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

NURUL IZWANIE BINTI RASLI

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

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

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Signature : .....
Supervisor : Rohaida Che Man
Date : .....

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

NURUL IZWANIE BINTI RASLI

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

December 2010

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

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To my beloved family and friends

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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ABSTRACT

Xylose is a monosaccharide containing five carbon atoms, including an aldehyde functional group. It is a pentose sugar which has chemical formula $C_5H_{10}O_5$. Hemicellulose is present in plant cell walls and is associated with the cellulose. Its chemical formula is $(C_5H_8O_4)n$ and in some cases is $(C_6H_{10}O_5)n$. It is possible to hydrolyze hemicellulose by several processes (enzymatic, physical and chemical) for producing monomer sugars with great purity and high yield. The aim of this study is to optimize the xylose production from sugarcane bagasse by manipulating the temperature, agitation rate and enzyme concentration using Response Surface Methodology (RSM) based on central composite design (CCD). In this study, producing xylose from sugarcane bagasse contributes to reduce the environmental impact and bioprocess cost. Alkaline and acid hydrolysis method was used for the pretreatment of sugarcane bagasse. After the pretreatment, the screening process was constructed to determine the best range of parameters to be used in optimization process. Seventeen experiments have been arranged by RSM for optimization. The optimized conditions of parameters were 50°C of temperature, 180 rpm of agitation rate and 2 mg/ml of enzyme concentration with the predicted xylose production was 0.367 mg/ml. The actual xylose production was 0.373 mg/ml. Before the optimization, the xylose production was 0.228 mg/ml. As a conclusion, the optimization of xylose production from sugarcane bagasse by using RSM was successfully done with 63.6% of increment.

ABSTRAK

Xilosa adalah monosakarida yang mengandungi lima atom karbon, termasuk kumpulan berfungsi aldehid. Ia adalah gula pentosa yang mengandungi formula kimia $C_5H_{10}O_5$. Hemiselulosa hadir dalam dinding sel tumbuhan dan bercampur dengan selulosa. Formula kimianya adalah $(C_5H_8O_4)n$ dan dalam beberapa kes adalah $(C_6H_{10}O_5)n$. Adalah mungkin untuk menghidrolisis hemiselulosa oleh beberapa proses (enzimatik, fizikal dan kimia) untuk menghasilkan gula monomer dengan ketulenan dan hasil yang tinggi. Tujuan kajian ini adalah untuk mengoptimumkan penghasilan xilosa daripada hampas tebu dengan memanipulasikan suhu, kadar agitasi dan kepekatan enzim menggunakan Kaedah Tindak Balas Permukaan (RSM) berdasarkan Rekabentuk Komposit Pusat (CCD). Dalam kajian ini, xilosa dihasilkan daripada hampas tebu dapat mengurangkan kesan pada persekitaran dan kos bioproses. Kaedah alkali dan asid hidrolisis digunakan untuk pra-rawatan hampas tebu. Setelah dirawat, proses saringan telah dilakukan untuk menentukan julat terbaik pembolehubah - pembolahubah yang akan digunakan dalam proses pengoptimuman. Sebanyak 17 eksperimen telah ditetapkan oleh RSM untuk pengoptimuman. Kondusi pembolehubah – pembolehubah yang telah dioptimumkan adalah pada suhu 50 °C, kadar agitasi 180 rpm dan kepekatan enzim sebanyak 2 mg/ml dengan penghasilan xilosa yang diramalkan adalah 0.367 mg/ml. Penghasilan xilosa yang sebenar adalah 0.373 mg/ml. Sebelum pengoptimuman, penghasilan xilosa adalah 0.228 mg/ml. Kesimpulannya, pengoptimuman penghasilan xilosa daripada hampas tebu dengan menggunakan RSM telah berjaya dilakukan dengan peningkatan sebanyak 63.6%.

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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
OD	-	Optical density
rpm	-	revolution per minute
RSM	-	Response Surface Methodology
°C	-	Degree Celcius
µL	-	microlitre
%	-	Percentage

CHAPTER 1

INTRODUCTION

1.1 Overview

Among several potential sources of biomass, the sugarcane bagasse has been one of the most promising industrial residues obtained from the sugar and alcohol industries (Saska & Ozer, 1994). In recent years, an increasing effort has been made towards a more efficient utilization of renewable agro-industrial residues, including sugarcane bagasse. Lignocellulosics are an abundant and inexpensive source of carbohydrates that can be used to produce high-value chemicals (Carvalho *et al*., 2005). Biotechnological production of xylitol could be economically attractive using hemicellulosic hydrolysates as potential substrates, instead of pure xylose, to reduce the cost of production (Pessoa *et al.,* 1996). Sugarcane bagasse is composed approximately of 40% cellulose, 24% hemicellulose and 25% lignin.

Hemicellulose is a plant cell wall polysaccharide and in some plants, comprises up to 40% of the total dry material. In Brazil, the bagasse is a particularly convenient source for carbohydrate conversion because it is produced in large amounts (5.8×10^7 tonne in a year). Each ton of milled sugarcane gives 180-280 kg of bagasse residues (Pessoa *et al*., 1997). It is possible to hydrolyse hemicellulosic materials by several processes (enzymatic, physical and chemical) for producing monomer sugars with great purity and high yield. The hemicellulosic fraction can be easily hydrolysed by acid treatment. If cellulose and hemicellulose were utilized in an efficient hydrolysis process, the hemicellulose would be completly hydrolyzed to D-xylose (50-70% w w-1) and L-

arabinose (5-15% w w-1) as well as the cellulose would be converted to glucose (Puls and Schuseil, 1993). Hemicellulose can be converted to carbohydrates, particularly simple sugar. The dilute acid hydrolysis of bagasse hemicellulose to produce xylose, arabinose, glucose, acid-soluble lignin (ASL) and furfural were conducted using a temperature-controlled digester (Lavarack *et al*., 2002). However, the bagasse needs to undergo pretreatment process to increase the yield of production.

Pretreatment is one of the most important steps in the process in the production of ethanol from lignocellulosic materials. The most common chemical pretreatment methods used for cellulosic feedstock are dilute acid, alkaline, organic solvent, ammonia, sulfur dioxide of other chemicals to make the biomass more digestible by enzymes. The main goal of pretreatment is to alter or remove structural and compositional impediments to hydrolysis and subsequent degradation processes in order to enhance digestibility, improve the rate of enzyme hydrolysis and increase yields of intended products (Hendriks and Zeeman, 2009). These methods cause mechanical, physical, chemical or biological changes in the plant biomass in order to achieve the desired products.

The complete hydrolysis of hemicellulose into monosaccharides requires the concerted action of several enzymes. These include xylanases, galactanases, mannanases, as well as glycosidases xylosidase, galactosidase and mannosidase. The used of xylanase enzyme improving the yield of xylose production from hemicelluloses from 70-74% (Ohgren *et al*., 2007).

Xylose is a hemicellulosic sugar mainly used for its bioconversion to xylitol. It is a major product of the hydrolysis of hemicellulose from many plant materials. Rahman *et al.*, (2007) had done a research entitle optimization studies on acid hydrolysis of oil palm empty fruit bunch fiber for production of xylose. The objective of the study was to determine the effect of H_2SO_4 concentration, reaction temperature and reaction time for production of xylose. Batch reactions were carried out under various reaction temperature, reaction time and acid concentrations. Response Surface Methodology (RSM) was followed to optimize the hydrolysis process in order to obtain high xylose yield. The optimum reaction temperature, reaction time and acid concentration found were 119 C, 60 min and 2%, respectively. Under these conditions xylose yield and selectivity were found to be 91.27% and 17.97 g/g, respectively.

1.2 Problem Statement

Xylose is highly demand in industry and expensive sugar. It is relatively expensive by about $7/kg (Leathers, 2003) comparatively with other natural sweeteners. It is found in the fibers of many fruits and vegetables, including various berries, corn husks, oats, and mushrooms. It also can be found in chewing gum, toothpaste and corn sweeteners.

Xylose is used to produce xylitol, which is a sugar alcohol sweetener used as a naturally occurring sugar substitute. Xylitol has been used in foods since the 1960's. In the U.S., xylitol is approved as a food additive in unlimited quantity for foods with special dietary purposes. It is suitable for diabetes, recommended for oral health and paranteral nutrition (Makinen, 2000). On an industrial scale, xylitol is currently produced through chemical reduction of xylose derived from birchwood chips and sugarcane baggase hemicelluloses hydrolysate. The chemical process is expensive because of the high temperature and pressure required for hydrogenation of xylose. Furthermore, extensive steps for separation and purification had increased the cost of production. Instead of using pure xylose, the biotechnological production of xylose could be economic and attractive method, which could reduce the cost of production.

Xylose also can be fermented to ethanol by many bacteria, yeasts and fungi. Production of ethanol from sugars or starch from sugarcane and cereals, respectively, impacts negatively on the economics of the process, thus making ethanol more expensive compared with fossil fuels. Hence, the technology development focus for the production of ethanol has shifted towards the utilization of residual lignocellulosic materials to lower production costs (Howard *et al*., 2003).

Besides, open burning of bagasse is one of the topics in environmental issues. Instead of burning for disposal purpose, sugarcane baggase can be reuse for xylose and others production. This could improve the waste management process of the country.

1.3 Objective

The aim of this study is to optimize the production of xylose from sugarcane bagasse using Respond Surface Methodology (RSM).

1.4 Scope of Research

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

- To study the effect of temperature on xylose production from sugarcane bagasse.
- To study the effect of agitation rate on xylose production from sugarcane bagasse.
- To study the effect of enzyme concentration on xylose production from sugarcane bagasse.
- To optimize the production of xylose from sugarcane bagasse by using Response Surface Methodology (RSM).

1.5 Rationale and Significance

The rationale of using bagasse in xylose production is based on commercially unproven and new technology and hence with considerable potential risks as well as direct and indirect benefits, arising from adaptive technological research. It is directly linked and interrelated with agriculture as well as the sugar industry. Besides, this method use innovative characteristic by doing experimentation with new ideas possible.

The rational and significance of this study is to reduce the air pollution from sugarcane bagasse open burning. Usually, the agriculture waste is dispose by burning but nowadays, the waste had been reuse to become other beneficial product such as diesel, sugar and ethanol. In this study, sugarcane bagasse will be use as a source of xylose production which has high demand in industry. Besides, sugarcane bagasse is very economical source since it is a waste product.

One of natural fibres with high availability is sugarcane bagasse, a residue of the sugarcane milling process. In Malaysia, the annual production of sugarcane reaches a million tonnes, which is less than 0.1% of the world annual production (Wirawan *et al*., 2010) Nearly 30% of that number will turn into bagasse when it is crushed in a sugar factory. This procedure produces a large volume of bagasse wastes that may have an extremely harmful effect upon the environment if not suitably treated (Reis, 2006). Moreover, the stock is abundant, and the price of sugarcane bagasse is less expensive than that of other natural fibres (Vazquez *et al.,* 1999)

CHAPTER 2

LITERATURE REVIEW

2.1 Sugarcane

Sugarcane (*saccharum officinarum*) is a grass that is harvested for its sucrose content. Sugarcane is any of six to thirty-seven species (depending on taxonomic system) of tall perennial grasses of the *genus saccharum* (family *poaceae*, tribe *andropogoneae*). Native to warm temperate to tropical regions of Asia, they have stout, jointed and fibrous stalks that are rich in sugar and measure two to six meters (six to nineteen feet) tall. All sugar cane species interbreed and the major commercial cultivars are complex hybrids. Brazil produces about one-third of the world's sugarcane. After extraction of sugar from the sugarcane, the plant material that remains is termed bagasse. Currently, the bagasse production in the United States is about 8.6 million tons per year. Sugarcane bagasse found at sugar mills contain both relatively easy and hard to degrade materials. The easily degraded materials appear to be from the leaf matter and the hard to degrade from the rind (Fox, 1987). Bagasse is cheap, readily available and has high carbon content (Martín *et al*, 2002).

Sugarcane bagasse is composed approximately of 40% cellulose, 24% hemicelluloses and 25% lignin. In plant cells, including sugarcane plants, a secondary wall, consisting of three layers (S1, S2 and S3) is surrounded by a thin primary wall. The secondary wall is surrounded by lignin. The S1 and S3 layers contain mainly amorphous cellulose and hemicellulose. The S2 layer contains crystalline cellulose. However, amorphous regions also exist in the cellulose. Amorphous cellulose, hemicelluloses and

lignin are present between the layers (S1, S2 and S3) (Fox, 1987). An illustration of the structure of the cell wall with its component organization is shown in Figure 2.1. At the sugarcane bagasse, the basic composition is 40% cellulose, 24% hemicelluloses and 25% lignin. S1 and S3 layers have more amorphous cellulose and hemicellulose contents. S2 layer has more crystalline cellulose regions (Bidlack *et al*, 1992).

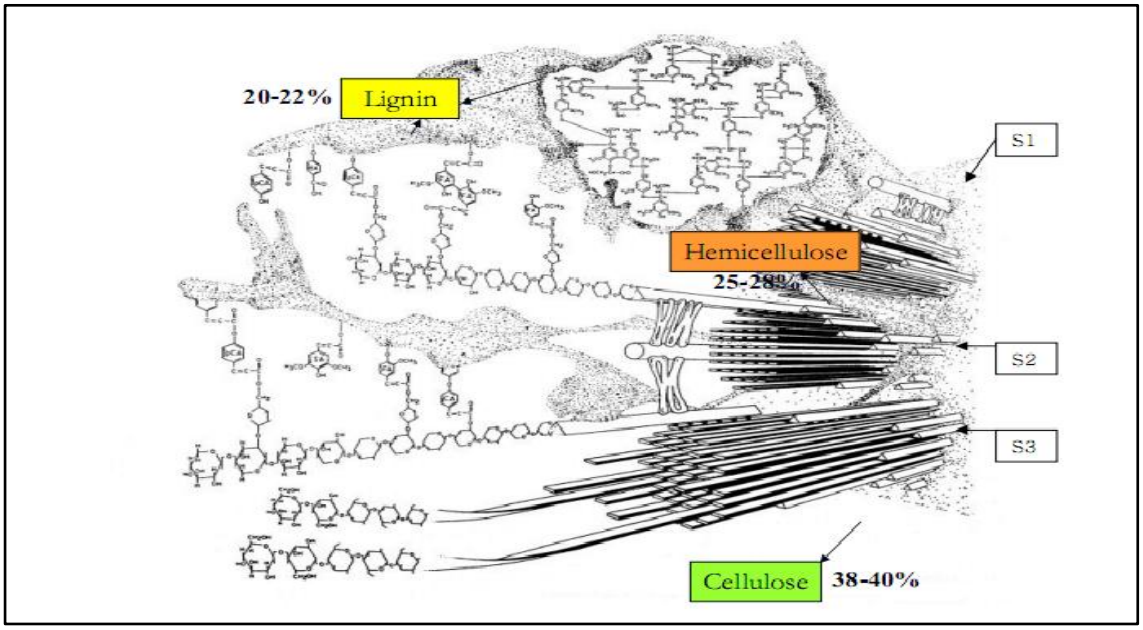

Figure 2.1: Secondary Cell-Wall (CW) Structure of cellulose, hemicellulose and lignin in lignocellulosic materials.

According to Brienzo *et al*., (2009), sugar mills generate approximately 135 kg of bagasse (dry weight) per metric ton of sugarcane. Bagasse is a rich source of not only cellulose, but also hemicellulose, represented by L-arabino-(4-O-methyl-D-glucurono)-D-xylan. The two polysaccharides represent about 70% of bagasse. Nowadays about 50% of generated sugarcane bagasse is used to generate heat and power to run the sugar mills and ethanol plants. The remaining portion is usually stockpiled. However, because the heating value of carbohydrates is approximately half of that of lignin, it would be beneficial to develop a more economical use of carbohydrates.

In the Brazilian economic context an effective utilization of sugarcane bagasse for bioproduction is very important. More than 60,000,000 tons of bagasse containing 50% moisture can be produced annually during the ethanol production season. This waste has been used as a raw material to produce hydroxymethyl furfural, paper pulp, acoustic boards, pressed woods and agricultural mulch (Dominguez *et al.*, 1996). About 70% of the dry mass in lignocellulosic biomass consists of cellulose and hemicellulose. If these two carbohydrates were utilized in an efficient hydrolysis process, the hemicellulose would be completely hydrolyzed to D-xylose (50-70% w/w) and L-arabinose (5-15% w/w), while the cellulose would be converted to glucose (Cao *et al.*, 1995).

Lavarack *et al*., (2002) studied that the acid hydrolysis of sugarcane bagasse hemicelluloses could produce xylose, arabinose, glucose and other products. The experimental were conducted using a temperature-controlled digester. The reaction conditions varied at temperature (80–200^{o}C), mass ratio of solid to liquid (1:5–1:20), type of bagasse material (bagasse or bagacillo), concentration of acid (0.25–8 wt% of liquid), type of acid (hydrochloric or sulphuric) and reaction time (10–2000 min). Kinetic modelling of the global rates of formation of products was performed. The most accurate kinetic model of the global reaction for the decomposition of xylan was a simple series hydrolysis of xylan to xylose followed by xylose decomposition. Similar schemes were used to model the production of arabinose, glucose and furfural from the hemicellulose. The yield of xylose is about 80% of the theoretical xylose available from the bagasse.

2.2 Hemicellulose

The term hemicellulose refers to a group of homo- and heteropolymers consisting largely of anhydro-β-(1, 4)-D-xylopyranose, mannopyranose, glucopyranose, and galactopyranose main chains with a number of substituent. Hemicellulose is present in plant cell walls and is associated with the cellulose. It is a complex polysaccharide that is soluble in both alkali and acid solutions. Its chemical formula is $(C_5H_8O_4)n$ and in some cases is $(C_6H_{10}O_5)n$. Hemicellulose is a heterogeneous polymer, unlike cellulose and is usually composed of 50 to 200 monomeric units of a few simple sugars. Xylose, a C5 sugar is the most abundant component in hemicellulose. Xylan contains of D-xylose units and is linked from the number one to the number four carbon of each residue. Arabinose is normally the next most plentiful component in hemicellulose. Minor components, including mannose, galactose and uronic acids may also be present (Jeffries *et al*., 1994).

Hemicelluloses are generally found in association with cellulose in the secondary walls of plants, but they are also present in the primary walls. The principal component of hardwood hemicellulose is glucuronoxylan and glucomannan in the softwoods. Some hemicelluloses contain both glucomannans and galactoglucomannans. The glucomannans contain D-glucose and D-mannose units in a ratio of 30:70. The galactoglucomannans contain D-galactose, D-glucose and D-mannose units in a ratio of 2:10:30 (Paster, 2003). Hemicellulose utilization is considered difficult because the branched structure of hemicellulose slows enzymatic hydrolysis. Among biomass components, hemicelluloses which are mainly composed of xylans, provide an important source of interesting molecules such as xylose and xylo-oligosaccharides which have potential applications in different areas, notably in chemical, food and pharmaceutical industries (Fooks *et al*., 1999).

Herrera *et al*., (2003) studied that xylose can be convert from sorghum straw by using hydrochloric acid. The main composition of the sorghum straw was cellulose, 35%; xylan, 19%; hemicelluloses, 24% and lignin, 25%. The objective of the research was to study the xylose production by hydrolysis of sorghum straw with hydrochloric acid at 122^{o}C. Several concentrations of HCl (2 - 6%) and reaction time (0 - 300 min) were evaluated. Kinetic parameters of mathematical models for predicting the concentration of xylose, glucose, acetic acid and furfural in the hydrolysates were found. Optimal conditions for hydrolysis were 6% HCl at 122^{o}C for 70 min, which yielded a solution with 162 g xylose/L, 38 g glucose/L, 20 g furfural/L and 19 g acetic acid/L.

Figure 2.2 shows the composition of pectin, cellulose and hemicelluloses in primary cell wall of a plant. Cellulose and hemicellulose are arranged into at least three layers in a matrix of pectin polymers.

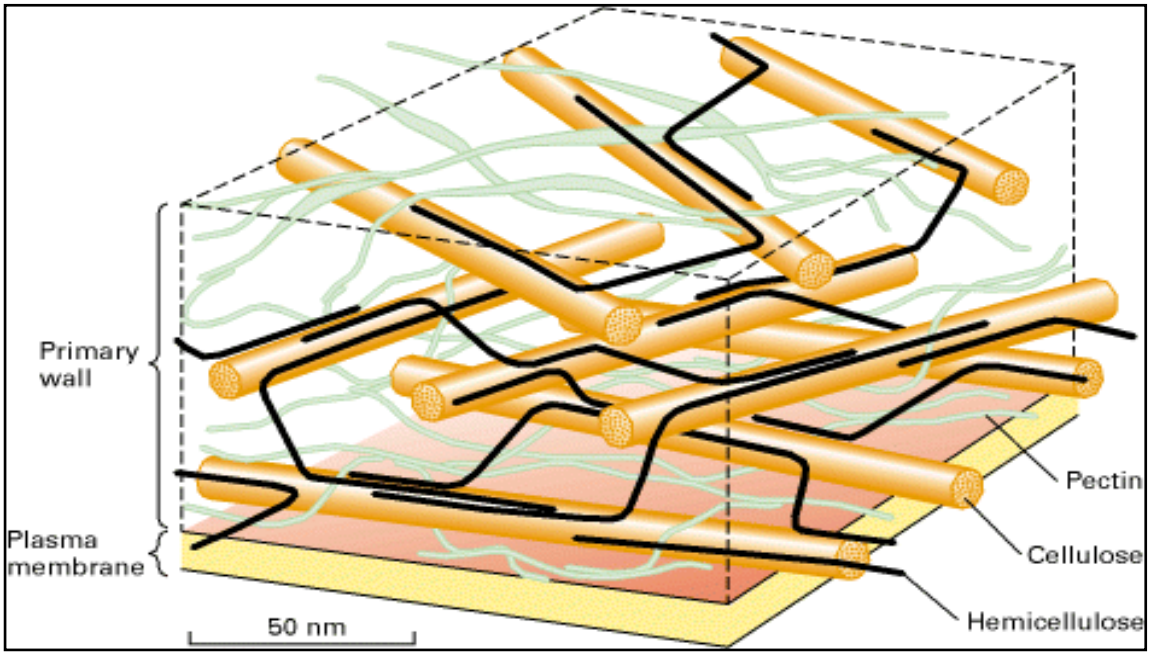

Figure 2.2: Composition of pectin, cellulose and hemicelluloses in plant cell wall.

2.3 Lignin

Lignin are a highly branched polymers formed in plant cell walls (Pareek *et al.*, 2000). Lignin resists the growth of microorganisms and stores more solar energy than either cellulose or hemicelluloses (Hu, 2002). The structure of lignin is complex, disordered, and random and consists mainly of ether linked aromatic ring structures, which adds elasticity to the cellulose and hemicellulose matrices (Paster, 2003). In pulp industries, the emphasis is removal not utilization of lignin. However, lignin is in the spotlight as a useful source for phenolic compounds for plastics and other materials (Wallis, 1971).

Lignin is mainly composed of phenylpropane or C9 units. Three different types of C9 units are present in lignin (Figure 2.3). These are p-hydroxyphenylpropane, guaiacylpropane and syringylpropane units (Gratzl, 2000). In hard wood, lignin consists mainly of guaiacylpropane and syringylpropane units with a small amount of p-hydroxyphenylpropane units. Lignin is composed principally of guaiacylpropane units with traces of p-hydroxyphenylpropane units in soft wood (Baucher, 1998). In grasses, lignin is composed of both guaiacylpropane and syringylpropane units. P-hydroxyphenylpropane units exist as a minor component of lignin in grasses (Grabber *et al.*, 2004).

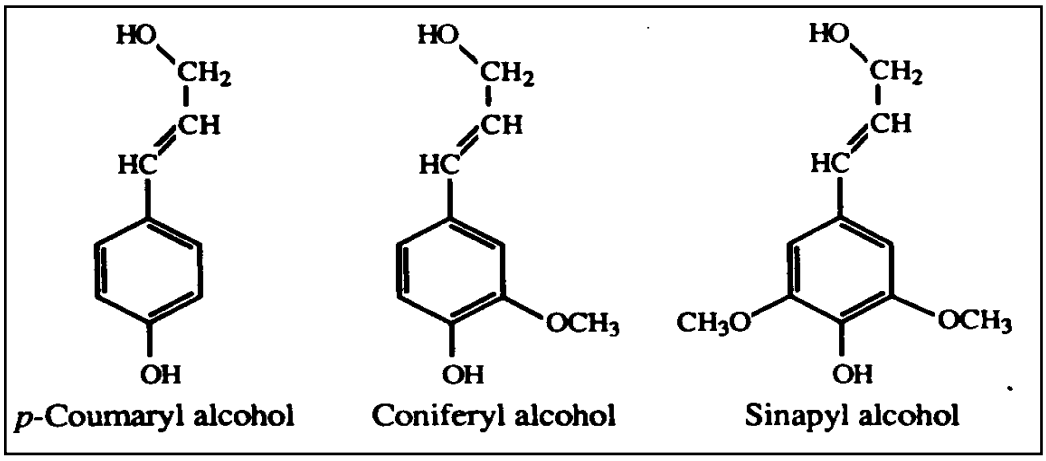

Figure 2.3: Three different types of C9 units are present in lignin.

2.4 Cellulose

Cellulose is the most abundantly available carbohydrate polymer in nature (Imai *et al*., 2003) and has therefore long been pursued as a source of providing plentiful food and energy resources. Cellulose is a complex carbohydrate polymer comprising of d-glucose units linked together by β-1,4 bonds. It comprises approximately 45% of dry wood weight. Cellulose can be hydrolyzed enzymatically or with acid. Cellulose, the major fraction of lignocellulosic biomass, can be hydrolyzed to glucose by cellulase enzymes (Jeya *et al.*, 2009). Cellulose in the primary walls of dividing and elongating cells fulfills severa1 functions, the most obvious being to provide strength. In most primary walls, cellulose exists as elementary fibrils that form a complex with xyloglucan (Hayashi, 1989).

Cellulose is the substance that makes up most of a plant's cell walls. Aside from being the primary building material for plants, cellulose has many others uses. According to how it is treated, cellulose can be used to make paper, film, explosives, and plastics, in addition to having many other industrial uses. A plant uses glucose to make cellulose when it links many simple units of glucose together to form long chains. These long chains are called polysaccharides and form very long molecules that plants use to build the walls (http://www.scienceclarified.com).

Figure 2.4 illustrated the Scanning Electron Micrograph (SEM) of cellulose in wood cell wall.

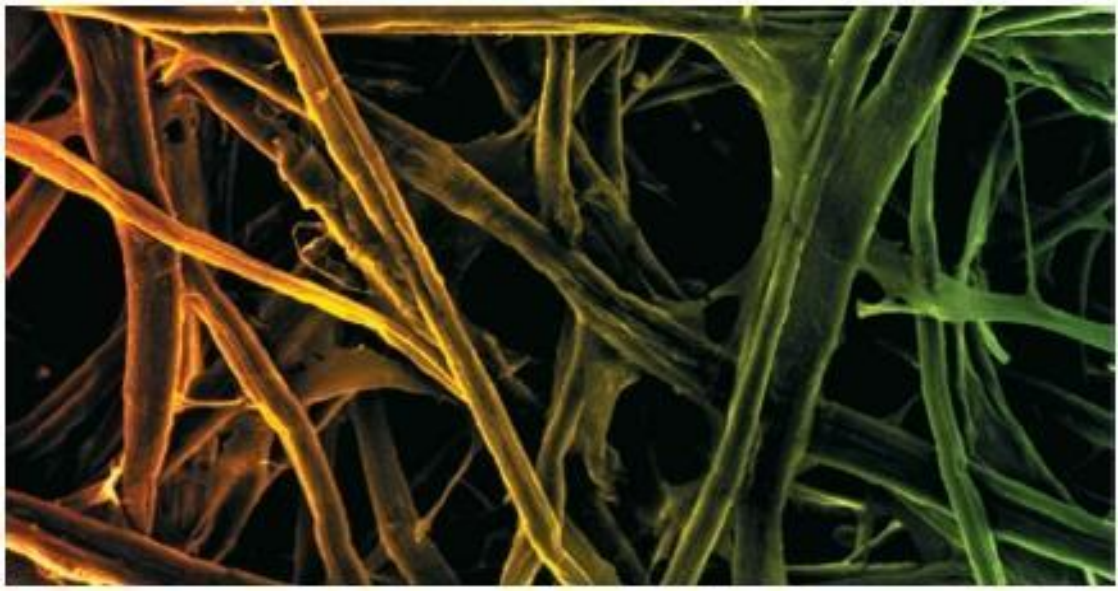

Figure 2.4: Scanning electron micrograph of wood cellulose.

2.5 Acid Hydrolysis

In general, acid treatment is effective in solubilizing the hemicellulosic component of biomass. Proper combinations of pH, temperature, and reaction time can result in high yields of sugars, primarily xylose from hemicellulose (Elander and Hsu, 1995). Acid hydrolysis has been investigated as a possible process for treating lignocellulosic materials such as wood chips, rice straw, sugar beet pulp and wheat straw. According to Parisi (1989), the mineral acids act simply and rapidly as reaction catalyzers of polysaccharide fractions. Sugarcane bagasse can be hydrolyzed using dilute acid to obtain a mixture of sugars with xylose as the major component. However, in the hydrolyzate some by-products generated in the hydrolysis, such as acetic acid, furfural, phenolic compounds, or lignin degradation products, can be present. These are potential inhibitors of a microbiological utilization of this hydrolyzate (Dominguez *et al.*, 1996).

Processes such as two-stage acid hydrolysis can be employed to produce xylose and glucose (Beck, 1986). Treatment with dilute sulphuric acid at moderate temperatures (the first stage of acid hydrolysis) has proven to be an efficient means of producing xylose from hemicellulose (Roberto *et. al.*, 1994). In the second stage more drastic reaction conditions are employed and glucose can be produced from cellulose hydrolysis (Gregg and Saddler, 1995).

The main advantages of the dilute acid treatment of biomass include the production of a soluble pentose stream that can be physically separated from the particulate residue. Secondly, a substantially increased rate of enzymatic hydrolysis of the residual cellulose portion results, in large part, to the acid-induced fiber porosity. On the other hand, acid treatment produces furfural that is toxic to many micro-organisms and the residual acid must be neutralized (Hespell *et al.*, 1997). The non glucose carbohydrate portion of the lignocellulosic biomass is much more sensitive to acid hydrolysis than the glucose portion. Reasonable amounts (over 70%) of hemicellulosic sugars, especially xylose, can be easily obtained by acid hydrolysis at temperatures between 100 and 160^{o}C (Wayman, 1986). The hydrolysis of hemicellulose is accelerated at elevated temperatures owing to relatively high activation energy in the solid-liquid phase reaction. At high temperatures part of the xylose released from hemicellulose can be degraded rapidly and cellulose in the amorphous region can yield glucose (Banerjee, 1989).

A study of acid hydrolysis of hemicellulose from sugarcane bagasse by Pessoa *et al.*, (1997), state that hydrolysis of the hemicellulosic fraction of sugarcane bagasse by sulphuric acid was performed in laboratory (25 mL) and semi-pilot (25 L) reactors under different conditions of temperature, time and acid concentration. On the laboratory scale, the three highest recovery yields were obtained at: 140ºC for 10 min (yield=73.4%); 140ºC for 20 min with (yield=73.9%) and 150ºC for 20 min with (yield=71.8%). These conditions were also used for hydrolysis in a semi-pilot reactor, and the highest xylose recovery yield (83.3%) was obtained at 140ºC for 20 min.

Gamez *et al*., (2006) studied in the hydrolysis of sugarcane bagasse using phosphoric acid stated that samples of sugarcane bagasse were hydrolysed with phosphoric acid under mild conditions (H_3PO_4, 2–6%; time, 0–300 min and 122 °C) to study the feasibility of using the liquid phase as fermentation media. Solid yield, sugar concentrations and decomposition product concentrations were measured. The composition of hydrolysates, their purity and the ratio sugars/inhibitors were analyzed. Kinetic models were developed to describe the course of products of the acid hydrolysis. The course of xylose, glucose, arabinose, acetic acid and furfural were satisfactorily described by the models. The optimal conditions selected were 122 °C, 4% of H_3PO_4 and 300 min. Using these conditions, 17.6 g of xylose/L; 2.6 g of arabinose/L; 3.0 g of glucose/L, 1.2 g furfural/L and 4.0 g acetic acid/L were obtained. The efficiency in these conditions was 4.46 g sugars/g inhibitors and the mass fraction of sugars in dissolved solids in liquid phase was superior to 55%.

2.6 Alkaline hydrolysis

Chemical treatment involves the use of alkaline, acidic or oxidative agents. Compared with acid or oxidative reagents, alkali treatment appears to be the most promising in breaking the ester bonds between lignin, hemicelluose and cellulose by avoiding fragmentation of the hemicellulose polymer (Doner *et al*., 2001). Alkaline pretreatment refers to the application of alkaline solutions to remove lignin and various uronic acid substations on hemicellulose that lower the accessibility of enzyme to the hemicellulose and cellulose (Chang and Holtzapple, 2000). In alkaline hydrolysis, bases such as NaOH and NH_3 are used for pretreatment. The effects include increased porosity, larger internal surface area and decrease of the degree of polymerization, separation of structural linkages between lignin and carbohydrates and disruption of the lignin structure.

It is well known that alkaline pretreatment provides the effective delignification and chemical swelling of the fibrous cellulose (Zhao *et al*., 2009). However, Hsu reported that alkaline pretreatment is generally more effective on agricultural residues and herbaceous crops than on wood materials (Hsu, 1996). These effects enhance the accessibility of enzymes and the digestibility of holocellulose components because of the solvation and the saphonication during alkaline pretreatment. At the same time, the alkaline pretreatment can also cause condensation of lignin and modification of the crystal structure, which can introduce unwanted effects for lignin removal and cellulose degradation (Gregg & Saddler, 1996).

Curreli *et al*., (1997) studied that a mild alkaline/oxidative pretreatment of wheat straw prior to enzymic hydrolysis. It consists of a first alkaline (1% NaOH for 24 h) and a second alkaline/oxidative step (1% NaOH and 0.3% H202 for 24 h), which solubilises and oxidises lignin to minor polluting compounds. The entire process was carried out at low temperature (25-40°C) using a low concentration of chemicals, resulting in a relatively low cost and waste liquors containing only trace amounts of dangerous pollutants derived from lignin. Recovery of cellulose after the double pretreatment reached 90% of that contained in the starting material, with a concomitant 81% degradation of lignin. The action of a commercial cellulase on the cellulose obtained produced syrup with a high concentration of glucose (220 mg/ml).

2.7 Enzymatic Hydrolysis

Enzymatic hydrolysis is a process by which enzymes (biological catalysts) are used to break down starch, cellulose or hemicelluloses into sugar. Glucose and xylose can be recovered with high yield using steam pretreatment and subsequent enzymatic hydrolysis (Öhgren *et al*., 2005).

The high cost of enzymes presents a significant barrier to commercialization of bio-based products. In the simplest terms, the cost is a function of the large amount of enzyme protein required to break down polymeric sugars in cellulose and hemicellulose to fermentable monomers. In recent years, significant effort has been expended to reduce the cost by focusing on improving the efficiency of known enzymes, identification of new, more active enzymes, creating enzyme mixes optimized for selected pretreated substrates, and minimization of enzyme production costs (Merino and Cherry, 2007).

Xylanase is a hydrolase enzyme and use to breakdown the xylan into xylose. In biochemistry, a hydrolase is an enzyme that catalyzes the hydrolysis of a chemical bond. Xylanase is an enzyme which degrades the linear polysaccharide beta-1, 4-xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls.

A study of enzymatic hydrolysis of ammonia-treated rice straw by Sulbaran-De-Ferrer *et al.,* (2003) has stated that rice straw pretreated with liquid anhydrous ammonia was hydrolyzed with cellulase, cellobiase, and hemicellulase. Ammonia-processing conditions were 1.5 g of NH3/g of dry matter, 85°C, and several sample moisture contents. There were four ammonia addition time (min) - processing time (min) combinations. Sugars produced were analyzed as reducing sugars (dinitrosalicylic acid method) and by high-performance liquid chromatography. Production of monosaccharides was greater at higher moisture content and was processing time dependent. Glucose was the monosaccharide produced in greater amounts, 56.0%, followed by xylose, arabinose, and fructose, with 35.8, 6.6, and 1.4%, respectively.

Adsul *et al*., (2005) studied in enzymatic hydrolysis of delignified bagasse polysaccharides stated that the bagasse samples were hydrolyzed by cellulase and xylanase enzymes, produced earlier by *Penicillium janthinellum NCIM 1171* in the same bagasse polysaccharides production medium. The hydrolysis was carried out by using different concentrations of the enzymes at two different temperatures, 30 and 50 °C, taking hydrolysis of Avicel as control. It was found that while the maximum hydrolysis for Avicel was 70% that of some of the bagasse polysaccharides was as high as 95%. The products of hydrolysis were glucose, xylose, and arabinose, as confirmed by high pressure ion chromatography (HPIC). The initial rates of hydrolysis was found to be much higher for the bagasse polysaccharides and in some cases about 90% of the hydrolysis occurred within 20 h. The delignified bagasse medium appears to be a facile medium for the combined hydrolytic action of the cellulase and xylanase enzymes.

2.8 Production and Properties of Xylose

Xylose is a monosaccharide containing five carbon atoms and including an aldehyde functional group. It has chemical formula $C_5H_{10}O_5$. Xylose is found in the embryos of most edible plants. With its free carbonyl group, it is a reducing sugar. This pentose can be used as a substrate to produce a wide variety of compounds or fuels by chemical or biotechnological processes. Xylose is one of the eight essential sugars needed to optimal health and functioning in humans. Xylose has both antibacterial and antifungal properties and like the other glyconutrients aids in communication between cells (www.gluconutrient.biz).

Xylose may help prevent cancer in the digestive system. In some patients with colitis, diabetes and other digestive diseases, the absorption of xylose is decreased. So for many diabetics, xylose or the commercial derivative can be used as a sugar substitute. Reduction of xylose by catalytic hydrogenation produces the sugar substitute xylitol. Conversion of xylose from agro industrial residues into xylitol through biotechnological pathways as an economical alternative to the chemical process currently used has been extensively studied (Roberto *et al.*, 1994).

Conversion of xylose from agro industrial residues into xylitol through biotechnological pathways as an economical alternative to the chemical process currently used has been extensively studied. Although xylose was the main sugar obtained from hemicellulose, other byproducts such as glucose, acetic acid, furfural and others, were also produced in low amounts during the hydrolysis process (Silva *et al.*, 1998). It was also reported that amount of sugar released during hydrolysis, depended on type of raw material and operating conditions of the experiment.

The main application of xylose is its bioconversion to xylitol, a functional sweetener with important technological properties like anticarcinogenicity, low caloric value and negative heat of dissolution (Kim *et al*., 1999). The economic interest in xylitol production can be enhanced if the needed xylose solutions can be obtained from the hydrolysis of low-cost lignocellulosic wastes.

In a study of production of xylose from oil palm empty fruit bunch fiber using sulfuric acid by Rahman *et al.,* (2006) the batch hydrolysis of oil palm empty fruit bunch fiber was performed at operating temperature 120 °C using various concentration of sulfuric acid (2–6%) and reaction time (0–90 min). Concentration of xylose, glucose, furfural and acetic acid in the resulting hydrolysate were determined. Kinetic parameters of mathematical models were obtained in order to predict concentration of xylose, glucose, furfural, acetic acid in the hydrolysate and to optimize the process. Optimum H_2SO_4 concentration and reaction time obtained under operating temperature of 120 °C was 6% and 15 min, respectively. Optimum concentration of xylose, glucose, furfural and acetic acid found in the hydrolysate were 29.4, 2.34, 0.87 and 1.25 (g/l), respectively.

2.9 Factor Affecting the Production of Reducing Sugar

Sugars that contain aldehyde groups that are oxidised to carboxylic acids are classified as reducing sugars (http://www.chem.ucalgary.ca, 2010). Examples of reducing sugar are glucose, fructose, glyceraldehydes, lactose, xylose, arabinose and maltose. The maximum production of reducing sugar is affected by various conditions of parameter such as effect of agitation time, substrate concentration, pH and so on. In this study, the effect of temperature, agitation rate and enzyme concentration in xylose production from sugarcane bagasse were investigated. Some literature reviews reported on these conditions on production of reducing sugar.

2.9.1 Effect of Temperature on Production of Reducing Sugar

Fu *et al*., (2010) had studied the effect of enzymatic reaction temperature on the hydrolysis performance in Hydrolysis of microalgae cell walls for production of reducing sugar and lipid extraction. According the experimental results, the hydrolysis yield increased with temperature from 40 to 50 °C, and then decreased dramatically at 60 °C in six hours as presented in Fig. 2.4. This decrease of activity was caused by deformation of exoglucanase at around 60 °C. Therefore, 50 °C was chosen as the optimal reaction temperature based on hydrolysis performance. Figure 2.5 shows the result of the effects of reaction temperature on hydrolysis yield under conditions of pH 4.6, microalgae concentration: 20 g/l.

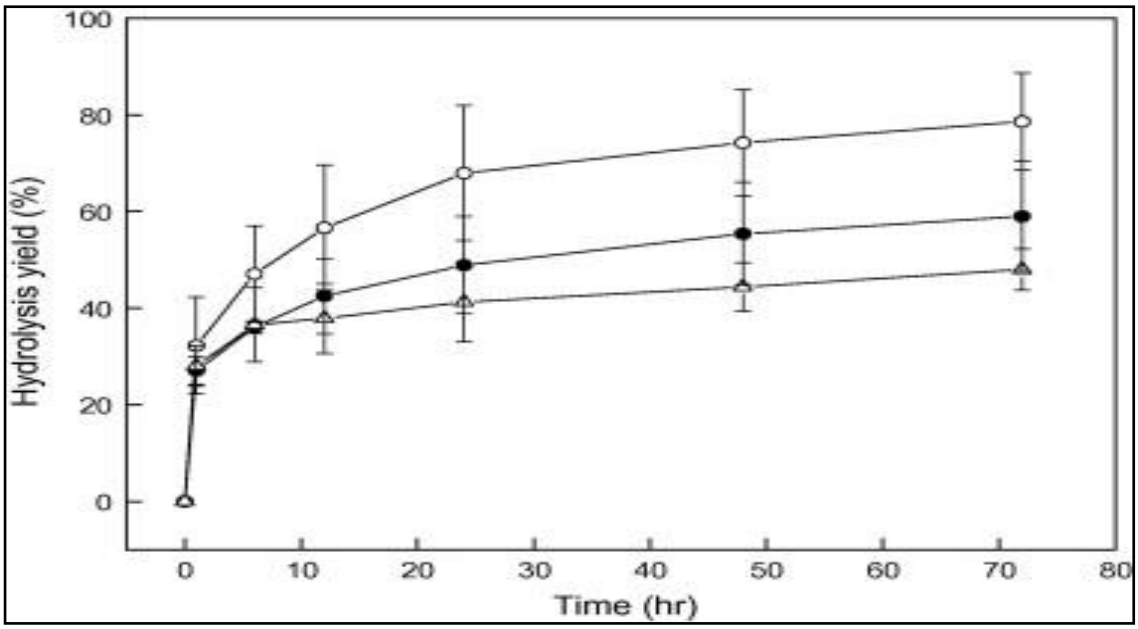

Figure 2.5: Effects of reaction temperature on hydrolysis yield under conditions of pH 4.6, microalgae concentration: 20 g/l. (Temperature: (●) 40 °C; (○) 50 °C; (Δ) 60 °C)

2.9.2 Effect of Agitation Rate on Production of Reducing Sugar

Feng *et al.*, (2002) studied the effect of agitation rate on reducing sugar production in a batch fermenter. A higher agitation speed increased the amount of dissolved oxygen and dispersion of macromolecules in the medium. Therefore, the greater growth and better production was noted in this study. However, the shearing effect induced by the higher agitation speed on the cells and enzyme inactivation may contribute negatively towards cell growth and enzyme stability. Oxygen limitation is thought to have contributed to the lower growth and enzyme activity observed at 450 rpm. The study indicated that 600 rpm was best for *B. Licheniformis NK-27* for the production. Figure 2.6 shows the production by *Bacillus Licheniformis NK-27* at different agitation speed.

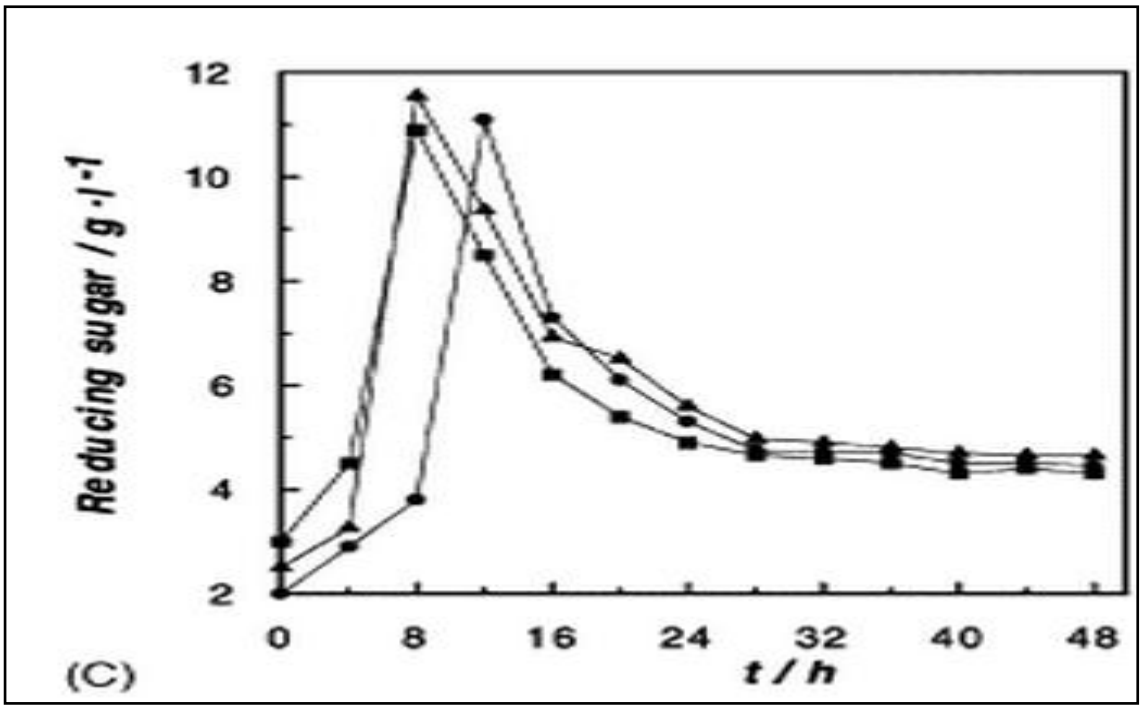

Figure 2.6: Production by *Bacillus Licheniformis NK-27* at different agitation speed of 450 rpm (▲), 600 rpm (•), and 750 rpm (▪).

2.9.3 Effect of Enzyme Concentration on Production of Reducing Sugar

Enzymatic hydrolysis of maize straw polysaccharides was investigated for the production of reducing sugars by Chen *et al*., (2008). Enzymatic hydrolysis of pretreated maize straw sample at 80 g/l substrate concentration, using different enzyme dosages (presented as FPU/g substrate), is shown in Figure 2.7. In experiments it is found that liquefaction of the substrate usually took 3–4 h, but it took about 12 h to liquefy the substrate at an enzyme dosage of 7 FPU/g substrate, indicating obviously insufficient enzyme activities at this level. For each enzyme dosage, hydrolysis yield increased sharply for the first 12 h, and then more slowly from 12 to 48 h. The optimal enzyme dosage was identified as 20 FPU/g substrate, i.e. an enzyme complex including cellulase of 20 FPU/g substrate and cellobiase of 10 FPU/g substrate, and further increase in enzyme dosage did not produce a corresponding increase in the hydrolysis yield.

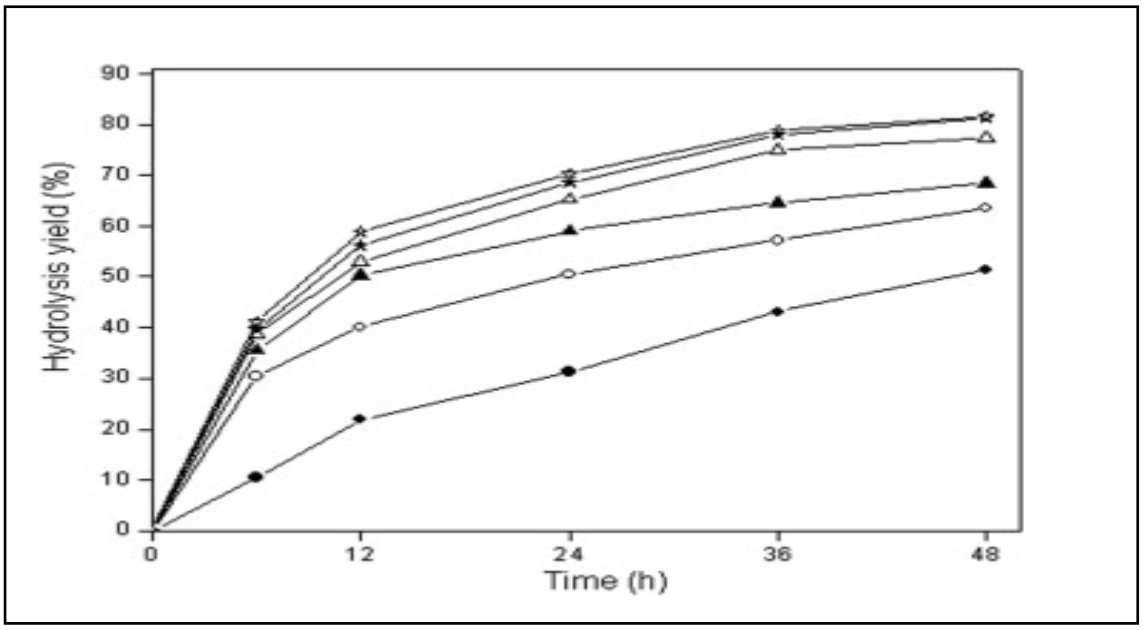

Figure 2.7: Effects of enzyme dosage (presented as filter paper activity per gram of substrate, FPU/g substrate) on the enzymatic hydrolysis. (•) 7 FPU/g substrate; (○) 10 FPU/g substrate; (▲) 13 FPU/g substrate; (Δ) 17 FPU/g substrate; (★) 20 FPU/g substrate; (☆) 23 FPU/g substrate.

2.10 Response Surface Methodology

Response surface methodology (RSM) is a collection of tools developed in the 1950s for the purpose of determining optimum operating conditions in applications in the chemical industry (Myers *et al*., 1989). Response surface methodology (RSM) can be defines as a statistical method that uses quantitative data from appropriate experiments to determine & simultaneously solve multivarient equations. Factors to be consider in this methodology are critical factors are known, region of interest, where factor levels influencing product is known. Factors vary continuously through out the experimental range tested and a mathematical function relates the factors to the measured response and the response defined by the function is a smooth curve.

RSM is a combination of mathematical and statistical techniques that is useful for analyzing the effects of several independent variables on the system response without the need of a predetermined relationship between the objective function and the variables (Draper and John, 1988). In fact, the relationship between the response and the independent variables is usually unknown in a process; therefore the first step in RSM is to approximate the function (response) through analyzing factors (independent variables). Usually, this process employs a low-order polynomial equation in a pre-determined region of the independent variables. If there is a curvature in the response, then a polynomial of higher degree, such as a second-order model, must be used to approximate the response, which is later analyzed to locate the optimum values of independent variables for the best response value (Ismail *et al*., 1999). Using RSM requires special precautions to be taken to determine all critical variables sufficiently as well as not to work with too many variables over wide ranges (Vining and Myers, 1991).

Aktas *et al*., (2006) state that *Kluyveromyces marxianus* Y-8281 yeast culture was utilized for the biological treatment of deproteinated whey wastewater in a batch system. Removal of lactose was optimized by the utilization of response surface methodology, RSM. The empirical model developed through RSM in terms of effective operational factors of medium pH, temperature, lactose and ammonia concentrations was found adequate to describe the treatment of deproteinated whey. Through the analysis, medium pH and temperature were found to be the most significant factors and an increment in both had a positive effect on lactose utilization, while lactose and ammonia concentrations had the least weight within the ranges investigated. Based on contour plots and variance analysis, optimum operational conditions for maximizing lactose removal were found to be 31 °C, 45 g/L whey powder concentration, 4 g/L total ammonium salt concentration and medium pH 6. Under the optimum operating conditions determined, 95% lactose removal was achieved after 18 h fermentation.

Marques *et al*., (2007) also studied the optimization in modelling of the high pressure–temperature effects on naringin hydrolysis based on response surface methodology. The aim of this study was the modelling, under high pressure, of naringin hydrolysis by naringinase. Response surface methodology (RSM) was used to compare the effects of the selected variables on the bioconversion under study. The combined action of temperature (13 – 61 °C) and pressure (80 – 216 MPa) on the catalytic activity of naringinase was investigated at pH 4.0 using naringin as the substrate. The choice of experimental domains resulted from preliminary studies. Naringinase activity, for naringin hydrolysis at pH 4.0, could be described by a convex surface with a maximum of 0.13 mM min^{-1}, at 41 °C and 158 MPa. After 1 h of reaction time, reducing sugars production could also be described by a convex surface, with a maximum reducing sugars concentration of 8 mM at 38 °C and 168 MPa. The interaction temperature – pressure had a significant effect on both naringinase activity and reducing sugar formation after 1 h. Under the optimized conditions, the naringin hydrolysis by naringinase was evaluated.

CHAPTER 3

METHODOLOGY

3.1 Procedure on Optimization of Xylose Production From Sugarcane Bagasse Using Response Surface Methodology

In this research, the procedures have been divided into four steps in order to achieve the objective of the research. Firstly, the xylose standard curve was constructed by plotting optical density versus concentration of xylose. The wavelength used is 540 nm. Next, the alkaline and acidic pretreatment process was done to remove lignin from the sugarcane bagasse and improved the efficiency of the production. After that, the screening process was done to obtain the best range of parameters (temperature, agitation rate and enzyme's concentration) using conventional method. Finally, the optimization on xylose production using Response Surface Methodology (RSM) was conducted. The overall process of experiment for production of xylose from sugarcane bagasse was shown in Figure 3.1.

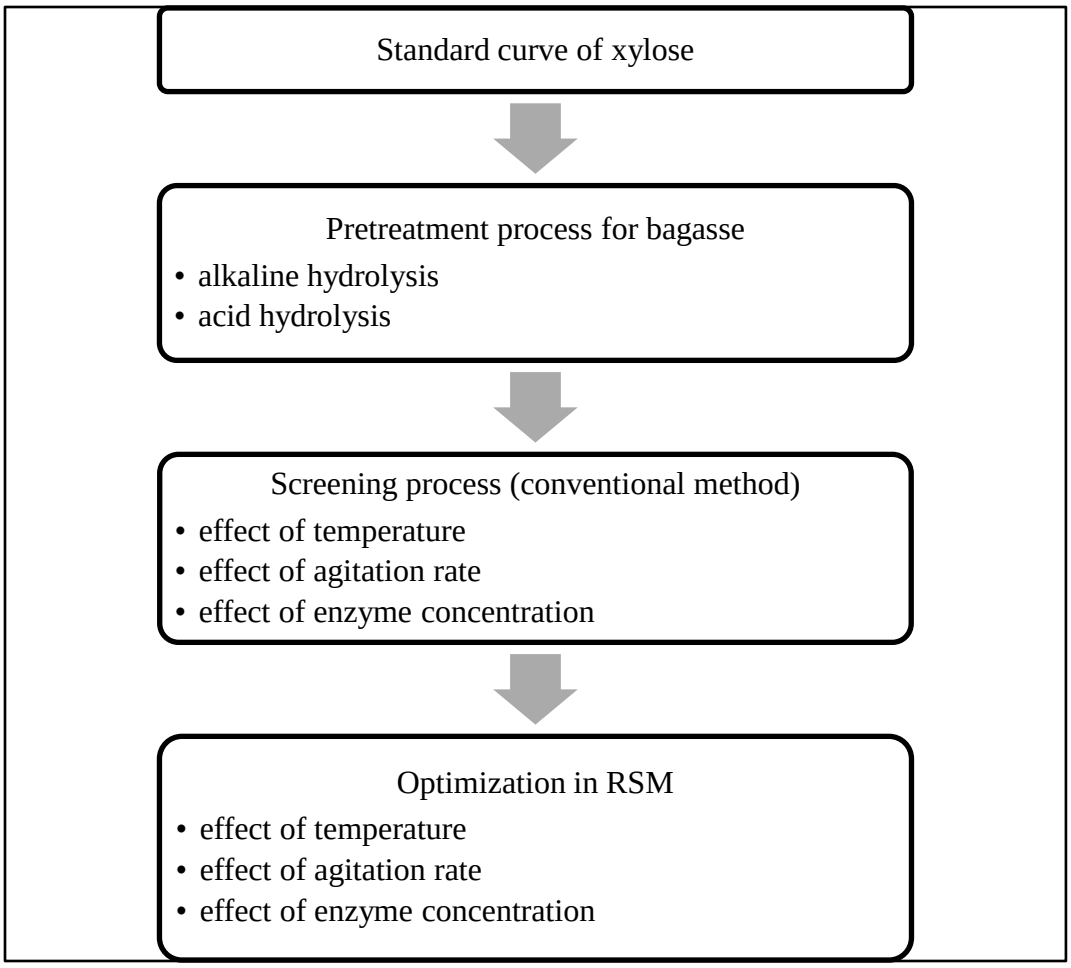

Figure 3.1: Process flow for xylose production from sugarcane bagasse.

3.2 Chemicals, Apparatus and Equipments

In this research, all chemicals, apparatus and equipments used were supplied by Faculty of Chemical and Natural Resources Engineering. The chemicals used were bought from Sigma, Merck and Fluka companies. In this research, the chemicals used were sodium hydroxide, sulphuric acid, D-xylose, Dinitrosalicyclic (DNS) reagent, citric buffer and xylanase enzymes from *Thermomyces lanuginosus*. Apparatus used were volumetric flasks, conical flasks, beakers, measuring cylinders, glass funnel and micropipette. Equipments used were analytical balance, water bath and UV-Vis Spectrophotometer Hitachi U-1800.

3.3 Pretreatment Process

The sugarcane bagasse was obtained from sugarcane juice stall at Perak. The bagasse was dried by sunlight for a day to get rid the water contain in the bagasse. Next, the dried bagasse was grinded by using a blender to obtain the small size of bagasse. The purpose of this step is to increase the surface area of the bagasse. High surface area could increase the efficiency of the pretreatment and enzymatic hydrolysis process.

The bagasse was treated with alkaline and acid hydrolysis pretreatment. Firstly, the bagasse was treated with 1% of sodium hydroxide in water bath for 2 hours. After that, the bagasse was cooled down to the room temperature and rinsed with distilled water to remove the sodium hydroxide. Next, the filtered bagasse was treated with 1% of acid sulphuric and incubates it for 90 minutes. The purpose of these pretreatment is to remove lignin in the bagasse. Lastly the bagasse was incubated in the oven at $75^{o}C$ for 5 hours. Figure 3.2 to Figure 3.7 shows the pictures during the pretreatment processes.

Figure 3.2: The sugarcane bagasse was dried to the sunlight.

Figure 3.3: The grinded bagasse after dried for a day.

Figure 3.4: Sugarcane bagasse during the alkaline hydrolysis before incubates in water bath.

Figure 3.5: Sugarcane bagasse during the alkaline hydrolysis after incubated in water bath.

Figure 3.6: Sugarcane bagasse during acid hydrolysis with 1% sulphuric acid.

Figure 3.7: Sugarcane bagasse contain hemicelluloses after incubated in oven.

3.4 Standard Curve of Xylose

The standard curve of xylose has been constructed as guidance to determine the xylose concentration obtained after the enzymatic hydrolysis. The different concentration of xylose was prepared by dissolved the commercial xylose into 10 ml of citric buffer. The concentration of xylose was varies at 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2 mg/ml. Each concentration (2 ml) was added with 2 ml of DNS reagent and was tested using the UV-Vis Spectrophotometer at 540 nm. The graph of optical density (OD) versus concentration of xylose was plotted.

3.5 Screening Process for Temperature, Agitation Rate and Enzyme Concentration on Xylose Production Using Conventioanal Method.

The enzymatic hydrolysis was used to produce xylose from xylan which contain in hemicelluloses inside the sugarcane bagasse. The process has been done by manipulating one parameter and the other two parameters were kept at constant. The best ranges was selected in each parameter and used in optimization process.

The substrate was weight to 0.5 g using analytical balance and mixed with 25 ml of citric buffer in a conical flask. Then, 250 µl enzyme was added to the mixture. The enzyme used in this experiment was xylanase from *Thermomyces lanuginosus.*

3.5.1 Effect of Temperature on Xylose Production

The enzymatic hydrolysis method was conducted using different temperature at 15, 25, 35, 45, 55 and 65°C and the other two parameters which were agitation rate and enzyme concentration were remain constant at 150 rpm and 1.5 mg/ml respectively. The process was conducted in an incubator shaker for 4 hours.

3.5.2 Effect of Agitation Rate on Xylose Production

The experiment was repeated using optimal temperature value obtained (55°C) but in different agitation rate (60, 90, 120, 150,180 and 210) and the other two parameters which were temperature and enzyme concentration were remains constant at 55°C and 1.5 mg/ml respectively. The process was conducted in an incubator shaker for 4 hours.

3.5.1 Effect of Enzyme Concentration on Xylose Production

The experiment was repeated using optimal temperature value and agitation rate obtained which were 55°C and 150 rpm respectively but in different enzyme concentration (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/ml). The process was conducted in an incubator shaker for 4 hours.

3.6 Reducing Sugar Analysis

Xylose concentration was measured and determined using Dinitrosalicyclis acid (DNS) method (Miller, 1959). This method measured the concentration of xylose, which was release as a result of enzymatic hydrolysis process. Sample (2 ml) was added to 2 ml of DNS solution and incubated in water bath at 90°C for 5 minutes. The DNS reagent contains 10 g of DNS acid, 2 g of phenol, 0.5 g of sodium sulfite, 182 g of potassium sodium tartarate and 10 g of sodium hydroxide. These chemicals were dissolved in one liter of distilled water

The mixture was incubated in water bath for 5 minutes. Next, the mixture was let to be cooled down to the room temperature and the absorbance of the sample was analyzed by using UV-VIS Spectrophotometer at 540 nm. Figure 3.8 illustrated the differences before and after DNS reaction

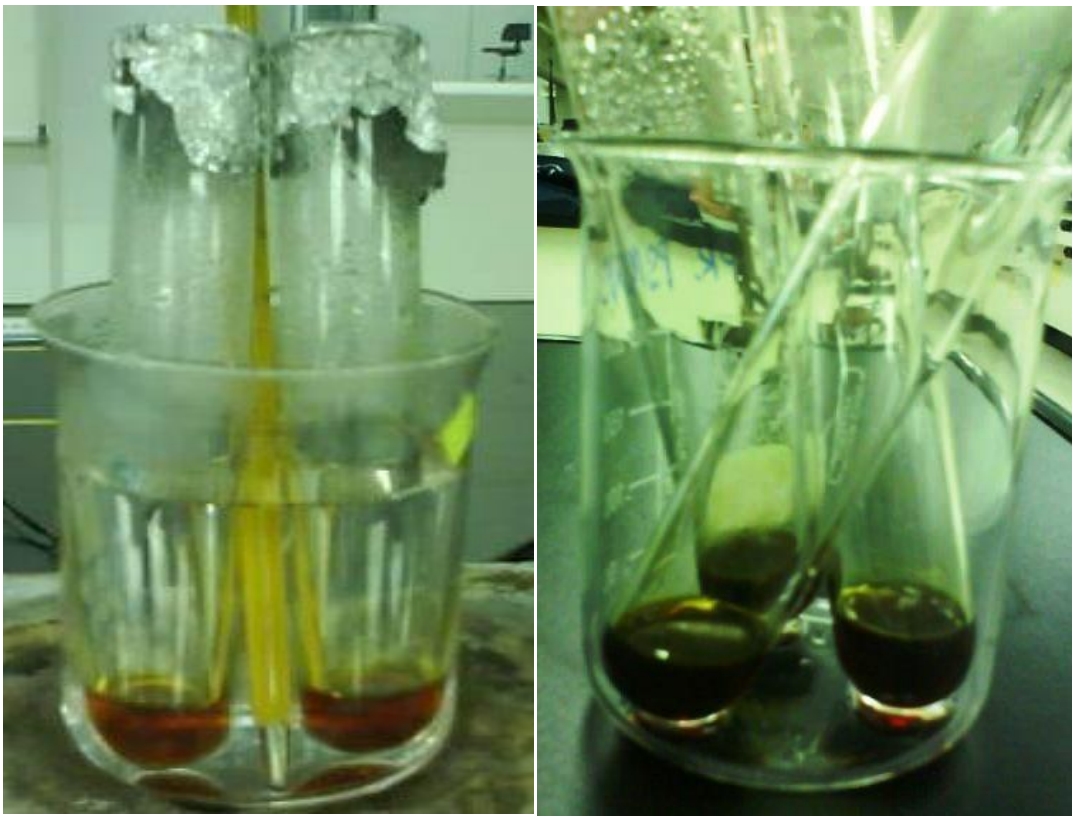

Figure 3.8: Comparison between of sample before and after DNS reaction.

3.7 Optimization of Xylose Production

The production of xylose from sugarcane bagasse was optimized by Response Surface Methodology (RSM). The temperature, agitation rate and enzyme concentrations were manipulated by central composite design (CCD). The design of experiment by RSM was shown in Table 3.1. There were 17 experiments was arranged by RSM for the optimization.

Table 3.1: Experimental ranges of the independent variables with different levels

Independent variables	**Range and levels**	
	Low	**High**
Temperature (^{o}C)	50	60
Agitation rate (rpm)	120	180
Enzyme's concentration	1	2

CHAPTER 4

RESULT AND DISCUSSION

4.1 Standard Curve of Xylose

Xylose standard curve is used to identify the concentration of xylose in an unknown sample by comparing the unknown to a set of standard samples of known concentration. The absorbance value is obtained by using UV-Spectrophotometer were plotted against the concentration of xylose. The following Equation 4.1 was obtained.

$$y = 0.902x \qquad \textbf{(Equation 4.1)}$$

with $R^2 = 0.977$

Where:

y = absorbance value

x = concentration of xylose (mg/ml)

Figure 4.1 shows the standard curve of xylose for the experiment. The graph was plotted with absorbance versus concentration of xylose.

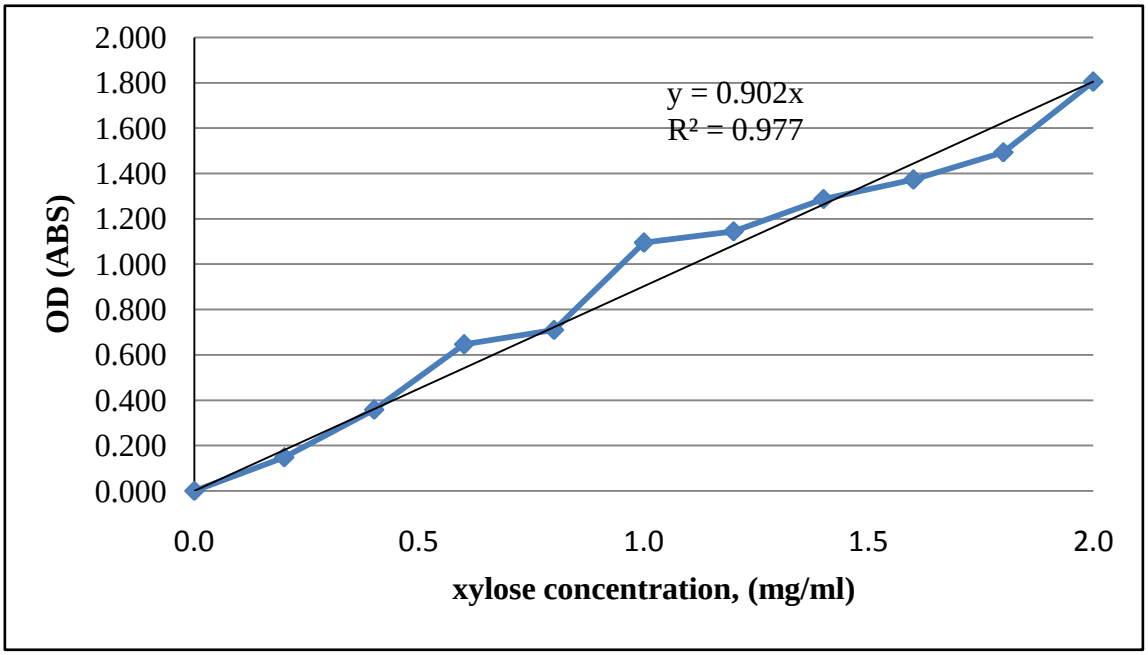

Figure 4.1: The standard curve of xylose.

4.2 Screening Process of Temperature, Agitation Rate and Enzyme Concentration on Xylose Production Using Conventional Method

The screening process is carried out to obtain the best range of parameter of studies in xylose production for the optimization process. The parameters studied in this research were the temperature, agitation rate and enzyme concentration. The substrate in this whole discussion is referring to hemicelluloses from sugarcane bagasse and the enzyme refers to xylanase enzyme from *Thermomyces lanuginosus*. The screening process was a conventional method which had been done by varying two parameters and made it constant for another one. Each parameter, the low value and the high value for optimization process would be selected before and after the maximum value from the screening process.

4.2.1 The Effect of Temperature on Xylose Production

The effect of temperature was studied conventionally to obtain the best range of temperature on production of xylose. The temperature was manipulated at 15, 25, 35, 45, 55 and 65 while the other two parameters, agitation rate and enzyme concentration were kept constant at 150 rpm and 1.5 mg/ml respectively. Figure 4.2 shows the graph plotted of the concentration of xylose at different temperature.

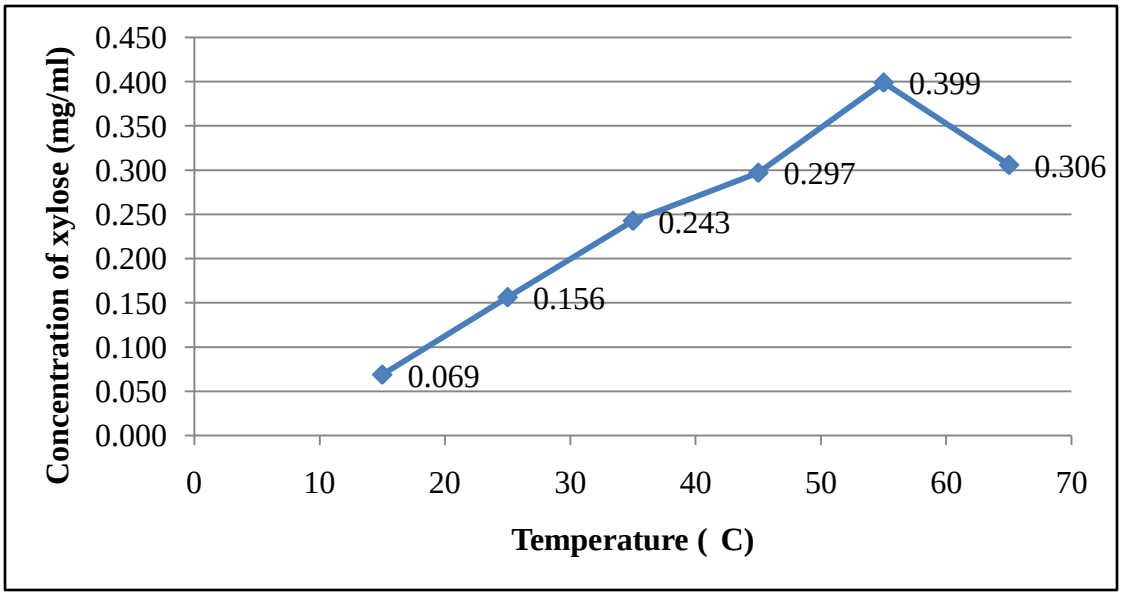

Figure 4.2: The effect of temperature on xylose production.

From the graph, the concentration of xylose increase from 0.069 mg/ml to 0.399 mg/ml at 15^{o}C to 55^{o}C but declined to 0.306 mg/ml at 65^{o}C. The peak is at 55^{o}C where the concentration of xylose is 0.399 mg/ml. The graph shows that the xylose concentration increased with the increasing of the temperature. The best range of temperature chosen was from 50^{o}C to 60^{o}C.

This pattern of graph was supported by research on xylose production from sugarcane bagasse by response surface methodology (Paiva *et al*., 2009). The research reported that as the temperature increase, the production of xylose also increased. This is due to the enzymatic hydrolysis process which had been affected by environment factor such as temperature and pH. Enzymes activity is optimum at certain temperature and begins to denature over the optimal temperature. Increasing temperature has two major effects on enzymes activity which are the catalytic rate increases and ultimately denaturation occurs (Thomas and Scopes, 1998).

4.2.2 The Effect of Agitation Rate on Xylose Production

The effect of agitation rate was studied conventionally to obtain the best range of agitation rate on production of xylose. The agitation rate was manipulated at 60, 90, 120, 150, 180 and 210 rpm while the temperature and enzyme concentration were kept constant at 55^{o}C and 1.5 mg/ml respectively. The graph plotted in Figure 4.3 illustrated the concentration of xylose at different agitation rate.

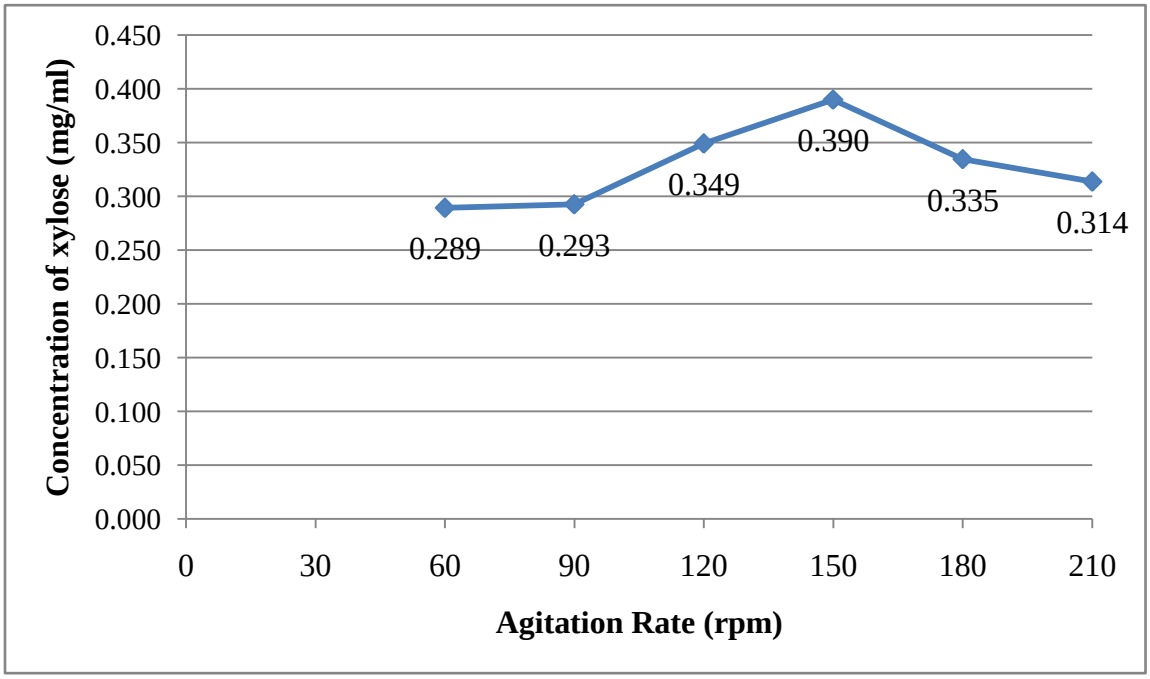

Figure 4.3: The effect of agitation rate on xylose production.

The graph shows that the agitation rate does give effect to the xylose concentration. From the graph, the concentration of xylose increase from 0.289 mg/ml to 0.390 mg/ml at 60 to 150 rpm but declined to 0.314 mg/ml at 210 rpm. The peak is at 150 rpm where the concentration of xylose is 0.390 mg/ml. The graph shows that the xylose concentration increased with the increasing of the agitation rate. However, the xylose concentration was decreased after 150 rpm. The best range of agitation rate chosen was from 120 rpm to 180 rpm.

This pattern of graph was supported by research on effect of agitation speed on morphological changes in *Aspergillus niger* hyphae during production of tannase. Based on the SEM and TEM studies, it was found that there were significant correlations between the speed of agitation and the hyphal morphology including its internal structures and the activity of enzyme. Low production of xylose at high agitation rate is cause by the lower enzyme activity. Enzyme activity was low because of the shear stress at high agitation rate (Lejeune and Baron, 1995). Shear stress is force acting at the surface area.

4.2.3 The Effect of Enzyme Concentration on Xylose Production

The effect of enzyme concentration was studied conventionally to get the best range on xylose production. The enzyme concentration was manipulated at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/ml while the temperature and agitation rate were kept constant at 55^{o}C and 150 rpm respectively. The graph plotted in Figure 4.4 shows the concentration of xylose at different enzyme concentration.

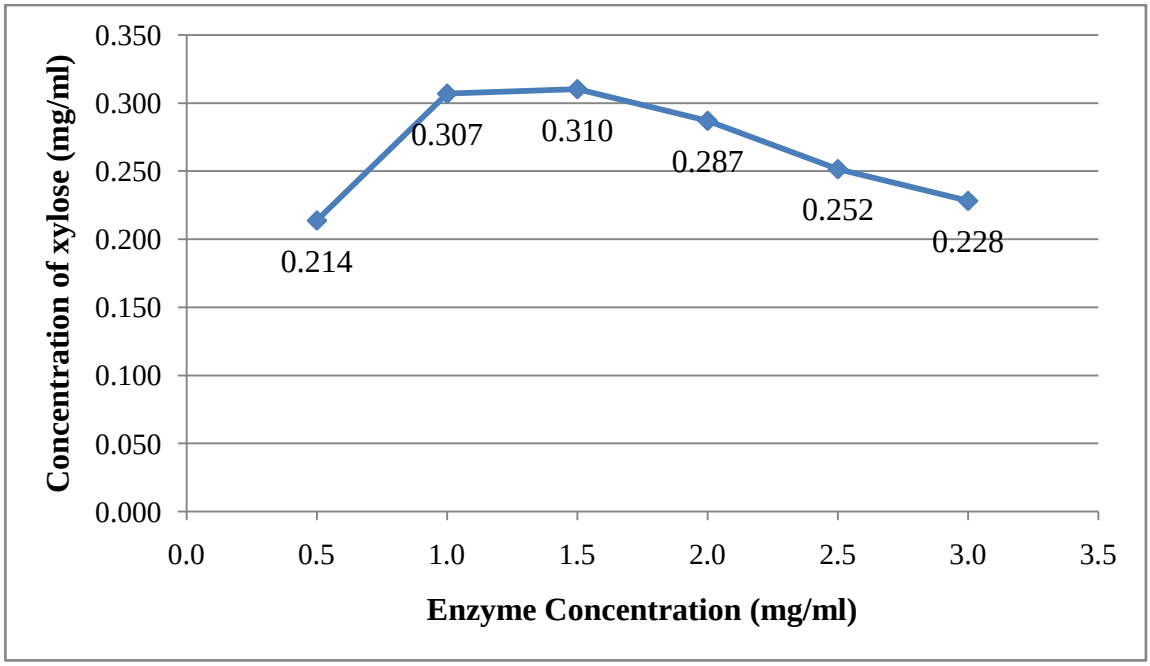

Figure 4.4: The concentration of xylose at different enzyme concentration.

From the graph, the highest xylose concentration was obtained at 1.5 mg/ml enzyme concentration with the xylose production was 0.310 mg/ml. However, the production was declined from 0.310 mg/ml to 0.228 mg/ml at 1.5 mg/ml to 3.00 mg/ml of xylanase concentration. The best range of enzyme concentration chosen was 1 mg/ml to 2 mg/ml.

This pattern of graph was supported by research on kinetics of enzymatic high-solid hydrolysis of lignocellulosic biomass studied by using calorimetry (Olsen *et al*., 2010). The research stated that at low conversion, catalytic efficiency decreases with increasing enzyme dosage for substrates. The overall hydrolysis rate is limited by the availability of the substrate.

Rate of enzyme hydrolysis is dependent only on the number of available reaction sites and amount of enzyme available to occupy these sites (Draude *et al*., 2001). Enzymatic hydrolysis is a process by which enzymes are used to break down starch, cellulose or hemicelluloses into sugar. In this study, xylanase was used to break down xylan from hemicelluloses into xylose.

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 xylose.

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)	50	60
Agitation rate (rpm)	120	180
Enzyme concentration (mg/ml)	1	2

4.3 Analysis of Temperature, Agitation Rate and Enzyme Concentration on Xylose Production Using Response Surface Methodology (RSM)

The Design-Expert package (Version 6.0.8, 2002; 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.

The regression equation Equation 4.2 was obtained from the analysis of variance (ANOVA) in optimization using Response Surface Methodology (RSM).

$$\textit{xylose concentration} = + 0.16 + 0.010A + 0.035B + 0.013C + 0.048A^2 + 0.076B^2 - 0.012C^2 - 0.044AB - 7.625E - 003AC + 4.375E - 003BC \quad \textbf{(Equation 4.2)}$$

The term A indicates the coded value for the temperature, B is the coded value for agitation rate and C is the coded value for enzyme concentration. The combination data from RSM and the responses were shown in Table 4.2. The highest xylose production was at Standard 12 which produced 0.423 mg/ml of xylose. The parameters for the Standard 12 were 55^{o}C of temperature, 200.45 rpm of agitation rate and 1.5 mg/ml of enzyme. The lowest value of xylose production was at Standard 13 which produced 0.107 mg/ml of xylose. The parameters for the Standard 13 were at 55^{o}C of temperature, 150 rpm of agitation rate and 0.66 mg/ml of enzyme.

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

Standard	Factor 1 Temperature (^{o}C)	Factor 2 Agitation rate (rpm)	Factor 3 Enzyme Concentration (mg/ml)	Response 1 Xylose Concentration (mg/ml)
1	50.00	120.00	1.00	0.167
2	60.00	120.00	1.00	0.284
3	50.00	180.00	1.00	0.315
4	60.00	180.00	1.00	0.281
5	50.00	120.00	2.00	0.186
6	60.00	120.00	2.00	0.298
7	50.00	180.00	2.00	0.377
8	60.00	180.00	2.00	0.287
9	46.59	150.00	1.50	0.290
10	63.41	150.00	1.50	0.309
11	55.00	99.55	1.50	0.334
12	55.00	200.45	1.50	0.423
13	55.00	150.00	0.66	0.107
14	55.00	150.00	2.34	0.153
15	55.00	150.00	1.50	0.139
16	55.00	150.00	1.50	0.176
17	55.00	150.00	1.50	0.171

The elevation (residual) between actual data (experimental data) and predicted data was shown in Table 4.3.

Table 4.3: The residual between actual and predicted value

Diagnostics Case Statistics			
Standard Order	**Actual Value**	**Predicted Value**	**Residual**
1	0.167	0.170	-0.003
2	0.284	0.293	-0.009
3	0.315	0.319	-0.004
4	0.281	0.266	0.015
5	0.186	0.202	-0.016
6	0.298	0.295	0.003
7	0.377	0.369	0.008
8	0.287	0.285	0.002
9	0.290	0.282	0.008
10	0.309	0.316	-0.007
11	0.334	0.319	0.015
12	0.423	0.436	-0.013
13	0.107	0.107	0.000
14	0.153	0.151	0.002
15	0.139	0.162	-0.023
16	0.176	0.162	0.014
17	0.171	0.162	0.009

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 xylose production

Source	**F Value**	**Prob > F**	
Model	51.14	< 0.0001	*significant*
A	4.83	0.0640	
B	57.98	0.0001	
C	8.19	0.0243	
A^2	92.38	< 0.0001	
B^2	230.16	< 0.0001	
C^2	5.37	0.0536	
AB	54.74	0.0001	
AC	1.63	0.2418	
BC	0.54	0.4870	
Residual			
Lack of Fit	0.59	0.7265	*not significant*
Pure Error			
Cor Total			

Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case, the liner term of agitation rate (B), enzyme concentration (C), squared terms of temperature (A^2), agitation rate (B^2) and the interaction term of temperature and agitation rate (AB) are significant model. Values greater than 0.1 indicate the model terms are not significant. The interaction term of temperature and enzyme concentration (AC) is not significant as well as agitation rate and enzyme concentration (BC). If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

The "Lack of Fit F-value" of 0.59 implies the Lack of Fit is not significant relative to the pure error. There is a 72.65% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good to ensure the model to be fit. The "Pred R-Squared" of 0.9148 is in reasonable agreement with the "Adj R-Squared" of 0.9658. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 25.424 indicates an adequate signal. This model can be used to navigate the design space.

3D Response Surface will result in a three dimensional display of the response surface. The 3D response surface is the graphical representation of regression equation. In addition, all the mutual interactions among the tested variables are significant. Through the three-dimensional plot it is very easy and convenient to understand the interactions between two variables and to locate their optimum ranges. Figure 4.5 to 4.7 shows the 3D interaction between temperature, agitation rate and enzyme concentration based on the seventeen experiments carried out previously.

Figure 4.5 shows the combined effect of the temperature and agitation rate on the xylose production.

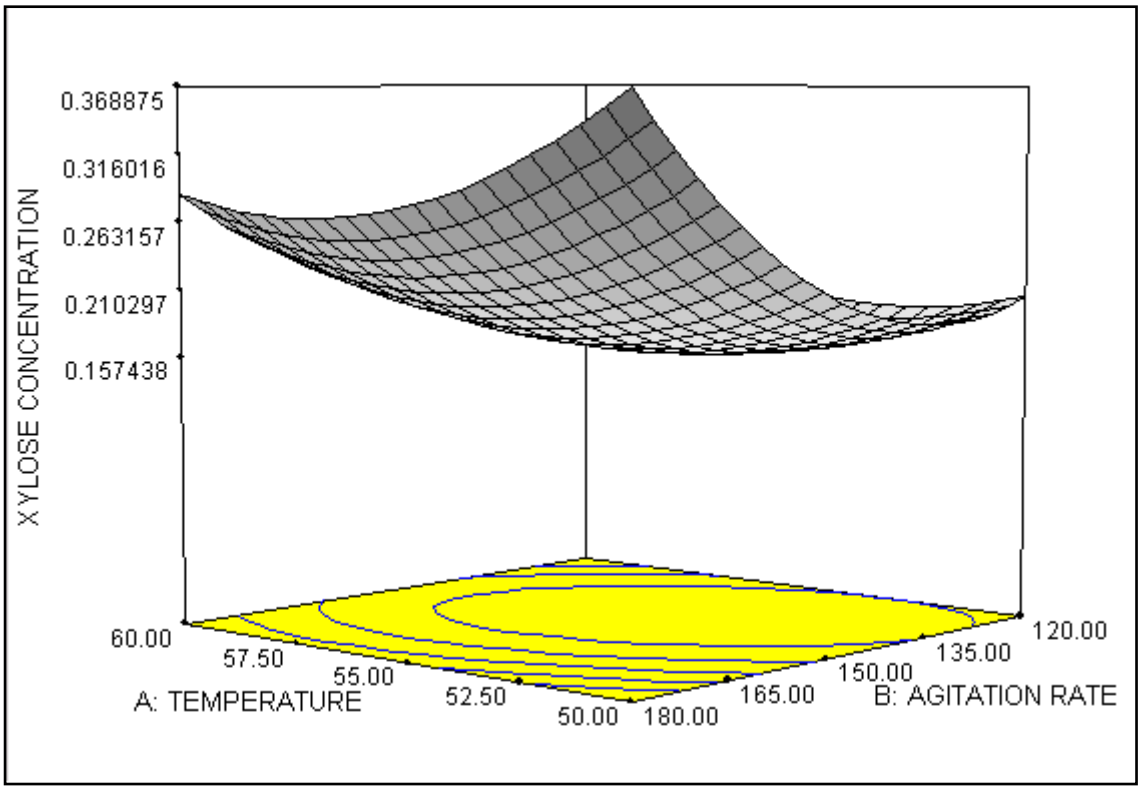

Figure 4.5: Response surface plot of the combined effect of temperature and agitation rate on the xylose production

Based on the Figure 4.5, the optimum value of temperature was detected at 50^{o}C with 0.369 mg/ml of xylose production. The xylose production decrease as the temperature increased. Similar result reported in the study of enzymatic synthesis of fruit flavor esters by immobilized lipase from *Rhizopus oligosporus* optimized with Response Surface Methodology (Mahapatra *et al*., 2009). In lipase-catalyzed reactions, temperature significantly influences both the initial rate of the reaction and stability of the enzyme. The study reported that when the temperature increased, the synthesis of flavor ester decreased. This indicated that the enzyme tertiary structure might have been disrupted causing it to denature at higher temperatures (Romero *et al*., 2005).

Figure 4.6 shows the combined effect of the temperature and enzyme concentration on the xylose production.

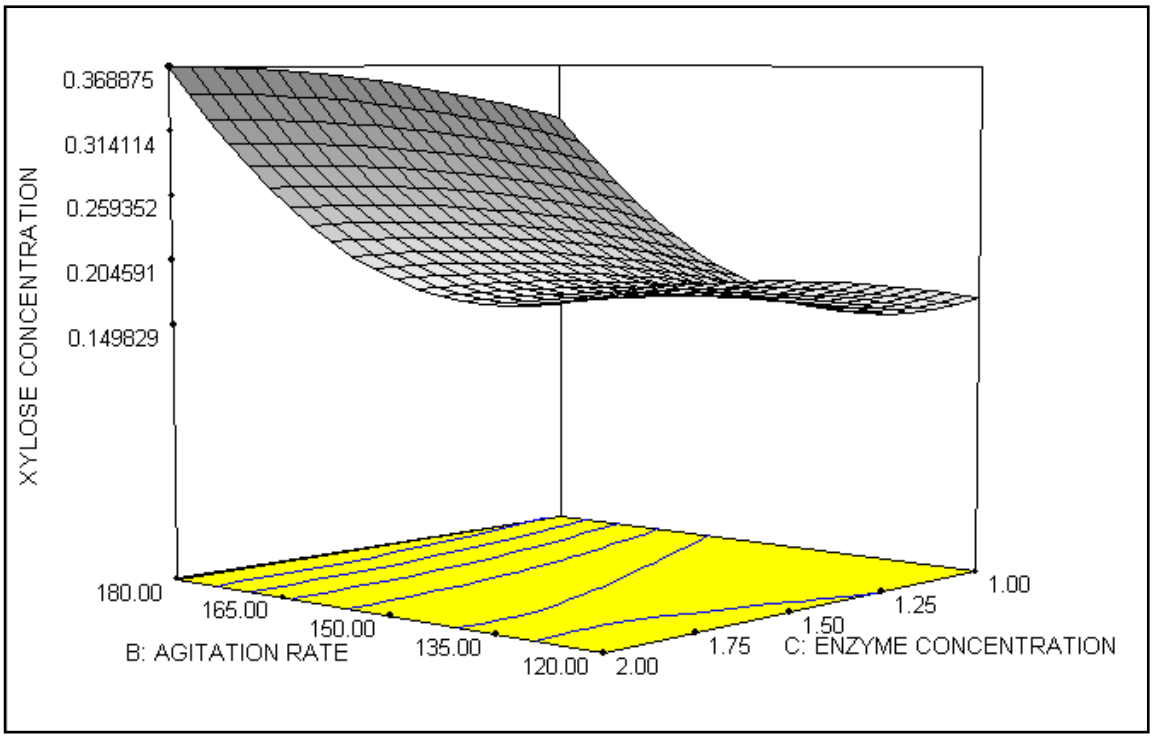

Figure 4.6: Response surface plot of the combined effect of agitation rate and enzyme concentration on the xylose production

From Figure 4.6, at 180 rpm of agitation rate, the xylose production was measured 0.369 mg/ml. The xylose production increased as the agitation rate increased. Johns *et al*, (1994) stated that high agitation rate gave superior result compared to the low agitation rate on hyaluronic acid production. Agitation enables an even and effective distribution of the spore suspension, water required for moisture control and of any other nutrient solutions (Suryanarayan, 2003). These statements proved that the agitation rate does give effect on the production. As the agitation rate increase, the production of xylose also increases.

Figure 4.7 shows the combined effect of the agitation rate and enzyme concentration on the xylose production.

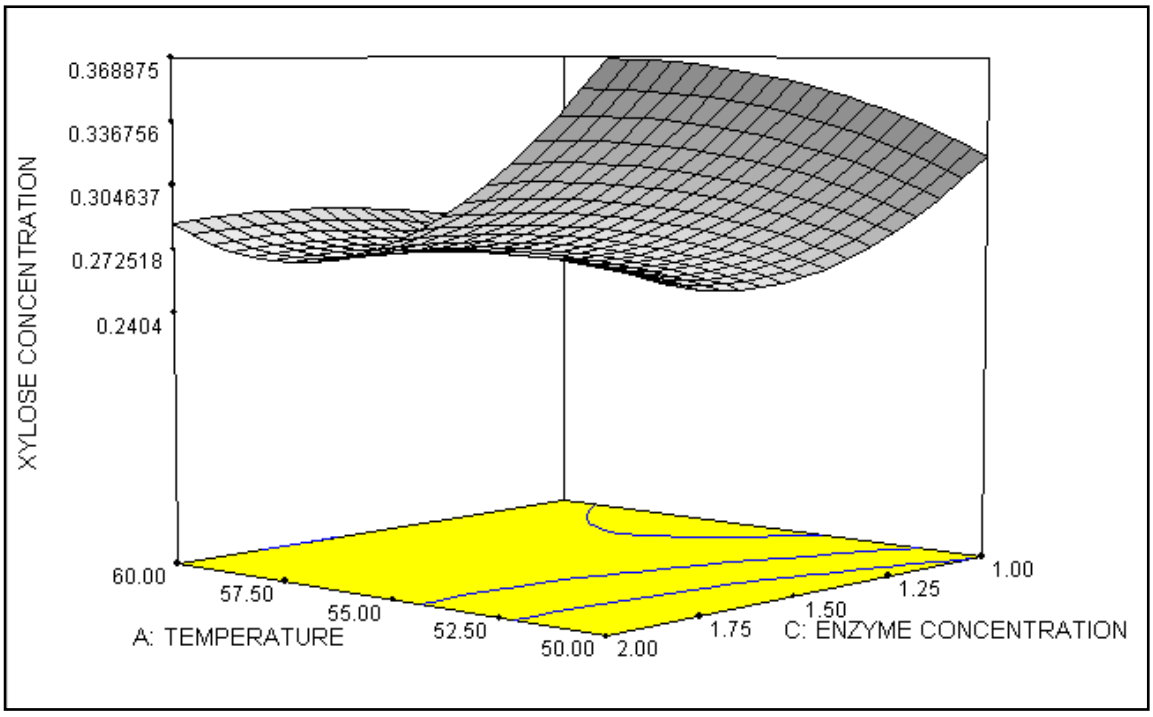

Figure 4.7: Response surface plot of the combined effect of temperature and enzyme concentration on the xylose production

The graph shows that the xylose production increased as the enzyme concentration increased. The enzyme concentration was at 2 mg/ml with the 0.369 mg/ml of xylose production. Similar result was supported by research on the effects of enzyme concentration, temperature and incubation time on nitrogen content and degree of hydrolysis of protein precipitate from cockle (*Anadara granosa*) meat wash water by Haslaniza *et al*. (2010). The research reported that increasing bromelain concentration from 0 to 2.5% produced an increase in nitrogen content and degree of hydrolysis. This proved as the enzyme concentration increase, the production also increase.

4.4 Optimization of Temperature, Agitation Rate and Enzyme Concentration on Xylose Production using Response Surface Methodology (RSM)

The result from the optimization step using Response Surface Methodology (RSM) showed that the optimized condition for maximum xylose production were 50°C of temperature, 180 rpm of agitation rate and 2.00 mg/ml of enzyme concentration. The optimize xylose production predicted by the model was 0.367mg/ml. An experiment need to be done in order to validate these optimized conditions. The xylose production obtained from the experimental was 0.373 mg/ml. Table 4.5 shows the optimization of xylose production from sugarcane bagasse using RSM.

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

Parameter	**Before optimization**		**After optimization**		
	Value	**Xylose concentration (mg/ml)**	**Value**	**Xylose concentration (mg/ml)**	
				Predicted	**Actual**
Temperature	55^{o}C	0.228	50^{o}C	0.367	0.373
Agitation rate	150 rpm		180 rpm		
Enzyme concentration	3 mg/ml		2 mg/ml		

Table 4.5 shown the optimized condition of the parameters in this research. Before the optimization, the conditions were 55^{o}C, 150 rpm and 3 mg/ml of enzyme concentration with 0.228 mg/ml of xylose production. After the optimization, the temperature had been reduced to 50^{o}C, 180 rpm of agitation rate and the enzyme concentration also had been reduced to 2 mg/ml. Thus, the reducing of enzyme consumption could reduce of the cost of production. The predicted xylose concentration after the optimization was 0.367 mg/ml and the actual value of xylose concentration from validation experiment was 0.373 mg/ml. The increment of xylose production was 63.6 %. As a conclusion, the objective of this study to optimize the production of xylose from sugarcane bagasse using Response Surface Methodology (RSM) was achieved.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study on optimization of xylose production from sugarcane bagasse using Response Surface Methodology (RSM) was successfully achieved 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 xylose production showed that the best range of temperature was between 50°C - 60°C. The best range for agitation rate was between 120 rpm – 180 rpm and the best range for enzyme concentration was between 1 – 2 mg/ml. These ranges of data obtained from the conventional method were used for optimization process using Response Surface Methodology (RSM).

After optimization and validation, the optimized conditions on xylose production were 50°C of temperature, 180 rpm of agitation rate and 2.0 mg/ml of enzyme concentration. The xylose production was 0.373 mg/ml compared to predicted value, 0.367mg/ml. Before optimization, the xylose yielded was 0.228 mg/ml. The percentage increment was 63.6%. As a conclusion, the objective of this research to optimize the xylose production from sugarcane bagasse using Response Surface Methodology (RSM) was successfully conducted.

5.2 Recommendation

Most of biomass residue contains hemicelluloses which can produce xylose or other simple sugar. Various agricultural residues, such as corn fiber, corn stover, wheat straw and rice straw contain about 20–40% hemicellulose, the second most abundant polysaccharide in nature (Saha, 2003). These substrates could be use to replace sugarcane bagasse in the future studies on xylose production.

Other parameter such as pH, agitation time, acid concentration and substrate concentration also can be studied in order to optimize the xylose production. Rahman *et al.*, (2006) stated that the effect of H_2SO_4 concentration, reaction temperature and reaction time for production of xylose could be studied. Batch reactions were carried out under various reaction temperature, reaction time and acid concentrations and Response Surface Methodology (RSM) was followed to optimize the hydrolysis process in order to obtain high xylose yield.

Scale up to 19.19 g/L by using bioreactor also could be done to increase the production of the xylose. Rodrigues *et al*., (2010) studied that experiments could be conducted in laboratory and semi-pilot reactors to optimize the xylose recovery and to reduce the generation of sugar degradation products, as furfural and 5-hydroxymethylfurfural (HMF). The hydrolysis scale-up procedure was based on the H-Factor, that combines temperature and residence time and employs the Arrhenius equation to model the sulfuric acid concentration (100 mg acid/g dm) and activation energy (109 kJ/mol). This procedure allowed the mathematical estimation of the results through simulation of the conditions prevailing in the reactors with different designs.

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APPENDIX A

MATERIALS AND METHOD

Appendix A1

Preparation of Enzyme Concentration in Sodium Citrate Buffer

Mix in the following proportions to get the required concentration of xylanase

Xylanase Concentration (mg/ml)	Mass of xylose (mg)	Volume of buffer solution (ml)
0.5	50	100
1.0	100	100
1.5	150	100
2.0	200	100
2.5	250	100
3.0	300	100

APPENDIX B

RESULT AND DISCUSSION

Appendix B1

Experiment Data for Standard Curve of Xylose

Table B-1: Standard Curve of Xylose Concentration

XYLOSE CONCENTRATION (mg/ml)	OD reading (λ=540nm)
0.0	0.000
0.2	0.148
0.4	0.358
0.6	0.646
0.8	0.710
1.0	1.095
1.2	1.145
1.4	1.288
1.6	1.373
1.8	1.494
2.0	1.805

Appendix B2

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

Table B-2: Effect of Temperature on Xylose Production

TEMPERATURE (°C)	OD READING	XYLOSE CONCENTRATION (mg/ml)
15	0.062	0.069
25	0.141	0.156
35	0.219	0.243
45	0.268	0.297
55	0.360	0.399
65	0.276	0.306

Table B-3: Effect of Agitation Rate on Xylose Production

AGITATION RATE (rpm)	OD READING	XYLOSE CONCENTRATION (mg/ml)
60	0.261	0.289
90	0.264	0.293
120	0.315	0.349
150	0.352	0.390
180	0.302	0.335
210	0.283	0.314

Table B-4: Effect of Xylanase Concentration on Xylose Production

ENZYME CONCENTRATION (mg/ml)	OD READING	XYLOSE CONCENTRATION (mg/ml)
0.500	0.193	0.214
1.000	0.277	0.307
1.500	0.280	0.310
2.000	0.259	0.287
2.500	0.227	0.252
3.000	0.206	0.228

Appendix B3

Enzymatic hydrolysis using RSM suggested parameters

Table B-5: Experimental data based on RSM suggested parameters

Standard	Factor 1 Temperature (^{o}C)	Factor 2 Agitation rate (rpm)	Factor 3 Enzyme Concentration (mg/ml)	Response 1 Xylose Concentration (mg/ml)
1	50.00	120.00	1.00	0.167
2	60.00	120.00	1.00	0.284
3	50.00	180.00	1.00	0.315
4	60.00	180.00	1.00	0.281
5	50.00	120.00	2.00	0.186
6	60.00	120.00	2.00	0.298
7	50.00	180.00	2.00	0.377
8	60.00	180.00	2.00	0.287
9	46.59	150.00	1.50	0.290
10	63.41	150.00	1.50	0.309
11	55.00	99.55	1.50	0.334
12	55.00	200.45	1.50	0.423
13	55.00	150.00	0.66	0.107
14	55.00	150.00	2.34	0.153
15	55.00	150.00	1.50	0.139
16	55.00	150.00	1.50	0.176
17	55.00	150.00	1.50	0.171

Appendix B4

RSM Analysis

Predicted Vs. Actual Analysis

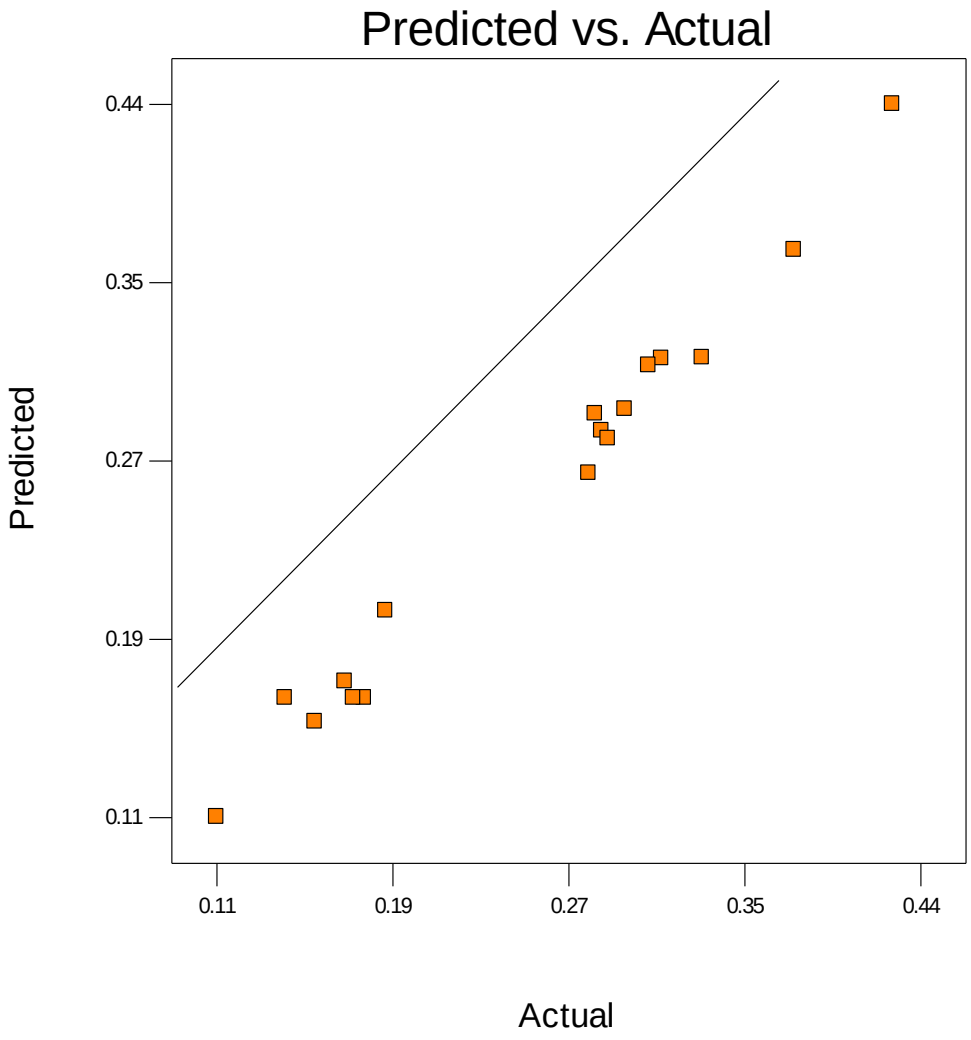

Figure B-1: Graph of Predicted Vs. Actual Analysis

Outlier T Analysis

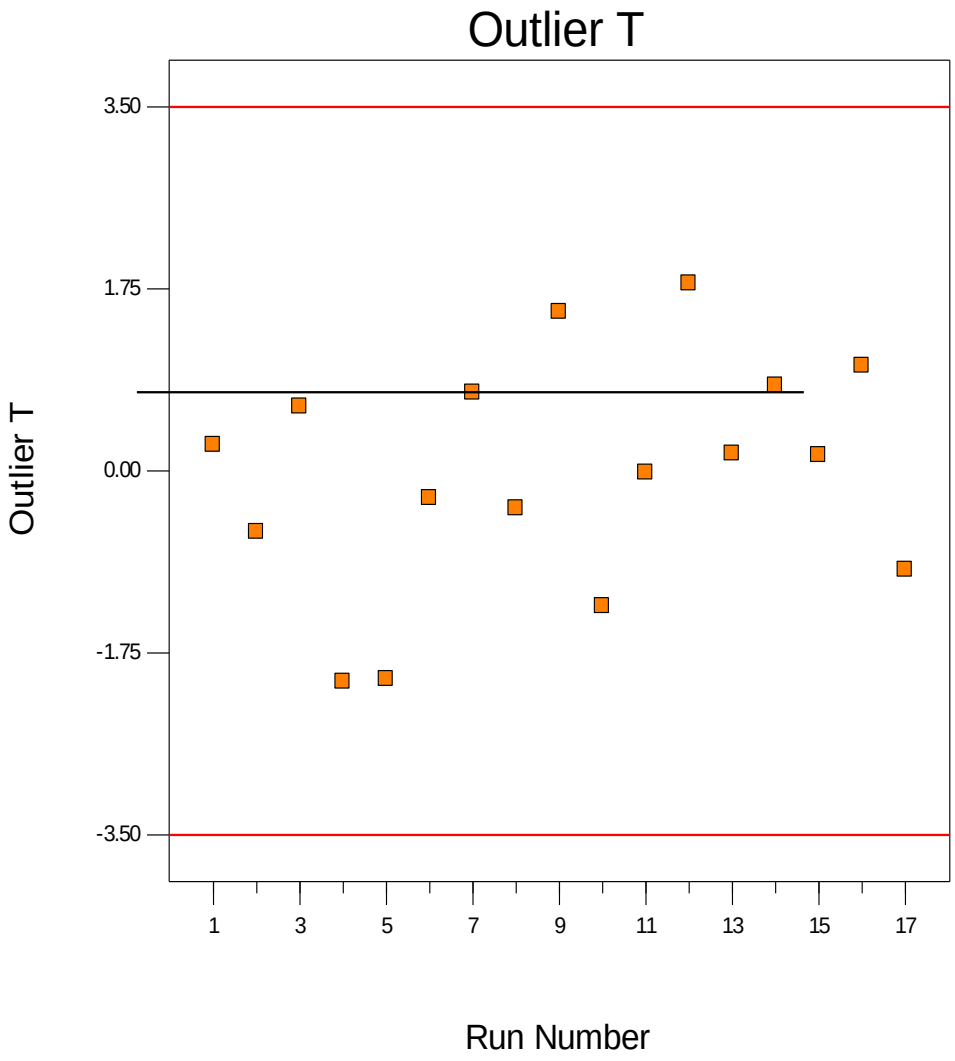

Figure B-2: Graph of Outlier T Analysis

Residuals Vs. Temperature Analysis

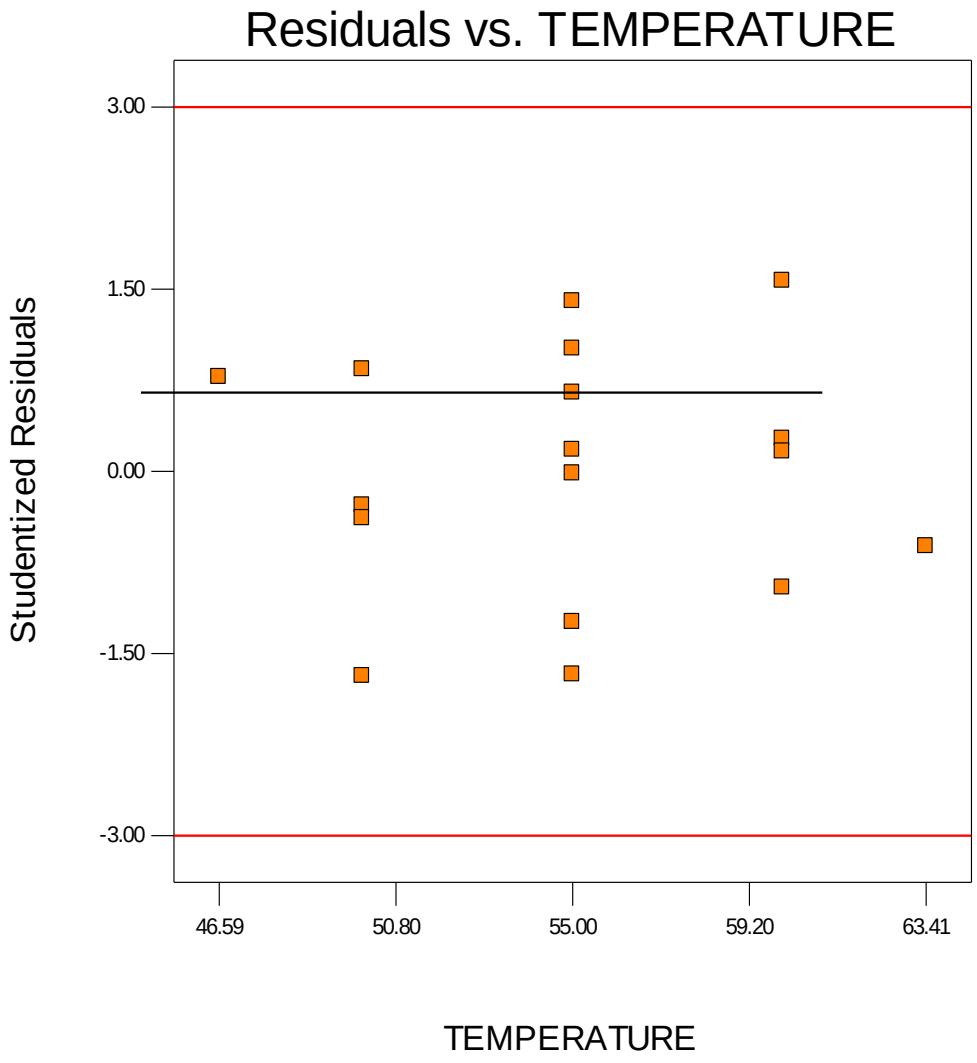

Figure B-3: Graph of Residuals Vs. Temperature

Residuals Vs. Agitation Rate Analysis

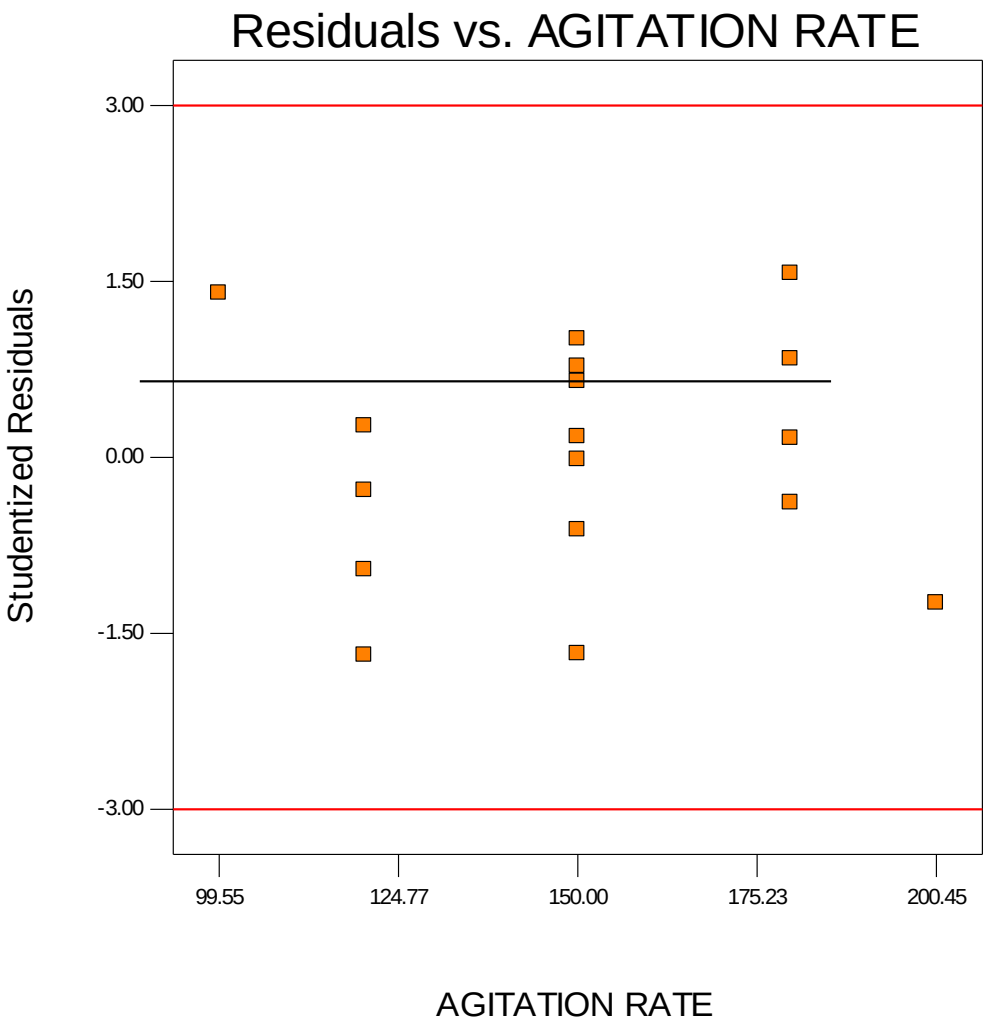

Figure B-4: Graph of Residuals Vs. Agitation Rate

Residuals Vs. Enzyme Concentration Analysis

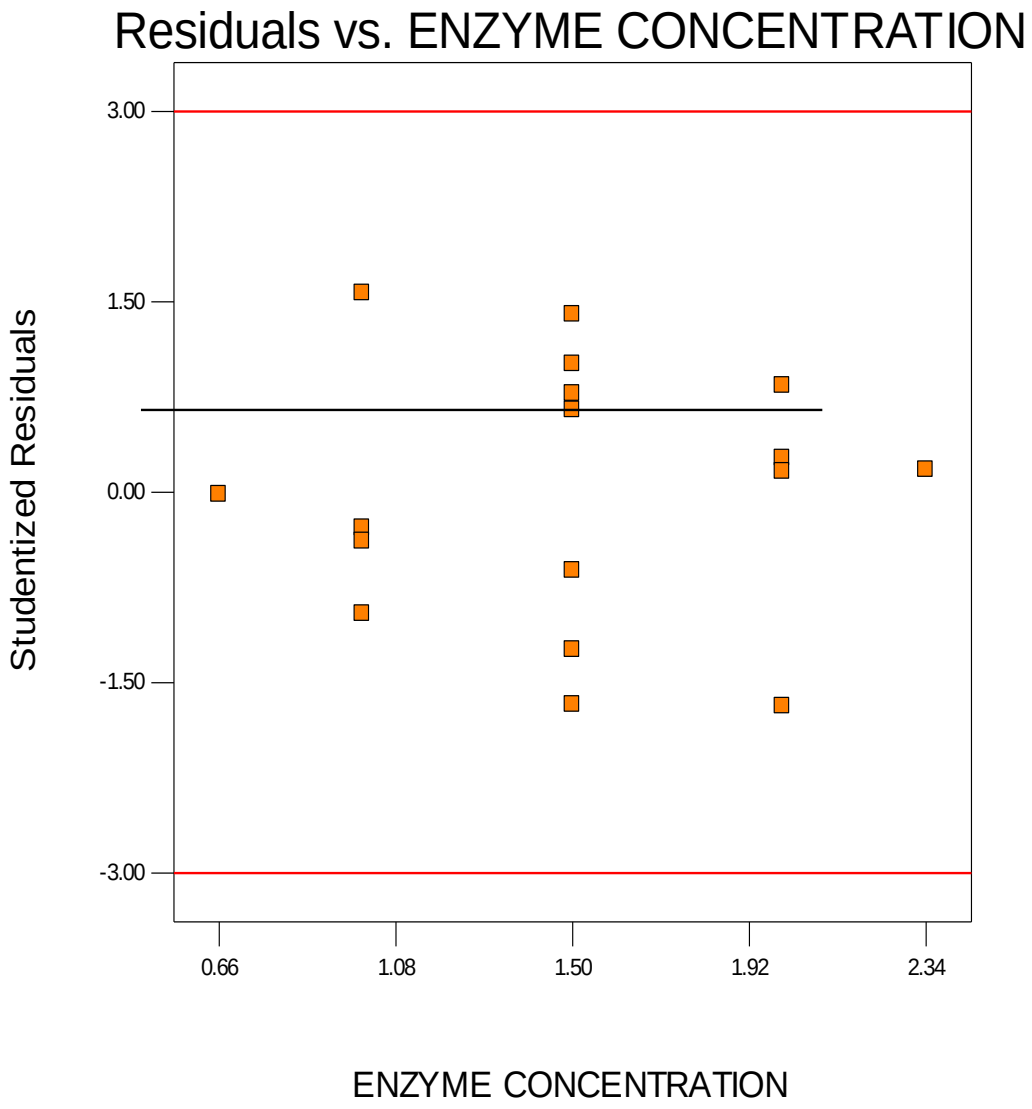

Figure B-5: Graph of Residuals Vs. Enzyme Concentration

Box-Cox Analysis

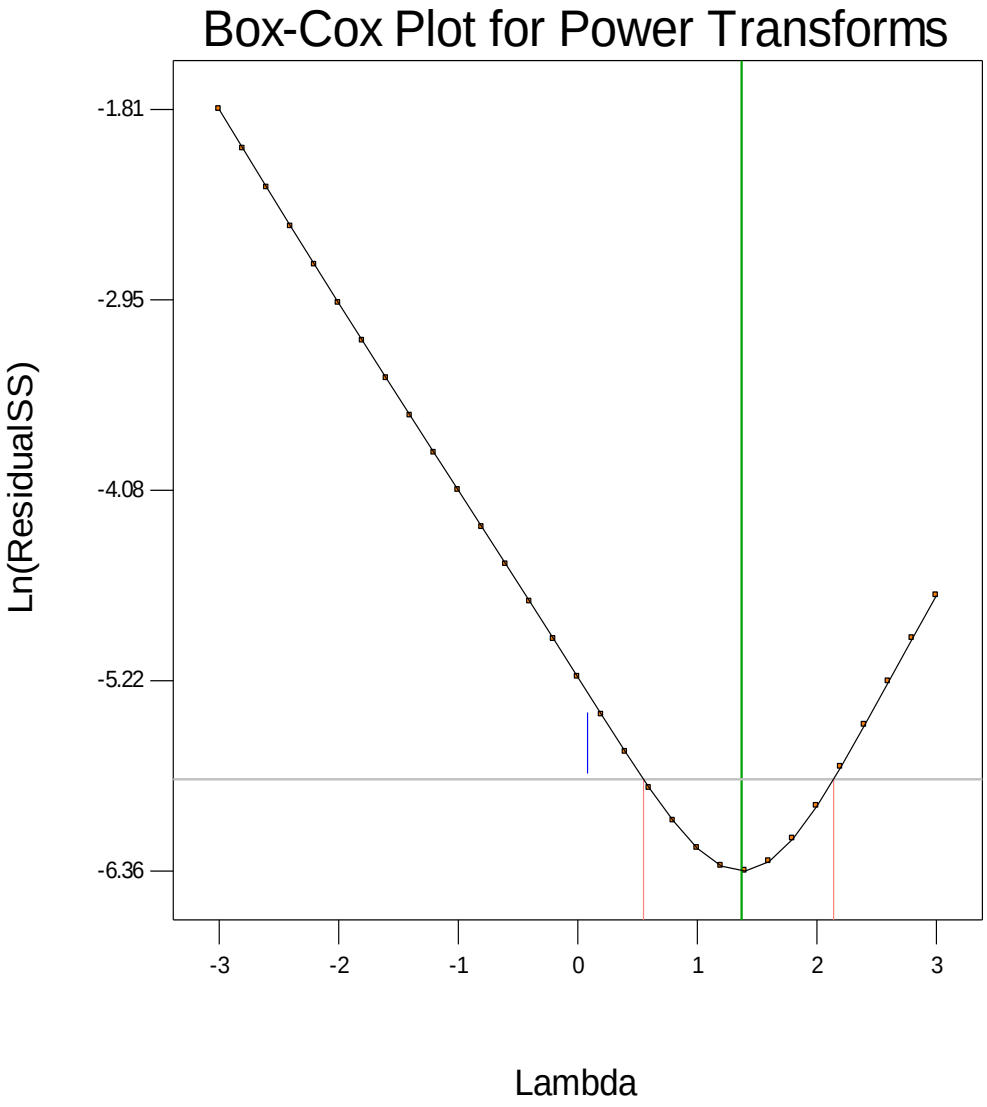

Figure B-6: Graph of Box-Cox Analysis