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Conversion of Lignocellulosic Material Into Fermentable Sugars

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Abstract

Enzymatic hydrolysis process of lignocellulosic biomass materials is difficult because of inherent structural features of biomass, which represents barriers that prevent complete hydrolysis; therefore, pretreatment techniques are necessary to render biomass highly digestible in enzymatic hydrolysis process. In this research, (non?) oxidative short-term lime pretreatment of willow wood was used. A weight of 11.40 g of willow wood was mixed with an excess of calcium hydroxide (0.4 g $Ca(OH)_2$/g raw biomass) and water loading (15 g/g raw biomass). Lime pretreatment was carried out for various periods of time including 1, 2, 3.5, 5 and 6 h, with temperatures at 100, 113, 130, 147 and 160^{0}C, and oxygen pressures as oxidativeagent (6, 9, 13.5, 17.8, 21 bar absolute). The optimization of both pretreatment and enzymatic hydrolysis were depended on the maximum overall yields of glucan and xylan after two processes of lime pretreatment and enzymatic hydrolysis. The optimal conditions of pretreatment were as follow: 1) 1.33 h, 147^{0}C, 17.8 bar absolute, 0.26 g $Ca(OH)_2$/g raw biomass. 2) 1.25 h, 155 ^{0}C, 21 bar absolute, 0.26 $Ca(OH)_2$/g raw biomass. Furthermore, the optimal values for low impact factors such as water loading was 15 g/g raw biomass and particle size was less than 3 mm. The optimal conditions of enzymatic hydrolysis were as follow: Cellulase enzymeloading was 0.1 g /g glucan in raw biomass, at substrate concentration of 50 g/L during 72 h of enzymatic hydrolysis The yield of enzymatic hydrolysis under these conditions were as follow: 96.00 g glucan/100 g of glucan in raw biomass, and 65.00 g xylan/100 g xylan in raw biomass.

Keywords: *lime pretreatment, enzymatic hydrolysis, lignocellulosic biomass, willow wood.*

1. Introduction

Lignocellulosic biomass is the most widely available source of carbohydrates, which can be converted in to biofuel; however, this feedstock is not readily digestible, to overcome this difficulty, lignocellulose structure must be modified through pretreatment [1].

There are three major components inlignocellulosic biomass which one, cellulose, hemicellulose and lignin. Cellulose and hemicellulose are not directly available for bioconversion due to their intimate association with lignin [2].

Enzymatic hydrolysis of lignocellulosic biomass has been shown to be limiting factor in the conversion of biomass to chemicals and fuels, this limitation is due to inherent structural features (i.e., acetyl content, lignin content, crystallinity, surface area, particle size and pore volume) of biomass, which represents the barriers that prevent complete hydrolysis, therefore to increase the enzymatic digestibility of lignocellulosic biomass, biomass must subject mechanically (reducing size $\leq$ 4 mm) and chemically (e.g., acid/ alkali treatment) pretreatments. The treated biomass will become more readily available to enzymatically hydrolysis of their sugars by cellulase and hemicellulase enzymes and the resulting sugars which can be fermented to ethanol or other biofuel by microorganisms via fermentation process [3].

Alkali pretreatment processes utilize lower temperatures and pressures compared to other pretreatment technologies. Alkali pretreatment may be carried out at ambient conditions, but pretreatment duration is measured in terms of hours or days rather than minutes or seconds [4].Compared with acid processes, alkaline processes have less sugar degradation and many of the caustic salts can be recovered and/or regenerated. Sodium, potassium, calcium, and ammonium hydroxide are suitable alkaline pretreatment agents. In lime pretreatment, calcium hydroxide $Ca(OH)_2$, water and an oxidizing agent (air or O_2) are mixed with the biomass at temperatures ranging from 40 to 160^0C for a period ranging from hours to weeks, two types of lime pretreatment which show high total sugar yields and they are currently used: short term and long term. Short-term lime pretreatment involves boiling the biomass with a different limeloading, temperatures and with or without oxygen at different pressure [5, 9].Long-term pretreatment involves using lower lime loading and temperatures (40-55^0C) for 4-6 weeks in the presence of air [6].

Hydrolysis of carbohydrates includes breaking the polymer of cellulose and himecelluloseto their monomers. Hydrolysis of cellulose gives glucose, whereas hydrolysis of hemicellulose results pentoses and hexoses. There are two basic methods to degrade the biomass to sugars: enzymatic hydrolysisand dilute acid hydrolysis, compared to dilute acid hydrolysis, enzymatic approachis promising because it can achieve high sugar yields and eliminate the need for largequantities of chemicals and the formation of inhibitory by-products during dilute acidhydrolysis. During the enzymatic hydrolysis we need the cellulose enzyme which is responsibleto catalyze cellulose degradation to glucose and the hemicellulose enzyme as additiveto improve the hydrolysis process, cellulaseenzyme is actually a complex mixture of severalenzymes including endoglucanase, exoglucanase and β-glucosidase [7]

The main purposes of this study can be summarized as follows: (1) Compositional analysis of willow wood by analyzing the biomass.(2) Study the effect the different conditions of short-term lime pretreatment on raw willow wood and also on the enzymatic hydrolysis process.(3) Optimize the pretreatment conditions (pretreatment time, oxygen pressure, temperature, lime loading, water loading, and particle size) to give the best yield of glucanand xylanfor both pretreatment and enzymatic hydrolysis, derivative empirical model for kinetic degradation of lignin and carbohydrate and optimize of enzyme loading and substrate concentration of enzymatic hydrolysis process.

2. Materials and Methods

Feedstock for this study was willow wood type Tordis ((Salix viminalis x Salix schwerinii) x Salix viminalis). From a private plantation. Approximately 5 bundles of tree stems of 2-3 inch diameter were harvested in January 2010 from private land in Saxony-Anhalt, Germany. A part of them was taken for debarking, drying and chipping and then chips were milled by wood mill to pass a 8.0 mm round screen. Then the milled feedstock was taken to reduce particle size pass a 2 mm round screen by using laboratory mill, the biomass was collected after comminution process, sieved to pass 20 mesh (0.850 mm) and 80 mesh (0.180 mm). Stack the sieves in the following order, starting at the bottom: the bottom pan, 80, 20-mesh sieve and lid of stack. The purpose is converting a variety of biomass samples into a uniform material suitable for compositional analysis in a reproducible way.Finally the samples are air-dried to a moisture content less than or equal to 10%.Neither the particles retained on the 20 mesh nor the ones passing the 80 mesh were used, only those less than 20 mesh more than 80 mesh ware used [8]. Because out this rang of particle size, there is a big deviation in carbohydrate and lignin contents. Once the samples were prepared, the particles of biomass was re-packaged into polyethylene bags (either completely filled or tightly wrapped to reduce evaporation into the headspace), and stored frozen at -20 ^{0}C. When needed, the biomass was directly used. These procedures are based on the NREL (National Renewable Energy Laboratory) standard procedure (Preparation of Samples for Compositional Analysis).

3. Experimental Setup and Operation

Short-term lime pretreatment process was performed in a system of a reactor constructed from 30-cm long, 38-mm inside diameter, 304 stainless steel pipe with a 285-mL volume. The reactor was supplied at both of ends by fittings made of stainless steel, see figure -1-.To get the temperature profile of reaction progress inside

reactor, we used a bimetal stem thermometer (Type K) was used and inserted inside the reactor through the pipe at the end of the reactor, thus the temperature can be recorded by USB Thermocouple Data Logger every 5 sec.

Fig. 1. Photograph of lime pretreatment reactor.

Oxygen used in the experiments was as compressed to the reactor at constant pressure (CP) in which oxygen was continuously provided during pretreatment at the desired pressure. CP was attained by using flexible tubing (1/8-inch stainless steel) connected to an oxygen tank. Raw biomass (11.4 g dry weight) and excess of calcium hydroxide (0.4 g/g dry biomass) were placed in a beaker size 250 ml. The mixture was thoroughly mixed with deionized water (15 g/g dry biomass),then the mixture was placed in the reactor. The mixing process during the reaction progress was carried out by using a system of motion which consisted of electrical motor and set of levers which can move the reactor toward left and right around 50 rpm. After tightly capping, the reactor was preheated to 75^0C by water bath, and then placed in the preheated oven. The pretreatment temperature was maintained by inserting the reactor in a temperature-controlled tube-oven at desired temperature, time ofexperiments begin when the temperature of the reactor reaches the desired temperature. Therefore we need some time between 30 to 40 minutes to achieve to desired temperature in most of the experiments. At the end of the pretreatment process, the reactor was cooled by pressurized air, depressurized slowly by the valve that is connected to the reactor, remove the reactor from the oven, cool it down in cold water and open it. The pretreated biomass was transferred to a convenient container using about 250 mL of deionized water. The slurry was then neutralized by titrating with 5.0-N HCl to measure unreacted lime. After that the solids were extensively washed with clear deionized water and filtered using a vacuum, filtration apparatus with What manglass fiber filter paper (particle retention = 2µm). Once filtered, the biomass to be analyzed for composition was air-dried at room temperature. The weight of the dry biomass and its moisture content were recorded to account for the pretreatment yield of solids. The biomass was stored at -20^0C until used for analysis or enzyme hydrolysis.

4. Compositional Analysis

Samples of raw and treated willow wood were prepared for compositional analysis. Then the biomass was extractedwith 95% ethanol for 24 h in a Soxhlet apparatus. The extracted samples were acid hydrolyzed in two stages to determine the carbohydrate and lignincontents, the lignin fractionates into acid-soluble material and acid-insoluble material, the acid soluble lignin is quantified by UV-visible spectroscopy and the insoluble lignin is determined by gravimetric analysis. The analysis ofcarbohydrates was performed by HPLC device by using Rezex RPM-Monosaccharide (RPM Pb^{+2}) column of HPLC device, with refractive index detection (Lab Index 2000L Refractive Index Detector). Ashcontent was determined by weighing the sample before andafter ashingat 575 ^{0}C± 25^0C (Fisher Scientific, programmablemuffle furnace).Table (1)shows the composition of the raw willow wood that is used in this study.

Table1,
composition of raw willow wood feedstock.

Components (g/ 100 g biomass)	Washed willow wood				Average	Std. Dev
Glucane	40.70	40.90	41.10	40.09	41.00	0.14
Xylane	17.70	16.60	15.60	16.70	16.65	0.13
Mannan	4..00	3.00	5.00	5.83	4.45	1.25
Galactan	2.00	1.90	2.00	2.00	2.00	0.05
Lignin	25.70	26.00	25.80	25.90	25.85	0.11
Extarctive	5.80	6.20	5.90	6.00	6.00	0.15
Ash	0.8	0.80	0.86	0.82	0.82	0.03
Acetyl	2.8	2.80	2.60	2.75	2.72	0.06
Total	98.6	98.50	99.66	99.00	99.50	

5. Results and Discussion

5.1.Lime Pretreatment

Oxidative short-term lime pretreatment is an effective method for improving lignocellulose digestibility, Partial removal of lignin allowed some of biomass of swelling, increased internal surface area, removes acetyl and the various uranic acid substitutions on hemicellulose and larger median pore volume, all of which enhanceenzyme accessibility to carbohydrate polymers. The experimental conditions for this study were selected as shown in Table (2).

Table 2,
pretreatment conditions.

Variables	conditions
Oxygen pressure	6-21 bar(absolute)
Temperature	100- 160 C^0
Time	1-6 h
Lime loading	0.4 $Ca(OH)_2$ / g dry biomass
Water loading	15 g H_2O / g dry biomass

Previous studies [9] showed that pretreatment time, temperature and oxygen pressure have greaterimpact than water loaded and lime loading on delignification; therefore , the pretreatment condition were conducted to hold the low impact variables constant while systematically change the high impact variables. After determined the high impact, the low impact then investigated, and the experiments started with excess lime loading of 0.4 g ($Ca(OH)_2$/g dry biomass) because normally the lime consumption is between 0.1-0.3g ($Ca(OH)_2$/g dry biomass) [1]. After completing of the pretreatment, back filtration with HCl to determine the excess lime and to calculate theoretical lime consumption.

To determine the desired loading of water, water loading must be provided in a sufficient quantity to make this mixture (water, wood particle and lime) slurry, we found the minimum water loading which make the mixture homogeneous was (15 g H_2O/g dry biomass) . Therefore, we supposed it as optimum loading.

To determine the high impact variables (temperature, time, oxygen pressure)a statistical design was used, and then the corresponding data was be collected and analyzed by appropriate statistical methods to achieve valid and objective conclusions. In the present work, the response surface methodology type (Central Composite Design) has been used to plan the experiments and subsequent analysis of the collected data. If the response is well-modeled by a linear function of the independent variables, then the approximating function is the first order model, but if there is a curvature in the system, then the polynomial is of higher degree such as the second-order expression.

As mentioned, the aims of this work involve the study of effect of temperature, oxygen pressure and pretreatment time on the (pretreatment yield and their content on the treated biomass).

On the other hand, the study also aims to determine which lime pretreatment conditions cause the greatest increase in pretreated willow wood digestibility which gives highest pretreatment yield (total sold mass recovery).

Pretreatment yields specify the quantity of the component (i.e., lignin, glucan or xylan) in the raw biomass was found in the pretreated biomass. Thus they assess pretreatment. Pretreatment yield (Yp) which also known recovery yield of total mass or yield of total solid. Table (3) shows the results of recovery yield of total mass (Yp) and total mass remaining. Generally, the tendency of more solubility is for high temperatures, long pretreatment times and high pressure. Also the rate of solubility increases along time at all temperatures and especially when increasing the oxygen pressure.

Table 3,
Pretreatment yield of total mass with pretreatment conditions.

Pretreatment conditions				Pretreatment yield	
Ex.No	**Temperature, C^0**	**Pressure, bar**	**Time, h**	**Recovery total mass. g**	**Y_P**
1	147	17.8	5.0	6.090	0.535
2	113	17.8	5.0	7.700	0.675
3	147	9.2	5.0	6.450	0.565
4	147	17.8	2.0	6.810	0.597
5	113	9.2	5.0	8.160	0.715
6	113	17.8	2.0	8.550	0.750
7	147	9.2	2.0	7.420	0.650
8	113	9.2	2.0	8.900	0.780
9	160	13.5	3.5	5.740	0.504
10	130	21.0	3.5	7.110	0.623
11	130	13.5	6.0	6.750	0.592
12	100	13.5	3.5	9.210	0.807
13	130	6.0	3.5	7.750	0.679
14	130	13.5	1.0	8.350	0.732
15	130	13.5	3.5	7.540	0.661
16	130	13.5	3.5	7.560	0.663
17	130	13.5	3.5	7.530	0.660
18	130	13.5	3.5	7.550	0.662

The pretreatment yields of interest include: glucan pretreatment yields, Y_G (i.e., glucan remaining in the solids after pretreatment), xylan pretreatment yields, Y_X (i.e., xylan remaining in the solids after pretreatment), and lignin pretreatment yields, Y_L (i.e., lignin remaining in the solids after pretreatment).Table(4) shows pretreatment yield of glucan and xylan. For all conditions, glucan yields were above 90 g glucan recovered/100 g glucan in raw biomass. In case of xylan, the degradation was much faster than glucan. In general, less carbohydrate was recovered when the solids underwent aggressive pretreatments (higher temperature, higher oxygen pressure and longer time).

Table 4,
Pretreatment yield of glucan and xylan with pretreatment conditions.

Pretreatment conditions				Pretreatment yield			
Ex. No	**Temperature, C^0**	**Pressure, bar**	**Time, h**	**Glucane recovered. g**	**Glucane yield**	**Xylan recovered. g**	**Xylan yield**
1	147	17.8	5.0	4.324	0.927	0.800	0.422
2	113	17.8	5.0	4.395	0.942	1.051	0.555
3	147	9.2	5.0	4.386	0.941	0.825	0.436
4	147	17.8	2.0	4.392	0.942	0.951	0.502
5	113	9.2	5.0	4.433	0.950	1.057	0.558
6	113	17.8	2.0	4.543	0.974	1.154	0.610
7	147	9.2	2.0	4.526	0.970	1.007	0.532
8	113	9.2	2.0	4.628	0.992	1.291	0.682
9	160	13.5	3.5	4.218	0.904	0.796	0.420
10	130	21.0	3.5	4.331	0.929	0.959	0.314
11	130	13.5	6.0	4.325	0.927	0.923	0.487
12	100	13.5	3.5	4.605	0.987	1.473	0.778
13	130	6.0	3.5	4.382	0.987	1.123	0.593
14	130	13.5	1.0	4.580	0.982	1.169	0.617
15	130	13.5	3.5	4.411	0.946	1.025	0.541
16	130	13.5	3.5	4.417	0.947	1.008	0.532
17	130	13.5	3.5	4.405	0.945	1.024	0.541
18	130	13.5	3.5	4.416	0.947	1.025	0.541

By using statistical program (STATISTICA-program software),relationships were obtained between the expected values of pretreatment yields of different components with pretreatment conditions as follows:

$$Y_L = +1.72070 - 6.884E-3 \times X_1 - 0.011937 \times X_2 + 8.54E-4 \times X_3 - 4.433E-5 \times X_1X_2 - 1.224E-5 \times X_1X_3 - 5.212E-6 \times X_2X_3 + 3.862E-7 \times X_1^2 + 3.795E-4 \times X_2^2 + 2.387E-7 \times X_3^2 \text{(R=98)} \quad \ldots(1)$$

$$Y_G = +1.23308 - 2.326E-3 \times X_1 + 5.563E-3 \times X_2 + 8.232E-4 \times X_3 - 3.266E-5 \times X_1X_2 + 2.2639E-6 \times X_1X_3 + 1.269E-6 \times X_2X_3 + 5.192E-6 \times X_1^2 - 1.213E-4 \times X_2^2 + 6.708E-7 \times X_3^2 \text{(R=87)} \quad \ldots(2)$$

$$Y_X = +2.461 - 0.023 \times X_1 + 0.0177X_2 - 1.047E-3 \times X_3 + 5.617E-5 \times X_1X_2 + 5.847E-8 \times X_1X_3 + 2.723E-5 \times X_2X_3 + 6.841E-5 \times X_1^2 - 1.508E-3 \times X_2^2 + 4.809E-7 \times X_3^2 \text{(R=89)} \quad \ldots(3)$$

Where: X_1:temperature , X_2:pressure, X_3:time .

These equations include all the terms regardless of their significance which was more 0.95. It can be seen that the model is in a good fit with high value of regression coefficient (R) ,and all of the statistical models of different components are statistically significant because they have P-values lower than 0.05. These equations are valid for temperatures between 112 and148C^0, pressure values ranging from 9 to 18 bar (absolute) and the pretreatment time between 2 and 5 hours (according with this type of central composite design).

5.2. Lignin Degradation

Delignification depends highly on temperature and the presence of oxygen. Thus higher temperature, oxygen pressure and longer pretreatment time result in much higher lignin degradation. Figures (2- A, 2-B, 2-C) represent the curves of lignin yields that were derived from statistical program, which based on the above equation, corresponding to varying pressure of oxygen and pretreatment while temperature was held at 113, 130 and 147^{0}C. The color gradient of these figures, which begins with the orange color and ends with dark blue, orange color indicate the highest undesirable lignin yield while dark blue represents the lowest desirable lignin yield. In these figures we observe the effect of the oxygen pressure and pretreatment time on the lignin yield at constant temperature. It can also be observed that increasing temperature leads to shifts of lignin yield toward more removal. The highest recorded delignification was obtained for the pretreatment at 147 ^{0}C, 17.8 bars and 5 h. The yield was 19.3 g of lignin remaining/100 g lignin in the raw biomass. Whereas a very poor delignification response was achieved for the pretreatment at 100.0 ^{0}C, 13.5 bar and 3.5 h, the lignin yield was 78 g of lignin remaining/100 g lignin in the raw biomass.

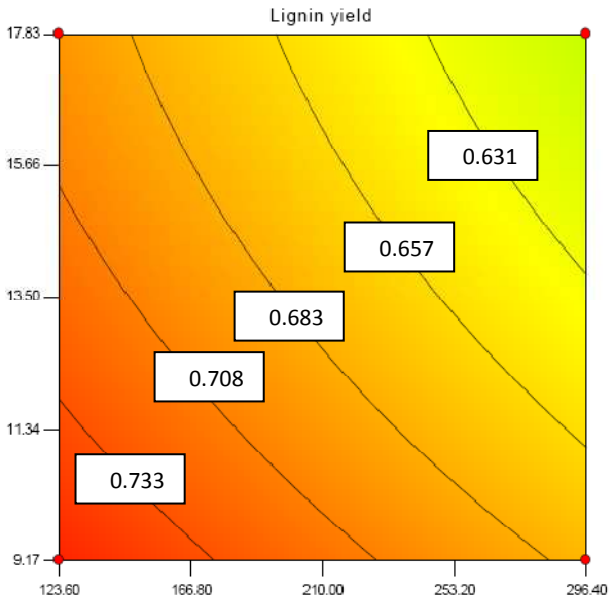

Fig. 2-A. X: time (min), Y: Pressure (bar), temperature 113 ^{0}C

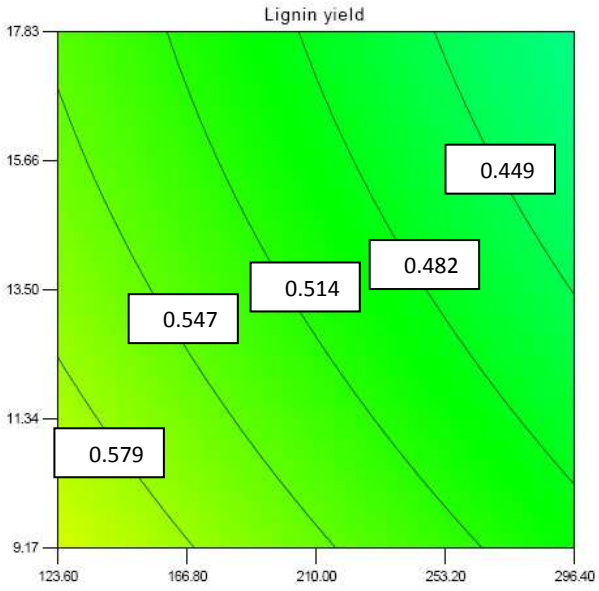

Fig. 2-B. X: time (min), Y: Pressure (bar), temperature 130 ^{0}C

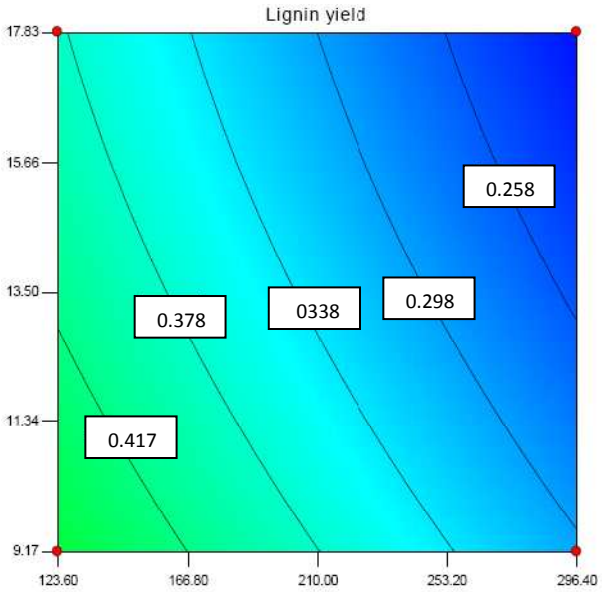

Fig. 2-C.X: time (min), Y: Pressure (bar), temperature 147 ^{0}C

5.3. Glucan Degradation

Glucan pretreatment yields were above 90 g glucan recovered/100 g glucan in raw biomass, for all operation conditions as shown in the figures (3-A,3-B,3-C),these figures show the relationship between the oxygen pressure and time of pretreatment on the glucan yield by holding the temperature at 113, 130 and147^{0}C. The color gradient, unlike lignin degradation, these figures which begins with orange color indicates the highest desirableglucan yield , and end with dark blue represents the lowest undesirable glucan yield, furthermore degradation within progress of pretreatment at increasing temperature and/or pressure. The highest observed glucan degradation was recorded for the pretreatment at160^{0}C and 13.5 bar 3.5 h (90.47 g glucan degraded/g glucan in raw biomass) while the lowest glucan degradation was at 113^{0}C, 9.2 bar and 2 h, and its yield was 99.3 g of glucan remaining/100 g glucan in the raw biomass. Generally, glucan degradation was very small in comparison with lignin and hemicellulose, because as soon as the pretreatment started, some rapid peeling degradation of glucan occurs. Fast stopping reactions follow and degradation of remaining glucan depends on the generation of newly accessible reducing-end groups [1].

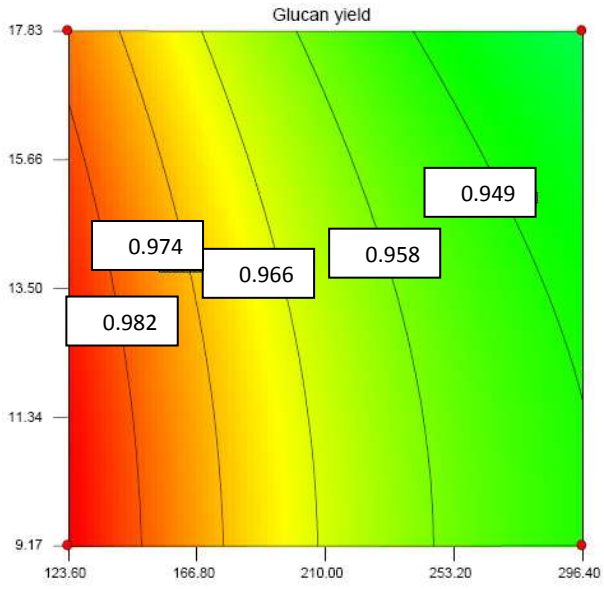

Fig.3- A, X: time (min), Y: Pressure (bar), temperature 113 ^{0}C

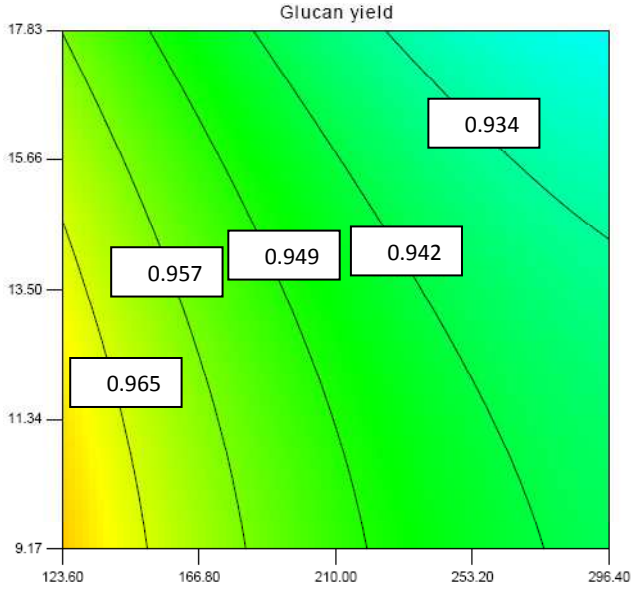

Fig.3- B, X: time (min), Y: Pressure (bar), temperature 130 ^{0}C

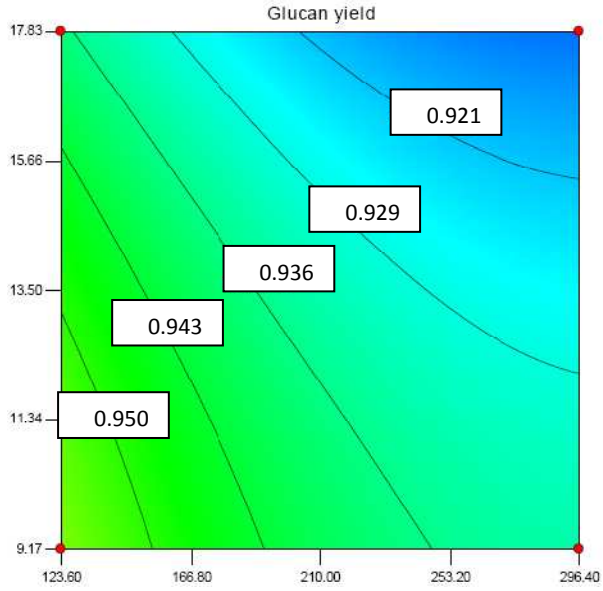

Fig. 3-C.X: time (min), Y: Pressure (bar), temperature 147 ^{0}C

5.4. Xylan Degradation

In a case of xylan, the degradation was much faster than glucan. At 113^{0}C, 31.5 bar and 3.5 h the yield was above 75 g xylan recovered/100 gxylan in the raw biomass, whereas the highest xylan degradations were observed for the pretreatment at 160^{0}C, 13.5 bar and 3.5 h, and 147^{0}C, 17.8 bar and5 h, the yield was 42 g of lignin remaining/100 g lignin in the raw biomass.Hemicellulose degradation is important, because the contribution of this carbohydratepolymer to the total carbohydrate yield is potentially significant. Theaverage yield of xylan degradation for all our conditions was approximately close to the average yield of lignin degradation (0.536 and 0.511, respectively). Similar tolignin and cellulose degradation, xylan degradation was triggered by higher temperaturesand oxygen pressures. Furthermore, xylan degradation was much more significantthan glucan degradation and behaves very similar to lignin degradation because of covalent bonds between hemicellulose and lignin in the cell wall [10].

Generally, Lignin content and crystallinity have major effects on biomass enzymatic digestibility, whereas acetyl content has a minor impact [7, 11]. Therefore, pretreatments that can significantly remove lignin or reduce crystallinity were particularly effective by taking into consideration the balance between the lignin removal and carbohydrate degradation.

6. Enzymatic Hydrolysis

6.1. Materials and Methods

At the end of oxidative short-term lime process, the pretreated biomass was neutralized, wash, and stored wet in the freezer at-20^{0}C. The substrates used in enzymatic hydrolysis are raw biomass (untreated) and pretreated-neutralized-washed willow wood. The materials used in enzymatic hydrolysis were: (Cellic CTec2 Cellulase) which was providedby Novozyme, Deionized water, sodium citrate buffer 0.05-M (PH 5) and sodium azide 10.0 g/L to prevent microbial contamination, the final volume was 10 mL and the incubation period of enzymatic hydrolysis was 72 h.

Enzymatic hydrolysis was performed in 20 mL Erlenmeyer flasks or scintillation vials (20 mL) in a static incubator (oven) set at 50 ^{0}C ±1 which contains multiple place magnetic stirrers inside. The temperature was set at 50 ^{0}C and the speed of magnetic stirrers at 200 rpm. The mixing process must be sufficient to keep solids in constant suspension for a period of 72 h, where most of glucan or xylan is hydrolyzed to their sugars from the solid pretreated biomass. The samples that subjected to enzymatic hydrolysis were withdrawn after 3 days and then boiled for 15 minute to denature the enzymes to prevent further hydrolysis. After that the samples were centrifuged at 4000 rpm for 20 minutes. Then the samples were diluted by using deionized water to get sugar concentration in the range of carbohydrate standard concentration. The required quantity of cellulase enzyme was calculated based on the amount of glucan in the raw biomass and the desired enzyme loading. The suggested enzyme trial dosage levels for initial investigation of a substrate were 30.0%w/w (g enzyme/g cellulose) and the amount of biomass to be weighed was calculated based on the moisture content and the glucan content to provide 0.1 g glucan for the reaction. The samples were filtered through a 0.2µm filter and subjected to glucose analysis by using HPLC analysis by using Phenomenex Rezex(RPM pd^{+2}) column, and RI detector. The measured sugars of hydrolysis samples were used to indicate biomass digestibility. All enzymatic hydrolysis experiments were conducted duplicate an dall steps of enzymatic hydrolysis process were based on(NREL).

6.2. Results and Dissection

It has been using the same statistical method which previously used in the pretreatment to find the relationship between overall total sugar yield(Y^T_S) and pretreatment conditions, in order to find the best overall yield of totalsugar (Y^T_S). Table (4) shows the results of different types of yields.

Table 4,
Different yields of overall process (Pretreatment and enzymatic hydrolysis).

Ex.No	Y_G%	Y_g%	Avar.Y_g	Y^T_G%	Glucan. g	Y_X%	Y_x%	Y^T_X%	Xylan. g	Y^T_S%
1		0.897								
1	92.00	0.910	0.903	83.076	3.873	43.317	0.840	36.386	0.688	0.400
2		0.942								
2	93.27	0.955	0.950	88.606	4.131	55.467	0.955	52.971	1.002	0.45
3		0.955								
3	94.00	0937	0.940	89.770	4.185	43.613	0.976	42.566	0.805	0.437
4		0.999								
4	94.34	0.988	0.994	93.700	4.371	50.185	0.992	49.784	0.943	0.466
5		0.815								
5	99.40	0.825	0.820	81.200	4.800	68.200	0.909	62.000	1.173	0.436
6		0.905								
6	97.20	0.922	0.914	88.841	4.141	60.961	0.920	56.084	1.061	0.456
7		0.955								
7	97.20	0.915	0.950	92,340	4.305	53.200	0.950	50.536	0.956	0.461
8		0.970								
8	95.12	0.973	0.972	92.456	4.310	55.867	0.990	55.308	1.047	0.470
9		0.965								
9	90.47	0.977	0.971	87.435	4.076	40.623	0.990	40.217	0.761	0.424
10		0.990								
10	93.00	0.991	0.990	92.070	4.292	49.181	0.990	48.690	0.868	0.452
11		0.970								
11	92.77	0.975	0.972	90.172	4.204	48.779	0.945	46.096	0.872	0.445
12		0.784								
12	98.80	0.774	0.979	77.000	4.590	78.610	0.909	71.456	1.353	0.434
13		0.930								
13	94.00	0.920	0.925	86.025	4.010	59.323	0.920	48.577	1.033	0.442
14		0.904								
14	98.87	0.931	0.918	87.714	4.090	61.800	0.956	59.087	1.118	0.457
15		0.950								
15	94.65	0.950	0.950	87.714	4.205	52.720	0.953	50.242	0.951	0.448
16		0.939								
16	94.75	0.937	0.938	89.065	4.152	53.249	0.942	50.160	0.950	0.445
17		0.949								
17	94.50	0.953	0.951	90.000	4.196	54.094	0.950	51.390	0.973	0.452
18		0.950								
18	94.70		0.950	90.000	4.196	54.147	0.940	50.898	0.963	0.450

where:
Y_G=Pretreatment yield of glucan (g glucan recovered/100 g glucan in raw biomass)
Y_g=Hydrolysis yield ogglucan (g glucan hydrolyzed/ g glucan in treated biomass)
Y^T_G=Overall yield glucan (g glucan hydrolyzed/100 g glucan in raw biomass)
Y_X=Pretreatment yield of xylose(g xylan hydrolyzed/100 g xylan in raw biomass)
Y_x=Hydrolysis yield of xylan(g xylan hydrolyzed/ g xylane in treated biomass)
Y^T_X =Overall yield of xylane (g xylan hydrolyzed/100 g xylan in raw biomass)
Y^T_S =Overall yield of total sugar (glucan+ xylan)/ g raw biomass)

Glucan (g) and xylan (g) representquantity of glucan and xylan are hydrolyzed after pretreatment and enzymatic hydrolysisfor total raw biomass (11.40 g).

Generally, for all samples that underwent to pretreatment conditions (pretreatment time, compressed oxygen, and temperature) have strongly affected through biomass enzymaticdigestibility. During enzymatic hydrolysis, glucan and xylan converted toglucose and xylose and trace of mannose. Since the enzymatic hydrolysis operation have took place inthe separate stage, thus, the recommended pretreatment conditions were chosen based on the glucan and xylan overall yield (i.e., yields after

the combined operations of pretreatment and enzymatic hydrolysis).The following equation represents the relationship (polynomial equation) between overall yield and pretreatment condition, that was driven from statistical model.

$$Y_S^T = +0.06431 + 2.51342E - 003 \times X_1 + 0.012445 \times X_2 + 0.093164 \times X_3 - 5.33365E - 5 \times X_1X_2 - 5.914E - 4 \times X_1X_3 - 1.6439E - 3 \times X_2X_3. \text{ (R=0.83, p-value} \leq 0.001) \quad \ldots(4)$$

Equation (4) includes all the terms regardless of their significance which was more than 0.95.The highest value recorded of practical experiments of overall yield of glucan was 93.700 g glucan hydrolyzed/100 g glucan in raw biomass (equivalent to the hydrolysis yield of 99.00 g glucan hydrolyzed/100 g glucan in treated biomass) also it had the overall total sugar yield 0.466 under pretreatment condition: 148^{0}C, 17,8 bar and 2.06 h. The experimentally calculated value of overall total sugar yield was almost close to predicted optimum value (0.468). This point represents the optimum point of overall process.

Through the study of the relationship between the enzymatic hydrolysis and the residuallignin content, it was noticed, that the enzymatic hydrolysis at 147.8^{0}C ,17.8 bar and 5 h was 90.30%, nevertheless the lignin content of the sample was lower than the rest samples but it has lower enzymatic hydrolysis. To realizethis phenomenon, cellulase enzyme that was supported from novozyme Cop. is a rather friendly with lignin at certain level of lignin. This type of enzyme contain GH61proteins were able to enhance enzymatic hydrolysis of glucane which hasa certain level of lignin, unlike of pure glucane , none of the GH61proteins were able to enhance the hydrolysis of pure glucan like filter paper. This lack of enhancement was also shown with other pure cellulose substrates such asavicel, phosphoric-acid swollen cellulose and carboxymethyl cellulose. Proteins GH61are capable to enhance hydrolysis of acid pretreated corn Stover and as it is well known that the samples which is conducted with acid pretreatment have large amount of lignin [12]. This mean that lignin content at 147.8 ^{0}C, 17.8bar and 5 his lower than lignin content which enhance cellulase activity.

Also we noticed the highest residual concentration of lignin that gives full hydrolyzed for glucan was approximately 0.15 g lignin remaining /g of pretreated biomass or lignin selectivity is equal 0.15 residual lignin /g of pretreated biomass, which is at 147.8^{0}C , 17,8 bar and 2.06h. This point is very important because of going on more than this point will increasecarbohydrate degradation without any benefit, and less than this point of lignindegradation will not get the full hydrolysis.

As shown in Figure (4), it is clear that the optimum point of total sugar yield (bold point) will be terminal at temperature 147.8^{0}C, oxygen pressure 17.8 bar and time 2.06 h, or in other mean this point lie on the circumference of circle. To complete whole region of my interested, the study examined the area around the previous point within temperature range 147.8-160^{0}C, oxygen pressure range 21-17.8 bar (absolute) and pretreatment time range 1-2 h. Extra experiments have been done in this region for points that have the potential to be a more optimal point with considering to reduced pretreatment time less than two hours and at the same time increasing the oxygen pressure and temperature.

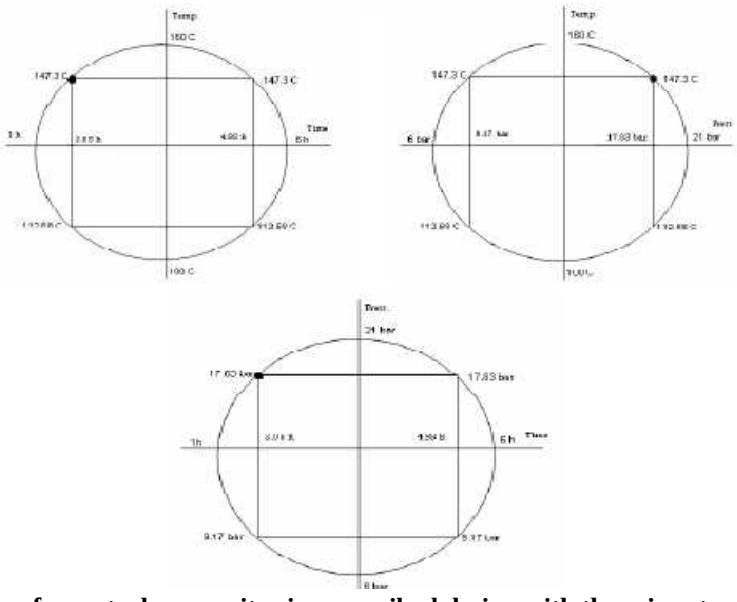

Fig. 4. Points of a central composite circumscribed design with three input parameters.

Table (6) shows that the best overall total sugar yield (Y^T_S) was (0.486) at 148^{0}C, 17,8 and time 1.33 h (80 minutes) and it was the optimum condition. Also it can be noticed that most of the values of overall total sugar yields in this table were approximately close.

Table 6,
The yields for extra experiments.

Ex.No	Pretreatment conditions			Yields				
	Temperature, C^0	Pressure, bar	Time, h	Y_G%	Y_g%	Y_X%	Y_x%	Y^T_S%
19	148	17.8	1.33	96.00	0.98	63.18	0.99	0.48
20	150	21.0	1	95.40	0.95	58.00	0.95	0.46
21	155	21.0	1.25	94.70	0.99	52.40	0.99	0.47
22	160	21.0	1	94.70	0.98	54.20	0.96	0.46

The effect of substrate concentration on the enzymatic hydrolysis process was studied in this research, to find the best high substrate concentration, the experiments were carried out at different concentrations (10, 20, 50 g/L) with the same concentration ofenzyme loading (30.0%(g enzyme/g cellulose). Glucose, xylose and mannose were measured after end of hydrolysis by HPLC device. The highest concentration of substrate that can be obtained with the same digestion efficiency of 10 g/L was 50 g/L. this result was compatible with other research [7]. Also the concentration of substrate more 50 g/L was noticed will not be suitable for enzymatic hydrolysis because the mixture will be heterogeneous.

In the previous experiments in this research, excess enzyme loading was used for eliminate any interference related to amount of enzyme and then to determine the lowest enzyme loading that gives same digestion efficiency of 30.0%w/w (g enzyme/g cellulose) at 50 g/L substrate concentration for points at (148^{0}C, 17,8 and time 1.33 h) and (155^{0}C, 21bar and time 1.25 h). Three type of enzyme loading (15.0%, 10.0%, 5.0% w/w (g enzyme/g cellulose) were tested. The lowest enzyme loading gives high digestion efficiency at (10.0%w/w (g enzyme/g cellulose) for both points.

At the final stage of this research, the low-impact variables such as lime loading and particle size were studied in order to reduce both the consumption of lime and energy comminution of biomass. The best lime loading was 0.26 g lime/g raw dry biomass and the best particle size was between 2-3 mm.

7. Conclusion

For high-lignin biomass, such as willow wood and poplar wood, oxidative short- termlime pretreatment was very effective for digestibility by enzymatic hydrolysis. Generally, oxidative short-term lime pretreatment showed less sugar degradation with more lignin removal. Glucan was preserved much more than xylan because xylan degradation was more related to lignin removal and deacetylation. The most important pretreatment glucanand xylan yield which gives very high digestibility at 147 ^{0}C, 17.8 bar and 1.33 h (80minute), and 155 ^{0}C, 21 bar, 1.25 h (75 minute) also they have lignin content lies between 15-16.5%.

A lower pretreatment yield of glucan and xylan were observed at 147.8 ^{0}C, 17.8 barand 5h (0.927 and 0.422, respectively) and 160 ^{0}C, 13.5 bar, 3.5 h (0.904 and 0.420, respectively). this mean, the pretreatment temperature is more dominant than pressure and pretreatment time. At temperature 100 ^{0}C, 13.5 bar and 3.5 h, the enzymatic hydrolysis of glucan was low (78%), because of poor delignification (22% lignin content).

A lonely criteria used in enzymatic hydrolysis is that the process is performing on 3-days to avoid the effect of glucan crystallinity. Accordingly, the lignin content was considered as the crucial factor.

This study found, that there are two of optimum points are: (1) 147.8 ^{0}C, 1.33 h, 17.8bar (absolute), 0.26 g Ca(OH)2 and 15 g water/g raw biomass (2) 155 ^{0}C, 1.25 h, 21.0bar (absolute), 0.26 g Ca(OH)2 and 15 g water/g raw biomass. The overall glucan and xylanyields(i.e., glucose and xylose recovered after both pretreatment and enzymatic hydrolysis, and expressed as equivalent glucan and xylan) that obtained in the first are:0.96 g glucan hydrolyzed/100 gglucan in raw biomass, and 0.63 g xylan hydrolyzed/100 gxylan in raw biomass, in the second point are:0.94 g glucan hydrolyzed/g glucan in raw biomass and 0.52 g xylan hydrolyzed/100 g xylan in raw biomass, respectively.

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تحويل المواد اللكنوسليلوزية الى سكريات قابلة للتخمر

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الخلاصة

ان عملية التحلل الانزيمي للمواد اللكنوسليلوزية هي عملية صعبة بسبب البنية الهيكلية للمواد اللكنوسليلوزية والتي تمنع من نفوذ الانزيمات الى داخل الكتلة الخشبية. في بحثنا هذا تم اختيار محلول هيدروكسيد الكالسيوم $Ca(OH)_2$ والاوكسجين المضغوط بوصفه عامل مؤكسدة في عملية المعالجة الاولية وخلال مدة قصيرة من الزمن ، وتم اختيار خشب الصفصاف انموذجا للمواد اللكنوسليلوزية او الكتلة حيوية . حيث تم مزج خشب الصفصاف (١١.٤ غم خشب خام) مع فائض من هيدروكسيد الكالسيوم (٠.٤ غم $Ca(OH)_2$/ غم كتلة الحيوية الخام) والماء الخالي من الاملاح (١٥ غم ماء / غم الكتلة الحيوية الخام). كانت مدة المعالجة الاولية متغيرة و لمدد مختلفة من الزمن (١، ٢، ٣.٥، ٥، ٦) ساعة ، ولدرجات حرارة مختلفة ايضا"والتي كانت بحدود (١٠٠، ١١٣، ١٣٠، ١٤٧، ١٦٠٠م˚) ، والأوكسجين المضغوط الذي استخدم عاملا مؤكسدا بضغط (٦، ٩، ١٣.٥،١٧.٨، ٢١) بار (مطلق) . كانت الظروف المثلى لكل من المعالجة الاولية والتحلل الإنزيمي تعتمد على أقصى قدر من العائدات الإجمالية للكلوكان (Glucan) والزيلان (Xylan) بعد عمليتي المعالجة الاولية والتحلل الانزيمي ، حيث كانت الظروف المثلى للمعالجة الاولية على النحو التالي: (١) ١.٣٣ ساعة ، ١٤٧٠م˚، 17.8 بار (المطلق)، ٠.٢٦ (هيدروكسيد الكالسيوم /غم كتلة حيوية خام). (٢) ١.٢٥ ساعة، ١٥٥ م˚، 21 بار(المطلق)، ٠.٢٦ (هيدروكسيد الكالسيوم /غم كتلة حيوية خام)، كما أن الظروف المثلى للعوامل قليلة التأثير مثل كمية الماء المستخدمة كانت ١٥ (غم ماء / غم كتلة حيوية خام) والحجم الحبيبي للخشب كان ما بين ٢- ٣ مم . وكانت الظروف المثلى لعملية التحلل الإنزيمي على وفق النحو الاتي: كمية انزيم السليليز (٠.١ غم/ غم كلوكان في الكتلة الحيوية الخام)، تركيز الكتلة الحيوية ٥٠ غم/ لتر وخلال ٧٢ ساعة من التحلل الإنزيمي، بعدها كان العائدالاجماليلعملية التحلل الانزيمي للكتلة الحيوية المعالجة على النحو التالي: ٩٦.٠٠ غم جلوكان / ١٠٠ غم من جلوكان في الكتلة الحيوية الخام، و٦٣.٠٠ غم زيلان / ١٠٠ غم زيلان في الكتلة الحيوية الخام.
