining solids is then hydrolysed in a 0.4 to 2 %w/w of dilute sulphuric acid solution at temperature of 200 to 215 °C. The sugar-rich liquid after hydrolysis is sent for fermentation. The solid residues which are mainly lignin and some of the residual cellulose can be used for heat and power generation (Chiaramonti, 2007).

Figure 2.4 shows the dilute acid hydrolysis process, the first-stage and the second-stage followed by the separate fermentation of the 5-carbons and 6-carbons monomeric sugars (Chandel *et al.*, 2007).

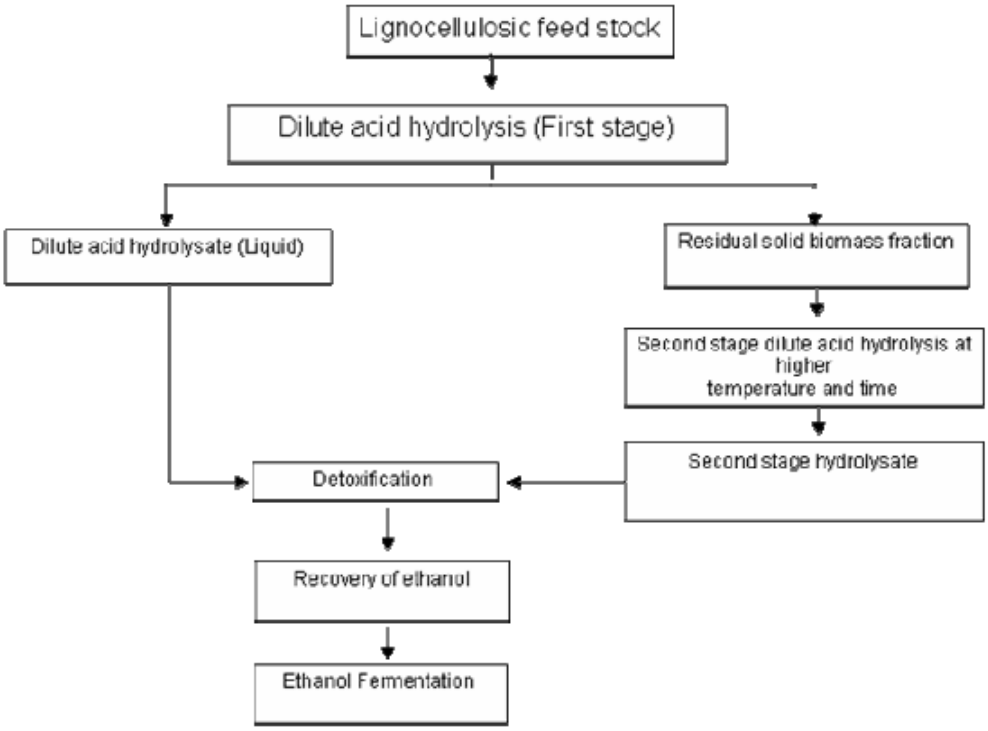

Figure 2.4: Dilute acid hydrolysis (first-stage and second-stage) and separate fermentation of pentose and hexose sugars to become ethanol (Chandel *et al.*, 2007)

2.8.2 Enzymatic Hydrolysis

Instead of using acid or chemical to hydrolyse the biomass into monomeric sugars, enzymes can also be used to break down the biomass. Enzymatic process is a hydrolysis process in which selected enzymes break the polymeric chain of the cellulose and hemicellulose leaving the monomeric sugars available for fermentation. It gives a higher hydrolysis yields compared to chemical processes (Chiaramonti, 2007).

A variety of microorganisms including bacteria and fungi may have the ability to degrade the cellulose part of the lignocellulosic biomass to glucose monomers. Basically, bacterial cellulases exist as discrete multi-enzyme complexes, called cellulosomes that consist of multiple subunits. The cellulolytic enzyme systems from the filamentous fungi, especially *Trichoderma reesei*, contain two types of exoglucanases, at least four type of endoglucanase, and one β-glucosidase. The hydrolysis of cellulose will need the synergistical catalysis of the above enzyme groups (Kumar *et al*., 2008). There are group of microorganisms which include *Clostridium*, *Cellulomonas, Trichoderma*, *Penicillium*, *Neurospora* and *Fusarium*, *Aspergillus* that show high cellulolytic and hemicellulolytic activity. They are also highly capable of fermenting monomeric sugars (Chandel *et al.*, 2007).

Biomass pretreatment is an essential step in enzymatic hydrolysis process. Pretreatment aims at increasing the surface accessible to enzymes by destroying the cell structure, breaking the lignin-hemicellulose sheath around cellulose and reducing the cellulose polymerisation and crystallinity (Chiaramonti, 2007). Only 20 % of the untreated biomass pore volume can be reached by the large cellulase enzymes molecules. Therefore, biomass pretreatment is important to improve the efficiency of the cellulose hydrolysis and subsequently increase the sugar yields.

The enzymatic hydrolysis will produce a sugar solution that is easily used for fermentation. But unfortunately, the cost of enzyme and the pretreatment process are high. Therefore, a cost-efficient pretreatment stage is a key to the success of the lignocellulosic biomass conversion process.

2.9 Fermentation of Sugars

The hydrolysis process breaks down the cellulose and hemicellulose part of the biomass into sugar solutions that can then be easily fermented into ethanol. The sugar syrup obtained after cellulosic hydrolysis is used for ethanol fermentation. Fermentation is performed by fermenting microorganisms (yeasts, bacteria, fungi) in the absence of oxygen according to the following main reactions shown in Equation 2.1:

$$C_6H_{12}O_6 \quad 2\ C_2H_5OH + 2\ CO_2 \qquad \text{Eq. (2.1)}$$

Glucose ($C_6H_{12}O_6$) will be degraded to form ethanol (C_2H_5OH) and carbon dioxide (CO_2) will be released during the fermentation process. *Saccharomices cerevisiae* is the typical yeast for hexose sugars fermentation. It is capable of converting only 6-carbon sugar to ethanol (Chiaramonti, 2007).

The fermentation of 5-carbon sugars (pentoses, such as arabinose and xylose) derived from lignocellulosic biomass is also possible, but not with the ordinary strains of yeasts. The chemical reaction is shown in Equation 2.2 (Chiaramonti, 2007):

$$3\ C_5H_{10}O_5 \quad 5\ C_2H_5OH + 5\ CO_2 \qquad \text{Eq. (2.2)}$$

Currently, the most promising yeasts that have the ability to use both the C-5 and C-6 sugars are *Pichia stipitis*, *Candida shehatae* and *Pachysolan tannophilus* (Chandel *et al.*, 2007).

Researches are now focusing on developing recombinant yeast, which can greatly improve the yield of ethanol production by metabolising all form of sugars, and reduce the cost of operation (Chandel *et al.*, 2007).

Upon completion of the fermentation process, the "ethanol broth" as an output will need to be dehydrated to remove the excess water from ethanol and this step is therefore called Ethanol Recovery. The other byproducts that include products like lignin are used to produce electricity that is required for the production of ethanol.

At presents, the entire process of biofuel production is less efficient and hence less cost effective following this route therefore scientists are working intensively to look for cheaper processes so that the process can become cost effective.

2.10 Hydrolysis Processes of this Study

In this study, dilute acid hydrolysis pretreatment is selected to pretreat or dissociate the cell components of the EFB before proceed to the enzymatic treatment which aims to degrade the cellulose part of EFB into glucose.

The cost and the effectiveness of the dilute acid hydrolysis made it a common method to use in the pretreatment of lignocellulosic biomass. Many factors will affect the efficiency and also the product of this acidic hydrolysis. Based on literature search, three variables namely the pretreatment temperature, acid concentration and pretreatment reaction time which mainly affected the acidic pretreatment process were

selected for optimisation in this study. Many researchers have studied the effect of these three factors on different type of lignocellulosic biomass including corn stover (Lloyd and Wyman, 2005), sugar cane bagasse (Aguilar *et al*., 2002) and agri-food waste (Campo *et al.*, 2006).

The operating temperature of hydrolysis selected varied between 80 and 120 °C and samples were collected at various time intervals in the range of 10 to 30 min using 2 to 6 %w/w of sulphuric acid. The operating conditions applied in this study are referred to the previous research (Rahman *et al*., 2006) which used EFB as substrate too but for the production of xylose sugar. Operating at low temperature aims to save energy and cost and the duration of pretreatment time is also less than 1 hour. High temperature will lead to the formation of degradation product. Dilute acid hydrolysis is the simplest method compared with others chemical or even the physical methods. Acid recovery is not needed after the treatment process and the hemicellulose can easily depolymerise to its subunits.

Following the pretreatment process, enzymatic hydrolysis is carried out to degrade the cellulose part of EFB. The enzymatic hydrolysis is a biological process, more environments friendly and will produce a sugar solution that is easily used for fermentation therefore was chosen in this study. Enzymatic hydrolysis of pretreated EFB is carries out by enzyme cellulase. Fungal genera like *Trichoderma* and *Aspergillus* are cellulase producers and crude enzymes produced by these microorganisms are commercially available (Immanuel *et al*., 2007).

Two operating variables during the enzymatic process, namely the enzymatic substrate concentration and the enzymatic reaction time are selected for optimisation in this study. These two variables are expected to have significance effect on the yield of glucose production during the degradation of cellulosic part of EFB. Since commercial

enzymes are used in this study, the operating temperature and pH chosen were based on the instruction manual of the products. According to the information sheet, the optimum temperature for cellulase complex, Celluclast® is in the range of 45 to 60 ºC and for *β*-glucosidase, Novozym® in the range of 45 to 70 ºC. For optimum pH, the range for Celluclast® is from 4.5 to 6.5 and for *β*-glucosidase, 2.5 to 6.5. Therefore, in this study, the temperature of enzymatic treatment process was fixed at 50 ºC. Buffer at pH 4.8 was used to maintain the operating condition with minimum dramatic fluctuations (Zhong *et al.*, 2007).

With the increase of substrate concentration, the production of sugars is expected increase accordingly. After the optimum condition reached, with the continuous adding substrate without additional loading of enzyme, the reaction will become flat and ultimately decreased. Stirring difficulties, reduction of the aqueous movable phase and end product inhibition might hinder the enzymatic hydrolysis at higher substrate concentration. Therefore the hydrolysis of pretreated EFB is carried out in the range of 24 to 72 hours and the substrate concentrations chosen were between 5 to 15 %w/v at a fixed of enzyme loading (Zhong *et al.*, 2007).

Through the design and analysis of the experiment which will be discussed later in Section 2.11, the yield of glucose can be optimised by experimental design and analysis using the statistical tool of RSM (response surface methodology).

2.11 Design and Analysis of Experiments

Design of experiment (DOE) is a structured, organised method used to determine the relationship between the different factors affecting a process and the output or response of that process. Further analysis is conducted based on the experimental results obtained. This statistical method of experimental design was first developed by the late Sir Ronald A. Fisher since 1920s (Montgometry, 1997).

DOE involves designing a set of experiments, in which all relevant factors are varied systematically. It helps to identify the optimal conditions, the factors that most influence the results, and those that do not, as well as the existence of interactions and synergies between factors when the results of these experiments are analysed. Experimental design methods require well-structured data matrices. When applied to a well-structured matrix, analysis of variance delivers accurate results, even when the matrix being analysed is quite small (Oehlert, 2000).

DOE is a strategy to gather empirical knowledge based on the analysis of experimental data and not on theoretical models. It can be applied whenever one intends to investigate a phenomenon for better understanding or improving the performance. There are three basic principles of experimental design which are replication, randomisation and blocking (Montgometry, 1997).

DOE is widely used in research and development, where a large proportion of the resources would go towards solving and optimisation of the problems. The key to minimising optimisation costs is to conduct as few experiments as possible. DOE requires only a small set of experiments and thus helps to reduce costs. Currently, the methods by Fisher (1920) represent the international standards for experimental and

analysis in business and applied science. A wide range of software is available in the market for design and analysis purposes.

2.11.1 Experimental Design and Analysis of this Study

Many parameters affect the hydrolysis of EFB to glucose. Therefore, efficient experimental design is needed to determine the effect of each or interrelation between the parameters in the hydrolysis processes. Consequently, the highest yield of glucose can be obtained through the optimised experimental design.

In this study, response surface methodology (RSM) is selected for design and analysis of the experimental data. The objective of RSM is to optimise the responses. It is compatible with the objective of this research (for optimised yield of glucose). There are many types of models or designs in the RSM for optimisation purpose. Central composite design (CCD) was chosen for this study because of the usefulness of this design for building a second-order model for the response variable without needing to use a complete three-level factorial experiment. Using more complicated design will increase the number of experiment runs and ultimately increase the cost and time involved. The detailed explanations of the RSM and CCD are discussed in Section 2.11.2 and 2.11.3.

2.11.2 Response Surface Methodology (RSM)

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analysing the significance or the influence where the independent variables have on the dependent variable or response. ANOVA and various diagnostic plots are using in the analysis of the significance of the models. The objective of RSM is to optimise the response (Montgometry, 1997). RSM explores the relationships between several explanatory variables and one or more response variables. It was first introduced by G. E. P. Box and K. B. Wilson in 1951 (Buyske, 2001).

RSM relies on a set of designed experiments to obtain an optimal response. These procedures are used to optimise a process. RSM is a statistical technique for the modelling and optimisation of multiple variables, which determines the optimum process conditions by combining experimental designs with interpolation by first or higher order polynomial equations through a sequential testing procedure (Ferreira *et al.*, 2008).

Many experiments have the goals of describing how the response varies as a function of the variables and determining treatments that give optimal responses, perhaps maxima, minima or attaining a specific target. An easy way to estimate a first-degree polynomial model is to use a factorial experiment or a fractional factorial designs. Factorial structures can be used for these kinds of experiments too. While treatment variables can be varied across a continuous range of values, other treatment designs may be more efficient. A more complicated design, such as a central composite design in RSM can be implemented to estimate a second-degree polynomial model, which is still only an approximation at best. The second-degree model can be used to optimise a response. Response surface methods are designs and models for working

with continuous treatments when finding optima or describing the response is the goal (Oehlert, 2000).

A response surface is the geometric representation obtained when a response variable is plotted as a function of one or more quantitative factors (Mason *et al*., 2003). A typical response surface can be represented graphically in Figure 2.5 (Montgometry, 1997).

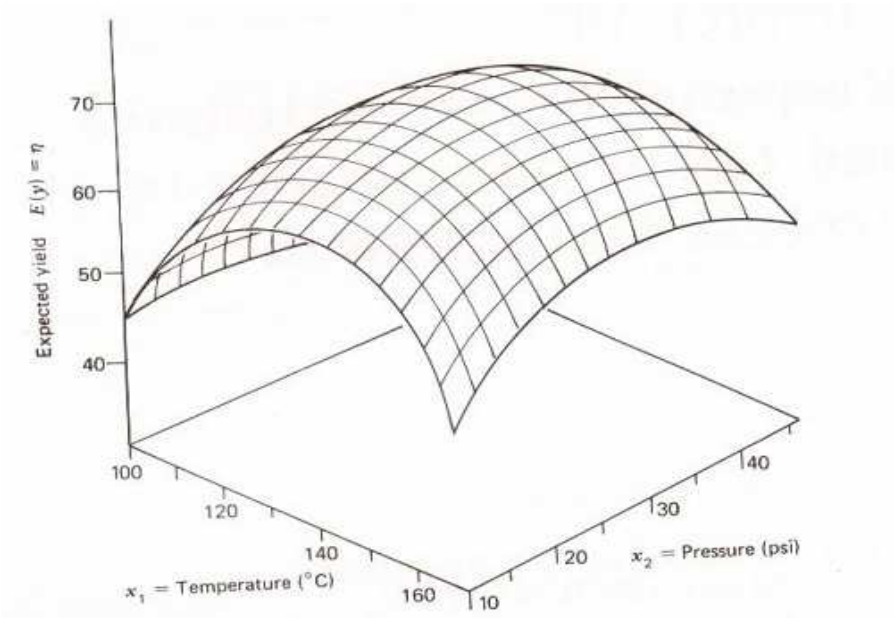

Figure 2.5: A three-dimensional response surface showing the expected yield as a function of temperature and pressure (Montgometry, 1997)

To visualise the shape of a response surface, contours plots are useful. A contour plot is a series of lines or curves that identify values of the factors for which the response is constant. Curves for several values, usually equally spaced of the response are plotted (Mason *et al*., 2003). Figure 2.6 shows an example of the contour plot. In the contour plot, lines of constant response are drawn in the x_1, x_2 plane. Each contour corresponds to a particular height of the response surface (Montgometry, 1997). A contour plot

shows the contours of the surface, that is, curves of x_1, x_2 pairs that have the same response value.

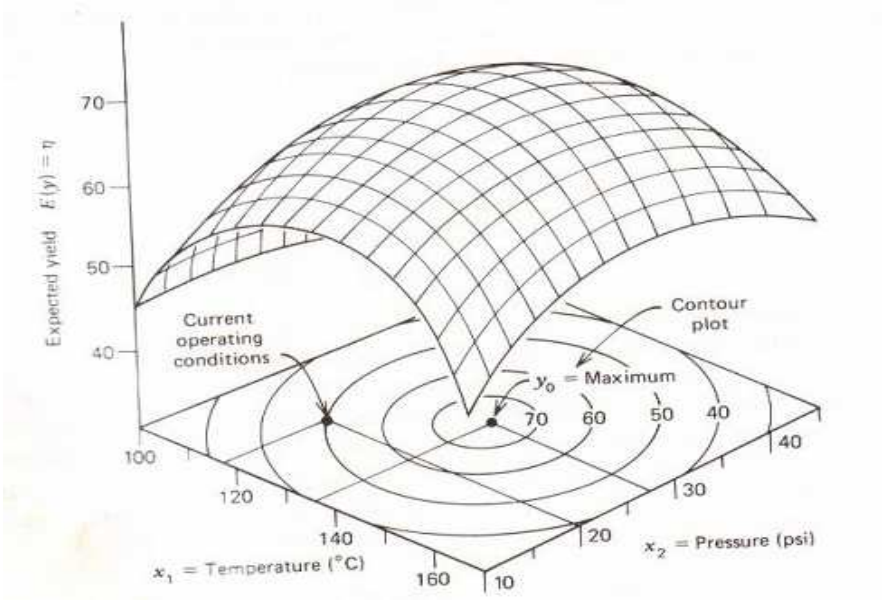

Figure 2.6: A contour plot of a response surface (Montgometry, 1997)

In most RSM, the form of the relationship between the response and the independent variable, *Y* is unknown. Thus the first step in RSM is to find a suitable approximation for the true functional relationship between *Y* and *q* set of independent variables (*X*). Usually, a low-order polynomial in some region of the independent variables is employed. If the response is well modeled by a linear function of the independent variables, then the approximating function is the first-order model as described by Equation 2.3 (Buyske, 2001).

$$Y = \beta_0 + \beta_1 X_1 + \ldots + \beta_q X_q$$

$$= \beta_0 + \sum_{i=1}^{q} \beta_i X_i \qquad \text{Eq. (2.3)}$$

If there is curvature in the system, then a polynomial of higher degree must be used, such as second-order model as described in Equation 2.4 (Buyske, 2001).

$$Y = \beta_0 + \sum_{i=1}^{q} \beta_i X_i + \sum_{i=1}^{q} \beta_i X_i^{\,2} + \sum_{i} \sum_{j>i} \beta_i X_i X_j \qquad \text{Eq. (2.4)}$$

RSM is a sequential procedure. Almost all RSM problems utilise one or both of these models. Often, if there is a little curvature in the system, the first-order model is appropriate to use. First-order designs collect data to fit first-order models. The standard first-order design is a 2^q factorial with center points. The (coded) low and high values for each variable are ±1; the center points are m observations taken with all variables at 0. This design has $2^q + m$ points. Second-order models are used when the portion of the response surface has curvature. A second-order model contains all the terms in the first-order, plus all quadratic terms and all cross product terms. In principal, third- or higher order models can also be used. But, this is rarely done, as second-order models are generally sufficient (Oehlert, 2000).

Experimental designs used in RSM must make tradeoffs between reducing variability and reducing the negative impact that can be caused by bias. By careful monitoring, RSM will be a useful method for industrial optimisation.

2.11.3 Central Composite Design (CCD)

There are several choices for second-order designs in the RSM. One of the most popular methods is the central composite design (CCD). It is useful for building a second-order model for the response variable without needing to use a complete three-level factorial experiment.

A CCD composed of three groups of design points which are factorial points, axial points, and center points. Figure 2.7 shows the CCD for factor number, $q = 2$ (Montgometry, 1997).

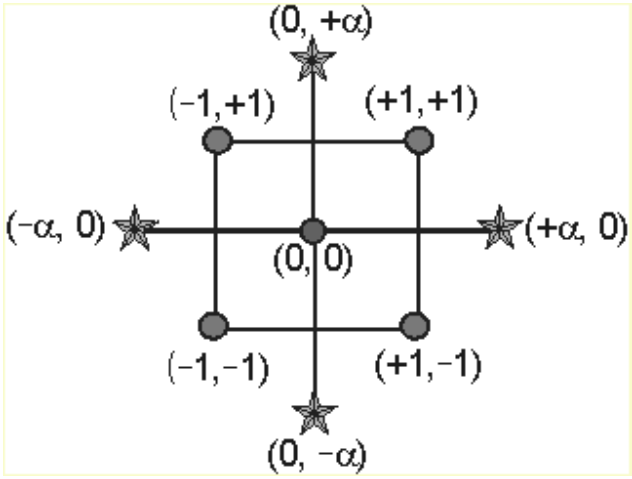

Figure 2.7: Central composite design for $q = 2$ (Montgometry, 1997)

Factorial points are the points from a 2^q design (two levels) with level coded as ±1 (Oehlert, 2000). The two-level factorial part of the design consists of all possible combinations of the +1 and -1 levels of the factors. For example, the two factor case there are four design points: (-1, -1) (+1, -1) (-1, +1) (+1, +1).

Center points, as implied by the name, are points with all levels set to coded level 0, the midpoint of each factor range, (0, 0). It is *m* points at the origin. Experimental runs whose values of each factor are the medians of the values used in the factorial portion. This point is often replicated in order to improve the precision of the experiment (Oehlert, 2000).

The axial or star points have one design variable at ±α (alpha) and all other design variables at 0; there are $2q$ axial points (Oehlert, 2000). It has all of the factors set to 0, the midpoint, except one factor, which has the value +/- alpha. For a two factor problem, the star points are (-α, 0) (+α, 0) (0, - α) (0, + α). The value for alpha is calculated in each design for both rotatability and orthogonality of blocks. Another position for the star points is at the face of the cube portion on the design. This is commonly referred to as a face-centered central composite design. After the designed experiment is performed, linear regression is used to obtain results. Coded variables above are often used when constructing this design.

The total number of experiment run in a CCD based on a complete 2^q factorial is $n = 2^q + 2q + m$. This count usually is less than 3^q, so that fewer observations are required than in the 3^q factorial. The CCD can be made rotatable by choosing $\alpha = F^{1/4}$, where F is the number of factorial points (Mason *et al*., 2003). The rotatability is a desirable property for response surface model because prior to the collection of data and the fitting of the response surface, the orientation of the design with respect to the surface is unknown. Thus, the exploration of the response surface is dependent on the orientation of the design.

The CCD can run whether in full or fractional factorial. CCD with full factorial is a design in which the factorial portion is a full factorial with all combinations of the factors at two levels. It means more experiments will need to be conducted in order to

get more precise results. But unfortunately, it is only applicable to design that has less factors interaction. Resolution V factorial designs allow independent estimation of the main effects and two factor interactions assuming that three factor and higher interactions are negligible. When the number of factors is 5 or greater, it is not necessary to run all combinations of factors. Therefore, the CCD with fractional factorial is more suitable to reduce the experiment runs. Besides the two CCD design above as mentioned, small central composite designs are also available when the number of factors is 3 or more. These designs are the minimal-point designs needed to estimate the terms in a second order model. Small central composite designs are however unbalanced minimal point designs. They are not rotatable and are extremely sensitive to outliers (Mason *et al*., 2003). Hence, the fractional CCD was selected as the design method in this study.

Through successful design and analysis of experiment using CCD and RSM, the optimised experimental conditions can be obtained and highest the yield of glucose could be obtained ultimately.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Introduction

Oil palms are planted in enormous amount throughout Malaysia. The lignocellulosic waste produced from oil palm related industries can be utilised in the good way not just overcome the waste disposal problem but also produce useful chemical like bioethanol. Therefore, empty fruit bunch (EFB) from palm oil mill was used as the raw material or analysis sample in this study to obtain the glucose that can be further converted to ethanol through fermentation process.

Various type of chemical materials were using in this study. These chemical reagents were purchased commercially and used in the different processes in this study. Besides, the enzymes using is the main element determining the successfulness of this research.

The overall methodology layout of this study is shown in Figure 3.1.

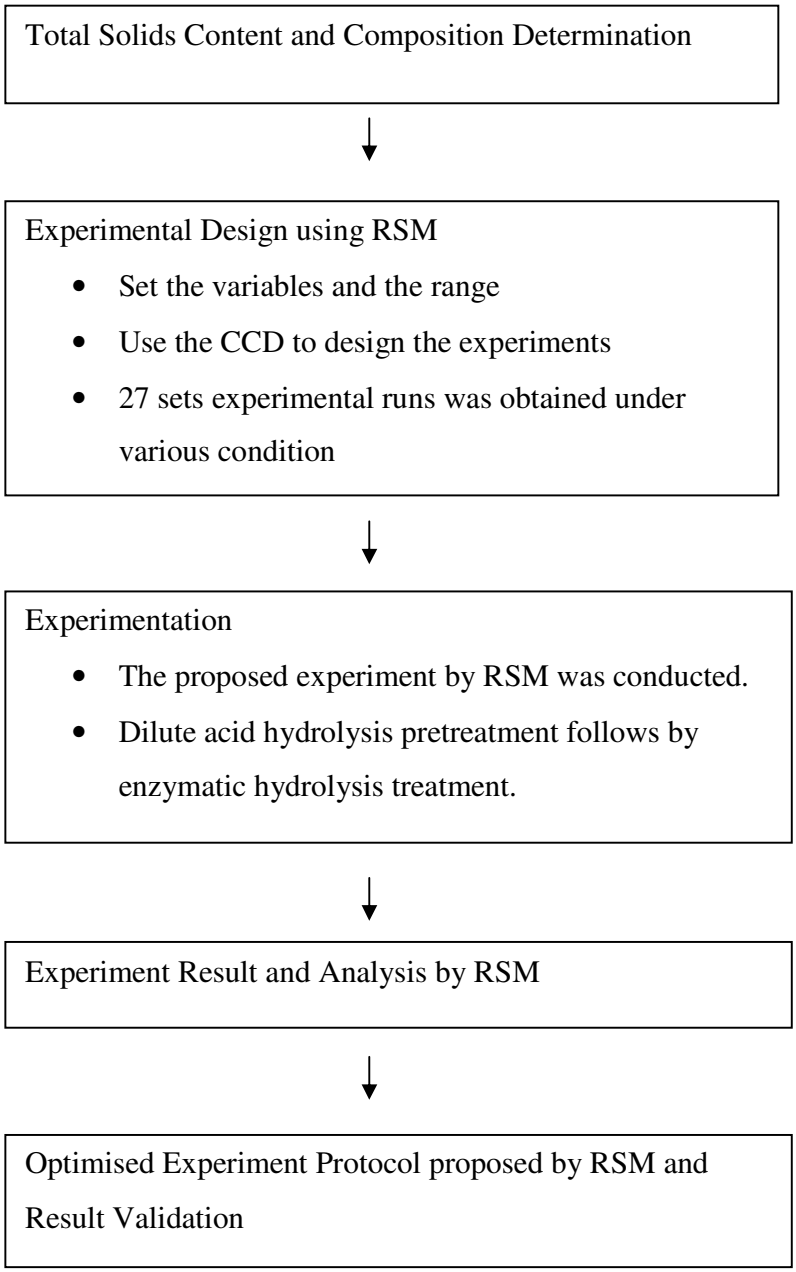

Figure 3.1: Flow of experiment

3.2 Materials

3.2.1 Raw material

The lignocellulosic biomass being used in this study was oil palm empty fruit bunch (EFB). It was collected from a local oil mill plant located at Kulai, Johor (Mahamurni Plantation Sdn. Bhd.). This company produced around three tones of EFB waste every day. Following sampling, the EFB was air-dried first at room temperature to avoid the fungus growth and then ground to particle size of less than 1mm using a hammer mill (Janke and Kunkel, IKA-Labortechnik, Germany). The grounded EFB biomass was then oven-dried at 105 ºC for overnight and stored in a desiccator until further use.

3.2.2 Chemicals and Reagents

All chemicals and reagents used as listed in Table 3.1 are of analytical grade. The reagents were used without further purification.

Table 3.1: Analytical reagents

Chemical	Supplier
Sulphuric Acid (435589, 95-98 %)	Sigma-Aldrich, USA
D (+)-Glucose anhydrous (1.08337.0250)	Merck, Germany
D (+)-Xylose (1.08689.0025, ≥ 99 %)	Merck, Germany
Calcium Carbonate (239216, 99 %)	Sigma-Aldrich, USA
Citric Acid anhydrous (1.818701.1000, ≥ 99 %)	Merck, Germany
Tri-Sodium Citrate dehydrate (1.06432.1000, 99-100.5 %)	Merck, Germany

3.2.3 Enzymes

The cellulase enzyme was purchased commercially from Sigma Aldrich Sdn Bhd. It is a commercial available cellulolytic complex, known as Celluclast® 1.5 L by Novozyme A/S (Denmark). It was produced by submerged fermentation of a selected strain of the fungus *Trichoderma reesei*. It catalyses the hydrolysis of cellulose to glucose, cellobiose, and/or higher glucose polymers. The enzyme was in aqueous solution and had a density of 1.2 $g.mL^{-1}$ at 25 °C.

Cellobiase (β-glucosidase) (Novozym® 188, Novozymes A/S, Denmark) was also added to supplement the β-glucosidase activity on the degradation of cellulose. Cellobiase enzyme was prepared by submerged fermentation of an *Aspergillus niger* microorganism. The cellobiase hydrolyses cellobiose to glucose. Accumulation of cellobiose in solution will affect the result of cellulose conversion.

3.3 Analytical Methods

High Performance Liquid Chromatography (HPLC, Perkin-Elmer 200) was selected to analyse the composition of carbohydrate in this research. Refractive Index, RI was used as the detector. All sample, standards and buffer were filtered through 0.2 µm regenerated cellulose filters prior to analysis. For monosaccharide determination, column Rezex RPM Monosaccaride Pb++ 8 % (Phenemenex, USA) was used. Pure deionised water (Milli Q, MILLIPORE, France) was used as mobile phase with a flow rate of 0.6 $mL.min^{-1}$ and oven temperature was maintained at 85 ºC. Sample volume was 50 µL and run time was set as 35 minutes.

The concentration of the glucose in the standard solutions and the hydrolysate after treatment processes were measured using the glucose analyser (YSI 2700 SELECT Biochemistry Analyser, YSI Life Sciences, Yellow Springs, OH). For glucose determination, the membrane of YSI 2365 in the Glucose (Dextrose) Membrane kit was used (YSI Life Sciences, Yellow Springs, OH). The reactive ingredients inside the membrane are immobilised glucose oxidase from *Aspergillus niger*. This was a direct reading (in $mg.mL^{-1}$) of dextrose in solution by the enzyme sensor. Glucose oxidase was immobilised in the YSI Dextrose Membrane.

3.4 Determination of Total Solids Content in EFB

The total solids content in EFB samples was determined according to the laboratory analytical procedure (LAP)-001 for Standard Method for the Determination

of Total Solids in Biomass (Ehrman, 1994). This method was intended to determine the amount of total solids remained after drying the solid sample at 105 ºC. The total solids of the sample must be done as soon as possible after sampling from plant (before storage) to avoid any moisture changes of the sample.

The convection oven procedure was used to determine the total solids content of EFB. This method involves drying a sample at 105°C ± 3°C in a convection oven (Memmert, Germany). Firstly, the weighing dish was predried at 105 ºC overnight before analysis. Then, the predried dish was cooled down in a desiccator. After being cooled down, the dish was weighed and recorded accurately. Approximately 1 to 5 g of fresh empty fruit bunch (EFB) was thoroughly mixed and weighed in the weighing dish prior to any preparation (as received). The weight of the sample plus weighing dish was recorded accordingly. The weighed sample in the dish was placed into a convection oven at 105 ºC for overnight. The sample was then removed from the oven and placed in a desiccator to allow cooling to room temperature. Then, the dish containing the oven-dried sample was weighed again and the percentage of the total solids was calculated according to Equation 3.1.

$$\% \text{ Total Solids} = \frac{\text{Weight of dried sample plus dish - Weight of dish}}{\text{Weight of sample as receive}} \text{ X } 100 \qquad \text{Eq. (3.1)}$$

Furthermore, the total moisture content of a sample can also calculated using Equation 3.2.

$$\text{Total Moisture (\%)} = 100\% - \text{Total solids content of sample (\%)} \qquad \text{Eq. (3.2)}$$

3.5 Determination of EFB Composition

The characterisation and analytical assays of the oven-dried EFB were performed following the laboratory analytical procedures (LAP) developed by the National Renewable Energy Laboratory (NREL, Golden, CO) (NREL, 1996). All experiments were carried out in triplicate; each value is presented as mean value.

3.5.1 Determination of Carbohydrate Content

The carbohydrate content of the EFB was determined using LAP-002, Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography (Ruiz and Ehrman, 1996). The standard method utilises two stages of acid hydrolysis processes to estimate the content of sugar in the biomass sample in percentage. The sample was taken through a primary stage of hydrolysis using 72 %w/w sulphuric acid and followed by a secondary stage of hydrolysis using 4 %w/w dilute sulphuric acid.

0.3 g of prepared sample (after milled and predried at 45 ºC for 4 hours) was weighed and placed in a 100 mL vessel. 3 mL of 72 % w/w sulphric acid was then added to the vessel containing EFB sample. A glass rod was used to stir the sample until the sample was thoroughly wetted. Sample was stirred for every 15 min to assure complete mixing and wetting. The vessel was then placed in the waterbath (Edelstahl Rostfrei, Deutschland) set at 30 ºC and left be hydrolysed for 2 hours.

Pure samples of glucose and xylose (0.3 g each, predried at 45 ºC for 4 hours) was weighed separately and placed in a 100 mL vessel. Acid was added and sugars were hydrolysed and stirred as described in the previous steps. The sugar recovery standards were taken through the remaining steps in the procedure in parallel with the samples. The calculated recovery of the standards was used to correct for losses due to the destruction of sugars during the hydrolysis process.

Upon completion of the two hour hydrolysis process, 84 mL of deionised water was added to dilute the acid concentration to 4 %w/w. The vessels were closed and sealed. The secondary hydrolysis process was done by autoclaving the sample in 4 %w/w H_2SO_4 at 121 ºC for 1 hour. The hydrolysate upon completion of autoclave cycle was then cooled for 20 min at room temperature before the seals removed.

These autoclaved solutions will be used for the determination of acid-insoluble residue (AIL, the residual solid, for acid-insoluble lignin determination) and acid-soluble lignin (ASL), in parallel with this carbohydrate determination. 20 mL aliquots of each hydrolysate were transferred to 50 mL Erlenmeyer flasks and neutralised with calcium carbonate to a pH between 5 and 6. Calcium carbonate was slowly added with frequent swirling to avoid problems of foaming and over-neutralisation.

The neutralised hydrolysate was filtered using a 3 mL syringe with a 0.2 µm filter (Sartorius Minisart® Syringe Filter, Sartorius Stedim Biotech. GmbH., Germany) attached. One portion of the hydrolysate was filtered directly into a sealable test tube for storage and reserved in case a repeat analysis is required. Then, a second portion was filtered directly into an autosampler vial of HPLC. The sugar content of EFB sample was then analysed using HPLC.

The importance of determining the carbohydrate content in this study was to calculate the yield of the glucose content after the completion of hydrolysis processes. Glucose yield can be calculated according to Equation 3.3.

$$\text{Glucose Yield (\%)} = \frac{\text{Glucose concentration after the hydrolysis}}{\text{*Total glucose content in sample}} \text{ X } 100 \qquad \text{Eq. (3.3)}$$

* This calculation is considered valid that assuming all glucan in the EFB was converted to glucose.

3.5.2 Determination of Acid Insoluble Lignin (AIL)

The residual solid following the two step acid hydrolysis processes of carbohydrate content determination was used to determine the AIL content in the EFB sample. This experiment was done according to the laboratory analytical procedure (LAP)-003 for the Determination of Acid-Insoluble Lignin in Biomass (Templeton and Ehrman, 1995).

Firstly, the crucible needed for analysis was labeled and ignited at 575 ºC to achieve a constant weight. Then, the ignited crucible was stored in a desiccator until needed. The hydrolysis solution, after the two step acid hydrolysis process was vacuum filtered through the ignited filtering crucible. The crucible and content was then dried at 105 ºC until constant weight was achieved. After heating, the crucible with sample was cooled in a desiccator and the weight was recorded as W_2. The crucible and the content was placed in the muffle furnace (Carbolite, United Kingkom) and ignited at 575 ºC for 4 hours. After that, the crucible was cooled in the desiccator. The remaining solid in the

crucible after ignition was weighing out as W_3. AIL content was then calculated based on Equation 3.4.

$$\% \text{ AIL} = \frac{W_2 - W_3}{\text{Initial sample weight} \times \frac{TS}{100}} \text{ X } 100 \qquad \text{Eq. (3.4)}$$

where,

AIL = acid insoluble lignin

W_2 = weight of crucible and sample after drying at 105 °C

W_3 = weight of crucible and sample after igniting at 575 ºC

TS = total solids

3.5.3 Determination of Acid Soluble Lignin (ASL)

A portion of the filtrate following the two-step hydrolysis processes was collected for the analysis of acid soluble lignin according to the LAP-004 for Determination of Acid-Soluble Lignin in Biomass (Ehrman, 1996). This procedure described a spectrophotometric method for determining the amount of lignin solubilised upon hydrolysis of a biomass sample.

The absorbance of the hydrolysate, *A* was measured at 205 nm using the 1 cm light path cuvette. A 4 %w/w solution of H_2SO_4 was used as reference blank. The

samples need to be diluted if the absorbance reading exceeds 0.7. The ASL content can be calculated based on Equation 3.5.

$$\% \text{ ASL} = \frac{\frac{\text{A}}{\text{b} \times \text{a}} \times df \times V \times \frac{1L}{1000\ mL}}{\text{Initial sample weight} \times \frac{\text{TS}}{100}} \text{ X } 100 \qquad \text{Eq. (3.5)}$$

where,

A = absorbance of hydrolysate at 205 nm
b = cell path length, 1 cm
a = absorptivity, 110 L/g-cm
df = dilution factor
V = filtrate volume, 87 mL
TS = total solids

3.5.4 Determination of Ash

The ash content of the EFB sample was determined using the laboratory analytical procedure (LAP-005) of Standard Method for Ash in Biomass. The ash is the inorganic residue left after ignition at 575 ºC.

The crucible was placed in the muffle furnace and ignited at 575 ºC until a constant weight was achieved. After cooled down to room temperature in the desiccator,

the crucible was weighed. The EFB sample was predried at 105 ºC prior to analysis. Subsequently, 0.5 to 1.0 g of the sample was weighed in the preheated crucible. The crucible and sample was then placed in the furnace and ignited for 4 hours. The weight was recorded after ignition to determine the ash content in the EFB sample according to Equation 3.6.

$$\% \text{ Ash} = \frac{\text{Weight of ash after ignition at } 575\,^{\circ}\text{C}}{\text{Initial weight of } 105\,^{\circ}\text{C dried sample}} \text{ X } 100 \qquad \text{Eq. (3.6)}$$

3.6 Design of the Experiment

For the experimental design, the statistical analysis of response surface methodology (RSM) was used to optimise the parameters for the combined acid and enzymatic hydrolysis processes.

A 2^5 half fraction rotatable central composite design (CCD) of RSM was adopted to design the experiment. From the design, five factors or variables were selected. There were the pretreatment temperature, acid concentration, pretreatment reaction time from the acid hydrolysis process and enzymatic substrate concentration and enzymatic reaction time from the enzymatic hydrolysis process. The actual and coded values for each component studied in the CCD are shown in Table 3.2.

Table 3.2: Experimental range and levels of independent process variables

Independent Variables	**Symbol**	**Range and Levels**				
		-α	**-1**	**0**	**+1**	**+α**
Pretreatment Temperature (°C)	X_1	60	80	100	120	140
Acid Concentration (%w/w)	X_2	0	2	4	6	8
Pretreatment Reaction Time (min)	X_3	0	10	20	30	40
Enzymatic Substrate Concentration (%w/v)	X_4	0	5	10	15	20
Enzymatic Reaction Time (hrs)	X_5	0	24	48	72	96

According to the half fraction of CCD, the total number of experiments was ½ $(2^q) + 2q + m$, where q is the number of independent variables and m is the number of repetitions of the experiments at the centre point. In this study, the total number of experiments of the 2^5 half fraction CCD with 1 centre points was 27 runs. There were two axial points on the axis of each design variable at a distance of 2 from the design centre.

Table 3.3 shows the design matrix constructed by the Design Expert Software Version 7.1.5 (Stat-Ease Inc., Minneapolis, USA). Experimental runs would follow the protocols as proposed by the software.

Table 3.3: Experimental design matrix

Runs	Variables				
	X_1 Temperature (°C)	X_2 Acid Conc (%w/w)	X_3 Pretreatment Time (min)	X_4 Sub Conc (%w/v)	X_5 Enz Reac Time (Hrs)
1	80	2	10	5	72
2	80	2	10	15	24
3	80	6	10	5	24
4	80	6	10	15	72
5	80	2	30	5	24
6	80	2	30	15	72
7	80	6	30	5	72
8	80	6	30	15	24
9	120	2	10	5	24
10	120	2	10	15	72
11	120	6	10	5	72
12	120	6	10	15	24
13	120	2	30	5	72
14	120	2	30	15	24
15	120	6	30	5	24
16	120	6	30	15	72
17	60	4	20	10	48
18	140	4	20	10	48
19	100	4	0	10	48
20	100	4	40	10	48
21	100	0	20	10	48
22	100	8	20	10	48
23	100	4	20	0	48
24	100	4	20	20	48
25	100	4	20	10	0
26	100	4	20	10	96
27	100	4	20	10	48

3.7 Experimentation

Twenty seven set experiments as listed in Table 3.3 were conducted. Each experiment involved dilute sulphuric acid hydrolysis pretreatment followed by the enzymatic hydrolysis treatment.

3.7.1 Dilute Acid Hydrolysis

Acid hydrolysis pretreatment of empty fruit bunch were carried out in a 125 mL Erlenmeyer flasks. The media consisted of 2 to 6 %w/w sulphuric acid using a charge of 1 g EFB fiber per 8 g liquor H_2SO_4 on dry basis. The acid was added at 1 / 8 (w/v) solid/liquid ratio. A temperature-controllable oven (Memmert, Germany) was used to conduct the hydrolysis process. The operating temperature of the hydrolysis was varied between 80 and 120 °C and samples were collected at various time intervals in the range of 10 to 30 min (Rahman *et al*., 2006).

Upon completion of reaction, the solids were separated from the aqueous solution by filtration. The liquid phase was analysed for glucose concentration using the glucose analyser. The water-insoluble solids (WIS) fraction was collected, washed with distilled water until no traces of acid could be detected and then dried in an oven at 50 ºC until constant weight. This pretreated WIS was used as the substrate in the subsequent enzymatic hydrolysis experiments.

3.7.2 Enzymatic Hydrolysis

The pretreated WIS fraction of EFB was enzymatically hydrolysed in a 20 mL Universal Bottles using the commercial cellulase enzyme, Celluclast®. Enzyme loading was maintained at 30 filter paper units (FPU) per gram substrate as illustrated in the previous study (Zhong *et al.*, 2007). Fungal *β*-glucosidase (Novozym 188) was added at 25 Units per gram substrate to supplement the cellobiase activity on the degradation of cellulose.

Enzymatic hydrolysis was performed in 5 mL of 0.1 M sodium citrate buffer (pH 4.8) at 50 °C and was shaked at 120 rpm using the waterbath shaker (Wisebath, Daihan Scientific Co. Ltd., Korea). The effects of substrate concentration (5 to 20 %w/v) and hydrolysis time (24 to 72 hrs) on the enzymatic hydrolysis of oil palm EFB were studied as proposed by the RSM design of experiment.

The 0.1 M sodium citrate buffer (pH 4.8) was prepared by dissolving 8.03 g of citric acid and 17.12 g sodium citrate in 1 L distilled water.

3.8 Analysis of the Experiment

The glucose yield results obtained from the 27 sets of experiments were analysed using RSM. The experimental results were input into the Design Expert software. Simulated results were then output by the software.

RSM is a sequential procedure. Usually, a low-order polynomial in some region of the independent variables is employed. If the response is well modeled by a linear function of the independent variables, then the approximating function is the first-order model as shown in Equation 3.7.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 \qquad \text{Eq (3.7)}$$

If there is curvature in the system or model, then a polynomial of higher degree must be used, such as the second-order model (Montgometry, 1997) as shown in Equation 3.8.

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 \\ & + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{45} X_4 X_5 + \beta_{11} X_1^{\,2} + \beta_{22} X_2^{\,2} \\ & + \beta_{33} X_3^{\,2} + \beta_{44} X_4^{\,2} + \beta_{55} X_5^{\,2} \end{aligned} \qquad \text{Eq. (3.8)}$$

where Y represents response variable (glucose yield), β_0 is interception coefficient, β_1, β_2, β_3, β_4 and β_5 are linear terms, β_{11}, β_{22}, $\beta_{33,}\beta_{44}$ and β_{55} are quadratic terms and X_1, X_2, $X_{3,}$ $X_{4,}$ and X_5 are independent variables studied (pretreatment temperature, acid concentration, pretreatment reaction time, enzymatic substrate concentration and enzymatic reaction time).

At the end, mathematical and statistical tools of RSM were then employed to verify the significance of the model.

3.9 Optimisation and Validation of the Results

The strategy of RSM contains four steps in optimising the variable conditions. Firstly, RSM procedure was designed to move into the optimum region. Then, RSM determined the behavior of the response in the optimum region; this was followed by the estimation of the optimum conditions of the process, and lastly followed by the verification step (Tanyildizi *et al*., 2005).

Following the design and analysis of the experiment, the optimised experiment protocols were proposed by the software. The five optimised condition of the variables were provided. The maximum yield of glucose can be obtained through the experimental conditions provided by the protocol. The experiments were conducted according to the protocol proposed. The results of the experiments were compared with the proposed yield to verify the significance of the model.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The total solids content and the composition of oil palm empty fruit bunch (EFB) were firstly discussed in Section 4.2 and Section 4.3. After that, the experimental design and analysis were carried out using Design Expert v.7.1.5 software (Stat-Ease Inc. Minneapolis MN, USA) as reported in Section 4.4 and subsections. The results of the experimental runs were analysed and the significance of each variable in the model was investigated through the ANOVA and the diagnostic plots. A predictive model was then obtained.

The optimisation points proposed by the software are useful in predicting the highest yield of glucose. Validation of the point is important to verify the significance of the model.

4.2 Total Solids Content of EFB

Analysis of total solids content of EFB was carried out following the National Renewable Energy Laboratory standard method (NREL,1996). Biomass can rapidly gain or lose moisture when in contact with the air. Hence the analysis of solids content was conducted as soon as samples were taken. From the analysis, the total solids content of EFB was found to be 48.66 %.

The moisture content of the sample can be calculated based on the total solids content according to Equation 3.2. It is a measure of the amount of water and other volatile component (volatile at 105 ºC) present in the sample. The total moisture content of EFB was found to be 51.34 %. It was likely the steam used in the sterilisation process during the processing of palm oil has resulted in the high moisture content in the EFB, which makes it unsuitable as fuel (Prasertsan and Prasertsan, 1996).

EFB is a type of biomass that comprises of hygroscopic materials which contain a large amount of moisture. Chemical analyses of all lignocellulosic biomass are typically reported based on dry weight basis to avoid bias. Therefore, the determination of the amount of solids present in the solid EFB is important. The analyses of the EFB composition were determined based on the total solids content in the EFB, hence was regardless of the water content in the EFB.

4.3 Composition of EFB

The main composition of the empty fruit bunch (EFB) is shown in Table 4.1. The composition analyses of EFB were carried out using quantitative acid hydrolysis method following the NREL standard method (1996) based on the total solids content of EFB.

Table 4.1: Main composition of oil palm empty fruit bunch

Main Composition (%)	**This Study**	**Rahman *et al.*, 2006**	**Std Dev**
Glucan	40.66	44.76	2.90
Xylan	24.95	22.10	2.02
Acid Insoluble Lignin (AIL)	10.71	11.70	0.70
Acid soluble Lignin (ASL)	2.24	Not determined	-
Ash	0.56	0.52	0.03
Others	20.88	20.92	0.03

The composition analyses of the EFB obtained from this study was comparable to that obtained by Rahman *et al.* (2006) which has used another standard method developed by Browning (1967). As shown by both study, glucan was found to be the main component in EFB. Glucose will be the product following the degradation of glucan from the cellulose of EFB. In the NREL method (1996), after the EFB is treated with concentrated acid, cellulose will be broken down to its subunit of glucose. Hence, it is assumed in this method that the glucose composition represents the cellulose component in the EFB. The cellulose content was found to be 40.66 % in this study. It is assumed that the cellulose was not further degraded to other byproducts. It is

important to fully utilise the major component of the EFB so that most component would be converted to become fermentable sugar in the bioethanol production.

Following the analysis of glucan in the EFB, 40.66 % of glucan in the EFB is assumed to be convertable to glucose. Hence, this glucose value will be used as the basis for the calculation of glucose yield following the hydrolysis processes, namely both the acid pretreatment and enzymatic treatment.

The EFB contained 24.95 % of xylan. It is the second major component in the EFB. Xylose is the main component derived from hemicellulose. Hemicellulose can be easily degraded to its monomeric sugar by dilute acid hydrolysis. Whereby, the total lignin content of EFB was 12.95 %. The ash content of EFB was measured as 0.56 %. Ash is the remaining solid of biomass after igniting at high temperature, 575 °C. Other components in EFB may include the other monomeric sugar derived from hemicellulose degradation and other extractives present in cellulosic biomass.

The composition analyses of EFB obtained from this study were very comparable to those obtained by Rahman *et al.* despite both studies used different standard method for the analyses.

4.4 Experimental Design and Statistical Analysis

The experimental design and analysis was carried out using Design Expert v.7.1.5 (Stat-Ease Inc. Minneapolis MN, USA) to study the effect of pretreatment temperature (X_1), acid concentration (X_2) and pretreatment reaction time (X_3) during the dilute acid hydrolysis pretreatment and enzymatic substrate concentration (X_4) and

enzymatic reaction time (X_5) during the enzymatic hydrolysis treatment for the conversion of EFB to glucose. Subsequently, the optimal factors of the above variables were investigated to obtain the maximum glucose yield from EFB.

The optimisation using statistical approach involved four major steps namely the selection of design of experiments, estimation of coefficients based on the mathematical model and prediction of the responses and finally the confirmation or validation of model adequacy (Tanyildizi *et al*., 2005).

4.4.1 Experimental Design

The experimental design applied in this study was a half fraction 2^5 factorial design. Table 4.2 shows the experimental range and levels of independent process variables for the experiments conducted in this study. There were five independent variables; X_1, X_2, X_3, X_4 and X_5. These factors were optimised using RSM to provide the maximum or optimised yield of glucose. A central composite design was employed to search for the optimal value of the 5 significant components. In order to ensure the design is rotatable, star points were set at +/- alpha (α) value of 2 in the design. The value for α is calculated in the design for both rotatability and orthogonality of blocks.

Table 4.2: Experimental range and levels of independent process variables

Independent Variables	**Symbol**	**Range and Levels**				
		-α	**-1**	**0**	**+1**	**+α**
Pretreatment Temperature (°C)	X_1	60	80	100	120	140
Acid Concentration (%w/w)	X_2	0	2	4	6	8
Pretreatment Reaction Time (min)	X_3	0	10	20	30	40
Enzymatic Substrate Concentration (%w/v)	X_4	0	5	10	15	20
Enzymatic Reaction Time (hrs)	X_5	0	24	48	72	96

Following the experimental design as proposed in Table 4.2, it translated into the experimentation protocol as summarised in Table 3.3 (Chapter 3). These experiments were then conducted to obtain the corresponding glucose yields as would be expressed as Response, glucose yield, *Y* (%).

4.4.2 Experimentation

Twenty seven set experiments as proposed by the Design Expert software were conducted according to the method as described by Section 3.7.1 and 3.7.2. The experimental design matrix is shown in Table 3.3, Chapter 3. The results obtained from these experiments are tabulated and reported in Table 4.3. Results are reported as Response *Y* (%) which represents the glucose yields obtained from different set of experiments. Yields were calculated based on the glucose concentration (in $mg.mL^{-1}$) measured following the combined hydrolysis processes and calculated based on Equation 3.3.

Table 4.3: Experimental designs and the summary of glucose yield results obtained from the combined acid pretreatment and enzymatic hydrolysis treatment

Runs	Variables					Response Y (%)
	X_1	X_2	X_3	X_4	X_5	
1	-1	-1	-1	-1	1	25.73
2	-1	-1	-1	1	-1	16.89
3	-1	1	-1	-1	-1	21.89
4	-1	1	-1	1	1	22.46
5	-1	-1	1	-1	-1	19.97
6	-1	-1	1	1	1	19.51
7	-1	1	1	-1	1	26.32
8	-1	1	1	1	-1	16.18
9	1	-1	-1	-1	-1	20.51
10	1	-1	-1	1	1	27.87
11	1	1	-1	-1	1	39.35
12	1	1	-1	1	-1	28.86
13	1	-1	1	-1	1	46.78
14	1	-1	1	1	-1	29.02
15	1	1	1	-1	-1	42.30
16	1	1	1	1	1	42.14
17	-2	0	0	0	0	28.53
18	2	0	0	0	0	46.97
19	0	0	-2	0	0	14.02
20	0	0	2	0	0	38.86
21	0	-2	0	0	0	15.32
22	0	2	0	0	0	27.79
23	0	0	0	-2	0	0.30
24	0	0	0	2	0	24.59
25	0	0	0	0	-2	0.33
26	0	0	0	0	2	30.74
27	0	0	0	0	0	24.84

* ± 2=± α

These results were then further analysed by RSM to provide the proposed optimal point with the corresponding optimised parameter values (X_1 to X_5) that would provide optimised glucose yield.

4.4.3 Experimental Results Analysis

Based on the experimentation matrix for the design and the experimentation results of glucose yields, Y (%) as outlined in Table 4.3, central composite design and response surface methodology were employed to analyse the interaction between the variables and the responses.

Further data analysis using RSM was conducted to determine the suitable model that best fit the experiment data. Table 4.4 shows the sum of square (SS) of the RSM sequential model. The corresponding statistical analyses for each model were also presented in Table 4.4.

Table 4.4: Sequential model sum of square

Source	Sum of Squares	Degree of Freedom	Mean Square	*F* Value	*p*-value
Mean	18049.22	1	18049.22		
Linear	1897.03	5	379.41	4.44	0.0065
2FI	243.88	10	24.39	0.17	0.9951
Quadratic	845.50	5	169.10	1.44	0.3322
Cubic	592.04	5	118.41	1.05	0.6266
Residual	113.02	1	113.02		
Total	21740.68	27	805.21		

The selection of a best model lies mainly on the *p*-value. If the *p*-value is very small (less than 0.05), then the factors (variables) in the model have significant effects on the responses. If more than one model shows the significant effect, then the model with the highest order of polynomial will be selected. From Table 4.4, only the linear model was found to be significant with *p*-value of 0.0065 (< 0.05). *p*-value or significance probability is the probability of obtaining a value for a statistic test that is as extreme as or more extreme than the observed value, assuming the null hypothesis is true means that there is no effect of the factor (Mason *et al*., 2003). More formally, the *p*-value is the smallest level of significance that would lead to rejection of the null hypothesis. It is customary to call the test statistic and the data significant when the null hypothesis is rejected (Montgometry, 1997). Small probability values call for rejection of the null hypothesis. The probability equals the proportion of the area under the curve of the *F*-distribution that lies beyond the observed *F* value. The *F* distribution itself is determined by the degrees of freedom associated with the variances being compared. It is a measurement of variance of data about the mean, based on the ratio of mean square of group variance due to error (Tabachnick *et al.*, 2007).

Table 4.5 shows more statistical analyses on the model. The model that has the maximised value of the adjusted R-squared and the predicted R-squared would be selected. Consistent to the result as indicated by the p-value, the linear model was found to have the largest value of adjusted R-squared (0.3982) and the predicted R-squared (0.2090).

Table 4.5 Statistics summary of the models

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS
Linear	9.24	0.5139	0.3982	0.2090	2919.92
2FI	11.87	0.5800	0.0072	-3.7701	17608.55
Quadratic	10.84	0.8090	0.1723	-4.2812	19495.42
Cubic	10.63	0.9694	0.2039	-39.9206	151056.80

R-squared (R^2) is a measure of the amount of reduction in the variability of response (Y) obtained using the regressor variables X_1, X_2, …., X_q in the model. However, a large value of R^2 (~1) does not necessarily imply that the model is a good one. Adding a variable to the model will always increase R^2, regardless of whether the additional variable is statistically significance or not. Thus, it is possible for models that have large values of R^2 to yield poor predictions of new observation of the mean response (Montgometry, 1997). Equation 4.3 shows the calculation for R^2. As R^2 always increase as more variables are added, so adjusted R^2 (R^2_{adj}) was chosen as a better parameter to evaluate the model.

$$R^2 = 1 - \frac{SS_{Residual}}{SS_{Total}} \qquad \text{Eq. (4.3)}$$

R^2_{adj}, as can be calculated by Equation 4.4, it is a measure of the amount of variation around the mean explained by the model. It is adjusted for the number of variables in the model. In general, the R^2_{adj} will not always increase as variables are added to the model. In fact, if unnecessary variables are added, the value of R^2_{adj} will often decrease (Montgometry, 1997).

$$R^2_{adj} = 1 - \frac{SS_{Residual} / df_{residual}}{SS_{Total} / df_{total}} = 1 - \left(\frac{df_{total}}{df_{residual}} \right)(1 - R^2) \qquad \text{Eq. (4.4)}$$

The predicted R-squared ($R^2_{prediction}$) estimates the amount of variation in the new data as explained by the model. $R^2_{prediction}$ can be calculated by Equation 4.5. A negative value of $R^2_{prediction}$ is undesirable as it suggests that the model consists of only the intercept that is a better predictor of the response than the variables of this model. The model that has the highest value of R^2_{adj} and $R^2_{prediction}$ would be selected.

$$R^2_{prediction} = 1 - \left(\frac{PRESS}{SS_{Total}} \right) \qquad \text{Eq. (4.5)}$$

The PRESS is the predicted residual error sum of squares. It provides a useful residual scaling which indicates how well the model fits the data (Montgometry, 1997). The PRESS is computed by first predicting each point to be from a model that contains all other points except the one in question. The squared residuals, which are the difference between the actual and predicted values, are then summed. The PRESS of the chosen model should be the smallest relative to the other models under consideration.

Based on these evaluating criteria, and from both the data analysis as summarised in Table 4.4 and 4.5, the linear model was found to be the best model that fit well with the experimental values and hence was suggested for the further analysis during the optimisation study.

4.4.4 ANOVA for Response Surface Linear Model

Apart of the statistical analyses as discussed in Section 4.4.3, the analysis of variance (ANOVA) was further conducted to check the fitness of the linear model with respect to each variable (X_1, X_2, X_3, X_4 and X_5) and the overall fitness of these variables.

ANOVA is an arithmetic procedure that tests the statistical significance of the mean differences and central tendency among different groups of scores (i.e. the experiment runs). The different groups of scores may correspond to different levels of a single experimental factor or to different combination of levels of two or more factors (Tabachnick, 2007). ANOVA separates or partitions the variation observable in a response variable into two basic components which are variation due to assignable causes and to uncontrolled or random variation. Assignable causes refer to known or suspected sources of variations from variates that are controlled (experimental factors) or measured (covariates) during the conduct of an experiment. Whereby, random variation includes the effects of all other sources not controlled or measured during the experiment (for example, measurement errors) (Mason *et al*., 2003). The curve fitting between the experimental results and the model was evaluated by regression analysis and the ANOVA analysis as shown in Table 4.6.

Table 4.6: The results of curve fitting between the model and experimental data evaluated by regression and ANOVA analysis

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value
Model	1897.03	5	379.41	4.44	0.0065
X_1	*873.31*	*1*	*873.31*	*10.22*	*0.0043*
X_2	*140.91*	*1*	*140.91*	*1.65*	*0.2131*
X_3	*325.15*	*1*	*325.15*	*3.81*	*0.0646*
X_4	*3.13*	*1*	*3.13*	*0.04*	*0.8501*
X_5	*554.54*	*1*	*554.54*	*6.49*	*0.0187*
Residual	1794.43	21	85.45	-	-
Total	3691.46	26	-	-	-

Based on Table 4.6, the first step in ANOVA was to define a suitable measure of variation which involved partitioning into components, due to assignable causes and to random variation, which can be accomplished. While they are many measures that could be used, the numerator of the sample variances is used for a variety of computational and theoretical reasons. This measure of variability is referred to as the total sum of squares (SS_T), from Table 4.6, SS_T is reported as 3691.46 as calculated from Equation 4.6 (Mason *et al*., 2003).

$$\text{Total Sum of Squares } (SS_T) = \text{Model Sum of Square} + SS_{Error}$$
$$= SS_{X_1} + SS_{X_2} + SS_{X_3} + SS_{X_4} + SS_{X_5} + SS_{Residual} \qquad \text{Eq. (4.6)}$$

where SS_{X_1}, SS_{X_2}, SS_{X_3}, SS_{X_4} and SS_{X_5} represent the individual sum of square of the five experimental factors that contributed to the total sum of squares. SS_{Error} or $SS_{Residual}$ represents the sum of square of error or residual which also contributed to the summation of SS_T.

From Equation 4.6, SS_T can be partitioned into components for the assignable causes (factor X_1, X_2, X_3, X_4 and X_5) and for random variation (uncontrolled experimental, residual or error). It is important to note that the total variance in the set of scores is partitioned into two sources, the experimental factors and error that may occur during the experiment runs. In this sense, it is known as an analysis of variance. As the effects of the individual factors are assessed by changes in the central tendencies of the groups, the interferences arose from ANOVA are about the differences in the central tendency. However, the sum of squares (SS) are not yet the variances. To become variances, they must be averaged. The denominators for averaging SS in ANOVA are called the degrees of freedom (df). df is partitioned following the same manner as the SS. The total degrees of freedom are the total number of the experimental runs, *N* minus 1. One degree was lost when the grand mean is estimated (Tabachnick, 2007). In this study, *N* equals to 27, therefore, there was a total df of 26 (27 – 1).

Variance is an 'averaged' sum of squares. Division of a SS by df produces variance, which is known as mean square (MS) in ANOVA. ANOVA produces three variances: one associates with the total variability among scores (MS_T); one with variability between groups or factors (MS_A); and one with variability within groups (residuals) (MS_E). The equations for mean squares are shown in Equation 4.7 (Tabachnick, 2007):

$$MS_T = \frac{SS_T}{df_T} \text{ or } MS_A = \frac{SS_A}{df_A} \text{ or } MS_E = \frac{SS_E}{df_E} \qquad \text{Eq. (4.7)}$$

MS_T is the total variance for the entire experiment runs across all groups. Therefore, it is generally not a useful quantity for ANOVA. The *F* distribution is a sampling distribution of the ratio of two variances, namely the MS_A and MS_E. *F*-value

is a measurement of variance of data about the mean, based on the ratio of mean square of the group variance (MS_A) to that due to error (MS_E) as indicated by Equation 4.8.

$$F\text{-value} = \frac{MS_A}{MS_E} \qquad \text{Eq. (4.8)}$$

In ANOVA, *F* ratio tests the null hypothesis by comparing the model variance to the residual (error) variance (Tabachnick, 2007). If the variances are close to each other, the *F*-value will be close to one and it is less likely that any of the factors have a significant effect on the response. It means that the null hypothesis is true. Once *F*-value is obtained, it is tested against the critical $F_{(\alpha,a-1,N-a)}$, value obtained from the *F* distribution table, with numerator df = $a - 1$ (df of residual) and denominator df = $N - a$ (df of model) at the desired alpha level (95 % or 0.05). If the *F*-value obtained is equal to or larger than the critical *F*, it means that the null hypothesis is rejected. There is a difference among the means in the groups. Any increased in the *F*-value would increases the probability of rejecting a false null hypothesis. Equation 4.8 indicates that *F*-value can be increased by an increase value in the numerator (MS_A) or by a decrease value in the denominator (MS_E) (Tabachnick, 2007).

In principle, *F*-value should be greater than the tabulated critical $F_{(\alpha,a-1,N-a)}$-value to ensure the model is a good prediction of the experiment results. From the ANOVA table, the *F*-value (4.44) was higher than the tabulated *F*-value ($F_{0.05,\,21,\,5}$ = 2.68). Therefore, it shows that the model is significant relative to the error.

Another way of testing the hypothesis of the experiment was by evaluating the *p*-value. One way to report the results of a hypothesis test is to state that the null hypothesis was or was not rejected at a spesific α-value or level of significance. *p*-value

is usually obtained via the use of statistical software. By tradition, a *p*-value of less than 0.05 are termed statistically significant, and those less than 0.01 are termed highly statistically significant (Oehlert, 2000). In this study, the *p*-value for the model was 0.0065. It means that there was only a 0.65 % of probability that a "Model *F*-value" was rejected due to errors. Variables that have the *p*-value of less than 0.05 indicate that the model terms are significant. The regression result was set at a confidence level of 95.

In addition, the significance of each coefficient was determined using the *p*-value. The statistical significance (*p*-value) of a result is an estimated measure of the degree to which it is "true". The lower *p*-level verify that the more significant of the observed relation between variables in the sample. Specifically, the *p*-value represents the probability of error that is involved in accepting the observed result as valid. From Table 4.6, the largest effects on the response or the glucose yield were found to be the linear term of pretreatment temperature (X_1) and enzymatic reaction time (X_5). Each of this linear term has the *p*-value of <0.05. However, for other terms where their *p*-values were greater than 0.05, it indicated that their confidence levels were probably <95%.

Table 4.7 shows the R-squared (R^2) table from the ANOVA. As discussed in Section 4.4.3, the regular R^2 can be artificially inflated by simply continuing to add terms to the model, even if the terms are not statistically significant. Therefore, the adjusted R-squared (R^2_{adj}) and predicted R-squared ($R^2_{prediction}$) values are more valuable in analysing the significance of the model. The R^2_{adj} basically plateaus when insignificant terms are added to the model, while the $R^2_{prediction}$ will decrease when there are too many insignificant terms.

Table 4.7: The R-squared table from ANOVA

Std. Dev.	9.24	**R-Squared**	0.5139
Mean	25.86	**Adj R-Squared**	0.3982
C.V. %	35.75	**Pred R-Squared**	0.2090
PRESS	2919.92	**Adeq Precision**	7.776

Theoretically, the R^2 values close to 1.0 indicate a perfect fit, a value lower than 1.0 indicates a lower degree of model fitting. However, there is no "cut-off" value applied for the R^2. In other words, if the R^2 value is much lower than 1.0, the model would not be rejected. The more important evaluation parameters lie on the statistical significance of the model.

The linear model selected was proven to be statistically significant, the R^2 value was rather low (0.5139), both the adjusted and predicted *R*-squared values of the model were also substantially low, namely 0.3982 and 0.2090 respectively. $R^2_{\text{prediction}}$ of 0.2090 was however in reasonable agreement with the R^2_{adj} of 0.3982. The lower values of R^2_{adj} and $R^2_{\text{prediction}}$ were not unexpected as the linear only contains two factors (X_1 and X_5) that were significant. According to Montgometry (1997), if unnecessary variables are added, the value of R^2_{adj} will often decrease

The adeq. precision from Table 4.7 measures the signal to noise ratio. It compares the range of the predicted values at the design points to the average prediction error. A ratio greater than 4 indicates adequate model discrimination and is desirable. The value of 7.776 in this analysis indicates an adequate signal. This model can be used to navigate the design space.

Std. Dev. estimation is the square root of the error mean square, $\sqrt{85.45} = 9.24$. It can be considered as an estimate of the standard deviation associated with the experiment. Whereby, C. V. is the coefficient of variance, it is defined as the standard of deviation expressed as a percentage of the mean. It was calculated by dividing the Std. Dev. by the Mean and multiplying by 100. The coefficient of variance measures the unexplained or residuals variability in the data as a percentage of the mean of the response variable (Montgometry, 1997). The C. V. of this design is 35.75 %.

Table 4.8 presents the post ANOVA of the regression results and the significance of regression coefficients of the glucose yield model. Coefficient estimate is the regression coefficient representing the expected change in response *Y* per unit change in each factor *X* when all other remaining factors are held constant. It is important for the determination of the mathematical regression coded model equation later. Standard error is the standard deviation associated with the coefficient estimate.

Table 4.8: Post ANOVA of regression results and the significance effect on regression coefficient for response (glucose yield)

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	25.86	1	1.78	22.16	29.55	
X_1	6.03	1	1.89	2.11	9.96	1.00
X_2	2.42	1	1.89	-1.50	6.35	1.00
X_3	3.68	1	1.89	-0.24	7.60	1.00
X_4	0.36	1	1.89	-3.56	4.29	1.00
X_5	4.81	1	1.89	0.88	8.73	1.00

The two columns of 95 % CI Low and 95 % CI High value represents the range that the true coefficient should be found within 95% of the time. If one the limit of 95 % CI Low and High is positive and the other is negative, then the null hypothesis could be true. It indicates that the factor has no effect to the model. From Table 4.8, the 95 % CI Low value of X_2, X_3 and X_4 were negative value (-1.50, -0.24 and -3.56 respectively) and the 95 % CI High value for these three factors were positive value (6.35, 7.60 and 4.29 respectively). It indicates these three factors were not affecting the model's yield. Besides, both of the 95 % CI Low and 95 % CI High value of factors X_1 and X_5 were positive value. It indicates these two factors will affect the model's yield. The results of 95 % CI Low and 95 % CI High value shows the same result as the *p*-value that only X_1 and X_5 are significant factors in the model analysis of glucose yield.

The variance inflation factor (VIF) measures how much the variance of the model was inflated by the lack of orthogonality in the design. The VIF value of 1 indicates that the factor is orthogonal to all other factors in the model. The values greater than 1 indicate that the factors are too correlated together and mean they are not independent. From Table 4.8, the VIF of all factors in the model were 1. It means that each of the factors are orthogonal to all others factors in the model.

Based on the Coefficient Estimate as tabulated in Table 4.8, the predictive model can now be derived and as shown in Equation 4.9.

$$Y = 25.86 + 6.03X_1 + 2.42X_2 + 3.68X_3 + 0.36X_4 + 4.81X_5 \qquad \text{Eq (4.9)}$$

where Y, the glucose yield, is expressed as a function of the pretreatment temperature (X_1), acid concentration (X_2), pretreatment reaction time (X_3), enzymatic substrate concentration (X_4) and enzymatic reaction time (X_5).

The predictive model is listed in coded terms (X_1, X_2, X_3 X_4 and X_5). The coded (or pseudo) equation is useful for identifying the relative significance of the factors by comparing the factor coefficients. This comparison cannot be made with the actual equation because the coefficients are scaled to accommodate the units of each factor. The equations give identical predictions. These equations, used for prediction, have no block effects. Blocking is a restriction on the randomisation of the experiment, used to reduce error. It is not a factor being studied. Therefore, blocks are only used to fit the observed experiments, not to make predictions.

4.4.5 Diagnostic Plots of Linear Model

The adequacy and validity of the design can be inspected using various diagnostic plot provided by Design Expert software. There are four main plots need to be focused on. The first one is the normal probability plot of the studentised residuals to check for normality of residuals. Then, the studentised residuals versus predicted values to check for constant error of the model design. After that, the externally studentised residuals plot to look for outliers or the influential values. Finally, the Box-Cox plot for power transformations.

The normal probability plot of the studentised residual is the most important diagnostic plot to determine the validity of design. Figure 4.1 shows the probability plot of the studentised residuals. The probability plot indicates whether the residuals follow a normal distribution. The data points should be approximately linear or follow a straight line and is expected that some moderate scatter was even with the normal data.

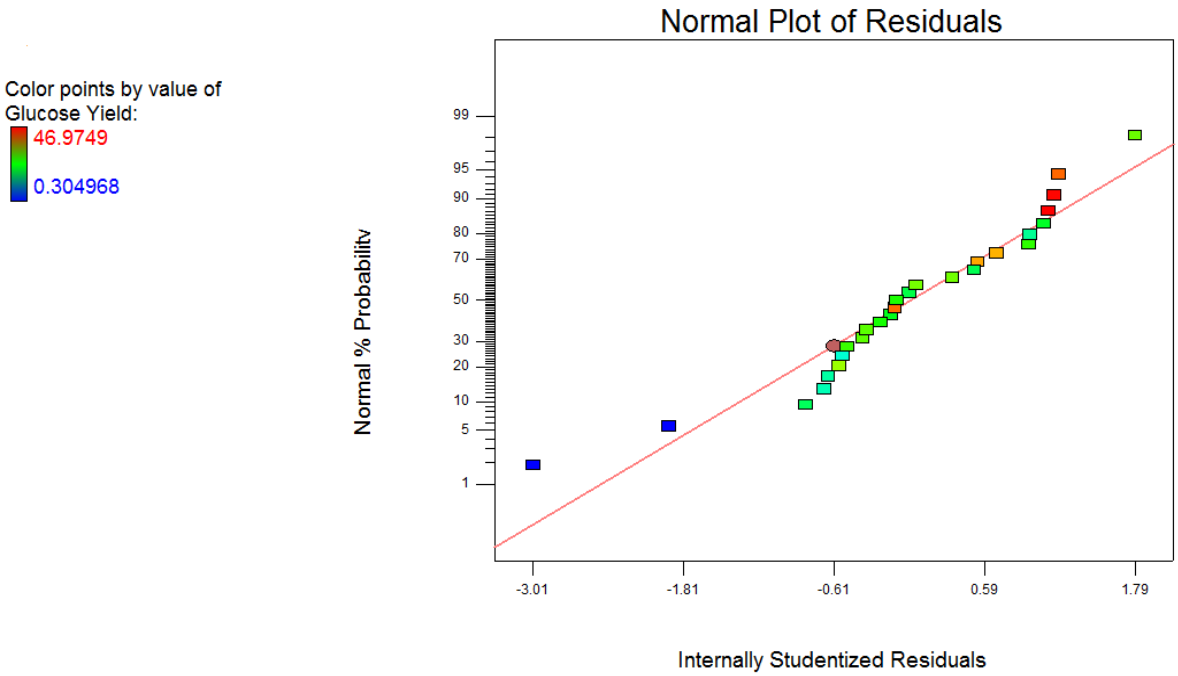

Figure 4.1: Normal probability plot of studentised residuals

A non-linear pattern indicates the non-normality in the error term, which may be corrected by a transformation. The plot in Figure 4.1 shows that the residuals are normally distributed along the line but with some data points scatter from the straight line. This can indicates the design is valid for estimation of glucose yield.

Another key diagnostic plot is the plot of residuals versus predicted values. This is a plot of the residuals versus the ascending predicted response values. The plot is used to test the assumption of constant variance. The plot should show a pattern of random scatter, and the points should be scattered inside the constant range of residuals across the graph. If megaphone pattern ("<") occur, this indicates the problem of non-constant variances of the residuals in the design and a transformation of the response is needed to improve the fit of model. Figure 4.2 shows that the points are randomly

scattered inside the constant range of residuals. This indicates that no transformation is needed.

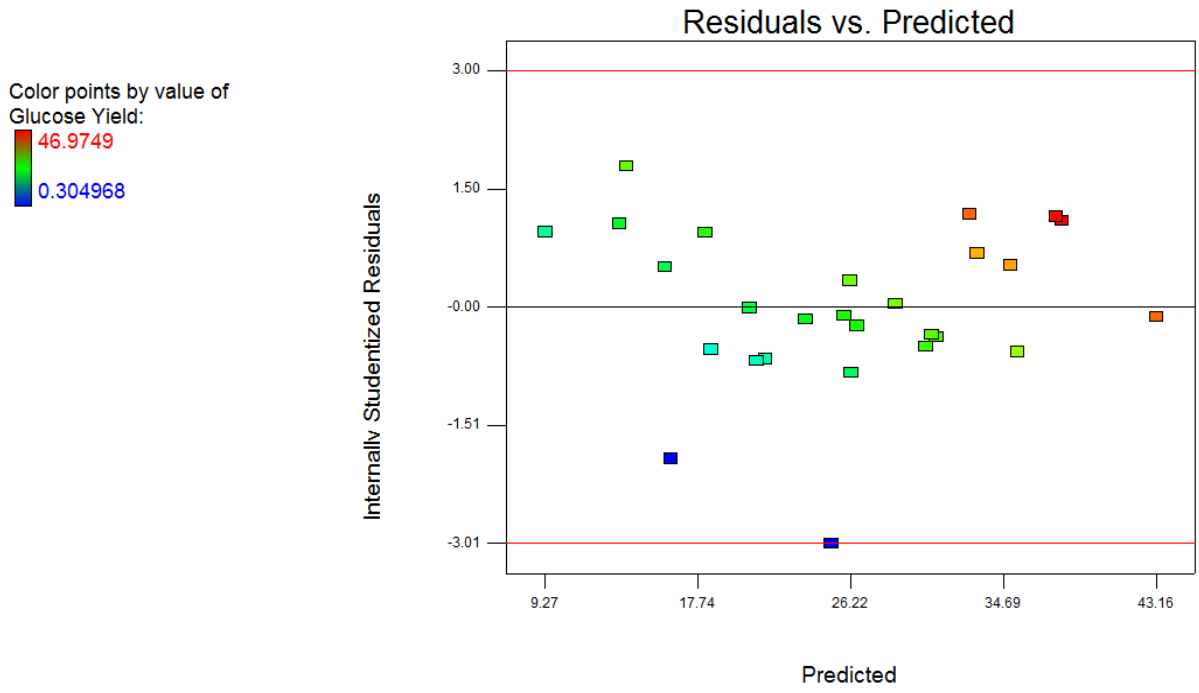

Figure 4.2: Plot of residuals versus predicted values

The externally studentised residual plot provides an easier way to identify abnormal (outlier) runs if any points stand out, which fall outside the red line (+3.58 and -3.58 standard deviation limits). These values provide measures of the influence, potential or actual, of an individual run. This graphical plot provides a better perspective on whether a case sticks out from the others. It shows how that data points from the runs fits in with the other points of this model.

The externally studentised residual is a measure of how much standard deviation the actual value deviates from the value predicted after deleting the point in question. Sometimes, The externally studentised residual is also called Outlier T, R-Student. Figure 4.3 represents the externally studentised residual plot. This plot shows that all

the points fall well within the limit range of standard deviation except one point from the run-23. This indicates no outliers or abnormal experiments were found in the design except for run-23. After repeating the experiment of run-23, no special cause was identified. Therefore, the data point remained in the data set.

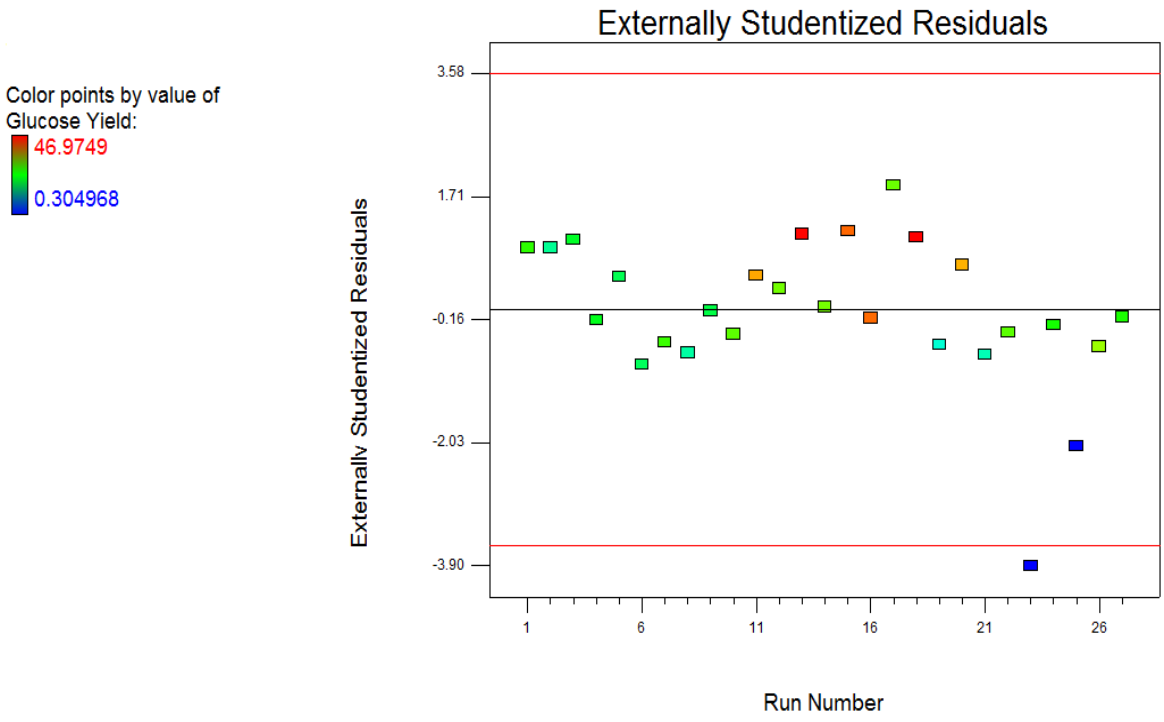

Figure 4.3: Externally studentised residuals plot

The last diagnostic plot is the Box-Cox plot as shown in Figure 4.4. The Box-Cox plot is a tool that provides a guideline for selecting the correct power law transformation for the response data. The red lines indicates the 95 % confidence limits. The lowest point on the Box Cox plot (blue line) represents the value of lambda (λ) that results in the minimum residual sum of squares in the transformed model. The potential for improvement is greatest when the range of the maximum to minimum response value is greater than 3. If the blue line points to a lambda value at 1, it symbolised no

transformation of the response. The green line indicates the best lambda value. A recommended transformation is listed based on the best lambda value, which is found at the minimum point of the curve generated by the natural log of the sum of squares of the residuals. If the 95 % confidence interval around this lambda includes 1, then no specific transformation is recommended by Design Expert software. The plot shows that the current lambda value of 1 fall fell within the 95 % confidence interval. Thus, no power transformation is recommended. The plot also shows the current lambda value (1) was very close to the best current value suggested (1.17). Thus, the non-transformed model is well accepted in good agreement.

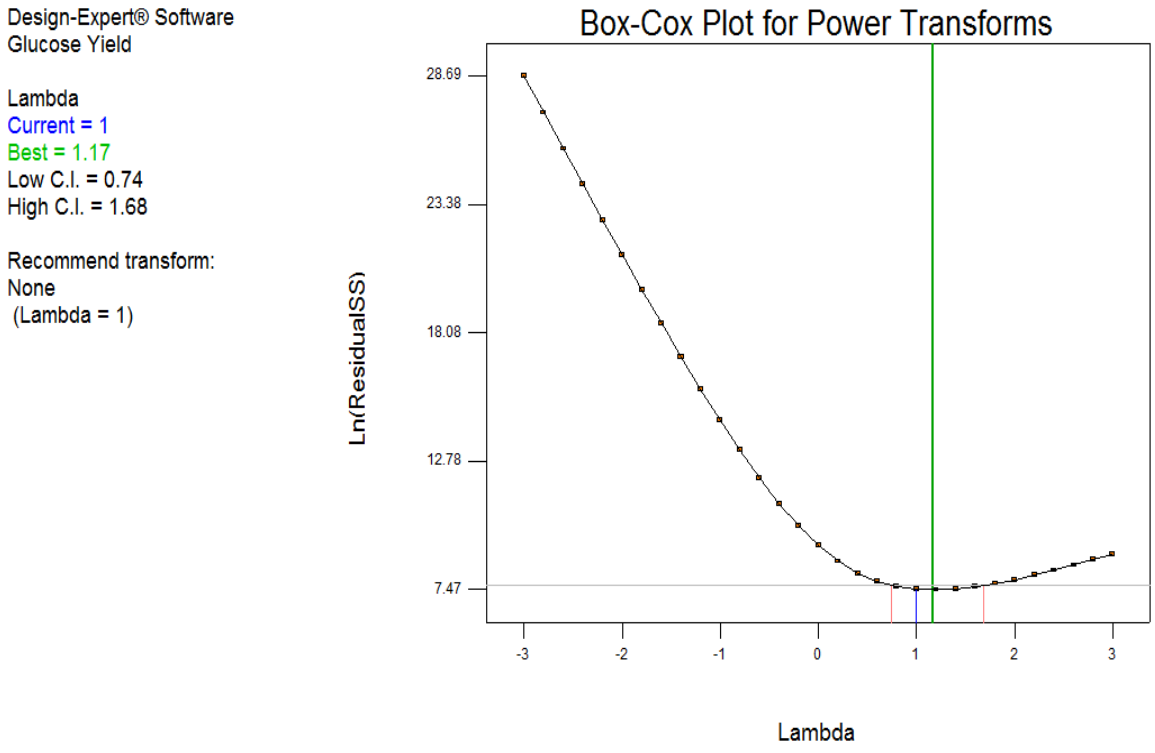

Figure 4.4: Box-Cox plot for power transformation

4.4.6 Graphs of LinearModel

The model graphs were used to intepret and evaluate the model. Estimation of the response of glucose yield with regards to the independent variables X_1, X_2, X_3, X_4 and X_5 in terms of response surfaces are shown in Figure 4.5 to 4.19. The figures show the one factor plot for representing the regression model of Equation 4.9. The figures illustrate the effect of pretreatment reaction temperature (X_1), acid concentration (X_2), pretreatment reaction time (X_3), enzymatic substrate concentration (X_4) and enzymatic reaction time (X_5) over the yield of glucose. Besides, three-dimensional surface and contour plot of the factors interaction will also be shown accordingly. A surface plot displays a three-dimensional view that may provide a clear picture of the response surface. Meanwhile, the contour plot is viewed as two-dimensional plane where all points that have the same response are connected to produce contour lines of constant responses.

The effect of the pretreatment reaction temperature (X_1) on response of glucose yield when acid concentration (X_2), pretreatment reaction time (X_3), enzymatic substrate concentration (X_4) and enzymatic reaction time (X_5) were selected at 4 %w/w, 20 min, 10 %w/v and 48 hrs respectively as the centre point is shown in Figure 4.5. From the figure, it can be interpreted that the maximum and minimum glucose yield of 31.89 % and 19.82 % can be obtained by conducting at 100 °C of pretreatment reaction temperature. It shows that the glucose yield increased with the increased of pretreatment temperature.

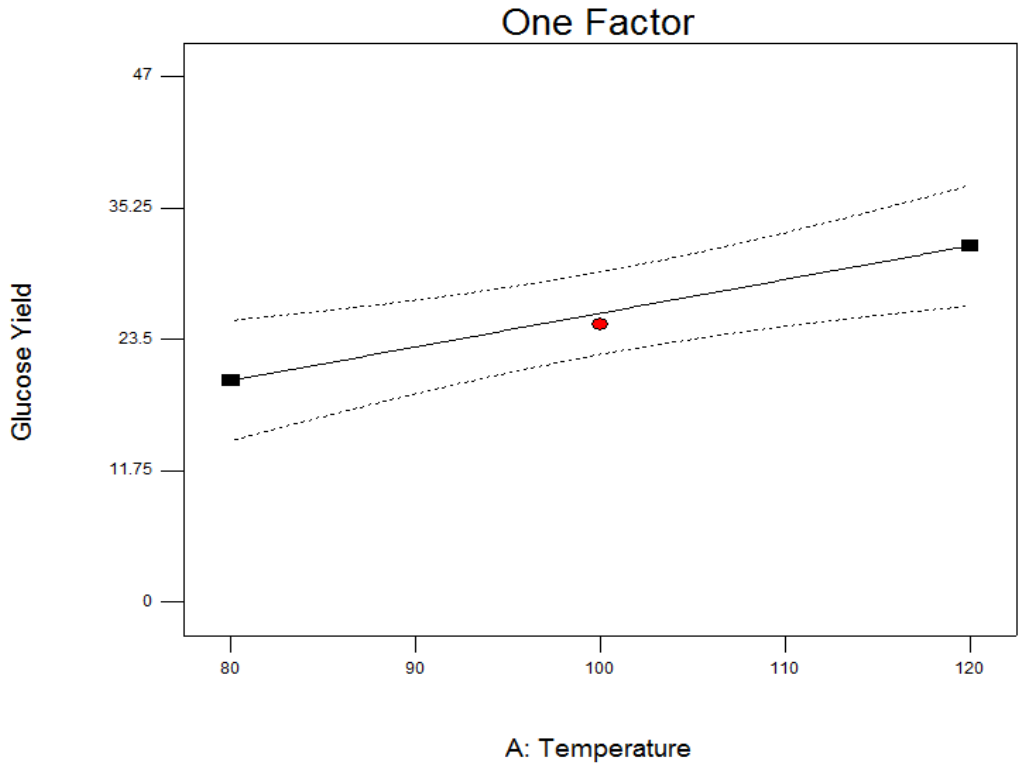

Figure 4.5: Effect of pretreatment temperature on glucose yield when acid concentration, pretreatment reaction time, enzymatic substrate concentration and enzymatic reaction time were selected at 4 %w/w, 20 min, 10 %w/v and 48 hrs respectively as the centre point

Figure 4.6 shows the effect of acid concentration on glucose yield when pretreatment temperature, pretreatment reaction time, enzymatic substrate concentration and enzymatic reaction time were selected at 100 °C, 20 min, 10 %w/v and 48 hrs respectively as the centre point. It can be interpreted that the increased in acid concentration slightly increased the glucose yield. As discussed previously in the ANOVA section (Section 4.4.4), the X_2, factor of acid concentration was not a significant factor in this experiment design. It is proven by Figure 4.6 which shows comparable maximum and minimum yields of glucose at 28.28 % and 23.43 %, respectively.

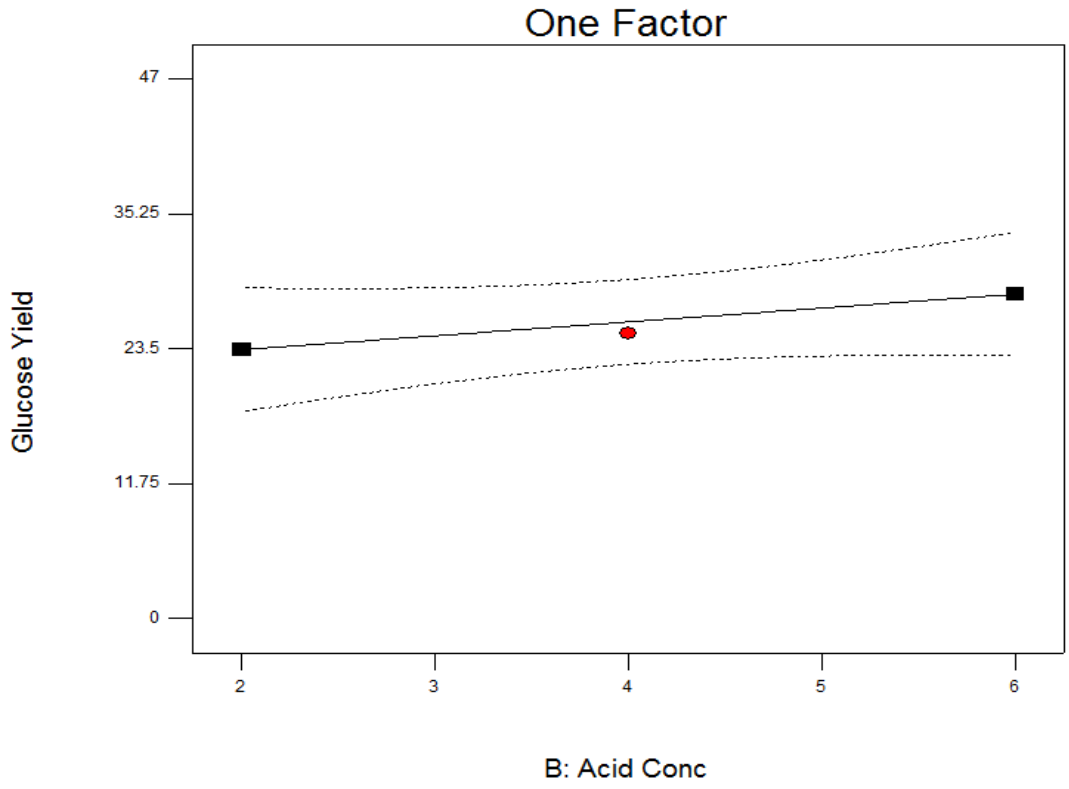

Figure 4.6: Effect of acid concentration on glucose yield when pretreatment temperature, pretreatment reaction time, enzymatic substrate concentration and enzymatic reaction time were selected at 100 °C, 20 min, 10 %w/v and 48 hrs respectively as the centre point

The response surface as a function of pretreatment temperature and acid concentration at pretreatment reaction time, enzymatic substrate concentration and enzymatic reaction time of 20 min, 10 %w/v and 48 hrs, respectively as centre point is shown in Figure 4.7. The corresponding contour plot of glucose yield is shown in Figure 4.8. From the figures, it can be interpreted that the yield of glucose increased with the increased in pretreatment temperature but inconsistence with the changes in acid concentration.

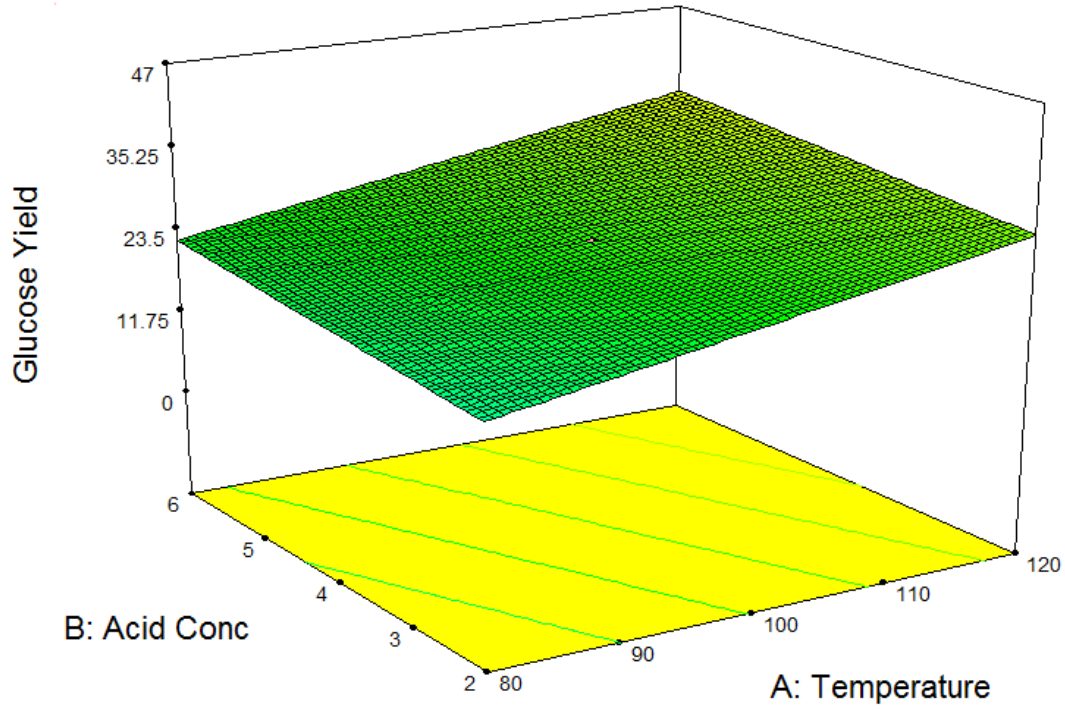

Figure 4.7: The response surface plot of glucose yield as a function of pretreatment temperature and acid concentration at pretreatment reaction time, enzymatic substrate concentration and enzymatic reaction time of 20 min, 10 %w/v and 48 hrs, respectively as the centre point

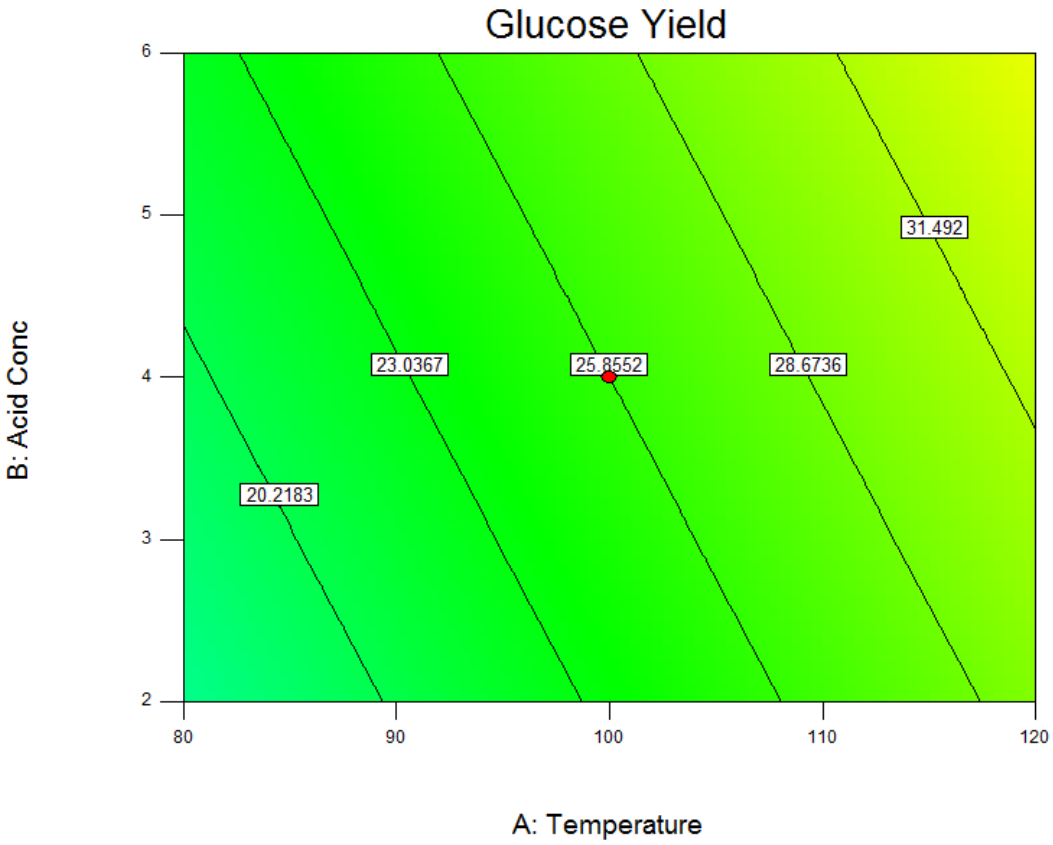

Figure 4.8: The response contour plot of glucose yield as a function of pretreatment temperature and acid concentration at pretreatment reaction time, enzymatic substrate concentration and enzymatic reaction time of 20 min, 10 %w/v and 48 hrs, respectively as the centre point

The effect of the pretreatment time on response of glucose yield when reaction temperature, acid concentration, enzymatic substrate concentration and enzymatic reaction time were selected at 100 °C, 4 %w/w, 10 %w/v and 48 hrs respectively as the centre point is shown in Figure 4.9. It can be interpreted that the increased in pretreatment time slightly increased the glucose yield. It shows the maximum and minimum yield of glucose at 29.54 % and 22.17 %, respectively.

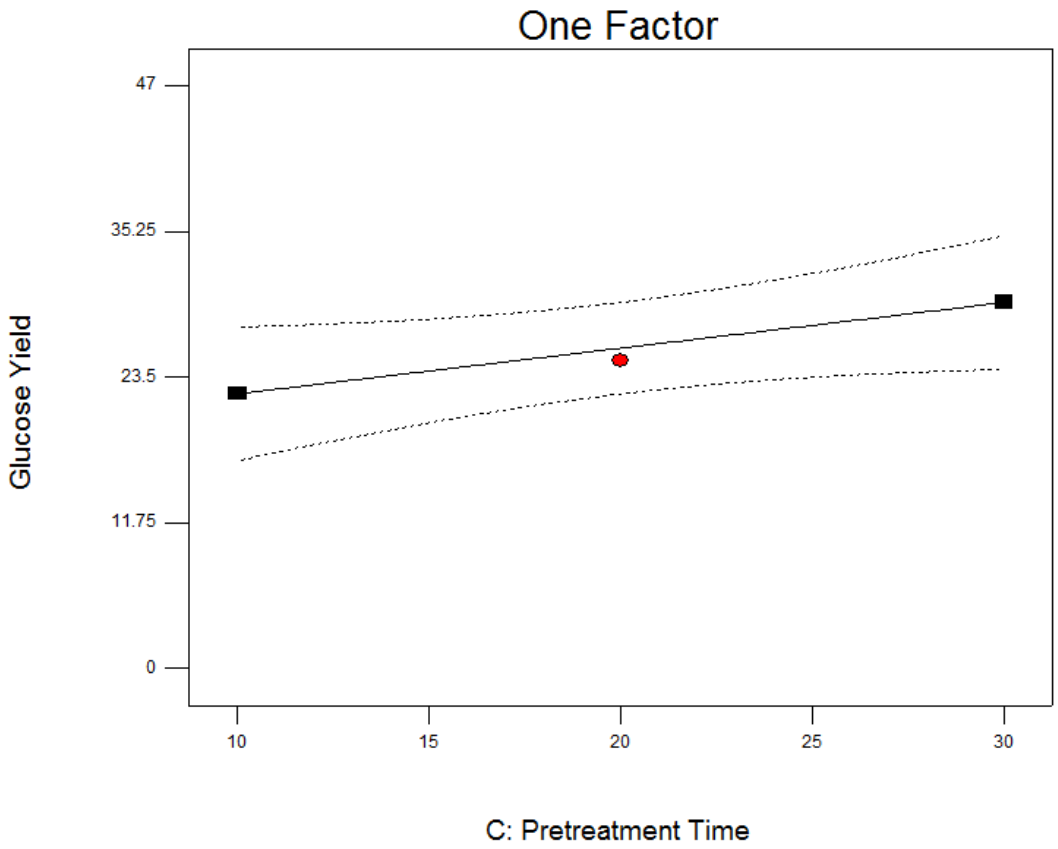

Figure 4.9: Effect of pretreatment reaction time on glucose yield when pretreatment temperature, acid concentration, enzymatic substrate concentration and enzymatic reaction time were selected at 100 °C, 4 %w/w, 10 %w/v and 48 hrs, respectively as the centre point

Figure 4.10 shows the response surface of glucose yield as a function of acid concentration and pretreatment reaction time at pretreatment temperature, enzymatic substrate concentration and enzymatic reaction time of 100 °C, 10 %w/v and 48 hrs, respectively as centre point. The corresponding contour plot is shown in Figure 4.11. From the figures, it can be interpreted that the increment in acid concentration and pretreatment reaction time showed no effect on the yield of glucose as the graphs show near to linear graph characteristic.

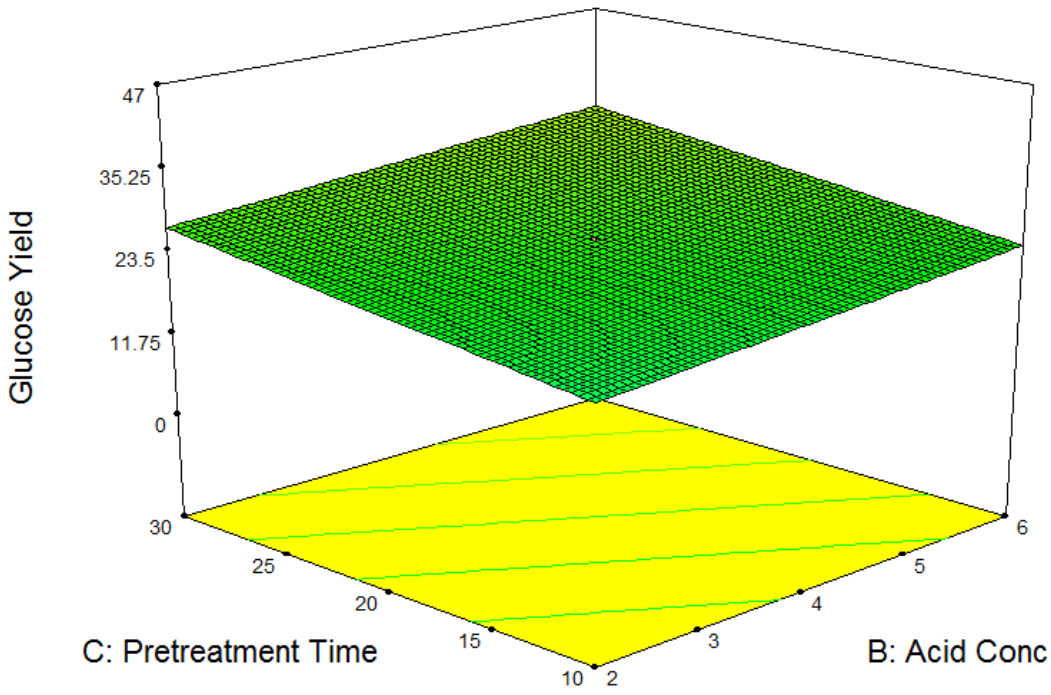

Figure 4.10: The response surface plot of glucose yield as a function of acid concentration and pretreatment reaction time at pretreatment temperature, enzymatic substrate concentration and enzymatic reaction time of 100 °C, 10 %w/v and 48 hrs, respectively as centre point

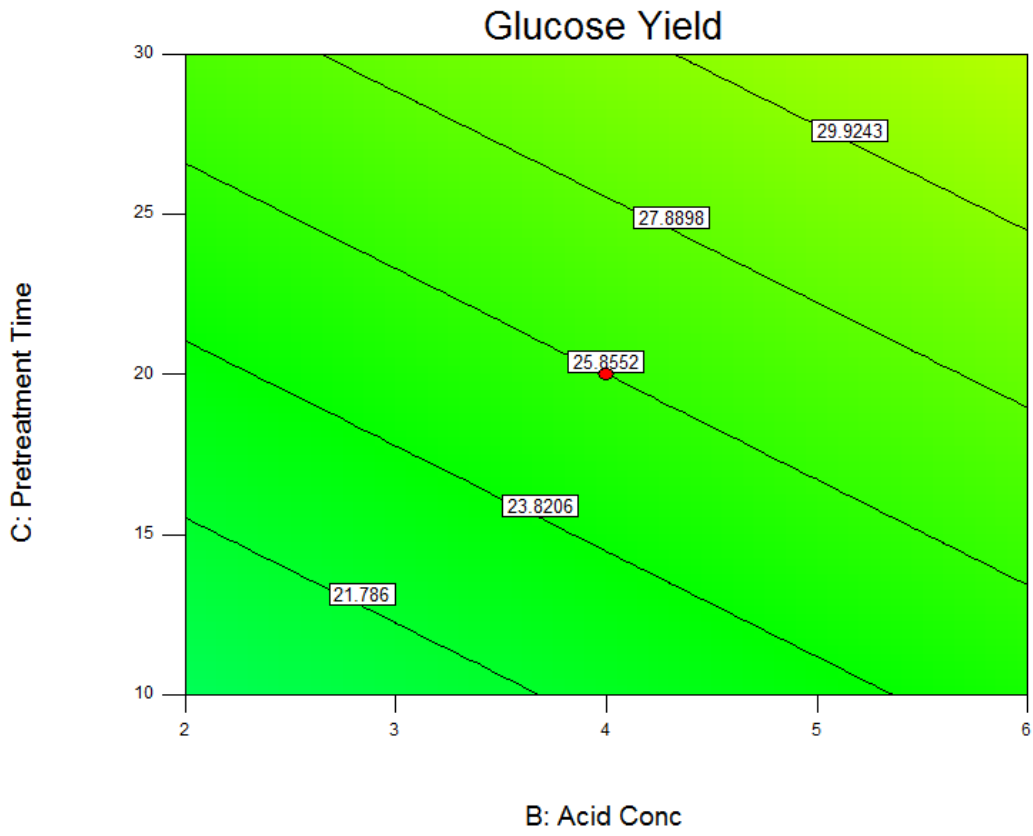

Figure 4.11: The response contour plot of glucose yield as a function of acid concentration and pretreatment reaction time at pretreatment temperature, enzymatic substrate concentration and enzymatic reaction time of 100 °C, 10 %w/v and 48 hrs, respectively as the centre point

Figure 4.12 shows the effect of enzymatic substrate concentration on glucose yield when pretreatment temperature, acid concentration, pretreatment reaction time and enzymatic reaction time were selected at 100 °C, 4 %w/w, 20 min and 48 hrs, respectively as the centre point. It can be interpreted that the increment in the enzymatic substrate concentration almost showed no effect on the glucose yield. The maximum and minimum yield of glucose was 26.22 % and 25.49 %, respectively.

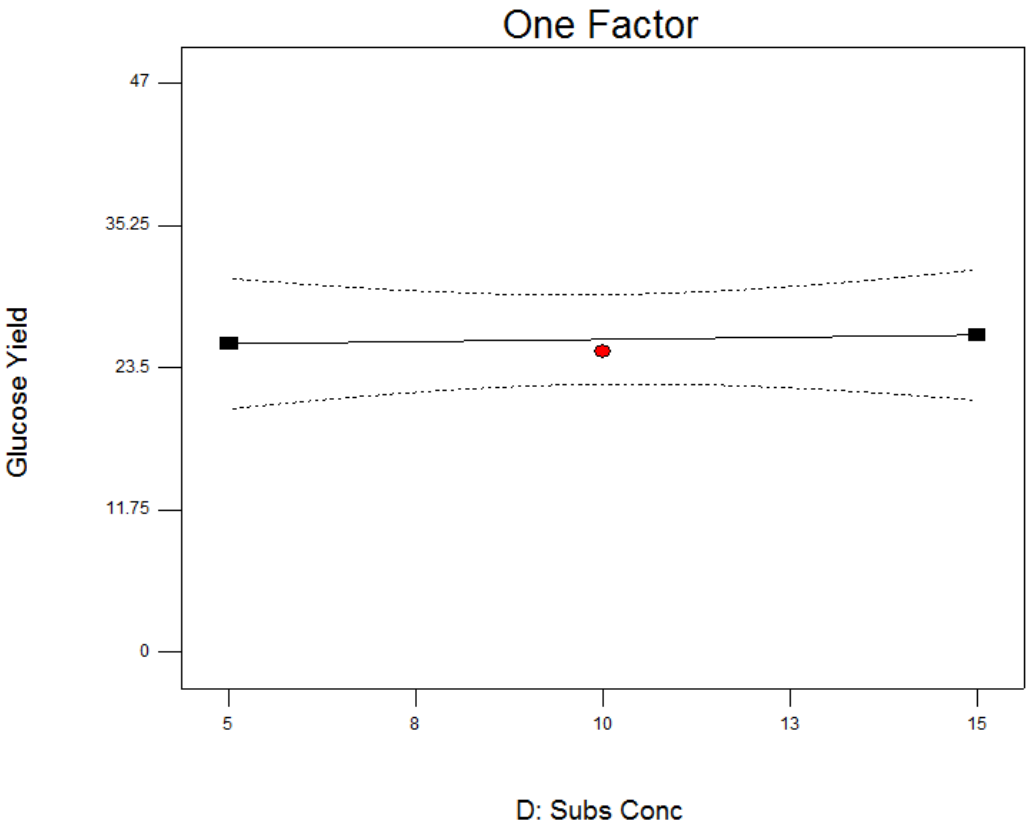

Figure 4.12: Effect of enzymatic substrate concentration on glucose yield when pretreatment temperature, acid concentration, pretreatment reaction time and enzymatic reaction time were selected at 100 °C, 4 %w/w, 20 min and 48 hrs, respectively as centre point

The response surface of glucose yield as a function of pretreatment reaction time and enzymatic substrate concentration at pretreatment temperature, acid concentration and enzymatic reaction time of 100 °C, 4 %w/w and 48 hrs, respectively as centre point is shown in Figure 4.13. The corresponding contour plot is shown in Figure 4.14. From the figures, it is shown that the yield of glucose has increased slightly with the increment in reaction time and enzymatic substrate.

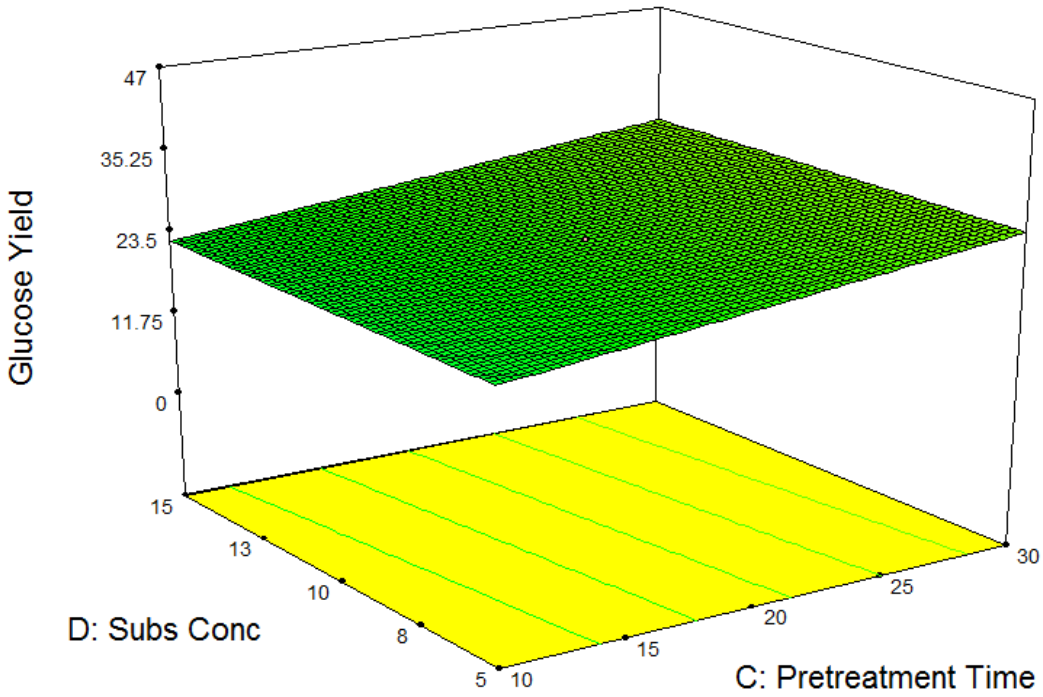

Figure 4.13: The response surface plot of glucose yield as a function of pretreatment reaction time and enzymatic substrate concentration at pretreatment temperature, acid concentration and enzymatic reaction time of 100 °C, 4 %w/w and 48 hrs, respectively as centre point

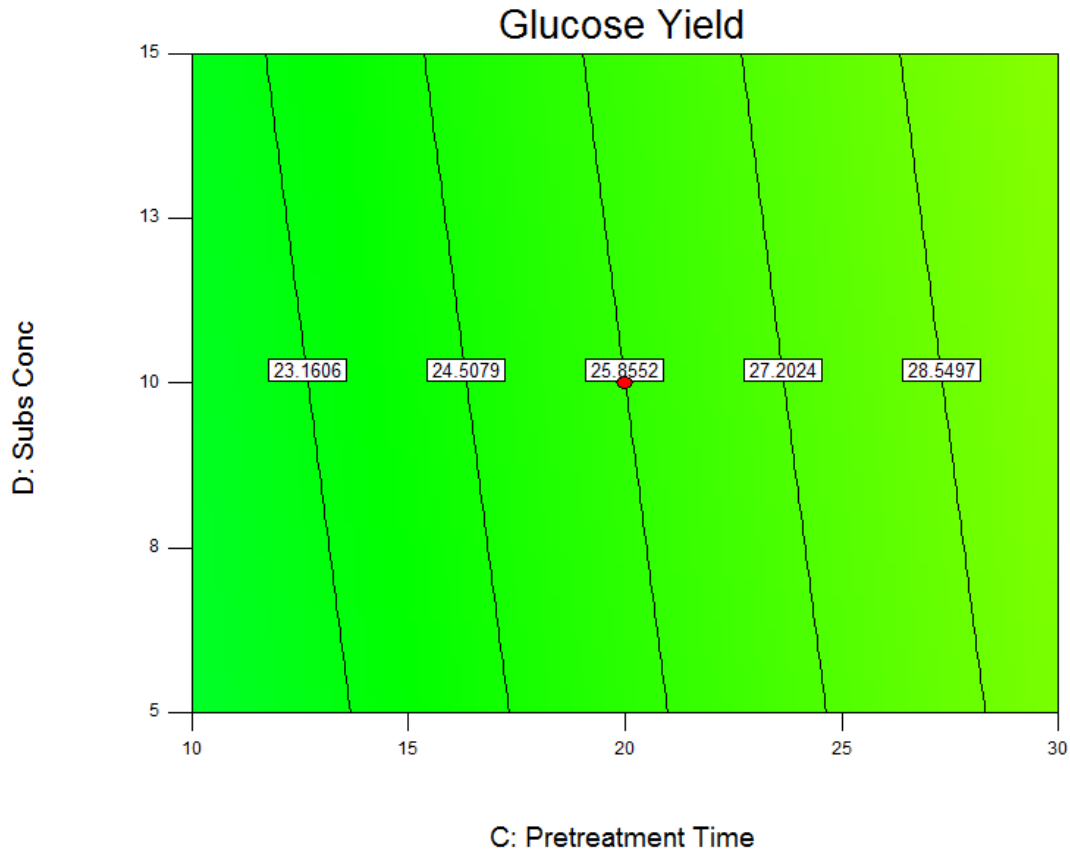

Figure 4.14: The response contour plot of glucose yield as a function of pretreatment reaction time and enzymatic substrate concentration at pretreatment temperature, acid concentration and enzymatic reaction time of 100 °C, 4 %w/w and 48 hrs, respectively as centre point

The effect of the enzymatic reaction time on the response of glucose yield when reaction temperature, acid concentration, pretreatment reaction time and enzymatic substrate concentration were selected at 100 °C, 4 %w/w, 20 min and 10 %w/v respectively as the centre point is shown in Figure 4.15. It can be interpreted that the increment in the enzymatic reaction time dramatically increased the glucose yield. It shows that the maximum and minimum yield of glucose was at 30.66 % and 21.05 %, respectively.

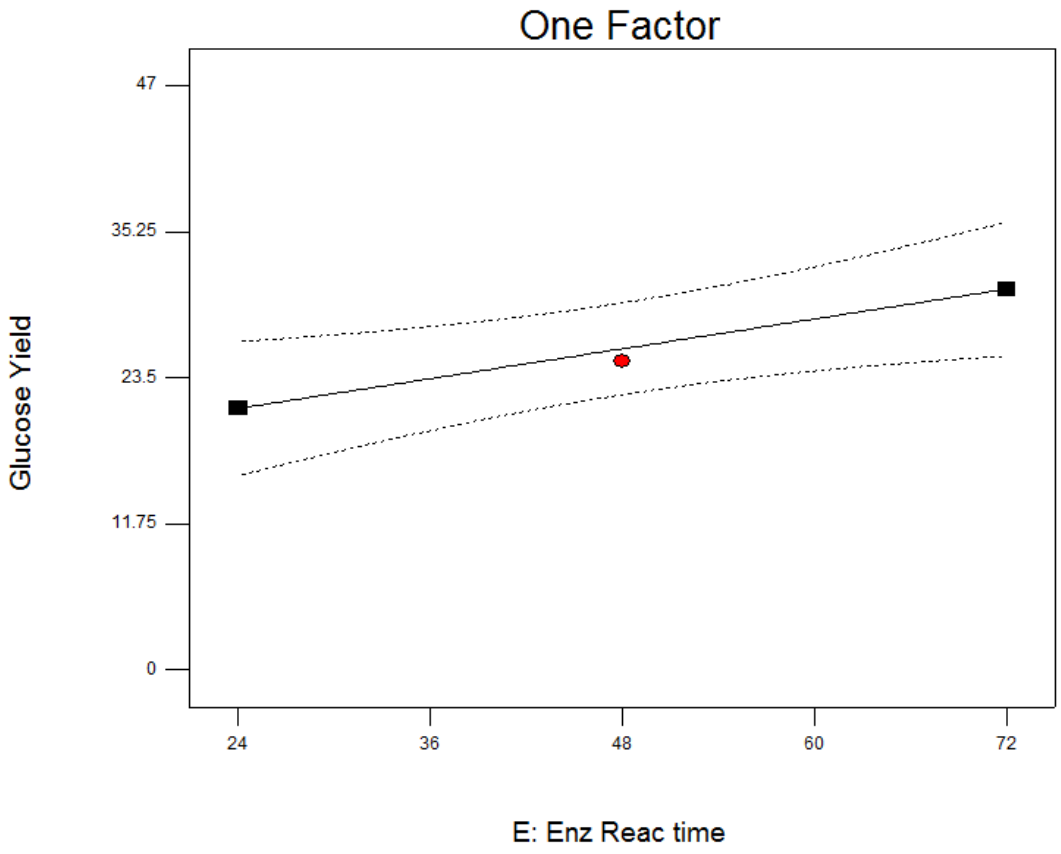

Figure 4.15: Effect of enzymatic reaction time on glucose yield when pretreatment temperature, acid concentration, pretreatment reaction time and enzymatic substrate concentration were selected at 100 °C, 4 %w/w, 20 min and 10 %w/v, respectively as centre point

Figure 4.16 shows the response surface of glucose yield as a function of enzymatic substrate concentration and enzymatic reaction time at pretreatment temperature, acid concentration and pretreatment reaction time of 100 °C, 4 %w/w and 20 min, respectively as centre point. The corresponding contour plot is shown in Figure 4.17. From the figures, it can be interpreted that the yield of glucose increased with the increased in enzymatic substrate concentration but almost no effect with the changes of acid concentration.

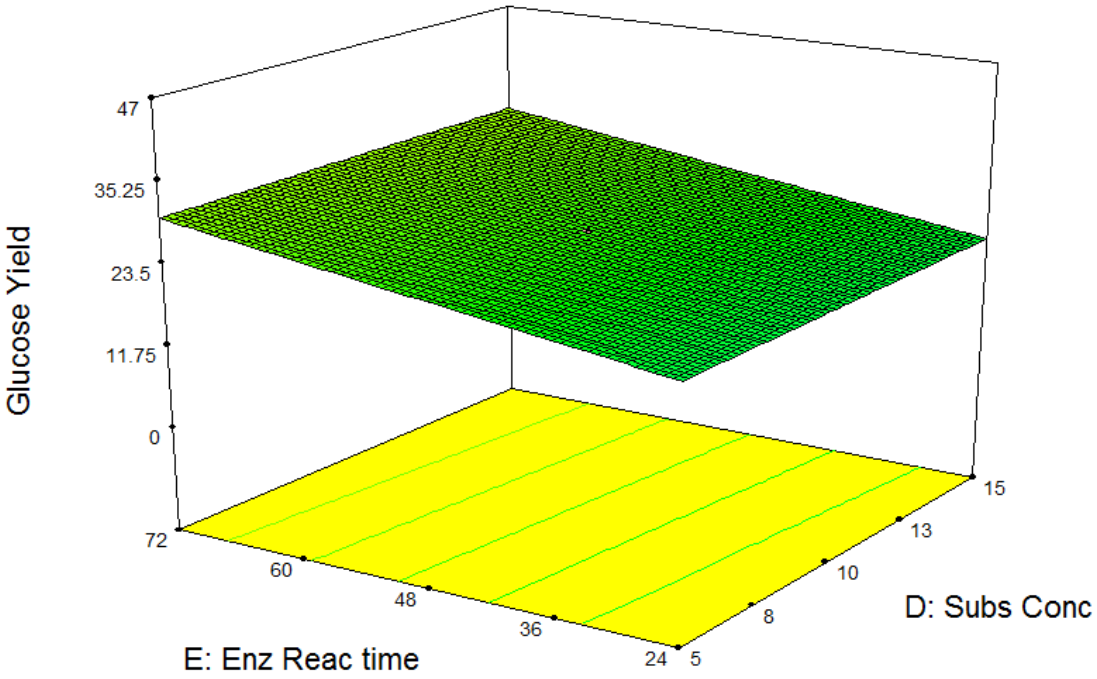

Figure 4.16: The response surface plot of glucose yield as a function of enzymatic substrate concentration and enzymatic reaction time at pretreatment temperature, acid concentration and pretreatment reaction time of 100 °C, 4 %w/w and 20 min, respectively as centre point

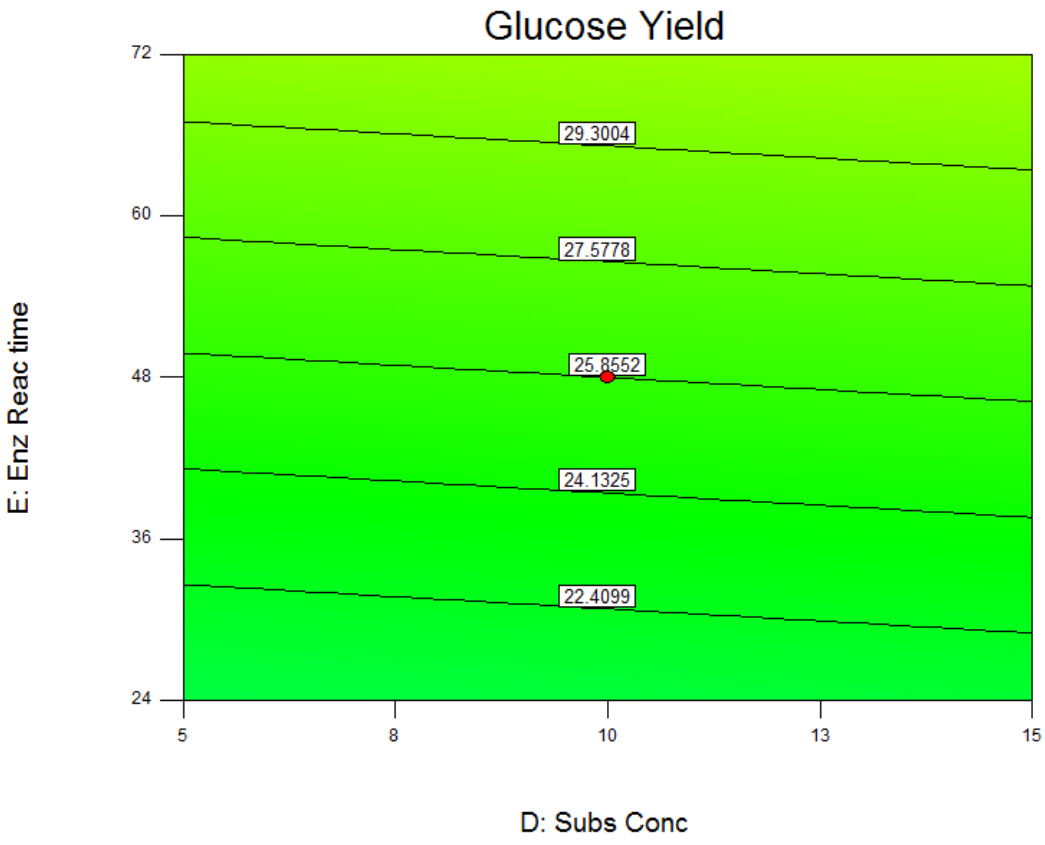

Figure 4.17: The response contour plot of glucose yield as a function of enzymatic substrate concentration and enzymatic reaction time at pretreatment temperature, acid concentration and pretreatment reaction time of 100 °C, 4 %w/w and 20 min, respectively as centre point

The response surface of glucose yield as a function of pretreatment temperature and enzymatic reaction time at acid concentration, pretreatment reaction time and enzymatic substrate concentration of 4 %w/w, 20 min and 10 %w/v, respectively as centre point is shown in Figure 4.18. The corresponding contour plot is shown in Figure 4.19. From the figures, it can be interpreted that the yield of glucose dramatically increased with the increment in pretreatment temperature and enzymatic reaction time. It is shown that these two factors significantly affect the response of glucose yield in this experimental design.

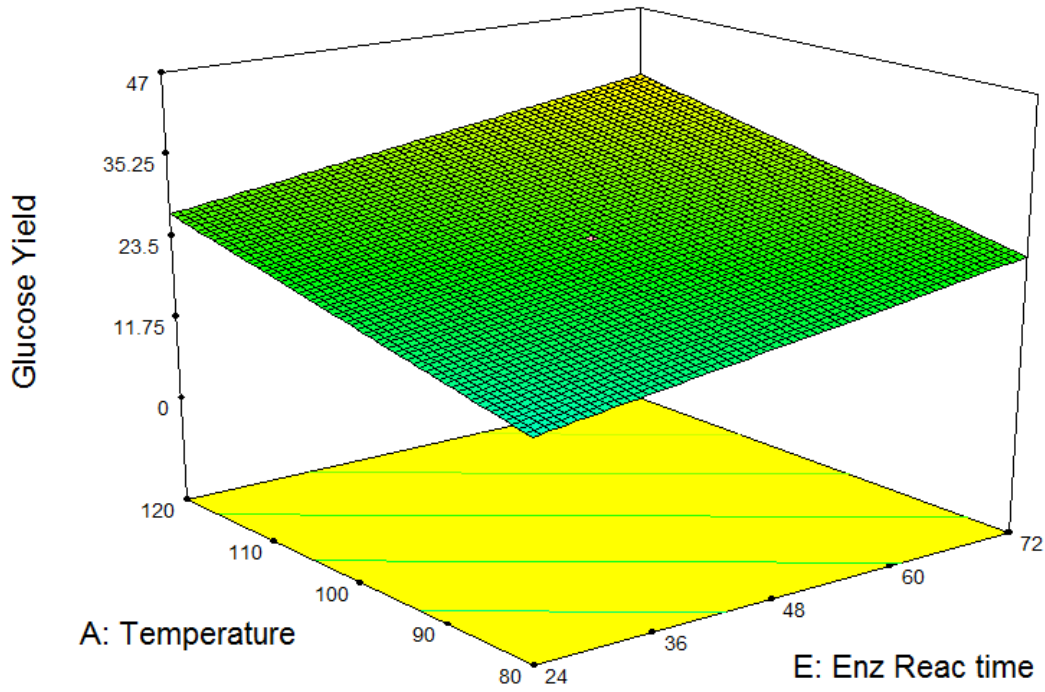

Figure 4.18: The response surface plot of glucose yield as a function of pretreatment temperature and enzymatic reaction time at acid concentration and pretreatment reaction time and enzymatic substrate concentration of 4 %w/w, 20 min and 10 %w/v, respectively as centre point

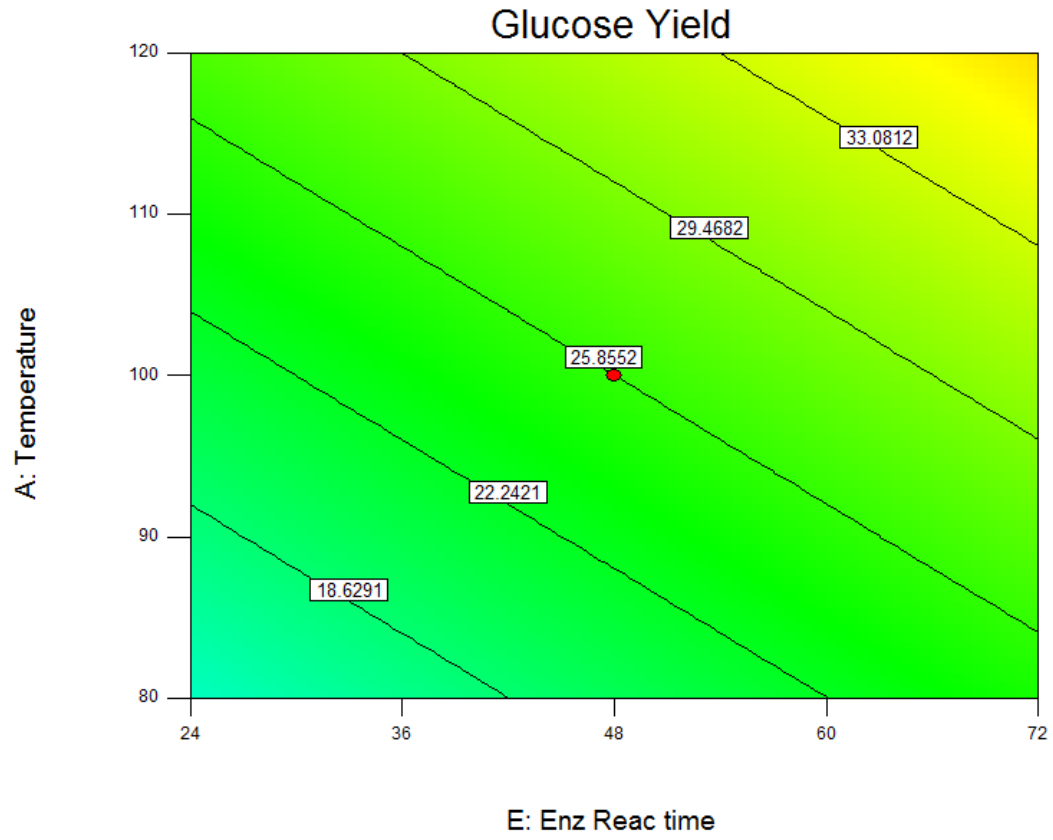

Figure 4.19: The response contour plot of glucose yield as a function of pretreatment temperature and enzymatic reaction time at acid concentration and pretreatment reaction time and enzymatic substrate concentration of 4 %w/w, 20 min and 10 %w/v, respectively as centre point

From all the plots as shown in Figure 4.5 to 4.19, it is shown that only the factors of X_1 and X_5, namely the pretreatment temperature and enzymatic reaction time, were significant in affecting the yield of glucose. These results were consistent to the statistical analysis as reported in Section 4.4.4. It means changing in the value of these two factors in the model will dramatically affect the final glucose yield. On the other hand, the other three factors that showed lack of significance can be omitted in future experimental runs.

4.4.7 Optimisation Point Prediction and Verification

Based on the linear model from the statistical design, numerical optimisation was conducted using Design Expert v.7.1.5. The variables were reset accordingly and the yield of glucose was set to maximise based on the finding of this study as shown in Table 4.9.

Table 4.9: Numerical setting for variables

Constrain Name	**Goal**	**Lower Limit**	**Upper Limit**
Pretreatment Temperature (°C)	is in range	80	140
Acid Concentration (%w/w)	is in range	2	8
Pretreatment Reaction Time (min)	is in range	10	40
Enzymatic Substrate Concentration (%w/v)	is in range	5	20
Enzymatic Reaction Time (hrs)	is in range	24	96
Glucose Yield (%)	maximise	0.30	46.97

The highest predicted yield of glucose of this suggested experimental condition from Equation 4.9 was 53.08 %. Figure 4.20 depicts the experimental condition suggested by the model with a desirability value of 1 (the maximum value). The desirability is an objective function that ranges from zero outside of the limits to one at the goal. It reflects the desirable ranges for each response. The desirable ranges are from zero to one which indicates least to most desirable, respectively. Further experimental work was conducted to validate the optimisation result so that an optimum operating condition of maximised glucose yield can be obtained.

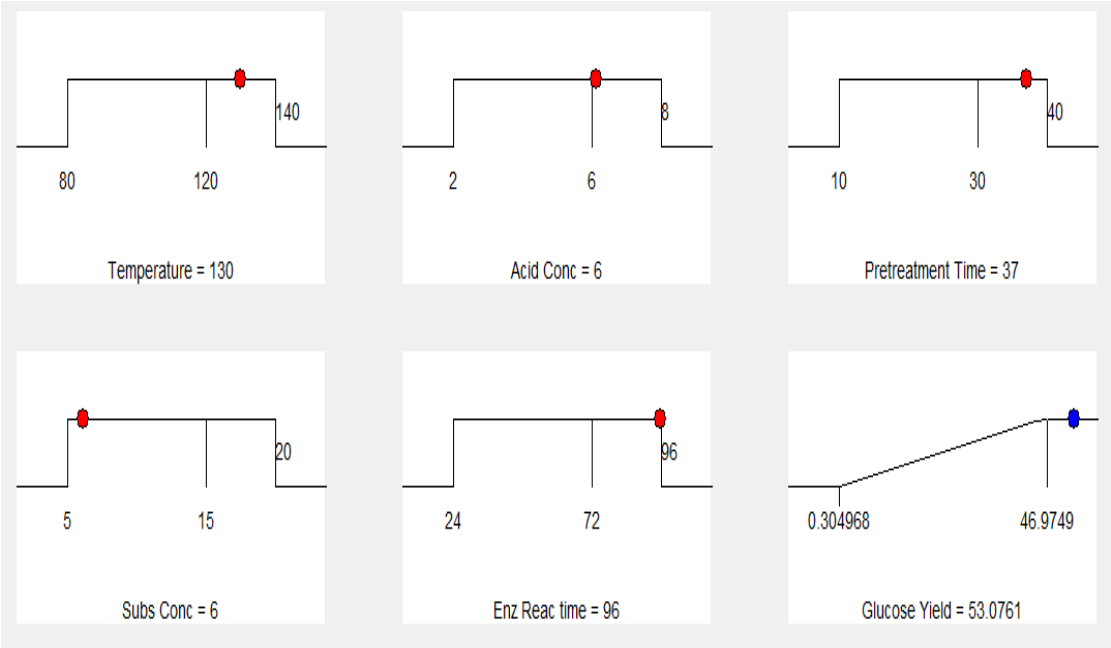

Figure 4.20: Optimum experimental conditions suggested by the model

The adequacy of the model for predicting the optimum yield of glucose was proven experimentally using the recommended experimental factors. The experimental glucose yield obtained from the recommended factors was 53.96 %, which is close to the predicted value (53.08%). As a conclusion, the experimental value was in good agreement with the model prediction.

Hence, the linear model resulted from the statistical design of CCD and RSM has successfully fitted the response of glucose yields. In other words, the model can be used to predict the glucose conversion from the cellulose in oil palm empty fruit bunch following the combined dilute acid pretreatment and enzymatic treatment.

Effort was attempted to compare the yield of glucose obtained from this study (53.96%) to other previous study. However, it is likely that the yield of glucose from

EFB is rather low so that such data has not been found in the literature. Most studies have reported the use of EFB for the production of xylose. For instances, Rahman *et al.* (2006) converted EFB to give xylose through dilute acid hydrolysis and obtained the highest yield of 86.22 %.

Hence, the yield of glucose obtained from this study is compared to other studies that used other biomass wastes to produce glucose. For instance, Zhong *et al.* (2007) studied the hydrolysis of soybean straw to give 51.22 % of glucose yield using ammonia and cellulose. The optimum condition involved the soaking of biomass waste in 10 %w/w ammonia liquor for 24 hrs at room temperature followed by the hydrolysis of 5 %w/v substrate for 36 hrs using cellulase. The result is quite comparable to the current study even though both studies involved different pretreatment methods.

In another comparison, the conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification could extract up to 75 % of all sugars in the raw material as conducted by Cara *et al.* (2008). The condition was 180 °C pretreatment temperature, 1 %w/w sulphuric acid concentration and 10 min of reaction time, shaked at 350 rpm and at a pressure of 3 MPa. The procedure was followed by 72 hrs of enzymatic saccharification. As the process involved much higher pretreatment temperature and pressure then this study, and the mixture was well-mixed, these factors accelerated the hydrolysis process and hence have also resulted in a much higher yield than this study. The high yield would be achieved at a higher production cost where careful optimisation would be needed to evaluate if the increased in the operating cost is worthy.

CHAPTER 5

CONCLUSION AND SUGGESTIONS

5.1 Conclusion

Combined acidic pretreatment and enzymatic hydrolysis of empty fruit bunch (EFB) was carried out with dilute sulfuric acid and selected cellulase enzyme under various operating conditions to obtain high concentration of glucose in the resulting hydrolysate. The glucose can then be further fermented by selected fungus strain to become the useful replacement fuel such as bioethanol. This study has optimised the dilute sulfuric acid pretreatment and the enzymatic treatment steps in a single, combined step using the statistical approach of RSM (response surface methodology) to improve the yield of fermentable glucose from EFB.

The EFB was analysed to contain 40.66 % of glycan that presumably or theoretically could be converted fully to give the glucose without formation of other by-product. This content was used as basis for calculation of glucose yield following the combined acidic pretreatment and enzymatic treatment. The extraction of glucose is

difficult and affected by many factors. Therefore, the yield of glucose obtained from the hydrolysis of EFB need be optimised using statistical approach. Five factors expected to affect the yield were selected based on literature information, there are the pretreatment temperature, acid concentration, pretreatment reaction time, enzymatic substrate concentration and enzymatic reaction time. Optimisation was conducted using 2^5 fractional factorial central composite designs. The linear model was found to be significant with p-value = 0.0065 and the highest value of adjusted R^2 being 0.3982.

Based on the statistical analysis conducted by RSM, the model obtained shows the correlation coefficient (R) of 0.7169 which indicates a high degree of correlation between the observed and predicted values of the glucose yield obtained from the model. The F value of 4.44 implies the model is significant. It was greater than the tabulated $F_{(\alpha, p-1, N-p)}$-value of 2.68 ($F_{0.05, 21, 5}$). Therefore, the analyses suggested that the model represents a good predict of the experiment results.

Based on the ANOVA results from RSM, two factors were identified to give significant effect on the yield of glucose, there are the linear terms of pretreatment temperature (X_1) and enzymatic reaction time (X_5) where both have implied the lowest p-value (<0.05). For other terms including the acid concentration (X_2), pretreatment reaction time (X_3) and enzymatic substrate concentration (X_4), their p-values were greater than 0.05 which indicated that the confidence level was probably not within 95% confidence level but lower. It means that in the future experimental set up, three of these factors can be omitted due to their lack of significance.

The predictive model obtained from the RSM is shown in Equation 5.1. The coded equation is useful for identifying the relative significance of the factors by comparing the factor coefficients.

$$Y = 25.86 + 6.03X_1 + 2.42X_2 + 3.68X_3 + 0.36X_4 + 4.81X_5 \quad \text{Eq. (5.1)}$$

where Y, the glucose yield, is expressed as a function of the pretreatment temperature (X_1), acid concentration (X_2), pretreatment reaction time (X_3), enzymatic substrate concentration (X_4) and enzymatic reaction time (X_5).

The 2^5 fractional factorial central composite design was adopted to optimise the above hydrolysis process. The experimental factors were reset and the yield of glucose was set to be maximised based on the finding of this study. The highest optimised glucose yield was predicted by the design as 53.08 %, with operating conditions of 130 °C of pretreatment temperature, 6 %w/w of acid concentration, 37 min of pretreatment reaction time, 6 %w/v of enzymatic substrate concentration and 96 hrs of enzymatic reaction time. An extra experiment run was conducted at these conditions to validate the model, a glucose yield of 53.96 % was obtained which was in good agreement with the model developed.

Not much literature was found for the production of glucose using the EFB as substrate. Therefore, comparison was done with other studies which used different lignocellulosic biomass as substrate. The highest yield obtained in this study was comparable to the optimised yield obtained by Zhong *et al.* (2007) which used soy bean straw as substrate (51.22 %). However Cara *et al.* (2008) obtained an even highest yield (75 %), most likely due to the use of much higher pretreatment temperature and pressure, and shaking being employed during the acid hydrolysis process.

Under controlled treatment conditions, oil palm empty fruit bunch can be utilised as a potential source of glucose (up to 53.96 %) for the production of bioethanol. Although there are more room for further optimisation of this process by means of

introducing improved strain or consortium of cellulose degrading microorganisms and other novel technologies to further increase the convertibility of cellulose to fermentable sugars.

5.2 Future Research

Many plant matter especially the biowaste have already been explored intensively for biofuel production worldwide. More extensive studies on such material instead of food source are highly desirable to mitigate the issue of soaring food prices.

This research provide alternative route for the production of fermentable glucose from oil palm agriculture waste that could subsequently be used as substrate for the fermentation of bioethanol. The main concern of this research is the low yield of glucose that renders the process less cost effective. The enzyme is the most expensive material in this research. Therefore, recycle or reuse of the enzyme becomes the main issue as the substrate (EFB) itself is a solid hence hinders the used of immobilised enzyme. Some studies such as that conducted by Tu *et al.* (2006) has attempted to recycle the soluble β-glucosidase using Eupergit C. Besides, screening of new strain or consortium of wild-type microorganisms for cellulase enzyme production can reduce the high cost of commercial cellulase enzymes. Genetically modified microorganism strain, even though could give higher specificity, is however difficult to be approved by the authority.

The composition of EFB consists of other sugars in small amount. Xylose is another main type of monomeric sugar in the EFB with typical content of approximately

24.95 %. Utilise xylase enzyme or combination of other polymeric sugars degrading enzymes can successfully increase the yield of monomeric sugars in the EFB hydrolysate and decrease the production of inhibitors. But integration of more enzymes requires more complicated experimental design and optimisation steps. The cost of other enzymes also becomes an issue to be considered.

Apart of the five operating factors as being considered in this study, many other factors may also affect the production of fermentable sugar or glucose. Therefore, the process can be further investigated and enhanced through the optimisation of incubation time, agitation speed, oxygen level, temperature and pH of incubation and others.

A technology of combining the hydrolysis processes with the fermentation process is currently applied on many lignocelluloses substrate for the production of bioethanol. This process is defined as Simultaneous Saccharification and Fermentation (SSF) (Chandel *et al.*, 2007). The SSF process could be applied for the conversion of EFB to provide bioethanol within one reactor. However this technology relies on new strain of genetically modified microorganism which can utilise both the cellulose and hemicelluloses as substrate. Careful selection of the operating conditions is also essential to ensure the success of the SSF process.

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APPENDIX A

PHOTOS OF STUDY

Figure: Bulky of EFB at the sampling site

Figure: Zoom in of EFB sample

Figure: EFB after grinding and oven-dried

Figure: Hydrolysate after enzymatic treatment

APPENDIX B

TREATMENT RESULTS

Std Run	X_1	X_2	X_3	X_4	X_5	12.12.08 ($mg.mL^{-1}$)	13.1.09 ($mg.mL^{-1}$)	13.2.09 ($mg.mL^{-1}$)	Final	Std Dev
1	80	10	2	5	72	5.740	4.920	5.030	5.230	0.445
2	80	10	2	15	24	7.710	10.890	12.300	10.300	2.351
3	80	10	6	5	24	4.650	5.710	2.990	4.450	1.371
4	80	10	6	15	72	14.700	9.560	16.840	13.700	3.742
5	80	30	2	5	24	5.090	3.981	3.109	4.060	0.993
6	80	30	2	15	72	13.980	9.020	12.700	11.900	2.575
7	80	30	6	5	72	6.450	4.950	4.650	5.350	0.964
8	80	30	6	15	24	10.370	7.590	11.650	9.870	2.076
9	120	10	2	5	24	5.680	3.930	2.900	4.170	1.405
10	120	10	2	15	72	16.200	16.06	18.740	17.000	1.509
11	120	10	6	5	72	7.250	7.430	9.320	8.000	1.147
12	120	10	6	15	24	16.100	17.950	18.750	17.600	1.359
13	120	30	2	5	72	9.110	8.690	10.730	9.510	1.077
14	120	30	2	15	24	16.900	17.630	18.570	17.700	0.837
15	120	30	6	5	24	8.7900	7.650	9.360	8.600	0.871
16	120	30	6	15	72	24.240	25.900	26.960	25.700	1.371
17	60	20	4	10	48	11.320	10.800	12.680	11.600	0.971
18	140	20	4	10	48	19.500	18.170	19.630	19.100	0.808
19	100	0	4	10	48	5.400	5.640	6.060	5.700	0.334
20	100	40	4	10	48	16.790	15.310	15.300	15.800	0.857
21	100	20	0	10	48	7.900	4.810	5.980	6.230	1.560
22	100	20	8	10	48	12.380	11.420	10.100	11.300	1.145
23	100	20	4	0	48	0.143	0.000	0.229	0.124	0.116
24	100	20	4	20	48	21.300	19.100	19.600	20.000	1.153
25	100	20	4	10	0	0.260	0.002	0.140	0.134	0.129
26	100	20	4	10	96	12.890	11.360	13.250	12.500	1.004
27	100	20	4	10	48	11.380	8.940	9.980	10.100	1.224

APPENDIX C

PUBLICATIONS

Published

Tan, C. W., Razali, F., Mat, R. and Lee, C. T. (2009). Optimization Studies on Combined Acid Hydrolysis Pretreatment and Enzymatic Treatment of Oil Palm Empty Fruit Bunch for Production of Glucose. *2nd International Conference on Biotechnology for the Wellness Industry*. 23-26 July 2009, PWTC, Kuala Lumpur, Malaysia.

Under Submission

Tan, C. W., Razali, F., Mat, R. and Lee, C. T. (2009). A Statistical Analysis on Dilute Acid Pretreatment and Enzymatic Treatment for the Hydrolysis of Oil Palm Empty Fruit Bunch to Glucose. *Journal of Biomass and Bioenergy*. Submitted on July 2009.

UNIVERSITI TEKNOLOGI MALAYSIA
Research Management Centre

PRELIMINARY IP SCREENING & TECHNOLOGY ASSESSMENT FORM

(To be completed by Project Leader submission of Final Report to RMC or whenever IP protection arrangement is required)

1. **PROJECT TITLE IDENTIFICATION** :

The Kinetic of Dilute-Acid Hydrolysis of Agrowaste for the Production of Reducing Sugar

Vote No: **78218**

2. **PROJECT LEADER** :

Name : PM Dr. Firdausi Razali

Address : FKKKSA UTM SKUDAI 81310 JOHOR.

Tel : 07 5535494 Fax : _____ e-mail : firdaus@fkkksa.utm.my

3. **DIRECT OUTPUT OF PROJECT** *(Please tick where applicable)*

Scientific Research	Applied Research	Product/Process Development
☐ Algorithm	☐ Method/Technique	☐ Product / Component
☐ Structure	☐ Demonstration / Prototype	☑ Process
☑ Data		☐ Software
☐ Other, please specify	☐ Other, please specify	☐ Other, please specify
_____	_____	Process optimisation_____
_____	_____	_____
_____	_____	_____

4. **INTELLECTUAL PROPERTY** *(Please tick where applicable)*

Not patentable	Technology protected by patents
☑ Patent search required	☐ Patent pending
☐ Patent search completed and clean	☐ Monograph available
☐ Invention remains confidential	☐ Inventor technology champion
☐ No publications pending	☐ Inventor team player
☐ No prior claims to the technology	☐ Industrial partner identified

5. LIST OF EQUIPMENT BOUGHT USING THIS VOT

WaterBath Shaker

6. STATEMENT OF ACCOUNT

a)	APPROVED FUNDING	RM : .....81000.00.....
b)	TOTAL SPENDING	RM : .....**77,689.69**.....
c)	BALANCE	RM : .....**3,310.31**.....

7. TECHNICAL DESCRIPTION AND PERSPECTIVE

Please tick an executive summary of the new technology product, process, etc., describing how it works. Include brief analysis that compares it with competitive technology and signals the one that it may replace. Identify potential technology user group and the strategic means for exploitation.

a) Technology Description

The hydrolysis of empty fruit bunch of palm tree to glucose using dilute acid pretreatment and enzyme treatment has been optimised. Response Surface Method was used to optimise both the pretreatment and enzymatic processes in order to obtain the highest glucose yield. An optimised glucose yield of 53.96 % was obtained at the operating condition of 130 °C pretreatment temperature, 6 %w/w sulphuric acid concentration, 37 min of pretreatment time and 96 hours of enzymatic treatment using 6 %w/v of substrate concentration.

b) Market Potential

c) Commercialisation Strategies

8. **RESEARCH PERFORMANCE EVALUATION**

a) FACULTY RESEARCH COORDINATOR

Research Status	( )	( )	( )	( )	( )	( )
Spending	( )	( )	( )	( )	( )	( )
Overall Status	( )	( )	( )	( )	( )	( )
	Excellent	Very Good	Good	Satisfactory	Fair	Weak

Comment/Recommendations :

Di - Sokong

.....

Signature and stamp of
JKPP Chairman
PROF. DR. ZULKEFLI B YAACOB
Deputy Dean (Research & Post Graduate Studies)
Faculty of Chemical & Natural Resources Engineering
Universiti Teknologi Malaysia
81310 UTM, Skudai, Johor

Name : .....

Date : 28/1/2010

b) RMC EVALUATION

Research Status	( )	( )	( )	( )	( )	( )
Spending	( )	( )	( )	( )	( )	( )
Overall Status	( )	( )	( )	( )	( )	( )
	Excellent	Very Good	Good	Satisfactory	Fair	Weak

Comments :-

Recommendations :

- ☐ Needs further research
- ☐ Patent application recommended
- ☐ Market without patent
- ☐ No tangible product. Report to be filed as reference

.....

Signature and Stamp of Dean /
Deputy Dean
Research Management Centre

Name : .....

Date : .....

BORANG FRGS – P3(R)

Kod Projek :	FRGS/FASA1-2009/(BIDANG)/(NAMA IPT)/(NO.RUJ. KPT)

FINAL REPORT
FUNDAMENTAL RESEARCH GRANT SCHEME (FRGS)

Laporan Akhir Skim Geran Penyelidikan Asas (FRGS) IPT
Pindaan 1/2009

A

RESEARCH TITLE :The Kinetic of Dilute-Acid Hydrolysis of Agrowaste for the Production of Reducing Sugar
Tajuk Penyelidikan

PROJECT LEADER : **PM DR. FIRDAUSI RAZALI**
Ketua Projek

PROJECT MEMBERS (including GRA) : 1. **PM DR. LEE CHEW TIN**
2. **PM DR. RAMLI MAT**
Ahli Projek

pr

B

ACHIEVEMENT PERCENTAGE			
Project progress according to milestones achieved up to this period	**0 - 50%**	**51 - 75%**	**76 - 100%**
Percentage			100%

RESEARCH FINDINGS		
Number of articles/ manuscripts/ books	**Indexed Journal**	***Non-Indexed Journal***
	0	0
Paper presentations	**International**	***National***
	1	0
Others (Please specify)		

HUMAN CAPITAL DEVELOPMENT

Human Capital	Number		Others (Please specify):
	On-going	Graduated	
PhD Student	0	0	
Masters Student	0	1	
Undergraduate Students	0	0	
Temporary Research Officer	0		
Temporary Research Assistant	0		
Total	1		

EXPENDITURE *(Perbelanjaan)*

C

Budget Approved *(Peruntukan diluluskan)* : **RM 81,000.00**
Amount Spent *(Jumlah Perbelanjaan)* : **RM 77,689.69**

Balance *(Baki)* : **RM 3,310.31**
Percentage of Amount Spent : 95.91 %
(Peratusan Belanja)

ADDITIONAL RESEARCH ACTIVITIES THAT CONTRIBUTE TOWARDS DEVELOPING SOFT AND HARD SKILLS
(Aktiviti Penyelidikan Sampingan yang menyumbang kepada pembangunan kemahiran insaniah)

D

International

Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit)	ICBWI 23-26 July 2009, PWTC, Kuala Lumpur	UTM

National

Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit)		

PROBLEMS / CONSTRAINTS IF ANY *(Masalah/ Kekangan sekiranya ada)*

E

RECOMMENDATION (*Cadangan Penambahbaikan*)

F

RESEARCH ABSTRACT – Not More Than 200 Words(*Abstrak Penyelidikan – Tidak Melebihi 200 patah perkataan*)

G

There is a growing incentive to identify alternative and ideally renewable energy sources. Among the sources, grains, plant matters and their wastes are commonly been used for biofuel production. Oil palm empty fruit bunch (EFB) is a type of lignocellulosic waste from palm oil mills. Fermentable sugars mainly the glucose from EFB can be further fermented for the production of bioethanol. This study aims to investigate the effect of the key parameters during dilute acid pretreatment and enzymatic treatment for the hydrolysis of EFB to produce optimised yield of glucose. Three parameters for the dilute acid pretreatment, namely the reaction temperature, acid concentration and reaction time and two parameters for the enzymatic treatment, namely the substrate concentration and treatment time were investigated to optimise the yield of glucose. Batch reactions were carried out under different combination of operational conditions as proposed by the experimental design produced by the RSM. An optimised glucose yield of 53.96 % was obtained at the operating condition of 130 °C pretreatment temperature, 6 %w/w sulphuric acid concentration, 37 min of pretreatment time and 96 hours of enzymatic treatment using 6 %w/v of substrate concentration. The optimised yield has also been validated through experiment work.

Date : *Tarikh* 28/01/2010

Project Leader's Signature:
Tandatangan Ketua Projek

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COMMENTS, IF ANY/ ENDORSEMENT BY RESEARCH MANAGEMENT CENTER (RMC)
(Komen, sekiranya ada/ Pengesahan oleh Pusat Pengurusan Penyelidikan)

H

.....

.....

.....

Name:
Nama:

Signature:
Tandatangan:

Date:
Tarikh: