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NAME: Mr. Teerayut Khunrong

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Vittaya Punsuvon, Ph.D.)

THESIS CO-ADVISOR

(Ms. Pinsuda Viravathana, Ph.D.)

THESIS CO-ADVISOR

(Mrs. Potjanart Suwanruji, Ph.D.)

THESIS CO-ADVISOR

(Ms. Pilanee Vaithanomsat, Ph.D.)

DEPARTMENT HEAD

(Assistant Professor Noojaree Prasitpan, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

OPTIMIZATION OF DELIGNIFICATION AND ENZYME HYDROLYSIS OF STEAM EXPLODED OIL PALM TRUNK FOR ETHANOL PRODUCTION

TEERAYUT KHUNRONG

A Thesis Submitted in Partial Fulfillment of
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Oil palm trunk chip, pretreated by steam explosion under selected condition, was used as a substrate for enzymatic hydrolysis. Response surface methodology (RSM) was used for optimization of delignification and hydrolysis processes. In delignification by NaOH and KOH, RSM was optimized according to the concentration of pulp, the concentration of alkaline base, reaction time and temperature. For enzyme hydrolysis, RSM was optimized according to temperature, hydrolysis duration, enzyme concentration and the concentration of pulp. Under these various delignification and hydrolysis conditions, the yield of glucose, alpha cellulose, removed lignin were determined. Comparative results with and without a delignification step, influenced on hydrolysis process, were presented. The optimum condition for NaOH and KOH delignification was 11%, 12% w/v of pulp concentration, 21.5%, 23.5% w/w of alkaline base concentration , 65 min reaction time, 78 ^{0}C, 80^{0}C temperature, respectively. These conditions gave 47.5% and 48% of glucose remaining in pulp after NaOH and KOH delignification, respectively. The optimum conditions for enzymatic hydrolysis of pulp obtained after NaOH and KOH delignification were 54, 65 FPU/g enzyme concentration, 50 h, 60 h hydrolysis duration, both 50 ^{0}C of temperature, and 2.5% pulp concentration, respectively. These conditions gave 85% of glucose yield for NaOH and 81% for KOH delignification. The hydrolysis process with delignification gave higher glucose yield than the hydrolysis process without delignification. The fermentation of 50 g/l glucose from enzyme hydrolysis gave maximum ethanol yield about 65%. Moreover, it was found that the alkaline delignification process prior to ethanol fermentation had no significant influence on the ethanol yield.

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
Df	=	Degree of freedom
CCD	=	Central composite design
R	=	Correlation coefficient
RF	=	Response factor
RSM	=	Response swurface methodology

OPTIMIZATION OF DELIGNIFICATION AND ENZYME HYDROLYSIS OF STEAM EXPLODED OIL PALM TRUNK FOR ETHANOL PRODUCTION

INTRODUCTION

The inevitable depletion of the World's petroleum supply and the increasing problem of greenhouse gas effects have resulted in an increasing worldwide interest in alternative nonpetroleum–based sources of energy. As the transportation sector is practically entirely dependent on oil and as it is responsible for half of the total CO_2 emission (Mieleuz, 2001), the increasing in market share of renewable biofuels includes ethanol fuel. The use of ethanol fuel will significantly reduce net carbon dioxide emission once it replaces fossil fuels because fermentation-derived ethanol is already a part of the global carbon cycle. However, to enhance the market position of the biofuel the production cost should be reduced. Nowadays, the raw material and enzyme production are the two main contributors to the overall costs, thus using high cellulose containing agricultural residues as a feedstock, for example, oil palm trunk, could result in cost reduction. Oil palm trunk is an abundant agricultural residue in the southern part of Thailand. The utilization has not been intensively studied. The increase of oil palm trunk every year could then cause pollution in the future thus development technology for ethanol production is urgently needed. The chemical component in oil palm trunk has been shown by Punsuvon *et al*. (2005) that oil palm trunk consists of three main structural components; 41% cellulose, 34% hemicellulose and 17% lignin. These carbohydrate fraction cellulose and hemicellulose more than 70% can be a potential raw material for fuel ethanol production. Due to structural features, such as the presence of lignin, acetyl groups, and cellulose crystallinity, lignocellulosic biomass must be pretreated to enhance its enzymatic digestibility before microbial conversion into liquid fuels (Kaar, 1995). The steam explosion has been shown to be a promising pretreatment method for fractionation of cellulose, hemicellulose and lignin. This fractionation method breaks down lignin structure and solubilizes the major part of hemicellulose fraction, thus enhancing enzymatic digestibility of the raw materials.

The main objectives of this study are

1. Optimize the condition of delignification process using the response surface methodology (RSM).
2. Optimize the condition of enzyme hydrolysis process using the response surface methodology (RSM).
3. Compare NaOH and KOH delignification with nondelignification of pulp in terms of sugar yield.
4. Study ethanol fermentation of the hydrolyzed glucose obtained from enzyme hydrolysis.

LITERATURE REVIEW

1. General Background

1.1 Oil palm trunk waste

The original of oil palm is in the tropical forest in West Africa. The obtained name is *Elaeis guineensis*. Its trunk looks like the coconut trunk and each of them contains brunch with 20 -30 kilograms weight.

Due to the palm oil is used as a raw material in many industries such as soap, cosmetic, detergent, vegetable oil and biodiesel. The oil palm is one of the most important plants in the world. Europe and America import palm oil from Africa and Asia countries. Nowadays oil palm becomes the economic plant of Malaysia, Indonesia and Thailand. In 2007 Thailand had 2.3 million rais of oil palm plantation mostly in the south and southwest. Usually, from the ages 3 years to 25 years, is the economical life of oil palm and after that, it is cut for replantation. The size of trunk usually is 15-18 meters in length and 46-60 centimeters in diameter. The trunk after cutting is agricultural waste causing problem in elimination but due to the trunk contains about 42% cellulose, 34.4% hemicellulose, 17.1% lignin and 7.3% other compounds, these agricultural wastes could make value added products (Pumiput, 2006).

2. The Chemical Composition of wood

Wood contains 4 main components: cellulose, hemicellulose, lignin and extractive. A simplified picture is that cellulose forms a skeleton which is surrounded by other substances functioning as matrix (hemicellulose) and encrusting (lignin) materials (Sjostrom, 1993).

2.1 Cellulose

Cellulose is the main constituent of wood. Approximately 40-45% of dry substance in most wood species is cellulose which is located predominantly in the secondary cell wall. The length of a native cellulose molecule is at least 5000 nm that corresponding to the chain with about 10,000 glucose units. Cellulose is a homopolymer composed of glucose units which are linked together by β-(1→4)-glycosidic bonds as shown in Figure 1. Cellulose molecules are completely linear and have a strong tendency to form intra and intermolecular hydrogen bonds. Bundles of cellulose molecule are thus aggregated together in the form of microfibrils, which highly order (crystalline) regions alternate with less ordered (amorphous) regions.

Properties of cellulose

1. Linear polysaccharides β-(1→4)-glycosidic linkage.
2. Repeating unit of glucose.
3. High molecular weight and high degree of polymerization (DP).
4. Cellulose is microfibril.
5. The same properties in hard and soft wood.
6. No side chain.

Cellulose could be used as a raw material for producing carboxymethyl cellulose (CMC), ethanol, and single cell protein (SCP).

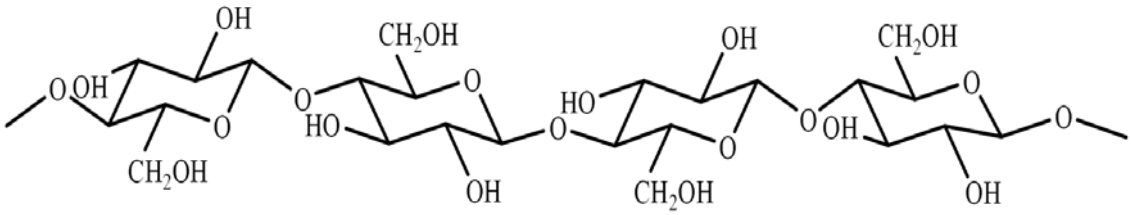

Figure 1 Structure of cellulose.

Source: Sjostrom (1993).

2.2 Hemicellulos

Hemicelluloses are originally believed to be the intermediates in the biosynthesis of cellulose. Hemicelluloses are heteropolymer composed of D-glucose, D-mannose, D-gaclactose, D-xylose, L-arabinose, and small amounts of L-rhamnose. Most hemicelluloses have a degree of polymerization of only 200 that are more easily hydrolyzed by acid than cellulose. The amount of hemicelluloses on dry weight of wood are usually between 20 to 30%. The composition and structure of the hemicellulose in softwoods differ in a characteristic way from those in hardwood. Oil palm trunk is the hard wood which consists mainly hemicellulose gluconoxylan or xylan and glucomannan.

Xylan (Figure 2) contains a backbone of D-xylose units linked β-(1→4)-glycosidic with acetyl groups at C-2 or C-3 of the xylose units, with average 7 acetyls per 10 xylose units. The xylan is substituted with side chains of 4-O-methylglucuronic acid units linked to the xylan backbone through a linking (1-2) with an average frequency of approximately a uronic acid group per 10 xylose units. Glucomanan is D-glucose and D-mannose units linked by β-(1→4)-glycosidic bond. The glucose: mannose ratio varies between 1:2 and 1:1, depending on the wood species.

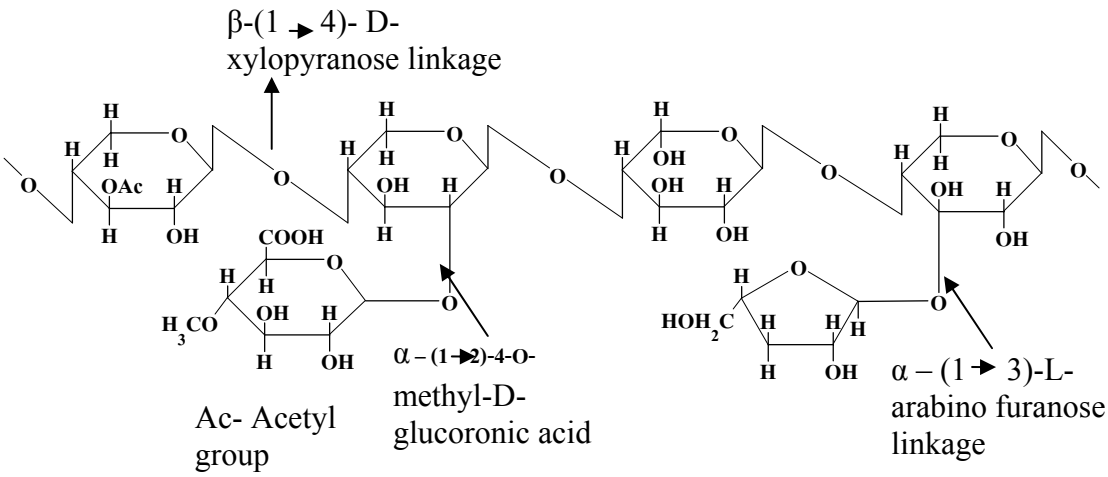

Figure 2 Structure of xylan.

Properties of hemicellulose

1**.** Repeating unit of different sugar**.**

2. Hemicelluloses are different in hard wood and soft wood.

3. Molecular weight is lower than cellulose.

5. Noncrystalline form.

6. More reactive than cellulose.

2.3 Lignin

Lignin is present in the cellular cell wall, conferring structural support and impermeability. Moreover, it resists against microbial attack and oxidative stress. Structurally, lignin is an amorphous heteropolymer, non-water soluble and optically inactive. It consists of phenylpropane units joined together by different types of linkages. The precursors of lignin are the three aromatic alcohols; coumaryl alcohol, coniferyl alcohol and sinapyl alcohols as shown in Figure 3, and the structure of lignin shown in Figure 4. Lignins from softwoods are mainly a polymerization product of coniferyl alcohol or guaiacyl lignin. Hard wood lignin is mainly sinapyl alcohol or syringyl-guaiacyl lignin.

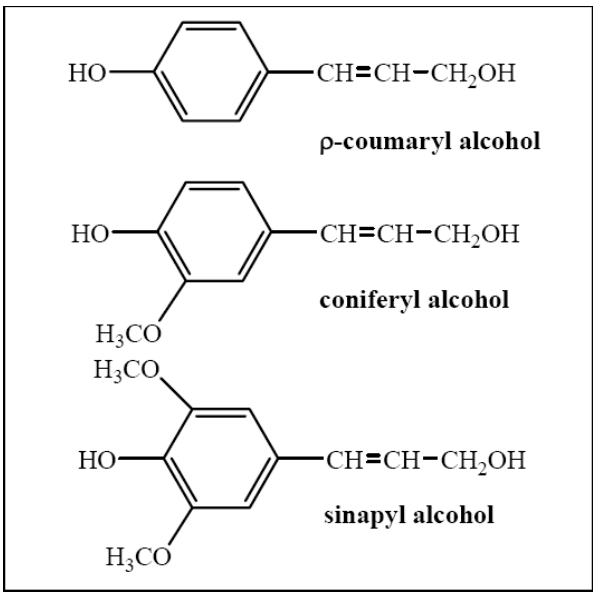

Figure 3 Structure of three precursors of lignin.

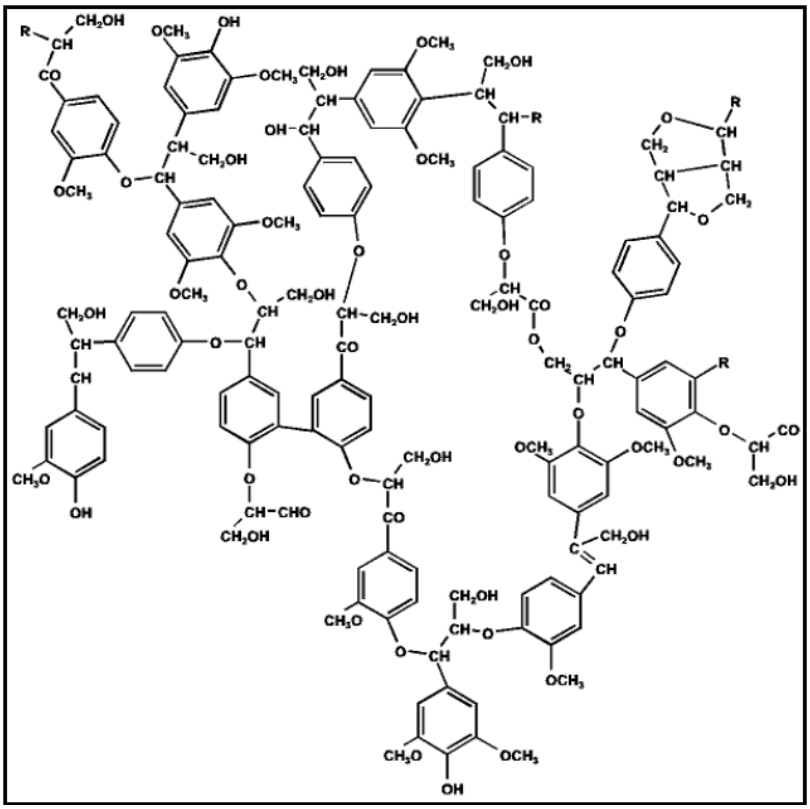

Figure 4 Part of lignin structure.

Source: Sjostrom (1993).

The function of lignin in plants is as an encrusting agent in the cellulose/hemicellulose matrix. It is often referred to the plant cell wall adhesive. Both lignin and extractive in plant reduce the digestibility in animals. Lignin is also associated with the hemicelluloses in some cases forming lignin-carbohydrate complexes that are resistant to hydrolysis even under the pulping condition.

2.4 Wood Extractive

Wood extractives are compounds of diverse nature with low to moderately high molecular weights which are soluble in organic solvents or water. They are a group of cell wall mainly consisting of fats, fatty acids, fatty alcohols, phenols, terpenes, steroids, resin acids, rosins, waxes, etc. These chemicals exist as monomers and polymers. They impart color, odor, taste, and occasionally decayed resistance to wood (Biermann, 1996).

2.5 Ash

Ash is inorganic content of a plant, which is an approximate measure of the mineral salts and other inorganic matters in the fiber after combustion at a temperature of 575±25°C. The inorganic content could be quite high in plants containing large amount of silica.

2.6 Monosaccharide

Most of the monosaccharide occurs as glycosides and as units in oligosaccharides and polysaccharides and only comparatively few of them are present free in plant. Glucose is the most abundant monosaccharide in nature. It could be prepared from cellulose and starch by acidic or enzymic hydrolysis of the other aldohexose. Mannose and galactose was important in hemicelluloses. The most common representatives of aldopentoses are xylose and arabinose. Fructose, which represented the only abundant ketose in plant, is present both free and in a combined state. Fructose is obviously not present in the cell wall polysaccharides of wood.

3. Steam Explosion Technique

Steam explosion technology is a method to defibrillate lignocellulose's materials that is studied about 60 years ago. The separation technique with steam explosion could be batch and continuous reactors. This technique could be applied in agriculture industry such as pulp and paper industry, cellulose industry and ethanol production industry. The steam explosion is a process that uses high temperature and high pressure of steam to fractionate hemicelluloses from biomass in a short period of time. The leaving part is cellulose and lignin. Lignin could be separated later by dissolving in alkaline solution. The parameters controlling the steam explosion technique are reaction temperature (T) and retention time (t). The relationship between temperature and time has been defined in one parameter as severity factor (R_0) (Ibrahim, 1998) as shown in the following equation:

$$R_0 = \int_0^t \exp[(T - 100/14.75)]dt \qquad (1)$$

Where R_0 = Severity factor

T = Reaction temperature, ^{0}C

t = Retention time, min

The main component and basis in operation steam explosion machine is shown in Figure 5 with these details.

1 = Water tank

2 = Heater tank

3 = Valve

4 = Reactor

5 = Receiver tank

6 = Valve

7 = Tube to release pressure

8 = Ball value

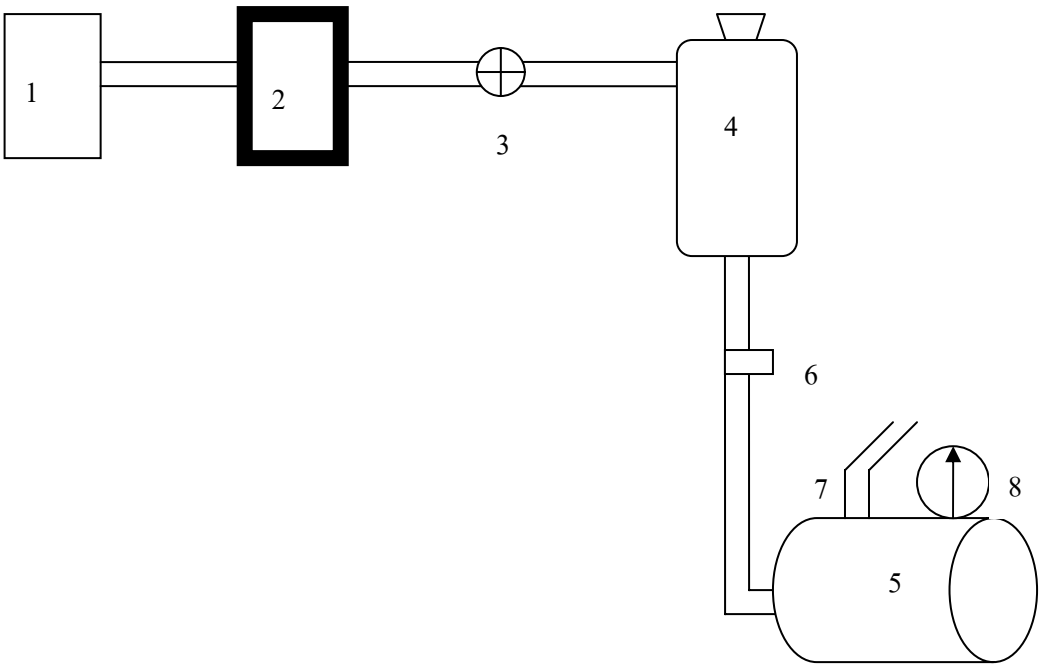

Figure 5 Main components of steam explosion machine.

The basic of operation on steam explosion is that water from machine 1 (water tank) is transferred to 2 (Heater tank) to produce steam. After that, steam is brought to valve 3 (valve) which controls the steam pressure before releasing to 4 (Reactor) that filled with chip. When open valve 3, steam enters the reactor 4. After that, the time is recorded for the explosion at constant pressure of steam until finish time. Thus, valve 6 connected between reactor 4 and receiver 5 (Receiver) is opened and the pulp is transferred into receiver 5. Then, pressure is released by open valve 7 until the pressure is zero on ball valve. After that, the receiver tank is opened and the pulp and solution are taken from the receiver tank.

Figure 6 shows the chemical mechanism of xylan during steam explosion. In the first reaction, high temperature and high pressure of steam change acetyl group in xylan molecule to acetic acid and the occurring acetic acid performs the hydrolysis and dehydration reactions to change xylan molecule to xylose, oligomer of xylose, furfural and 5-Hydroxymethylfurfural (HMF). In addition, the reaction breaks down lignin molecule to small molecules such as phenolic compounds.

The possible mechanism occurred in steam explosion is summarized from equations 2 to 6 where the steam explosion pretreatment could produce value added products as shown in Figure 6.

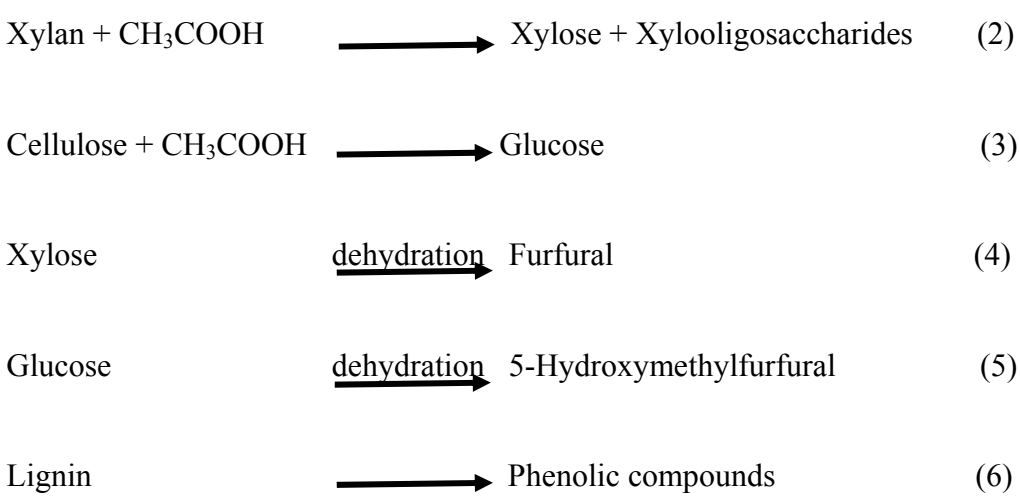

Figure 6 Possible mechanism reactions in steam explosion pretreatment.

The application of steam explosion machine is used for pretreatment of wood or agricultural waste to separate cellulose, hemicellulose and lignin for producing value added product from each component as shown in Figure 7.

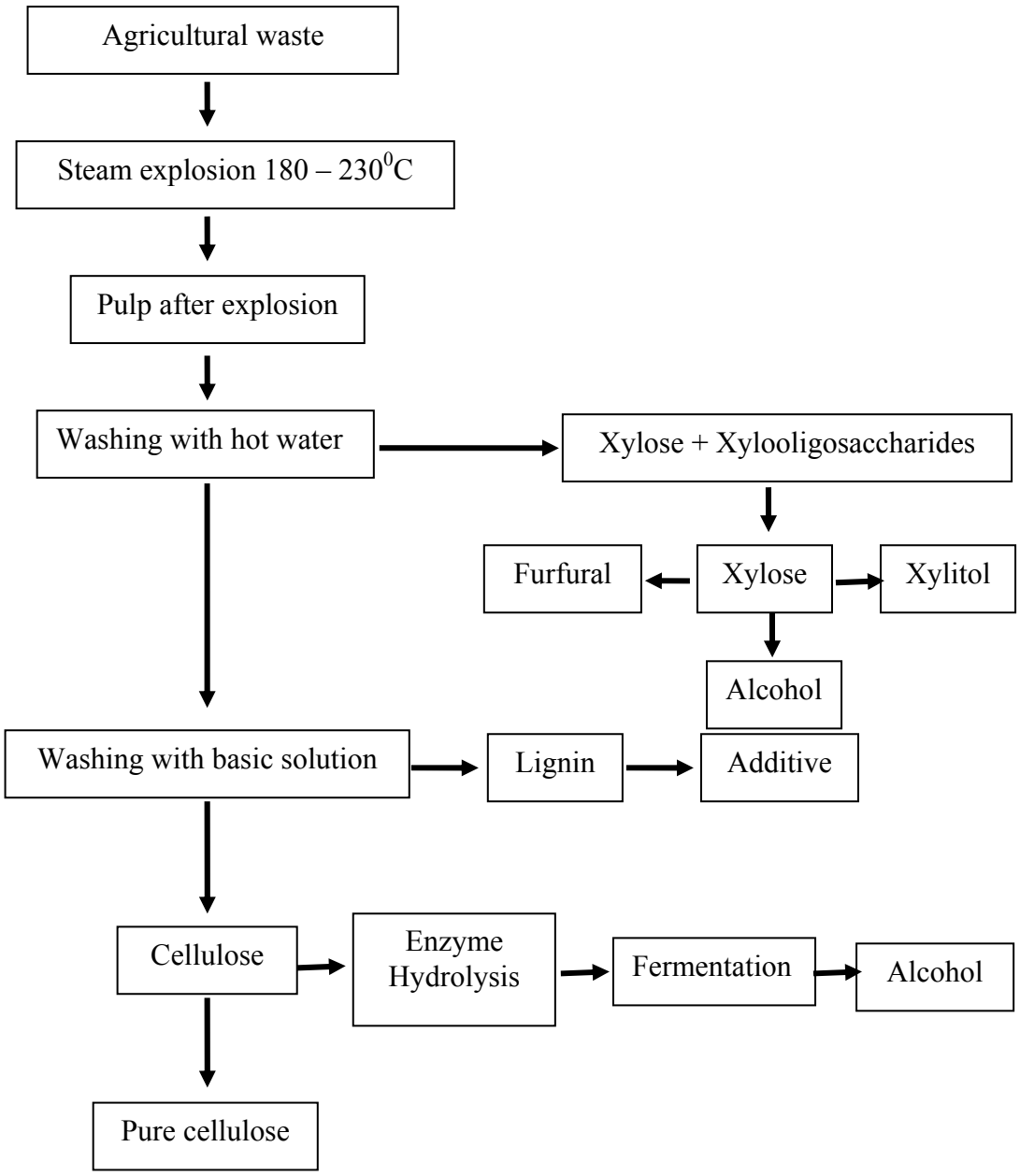

Figure 7 Applications of agricultural waste by steam explosion pretreatment machine

3.1 Experiment related with steam explosion pretreatment

Kaar *et al*. (1995) experimented the steam explosion of sugarcane bagasse as a pretreatment for ethanol production. In order to identify the optimum conditions of steam explosion, a range of operating temperatures at 188 – 243^{0}C and residence times at 0.5 - 44 min were applied. The results showed that pretreatment with steam explosion followed by enzyme hydrolysis had high efficiency in converting monosaccharide sugar to ethanol.

Nunes and Pourquie (1996) reported the steam explosion pretreatment and enzymatic hydrolysis of Eucalyptus wood. The comparison under conditions of acid and non acid impregnation of wood before steam explosion was experimented. The results demonstrated the same solubilization effect of both experiments. Ballesteros *et al*. (2004) reported that simultaneous saccharification and fermentation (SSF) process for ethanol production from various lignocellulosic woody (poplar and eucalyptus) and herbaceous (*Sorghum* sp. bagasse, wheat straw and *Brassica carinata* residue) materials had been assayed using the thermotolerant yeast strain. Biomass samples were previously treated in a steam explosion pilot plant to provide biomass with increased cellulose content relative to untreated materials and to enhance cellulase accessibility. SSF experiments were performed in laboratory conditions at 42^{0}C for 160 hours. The results showed that eucalyptus, wheat straw and sweet sorghum bagasse gave ethanol concentration at 17, 18 and 16.0 g/L respectively, in 72 hours of fermentation.

Nicoletta *et a*l. (2000) experimented the steam explosion of wheat straw. A fractionation of wheat straw components in a two-step chemical pretreatment was proposed. Hemicellulose was hydrolyzed by dilute H_2SO_4, allowing a substantial recovery of xylose. Lignin was removed by means of a mild alkaline/oxidative solubilization procedure, involving no sulphite or chlorine and its derivatives. The use of diluted reagents and relatively low temperatures was both cheap and environmentally friendly. The pretreated material was nearly pure cellulose, whose enzyme hydrolysis proceeded fast with high yields, that leading to high glucose syrup of remarkable purity.

Ballesteros *et al.* (2002) studied the enzyme hydrolysis of steam exploded herbaceous agricultural waste (*Brassica carinata*) at different particle sizes. The objective of this work was to evaluate the effect of particle size on steam explosion pretreatment of herbaceous lignocellulosic biomass. Hemicellulose and cellulose recovery and effectiveness of enzyme hydrolysis of the cellulosic residue was presented for the steam-exploded agricultural residue (*Brassica carinata*) with different particle sizes. The parameters tested were: particle size (2-5, 5-8 and 8-12 mm), temperature (190 and 210^0C), and residence time (4 and 8 min). The composition analysis of filtrate and water insoluble fibre after pretreatment and enzyme digestibility data were presented. The results showed that larger steam exploded particle (8-12 mm) resulted in higher cellulose and enzyme digestibility. The use of small particles in steam explosion would not be desirable in optimizing the effectiveness of the process improving economy.

Punsuvon *et al*. (2005) studied the fractionation of chemical components of oil palm trunk by steam explosion**.** The results showed optimal conditions for pretreatment at temperature 214°C for 2 min of steam explosion.

Ohgren *et al*. (2006) studied the ethanol fuel production from steam-pretreated corn stover using SSF at higher dry matter content. This study was performed on steam-pretreated corn stover at 5, 7.5 and 10% water-insoluble solids (WIS) with 2 g/L hexose fermenting *Saccharomyces cerevisiae*. The results showed that SSF at 10% WIS gave 74% of ethanol yield based on the glucose content in the raw material.

Ruiz *et al*. (2008) studied the steam explosion pretreatment prior to enzymatic hydrolysis of sunflower stalks. The stalks were subjected to steam explosion pretreatment in the temperatures ranging between 180^0C and 230^0C. The steam-exploded pulp was further hydrolyzed by enzyme. The result showed that after 96 hours of enzymatic reaction, a maximum hydrolysis yield of 72% was obtained after pretreatment at 220^0C, corresponding to a glucose concentration of 43.7 g/L in hydrolysis media. With regard to the filtrate analysis, most of the hemicellulosic derived sugars released during the steam pre-treatment were in the oligomeric form. The highest recovery was obtained at 210^0C of pretreatment

temperature. Moreover, the utilization of hemicellulosic-derived sugars as a fermentation substrate would improve the overall bioconversion of sunflower stalks into ethanol fuel.

4. Response Surface Methodology

4.1 Principle of Response Surface Methodology

Response surface methodology (RSM) is an empirical statistical technique employed for multiple regressions analysis by using quantitative data. It solves multivariable data which is obtained from properly designed experiments to solve multivariable equation simultaneously. The graphical representation of their function was called response surface, which is used to describe the individual and cumulative effect of the test variables and their subsequent effect on the response. Easy way to estimate response surface, factorial designs was the most useful scheme for the optimization of variables which is a limited number of experiments. A variety of factorial designs are a variable to accomplish this task. The most successful and best among them is the central composite design (CCD) which is accomplished by adding two experimental point along each coordinate axis to opposite side of the origin and at a distance equal to the semi diagonal of the hyper cube of the factorial design and new extreme values (low and high) for each factor added in this design. If the factorial is a full then

$$\alpha = [2^K]^{1/4} \quad (7)$$

Since the optimization of alkaline delignification and enzyme hydrolysis of steam exploded pulp investigated four factors such as the concentration of pulp, the concentration of alkaline solution, reaction time and temperature for delignification, and temperature, enzyme loading, reaction time and the concentration of pulp for enzyme hydrolysis, thus K = 4, So $\alpha = 2$.

Furthermore, the total number of experimental point (N) in a CCD could be calculated by following this equation

$$N = 2^K + 2K + X_0. \quad (8)$$

Where N is the number of experiment run, K is the number of variables and X_0 is the number of central points. Thus, for this design total number of experimental runs for delignification and for enzyme hydrolysis would be 31 (K = 4, X_0 = 6).

The data obtained from the central composite design is subjected to a second order multiple regression analysis to explain the behavior of the system using the least square regression methodology as equation:

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{14}X_1X_4 + e \quad 9$$

Where y is the predicted response, X_1, X_2, X_3, X_4, X_1^2, X_2^2, X_3^2, X_4^2 are independent variables in code values; b_o is the constant, b_1,b_2, b_3,b_4 are linear effect, b_{11}, b_{22}, b_{33}, b_{44} are squared effect and b_{12}, b_{23},b_{13},b_{14} are interaction effect. And e is the random error.

The analyses of results are performed with statistical and graphical analysis software. The software is used for regression analysis of the data obtained and to estimate the coefficient of regression equation. ANOVA (analysis of variance) which is statistical testing of the model in the form of linear term, squared term and interaction term is also utilized to test the significance of each term in the equation and goodness of fit of the regression model obtained. This response surface model is also used to predict the result by three dimensional surface plots and contour plots. Contour plots are the projection of the response surface as a two dimensional plane where as 3d surface plot is the projection of the response surface in a three dimensional plane.

4.2 Experimental design

A Central Composite Design (CCD) was applied with four design factors, namely, the concentration of pulp (%w/v), the concentration of alkaline solution (%w/w), reaction time (minute) and temperature (^{0}C) for delignification. In addition, a CCD was

applied with other four design factors, namely, reaction time (hour), temperature (^{0}C), enzyme loading (FPU/ g substrate) and the concentration of pulp (%w/v) for enzyme hydrolysis. A 2^4 full-factorial CCD for four independent variables at five levels was employed for batch process and the total number of experiment was 31 runs. The STATISTA software was used for regression and graphical analysis of the data obtained. The maximum values of the yield were taken as the response of the design experiment. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Once the experiments were performed, the response variables (lignin removal, alpha cellulose, glucose and glucose yield) were fitted a second – order model in order to correlate the response variable to the independent variable.

4.3 Experiments related with RSM method

Roberto *et al*. (2003) studied the dilute acid hydrolysis to recover xylose from rice straw in a semi-pilot reactor. Rice straw is consisted of pentose that could be used as a raw material for the production of many useful compounds. One of these was xylitol, with a potential application in the food and medical areas. The interest in biotechnological processes employing lignocellulosic residues was increased because this material was cheap, renewable and widespread sugar sources. The objective of the study was to determine the effects of H_2SO_4 concentration and reaction time on the production of sugars (xylose, glucose and arabinose) and on the reaction byproducts (furfural, HMF and acetic acid). Dilute sulfuric acid was used as a catalyst for the hydrolysis of rice straw at 121^{0}C in a 350-L batch hydrolysis reactor. Rationale for conducting this study was determined based on a central composite statistical design. Response surface methodology (RSM) was adopted to optimize the hydrolysis conditions aiming to attain high xylose selectivity. The optimum condition was 1% H_2SO_4 concentration for 27 min. This condition gave 77% of xylose yield and 5.0 g/g of selectivity.

Kunamneni *et al*. (2005) applied the response surface to optimize the enzymatic hydrolysis of maize starch for higher glucose production. Doses of pre-cooked α-amylase, post-cooked α-amylase, glucoamylase and saccharification temperature were examined to produce maximum conversion efficiency and all values were selected for optimization. Full

factorial composite experimental design and response surface methodology were used in the experiment design and result analysis. The optimum values for the tested variables were: 2.243 U of pre-cooked α-amylase /mg solids, 3.383 U of post-cooked 3.383 U of α-amylase /mg solids, 2.243 U of glucoamylase /mg solids at a saccharification temperature of 55.1^0C. The maximum conversion efficiency of 96.25% was achieved. This method was efficient because only 28 experiments were necessary for the assessment and also the model adequacy was very satisfactory.

Rahman *et al.* (2007) studied the acid hydrolysis of oil palm empty fruit bunch fiber for production of xylose. The objective was to determine the effect of H_2SO_4 concentration, reaction temperature and time on production of xylose. Batch reactions were carried out under various reaction temperatures, times and acid concentrations. The experiment was selected to optimize the hydrolysis process in order to obtain high xylose yield. The results showed that the optimum reaction temperature, time and acid concentration were 119^0C, 60 min and 2%, respectively. Under this condition xylose yield and selectivity were 91.27% and 17.97 g/g, respectively.

Leha *et al.* (2008) studied the optimization of oxygen delignification in production of totally chlorine-free cellulose pulp from oil palm empty fruit bunch fibre. The effects of oxygen delignification on prehydrolyzed-soda pulps produced from oil palm empty fruit bunch fibre was statistically investigated by RSM. Polynomial estimation models of 5 response variables namely percent yield, Kappa number, alpha cellulose, viscosity and brightness were developed. Each model comprised of four-independent variables: reaction temperature, reaction time, alkali charge, and initial Kappa number. The calculated optimum condition was 95^0C reaction temperature, 60 min reaction time, 2% alkali charge, and initial Kappa number of 6.6. This condition was capable of producing pulp with 98.1% yield, 2.4 Kappa number, 97.38% cellulose, 13.8 cPs pulp viscosity and 67.1% ISO brightness, which were proven close to the predicted values calculated from the estimation models.

5. Ethanol production from lignocelluloses materials

The techniques employed to produce bioethanol from lignocellulosic materials are subjected to the same economical demands as the more traditional sugar and starch processes, as the price of bioethanol must be competitive with that of petrol. Conversion of lignocellulosic materials to monomeric sugars and finally ethanol must thus be performed at low cost, while still achieving high yields. This can be done by developing processes that require limited amounts of chemicals, yeast and enzymes. To convert lignocellulosic materials to monomeric sugars, the material is first steam pretreated at a high temperature for a few minutes to facilitate subsequent enzymatic hydrolysis of cellulose, and to some extent also hemicelluloses to monomeric hexose and pentose sugars.

The bioconversion process from lignocellulose biomass to ethanol consists basically of three steps: pre-treatment, enzymatic hydrolysis and fermentation. Pretreatment is a necessary step to facilitate the enzymatic attack of lignocellulosic residues as shown in Figure 8. Steam explosion is recognized as an efficient pre-treatment method. The raw material is exploded to pressurized steam followed by rapid reduction in pressure resulting in substantial breakdown of the lignocellulosic structure, hydrolysis of the hemicellulosic fraction, depolymerization of the lignin components and defibration. Therefore, the accessibility of the cellulose components to degradation by enzymes is greatly increased.

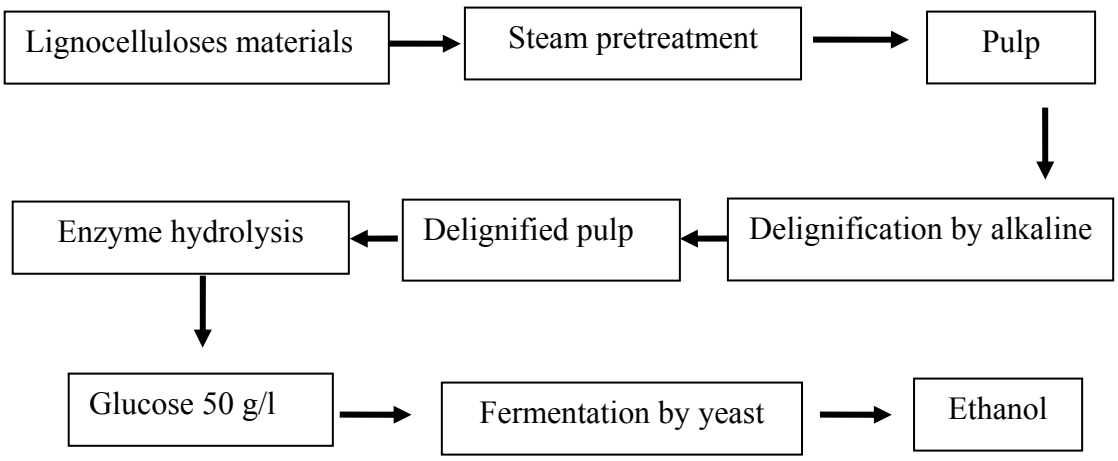

Figure 8 Ethanol production from lignocellulose.

5.1 Delignification step

The delignification of lignocellulose is the second step employed to increase the surface area of cellulose and can remove lignin to enhance the conversion of cellulose to glucose. Alkaline has a number of desirable characteristics as a deligninification reagent. It has high selectivity for reacting with lignin compared with other chemicals. Delignification with alkaline solution is a very useful method for the removal of lignin from pulp.

The goal for delignification is to make it easier for enzymatic hydrolysis. There are many ways of delinification the material; it could be done chemically, physically, biologically or as a combination of these. In this study the delignification was preformed with alkaline. The delignification increase the cellulose content and breaks down the lignin components in pulp obtained after steam explosion. Therefore, the accessibility of the cellulose components to be degraded by enzymes is greatly increased.

5.2 Enzyme hydrolysis step

Enzyme hydrolysis is a biochemical decomposition process that uses water to split chemical bonds of substances by enzyme. There are two types of hydrolysis process, acid and enzymatic. Each method had its advantages and disadvantages, but the overriding factor in the long run must be low energy requirement and low pollution. Enzymatic hydrolysis was not only energy sparing, because of the relatively mild reaction conditions but also avoided the use of toxic and corrosive chemicals.

In polysaccharides, monosaccharide molecules are linked together by glycosidic bonds. This bond can be cleaved by hydrolysis to yield monosaccharides by cellulase enzyme. Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria and protozoan that catalyzes the hydrolysis of cellulose.

The three mechanisms of reaction catalyzed by cellulases following these reactions:

1. Breakage of the non-covalent interactions present in the crystalline structure of cellulose (endo-cellulase).

2. Hydrolysis of the individual cellulose fibers into smaller sugars (exo-cellulase).

3. Hydrolysis of disaccharides and tetrasaccharides into glucose (beta-glucosidase).

The reaction mechanism of enzyme is shown in Figure 9.

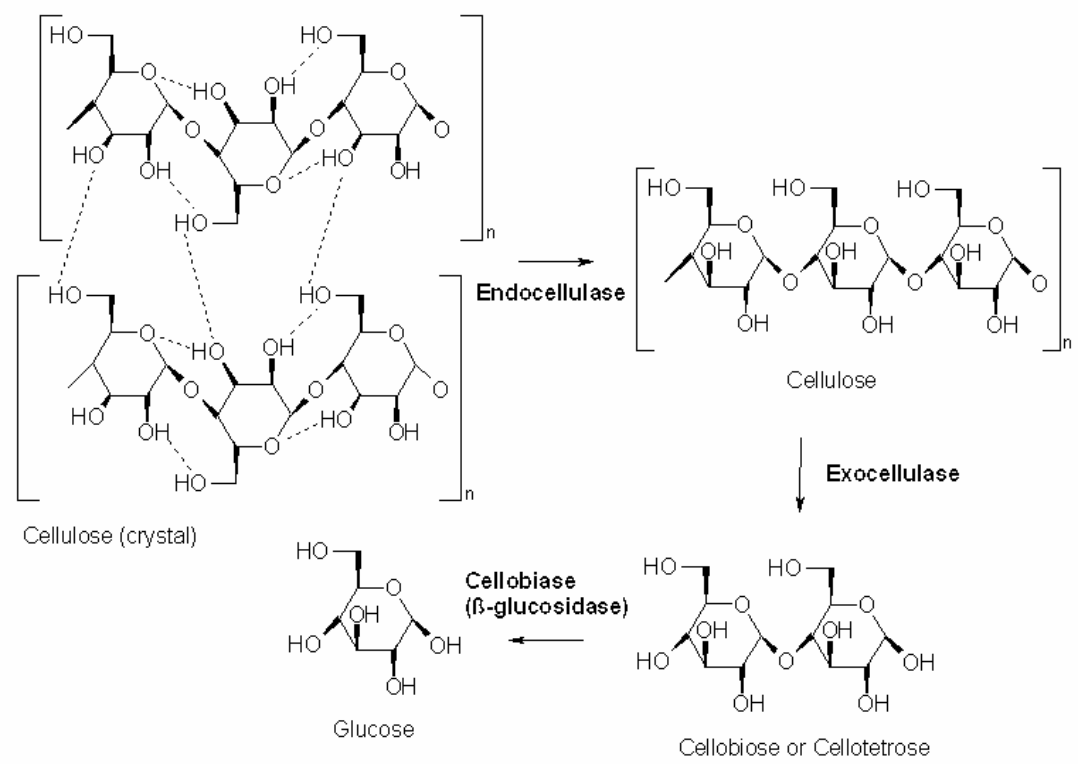

Figure 9 Mechanistic details of activity of cellulase.

5.3 Fermentation step

Ethanol fermentation is the biological process which sugars such as glucose, fructose, and sucrose are converted into ethanol and carbon dioxide as metabolic waste products. Yeasts carry out ethanol fermentation on sugars in the absence of oxygen. Because the process does not require oxygen, ethanol fermentation is classified as the

anaerobic process.The most common microorganism for this purpose is *S.cerevisiae.* The reaction of fermentation differs according to the sugar being used and the product produced. In the reaction shown below, the sugar is glucose ($C_6H_{12}O_6$), and the product is ethanol (C_2H_5OH)

The chemical equation below summarizes the ethanol fermentation, in which one hexose molecule is converted into two ethanol molecules and two carbon dioxide molecules:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 \qquad (8)$$

MATERIALS AND METHODS

1. Materials

1. Acetic acid, CH_3COOH 99.5% (J.T. Baker, USA.)
2. Barium hydroxide, $Ba(OH)_2$ (Merck, Germany)
3. Calcium nitrate**,** $Ca(NO_3)_2$ (Merck, Germany)
4. Ethyl alcohol, C_2H_5OH 99% (Merck, Germany)
5. D-Galactose, $C_6H_{12}O_6$ (Fluka, Switzerland)
6. D-Glucose, $C_6H_{12}O_6$ (Merck, Germany)
7. D-Mannose, $C_6H_{12}O_6$ (Fluka, Switzerland)
8. D-Xylose, $C_5H_{10}O_5$ (Fluka, Switzerland)
9. Ferric chloride, $FeCl_3$ (Fluka, Switzerland)
10. Inositol, $C_6H_{12}O_6$ (Fluka, Switzerland)
11. L-Arabinose, $C_5H_{10}O_5$ (Koch-light, Ltd., U.K.)
12. Magnesium sulphate, $MgSO_4$. $7H_2O$ (APS, Australia)
13. Potassium dihydrogen phosphate, KH_2PO_4 (APS, Australia)
14. Potassium hydroxide, KOH (Fluka, Switzerland)
15. Sulfuric acid, H_2SO_4 72% (J.T. Baker, USA.)
16. Sodium chloride, NaCl (APS, Australia)
17. Sodium borohydride, $NaBH_4$ (Merck, Germany)
18. Sodium hydroxide, NaOH (Fluka, Switzerland)
19. Sodium acetate, CH_3COONa (APS, Australia)

2. Equipments

1. Balance 4 digits (Precisa, 120A, USA.)
2. Gas chromatography instrument (Agilent Technique, 6890N, USA.)
3. Hot air oven (Binder, Germany)
4. Hot plate (Barndstead Electromal, EME6 0250/CEB, UK.)
5. High performance liquid chromatography (Shimadzu, Japan)

6. Screening (Somerville screen /size 0.15 mm, BUCHEL, BK-34, Netherlands)
7. Sieve 425 μm (D-42759, 40 meshes, Retsch, Germany)
8. Sieve 250 μm (60 mesh, Endocoris, England)
9. Steam explosion (Nitto Koatsu Company, Japan)
10. UV-spectrophotometer (Japan spectroscopic, 7800, Japan)
11. Water bath (Memmert, WB14, Germany)

3. Raw material

The 27-year old oil palm trunk from Krabri Province was used. The trunk was chipped by the chipper to 5X5 mm and shorter than 1 inch.

Figure 10 Oil palm trunk chip raw material.

4. Microorganism

Yeast *Saccharomyces cerevisiae* TISTR 5339 was obtained from Thailand Institute of Scientific and Technological Research (TISTR, Thailand).

5. Methods

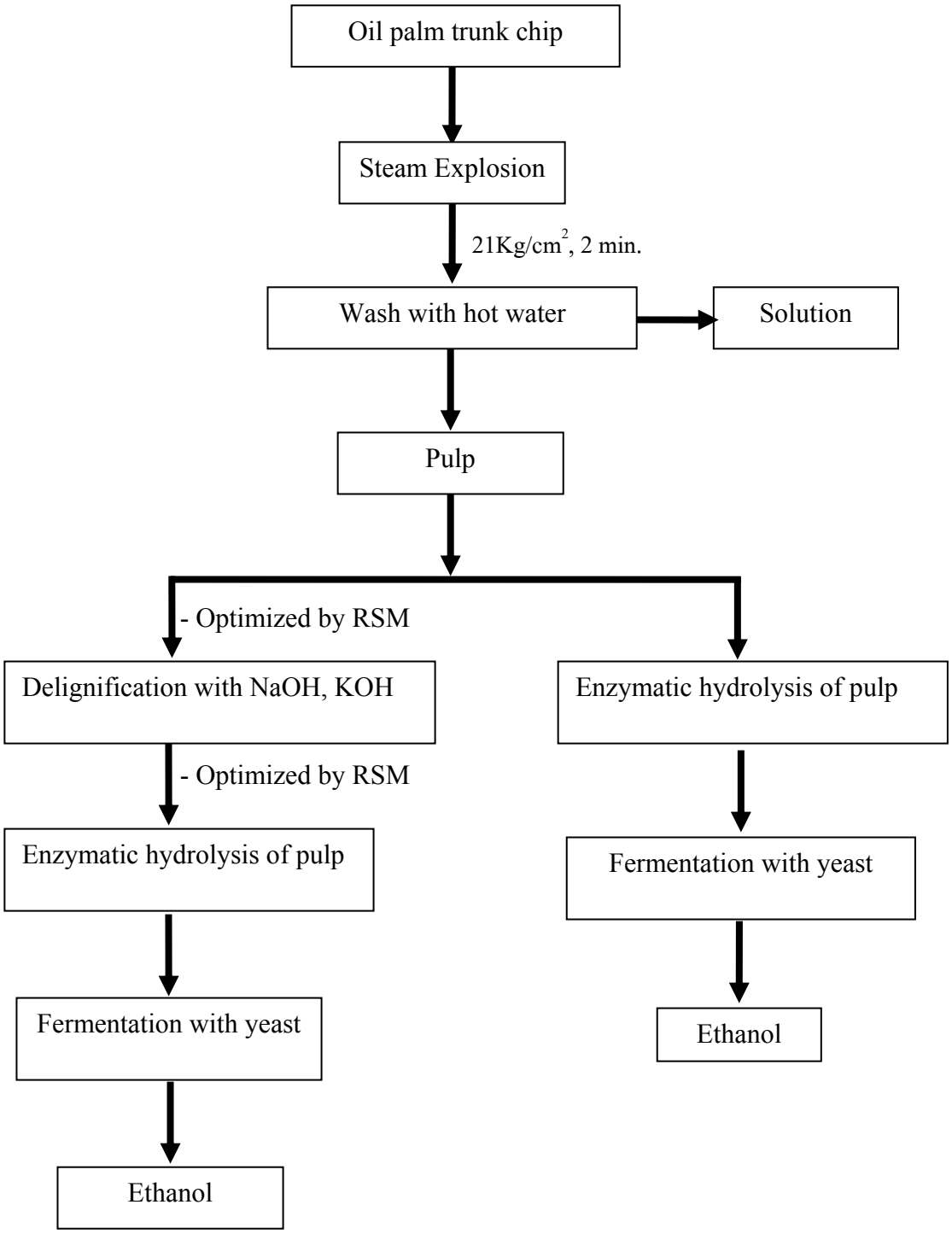

Figure 11 Experimental procedures for ethanol production from oil palm trunk chip.

5.1 Steam Explosion Pretreatment

An amount of 150 g of dry oil palm trunk chip sample were placed in 2.5 L batch digester (Nitto Koatsu Company, Japan). Heating was accomplished by direct steam injection into the digester and the hydrolysis temperature of steam at 214^0C for 2 min. The explosive discharge of the digester contents into a collecting tank was actuated by rapidly opening a value. The combined pulp slurry was collected and washed with hot water (80^0C) at a total volume of 2 L for 30 min. The pulp was filtered and dried at room temperature for the analysis of pulp yield percentage and chemical components. Another pulp was used to study the optimum condition in alcohol production.

5.2 Optimization of alkaline delignification of steam-exploded pulp

5.2.1 Experimental design of Plackett–Burman for alkaline delignification parameters

A Plackett and Burman design of the experiments was formulated for 4 factors. Each factor was tested at two levels, high (+1) and low (-1). The 4 factors tested were: concentration of pulp (6 and 12% w/v), concentration of alkaline solution (8 and 20%w/w), reaction time (30 and 60 min) and temperature (50 and 80^0C) as shown in Figure 12. The factor variables of experiments were shown in Table 1. After that, the reaction was carried out in a beaker under various maintained temperatures. Delignified pulp was recovered by filtration and after that pulp was washed several times with distilled water. The dried pulp obtained after oven drying was weighed and analyzed for alpha cellulose, glucose and lignin contents. The alpha cellulose, glucose and lignin removing value were used to optimize the screened factors.

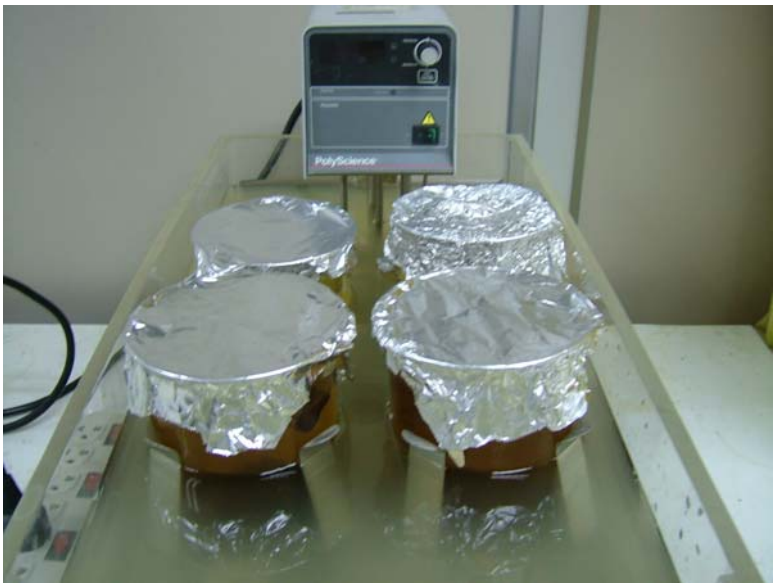

Figure 12 Alkaline delignification of steam-exploded pulp.

Table 1 Experimental design of Plackett–Burman (N=12) for significant influence on alkaline delignification.

Variables	Treatments											
	1	2	3	4	5	6	7	8	9	10	11	12
Concentration of pulp(% w/v)	12	12	12	6	12	6	6	6	12	6	12	6
Concentration of alkaline solution (% w/v)	20	20	8	20	20	20	8	8	8	20	8	8
Reaction time (min)	60	30	30	30	60	30	60	60	30	60	60	30
Temperature (^{0}C)	80	50	50	50	50	80	80	50	80	80	80	50

5.2.2 Central composite design in optimization of alkaline delignification of steam-exploded pulp

The central composite design (CCD) of the experiments was formulated at 4 factors. Each factor was tested at five levels (-α, -1, 0, +1, +α). The 4 factors tested were: concentration of pulp, X_1 (3, 6, 8, 12 and 15% w/v), concentration of alkaline solution, X_2 (2, 8, 14, 20, and 26%), reaction time, X_3 (15, 30, 45, and 60 min) and temperature, X_4 (35, 50, 65, 80, and 95 ^{0}C). The factor variables of experiments were shown in Table 2. Seven replicate runs at the centre (0, 0, 0) of the design were performed to allow the estimation of the pure error. Designs of a total 31 experiments were generated in Table 3. The analyzing response surface plots for lignin removal, alpha cellulose and glucose content were done by applying multiple regression analysis on the experimental data, following the second-order polynomial equation to evaluate the relationship between responses and variables. The variables were screened from Plackett–Burman design with confidence levels greater than 95% ($P \leq 0.05$) $R^2 > 0.75$ and optimized by three dimension and two dimension plot.

Table 2 Coded variable levels for central composite design in optimization of alkaline delignification of steam-exploded pulp.

Independent variable	Coded variable levels				
	- α	-1	0	1	α
Concentration of pulp, X_1(%w/v)	3	6	9	12	15
Concentration of alkaline solution , X_2(%w/w)	2	8	14	20	26
Reaction time, X_3(min)	15	30	45	60	75
Temperature, X_4(^{0}C)	35	50	65	80	95

Table 3 Design and response of the central composite design of alkaline delignification of steam-exploded pulp.

Treatments	X_1	X_2	X_3	X_4
1	1	-1	-1	-1
2	1	-1	-1	1
3	1	-1	1	-1
4	1	-1	1	1
5	1	1	-1	-1
6	1	1	-1	1
7	1	1	1	-1
8	1	1	1	1
9	-1	-1	-1	-1
10	-1	-1	-1	1
11	-1	-1	1	-1
12	-1	-1	1	1
13	-1	1	-1	-1
14	-1	1	-1	1
15	-1	1	1	-1
16	-1	1	1	1
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
21	0	0	0	0
22	0	0	0	0
23	-α	0	0	0
24	α	0	0	0
25	0	0	0	0
26	0	α	0	0
27	0	-α	0	0

Table 3 (Continued)

Treatments	X_1	X_2	X_3	X_4
27	0	-α	0	0
28	0	0	α	0
29	0	0	-α	0
30	0	0	0	α
31	0	0	0	-α

5.3 Optimization of enzyme hydrolysis of steam exploded pulp

5.3.1 Experimental design of Plackett–Burman for enzyme hydrolysis parameters

A Plackett and Burman design of the experiment was formulated at 4 factors. Each factor was tested at two levels, high (+1) and low (-1). The 4 factors tested were: reaction time, (30 and 70 hours), temperature (35 and 50 ^{0}C), enzyme loading (30 and 80 FPU/g substrate) and concentration of pulp (2 and 4 %w/v). The hydrolysis of steam exploded pulp was performed in 0.05M sodium citrate buffer (pH 4.8) on a rotary shaker with stirring rate at 200 rpm as shown in Figure 13. During the hydrolysis period, 10 ml of hydrolysis liquid was pipetted into beaker and the pH was adjusted to 7 with $Ba(OH)_2$ solution. This hydrolysis liquid was analyzed for glucose content by HPLC. The glucose yield was used for calculation the influence on each factor for optimizing the screened factors. The factor variables of experiments were shown in Table 4.

Figure 13 Enzyme hydrolysis of steam-exploded pulp.

Table 4 Experimental design of Plackett–Burman (N=12) for significant influence on enzyme hydrolysis.

Variables	Treatments											
	1	2	3	4	5	6	7	8	9	10	11	12
Reaction time (h)	70	70	70	30	70	30	30	30	70	30	30	70
Temperature (^{0}C)	50	50	35	50	35	50	50	35	35	50	35	35
Enzyme loading (FPU/substrate)	80	30	80	30	80	30	80	80	30	80	80	80
Concentration of pulp (%w/v)	2	4	2	2	2	4	4	4	4	4	4	2

5.3.2 Central composite design in optimization of enzyme hydrolysis of steam-exploded pulp

The central composite design (CCD) of the experiments was formulated for factors. Each factor was tested at five levels (-α, -1, 0, +1, α). The 4 factors tested were: reaction time, X_1 (10, 30, 50, 70, and 90 hours), temperature, X_2 (28, 35, 42.5, 50, and 57.5^{0}C), enzyme loading, X_3 (5, 30, 55, 80, and 105 FPU/ g substrate) and concentration of pulp (1, 2, 3, 4, and 5 % w/v). The factor variables of experiments were shown in Table 5. Six replicate runs at the centre (0, 0, 0) of the design were performed to allow the estimation of the pure error. A design of a total 31 experiments was generated in Table 6 (Montgomery, 2001).

The analyzing response surface plots for glucose yield were done by applying multiple regression analysis on the experimental data following the second-order polynomial equation to evaluate the relationships between responses and variables. The variables were screened from Plackett–Burman design with confidence levels greater than 95% ($P \leq 0.05$) $R^2 > 0.75$ and optimized by three dimension and two dimension plot.

Table 5 Independent variables and their levels for central composite design in optimization of enzyme hydrolysis of steam-exploded pulp.

Independent variable	Coded variable levels				
	- α	-1	0	1	α
Reaction time, X_1,(h)	10	30	50	70	90
Temperature, X_2,(^{0}C)	28	35	42.5	50	57.5
Enzyme loading ,X_3 (FPU/g substrate)	5	30	55	80	105
Concentration of pulp, X_4,(%w/v)	1	2	3	4	5

Table 6 Design and response of the central composite design of enzyme hydrolysis of steam-exploded pulp.

Treatments	X_1	X_2	X_3	X_4
1	1	-1	-1	-1
2	1	-1	-1	1
3	1	-1	1	-1
4	1	-1	1	1
5	1	1	-1	-1
6	1	1	-1	1
7	1	1	1	-1
8	1	1	1	1
9	-1	-1	-1	-1
10	-1	-1	-1	1
11	-1	-1	1	-1
12	-1	-1	1	1
13	-1	1	-1	-1
14	-1	1	-1	1
15	-1	1	1	-1
16	-1	1	1	1
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
21	0	0	0	0
22	0	0	0	0
23	-α	0	0	0
24	α	0	0	0
25	0	0	0	0
26	0	α	0	0
27	0	-α	0	0

Table 6 (Continued)

Treatments	X_1	X_2	X_3	X_4
28	0	0	α	0
29	0	0	-α	0
30	0	0	0	α
31	0	0	0	-α

5.4 Ethanol production by yeast fermentation

5.4.1 Fermentation with pure glucose

Yeast *S. cerevisiae* TISTR 5339 was grown on YNB agar and incubated at room temperature for 48 hours as shown in Figure 14. After that, the yeast was brought into 50 ml YNB broth in 250 ml of flask and incubated on a rotary shaker for 24 hours. The cells were separated by centrifugation at 5000 rpm for 10 min. After that, the cells were washed and suspended in distilled water.

The fermentation was carried out at a temperature of 30^0C, pH 5.0 and inoculum size 2.5% (v/v), in 150-ml Erlenmeyer flasks containing 100 ml appropriate media. The medium contained 50g/l pure glucose, 2.0 g/l KH_2PO_4, 1.0 g/l $MgSO_4$.$7H_2O$, 10.0 g/l yeast extract and 6.4 g/l urea. The flasks were sealed with a one-hole rubber stopper, in which a glass tube was connected to an air lock filled with 40% sulfuric acid solution. The reactor was maintained under anaerobic condition. The sample solution was picked at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 hours for the analysis of ethanol and residual glucose by GC and HPLC, respectively.

5.4.2 Fermentation with hydrolyzed glucose

The fermentation was carried out at a temperature of 30^0C, pH 5.0 and inoculum size 2.5% (v/v), in 150-ml Erlenmeyer flasks containing 100 ml appropriate media. The medium contained 50g/l hydrolyzed glucose, 2.0 g/l KH_2PO_4, 1.0 g/l $MgSO_4$

.7H_2O, 10.0 g/l yeast extract and 6.4 g/l urea. The flasks were sealed with a one-hole rubber stopper, in which a glass tube was connected to an air lock filled with 40% sulfuric acid solution as shown in Figure 15. The reactor was maintained under anaerobic conditions for appropriate a period of time according to section 5.4.1.The ethanol content from fermentation was analyzed by GC.

Figure 14 Preparation of yeast cells for fermentation (Plessas, 2007).

Figure 15 Fermentation of pure and hydrolyzed glucose solution.

5.5 Analysis methods

5.6.1 Analysis of Monosaccharide Content by HPLC

The monosaccharide content of the solid residue was determined based on the monomer content measured after two steps of acid hydrolysis. The first step of hydrolysis was performed with 4 ml of 72% (w/w) H_2SO_4 at 30^0C for 60 min. In the second step, the reaction mixture was performed in 3% (w/w) H_2SO_4 and subsequently autoclaved at 121^0C for 1 hour. The 10 ml of the filtrate were pipetted to the beaker and the pH was adjusted to 7 with $Ba(OH)_2$ solution. The samples were centrifuged at 8500 rpm to separate solution from solid. The solution was further filtered through 0.45 mm cellulose filter before injected onto the HPLC. The weight of glucose after enzymatic hydrolysis was calculated by dividing the glucose weight after enzyme hydrolysis with that before hydrolysis and multipling by 100.

Conditions

Equipment	:	High Performance Liquid Chromatography (Shimadzu, Japan)
Column	:	Aminex HPX-87C column(Bio- Rad)
Detector	:	Refractive index (RI) detector
Mobile phase	:	Deionized water
Oven temperature	:	80^0C
Flow rate	:	0.6 ml/min
Analysis method	:	Internal standard

5.6.2 Analysis of Ethanol Content by GC

A range of ethanol standard solutions were prepared at 50, 100, 150, 200, and 250 mg/l of ethanol. Then, 2 µl of the solution was injected in to GC and a chromatogram was recorded. The retention time of ethanol was found to be at 6.35 minutes. The area was plotted against the ethanol concentration to obtain a calibration curve. The

samples were centrifuged at 8500 rpm to separate solution from solid. The sample solution was filtered through 0.45 mm cellulose filter before injection onto the GC.

Conditions

Equipment	:	Gas Chromatography (Agilent Technologies, USA)
Column	:	HP-5 (bonded 5% phenyl, 95% dimethylpolysiloxane) capillary column 30m x 0.32mm ID, 0.25 μm film thickness
Carrier gas	:	He
Flow rate	:	2 ml/min
Pressure	:	14.54 psi
Detector	:	FID (Flame ionization detector) at 300^0C
Gradient	:	Oven temperature was set at 150^0C, and then raised at 10^0C /min to190^0C where it was held for 5 min. After that, the temperature was raised at 15^0C /min to 250^0C, where it was held for 15 min.
Injection	:	Standard and samples (2 μl) were injected using the split mode ratio 50:1, injection port held at 250^0C.

5.6.3 Analysis of Lignin Content by TAPPI 222 OM88 Method

The 1±0.1g of extractive free wood powder was weighed into beaker and hydrolyzed with 15 ml of 72%H_2SO_4 at 2^0C in cooling bath for 1 hour. The solution was continuously hydrolyzed at 25^0C for 2 hours to ensure the complete reaction. The solution was poured into 3% H_2SO_4 and made up to the final of 575 ml with deionized water. The solution was refluxed for 4 hours. The refluxed solution was stood overnight and filtered through a glass filter crucible No.4. The residue was washed with hot water and dried overnight at 100±5^0C in oven. After that, it was moved to a dessicator and left for 1 hour. The lignin content was calculated by dividing the lignin weight of wood powder and multiplying by 100.

5.6.4 Analysis of Alpha Cellulose by TAPPI 203 Method

The 1±0.1g of holocellulose was weighed into beaker with 75 ml of 17.5%NaOH. The temperature was controlled at 25^0C. The holocellulose was stirred until it was completely dispersed. The stirrer was rinsed for removing the adhered holocellulose with 25 ml of 17.5% NaOH. After that, the suspension was stirred with a glass rod and the temperature was maintained at 25 ^{0}C in a water bath. After 20 min from the first addition of the NaOH reagent, 100 ml of distilled water at 25^0C was added and stirred thoroughly with a glass rod. The beaker was stood in a water bath for 30 min so that the total extraction time was 60±5 min. The residual was filtered through a glass filtering crucible pore No.3 and washed with distilled water until the washing solution was neutral. The 40 ml of 10% acetic acid was poured into the crucible and held for 5 min. After that, the suction was released and the cellulose was placed overnight in the oven at 100±5^0C. Then, it was moved to dessicator, left for 1 hour and weighed. The alpha cellulose content was calculated by dividing the alpha cellulose weight with holocellulose and multiplying by 100.

5.7 Place of experiment

1. Enzyme and Waste Management Research Unit, Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University

2. Chemical Laboratory, Department of Chemistry, Kasetsart University

RESULTS AND DISCUSSION

1. Chemical components of oil palm trunk

Table 7 showed the chemical components of oil palm trunk. The oil palm trunk contained 68.87% (w/w) holocellulose, 37.14% (w/w) alpha cellulose, and 30.59 % (w /w) pentosan. Alpha cellulose was the polymer form of glucose. The oil palm trunk containing high amount of alpha cellulose indicated the possibility for using as raw material in ethanol production. In addition, oil palm trunk contained 22.32% (w/w) lignin and 8.56% (w/w) extractive. Halimahton and Rashid (1991) reported that the lignin content of oil palm trunk averaged at 20.6% (w/w) of dry sample weight. The extractive in ethanol/benzene and ash were 8.07% (w/w) and 8.50 % (w/w) based on dry weight of oil palm trunk, respectively.

Table 7 Chemical components of oil palm trunk.

Components	Percentage (w/w)
Lignin	22.32
Extractive in ethanol/benzene	8.56
Glucose	36.58
Xylose(hemicellulose)	30.42

2. Chemical components of steam-exploded oil palm trunk pulp

Table 8 Chemical components of steam-exploded oil palm trunk pulp.

Components	Percentage (w/w)
Lignin	38.46
Extractive in ethanol/benzene	8.56
Glucose	40.54
Xylose(hemicellulose)	9.36

The percentage of lignin, extractive, glucose and xylose of pulp after steam explosion were shown in Table 8. The pulp yield after steam explosion was 56% (w/w). The percentage of pentosan indicating the content of hemicellulose in oil palm trunk was decreased after the steam explosion pretreatment (from 30.42 to 9.36%). After steam explosion pretreatment, the steamed fiber was washed with hot water. The mixture of oligosaccharide and monosaccharide solution resulted from depolymerization of hemicellulose those were easily extracted from the exploded fiber by washing with hot water. Regarding the lignin content, higher values than those of raw material (initial lignin content in raw material 22.32%, Table 7) was obtained. This result indicated that the condensation of lignin could occur especially between sugars or sugar degradation products and other components from the extractive fraction (Cara, 2005; Nguyen1999). The glucose content of pretreated solid increased when compared to raw material because of hemicellulose solubilization (glucose in raw material = 36.58% w/w, glucose in pulp = 40.56 % w/w). Thus, this pulp obtained after steam explosion would further studied for ethanol production. The picture of pulp obtained after steam explosion was presented in Figure 16.

Figure 16 Pulp obtained after steam explosion.

The pulp obtained after steam explosion was further investigated for NaOH and KOH delignification. The pictures of pulp obtained after delignification were presented in Figures 17 and 18.

Figure 17 Pulp obtained after NaOH delignification.

Figure 18 Pulp obtained after KOH delignification.

3. Experimental design of Plackett–Burman for alkaline delignification parameters

Table 9 Analysis of variance showing significance of the variables on responses.

		Sum of square					
		Lignin removal		Glucose		Alpha cellulose	
Independent variables [1]	df	NaOH	KOH	NaOH	KOH	NaOH	KOH
Concentration of pulp	4	-2.654	13.40	-3.88	1.54	11.32	3.98
Concentration of alkaline solution	4	10.12**	15.00**	-14.54**	-2.00	2.43**	3.41
Reaction time	4	-2.56	-7.67	2. 05**	5.34	3.45**	14.39
Temperature	4	-2.28	-2.34	2.25	2.18	1.94	11.28

**significant at 5% level; significant at 10% level.
concentration of pulp at 2,4 %w/v, concentration of NaOH, KOH at 8, 20 %w/w, reaction time at 30, 60 min and temperature at 80, 50^0C for NaOH and KOH delignification.

From statistical analysis (Table 9), the concentration of pulp, concentration of alkaline solution, reaction time and temperature had significant effects on removed lignin, glucose and alpha cellulose ($p< 0.1$) but the concentration of alkaline solution both NaOH, KOH was the most important factor because it significantly affected removed lignin, alpha cellulose and glucose levels ($p < 0.05$).

4. Optimization of sodium hydroxide (NaOH) delignification of steam-exploded pulp

The NaOH delignification was experiment optimized. The percentages of removed lignin, alpha cellulose and glucose were compared between the experiment and the predicted values. The various experimental conditions and the experiment results were shown in Table 10.

Table 10 Experiment and predicted values of removed lignin glucose, and alpha cellulose and after NaOH delignification of steam-exploded pulp.

Treat-ments	X_1	X_2	X_3	X_4	Removed lignin (%)		Alpha cellulose (%)		Glucose (%)	
					P	E	P	E	P	E
1	12	20	60	80	50.42	49.22	43.21	45.40	45.12	45.23
2	12	20	60	50	55.54	55.06	51.34	50.19	49.23	49.93
3	12	20	30	80	36.64	37.99	44.85	43.50	43.42	43.68
4	12	20	30	50	43.54	44.22	49.30	49.02	49.84	49.09
5	12	8	60	80	34.62	35.62	42.41	43.32	42.36	42.38
6	12	8	60	50	40.56	39.78	38.90	40.30	40.25	40.05
7	12	8	30	80	30.54	32.02	47.48	47.41	47.45	47.47
8	12	8	30	50	35.65	36.45	51.43	50.11	49.54	49.57
9	6	20	60	80	43.21	43.69	41.43	39.02	38.22	38.47
10	6	20	60	50	50.53	50.02	34.45	31.72	30.65	30.86
11	6	20	30	80	34.65	34.87	40.49	41.03	40.62	40.80
12	6	20	30	50	40.06	41.33	40.43	43.02	42.78	42.14
13	6	8	60	80	32.57	30.77	47.32	48.12	48.50	48.17
14	6	8	60	50	29.54	29.65	52.54	50.21	49.33	49.36
15	6	8	30	80	32.45	30.09	40.42	40.02	38.11	38.03
16	6	8	30	50	34.56	35.47	52.31	50.15	48.76	48.28
17	8	14	20	26	51.43	50.38	40.54	41.31	43.60	43.34
18	8	14	20	26	52.34	51.46	46.36	45.56	44.33	44.92
19	8	14	20	26	50.48	50.05	47.40	46.31	44.56	44.05
20	8	14	20	26	50.65	52.44	34.51	35.75	44.63	44.94

Table 10 (Continued)

Treat-ments	X_1	X_2	X_3	X_4	Removed lignin (%)		Alpha cellulose (%)		Glucose (%)	
					P	E	P	E	P	E
21	8	14	20	26	50.32	51.43	34.76	34.54	44.32	44.34
22	8	14	20	26	53.34	52.55	50.41	52.55	43.23	43.88
24	35	14	20	26	29.34	30.66	30.48	30.66	43.64	43.26
25	95	14	20	26	24.66	25.06	26.09	25.06	39.43	39.87
26	8	30	20	26	24.54	23.65	50.78	49.66	47.96	47.25
27	8	75	20	26	60.33	60.66	50.64	49.66	48.49	48.96
28	8	14	2	26	24.56	25.88	46.75	44.05	43.70	43.93
29	8	14	26	26	46.54	45.25	51.30	50.03	48.85	48.67
30	8	14	20	3	23.45	24.66	50.47	50.47	49.90	49.06
31	8	14	20	15	30.45	31.44	45.65	45.41	44.98	44.76

The percentage of removed lignin, glucose, and alpha cellulose from the experiment were further applied to the linear regression by SPSS software using STATISTA software to compare with those from the prediction.

The application of RSM gave the predictive regression model of removed lignin, glucose and alpha cellulose left in pulp as shown in Table 11.

Table 11 Predictive regression models for removed lignin, alpha cellulose and glucose of pulp obtained after NaOH delignification.

Dependent variable	Predictive model	R^2
Removed lignin % (w/w)	$Y = -137.279 – 10.313X_1 + 0.977X_2 + 1.054X_3 + 2.854X_4 - 0.598X_1^2 - 0.045X_2^2 - 0.014X_3^2 - 0.022X_4^2 + 0.03X_1X_2 + 0.015X_1X_3 + 0.01X_1X_4 + 0.026X_2X_3 + 0.008X_2X_4 - 0.002X_3X_4$	0.977
Alpha cellulose % (w/w)	$Y = 61.939 - 1.755X_1 - 1.142X_2 + 0.289X_3 - 0.262X_4 + 0.043X_1^2 + 0.034X_2^2 + 0.005X_3^2 + 0.007X_4^2 + 0.154X_1X_2 - 0.013X_1X_3 - 0.001X_1X_4 - 0.006X_2X_3 - 0.013X_2X_4 - 0.01X_3X_4$	0.865
Glucose % (w/w)	$Y = 37.112 - 0.166X_1 - 0.959X_2 + 0.511X_3 + 0.104X_4 - 0.074X_1^2 - 0.005X_2^2 + 2.706X_3^2 + 001X_4^2 + 0.133X_1X_2 - 0.011X_1X_3 + 0.001X_1X_4 - 0.005X_2X_3 - 0.005X_2X_4 - 0.006X_3X_4$	0.875

Y was the response that represented the lignin removal, alpha cellulose and glucose contents and X_1, X_2, X_3 and X_4 were the coded values of test variables affecting the concentration of pulp (%w/v), concentration of NaOH (%w/w), reaction time (min) and temperature (^{0}C), respectively. The variables X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_3X_4 represented the interaction effects of concentration of pulp and NaOH, concentration of pulp and reaction time, concentration of pulp and temperature, respectively.

The effect of pulp concentration (%w/v), NaOH concentration (%w/w), reaction time (min) and temperature (^{0}C) on the coded values X_1, X_2, X_3 and X_4 in delignification process were investigated. The experiments were arranged according to the central composite design. The results were shown in Tables 10 and 11. The experimental data for removed lignin, glucose and alpha cellulose had correlation coefficients (R^2) of 0.977, 0.877 and 0.865 in NaOH delignification, respectively. The calculated models were 97.7, 87.7 and 86.6 % of removed lignin, glucose and alpha cellulose that attested the good fit of the

model. The correlation between the experimental and predicted values of the removed lignin (R^2 = 0.977) indicated good agreement between the experimental and predicted values. The correlation predicted values of glucose and alpha cellulose had correlation coefficients less than the removed lignin as shown in Table 11.

4.1 The response surface plot and contour plot of removed lignin

The relative effect of time and concentration of pulp with response to the lignin removal percentage was plotted on three and two dimensions as in Figure 19.

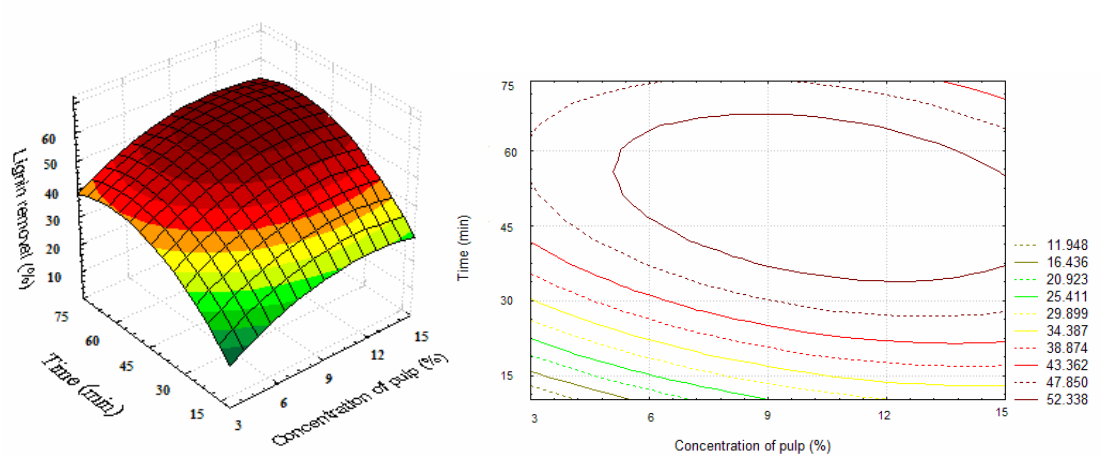

Figure 19 Response surface and contour plot for the effects of time and concentration of pulp on lignin removal percentage at 65^0C and 14 % (w/w) NaOH.

Figure 19 showed that increasing the reaction time and concentration of pulp could increase lignin removal percentage. The reaction time longer than 67 min had no significant effect on lignin removal. Likewise, the concentration of pulp higher than 14% (w/w) had no significant effect on lignin removal.

Response plot showed the optimized value at 65 min of reaction time and 12 % (w/v) of pulp concentration with 52.33% of lignin removal from pulp.

The relative effect of temperature and concentration of pulp with response to the lignin removal percentage was plotted on three and two dimensions as in Figure 20.

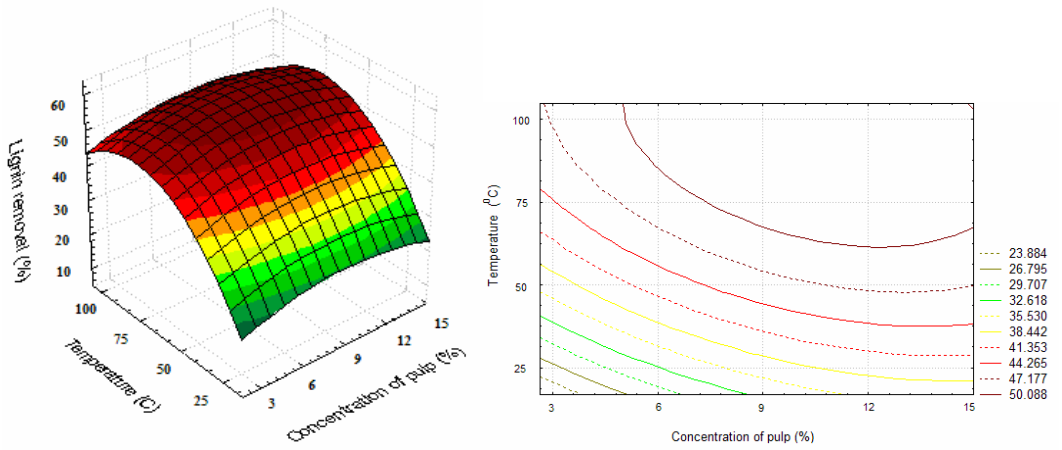

Figure 20 Response surface and contour plot for the effects of temperature and concentration of pulp on lignin removal percentage at 65 min and 14 % (w/w) NaOH.

Figure 20 showed that increasing the reaction temperature and concentration of pulp could increase lignin removal. The reaction temperature higher than 80^0C had no significant effect on lignin removal. Likewise, the concentration of pulp higher than 12.5% (w/w) had no significant effect on lignin removal.

Response plot indicated the optimized condition at 75^0C of temperature and 12.2 % (w/v) of pulp concentration with 50.08 % (w/w) of lignin removing from pulp.

The relative effect of concentration of pulp and NaOH with response to the lignin removal percentage was plotted on three and two dimensions as in Figure 21.

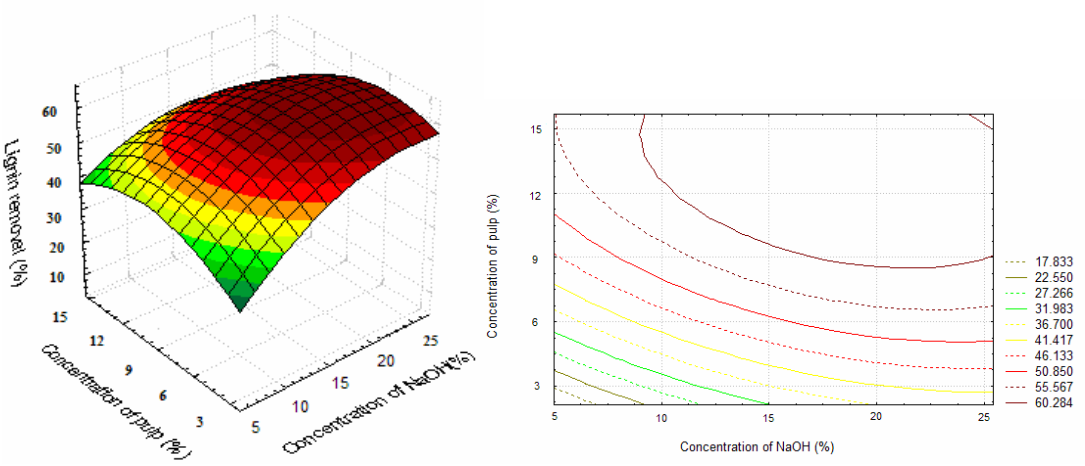

Figure 21 Response surface and contour plot for the effects of concentration of pulp and NaOH on lignin removal percentage at 45 min and 65^0C.

Figure 21 showed that increasing the concentration of NaOH and pulp could increase lignin removal. The concentration of pulp higher than 12.5% (w/w) had no significant effect on lignin removal.

Response plot indicated the optimized condition at 22.55 % (w/w) NaOH and 12.2% (w/v) of pulp concentration with 60.26% (w/w) lignin removal from pulp.

According to the mentioned results, the most optimum condition with highest 62.28 % (w/w) lignin removal was at 23% (w/w) NaOH, 11.2% w/v) of pulp concentration, 78^0C and 65 min.

3.2 The response surface and contour plot of alpha cellulose

The relative effect of NaOH and pulp concentration with response to alpha cellulose percentage in pulp was plotted on three and two dimensions as in Figure 22.

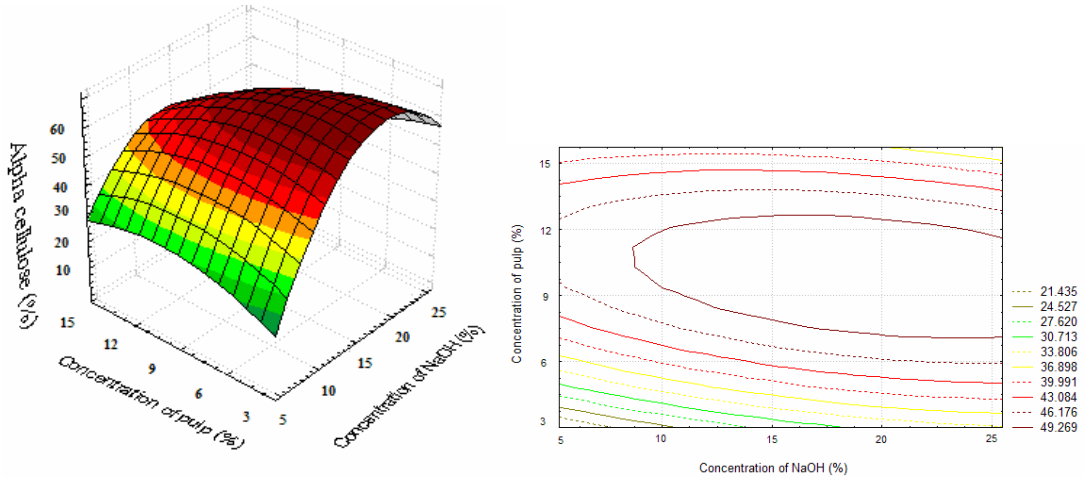

Figure 22 Response surface and contour plot for the effects of concentration of pulp and NaOH on alpha cellulose percentage at 45 min and 65^0C.

Figure 22 showed that increasing the concentration of NaOH and pulp could increase the alpha cellulose percentage in pulp after NaOH delignification. The concentration of NaOH higher than 23.5% (w/w) had no significant effect on the amount of alpha cellulose in pulp.

Response plot indicated the optimized condition at 22.5% (w/w) NaOH and 12.2 % (w/v) of pulp concentration that gave 49.26% of alpha cellulose left in pulp after NaOH delignification.

The relative effect of temperature and pulp concentration with response to alpha cellulose percentage in pulp was plotted on three and two dimensions as in Figure 23.

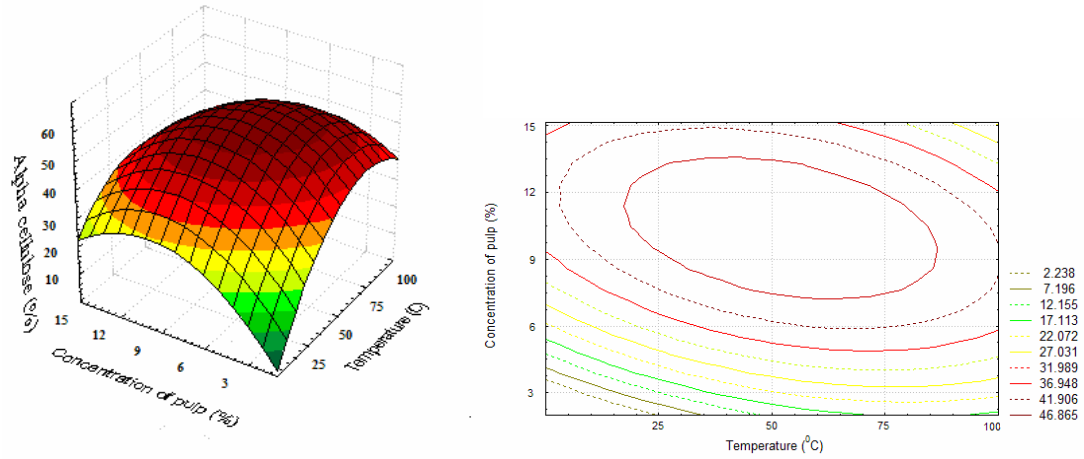

Figure 23 Response surface and contour for the effects of temperature and pulp concentration on alpha cellulose percentage at 45 min and 14 % (w/w) NaOH.

Figure 23 showed that the temperature and concentration of pulp could increase alpha cellulose percentage in pulp after NaOH delignification. The concentration of pulp higher than 13.5 % (w/w) had no significant effect on the amount of alpha cellulose in pulp.

Response plot indicated the optimized condition at 80 ^{0}C and 12.5 % (w/v) of concentration of pulp that gave 46.88% of alpha cellulose left in pulp after NaOH delignification.

The relative effect of time and pulp concentration with response to alpha cellulose percentage in pulp was plotted on three and two dimensions as in Figure 24.

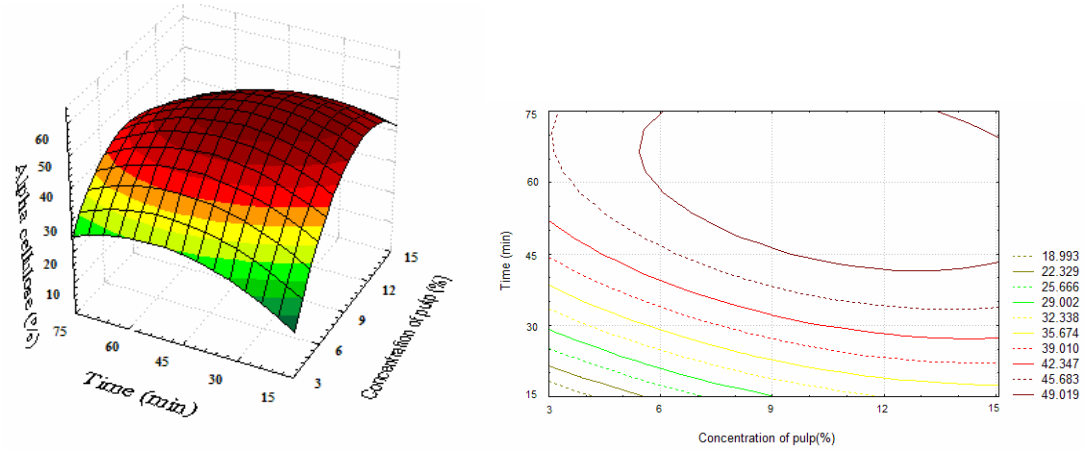

Figure 24 Response surface and contour plot for the effects of time and pulp concentration on alpha cellulose percentage at 65^0C and 14 % (w/w) NaOH.

Figure 24 showed that the time and pulp concentration could increase alpha cellulose percentage in pulp after NaOH delignification. The concentration of pulp higher than 14.50 % (w/w) had no significant effect on the amount of alpha cellulose percentage in pulp.

According to the mentioned results, the most optimum condition with the highest 49.26% (w/v) alpha cellulose at 21.50 % (w/w) NaOH, 11.20 % of (w/v) concentration of pulp, 78^0C and 65 min.

3.3 The response surface plot and contour plot of glucose

The relative effect of concentration of pulp and NaOH with response to glucose percentage in pulp was plotted on three and two dimensions as in Figure 25.

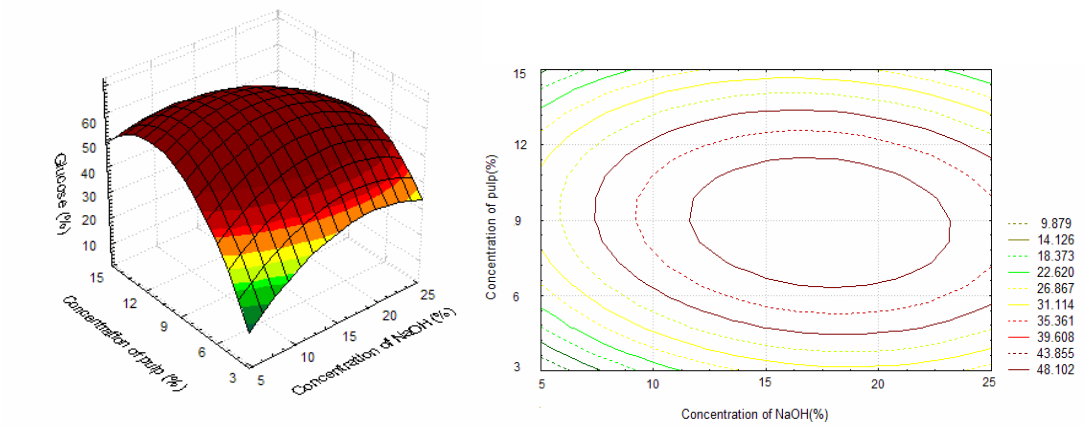

Figure 25 Response surface plot and contour for the effectsof concentration of pulp and NaOH on glucose percentage at 65^0C and 45min.

Figure 25 showed that the concentration of pulp and NaOH could increase glucose percentage in pulp after NaOH delignification. The concentrations of pulp higher than 13.50 % (w/w) had no significant effect on the amount of glucose percentage in pulp.

Response plot indicated the optimized condition at 12.50 % w/v of pulp concentration and 21.50% (w/v) NaOH gave 48.10% of glucose percentage pulp after NaOH delignification.

The relative effect on temperature and concentration of pulp with response to glucose percentage in pulp was plotted on three and two dimensions as in Figure 26.

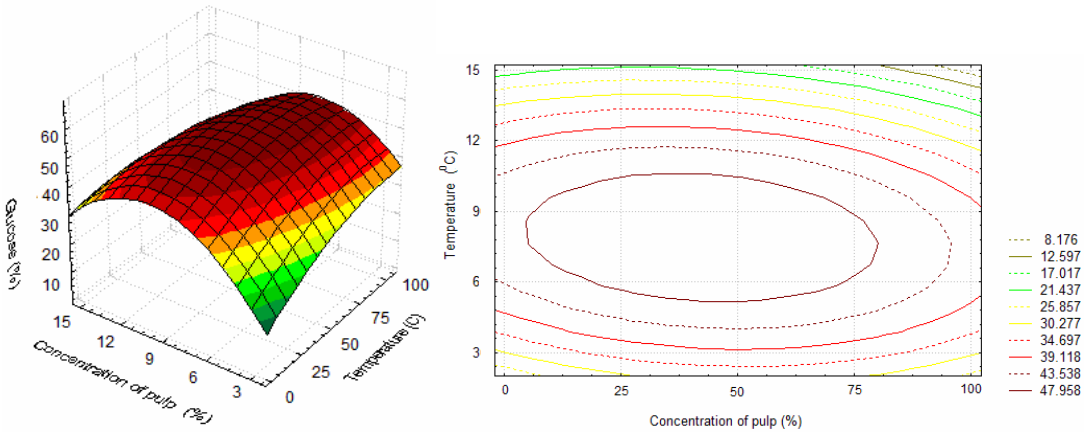

Figure 26 Response surface and contour plot for the effects of concentration of pulp and temperature on glucose percentage at 45 min and 14 % (w/w) NaOH.

Figure 26 showed that increasing temperature and concentration of NaOH could increase glucose percentage in pulp after NaOH delignification. The temperature higher than 80^0C had no significant effect on the amount of glucose in pulp after NaOH delignification.

Response plot indicated the optimized condition at 12.5 % w/v of pulp concentration and 80^0C that gave 47.95 % of glucose from pulp after NaOH delignification.

The relative effect of time and concentration of pulp with response to glucose percentage in pulp was plotted on three and two dimensions as in Figure 27.

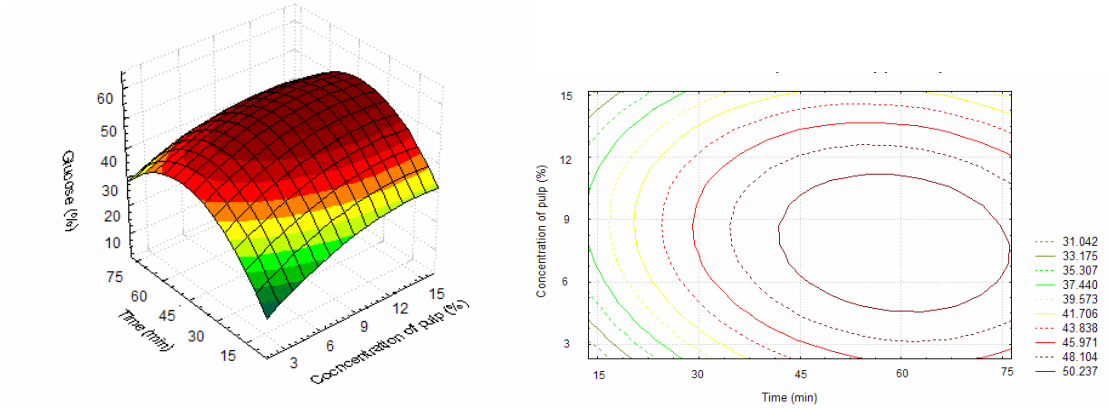

Figure 27 Response surface and contour plot for the effects of concentration of pulp and time on glucose percentage at 65^0C and 14 % (w/w) NaOH.

Figure 27 showed that increasing time and concentration of NaOH could increase glucose percentage in pulp after NaOH delignification. The time longer than 67 min had no significant effect on the amount of glucose in pulp.

Response plot indicated the optimized condition at 12.5 % w/v of pulp concentration and 65 min gave 50.23% of glucose in pulp after NaOH delignification.

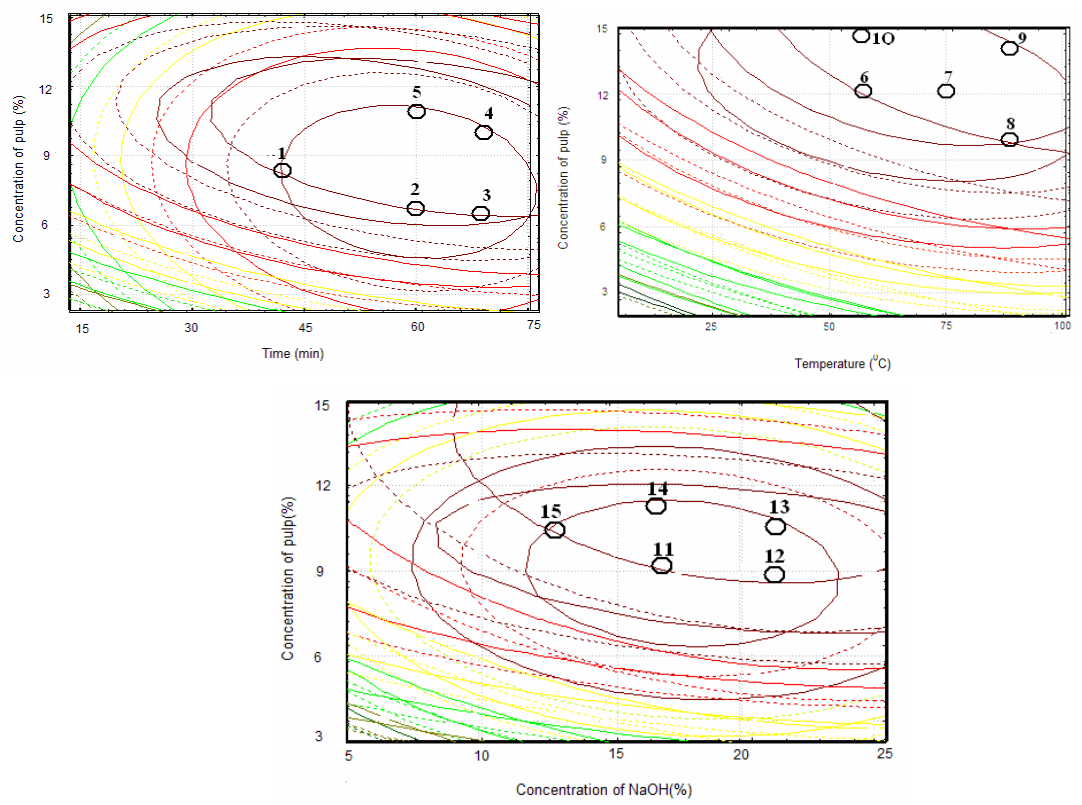

Figure 28 Contour plot of glucose percentage, lignin removal and alpha cellulose in pulp that effecting on concentration of pulp and time, concentration of pulp and temperature, concentration of pulp and NaOH.

Table 12 Experiment value of lignin removal, glucose and alpha cellulose after NaOH delignification.

Point	A	B	C	D	Lignin removal (%)	Glucose in pulp (%)	Alpha cellulose in pulp (%)
1	8	40	50	15	35.21	38.32	39.57
2	7.5	60	50	15	45.67	39.53	41.72
3	6.5	65	50	15	41.77	42.51	44.63
4	10	65	50	15	45.62	44.56	45.52
5	11	60	50	15	38.74	40.43	43.66
6	12	45	55	15	35.51	37.67	38.65
7	12	45	75	15	36.55	36.72	37.52
8	10	45	78	15	41.45	42.56	43.75
9	14	45	78	15	42.54	43.76	44.68
10	15	45	55	15	32.54	33.41	35.47
11	9	45	50	17	35.76	39.45	40.58
12	9	45	50	21.5	36.97	44.88	45.87
13	11	45	50	21.5	39.76	44.68	45.94
14	11	45	50	17	38.54	41.42	42.67
15	10	45	50	13	35.43	39.53	40.55

A = Concentration of pulp (%w/v)

B = Reaction time (min)

C = Temperature (^{0}C)

D = Concentration of NaOH (%w/w)

Tables 12 and 13 summarized the optimum condition and the comparison of lignin removal, glucose and alpha cellulose percentages between the prediction and experiment.

Table 13 Optimum condition and the comparison of predicted model and experiment values of after NaOH delignification.

Variables	Optimum condition	Lignin removal (%)		Glucose in pulp (%)		Alpha cellulose in pulp(%)	
		P	E	P	E	P	E
Concentration of pulp (%w/v)	11	59.13	58.68	47.5	49.18	50.33	51.75
Concentration of NaOH (%w/w)	21.5						
Reaction time (min)	65						
Temperature (^{0}C)	78						

Table 13 indicated that the optimum condition for NaOH delignification was obtained at 11% (w/v) of pulp concentration, 21% (w/w) NaOH, 65 min reaction time and 78^{0}C with the maximum percentage of glucose and alpha cellulose in pulp and lignin removal at 49.18% (w/w), 51.75% (w/w) and 58.68% (w/w), respectively.

4. Optimization of potassium hydroxide (KOH) delignification of steam-exploded pulp

The KOH delignification was optimized and the percentages of lignin removal, alpha cellulose and glucose compared from the experiment with the predicted values. The various experimental condition and the experiment results were shown in Table 14.

Table 14 Experiment and predicted values of percent lignin removal, alpha cellulose and glucose in pulp after KOH delignification of steam-exploded pulp.

Treat-ments	X_1	X_2	X_3	X_4	Lignin removal (%)		Alpha cellulose (%)		Glucose (%)	
					P	E	P	E	P	E
1	12	20	60	80	37.34	38.01	48.55	49.28	45.23	48.76
2	12	20	60	50	41.34	42.87	46.50	47.11	49.90	46.66
3	12	20	30	80	32.22	30.01	43.45	42.70	43.61	42.30
4	12	20	30	50	35.42	33.65	49.87	50.00	49.04	49.64
5	12	8	60	80	29.34	28.22	48.56	48.77	42.30	48.65
6	12	8	60	50	32.43	30.25	42.34	43.75	40.02	43.23
7	12	8	30	80	29.44	27.22	48.08	48.08	47.47	47.63
8	12	8	30	50	28.45	29.25	46.53	49.75	49.50	49.35
9	6	20	60	80	32.34	35.52	44.56	45.08	38.40	44.72
10	6	20	60	50	41.34	41.04	49.06	47.07	30.81	46.55
11	6	20	30	80	27.54	27.07	42.75	40.41	40.84	39.96
12	6	20	30	50	32.43	32.39	46.95	48.08	42.12	47.68
13	6	8	60	80	24.76	22.31	44.32	45.02	48.16	44.66
14	6	8	60	50	22.34	20.88	40.54	42.06	49.31	41.54
15	6	8	30	80	24.56	23.33	46.43	47.22	38.03	46.77
16	6	8	30	50	19.26	20.41	50.83	49.43	48.24	49.03
17	8	14	20	26	36.45	35.31	49.03	48.64	43.33	48.28
18	8	14	20	26	37.45	36.14	46.32	47.87	44.94	47.35
19	8	14	20	26	40.56	39.06	47.85	48.54	44.05	48.09
20	8	14	20	26	32.23	32.12	46.40	47.32	44.96	46.92
21	8	14	20	26	34.56	33.07	47.54	46.42	44.34	46.06
22	8	14	20	26	39.08	38.03	49.43	48.74	43.22	48.32
23	8	14	20	26	39.32	39.22	50.43	49.33	50.45	48.22
24	35	14	20	26	28.03	29.05	50.43	50.06	39.85	49.61
25	95	14	20	26	16.34	16.32	44.35	44.75	47.23	44.35

Table 14 (Continued)

Treat-ments	X_1	X_2	X_3	X_4	Lignin removal (%)		Alpha cellulose (%)		Glucose (%)	
					P	E	P	E	P	E
26	8	30	20	26	13.45	13.31	49.3	48.64	48.92	48.28
27	8	75	20	26	45.90	45.25	45.54	45.57	43.93	45.05
28	8	14	2	26	17.54	17.14	50.43	49.96	48.66	49.51
29	8	14	26	26	38.45	38.18	47.34	47.86	42.76	47.46
30	8	14	20	3	19.07	19.04	50.43	50.07	49.00	49.71
31	8	14	20	15	22.34	22.70	43.32	43.43	44.79	43.32

The percentage lignin removal, glucose and percent alpha cellulose from the experiment were further applied to the linear regression by SPSS software using STATISTA software to compare with those from the prediction.

The application of RSM gave the predictive regression model of lignin removal, glucose and percent alpha cellulose left in pulp as shown in Table 15

Table 15 Predictive regression models of lignin removal, alpha cellulose and glucose content of pulp obtained after KOH delignification.

Dependent variable	Predictive model	R^2
Lignin removal %(w/v)	$Y = -69.931 + 7.639X_1 + 0.343X_2 + 0.332X_3 + 1.420X_4 - 0.368X_1^2\ 0.022X_2^2 - 0.005X_3^2 - 0.013X_4^2 - 0.081X_1X_2 + 0.003X_1X_3 + 0.01X_1X_4 + 0.023X_2X_3 + 0.015X_2X_4 - 0.001X_3X_4$	0.877
Alpha cellulose % (w/v)	$Y = 39.979 - 0.138X_1 - 1.005X_2 + 0.438X_3 + 1.20X_4 - 0.088X_1^2 - 0.001X_2^2 + 0.005X_3^2 + 0.001X_4^2 + 0.134X_1X_2 - 0.006X_1X_3 + 0.01X_1X_4 - 0.003X_2X_3 - 0.005X_2X_4 - 0.007X_3X_4$	0.802
Glucose %(w/v)	$Y = 60.266 + 0.574X_1 - 1.398X_2 - 0.320X_3 + 0.091X_4 - 0.030X_1^2 - 0.001X_2^2 + 0.006X_3^2 - 002X_4^2 + 0.133X_1X_2 - 0.008X_1X_3 + 0.006X_1X_4 - 0.004X_2X_3 + 0.015X_2X_4 + 0.013X_3X_4$	0.890

Y was the response that representing the lignin removal, alpha cellulose and glucose content and X_1, X_2, X_3, and X_4 were the coded values of test variables effecting the concentration of pulp (%w/v), concentration of KOH (%w/w), reaction time (min) and temperature (^{0}C), respectively. The variables X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, and X_3X_4 represented the interaction effects of concentration of pulp and KOH, concentrations of pulp and reaction time, concentration of pulp and temperature, respectively.

The effect of pulp concentration, KOH concentration, reaction time and temperature on the coded values X_1, X_2, X_3 and X_4 in delignification process were investigated. The experiments were arranged according to the central composite design. The results were shown in Table 13 and 14. The experimental data for removed lignin, glucose and alpha cellulose had correlation coefficients (R^2) of 0.877, 0.890 and 0.802 in KOH delignification, respectively. The calculated models were 87.7, 89.0 and 80.2.0 % of lignin removal, glucose, and alpha cellulose that attested the good fit of the model. The correlation between

the experimental and predicted values of glucose (R^2 = 0.89) indicated good agreement between the experimental and predicted values of alpha cellulose. The correlation predicted values of glucose and lignin removal had correlation coefficients less than the alpha cellulose as shown in Table 15

5.1 The response surface plot and contour plot of removed lignin

The relative effect of time and concentration of pulp with response to the lignin removal percentage was plotted on three and two dimensions as in Figure 29.

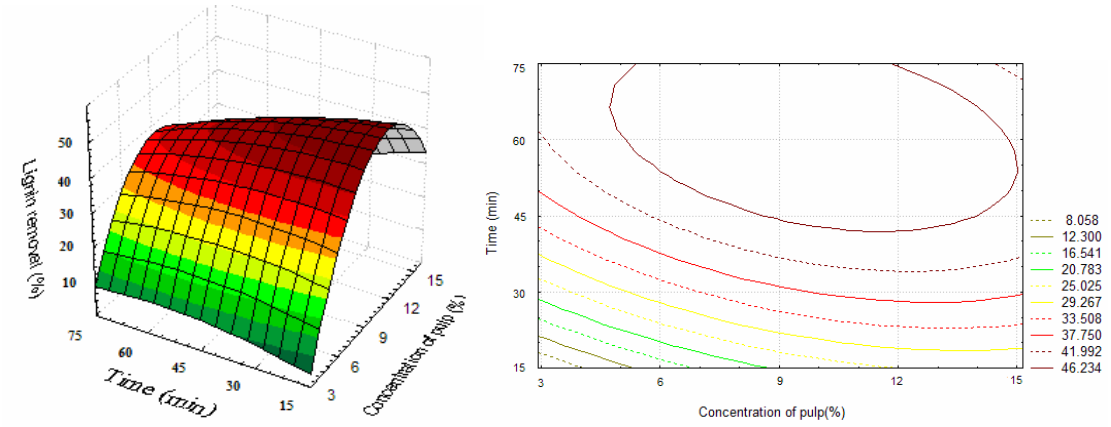

Figure 29 Response surface and contour plot for the effects of time and concentration of pulp on lignin removal percentage at 65^0C and 14 % (w/w) KOH.

Figure 29 showed that increasing reaction time and concentration of pulp could increase lignin removal percentage. The reaction time longer than 67 min had no significant effect on lignin removal. Likewise, the concentrations of pulp higher than 14% (w/w) no had significant effect on lignin removal.

Response plot indicated the optimized conditions at 65 min of reaction time and 12 % (w/v) of pulp concentration with 46.23% lignin removal from pulp after KOH delignification.

The relative effect of temperature and concentration of pulp with response to the lignin removal percentage was plotted on three and two dimensions as in Figure 30.

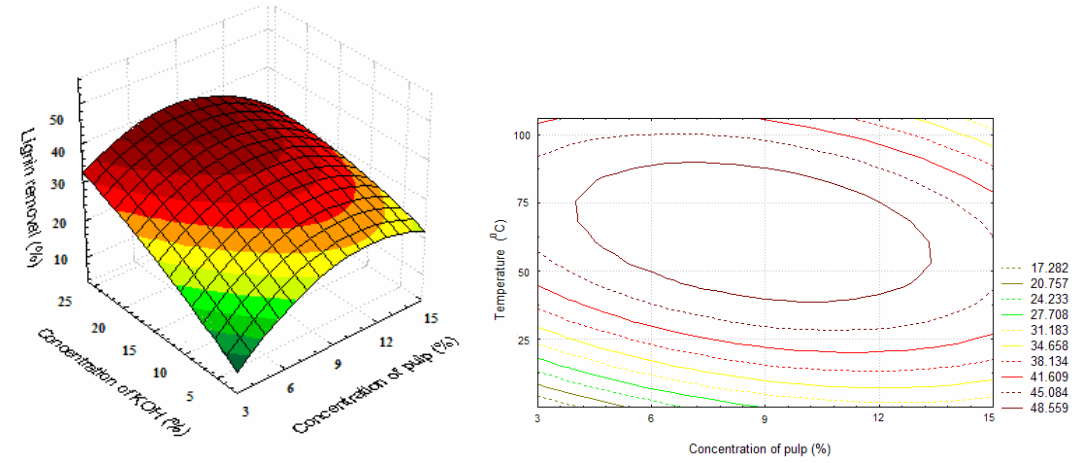

Figure 30 Response surface and contour plot for the effects of temperature and concentration of pulp on lignin removal percentage at 45 min and 14 % (w/w) KOH.

Figure 30 showed that increasing temperature and concentration of pulp could increase lignin removal percentage. The temperature higher than 80 ^{0}C had no significant effect on lignin removal percentage. Likewise, the concentration of pulp higher than 12% (w/w) had no significant effects on lignin removal after KOH delignification.

Response plot indicated the optimized condition at 73^{0}C and 10 % (w/v) of pulp concentration with 48.55% lignin removal from pulp after KOH delignification.

The relative effect of concentration of KOH and concentration of pulp with response to lignin removal percentage was plotted on three and two dimensions as in Figure 31.

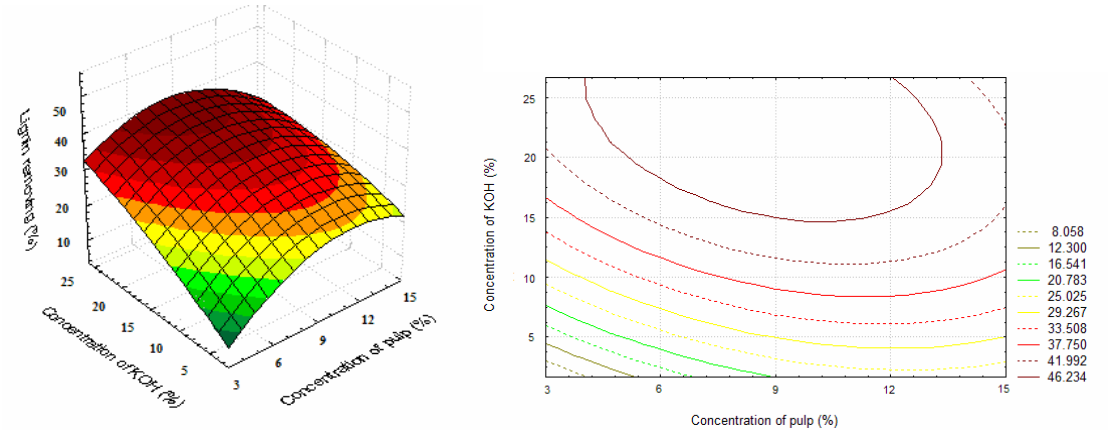

Figure 31 Response surface and contour plot for the effects of concentration of KOH and pulp on lignin removal percentage at 45 min and 65^0C.

Figure 31 showed that increasing concentration of pulp and KOH could increase lignin removal percentage. The concentration of KOH higher than 24 % (w/v) had no significant effected on lignin removal percentage. Likewise, the concentrations of pulp higher than 12% (w/w) had no significant effect on lignin removal after KOH delignification.

Response plot indicated the optimized condition at 17% (w/w) KOH and 10.50 % (w/v) of pulp concentration gave 46.23% (w/w) of lignin removal after KOH delignification.

According to the mentioned results, the most optimum condition with highest 48.55% (w/w) lignin removal was at 23% (w/w) NaOH, 11.2% (w/v) of pulp concentration, 80^0C and 65 min.

4.2 The response surface and contour plot of alpha cellulose

The relative effect on temperature and concentration of pulp with response to alpha cellulose percentage was plotted on three and two dimensions as in Figure 32.

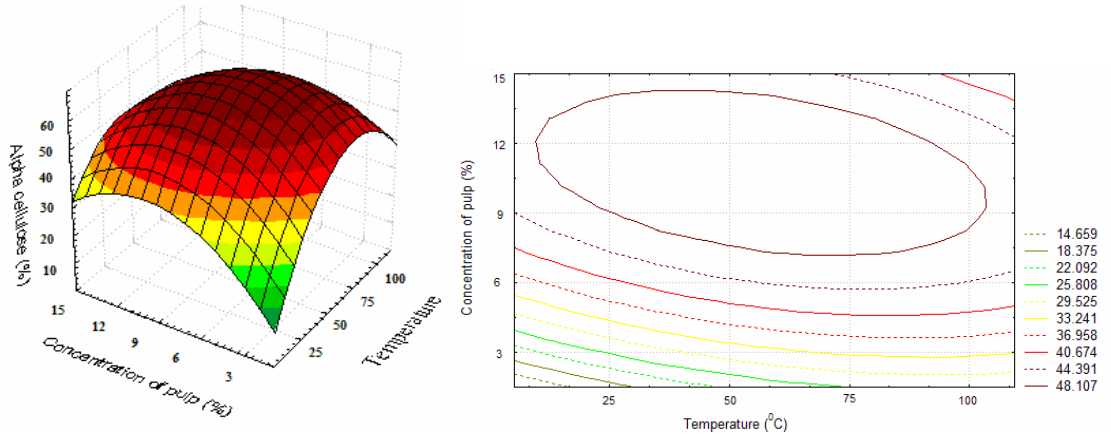

Figure 32 Response surface and contour plot for the effects of temperature and concentration of pulp on alpha cellulose percentage at 45 min and 14 % (w/w) KOH.

Figure 32 showed that increasing temperature and concentration of pulp could increase alpha cellulose percentage. The temperature higher than 80 ^{0}C had no significant effects on alpha cellulose percentage. Likewise, the concentration of pulp more than 12% (w/w) had no significant effects on alpha cellulose percentage after KOH delignification.

Response plot indicated the optimized condition at 78 ^{0}C of temperature and 12 % (w/v) of pulp concentration that gave 48.10% (w/w) of alpha cellulose after KOH delignification.

The relative effect of time and concentration of pulp with response to alpha cellulose percentage was plotted on three and two dimensions as in Figure 33.

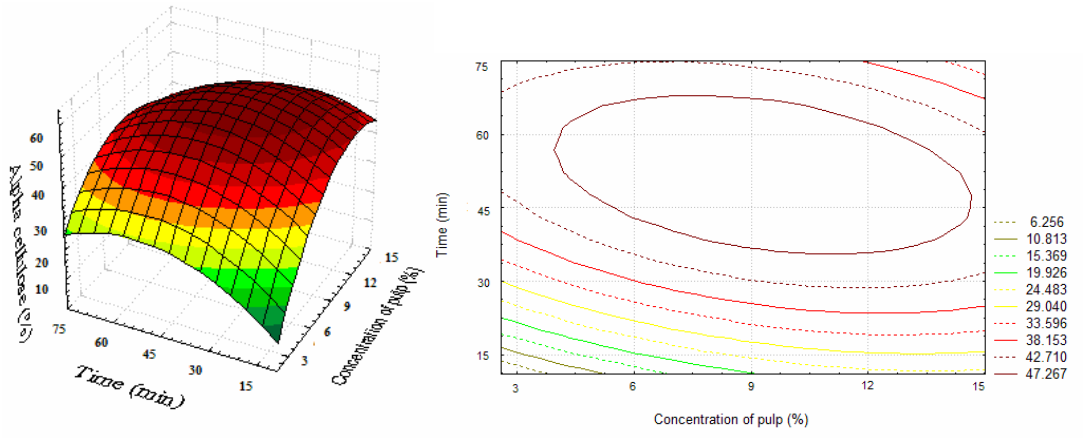

Figure 33 Response surface and contour plot for the effects of time and concentration of pulp on alpha cellulose percentage at 65^0C and 14 % (w/w) KOH.

Figure 33 showed that increasing time and concentration of pulp could increase alpha cellulose content left in pulp. The time longer than 60 min had no significant effects on alpha cellulose. Likewise, the concentration of pulp more than 12% (w/w) had no significant effect on the amount of alpha cellulose left in pulp.

Response plot indicated the optimized condition at 70 min and 12 % (w/v) of pulp concentration that give 47.26 % (w/w) of alpha cellulose after KOH delignification.

The relative effect of concentration of pulp and KOH with response to alpha cellulose percentage was plotted on three and two dimensions as in Figure 34.

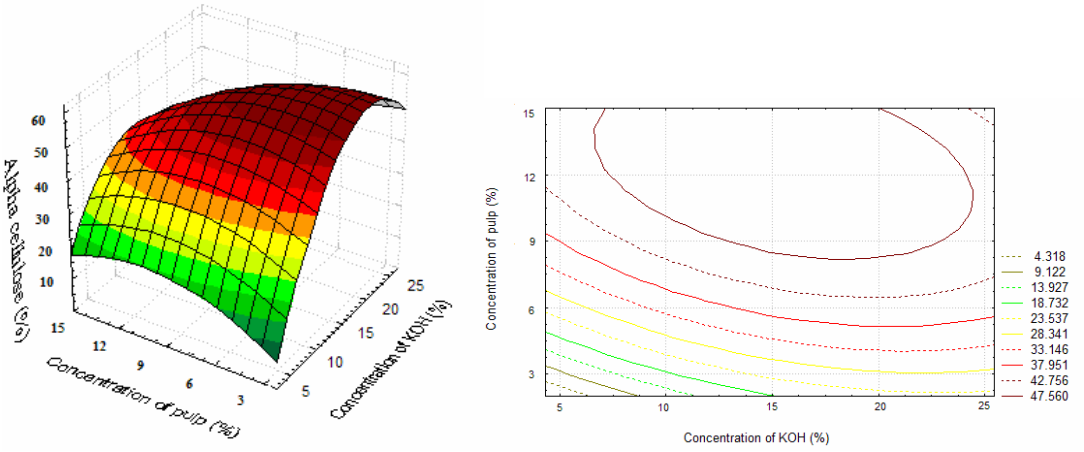

Figure 34 Response surface and contour plot for the effects of concentration of KOH and pulp on alpha cellulose percentage at 65^0C and 45 min.

Figure 34 showed that increasing concentration of pulp and KOH could increase alpha cellulose content in pulp. The concentration of KOH higher than 23 % (w/v) had no significant effect on alpha cellulose content in pulp. Likewise, the concentration of pulp higher than 12% (w/w) had no significant effect on the amount of alpha cellulose in pulp after KOH delignification.

Response plot indicated the optimized condition at 20% (w/w) of KOH concentration and 9% (w/v) of pulp concentration that give 47.56% of alpha cellulose pulp after KOH delignification.

5.3 The response surface and contour plot of glucose

The relative effect on concentration of pulp and temperature with response to glucose percentage was plotted on three and two dimensions as in Figure 35.

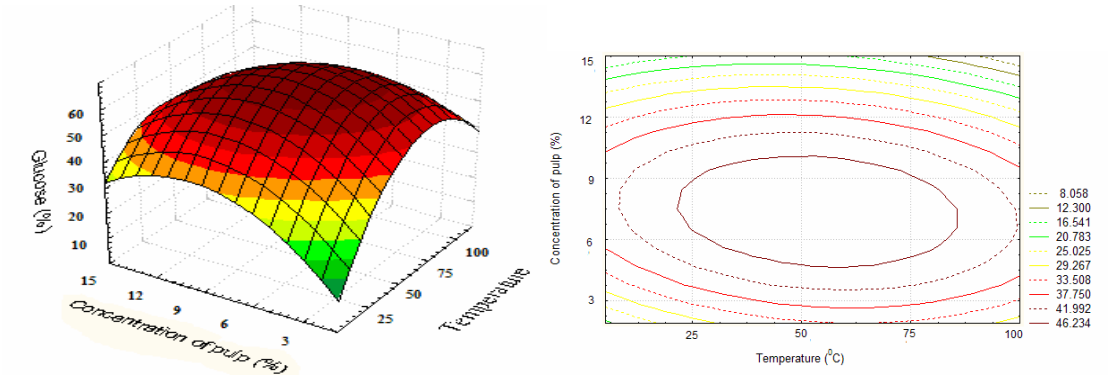

Figure 35 Response surface and contour plot for the effected of temperature and concentration of pulp on glucose percentage at 45 min and 14 % (w/w) KOH.

Figure 35 showed that increasing concentration of pulp and KOH could increase glucose in pulp. The concentration of KOH higher than 23 % (w/v) had no significant effect on glucose in pulp. Likewise, the concentration of pulp higher than 12% (w/w) had no significant effect on the amount of glucose in pulp after KOH delignification.

Response plot indicated the optimized condition at 78^0C and 12 % (w/v) of pulp concentration that gave 47.30 % (w/w) of glucose in pulp after KOH delignification.

The relative effect of concentration of pulp and time with response to glucose percentage was plotted on three and two dimensions as in Figure 36.

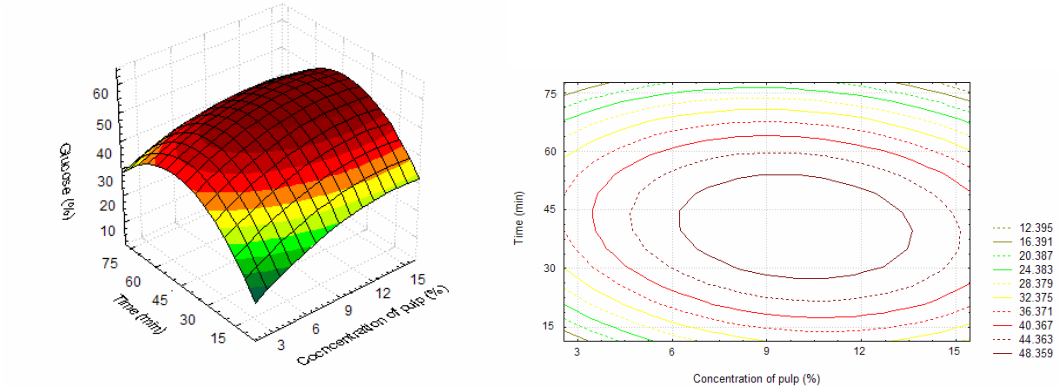

Figure 36 Response surface and contour plot for the effects of concentration of pulp and time on glucose percentage at 65 ^{0}C and 14 % (w/w) KOH.

Figure 36 showed that increasing time and concentration of pulp could increase glucose percentage in pulp. The time longer than 60 min had no significant effect on glucose percentage in pulp. Likewise, the concentration of pulp more than 12% (w/w) had no significant effect the amount of glucose in pulp after KOH delignification.

Response plot indicated the optimized condition at 12 % (w/v) of pulp concentration and 70 min of reaction time that gave 48.35 % (w/w) of glucose in pulp after KOH delignification.

The relative effect of concentration of pulp and KOH with response to glucose percentage was plotted on three and two dimensions as in Figure 37.

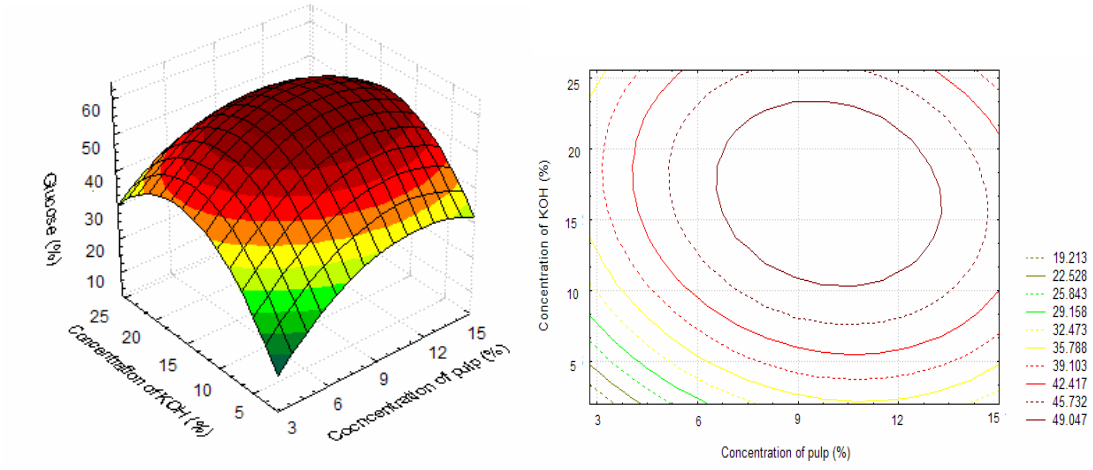

Figure 37 Response surface and contour plot for the effects of concentration of pulp and KOH on glucose percentage at 65^0C and 45 min.

Figure 37 showed that increasing concentration of KOH and pulp could increase glucose percentage in pulp. The concentration of pulp higher than 12.5% (w/w) had significant effect on the amount of glucose in pulp. On the other, the concentration of KOH higher than 23.4% (w/w) had no significant effect on the amount of glucose in pulp affer KOH delignification.

Response plot indicated the optimized condition at 10 % (w/v) of pulp concentration and 13.5 % (w/v) of KOH concentration that gave 49.04 of glucose in pulp after KOH delignification.

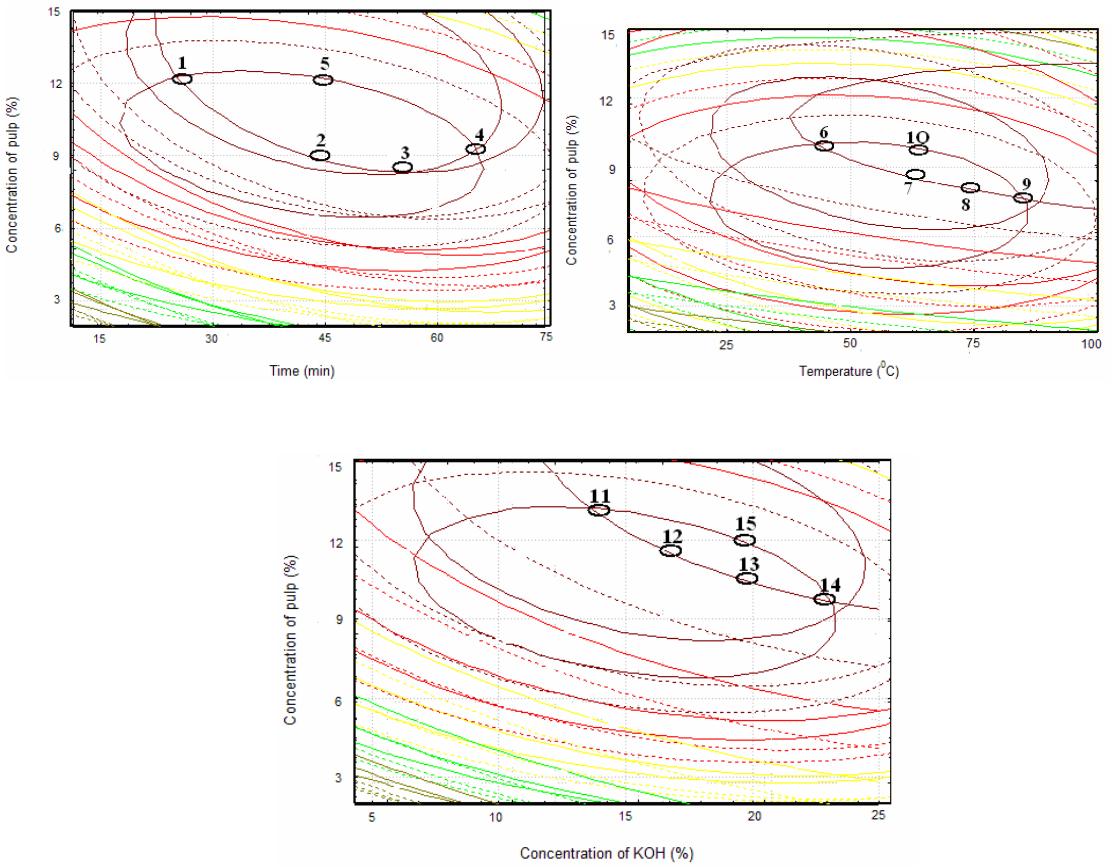

Figure 38 Contour plot of glucose, lignin removal and alpha cellulose percentage in pulp that affected the concentration of pulp and time, concentration of pulp and temperature, concentration of pulp and KOH.

Table 16 Experiment values of lignin removal, glucose and alpha cellulose of KOH delignification.

Point	A	B	C	D	Lignin removal (%)	Glucose in pulp (%)	Alpha cellulose in pulp (%)
1	12	25	50	15	25.46	41.46	42.56
2	9	45	50	15	26.53	41.46	41.34
3	8	55	50	15	28.43	40.56	40.49
4	9	65	50	15	29.57	42.52	42.46
5	12	45	50	15	26.78	40.03	41.05
6	10	45	45	15	25.43	38.92	39.82
7	8.5	45	60	15	27.54	39.04	40.32
8	8	45	75	15	30.78	42.09	43.49
9	7	45	80	15	34.32	44.34	44.48
10	8.5	45	60	15	30.92	35.84	36.94
11	13	45	50	13.5	29.04	37.96	38.94
12	11	45	50	16.5	27.54	36.54	38.99
13	10	45	50	20	25.04	39.07	40.83
14	9.5	45	50	23.5	31.45	43.98	44.96
15	10	45	50	20	29.86	40.86	41.89

A = Concentration of pulp (%w/v)

B = Reaction time (min)

C = Temperature (^{0}C)

D = Concentration of KOH (%w/w)

Tables 16 and 17 summarized the optimum condition and comparison of lignin removal, glucose percentage and alpha cellulose percentages between the predicted and experiment.

Table 17 Optimum condition and comparison of predicted and experiment values of KOH delignification.

Variables	Optimum condition	Lignin removal (%)		Glucose in pulp (%)		Alpha cellulose in pulp (%)	
		P	E	P	E	P	E
Concentration of pulp (%w/v)	12	59.13	47.62	47.62	48.50	48.50	52.75
Concentration of KOH (%w/w)	23.5						
Reaction time (min)	65						
Temperature (^{0}C)	80						

Table 17 indicated that the optimum condition for KOH delignification was obtained at 12 % (w/v) of pulp concentration, 23.5 % (w/w) of KOH, 65 min of reaction time and 80^{0}C with the maximum percentages of glucose, alpha cellulose and removed lignin removed pulp at 48.50 % (w/w), 52.75 % (w/w) and 48.68 % (w/w), respectively.

6. Experimental design of Plackett–Burman for enzyme hydrolysis of pulp obtained after alkaline delignification

Table 18 Analysis of variance showing significance of the variables on responses.

		Sum of square	
		Glucose yield	
Independent variables	df	NaOH	KOH
Reaction	4	3.94	2.53
Temperature	4	3.90	1.77
Enzyme loading	4	11.87**	10.58**
Concentration of pulp	4	1.73	4.99

**significant at 5% level; significant at 10% level.
reaction time at 10, 90 hours, temperature at 35, 50 ^{0}C, enzyme loading at 30,105 FPU/ g substrate, concentration of pulp at 2, 5 % w/v

From the statistical analysis (Table 18), reaction time, temperature, enzyme loading and concentration of pulp had a significant effect on glucose yield ($p < 0.1$) but the enzyme loading was the most important factor because it significantly affected glucose yield for both NaOH and KOH ($p < 0.05$).

7. Optimization of enzyme hydrolysis of pulp obtained after NaOH delignification

The enzyme hydrolysis of pulp obtained after NaOH delignification wasoptimized. The percentage of glucose yield from the prediction and experiment were compared. The 31 various experimental conditions and the experiment were shown in Table 19.

Table 19 Experiment and predicted values of glucose yield from enzyme hydrolysis of pulp NaOH- delignified pulp.

Treatments	X_1	X_2	X_3	X_4	Glucose yield (%)	
					Experiment	Predicted
1	70	50	80	80	86.2	86.03
2	70	50	80	30	75.4	75.34
3	70	50	30	80	80.0	80.04
4	70	50	30	30	63.9	64.26
5	70	35	80	80	46.7	46.24
6	70	35	80	30	23.0	23.23
7	70	35	30	80	35.4	35.18
8	70	35	30	30	20.6	20.06
9	30	50	80	80	80.3	80.85
10	30	50	80	30	71.6	70.243
11	30	50	30	80	76.4	76.36
12	30	50	30	30	58.6	58.29
13	30	35	80	80	41.4	41.30
14	30	35	80	30	18.8	18.84
15	30	35	30	80	27.33	27.34
16	30	35	30	30	10.55	10.98
17	50	42.5	55	3	89.87	89.26
18	50	42.5	55	3	79.3	79.23
19	50	42.5	55	3	80.70	80.72
20	50	42.5	55	3	80.49	80.14
21	50	42.5	55	3	81.31	81.66
22	50	42.5	55	3	81.62	81.97
23	50	42.5	55	3	81.45	81.89
24	90	42.5	55	3	83.78	83.46
25	10	42.5	55	3	10.37	10.04

Table 19 (Continued)

Treatments	X_1	X_2	X_3	X_4	Glucose yield (%)	
					Experiment	Predicted
26	50	57.5	55	3	15.45	15.42
27	50	28	55	3	50.56	50.51
28	50	42.5	105	3	77.67	77.63
29	50	42.5	5	3	21.35	21.35
30	50	42.5	55	5	80.22	81.20
31	50	42.5	55	1	79.83	79.78

The glucose yield from the experiment was applied to linear regression by SPSS software using STATISTA software to compare with that from the prediction.

7. Optimization of enzyme hydrolysis of pulp obtained after KOH delignification

The enzyme hydrolysis of pulp obtained after KOH delignification was optimized. The percentage of glucose yield from the prediction and experiment were compared. The 31 various experimental conditions and the experiment were shown in Table 20.

Table 20 Experiment and predicted values of glucose yield from enzyme hydrolysis of pulp obtained after KOH- delignified pulp.

Treatments	X_1	X_2	X_3	X_4	Glucose yield (%)	
					Experiment	Predicted
1	70	50	80	80	80.15	79.84
2	70	50	80	30	69.55	69.08
3	70	50	30	80	74.24	74.55
4	70	50	30	30	54.45	55.44
5	70	35	80	80	37.66	37.42
6	70	35	80	30	16.87	16.18
7	70	35	30	80	30.35	30.90
8	70	35	30	30	14.54	14.30
9	30	50	80	80	74.45	74.48
10	30	50	80	30	63.8	63.37
11	30	50	30	80	70.15	70.43
12	30	50	30	30	53.86	53.65
13	30	35	80	80	34.97	34.74
14	30	35	80	30	12.24	12.55
15	30	35	30	80	20.13	20.14
16	30	35	30	30	4.44	4.55
17	50	42.5	55	3	74.27	74.28
18	50	42.5	55	3	73.39	73.39
19	50	42.5	55	3	74.46	74.84
20	50	42.5	55	3	75.28	75.22
21	50	42.5	55	3	75.38	75.31
22	50	42.5	55	3	75.56	75.46
23	50	42.5	55	3	75.36	75.24
24	90	42.5	55	3	77.08	77.39
25	10	42.5	55	3	6.39	6.37

Table 20 (Continued)

Treatments	X_1	X_2	X_3	X_4	Glucose yield (%)	
					Experiment	Predicted
26	50	57.5	55	3	11.75	11.79
27	50	28	55	3	45.13	45.10
28	50	42.5	105	3	73.05	73.07
29	50	42.5	5	3	15.27	15.35
30	50	42.5	55	5	74.48	74.84
31	50	42.5	55	1	62.27	62.43

Table 21 Predictive regression models for glucose yield from the enzyme hydrolysis of pulp obtained after NaOH and KOH delignification.

Dependent variables	Predictive model	R^2
Glucose yield (%) after NaOH delignification	$Y = 49.956 + 2.654X_1 + 20.429X_2 + 1.310X_3 - 6.477X_4 - 0.021X_1^2 - 0.228X_2^2 - 0.012X_3^2 - 1.134X_4^2 - 0.005X_1X_2 + 0.001X_1X_3 + 0.003X_1X_4 + 0.002X_2X_3 + 0.225X_2X_4 + 0.002X_3X_4$	0.867
Glucose yield (%) after KOH delignification	$Y = 51.956 + 2.654X_1 + 20.332X_2 + 1.310X_3 - 6.477X_4 - 0.021X_1^2 - 0.224X_2^2 - 0.012X_3^2 - 1.835X_4^2 - 0.005X_1X_2 + 0.001X_1X_3 + 0.003X_1X_4 + 0.002X_2X_3 + 0.225X_2X_4 + 0.003X_3X_4$	0.935

The predictive equation derived from RSM was shown in Table 21. The effects of reaction time (hour), temperature (^{0}C), enzyme loading (FPU/g substrate) and concentration of pulp on enzyme hydrolysis process were investigated. The experiments were arranged according to the central composite design as indicated in Table 20. The predicted and experimental glucose yields (%) obtained from 31 treatments according to the codes were demonstrated in Table 20. The experimental glucose yield had correlation coefficients (R^2)

of 0.867 and 0.935 for NaOH and KOH delignification, respectively. The correlation between the experimental and predicted values of NaOH and KOH delignification indicated good agreement between the experimental and predicted values of glucose yield. After that, the experimental glucose yield was applied in linear regression by SPSS software using STATISTICA software to compare with that from the prediction. The application of RSM gave the predictive regression model of glucose yield as shown in Table 21.

Y was the response representing the glucose yield and X_1, X_2, X_3 and X_4 were the coded values of the test variables of reaction time (hour), temperature (^{0}C), enzyme loading (FPU/g substrate) and concentration of pulp, respectively. The variables X_1X_2, X_1X_3, X_1X_4, X_2X_2, X_2X_4 and X_3X_4 represented interaction effects of reaction time (hour) and temperature, reaction time and enzyme loading, reaction time and concentration of pulp, temperature and enzyme loading, and reaction time and concentration of pulp, respectively.

7.1 The response surface plot and contour plot for the glucose yield percentage in the hydrolyzed cellulose solution after NaOH delignification of steam-exploded pulp.

The relative effect of time and concentration of pulp with response to the glucose yield in the hydrolyzed cellulose solution was plotted on three and two dimensions as in Figure 39.

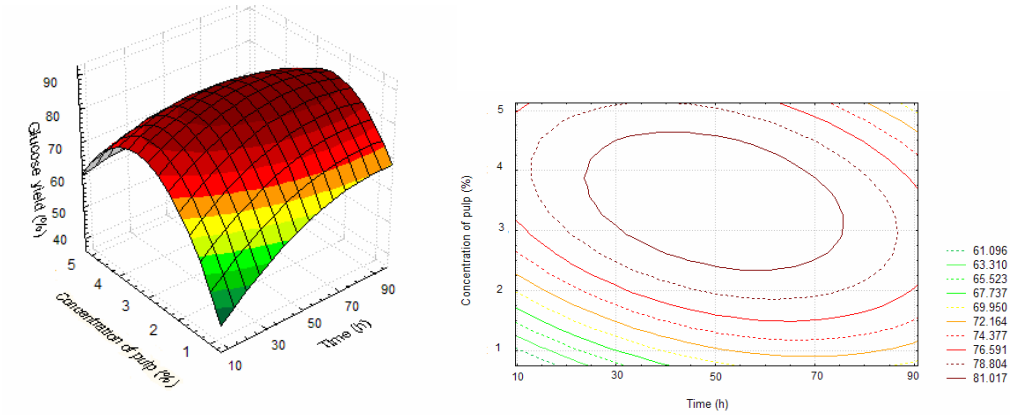

Figure 39 Response surface and contour plot for the effects of time and pulp concentration on the glucose yield in the hydrolyzed cellulose solution at 65^0C and 55 (FPU/ g substrate) of enzyme loading

Figure 39 showed that when time and concentration of pulp increased, the glucose yield in hydrolyzed cellulose solution also increased. The concentration of pulp higher than 4.5% (w/w) had no significant effect on the amount of glucose in hydrolyzed cellulose solution.

Response plot indicated that the optimized enzyme hydrolysis condition at 3 % (w/v) of pulp concentration and 65 hours of reaction of time gave 81.01 % (w/w) of glucose yield in hydrolyzed cellulose solution.

The relative effect of time and concentration of pulp with response to the glucose yield in the hydrolyzed cellulose solution was plotted on three and two dimensions as in Figure 40.

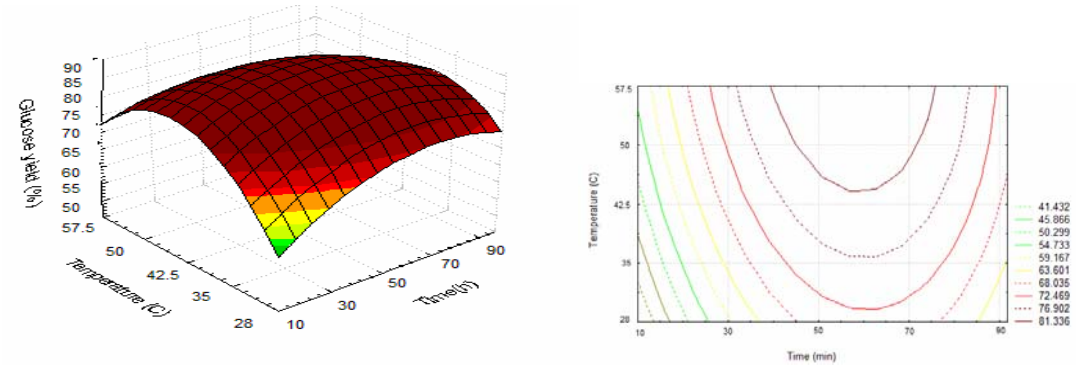

Figure 40 Response surface and contour plot for the effect of time and temperature on the glucose yield in the hydrolyzed cellulose solution at 2.5 % (w/v) and 55 (FPU/ g substrate) of enzyme loading

Figure 40 showed that increasing time and temperature could increase the glucose yield in hydrolyzed cellulose solution. The temperature higher than 50^0C had no significant effect on the amount of glucose in hydrolyzed cellulose solution.

Response plot indicated that gave the optimized condition at 44^0C of temperature and 65 hours of reaction of time could give 81.33% (w/w) of percent glucose yield in hydrolyzed cellulose solution after enzyme hydrolysis of pulp obtained after NaOH delignification.

The relative effect of time and enzyme loading with response to the glucose yield in the hydrolyzed cellulose solution was plotted on three and two dimensions as in Figure 41.

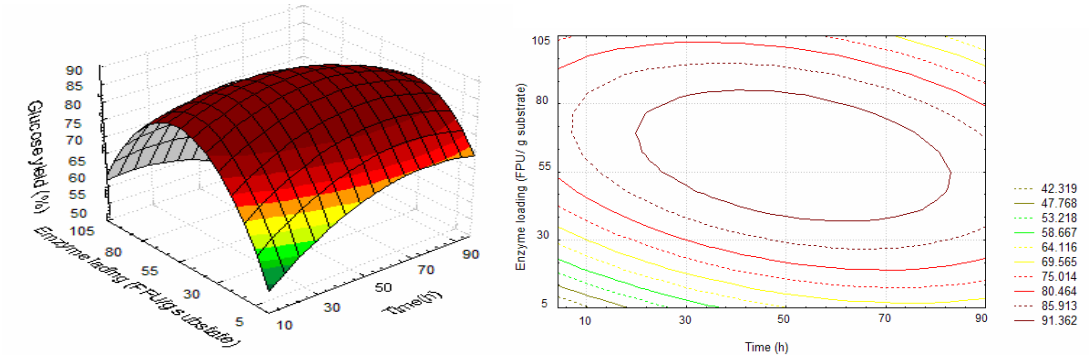

Figure 41 Response surface and contour plot for the effect of time and enzyme loading on the glucose yield in the hydrolyzed cellulose solution at 3 % (w/v) of pulp concentration and 55 (FPU/ g substrate) enzyme loading.

Figure 41 demonstrated that increasing time and enzyme loading could increase the glucose yield in hydrolyzed cellulose solution. The enzyme loading higher than 65 (FPU/ g substrate) had no significant effects the amount of glucose in hydrolyzed cellulose solution.

Response plot indicated that the optimized conditions at 54 (FPU/ g substrate) of enzyme loading and 50 hours of reaction time gave 91.36 (w/v) of glucose yield in hydrolyzed cellulose solution after enzyme hydrolysis of pulp obtained after NaOH delignification.

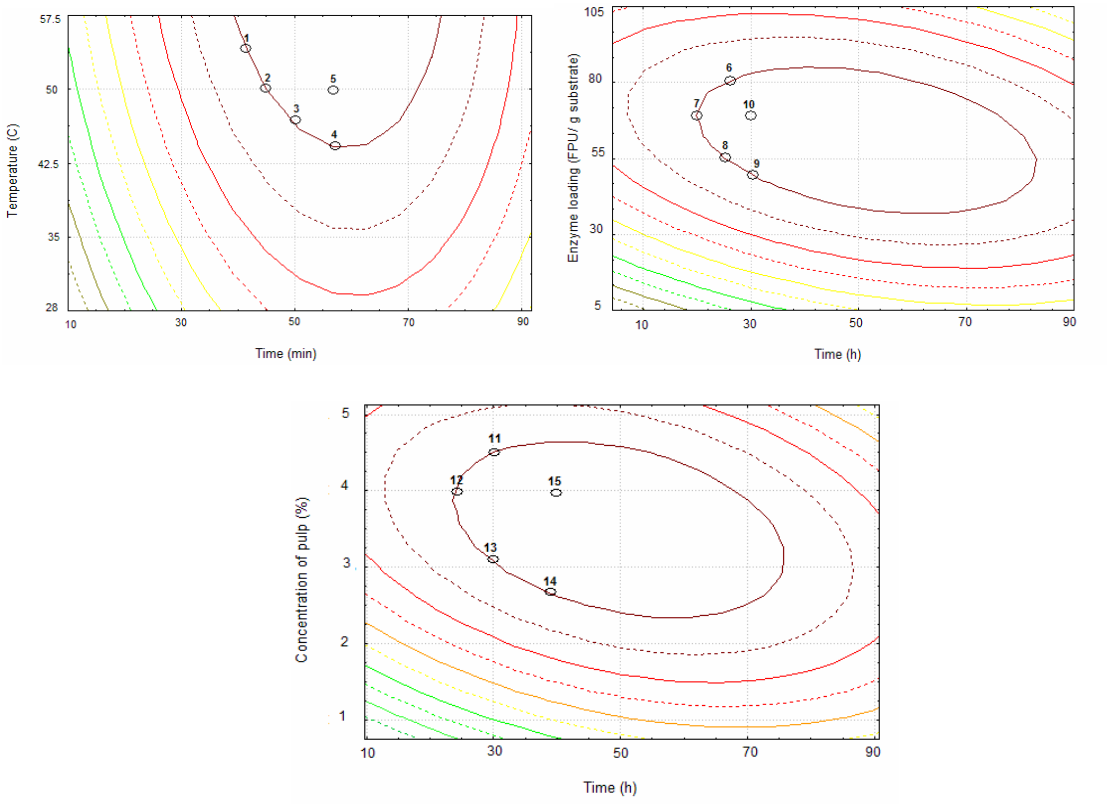

Figure 42 Contour plot for the effect of time and enzyme loading, time and pulp concentration, time and temperature on the glucose yield in the hydrolyzed cellulose solution.

Table 22 Experiment value of glucose yield in the hydrolyzed cellulose solution after NaOH delignification.

Point	Temperature, ^{0}C	Time, hours	Enzyme loading, FPU/g substrate	Concentration of pulp, % (w/v)	Glucose yield, % (w/v)
1	53	40	55	3	66.02
2	50	45	55	3	67.31
3	45	50	55	3	69.43
4	43	55	55	3	70.41
5	50	50	55	3	76.49
6	50	25	80	3	71.49
7	50	20	65	3	70.65
8	50	25	54	3	74.24
9	50	30	50	3	68.55
10	50	30	65	3	67.95
11	50	30	55	4.5	65.02
12	50	25	55	4	70.94
13	50	30	55	3	71.29
14	50	40	55	2.5	75.98
15	50	40	55	4	68.43

Table 23 Comparison of glucose yield percentage in solution of NaOH delginified and non-delignified pulp after enzyme hydrolysis.

Variables	Optimum condition	Glucose from delignified pulp	Glucose from non-delignified pulp
Temperature, ^{0}C	50		
Time, hours.	50	85	75
Enzyme loading, FPU/g	54		
Concentration of pulp,%w/v	2.5		

Table 23 compared the glucose yield in the hydrolyzed cellulose after enzyme hydrolysis of delignified and non-delignified pulp. Under the same hydrolysis condition, the glucose yield obtained from delignified pulp was higher than that from non-delignified pulp.

7.2 The response surface plot and contour plot for the percentage glucose yield in the hydrolyzed cellulose solution after KOH delignification of steam-exploded pulp.

The relative effect of time and concentration of pulp with response to the glucose yield in the hydrolyzed cellulose solution was plotted on three and two dimensions as in Figure 43.

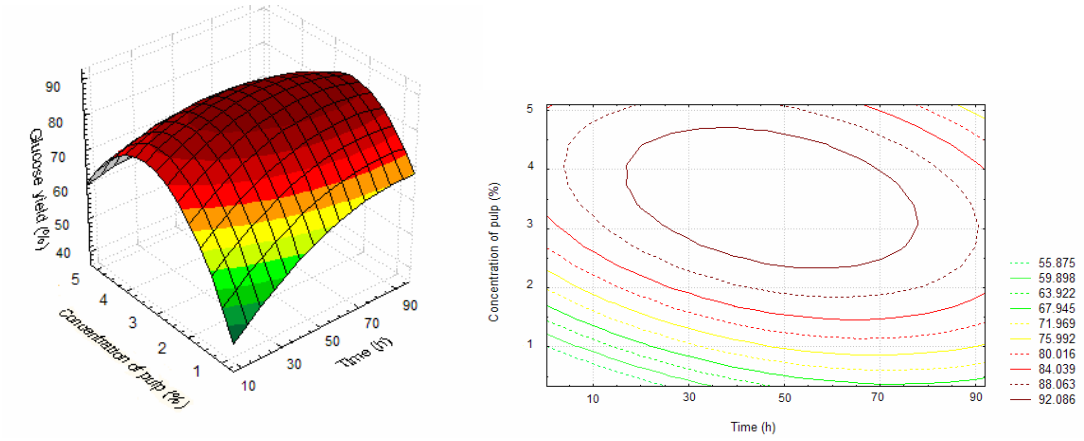

Figure 43 Response surface and contour plot for the effect of time and pulp concentration of the glucose yield in the hydrolyzed cellulose solution at 42.5^{0}C and 55 (FPU/ g substrate) of enzyme loading

Figure 43 demonstrated that increasing time and pulp concentration could increase the glucose yield in hydrolyzed cellulose solution. The reaction time longer than 75 hours had no significant effect on the amount of glucose in hydrolyzed cellulose solution.

Response indicated that the optimized condition at 3% (w/v) of pulp concentration and 65 hours of reaction time gave 92.08 % w/v of glucose yield in

hydrolyzed cellulose solution after enzyme hydrolysis of pulp obtained after KOH delignification.

The relative effect of time and temperature with response to the glucose yield in the hydrolyzed cellulose solution was plotted on three and two dimensions as in Figure 44.

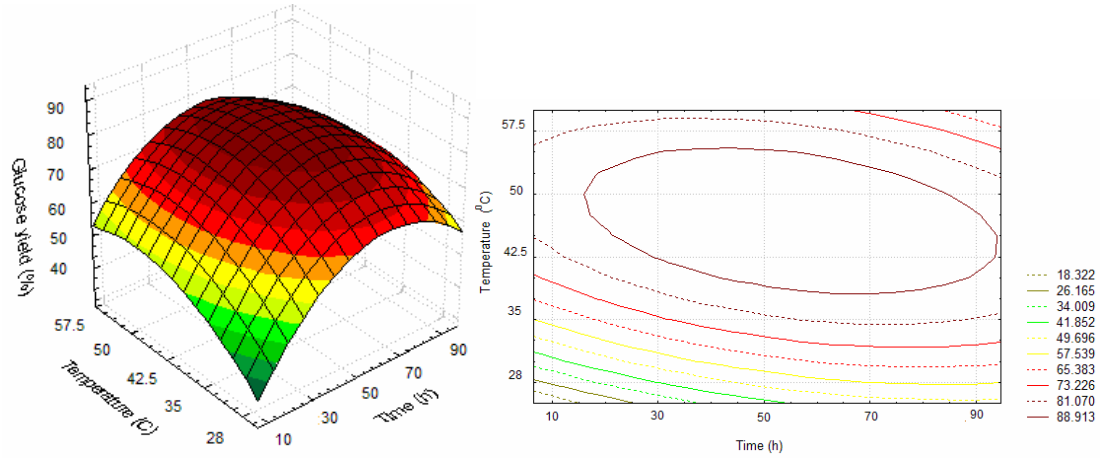

Figure 44 Response surface and contour plot the effect of time and temperature on the glucose yield in the hydrolyzed cellulose solution at 3 % (w/v) of pulp concentration and 55 (FPU/ g substrate) of enzyme loading.

Figure 44 indicate that increasing time and temperature could increase the glucose yield in hydrolyzed cellulose solution. The temperature higher than 50^0C had no significant effect on the amount of glucose in hydrolyzed cellulose solution.

Response indicated the optimized condition at 50^0C and 65 hours of reaction time that gave 88.91 % (w/v) of glucose yield in hydrolyzed cellulose solution after enzyme hydrolysis of pulp obtained after KOH delignification.

The relative effect of time and enzyme loading with response to the glucose yield in the hydrolyzed cellulose solution was plotted on three and two dimensions as in Figure 45.

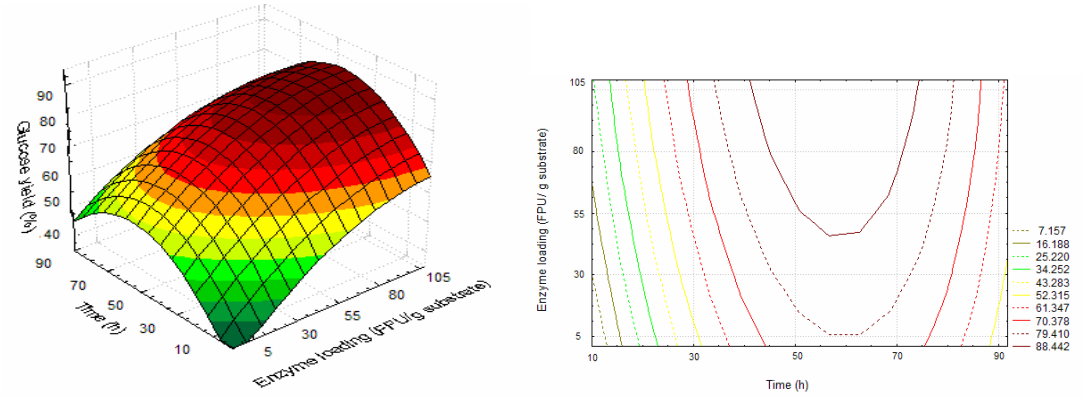

Figure 45 Response surface and contour plot for the effect of time and enzyme loading on the glucose yield in the hydrolyzed cellulose solution at 2.5 % (w/v) of pulp concentration and 42.5^{0}C

Figure 45 showed that increasing time and enzyme loading could increase the glucose yield in hydrolyzed cellulose solution. The enzyme loading more than 85 (FPU/g substrate) had no significant effect on the amount of glucose in hydrolyzed cellulose solution.

Response indicated the optimized condition at 54.5 (FPU/ g substrate) of enzyme loading and 66 hours of reaction time that could gave 88.44 % (w/v) of glucose yield in hydrolyzed cellulose solution after enzyme hydrolysis of pulp obtained after KOH delignification.

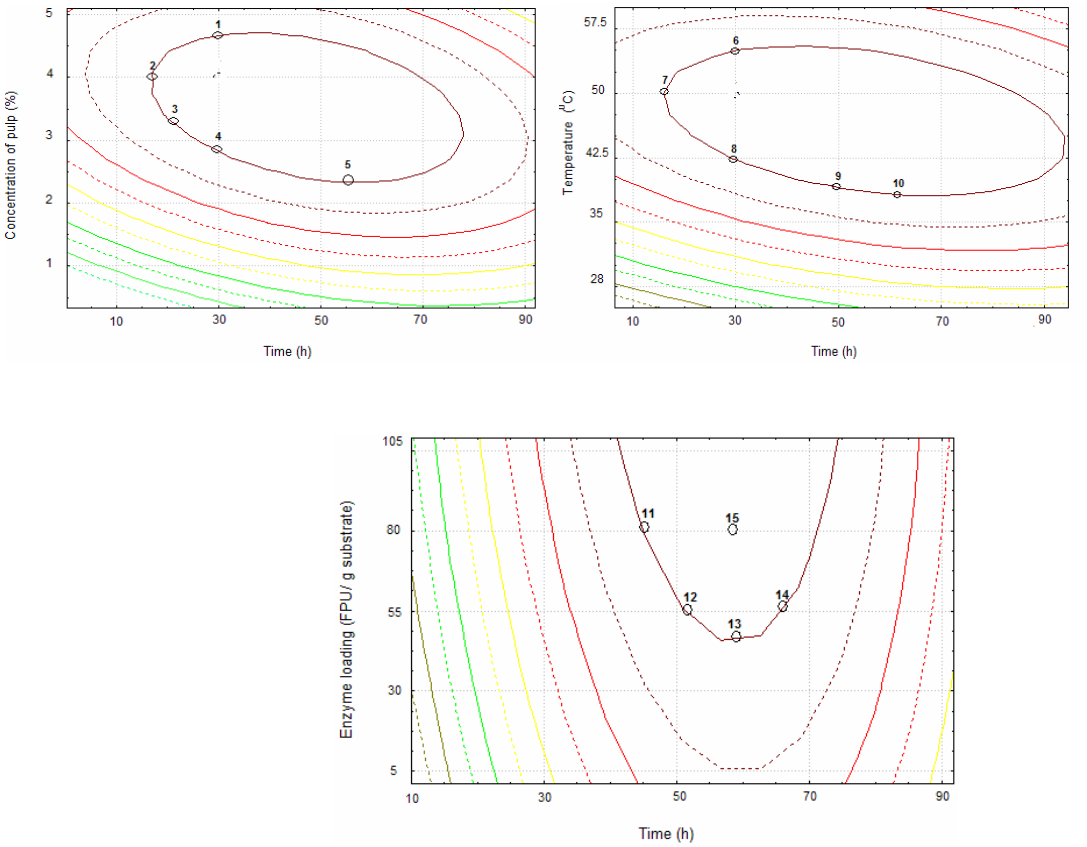

Figure 46 Contour plot for the effect of time and enzyme loading, time and pulp concentration, time and temperature on the glucose yield in the hydrolyzed cellulose solution.

Table 24 Experiment value of glucose yield in the hydrolyzed cellulose solution from KOH delignification.

Point	Temperature, ^{0}C	Time, hours	Enzyme loading, FPU/g substrate	Concentra-tion of pulp, % (w/v)	Glucose yield, % (w/w)
1	53	30	55	4.5	62.87
2	50	20	55	4	65.23
3	45	25	55	2.5	70.89
4	43	30	55	2.5	72.75
5	50	55	55	3	65.31
6	54	30	80	3	66.02
7	50	20	55	3	73.28
8	42.5	30	55	3	64.32
9	40	50	55	3	60.28
10	60	40	55	3	62.06
11	42.5	45	80	3	63.21
12	42.5	50	55	3	67.82
13	42.5	50	60	3	69.03
14	42.5	60	65	3	72.91
15	42.5	55	80	3	65.92

Table 25 Comparison of glucose yield percentage in solution of KOH and without delignified pulp after enzyme hydrolysis.

Variables	Optimum condition	Glucose from delignified pulp	Glucose from non-delignified pulp
Temperature, ^{0}C	50		
Time, h.	60	81	73
Enzyme loading, FPU/g substrate	65		
Concentration of pulp, % w/v	2.5		

Table 25 compared the glucose yield in the cellulose hydrolyzed after enzyme hydrolysis of delignified and non-delignified pulp. Under the same hydrolysis condition, the glucose yield obtained from delignified pulp was higher than that from non-delignified pulp.

Table 26 Comparison of glucose yield percentage in solution of NaOH and KOH delignified pulp after enzyme hydrolysis.

Variables	Optimum conditions		Percent glucose yield of delinified pulp	
	NaOH	KOH	NaOH	KOH
Temperature, ^{0}C	50	50		
Time, h.	50	60		
Enzyme loading, FPU/g substrate	54	65	85	81
Concentration of pulp, % w/v	2.5	2.5		

Table 26 showed the comparison of glucose after the enzyme hydrolysis of NaOH and KOH delignified pulp. The optimum conditions of enzyme hydrolysis for NaOH and KOH delignified pulp were 54, 65 (FPU/g substrate) of enzyme loading, 50, 60 hours, 50^0C, and 2.5% (w/v) pulp concentration, respectively. The enzymatic hydrolysis of delignified bagasse polysaccharides produced over 95% hydrolysis within 48 hour at 50 ^{0}C as reported by Adsul *et al.* (2005). The maximum glucose yield obtained were 85% and 81% from NaOH and KOH delignified pulp, respectively. The results also indicated that NaOH was a better alkaline solution in delignification. Furthermore, the pulp obtained from NaOH delignification used shorter hydrolysis time and less amount of enzyme loading.

The enzyme hydrolysis was reported the better glucose yield (57.8 %) when compared with the enzyme saccharification of pretreated sunflower stalks as shown by Sharma (2002).

7. Ethanol fermentation

7.1. Production of ethanol from pure glucose

S. cerevisiae TISTR 5339 was grown in YMB containing 50.0 g/l glucose, 20.0 g/l peptone and 10.0 g/l yeast extract. The experiment was performed at room temperature. The results of ethanol production were shown in Table 27.

Table 27 Production of ethanol from 50 g/l pure glucose by *S. cerevisiae* TISTR 5339.

Time (h)	Residual glucose (g/l)	Consumed glucose (g/l)	Ethanol Concentration (g/l)	Ethanol yield* (%)
0	50	0	0	0
6	40.52	9.48	4.62	18.5
12	33.21	16.79	8.05	32.23
18	25.16	24.84	12.15	48.62
24	17.35	32.65	16.08	64.35
30	10.30	39.70	19.46	77.84
36	3.21	46.79	21.92	87.68
42	0	50	21.81	87.25
48	0	50	21.75	87.10
54	0	50	21.04	86.66

*The ethanol yield means ethanol from experiment divided by the theoretical ethanol (g/g) and multipled by 100.

Table 27 showed production of ethanol from 50 g/l pure glucose by *S. cerevisiae* TISTR 5339. The result showed that within 36 hours of fermentation, the highest ethanol concentration was obtained at 21.92 g/l. The ethanol yield calculated from the experiment was divided by the theoretical ethanol and multiple by one hundred was 87.68%. This result indicated that *S. cerevisiae* TISTR 5339 had 87.68% in capability to change pure glucose to ethanol.

In addition, the result of residual glucose concentration and ethanol concentration were plotted related with fermentation time as showed in Figure 67. Residual glucose concentration decreased to zero at 42 hours of fermentation time which correspond to 21.81g/l of ethanol concentration. The concentration of ethanol began constant from 36 hours to 54 hours of fermentation time.

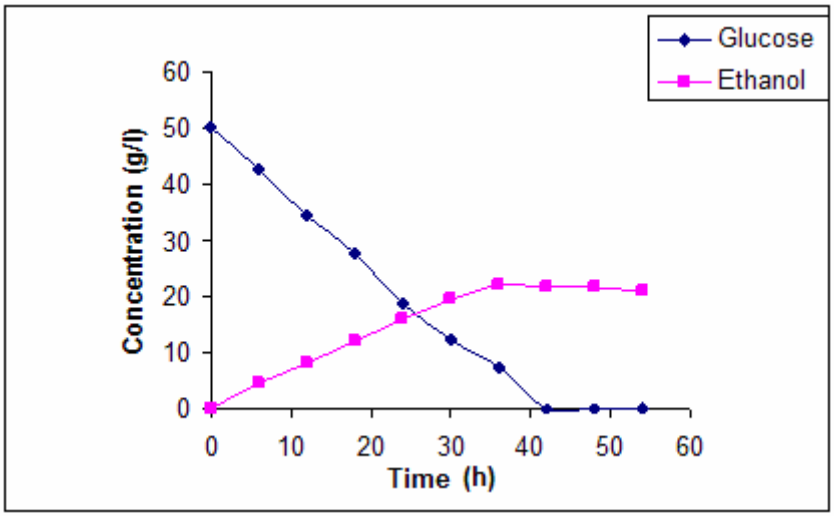

Figure 47 Comparison of resulting for occurring ethanol and reduce glucose concentration after 50 g/l pure glucose fermentation.

7.2 Production of ethanol from hydrolyzed solution of NaOH and KOH delignified pulp and non delignified pulp.

The hydrolyzed solution from three samples of pulp were concentrated to 50g/l of glucose. *S. cerevisiae* TISTR 5339 was applied in the same amount as in **7.1** but pure glucose was replaced with the hydrolyzed solution. The fermentation was finished within 36 hours. After that, the solution was analyzed for glucose and ethanol as shown in Table 28.

Table 28 Production of ethanol from hydrolyzed solution of NaOH and KOH delignified pulp and non- delignified pulp.

Hydrolyzed solution	Residual glucose (g/l)	Consumed glucose (g/l)	Ethanol concentration (g/l)	Ethanol yield (%)
From NaOH delignification	4.74	45.26	16.35	65.40
From KOH delignification	4.86	45.14	16.42	65.70
Without delignification	4.97	45.03	16.10	65.00

Table 28 showed all hydrolyzed solution had nearly ethanol yield. This results also showed delignification by NaOH and KOH had no effect on fermentation step. The maximum ethanol yield (65%) was also found in all hydrolyzed solution. Cara. (2007) carried out fermentation of enzyme hydrolysis steam exploded olive tree pruning by *S. cerevisiae* DER-CIEMAT 1701 in a fermenter that gave ethanol yield of 68.3%. The fermentation of enzyme hydrolysis has been reported better fermentation efficiencies in comparison to acid hydrolysis lignocellulose as Singh. (1984) Chung and Lee (1985) while studying fermentation of acid hydrolyzed baggage and acid hydrolyzed saw dust by *S. cerevisiae* in fermenter reported low fermentation efficiencies due to accumulation of toxic byproduct in the form of furfural and 5-hydroxy methyl furfural. When the ethanol yield obtained from pure glucose and hydrolyzed solution (Table 27 and Table 28) was compared, it showed that the hydrolyzed solution gave lower ethanol yield. This could be because the hydrolyzed solution contained toxic substances such as furfural, 5-hydroxy methyl furfural and acetic acid derived from steam explosion process. These substances can inhibit the fermentation of yeast.

CONCLUSIONS

The production of ethanol from glucose solution obtained from the steam explosion of oil palm trunk followed all of these steps: analysis of chemical components in oil palm trunk, optimization of alkaline delignification of pulp obtained after steam explosion, optimization of the enzyme hydrolysis of delignified pulp and optimization the ethanol production of glucose solution from oil palm trunk by the fermenting yeast *S. cerevisiae*. All the results were concluded:

1. The chemical components of oil palm trunk were 68.87 % (w/w) holocellulose, 37.14% (w/w) alphacellulose, 30.59 % (w/w) pentosan, 22.32% (w/w) lignin, 8.07% (w/w) extractive in ethanol /benzene and 8.56%(w/w) ash based on raw material weight.

2. The optimum condition of NaOH delignification was 11 % (w/v) of pulp concentration, 21.5% (w/w) of NaOH concentration, 65 min of reaction time and 78^0C of temperature. This condition gave product containing 47.50% (w/w) glucose and 50.33% (w/w) cellulose pulp, with 59.75% (w/w) lignin removal. The linear regression of glucose, alpha cellulose and lignin removing was shown below:

$$Y_{glucose} = 37.112 - 0.166\ X_1 - 0.959X_2 + 0.511X_3 + 0.104X_4 - 0.074X_1^2 - 0.005X_2^2 + 2.706X_3^2 + 001X_4^2 + 0.133X_1X_2 - 0.011X_1X_3 + 0.001X_1X_4 - 0.005X_2X_3 - 0.005X_2X_4 - 0.006X_3X_4$$

$$Y_{alpha\ cellulose} = 61.939 - 1.755X_1 - 1.142X_2 + 0.289X_3 - 0.262X_4 + 0.043X_1^2 + 0.034X_2^2 + 0.005X_3^2 + 0.007X_4^2 + 0.154X_1X_2 - 0.013X_1X_3 - 0.001X_1X_4 - 0.006X_2X_3 - 0.013X_2X_4 - 0.01X_3X_4$$

$$Y_{lignin\ removing} = -137.279 - 10.313X_1 + 0.977X_2 + 1.054X_3 + 2.854X_4 - 0.598X_1^2 - 0.045X_2^2 - 0.014X_3^2 - 0.022X_4^2 + 0.03X_1X_2 + 0.015X_1X_3 + 0.01X_1X_4 + 0.026X_2X_3 + 0.03X_3X_4$$

For KOH delignification, the optimum condition was 12 % (w/v) of pulp concentration, 23.5% (w/w) KOH, 65 min of reaction time and 80^0C. This condition gave containing 48.95% (w/w) glucose and 52.75% (w/w) cellulose pulp, with 48.68% (w/w) lignin removal. The linear regression of glucose, alpha cellulose and lignin removing was shown below:

$Y_{glucose} = 60.266 + 0.574X_1 - 1.398X_2 - 0.320X_3 + 0.091X_4 - 0.030X_1^2 - 0.001X_2^2 + 0.006X_3^2 - 002X_4^2 + 0.133X_1X_2 - 0.008X_1X_3 + 0.006X_1X_4 - 0.004X_2X_3 + 0.015X_2X_4 + 0.013X_3X_4$

$Y_{alpha\ cellulose} = 39.979 - 0.138X_1 - 1.005X_2 + 0.438X_3 + 1.20X_4 - 0.088X_1^2 - 0.001X_2^2 + 0.005X_3^2 + 0.001X_4^2 + 0.134X_1X_2 - 0.006X_1X_3 + 0.01X_1X_4 - 0.003X_2X_3 - 0.005X_2X_4 - 0.007X_3X_4$

$Y_{lignin\ removing} = -69.931 + 7.639X_1 + 0.343X_2 + 0.332X_3 + 1.420X_4 - 0.368X_1^2\ 0.022X_2^2 - 0.005X_3^2 - 0.013X_4^2 - 0.081X_1X_2 + 0.003X_1X_3 + 0.01X_1X_4 + 0.023X_2X_3 + 0.015X_2X_4 - 0.001X_3X_4$

3. The optimum condition of enzyme hydrolysis of pulp obtained after NaOH delignification was 54 (FPU/g substrate) of enzyme loading, 50 hours hydrolysis time, 50^0C, and 2.5 % (w/v) of pulp concentration. This condition gave the solution containing 85% of glucose yield. For pulp obtained after KOH delignification, the optimum was 65(FPU/g substrate) of enzyme loading, 60 hours hydrolysis time, 50^0C of temperature, and 2.5 % (w/v) of pulp concentration. This condition gave the solution containing 81% of glucose. The hydrolysis of delignified pulp gave the solution with higher glucose than that obtained from the non delinified pulp. The linear regression of enzyme hydrolysis for NaOH and KOH delignified pulp were shown below:

$Y_{glucose\ yield} = -49.956 + 2.654X_1 + 20.429X_2 + 1.310X_3 - 6.477X_4 - 0.021X_1^2 - 0.228X_2^2 - 0.012X_3^2 - 1.134X_4^2 - 0.005X_1X_2 + 0.001X_1X_3 + 0.003X_1X_4 + 0.002X_2X_3 + 0.225X_2X_4 + 0.002X_3X_4$.

$Y_{glucose\ yield} = -51.956 + 2.654X_1 + 20.332X_2 + 1.410X_3 - 6.477X_4 - 0.021X_1^2 - 0.224X_2^2 - 0.012X_3^2\ 1.835X_4^2 - 0.004X_1X_2 + 0.001X_1X_3 + 0.003X_1X_4 + 0.002X_2X_3 + 0.225X_2X_4 + 0.003X_3X_4$

4. The maximum glucose obtained from NaOH and KOH delignified pulp were 47.5% and 48% (w/w), respectively. Comparison of delignification conditions between NaOH and KOH showed that NaOH delignification consumed less alkaline concentration and lower temperature. This might be because NaOH was stronger alkaline than KOH. The effect of NaOH and KOH delignification on enzymatic hydrolysis was also tested. The results demonstrated that glucose released after enzymatic hydrolysis were 85% and 81%

from the NaOH and KOH delignified pulp, respectively. Thus, it could be concluded that the delignification step with NaOH solution was necessary prior to the enzymatic hydrolysis in order to improve the amount a released glucose.

5. The fermentation of 50 g/l glucose from all hydrolyzed solution resulted in similar ethanol yield. Moreover, it was found that the alkaline delignification process prior to the ethanol fermentation had no significant influence on the ethanol yield, even though the enzyme hydrolysis step was improved with higher released glucose.

6. The ethanol production from oil palm trunk had 3 main steps: delignification, enzymatic hydrolysis, and fermentation process. The delignification had optimum condition at 11% w/v of pulp concentration, 21.5% w/w NaOH, 65 min and 78^0C. The optimum condition of enzyme hydrolysis of pulp obtained after NaOH delignification was 54 (FPU/g substrate) of enzyme loading, 50 hours, 50^0C, and 2.5 % (w/v) of pulp concentration. This condition gave the solution containing 85% of glucose yield. The fermentation of 50 g/l glucose from enzyme hydrolysis gave maximum ethanol yield about 65% (16.35 g/l, ethanol concentration).

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APPENDICES

Appendix A

Method of analysis

APPENDIX method A1 Analysis of Moisture Content

1. Weigh a sample approximately 2 ±0.1g (A) in a tarred weighing bottle.

2. Dry for 2 hours in an oven at 100^0C, cool in desiccators, replace stopper and open the stopper momentarily to equalize the air pressure and weigh the sample.

3. Return bottle to the oven for 1 hours, repeat the cooling and weighing as above for successive hourly periods until constant weight (B) was reached.

4. The moisture content could be calculated as follow:

$$\text{Percent of Moisture Content} = [(A-B)/A]100$$

Where A = weight of sample, g

B = Oven –dried weight of test specimen, g

APPENDIX method A2 Analysis of Ash Content

1. Carefully clean crucible and ignite in a muffle furnace at 525^0C for 30- 60 min. After ignition, cool slightly and then place in the desiccator, then cool to room temperature.

2. Transfer the test sample to the crucible, with the lid agar, gently carbonize the specimen in the crucible on the furnace at about 100^0C. Raise the temperature to 525^0C. Sample must be charred, not burned so that the temperature of the sample does not exceed 525^0C.

3. When the sample is completely combusted as indicated by the absence of black particles, remove the crucible from the furnace, replace the cover, and allow to cool, then place in the desiccators and cool to room temperature.

Calculation

$$\text{Percent of Ash Content} = (AX\ B)/\ 100$$

Where A = weight of ash, g

B = weight of test sample, g moisture - free

APPENDIX method A3 Analysis of Holocellulose Content

1. Transfer 3 g of dry pulp sample were to 250-ml Erlenmeyer flasks with 160 ml of distilled water, 0.5 ml of acetic acid and 1.5 g of NaCl.

2. The samples were heated in a water bath at 70-80^{0}C with agitation at each 10 min.

3. After 60 min reaction, add 0.5 ml of CH_3COOH and 1.5 g of NaCl.

4. Repeat the addition at each 60 min until the final time of 4 hour of reaction. At the end of the fourth hour, put the Erlenmeyer flask in an ice bath to reach 10^{0}C.

5. Filter the samples in crucibles of porosity 2.

6. Wash the residue with hot distilled water under suction and then with acetone

7. Dry the crucible with lignin in an oven at 105^{0}C to constant weight. Cool in desiccators and weight.

$$\text{Percent of Holocellulose Content} = (W_1/W_2)\ 100$$

Where W_1 = weight of holocellulose, g

W_2 = weight of dry pulp, g

APPENDIX method A5 Analysis of Pentosans Content

1. Place the test sample in a boiling flask and add 20g NaCl, 100 ml of 3.85N HCL and a few boiling stones. Connect the flask to the distillation apparatus and mark the acid level in the flask. Add 250 ml of 3.85N HCl to the separate funnel.

2. Apply heat and distill the acid at a uniform rate of about 2.5ml per min. Collect the distillate in a 250 ml volumetric flask immersed in an ice bath.

3. During distillation, maintain a constant volume of 100 ml in the boiling flask by adding HCL from the separatory funnel, or in 25ml increments every 10 min. Continue the distillation for 90 min, in which time 225ml of distillate should be collected.

4. Bring the temperature of the distillate to about 20^{0}C, add 3.85 N HCL to the 250 ml mark and mix thoroughly. Pipet 5 ml of distillate into a 50 ml volumetric flask. Add 25 ml of orcinol reagent, mix, and place the flask in the water bath at 25^{0}C.

5. After 60 min, add ethanol up to the 50 ml mark, mix, and return to the water bath; then after another 60 min, measure the absorbance of the solution with a spectrophotometer at 630 nm. To avoid corrosion of the instrument by HCL fumes, the cells or cuvettes should be covered.

6. Read the number of milligram of xylan in the test specimen from a previously prepared.

$$\text{Percent of Pentosans Content} = (A/B)\ 100$$

Where A = xylan in test specimen, g

B = oven-dried weight of test specimen, g

APPENDIX method A6 Analysis of Monosaccharide Content

Retention time value of monosaccharide

1. Prepare each of sugar standard and internal standard (inositol) at 100 mg/l.
2. The solution is filtered through 0.45 mm cellulose acetate filter (Millipore).
3. Inject 20µl of sugar standards and internal standard onto HPLC.

Response Factor (RF) for each of sugar standard

1. Prepare a series of glucose, xylose, arabinose, mannose and galactose standards in the range of 20-100 mg/l.
2. Prepare 1000 mg/l of internal standard.
3. Filter the sugar solutions though on 0.45 mm cellulose acetate filter before injection onto the HPLC.
4. Pipette 0.9 ml of standard glucose and 0.1 ml internal standard into vials and inject 20µl of sample onto the HPLC.
5. Calculate RF of each sugar standard and internal standard and then calculate the RF. Sample aliquots are filtered through a 0.45 µm poly(tetrafluoroethylene) filter prior to injection.

6. Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.45 μm filter into an auto sampler vial. Seal and label the vial. Prepare each sample in duplicate if desired. If it is suspected that the sample concentrations may exceed the calibration range, dilute the samples as needed and record the dilution. The concentrations should be corrected for dilution after running. If necessary, neutralized samples may be stored in the refrigerator for three or four days. After this time, the samples should be considered compromised.

Response Factor calculation for each sugar standard and internal standard

$$RF = \frac{As \ X \ Ci}{Ai \ X \ Cs}$$

Where RF = Response Factor for each sugar standard

Cs = Concentration of sample, mg/l

Ci = Concentration of internal standard, mg/l

As = Peak area of sugar sample

Ai = Peak area of internal standard

APPENDIX method A7 Filter Paper Assay for Cellulase

Substrate: Whatman No. 1 filter paper strip, 1.0 x 6.0 cm (= 50 mg)

Procedure

1. Add 1.0 ml of 0.05 M Na-citrate, pH 4.8, to a test tube of volume at least 25 ml.

2. Add 0.5 ml of enzyme and dilute in citrate buffer. At least two dilutions must be made of each enzyme sample. One dilution should release slightly more and one slightly less than 2.0 mg of glucose (= reducing sugars as glucose) in the reaction condition.

3. Temperate to 50^0C, add one filter paper strip, mix (NB! it does not matter if a small part of the paper is above the liquid surface, but if the paper "winds" up the tube it must be pushed down again), incubate at 50^0C, 60 min.

4. Add 3.0 ml of DNS and mix thoughly. Transfer tube to a rack on the table.

5. Boil for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks, glucose standards and the spectro zero should be boiled together. After boiling, transfer to a cold water bath.

6. Add 20 ml of deionized water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion.

7. When the pulp has settled well, after at least 20 min, the color formed is measured against the spectro zero at 540 nm. If the paper pulp does not settle, it will do so after stirring with a glass rod. (The necessity for stirring can be seen after only a few minutes of settling time).

Unit Calculation

1. Construct a linear glucose using the absolute of glucose (mg/0.5 ml) plotted against A 540.

2. Use this standard to translate the absorbance values of the sample (after subtraction of enzyme blank) into glucose (= mg glucose produced during the reaction) translate the dilutions used into enzyme concentration:

3. Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by plotting glucose liberated against enzyme concentration on semi logarithmic graph paper.

Concentration = 1/dilution (volume of enzyme in dilution/ total volume of dilution)

Calculation FPU: FPU = 0.37/ enzyme concentration to released 2.0 mg glucose, (units ml^{-1})

APPENDIX method A8 Fermentation.

Microorganism and medium

S. cerevisiae TISTR 5339 was obtained from Thailand Institute of Scientific and Technological Research (TISTR, Thailand). It was maintained on a medium containing 20.0 g/l glucose, 20.0 g/l peptone and 10.0 g/l yeast extract at 4^0C. The yeast was subcultured every month at 30^0C. The growth medium of the yeast consisted of 10.0 g/l yeast extract, 6.4 g/l urea, 2.0 g/l KH_2PO_4, 1.0 g/l, and $MgSO_4$.$7H_2O$ and 20.0 g/l glucose.

Appendix B

Analysis of variance

APPENDIX Table B1 Approximate of Retention time and Response Factor (RF) for each sugar.

Sugars	Retention time	Mean RF
Glucose	11.32	0.94
Xylose	12.45	0.85
Galactose	13.17	0.91
Mannose	13.62	1.00
Arabinose	19.20	0.80
Inositol	15.98	1.20

APPENDIX Table B2 Analysis of variance (ANOVA) for lignin removal of pulp obtained after NaOH delignification.

Source	Sum of square	DF	Mean square	F-value	P-value
Model	9305.27	14	664.66	15.90	0.0078
Residual	668.64	16	41.79		
Lack of fit	500.43	10	50.04	1.78	0.0056
Pure error	168.21	6	28.03		
Total	9973.91	30			
R^2	0.977				

APPENDIX Table B3 Analysis of variance (ANOVA) for alpha cellulose in pulp after NaOH delignification.

Source	Sum of square	DF	Mean square	F-value	P-value
Model	9350.37	14	667.88	16.49	0.0012
Residual	647.96	16	40.49		
Lack of fit	490.96	10	49.09	1.87	0.0045
Pure error	157	6	26.16		
Total	9998.33	30			
R^2	0.865				

APPENDIX Table B4 Analysis of variance (ANOVA) for glucose in of pulp after NaOH delignification.

Source	Sum of square	DF	Mean square	F-value	P-value
Model	10005.23	14	714.65	15.69	0.0078
Residual	728.64	16	45.54		
Lack of fit	530.43	10	53.043	1.60	0.0056
Pure error	198.21	6	33.035		
Total	10733.87	30			
R^2	0.875				

APPENDIX Table B5 Analysis of variance (ANOVA) for lignin removal of pulp after KOH delignification.

Source	Sum of square	DF	Mean square	F-value	P-value
Model	9085.26	14	700.37	7.63	0.0078
Residual	1468.64	16	91.79		
Lack of fit	800.43	10	80.04	0.71	0.0056
Pure error	668.21	6	111.36		
Total	11273.90	30			
R^2	0.877				

APPENDIX Table B6 Analysis of variance (ANOVA) for alpha cellulose in pulp after KOH delignification

Source	Sum of square	DF	Mean square	F-value	P-value
Model	8323.4	14	594.5286	8.310421	0.0078
Residual	1144.642	16	71.54013		
Lack of fit	900.43	10	90.043	2.21225	0.0056
Pure error	244.212	6	40.702		
Total	9468.042	30			
R^2	0.802				

APPENDIX Table B7 Analysis of variance (ANOVA) for glucose in pulp obtained after KOH delignification.

Source	Sum of square	DF	Mean square	F-value	P-value
Model	7935.29	14	566.8064	10.88625	0.0078
Residual	833.06	16	52.06625		
Lack of fit	523.45	10	52.345	1.014405	0.0056
Pure error	309.61	6	51.60167		
Total	8768.35	30			
R^2	0.890				

APPENDIX Table B8 Analysis of variance (ANOVA) for glucose yield from the enzyme hydrolysis of NaOH delignified pulp.

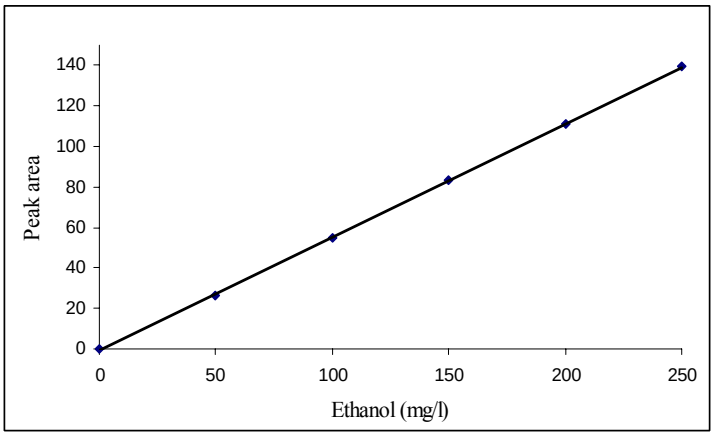
Source	Sum of square	DF	Mean square	F-value	P-value
Model	9235.43	14	659.6736	15.01583	0.0078
Residual	702.91	16	43.93188		
Lack of fit	540.45	10	54.045	1.995999	0.0056
Pure error	162.46	6	27.07667		
Total	9938.34	30			
R^2	0.867				

APPENDIX Table B9 Analysis of variance (ANOVA) for glucose yield from the enzyme hydrolysis of NaOH delignified pulp.

Source	Sum of square	DF	Mean square	F-value	P-value
Model	9235.43	14	659.6736	15.01583	0.0078
Residual	702.91	16	43.93188		
Lack of fit	540.45	10	54.045	1.995999	0.0056
Pure error	162.46	6	27.07667		
Total	9938.34	30			
R^2	0.935				

Appendix C

Calibration curve



Ethanol content

Correlation = 0.9987

Formulation : y = mx + b

: m = 0.559

: b = -0.7306

: x = Peak area

: y = Ethanol

APPENDIX Figure C1 Calibration curve of standard ethanol.

CURRICULUM VITAE

NAME	:	Teerayut Khunrong
BIRTH DATE	:	April, 17th 1976
BIRTH PLACE	:	Phatthalung, Thailand
EDUCATION	:	B.Sc.(Chemistry) Thaksin Univ. (2004)
POSITION/TITLE	:	-
WORK PLACE	:	Kasetsart Agricultural and Agro-industrial Product Improvement Institute (KAPI)
ORAL PRESENTATION AT CONFERENCE	:	Production of Ethanol from Pulp Obtained by Steam Explosion Pretreatment of Oil Palm Trunk