OPTIMIZATION OF GLUCOSE PRODUCTION FROM SUGARCANE BAGASSE USING RESPONSE SURFACE METHODOLOGY (RSM)

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JUDUL: **OPTIMIZATION OF GLUCOSE PRODUCTION FROM SUGARCANE BAGASSE USING RESPONSE SURFACE METHODOLOGY (RSM)**

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OPTIMIZATION OF GLUCOSE PRODUCTION FROM SUGARCANE BAGASSE USING RESPONSE SURFACE METHODOLOGY (RSM)

ZAIDATUL AKMAL BINTI MOHD MUSTAPHA

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

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

APRIL 2010

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

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Special Dedication to:

My mom, Puan Robiaton Mohamad,
My dad, Encik Mohd Mustapha Awang,
My family members,
My fellow lecturers,
My friends and
My fellow colleagues.

For all your care, support and believe in me.

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ABSTRACT

Glucose benefits much for industry, medical field and researches. Old method in producing glucose involved chemical process throughout the procedures. This method is not environmental friendly. Research showed that the production of glucose was successfully obtained from plant biomass. The purpose of this study was to optimize the glucose production from sugarcane bagasse using Response Surface Methodology (RSM). Bagasse is the sugarcane residue after juice extraction. The parameters used in this research were temperature, substrate (cellulose from bagasse) dose and enzyme (cellulase from *Aspergillus niger*) dose. The bagasse was treated with alkali before enzymatic hydrolysis procedure took place for glucose production. Screening process conducted in this study was to determine the best range of parameters involved and this range would be used for the optimization using RSM. Seventeen experiments have been arranged by RSM for analysis. RSM predicted the best conditions of parameters were 45°C of temperature, 1.3 g of substrate dose and 0.8 g of enzyme dose with the glucose production was 5.8672 g/L. The validation of experiment showed that glucose production was 5.725 g/L compared to predicted value, 5.8672 g/L. Before optimization, the production of glucose was 1.010 g/L with conditions 45°C of temperature, 2.0 g of substrate dose and 1.0 g of enzyme dose. The percentage of increment was 82.36%. From these observation and analysis, it can be concluded that the objective of this research to optimize the glucose production from sugarcane bagasse using Response Surface Methodology (RSM) was successfully conducted.

ABSTRAK

Glukosa mempunyai banyak kegunaan dalam industri, bidang perubatan and pengkaji. Kaedah lama dalam penghasilan glukosa dari bahan mentah sehingga terhasilnya glukosa keseluruhannya melibatkan proses tindak balas kimia. Kaedah ini tidak mengutamakan alam sekitar. Kajian terdahulu menunjukkan penghasilan glukosa dari serat tumbuhan telah berjaya dilakukan. Kajian ini bertujuan untuk mengoptimumkan penghasilan glukosa daripada hampas tebu menggunakan Kaedah Tindak Balas Permukaan (RSM). Hampas tebu adalah sisa-sisa daripada tebu selepas penghasilan air tebu. Pembolehubah-pembolehubah yang digunakan dalam kajian ini adalah suhu, dos substrat (selulosa daripada hampas tebu) dan dos enzim (enzim selulase daripada *Aspergillus niger*). Hampas tebu tersebut telah dirawat menggunakan alkali sebelum melalui proses hidrolisis menggunakan enzim dalam penghasilan glukosa. Proses saringan yang dijalankan dalam kajian ini bertujuan untuk mendapatkan lingkungan parameter terbaik untuk dioptimumkan di dalam RSM. Sebanyak 17 eksperimen telah disusun oleh RSM untuk dianalisis. RSM meramalkan pembolehubah terbaik untuk penghasilan glukosa adalah pada suhu 45°C, 1.3 g dos substrat dan 0.8 g dos enzim dengan penghasilan glukosa adalah 5.8672 g/L. Eksperimen telah dijalankan dan didapati penghasilan glukosa adalah sebanyak 5.725 g/L berbanding yang diramalkan iaitu sebanyak 5.8672 g/L. Sebelum proses ini dioptimumkan, penghasilan glukosa hanyalah sebanyak 1.010 g/L dengan suhu 45°C, 2.0 g dos substrat and 1.0 g dos enzim. Peratus kenaikan penghasilan glukosa adalah sebanyak 82.36%. Berdasarkan pemerhatian dan analisis, dapat disimpulkan bahawa objektif kajian ini untuk mengoptimumkan penghasilan glukosa daripada hampas tebu menggunakan RSM telah berjaya dilakukan.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of Variance
CCD	-	Central Composite Design
CCRD	-	Central Composite Rotable Design
DNS	-	Dinitrosalicylic
g	-	gram
g/L	-	Gram per Litre
L	-	Litre
mg	-	miligram
mL	-	mililitre
RSM	-	Response Surface Methodology
°C	-	Degree Celcius
µL	-	microlitre
%	-	Percentage

CHAPTER 1

INTRODUCTION

1.1 Introduction

Sugarcane or its scientific name *Saccaharum officinarum* is one type of the lignocellulosic biomass which is abundant and can be easily found. The solid residue left after extraction of the juice is called sugarcane bagasse or generally called as bagasse. It has high potential or the production of bioethanol, biohydrogen or other biofuel. The use of sugarcane bagasse in chemistry and biotechnology has been recently reviewed (Pandey *et al*., 2000).

Generally, in the bagasse specifically or other biomass's cell wall, it contains lignin, hemicelluloses and celluloses. Lignin can be hydrolyzed into poly aromatic hydrophobic structure for renewable solid fuel, while hemicelluloses can be hydrolyzed into five carbon (C_5) sugars for bio-refineries or enzymes production. In this research, the desired component is the cellulose which can be hydrolysed into six carbon (C_6) sugars which is glucose for bioethanol or other renewable liquid fuel production.

Generally, there are two main procedures in order to produce glucose from sugarcane bagasse. The first one is the pretreatment of the bagasse and the second one is the enzymatic hydrolysis itself. Many pretreatment methods have been reported and several detailed review papers have been published (Sun and Cheng, 2002). The pretreatment process indicates positive impact on the cellulose hydrolysis and consequently the glucose yields. The purpose of the pretreatment is to separate lignin and hemicelluloses from cellulose, reduce cellulose crystallinity and increase the porosity of the lignocellulosic materials so that cellulose hydrolysis can be significantly improved (Kuo and Lee, 2009).

Hydrolyzing the cellulose into glucose is performed by using specific enzyme in certain conditions such as temperature, the concentration of enzyme, the quantity of cellulose, pH and time. These conditions could be optimized using Response Surface Methodology (RSM) in order to get the optimum data. The enzymatic hydrolysis had been done on waste paper office using three commercial cellulases, Acremonium cellulase, Meicelase and Cellulosin T2 (Park *et al*., 2002). Waste paper office contains cellulose. This is one of the methods to reduce the waste paper office and consequently turn it into energy sources.

The optimization in RSM has demonstrated the use of a central composite factorial design by determining the optimum conditions leading to the high yield of enzyme production. Thus, smaller and less time consuming experimental designs could generally suffice for the optimization of many fermentation processes (Adinarayana and Ellaiah, 2002). With the aid of the experimental design and response surface methodology, the optimal concentrations of sugarcane molasses, bacteriological peptone and yeast extract Prodex Lac SD® for the production of glucosyltransferase by *Erwinia* sp. D12 were found to be 160 g/L, 20 g/L and 15 g/L, respectively (Kawaguti *et al*., 2006).

1.2 Problem Statement

The bagasse is the major residue from the sugar and alcohol industry. It can be a source of pollutant if it is dumped before treatment. The bagasse has no value to those industries because it is considered as a waste. As an engineer, everything has its own value including the wastes and a research is needed in order to develop the potential. A lot of studies have been done on treating the bagasse into something valuable.

The depleting fossil fuel resources and the environmental problems with greenhouse gases have sprung forth the awareness of the importance of renewable and cleaner sources of energy, such as biogas produced from lignocellulosic biomass. Sugarcane bagasse is a cheap and abundant raw material which can be used for this purpose (Carvalho, 2009).

From previous studies, the bagasse is found out to be a fuel source as it can produce more heat energy to supply in sugar and alcohol industries themselves (Sendelius, 2005). Besides, the researchers also found out that the bagasse can also produce glucose as it contains a lot of cellulose in its fibre. The major challenge in this research is to convert economically the bagasse into glucose. This research will focus on enzymatic hydrolysis process with various parameters to find out the most economic and optimum conversion.

1.3 Objective

The research was proposed to optimize the production of glucose from sugarcane bagasse using Response Surface Methodology (RSM).

1.4 Scopes of Study

In order to achieve the stated objective, the following scopes of study have been identified:

a) To study the effect of temperature on production of glucose.
b) To study the effect of cellulose dose (substrate) on production of glucose.
c) To study the effect of cellulase dose (enzyme) on production of glucose.
d) To optimize the glucose production using Response Surface Methodology (RSM).

1.5 Rationale and Significance

As mentioned previously, almost the entire quantity of the bagasse produced is used by the sugar mills themselves as fuels or boilers, which is a necessity-based on economical and efficient application. However, process such as production of enzymes and other products utilizing the bagasse as solid substrate would need relatively a small fraction of total bagasse. This may not affect its supply to the sugar mills and thus appears attractive for bioprocess (Pandey *et al.*, 2000).

About 32% of bagasse is produced from every tone of sugarcane that has been produced. The total plantation area of sugarcane bagasse in Malaysia is nearly 34 500 acre. About 1 111 500 tonnes of sugarcane is produced in 2002, hence the bagasse can be easily obtained in Malaysia (Lee and Mariatti, 2008). This research could be done without any problem in looking for raw materials.

This study is to optimize the production of glucose from sugarcane bagasse using Response Surface Methodology. This optimization is to find the best parameters in converting the bagasse into glucose economically. This leads to achieve the 'waste into wealth' industrial concept nowadays. More money can be generated from the waste. This research comply this recent needs.

The importance of this research is the glucose as the raw material of ethanol production or other biofuels. The other significance of the study is to reduce the environmental pollution as it is a worldwide threat to public health has given rise to a new massive industry for environmental restoration.

CHAPTER 2

LITERATURE REVIEW

2.1 Sugarcane Bagasse

Sugarcane is one of the agricultural products that have many benefits to human usage and also other living things (Pandey *et al*., 2000). Sugarcane is used as a main source in producing sugar for food and beverages. In sugar and alcohol industry, sugarcane bagasse is generated as a waste. This bagasse could be a pollutant to the environment if it is just disposed without treatment. Generally, the biomass composed of cellulose, polyoses, lignin, hemicelluloses, small amounts of extractives and mineral substances. In the cell wall of biomass, it consists of lignin, hemicelluloses and cellulose. The sugars of which they are made are linked together in long chains called polysaccharides, which form the structural portion of plant cell walls.

Unraveling these complex polymeric structures is the key to economic biorefining. Cellulose microfibrils consist of a crystalline structure of thousands of strands, each of which contains hundreds of glucose sugar molecules. These microfibrils are wrapped in a sheath of hemicelluloses and lignin, which protects the cellulose from

microbial attack. Hemicelluloses are relatively easy to break down using pretreatment step. It also disrupts the hemicelluloses or lignin sheath around the cellulose, making the cellulose accessible to further hydrolysis. The hydrolysis of the lignocellulosic biomass will liberate C_6 fermentable sugars will be carried out via enzymatic hydrolysis (Camassola and Dillon, 2009). Figure 2.1 below shows the structure of cellulose microfibrils.

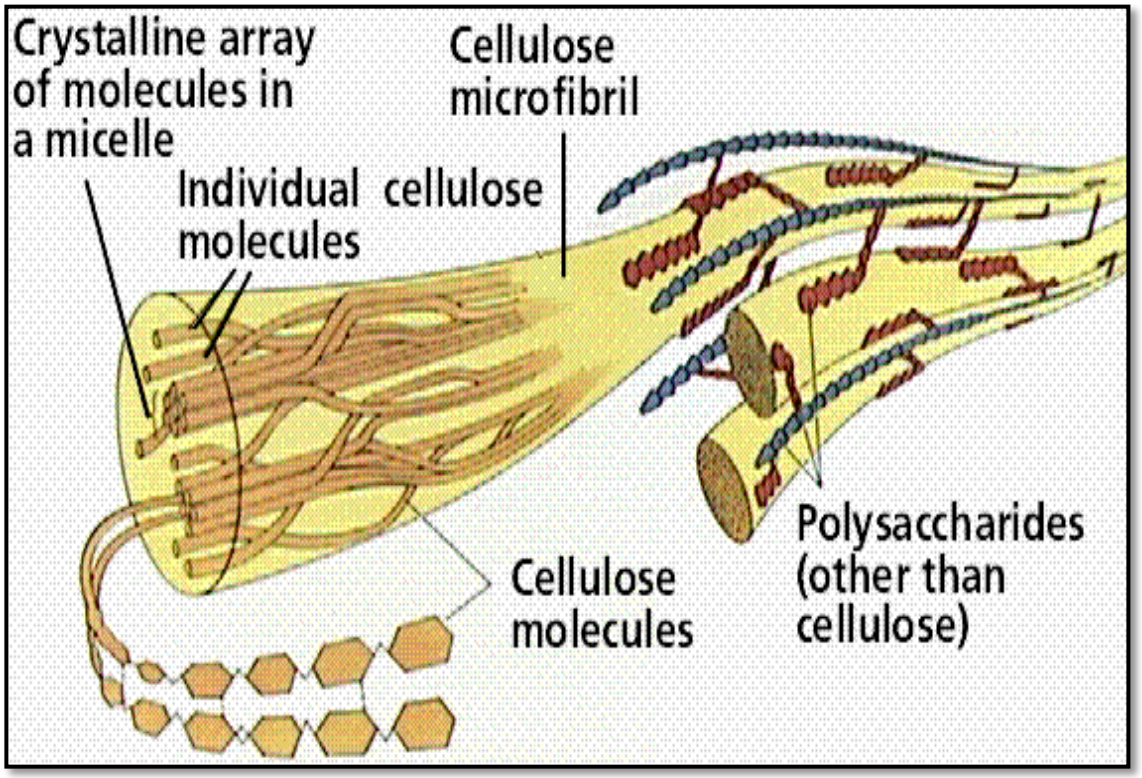

Figure 2.1: The structure of cellulose microfibrils

In this study, the bagasse is chosen as it consists of approximately 50% cellulose and 25% each of hemicelluloses and lignin. Chemically, bagasse contains about 50% α-cellulose, 20% pentosans and 2.4% ash. Because of its low ash content, bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11% respectively (Pandey *et al.*, 2000). Low ash content will enhance the enzymatic hydrolysis process. Sugarcane bagasse ash, a byproduct of

sugar and alcohol production, is a potential pozzolanic material which is used as partial replacement of Portland cements in mortars and concrete (Cordeiro *et al.*, 2009). Figure 2.2 shows the structure of cellulose in the plant cell wall.

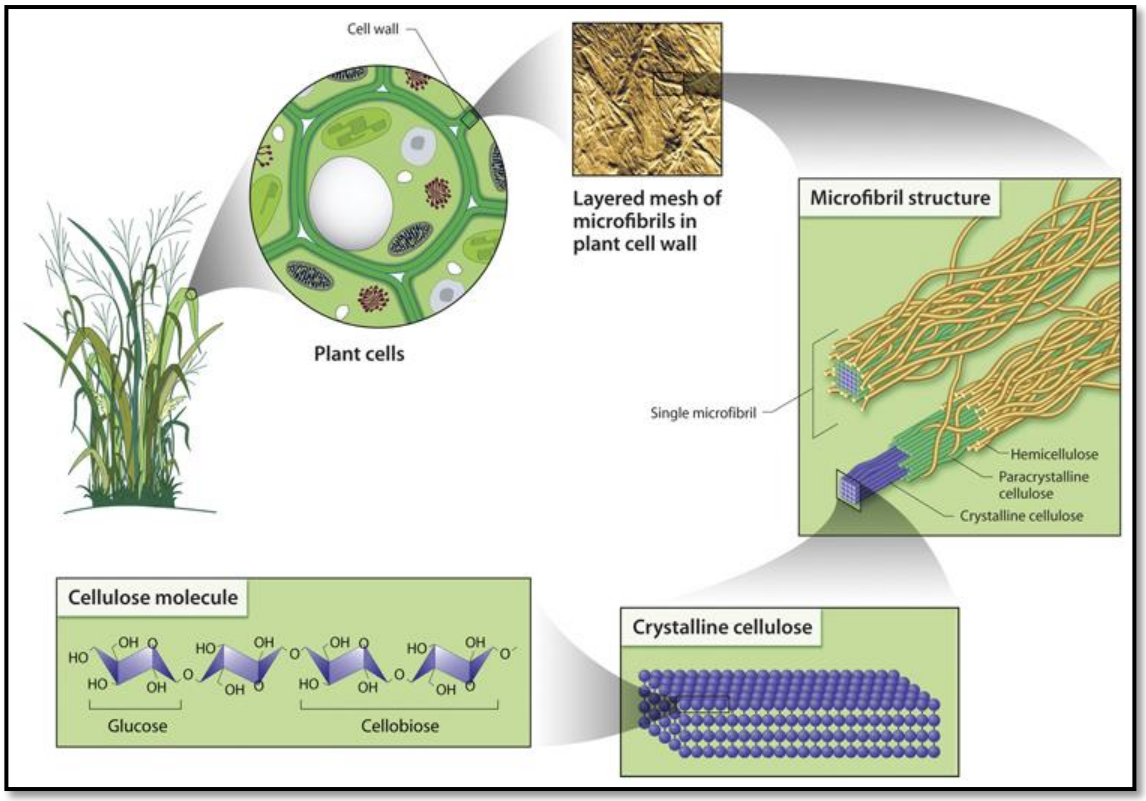

Figure 2.2: Zooming into the views inside the plant cells; structure of cellulose molecule

2.2 Pretreatment Method

Before the enzymatic hydrolysis is performed, the bagasse must be pretreated first to improve its digestibility and easy access for microbial attack (by removing core and noncore lignin fractions) (Pandey *et al*., 2000). Direct use of bagasse is not susceptible to exploit as substrate for cellulose (Javed *et al*., 2007).

Generally, the pretreatment process involves employing high temperature, pressure, acids or alkaline and organic solvents to disrupt the lignin seal and cellulose crystalline structure of lignocellulosic material. There are a few methods of pretreatments such as dilute acid pretreatments, alkali pretreatment, steam pretreatments, gamma radiation and enzymatic pretreatment.

Most of pretreatment methods have their own drawbacks in large scale application. For example, the dilute acid process generates toxic byproducts, such as furfural and aldehydes, which not only significantly reduces the sugar yield but also poisons enzymatic hydrolysis and biofuels fermentation. Steam explosion, operated at high temperature and pressure to achieve fibrillation, requires costly capital investment for equipments (Kuo and Lee, 2009).

In this study, the chemical pretreatment (alkaline pretreatment- e.g., treatment with alkali such as sodium hydroxide solution) is found to be effective and economical. Rodriguez-Vazguez *et al.* (1992) treated bagasse (pith) with a solution of sodium hydroxide in such a low volume that no free liquid was present. They referred it as dry pretreatment and compared it with wet pretreatment. Maximum digestibility with dry and wet pretreated bagasse was 75% and 71% respectively.

Schimper *et al*., (2009) also applied alkali pretreatment in the study on fabrics. Fabrics were pre-treated in one step which consists of immersing in alkali solutions for 1 or 2 min at a liquor ratio of 1:3 (w/v). No tension was applied to the fabrics. A range of concentrations (0 - 4.9 mol/L) was used in the impregnation solution. After squeezing in a padder, samples were rinsed two times with deionized water and then neutralized in a solution of 20 mmol/L acetic acid for five minutes. The alkali uptake was found to be between 100 and 250%, depending on alkali concentration. After neutralization the fabrics were exposed to enzyme for enzymatic hydrolysis.

2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis is a catalytic decomposition of a chemical compound by reaction with water, such as the conversion of cellulosic material into fermentable sugars by the addition of specific enzymes. In this study, the cellulosic material used is sugarcane bagasse. It will be hydrolyzed into fermentable sugar which is glucose by addition of cellulase as the specific enzyme. Figure 2.3 shows the general pathway of enzymatic hydrolysis.

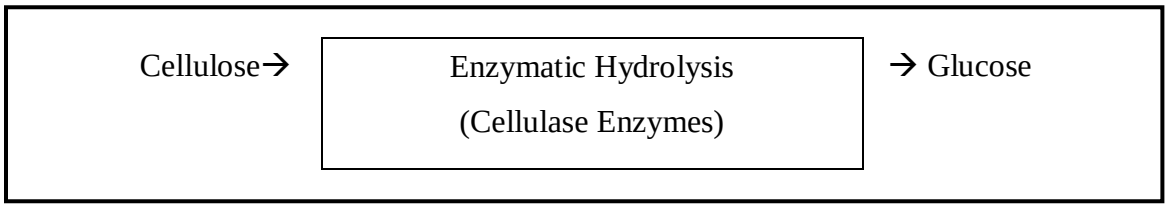

Figure 2.3: General pathway of enzymatic hydrolysis

This method is chosen as its utility cost is the lowest if compared to acid and alkaline hydrolysis. Sun and Cheng (2002) reported the enzymatic hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45-50°C) and does not have corrosion problem.

Enzymatic hydrolysis of cellulose has become an important research area due to the potential use of cellulosic biomass as feedstock for fermentation into ethanol. The enzymatic breakdown of cellulose to fermentable sugars is done by enzymatic hydrolysis of the glucosidic bonds. The reaction is thus a two-substrate reaction involving both cellulose and water. While there has been considerable interest in the cellulose–enzyme interactions as well as on the cellulose composition, limited attention has been paid to the role of water in the process. Felby *et al*., (2008) investigated the cellulose-water interactions during enzymatic hydrolysis. During the initial enzymatic hydrolysis of cellulose, the action of the enzyme system is a breakdown and loosening of the cellulose introducing more water into the structure and providing better access for the enzymes.

Cellulases enzymes refer to enzymatic hydrolytic system consist of three different enzymes and act in specific way. First, an endoglucanase attacks one of the cellulose chains within the crystal structure, breaking the strand via hydrolysis and thereby exposing two new chain ends. During hydrolysis process, a molecule of water is consumed and one of the chain ends chemistry becomes “reducing” and the other “non-reducing”. Then an exoglucanase attaches to a loose chain end, physically pulls the cellulose chain away from the crystal structure and then proceeds to work its way down the chain, breaking off cellobiose (a dimeric sugar comprised of two glucose molecules) as it goes. Actually, there are two types of exoglucanase – a cellobiohydrolase I (CBH I) attaches to the “reducing” end and a cellobiohydrolase II (CBH II) attaches to the “non-reducing” end. Finally, a betaglucosidase splits the cellobiose molecule into two

separate glucose molecules, making them available for processing into chemicals or fuels (Lenting and Warmoeskerken, 2001).

The process in enzymatic hydrolysis had been reported in literature review over the years. Enzymatic hydrolysis on corn stalks was reported by Dale *et al*., (1995). The hydrolysis of the corn stalks with the cellulase was measured by taking 0.1 g of cellulose in the corn stalks sample added to 9.9 ml water. Then, 66.7 μL of cellulase enzyme was added to the solution, and the sample was allowed to digest at 50°C. Final glucose concentrations of 4.8 g/L glucose, 3.2 g/L cellobiose, and 2.6 g/L xylose were noted.

The enzymatic hydrolysis on waste paper office was done using three commercial cellulases; Acremonium cellulase, Meicelase and Cellulosin T2. The glucose percentage was measured more than 90% from various waste papers. The research was reported by Park *et al*. (2002). Najafpour and Shan (2002) investigated the enzymatic hydrolysis of molasses. The study was to increase the amount of fermentable sugar for ethanol production. The fermentable sugar content of molasses by enzymatic hydrolysis was increased from 194 to 611 g/L. The obtained sugar enriched molasses represents a better quality of feed stock for fermentation industries such as ethanol production.

2.4 Production of Glucose

A number of studies in production of glucose have been developed over the years. The productions of glucose involved enzymatic hydrolysis or by chemical hydrolysis have been reported in the journals. Chemical hydrolysis, usually acid hydrolysis, is one of the viable methods currently being developed as a promising means of producing sugar from cellulose. The hydrolysis of cellulose in mineral acids is strongly affected by the acid concentration and temperature (Sun *et al*., 2009).

Katz and Reese (1968) reported that the enzymatic hydrolysis of commercial cellulose (Solka Floc) can give concentrations of glucose (30%) comparable to those obtained in the enzymatic hydrolysis of starch. The glucose production was under optimal condition for 70 hours and enzymes used were cellulase from *Trichoderma viride* plus β-glucosidase. The incubation was at pH 4.5 and temperature 40°C. It approves that enzymatic hydrolysis on cellulose can produce glucose.

Mosier *et al*., (2002) studied about the characterization of acid catalytic domains for cellulose hydrolysis and glucose degradation. The hydrolysis of cellulose in corn fiber was tested using maleic and sulfuric acids at 50 mM concentrations. The corn fiber was first pretreated by pressure cooking it in water, followed by addition of the appropriate amount of acid. After hydrolysis at 160°C for 30 minutes, chromatograms of the liquid recovered from hydrolysis show that significant glucose has been generated. The result showed that the highest yield of glucose was obtained by using maleic acid which is 26 g and by using sulfuric acid, the glucose was only 22.3 g. Maleic acid possesed superior selectivity for the production of fermentable sugars from cellulose more than than sulfuric acid.

2.5 The Application of Glucose in Industry

Glucose benefits much for industry, medical field and researches. Numbers of applications have been reported up to now. Shen and Xia (2006) produced lactic acid from industrial waste corn cob. The corn cob was hydrolyzed by cellulase and cellobiase. The cellulosic hydrolysate contained 52.4 g L^{-1} of glucose and was used as carbon source for lactic acid fermentation by cells of *Lactobacillus delbrueckii* ZU-S2 immobilized in calcium alginate gel beads. The final concentration of lactic acid and the yield of lactic acid from glucose were 48.7 g L^{-1} and 95.2%, respectively, which were comparative to the results of pure glucose fermentation.

From medical field, the increases in glucose production that occur during infusion of epinephrine may result in part from a direct effect of epinephrine on the liver as well as from effects of epinephrine on insulin secretion, glucagon secretion, and gluconeogenic precursor availability. The decreases in glucose clearance observed during infusion of epinephrine may be due in part to direct inhibition of tissue glucose uptake by epinephrine and to inhibition of tissue glucose uptake secondary to effects of epinephrine on plasma insulin and free fatty acid concentrations. Furthermore, when assessment of these adrenergic mechanisms is attempted in vivo by infusing either an alpha or beta adrenergic antagonist along with epinephrine, alteration in circulating hormone and substrate concentrations (e.g., insulin and glucagon, or insulin and free fatty acids) occur that may have opposing effects on glucose production or clearance (Rizza *et al*., 1980).

The concept of mass balance was used to analyze the metabolic pathways of citrate production by *Candida lipolvtica* from glucose (Alba and Matsuoka, 1979). Specific rates of glucose consumption, citrate and isocitrate productions, carbon dioxide evolution and cellular syntheses of protein and carbohydrate were observed in an NH_4^+

limited chemostat culture. These data permitted one to assess the carbon flux *in vivo* by solving simultaneous carbon balance equations with respect to intermediary metabolite pools in the steady state.

2.6 Factor Affecting the Production of Reducing Sugar

A reducing sugar is any sugar that has an aldehyde or a ketone group. This includes glucose, fructose, glyceraldehyde and galactose. The maximum production of reducing sugar is affected by various conditions such as effect of pH, revolution per minute (rpm), time and so on. In this experiment, the effect of temperature, effect of substrate dose and effect of enzyme dose in glucose production from sugarcane bagasse were investigated. Some literature reviews reported on these conditions on production of reducing sugar.

2.6.1 Effect of Temperature on Reducing Sugar Production

The effects of temperature in the range 40°C to 60°C were investigated on the enzymatic hydrolysis of steam-pretreated willow to obtain the optimal hydrolysis conditions for production of glucose (Eklund *et al*., 1990). The temperature affects both the initial hydrolysis rate and the final glucose yield. The highest glucose yield was obtained at 40°C. The yield will decrease with increasing temperature, due to the increased of enzyme deactivation at higher temperatures, while the initial reaction rate increased with increasing temperatures.

Chen and Jin (2006) studied on the enzymatic hydrolysis of crystalline cellulose. The crystalline cellulose was suspended in acetic acid buffer (pH 4.8) and cellulase enzyme from *Penicillium decumbens* was added into the slurry. Enzymatic hydrolysis experiments were performed separately at 30, 35, 40, 45 and 50°C. Based on these results it was concluded that 40°C was the best temperature for *Penicillium decumbens* cellulase enzymatic saccharification of crystalline cellulose.

The effect of temperature in the range of 30°C to 70°C on the production of reducing sugar was also shown in the study by Chotineeranat *et al*. (2004). In the production of reducing sugar from cassava pulp, two types of enzymes were used. At 50°C, by using α-amylase, the highest amount of reducing sugar produced was 2.0 mg/mL while by using glucoamylase, the amount of reducing sugar produced was 8.8 mg/mL. The glucoamylase showed the highest amount of reducing sugar at 60°C. Figure 2.4 shows the reducing sugar obtained at different temperatures for both α-amylase and glucoamylase.

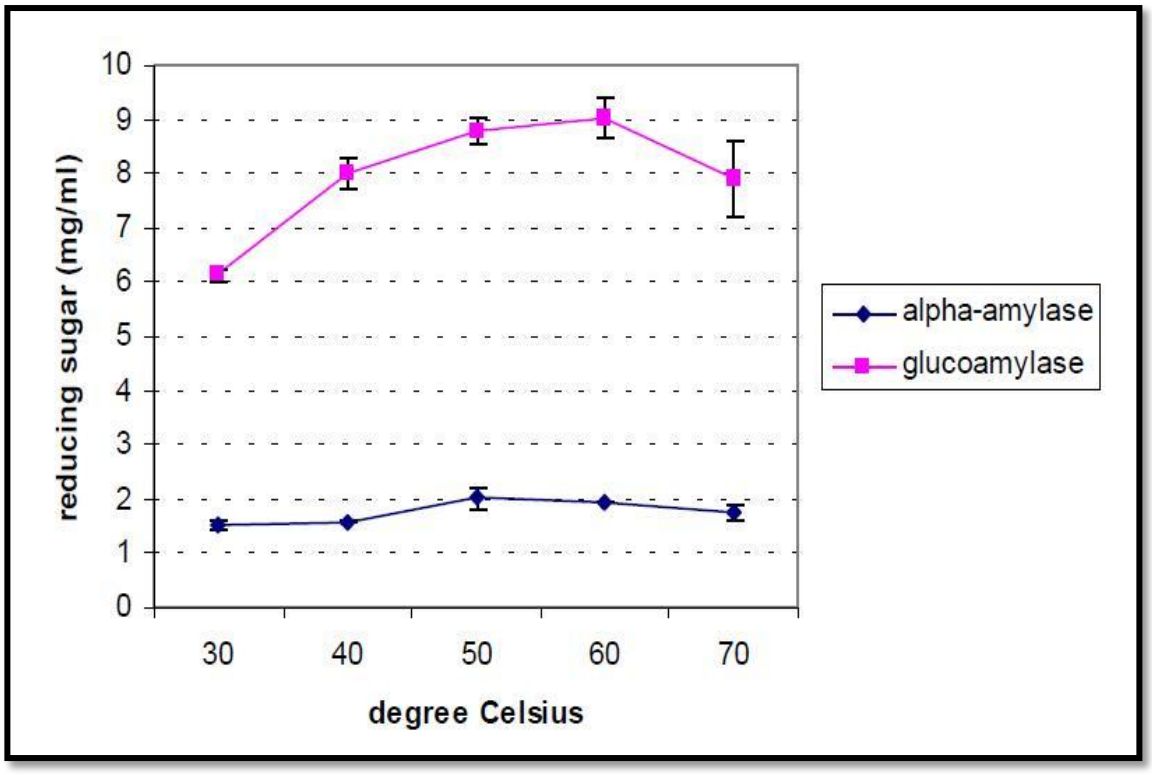

Figure 2.4: Effect of temperature on the production of reducing sugar from cassava pulp

2.6.2 Effect of Substrate Dose on Reducing Sugar Production

Fungsin *et al*., (2009) studied on the conversion of cassava waste into sugar using *Aspergillus niger* and *Trichoderma reesei* for ethanol production. In order to find the maximum concentration of reducing sugar, the amount of cassava waste was varying at 15, 30, 45, 60 and 70% (w/v) (wet weight) in the cultivation medium. The cassava waste acts as the substrate in this research. The research reported that 42.87 g/L of reducing sugar was obtained when 70% (w/v) of cassava waste used.

The effects of substrate concentration on enzymatic hydrolysis were investigated at a fixed ratio of cellulase from *Trichoderma reesei* to substrate (corncob). As shown in Figure 2.5, reducing sugar concentration and hydrolysis yield showed an opposite

variation trend, with substrate concentration increasing, the reducing sugar concentration increased but the hydrolysis yield decreased. This may be due to the end product feedback inhibition caused by high reducing sugar concentration (Chen *et al.*, 2007).

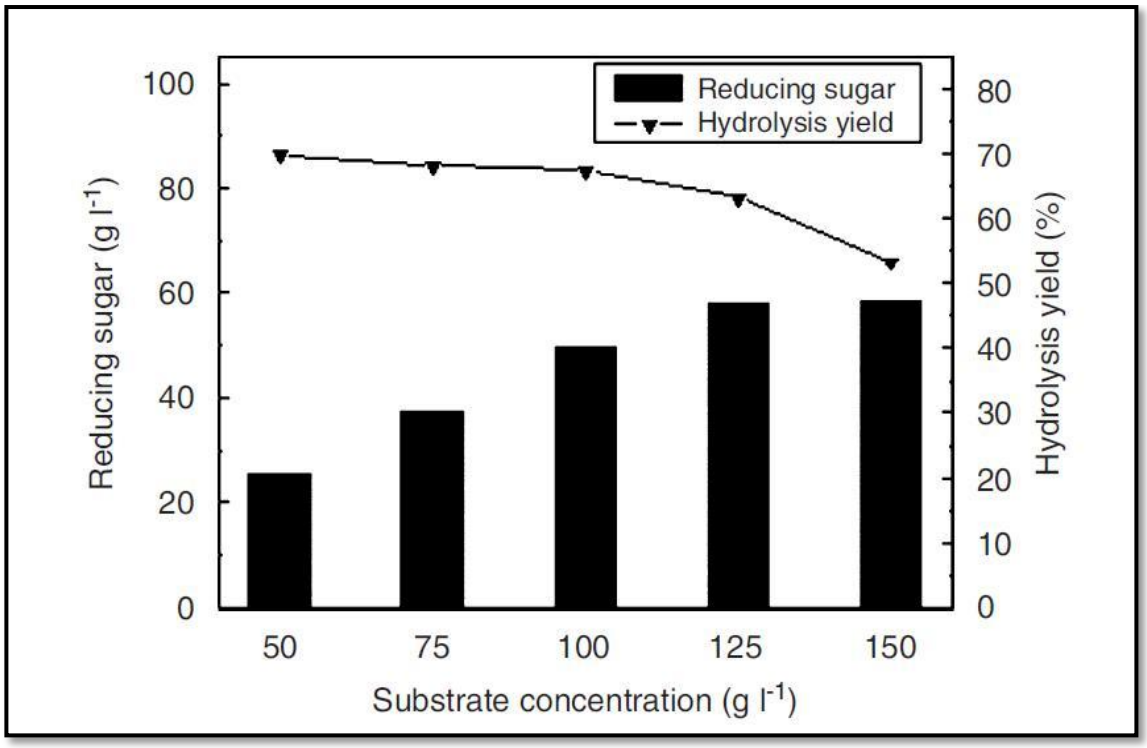

Figure 2.5: Effects of substrate concentration on the enzymatic hydrolysis of corncob

2.6.3 Effect of Enzyme Dose on Reducing Sugar Production

In the research of enzymatic hydrolysis on waste paper office, it is reported that the highest glucose concentration was reported about 2.6 g/L when enzyme was 10% loading. Figure 2.6 shows the glucose concentration obtained when enzyme dose is increased. The black bar indicated the glucose concentration. This graph shows the increasing of glucose concentration when the enzyme dose is increased (Park *et al.*, 2002).

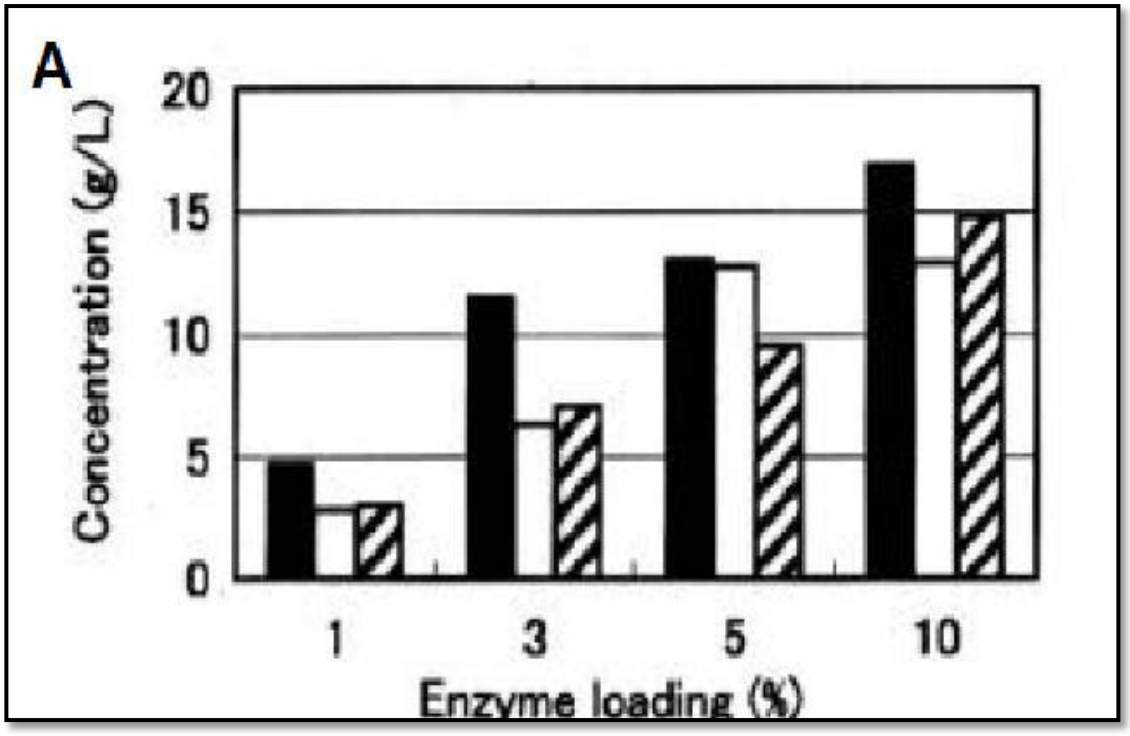

Figure 2.6: Effect of the percentage of enzyme loading in glucose concentration

In other research, Ouyang *et al* .(2009) also studied the effect of enzyme dosage on reducing sugar production, with the aim of manufaturing chemicals and energy. The substrate dosage (waste corncob residue) was fixed and the enzyme dosage was varied. The cellulase was produced from *Trichoderma reesei.* The study reported reducing sugar production during the hydrolysis of corncob residue at different enzyme dosage increased as the enzyme increased.

2.7 Reducing Sugar Analysis

Reducing sugar analysis is a method to test the presence and to measure the amount of the reducing sugar in a solution. Zoecklein (1995) reported that reducing sugar may be determined by chemical, enzymatic and high liquid performance chromatoghraphic (HPLC).

Chemical method involves chemical reaction between reducing sugar with copper (II) in alkaline solution. Benedict's reagent and Fehling's solution are test reagents used to test the presence of a reducing sugar. The reducing sugar reduces Cu^{2+} ions in these test solutions to Cu^{+}, which then forms a brick red copper (I) oxide precipitate. The solution should progress in the colors of blue (with no glucose present), green, yellow, orange, red, and then brick red or brown (with high glucose present). A color change would signify the presence of glucose (Benedict, 1908).

The 3,5-Dinitrosalicylic acid (DNS reagent) is another test reagent that allows quantitative spectrophotometric measurement of the amount of reducing sugar present. The 3,5-dinitrosalicylic acid reagent was first used by Sumner (1925) for the determination of sugar in urine. In the recommended procedure, 1 ml of sample and 3 ml DNS reagent were mixed, boiled for 5 min, cooled and diluted with distilled water to a final volume of 25, 50 or 100 ml in order to bring the colour developed into a readable range (Bailey, 1988). The darkest colour indicates high reducing sugar present.

2.8 Optimization using Response Surface Methodology (RSM)

Response Surface Methodology (RSM) is used in optimizing the conditions of tested variables in maximizing the response of an experiment. Many reports revealed by using RSM, the response is maximized. Beside, the period of research also decreased. In other ways, RSM helps in saving time and money.

Liu *et al*., (2003) did a research on *Optimization of Glucose Oxidase (GOD) Production by Aspergillus niger in a Benchtop Bioreactor Using Response Surface Methodology* (RSM). RSM was applied to optimize the speed of agitation and the rate of aeration for the maximum production of glucose oxidase. A 2^2 central composite design using RSM was applied in the investigation. The maximum level of GOD was achieved when the speed of agitation and the rate of aeration were 756 rev min)L and 0.9 v/v/m, respectively. The glucose oxidase was reported about 4.7 U/mL compared to before optimization, the glucose oxidase was only 2.5 U/mL.

Adinarayana and Ellaiah (2002) have demonstrated the use of central composite factorial design by determining the conditions leading to the high yield of enzyme production in their research entitled 'Response Surface Optimization Of The Critical Medium Components For The Production Of Alkaline Protease By A Newly Isolate *Bacillus* sp.' The optimum values for the tested variables for the maximum alkaline protease production were; glucose 7.798 (g/L), peptone 9.548 (g/L) and salt solution 8.757%. The maximum alkaline protease production was 4,98,123 PU/L. This method was efficient with only 20 experiments were necessary to assess these conditions and model adequacy was very satisfactory, as the coefficient of determination was 0.941. From the research, it can be concluded that smaller and less time consuming experimental designs could generally suffice for the optimization of many fermentation processes.

Statistically based experimental design was employed for the optimization of fermentation conditions for maximum production of enzyme tannase from *Aspergillus niger*. Central composite rotatable design (CCRD) falling under response surface methodology (RSM) was used. Based on the results of 'one-at-a-time' approach in submerged fermentation, the most influencing factors for tannase production from *A. niger* were concentrations of tannic acid, concentration of sodium nitrate, agitation rate and incubation period. Hence, to achieve the maximum yield of tannase, interaction of these factors was studied at optimum pH of 5.0 by RSM. The optimum values of parameters obtained through RSM were 5% tannic acid, 0.8% sodium nitrate, 5.0 pH, 5 x I07 spores/50mL inoculum density, 150 rpm agitation and incubation period of 48 h which resulted in production of 19.7 U/mL of the enzyme. This activity was almost double as compared to the amount obtained by 'one- at- a- time' approach (9.8 U/mL) (Sharma *et al*., 2007).

CHAPTER 3

METHODOLOGY

3.1 Procedures on Optimization of Glucose Production from Sugarcane Bagasse Using Response Surface Methodology (RSM)

In this research, the procedures have been divided into four steps in order to achieve the objective of the research. Firstly, the glucose calibration curve was obtained by plotting optical density at 540 nm versus concentration of glucose. In the second step, the alkali pretreatment process was done on the sugarcane baggase to remove lignin and hemicelluloses. Then, the screening process was done to get the best range of parameters (temperature, substrate dose and enzyme dose) using conventional method. Enzymatic hydrolysis was applied in this step. Lastly, the optimization on glucose production using Response Surface Methodology (RSM) was carried out. Figure 3.1 shows the overall process of experiment for production of glucose from sugarcane bagasse using RSM.

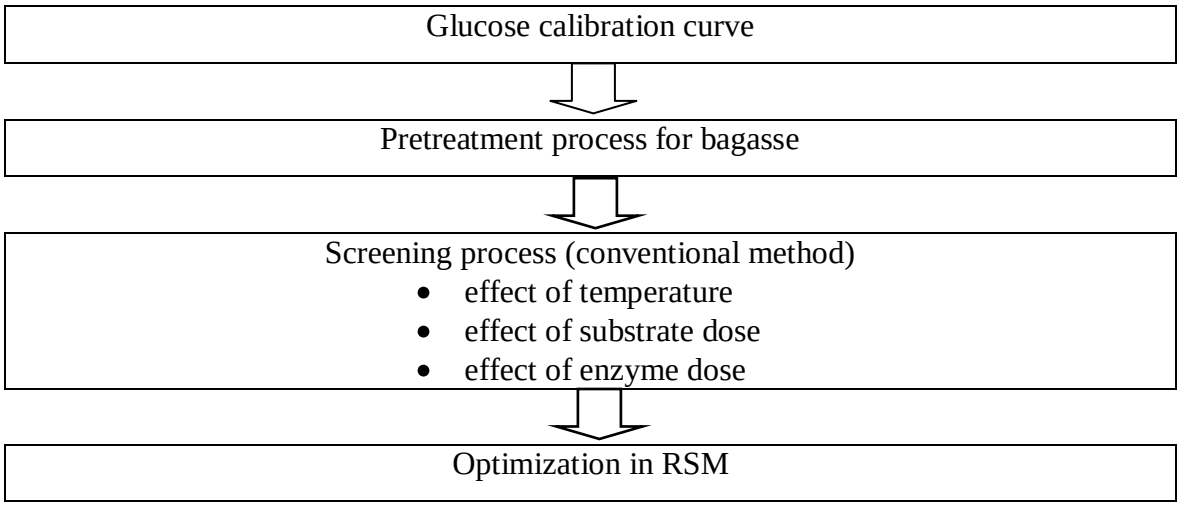

Figure 3.1 Process flow on optimization of glucose production from sugarcane bagasse using Response Surface Methodology

3.2 Chemicals, Apparatus and Equipments

In this research, all chemicals, apparatus and equipments were supplied by Faculty of Chemical and Natural Resources Engineering. The chemicals used were bought from Sigma, Merck and Fluka companies. Chemicals used in this research were sodium hydroxide, hydrogen peroxide, D-glucose, Dinitrosalicyclic (DNS) reagent, acetate buffer and cellulase enzymes from *Aspergillus niger*.

Apparatus used were volumetric flasks, conical flasks, beakers, measuring cylinders, glass funnel and pipette. Equipments used were analytical balance, autoclave Hirayama, shaking water bath and UV-Vis Spectrophotometer Hitachi U-1800.

3.3 Pretreatment Process

Bagasse was collected from Tanah Putih village in Kuantan and let to dry before pretreatment process begins. Baggase is high in moisture. It is important to ensure the baggase is fully dried before proceeding to the next steps. In the pretreatment process, the dried bagasse was grinded in the blender and sieved to attain 0.5 mm size of mesh powder. The smaller size of bagasse would increase surface area for interaction of enzyme in enzymatic hydrolysis step.

It was treated with hydrogen peroxide and sodium hydroxide (H_2O_2 + 1.5% NaOH) separately with 1:10 (w/v) ratio in an autoclave at 121°C for 15 minutes. After treatment, the baggase were washed with distilled water to neutralize the effects of chemical. Lastly, the baggase were dried in an oven for 12 hours at 75°C. The dried baggase should be stored in bottles before use for the enzymatic hydrolysis process. The dried baggase contained cellulose, which is the substrate needed in this research. The total amounts of substrates after treatment were 400 g. Figure 3.2 to 3.6 illustrate the alkaline pretreatment process.

Figure 3.2: Grinded sugarcane bagasse before pretreatment process

Figure 3.3: Sieving process of the bagasse to attain 0.5 mm size of mesh powder

Figure 3.4: Mixture of bagasse and alkali solution before autoclave

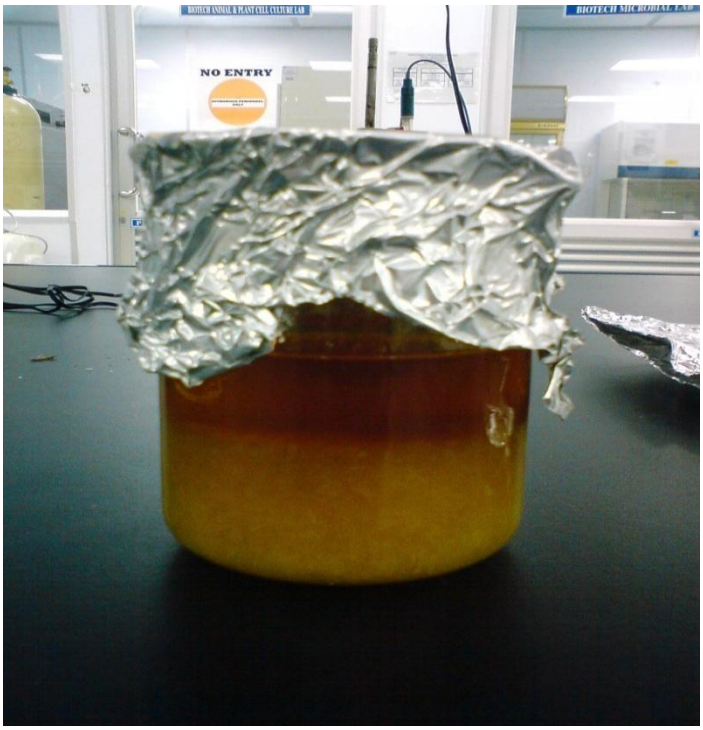

Figure 3.5: Mixture of bagasse and alkali solution after autoclave

Figure 3.6: The cellulose obtained after alkaline pretreatment

3.4 Glucose Calibration Curve

The calibration curve had been plotted to serve as guideline for glucose concentration. Fifty milligrams of commercial D-glucose was dissolved in 50 mL acetate buffer solution (0.1 M) to prepare the stock solution. The different concentration of solution 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL were prepared from the stock solution. For each concentration, three milliliters were added with three milliliters of DNS reagent. The samples were tested using the UV-Vis Spectrophotometer at 540 nm. The graph of Optical Density (OD) versus concentration of glucose was plotted.

3.5 Screening Process for Temperature, Substrate Dose and Enzyme Dose on Glucose Production Using Conventional Method

In the enzymatic hydrolysis, the cellulose will be converted into glucose using cellulase enzyme. The process had been done by varying one parameter and made it constant for another two. For each parameter, the best ranges would be selected in the study and will be used in optimization process.

3.5.1 Effect of Temperature on Glucose Production

Two gram of dried substrate was placed in glass vessel contained 0.56 g of cellulase enzyme from *Aspergillus niger* and then was mixed with 100 mL of 0.1 M acetate buffer solution. The vessel holding approximately 100 g of material at pH 4.8 was then kept in shaking water bath at 30°C and it was continuously shaken for 48 hours at 100 rpm. The experiment was repeated for 35, 40, 45 and 50°C. The sample was analyzed using Dinitrosalicyclic Colorimetric (DNS) method. The graph of glucose concentration versus temperature was plotted.

3.5.2 Effect of Substrate Dose on Glucose Production

The experiment was continued with the effect of substrate dose. The enzyme dose was set constant at 0.56 g in this experiment. The varying dose of substrate (1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 g) were used in this experiment. The mixture of enzyme and substrate were dissolved in 100 mL of acetate buffer solution. Then, it was kept in water bath at constant temperature 45°C for 48 hours at 100 rpm. The sample was analyzed

using DNS method. The graph of glucose concentration versus substrate dose was plotted.

3.5.3 Effect of Enzyme Dose on Glucose Production

The experiment was continued with the effect of enzyme dose. A constant dose of substrate, 2 g were mixed with different dose of enzyme which were 0.2, 0.4, 0.6, 0.8 and 1.0 g. The same procedures of dissolving the mixtures in 100 mL buffer solution and kept in water bath at constant temperature 45°C for 48 hours at 100 rpm. The sample was analyzed using DNS method. The graph of glucose concentration versus enzyme dose was plotted.

3.6 Optimization of Temperature, Substrate Dose and Enzyme Dose on Glucose Production Using Response Surface Methodology (RSM)

After the screening procedure was completed, the optimization was done by using the Response Surface Methodology (RSM). The low values and high values from each parameter were selected from the screening process. There were 17 experiments that was arranged by RSM need to be carried out. Table 3.1 shows the values that were used during optimization in RSM.

Table 3.1: Low and high values for optimization in Response Surface Methodology

Experimental range and levels of the independent variables		
Variables	**Range and levels**	
	Low	**High**
Temperature (°C)	45	55
Substrate Dose (g)	1.3	1.9
Quantity of enzyme (g)	0.4	0.8

3.7 Dinitrosalicyclic Colorimetric Method (DNS Assay)

After experiments, the materials inside the vessels were analyzed with Dinitrosalicyclic Colorimetric Method (DNS Assay) in order to test the concentration of glucose in each experiment. DNS reagent consists of 10 g of DNS acid, 2 g of phenol, 0.5 g of sodium sulfite and 10 g of sodium hydroxide. These chemicals were dissolved in one liter distilled water. The DNS reagent should be stored in dark place. Three milliliters of DNS reagent was added to 3 ml of samples in capped test tube. The mixture was heated at 90°C for 10 minutes to develop the red-brown colour. After cooling to room temperature in cold water bath, the absorbance was recorded with a UV-Vis Spectrophotometer at 540 nm of wavelength with distilled water as the blank. Figure 3.7 and 3.8 show the existence of reducing sugar in this hydrolysis. The red brown colour showed the presence of reducing sugar in the solution.

Figure 3.7: The glucose plus DNS reagent before heating

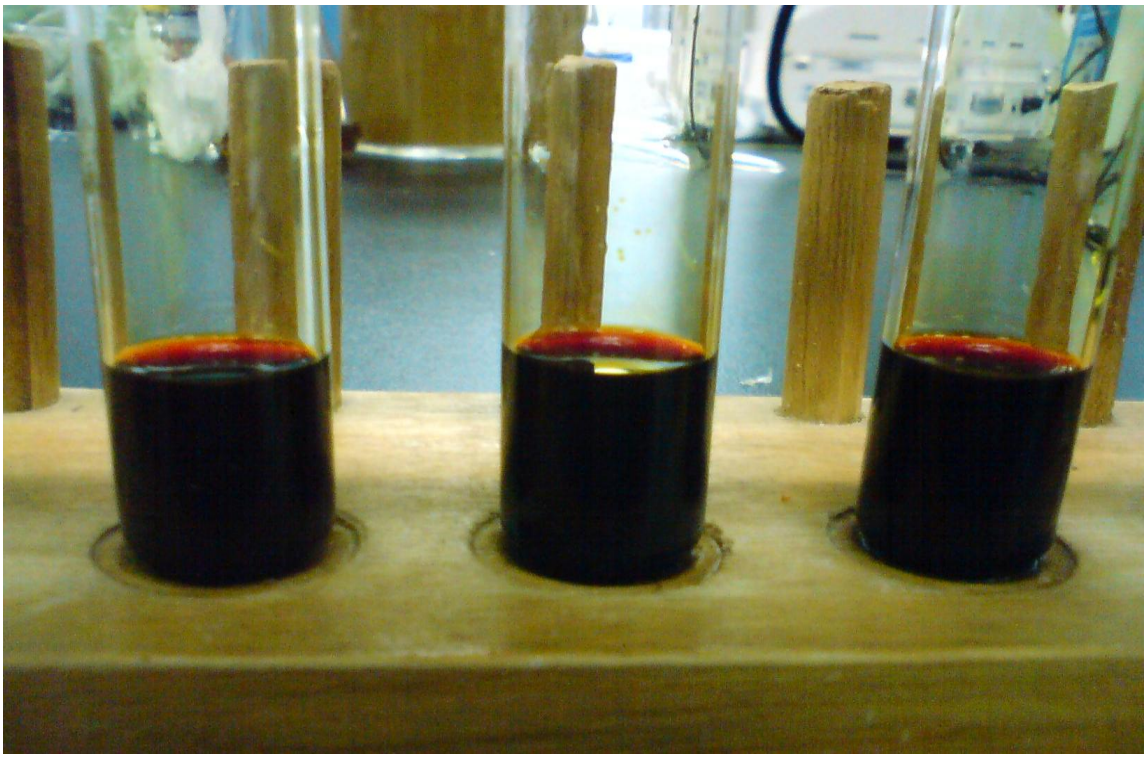

Figure 3.8: The glucose plus DNS reagent after heating

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Glucose Calibration Curve

Glucose calibration curve is used to determine the concentration of glucose in an unknown sample by comparing the unknown to a set of standard samples of known concentration. The absorbance values obtained by using the UV-Spectrophotometer were plotted against the concentration of glucose. The following Equation 4.1 was obtained.

$$y = 0.5415x - 0.3854 \quad \textbf{(Equation 4.1)}$$
$$with\ R^2 = 0.9922$$

Where:

y= absorbance values

x= concentration of glucose (g/L)

Figure 4.1 below shows the calibration curve of glucose for this experiment. The graph was plotted with absorbance versus concentration of glucose.

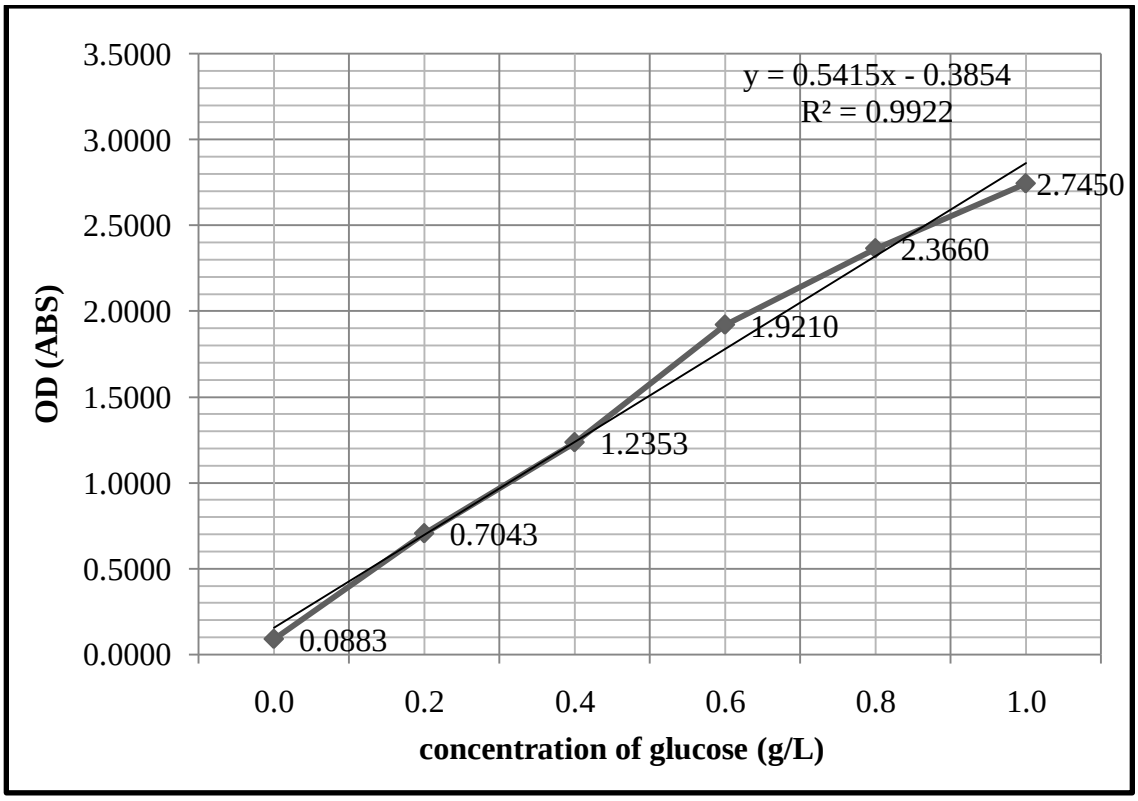

Figure 4.1: The calibration curve of glucose

4.2 Pretreatment Process

The bagasse has been treated with hydrogen peroxide + 1.5% sodium hydroxide to remove the lignin and the hemicelluloses and remain celluloses. The chemical pretreatments solubilized the lignin and the hemicellulose in biomass. This process is known as Xylan Delignification Process (XDP) (Dale *et a.l*, 1995).

Alkali pretreatment refers to the application of alkaline solutions such as sodium hydroxide, lime or ammonia to remove lignin and a part of the hemicelluloses and efficiently increases the accessibility of enzyme to the cellulose (Taherzadeh and Karimi, 2008). Higher temperatures and shorter reactions times were also shown to effectively pretreat lignocellulose with alkali (Mosier *et al*., 2005). About 400 g of cellulose has been obtained overall. The experiment has been continued by enzymatic hydrolysis to produce glucose.

4.3 Screening Process of Temperature, Substrate Dose and Enzyme Dose Using Conventional Method

The screening process need to be done to get the best range of parameters of studies in glucose production for next optimization process. The parameters studied in this research were the temperature, substrate dosage and enzyme dosage. The substrate in this whole discussion is referring to cellulose from sugarcane bagasse and the enzyme refers to cellulase enzyme from *Aspergillus niger*. This screening process was a conventional method. The process had been done by varying two parameters and made it constant for another one. For each parameter, the low value and the high value for optimization process would be selected before and after the maximum peak from the screening process.

4.3.1 The Effect of Temperature on Glucose Production

The effect of temperature was studied conventionally to get the best range of temperature on production of glucose. The temperature was manipulated at 35, 40, 45, 50 and 55°C while the other two parameters, substrate dose and enzyme dose were kept constant at 2.0 g and 0.56 g respectively. Figure 4.2 shows the graph plotted of the concentration of glucose at different temperature.

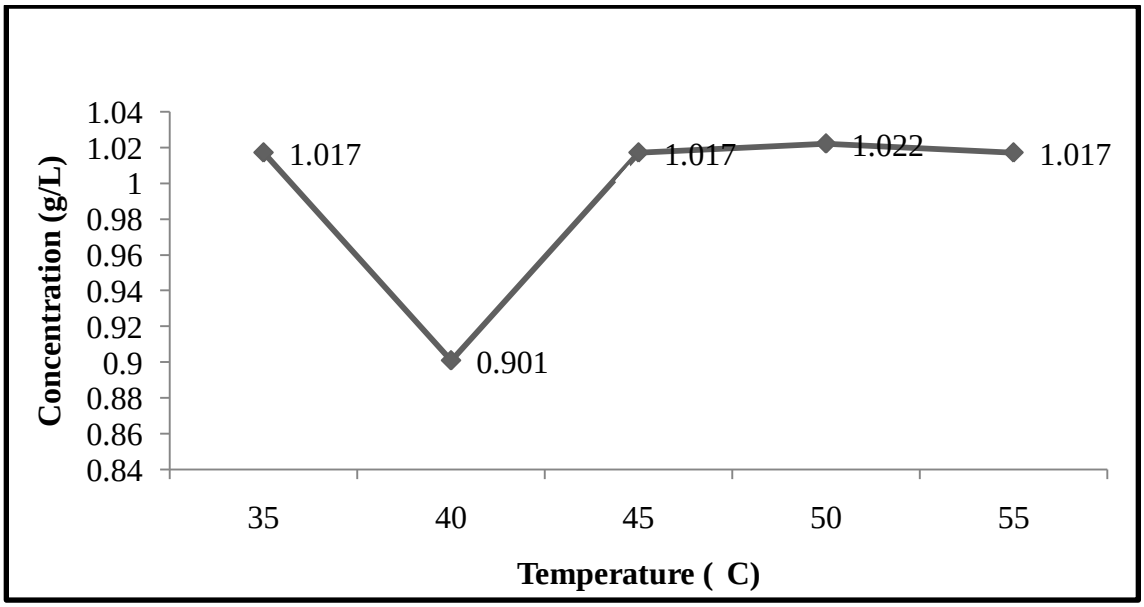

Figure 4.2: The effect of temperature on glucose production

From the graph, the concentration of glucose declined from 1.017 g/L to 0.901 g/L at 35°C to 40°C. From 45°C - 55°C, the concentration of glucose is about constant. From the graph, the peak is at 50°C where the concentration of glucose is 1.022 g/L. The graph shows that the glucose concentration increased with the increasing of the temperature. The best range of temperature chosen was from 45-55°C. This pattern of graph is supported by research on bioconverting lignocellulosic materials into ethanol in the simultaneous saccharification and fermentation system using cellulase from

Penicillium decumbens (Chen and Jin, 2006). The research reported that as the temperature increased, the production of reducing sugar also increased.

Increasing temperature has two major effects on enzymes. The first effect is the catalytic rate increases and the other one is ultimately enzyme denaturation occurs (Thomas and Scopes, 1998). As the temperature rises, reacting molecules have more and more kinetic energy. This increases the chances of a successful collision and so the rate increases. There is a certain temperature at which an enzyme's catalytic activity is at its greatest. Above this temperature the enzyme structure begins to break down (Najafpour and Shan, 2002). Denaturation of enzyme will stop the catalytic reaction.

4.3.2 The Effect of Substrate Dose on Glucose Production

The effect of substrate dose was studied conventionally to get the best range. The substrate dose was manipulated at 1.0, 1.2, 1.4, 1.6 and 1.8 g while the temperature and enzyme dose were kept constant at 45°C and 0.56 g respectively. The graph plotted in Figure 4.3 shows the concentration of glucose at different substrate dose.

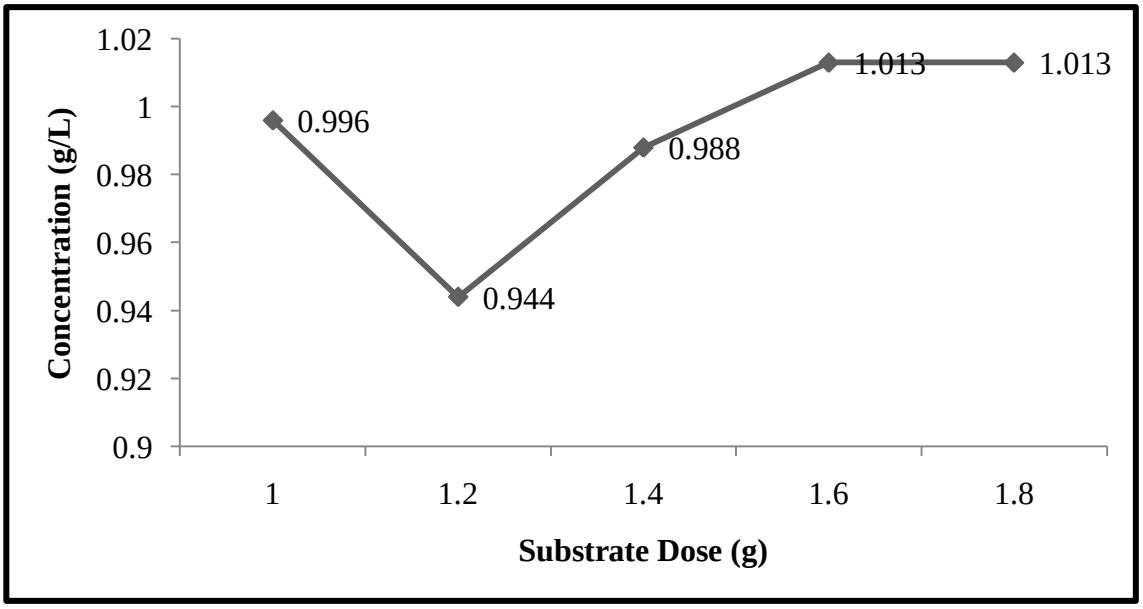

Figure 4.3: The effect of substrate dose on glucose production

The graph shows that increasing the substrate dose will also increase the glucose concentration. At high dose (1.6 g - 1.8 g), the glucose production was constant at 1.013 g/L. The best range of substrate dose was selected from 1.3 g to 1.9 g as the peak value was at 1.6 g. This pattern of graph was supported by Fungsin *et al*. (2009) on the study of the bioconversion of cassava waste into sugars by using *Aspergillus niger* TISTR 3352 and *Trichoderma reesei* TISTR 3080. The research reported that when maximum substrate dose was hydrolyzed using those microbial enzyme, maximum reducing sugar was obtained. This shows that introducing high dose of substrate in the enzymatic hydrolysis will produce high production of reducing sugar.

4.3.3 The Effect of Enzyme Dose on Glucose Production

The effect of enzyme dose was studied conventionally to get the best range. The enzyme dose was manipulated at 0.2, 0.4, 0.6, 0.8 and 1.0 g while the temperature and substrate dose were kept constant at 45°C and 2.0 g respectively. Figure 4.4 shows the concentration of glucose at different enzyme dose.

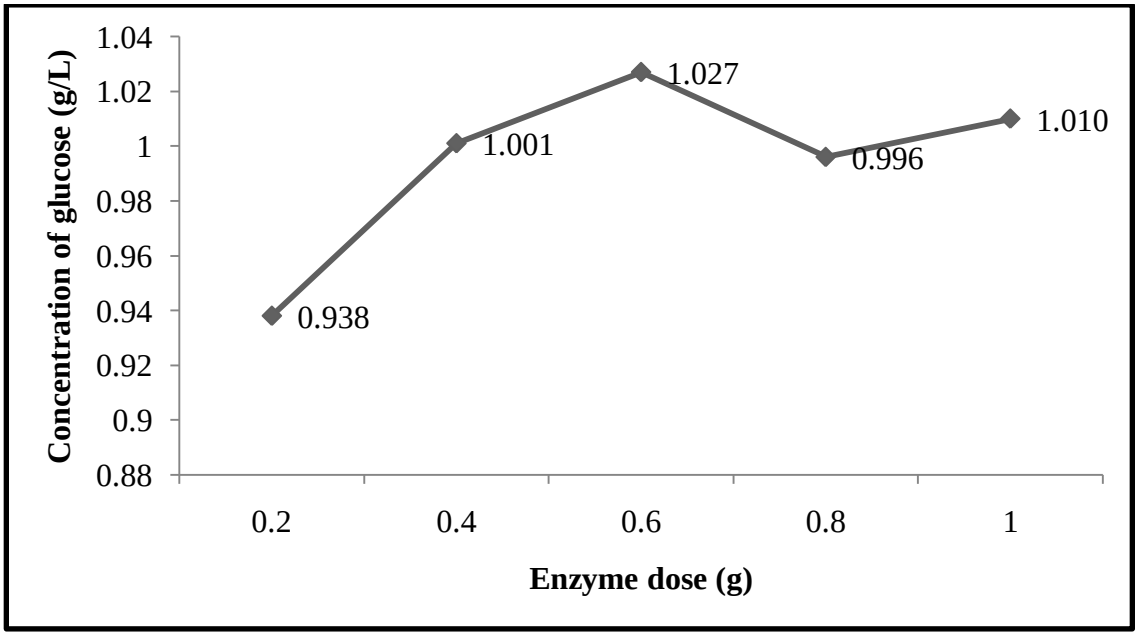

Figure 4.4: The effect of enzyme dose on glucose production

From the graph, the highest glucose production was detected at 0.6 g of enzyme dose with the glucose production was 1.027 g/L. The graph shows that high enzyme dose in the enzymatic hydrolysis will increase the production of glucose. The best range of enzyme dose was selected from 0.4 g to 0.8 g. From previous study, the high glucose yield was obtained when the high enzyme dose was introduced (Najafpour and Shan, 2002). The study investigated the enzymatic hydrolysis of molasses using glucoamylase as the enzyme. The study reported that the enzymatic hydrolysis reaction rate (the production rate of reducing sugar) increased proportionally with the amount of

glucoamylase present. The pattern of graph in the previous study supported the effect of enzyme dose on glucose production from sugarcane bagasse.

Table 4.1 shows the low and high values obtained from screening process using conventional method. As a conclusion, the low and high values would be used in the Response Surface Methodology (RSM) to determine the optimized conditions for optimum production of glucose.

Table 4.1: The low and high values from screening process

Experimental range and levels of the independent variables		
Variables	**Range and levels**	
	low	**high**
Temperature (°C)	45	55
Substrate Dose (g)	1.3	1.9
Enzyme Dose (g)	0.4	0.8

4.4 Analysis of Temperature, Substrate Dose and Enzyme Dose using Response Surface Methodology (RSM)

The Design-Expert package (Version 7.0.2, 2006; Stat-Ease, Minneapolis, MN, USA) was employed for regression analysis of the data and for estimation of the coefficients of the regression equation. For this study, The Central Composite Designs (CCD) with one axial (star) point, three center point and one replicate at the center point were employed to fit the second order polynomial model with 17 experiments were required for this procedure.

By using RSM, the regression equation (Equation 4.2) was obtained from the analysis of variance (ANOVA).

$$\begin{aligned} \textit{concentration of glucose} = {} & + 5.35953 + 0.014873A - 2.75945B \\ & + 6.56863C + 4.17203E\text{-}004A^2 \\ & + 0.93103B^2 + 0.12375C^2 + 5.08333E \\ & - 003AB - 0.11062AC - 0.61458BC \end{aligned} \quad \textbf{(Equation 4.2)}$$

The term A indicates the coded value for the temperature, B is the coded value for substrate dose and C is the coded value for enzyme dose. The highest glucose production was at Standard 12 which produced 5.877 g/L glucose. The parameters for the Standard 12 were 50°C of temperature, 2.1 g of substrate dose and 0.6 g of enzyme dose.

The lowest value of glucose production was at Standard 1 which produced 5.478 g/L glucose. The parameters for the Standard 1 were at 45°C of temperature, 1.30 g of

substrate dose and 0.40 g of enzyme dose. The combination data from RSM and the responses were illustrated as in Table 4.2.

Table 4.2: Combination data arranged by Response Surface Methodology (RSM)

Std	Factor 1 Temperature (°C)	Factor 2 Substrate Dose (g)	Factor 3 Enzyme Dose (g)	Response 1 Glucose Concentration (g/L)
1	45.00	1.30	0.40	5.478
2	55.00	1.30	0.40	5.700
3	45.00	1.90	0.40	5.630
4	55.00	1.90	0.40	5.843
5	45.00	1.30	0.80	5.860
6	55.00	1.30	0.80	5.600
7	45.00	1.90	0.80	5.825
8	55.00	1.90	0.80	5.635
9	41.59	1.60	0.60	5.658
10	58.41	1.60	0.60	5.600
11	50.00	1.10	0.60	5.796
12	50.00	2.10	0.60	5.877
13	50.00	1.60	0.26	5.529
14	50.00	1.60	0.94	5.698
15	50.00	1.60	0.60	5.598
16	50.00	1.60	0.60	5.598
17	50.00	1.60	0.60	5.598

The elevation (residual) between actual data (experimental data) and predicted data was shown in Table 4.3. The predicted data was a comparison to the actual data from the experiment.

Table 4.3: The Residual Between Actual and Predicted Value

Diagnostics Case Statistics			
Standard order	**Actual value**	**Predicted value**	**Residual**
1	5.48	5.49	-0.0156
2	5.70	5.68	0.0169
3	5.63	5.62	0.0147
4	5.84	5.84	0.0077
5	5.86	5.87	-0.0096
6	5.60	5.62	-0.0166
7	5.83	5.84	-0.0188
8	5.64	5.62	0.0137
9	5.66	5.64	0.0165
10	5.60	5.61	-0.0138
11	5.80	5.78	0.0139
12	5.88	5.89	-0.0113
13	5.53	5.54	-0.0150
14	5.70	5.68	0.0177
15	5.60	5.60	-0.0002
16	5.60	5.60	-0.0002
17	5.60	5.60	-0.0002

The results of the second order response surface model in the form of analysis of variance (ANOVA) are given in the Table 4.4.

Table 4.4 : Analysis of Variance (ANOVA) for response surface quadratic model of glucose production

Source	**Sum of Squares**	**DF**	**Mean Squares**	**F Value**	**Prob > F**	
Model	0.23	9	0.026	***59.24***	< 0.0001	*significant*
A	9.28E-04	1	9.28E-04	2.14	0.1872	
B	0.014	1	0.014	31.37	0.0008	
C	0.022	1	0.022	51.63	0.0002	
A^2	1.23E-03	1	1.23E-03	2.83	0.1367	
B^2	0.079	1	0.079	182.35	< 0.0001	
C^2	2.76E-04	1	2.76E-04	0.64	0.4512	
AB	4.65E-04	1	4.65E-04	1.07	0.335	
AC	0.098	1	0.098	225.54	< 0.0001	
BC	0.011	1	0.011	25.06	0.0016	
Residual	3.04E-03	7	4.34E-04			
Lack of Fit	3.04E-03	5	6.08E-04			
Pure Error	0	2	0			
Cor Total	0.23	16				

Values of Prob>F less than 0.05 indicate model terms are significant. In this case, the liner term of substrate dose (B), enzyme dose (C), squared terms of substrate dose (B^2), the interaction term of temperature and enzyme dose (AC) as well as the interaction term of substrate and dose enzyme dose (BC) are significant model. The term A, A^2, C^2 and interaction term of AB are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

The Fisher *F*-test with a very low probability value Prob>F = <0.0001 demonstrate a high significance for the model. The goodness of fit of the model was checked by the determination coefficient, R^2. In this case, the value of the determination coefficient R^2 = 0.9870 indicates that only 1.3% of the total variations are not explained by the model. The value of the adjusted determination coefficient (Adj. R^2 = 0.9704) is also high to advocate for a high significance of the model. A higher value of the correlation coefficient, *R*=0.8963, justifies an excellent correlation between the independent variables. The 'Pred $R^{2'}$ of 0.8963 is in reasonable agreement with the 'Adj R^{2}' of 0.9704. 'Adeq Precision' measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 24.697 indicates an adequate signal. This model can be used to navigate the design space. The application of response surface methodology produced the Equation 4.2, which is a relation between the concentration of glucose and the test variables.

The yield values for different concentrations of the variables can also be predicted from the respective response surface contour plots. The three-dimensional (3D) graphical represent the system behavior, called the response surface, was used to describe the individual and the cumulative effects of the variables as well as the mutual interactions between the independent variables and the dependent variables (Li *et al.*, 2007). Figures 4.5 – 4.7 show the 3D interaction between temperature, substrate dose and enzyme dose based on the seventeen experiments carried out previously.

Figure 4.5 shows the combined effect of enzyme dose and the temperature on the production of glucose.

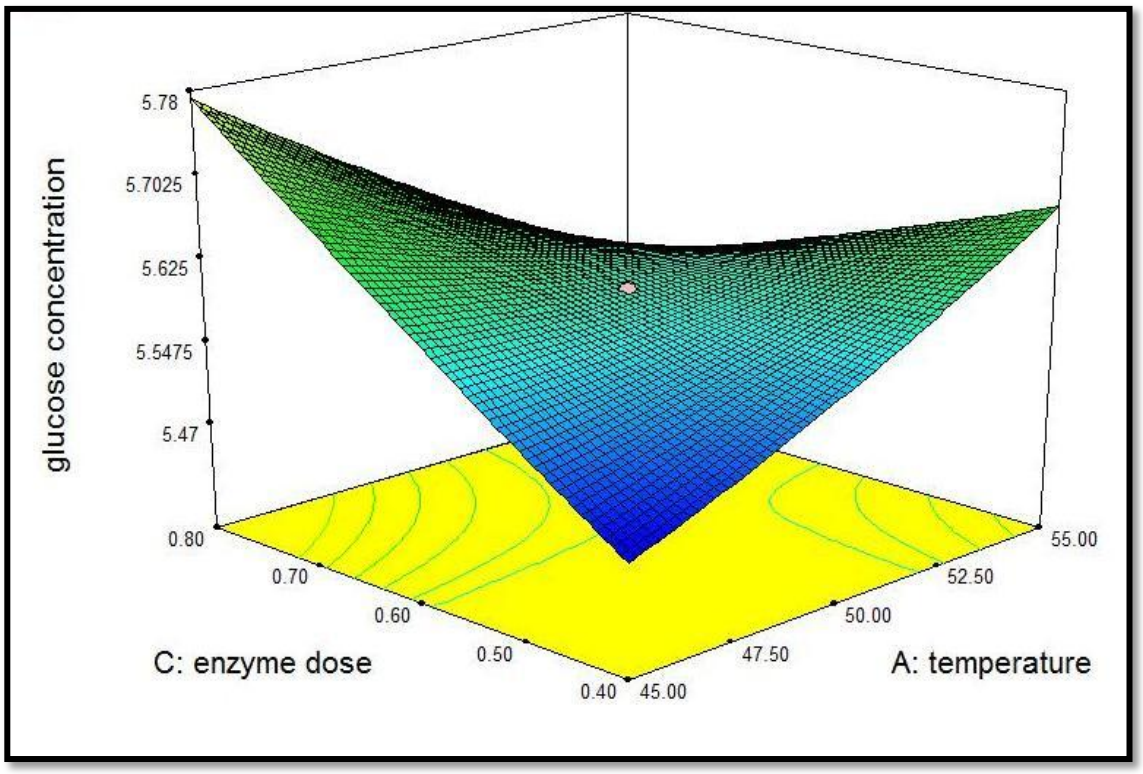

Figure 4.5: Response surface plot of the combined effect of enzyme dose and the temperature on the production of glucose

The graph shows that the glucose production increased when the temperature was increased. The optimum temperature was at 55°C with the glucose production was about 5.78 g/L. Similar result was supported by Le Man *et al*. (2010) in the research of ethanol production from Korean food waste leachate by using *Saccharomyces cerevisiae* as enzyme. The research aimed to optimize the temperature for the production. The research reported that by increasing the temperature, the ethanol concentration also increased.

Figure 4.6 shows the combined effect of substrate dose and the temperature on the production of glucose.

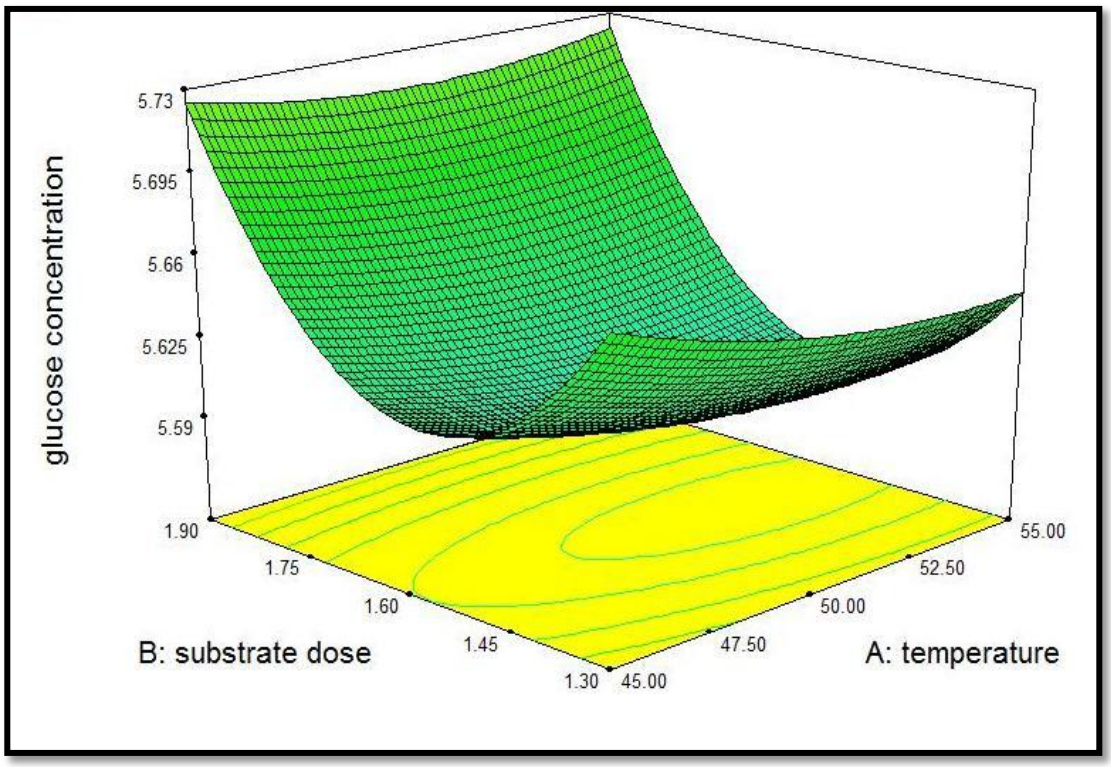

Figure 4.6: Response surface plot of the combined effect of substrate dose and the temperature on the production of glucose

Based on the Figure 4.6, the optimum value of substrate dose was positioned at the green yellowish area. The glucose production was recorded about 5.73 g/L (high value) at 1.9 g of substrate dose (high dose). The glucose production increased as the substrate dose increased. Similar result reported in the study of enzymatic hydrolysis of corncob and ethanol production (Chen *et al*., 2007). Cellulase from *Trichoderma reesei* ZU-02 was used in the hydrolysis of corncob. The study reported that when the substrate concentration increased, the reducing sugar concentration also increased.

Figure 4.7 shows the combined effect of enzyme dose and the substrate dose on the production of glucose.

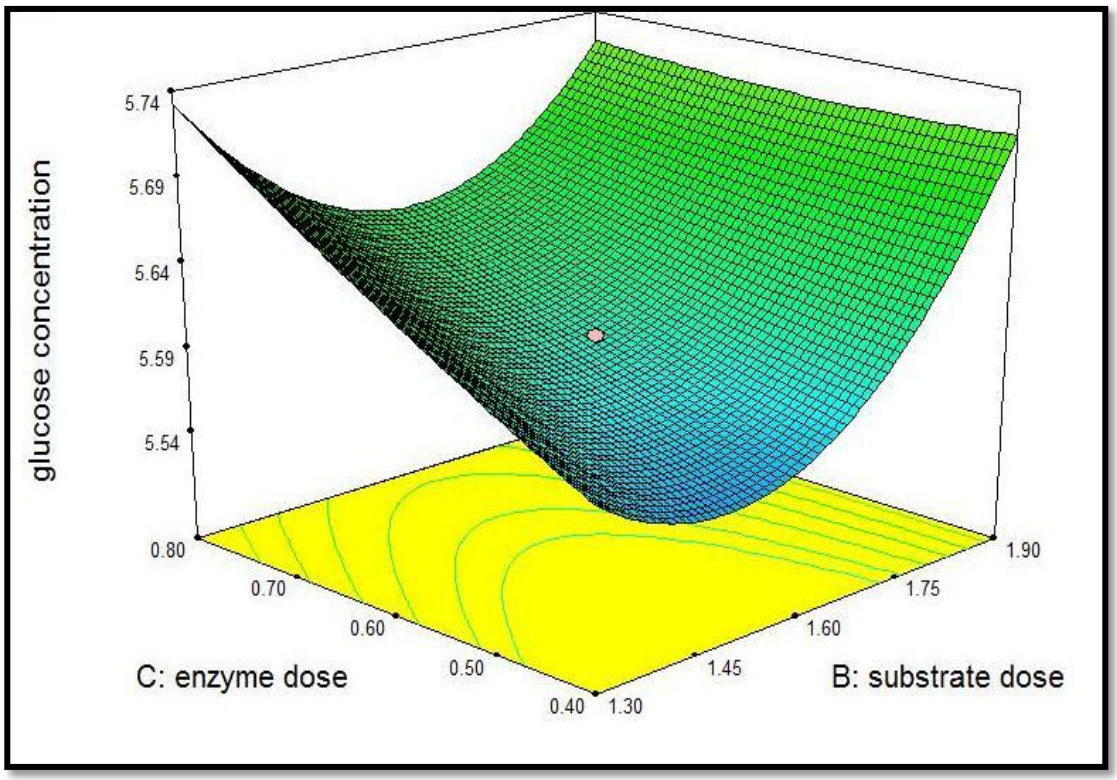

Figure 4.7: Response surface plot of the combined effect of enzyme dose and substrate dose on the production of glucose

From Figure 4.7, at 0.80 g of enzyme dose (high dose), the glucose production was measured at 5.78 g/L. The glucose production increased proportionally with the enzyme dose. The enzymatic hydrolysis on pure cellulose (Avicel PH-101) using Spezyme® CP cellulase enzyme in the previous study showed the effect of enzyme dose on glucose production (Kumar and Wyman, 2008). The study reported that as the mass of cellulase increased, the glucose production also increased. Saha and Cotta (2006) also supported the study through the research on ethanol production from wheat straw via enzymatic saccharification. The effect of enzyme dose was studied to see the sugar yield for the ethanol production. The enzymes used were Celluclast 1.5 L, Novozyme

188 and Viscostar 150 L. The study described that the sugar yield was better with higher enzyme dose. These proved that as the enzyme dose increased, the production of glucose also increased.

Theoretically, if there is no response after some addition of enzyme to fixed amount of substrate, it indicates that the amount of enzyme is not sufficient to the substrate. More addition of enzyme is needed to provide more active site to the substrate. The substrate will undergo reaction if enzyme dose is sufficient (Rupert, 1962)

4.5 Optimization of Temperature, Substrate Dose and Enzyme Dose using Response Surface Methodology (RSM) on Glucose Production

The result from the optimization step using Response Surface Methodology (RSM) showed that the optimized condition for maximum glucose production were 45°C of temperature, 1.3 g of substrate dose and 0.8 g of enzyme dose. The optimize glucose production predicted by the model was 5.8672 g/L. An experiment need to be done in order to validate this optimized condition. The glucose production obtained from the experimental was 5.725 g/L. Table 4.5 shows the optimization of glucose production from sugarcane bagasse using RSM.

Table 4.5: Summary of optimization of glucose concentration using Response Surface Methodology

Parameter	Before optimization		After optimization		
	Value	Glucose concentration (g/L)	Value	Glucose concentration (g/L)	
				Predicted	Actual
Temperature	45°C	1.0100	45°C	5.8672	5.7250
Substrate dose	2.0 g		1.3 g		
Enzyme dose	1.0 g		0.8 g		

From the table, the tested parameters for glucose production were optimized. Before the optimization process, the parameters were 45°C of temperature, 2.0 g of substrate dose and 1.0 g of enzyme dose with the glucose production was only 1.010 g/L. After optimization and validation, the glucose production increased to 5.725 g/L. The percentage increment was 82.36% compared to the value before optimization. At same temperature, the substrate dose decreased from 2.0 g to 1.3 g. For the enzyme dose, it also decreased from 1.0 g to 0.8 g. As a conclusion, the objective of this research to optimize the glucose production from sugarcane bagasse using Response Surface Methodology (RSM) was achieved.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This research on optimization of glucose production from sugarcane bagasse using Response Surface Methodology (RSM) was successfully met the objective requirement. This research gave the view of differences between the conventional method and by using RSM.

The conventional method for screening of the parameters on glucose production showed that the best range of temperature was between 45°C - 55°C. The best range for substrate dose was between 1.3 g – 1.9 g and the best range for enzyme dose was between 0.4 – 0.8 g. These ranges of data were being used for optimization process using Response Surface Methodology (RSM).

After optimization and validation, the optimized conditions on glucose production were 45°C of temperature, 1.3 g of substrate dose and 0.8 g of enzyme dose. The glucose production was 5.725 g/L compared to predicted value, 5.8672 g/L. Before optimization (the conventional method), the glucose yielded was only 1.010 g/L. The percentage increment was 82.36%. From these data, it can be concluded that the objective of this research to optimize the glucose production from sugarcane bagasse using Response Surface Methodology (RSM) was successfully conducted.

5.2 Recommendations

After the pretreatment process, the cellulose structure should be viewed through Scanning Electron Microscope (SEM). By viewing this structure, it will give the idea about the effectiveness of the pretreatment. The amount of cellulose produced also should be purified and measured.

In cellulose breakdown using enzymatic hydrolysis, the cellulose should be supplemented with β-glucosidase for the outstanding glucose production. This is because the mixture of enzyme will enhance the glucose production (Kumar and Wyman, 2008). The variables tested may be changed with other parameters such as effect of time, effect of pH and effect of revolution per minute (rpm).

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APPENDIX A

MATERIALS AND METHOD

Appendix A1

Preparation of acetate buffer 0.1 M

Acetate buffer solutions pH 3 - 6

Make up the following solutions

(1) 0.1M acetic acid

(2) 0.1M sodium acetate (tri-hydrate) (13.6g / l)

Mix in the following proportions to get the required pH

pH	Volume of 0.1M acetic acid	Volume of 0.1M sodium acetate
3	982.3 ml	17.7 ml
4	847.0 ml	153.0 ml
5	357.0 ml	643.0 ml
6	52.2 ml	947.8 ml

APPENDIX B

RESULT AND DISCUSSION

Appendix B1

Experiment Data for Glucose Calibration Curve

T**able B-1:** Glucose Calibration Curve

Concentration (mg/mL)	**OD (Abs)**			
	1	**2**	**3**	**Avg**
0.0	0.089	0.088	0.088	0.0883
0.2	0.704	0.704	0.705	0.7043
0.4	1.235	1.232	1.239	1.2353
0.6	1.921	1.921	1.921	1.9210
0.8	2.366	2.366	2.366	2.3660
1.0	2.745	2.745	2.745	2.7450

Appendix B2

Screening Process the Effect of Parameters on Glucose Production (Conventional Method)

T**able B-2:** Effect of Temperature on Glucose Production

Temperature (°C)	Concentration of glucose (g/L)
35	1.017
40	0.901
45	1.017
50	1.022
55	1.017

T**able B-3:** Effect of Substrate Dose on Glucose Production

Substrate Dose (g)	Concentration of glucose (g/L)
1.0	0.996
1.2	0.944
1.4	0.988
1.6	1.013
1.8	1.013
2.0	1.020

T**able B-4:** Effect of Enzyme Dose on Glucose Production

Enzyme Dose (g)	**Concentration of glucose (g/L)**
0.2	0.938
0.4	1.001
0.6	1.027
0.8	0.996
1.0	1.010

Appendix B3

Enzymatic hydrolysis using RSM suggested parameters

Table B-5: Experiment data based on RSM suggested parameter

Std	Factor 1 Temperature (°C)	Factor 2 Substrate Dose (g)	Factor 3 Enzyme Dose (g)	OD 1	OD 2	OD 3	AVG OD	Response 1 Glucose Concentration (g/L)
1	45.00	1.30	0.40	2.620	2.537	2.585	2.581	5.478
2	55.00	1.30	0.40	2.694	2.716	2.694	2.701	5.700
3	45.00	1.90	0.40	2.660	2.660	2.669	2.663	5.630
4	55.00	1.90	0.40	2.797	2.769	2.769	2.778	5.843
5	45.00	1.30	0.80	2.787	2.787	2.787	2.787	5.860
6	55.00	1.30	0.80	2.585	2.658	2.721	2.655	5.600
7	45.00	1.90	0.80	2.769	2.769	2.769	2.769	5.825
8	55.00	1.90	0.80	2.668	2.665	2.665	2.666	5.635
9	41.59	1.60	0.60	2.678	2.699	2.658	2.678	5.658
10	58.41	1.60	0.60	2.585	2.678	2.678	2.647	5.600
11	50.00	1.10	0.60	2.745	2.745	2.769	2.753	5.796
12	50.00	2.10	0.60	2.797	2.797	2.797	2.797	5.877
13	50.00	1.60	0.26	2.620	2.585	2.620	2.608	5.529
14	50.00	1.60	0.94	2.699	2.701	2.700	2.700	5.698
15	50.00	1.60	0.60	2.678	2.658	2.602	2.646	5.598
16	50.00	1.60	0.60	2.678	2.658	2.602	2.646	5.598
17	50.00	1.60	0.60	2.678	2.658	2.602	2.646	5.598

Appendix B4

RSM Analysis

Predicted Vs. Actual Analysis

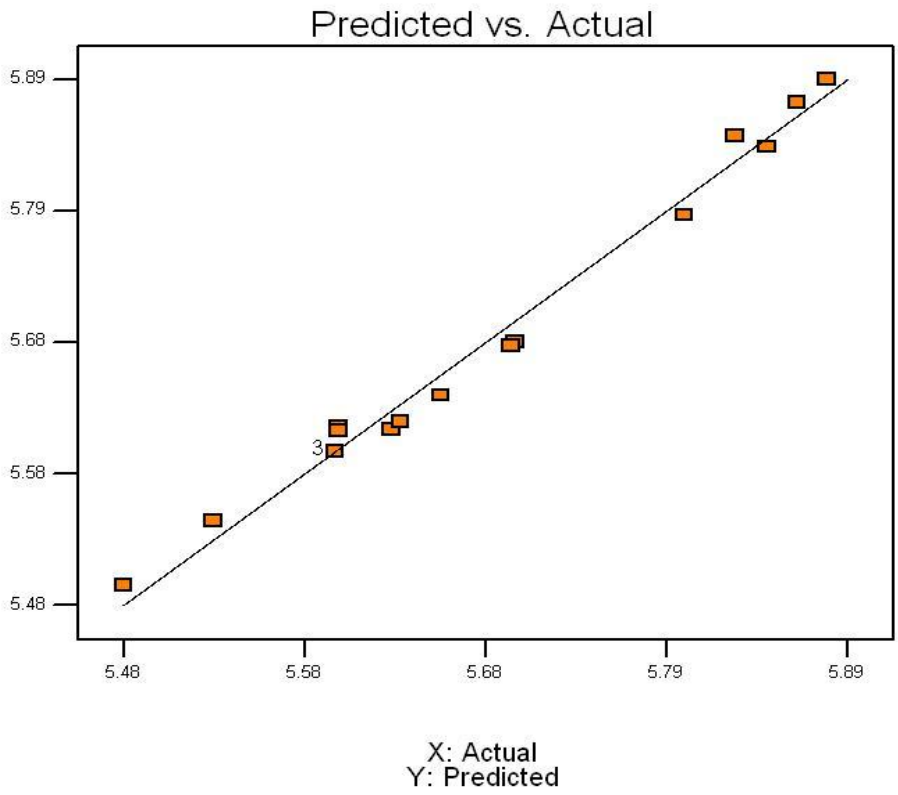

Figure B-1: Graph of Predicted Vs. Actual Analysis

Outlier T Analysis

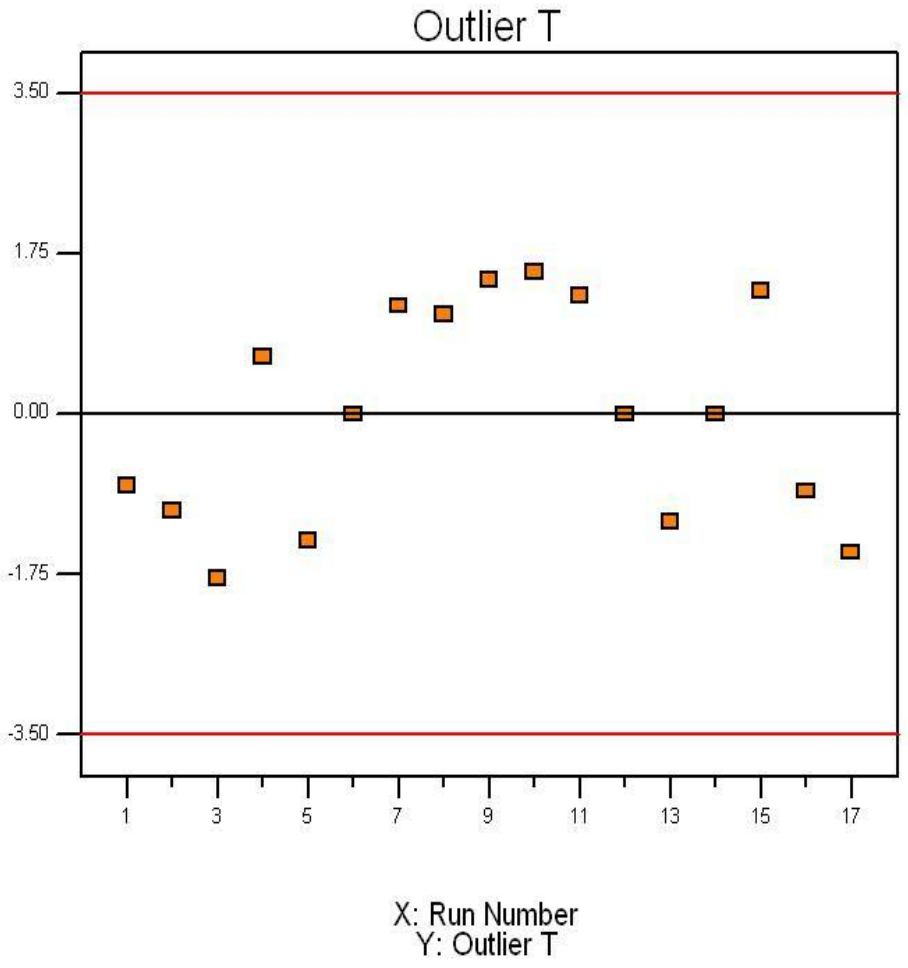

Figure B-2: Graph of Outlier T Analysis

Residuals vs. Temperature Analysis

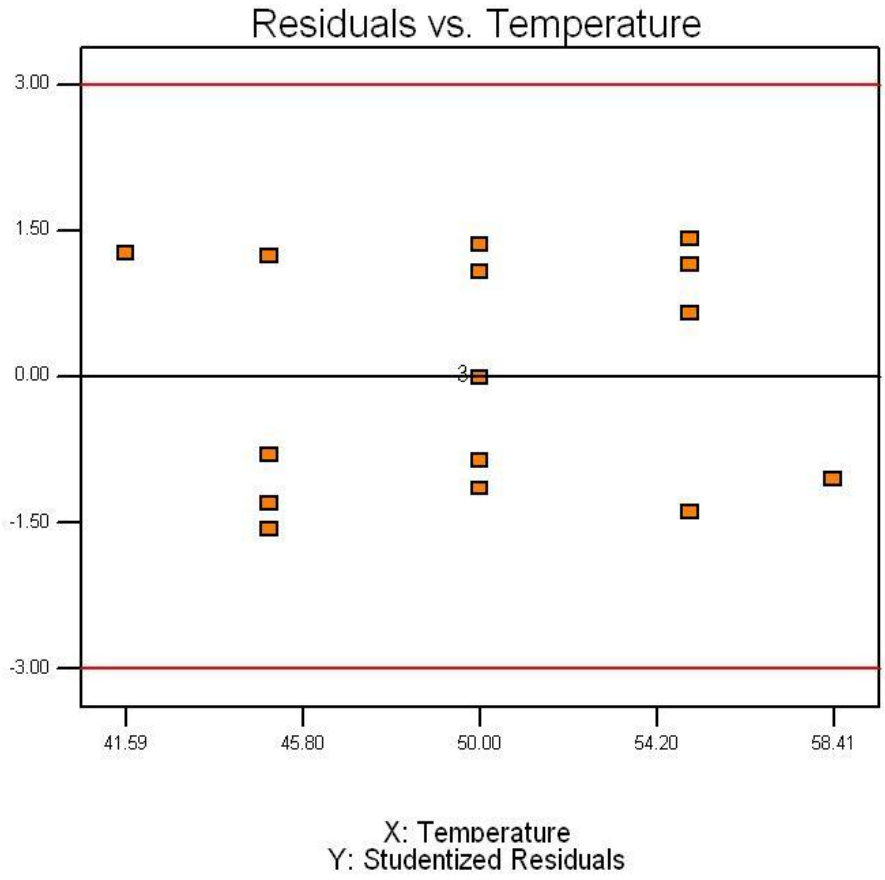

Figure B-3: Graph of Residuals vs. Temperature Analysis

Residuals vs. Substrate Dose Analysis

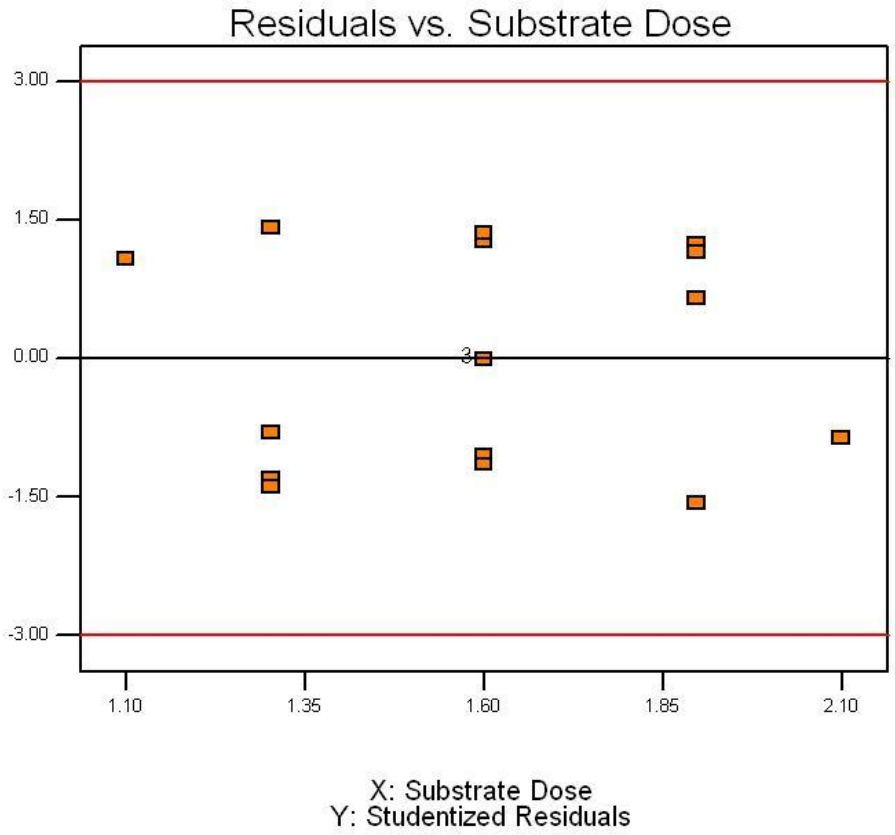

Figure B-4: Graph of Residuals vs. Substrate Dose Analysis

Residuals vs. Enzyme Dose Analysis

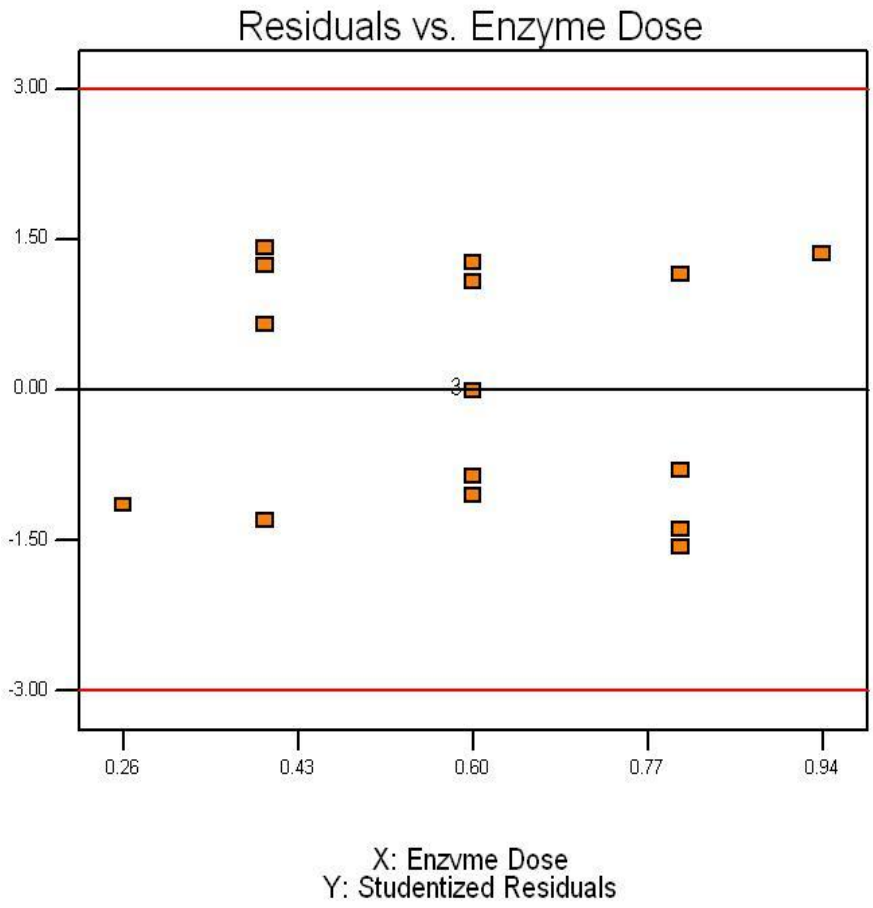

Figure B-5: Graph of Residuals vs. Enzyme Dose Analysis

Box-Cox Analysis

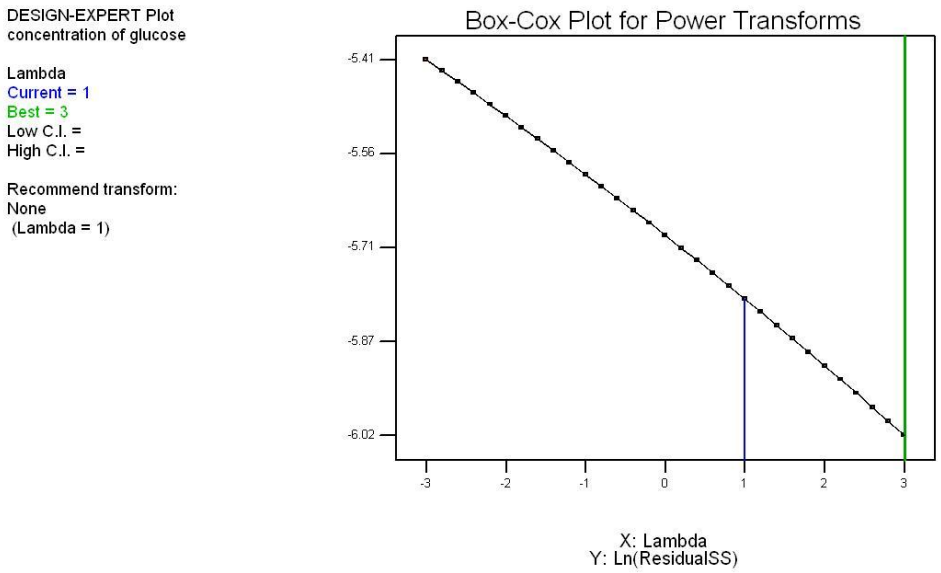

Figure B-6: Graph of Box-Cox Analysis

Optimization Data using RSM

Figure B-7: Optimization Data with Desirability 0.982