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DETERMINING THE ENZYME ACCESSIBILITY OF AMMONIA PRETREATED LIGNOCELLULOSIC SUBSTRATES BY SIMON'S STAIN METHOD

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ABSTRACT

The promising technology to assess the susceptibility of ammonia-treated of lignocellulosic biomass potential to enzymatic hydrolysis in bioconversion process to biofuels is Simon's Stain measurement. Fundamentally, the porosity and the overall surface area of lignocellulosic substrate will successfully increase by the pretreatment process; as the major structural feature influencing the hydrolysis of pretreated substrates by cellulases. The modified of Simon's Stain method was used in this study by decreasing the processing time from >50 to 6 hours, is the semi quantitative method for estimating the available surface area of ammonia-treated OPEFB and SSB. The materials, oil palm empty fruit bunches (OPEFB) and sweet sorghum bagasse (SSB), were soaked into aqueous ammonia for 1, 2 and 7 days in room condition. The data shown, the maximum dye adsorbed on the ammonia-treated materials was increasing as raise the pretreated-days as well. The total dye adsorption correlated well with the enzymatic hydrolysis yields resulting in good correlation coefficient (R^2). This method proved to be an effective tool for assessing the potential of cellulase to hydrolyze the lignocellulosic substrate.

Keywords: simon's stain method, OPEFB, SSB, soaking with aqueous ammonia, cellulase, adsorption.

INTRODUCTION

The bioconversion of lignocellulosic biomass to ethanol is a significant interested from a couple years ago. The research has arisen regarding to alter the matrix of lignocellulosic biomass become easily to completely hydrolyze by cellulase enzyme. Fundamentally, there are several factors considerable influence the affectivity of cellulase enzyme degradation: a) cellulose crystallinity, b) lignin content and distribution, c) the degree of polymerization of cellulose, d) pores size and distribution, e) the available surface area of cellulose, and f) the degree of swelling of fiber (*1-2*). The efficiency of enzymatic hydrolyze will increase when the enzyme pores size of substrate cellulose are large enough to accommodate enzyme component in both large and small size. Then, the large size enzyme components will reject by fiber and the synergistic action of enzyme will reduced when the pores size is too small. The effective pore size of fiber is about 40 – 50 Å or 4 – 5 nm(*3*). Therefore, need a pretreatment process prior to enzymatic hydrolysis in order to open the pores size of fibers.

Many researches proved pretreatment with ammonia were the effective methods to enhance the characteristic of lignocellulosic(*4-10*). The pores size of fiber will increase as an affect of ammonia action to the fiber by removed lignin content and decrease the degree of crystalinity of cellulose. Different with strong base pretreatment (such as NaOH), ammonia treatment in ambient temperature is a partial removal lignin. The lignin removal possibility is not as good as strong base treatment, which is totally removing lignin in lignocellulosic biomass. However, OPEFB was the most abundant waste in Indonesia and SSB is a potential sugar-juice for the future, which is limited information about the effectiveness of ammonia-treated into these materials. One of the aims of this research is finding the affect of ammonia-treated from OPEFB and SSB in order to alter these materials to ethanol-bioconversion process.

To date, there are several methods has been published to determine the accessibility of cellulose. Nitrogen absorption and solute exclusion (SE) are the most quantitative methods for measuring the lignocellulosic biomass porosity. Even though both of these methods are relative accurate to measure the "internal" porosity, however, it require a lengthy procedure and investment in time(*1*). The other effective and rapid methods to count the substrate swelling potential after pretreatment step is water retention value (WRV) and Simon's stain (SS).

A semi-quantitative Simon's stain method used two-color differential dye for the microscopic investigation mechanical damage of beaten fibers (*11-12*).These dyes consist of a low- and high-molecular weight polymeric mixture of direct blue (DB) and direct orange (DO) dye with a different affinity to cellulose. The DB dye ($C_{34}H_{28}N_6O_{16}S_4$) has a low affinity to cellulose and a molecular weight of 992.82 with a pore size of 1 nm. Meanwhile, the DO orange contains low and high molecular weight (LMW and HMW) molecules with the weight ratio was about 20:80 (HMW and LMW, respectively). The DO dye has a high affinity toward to cellulose and a pore size of 5 – 35 nm (see Figure-1). Although the LMW is 80% in the total of orange dye, it makes no contribution to the stain (*13*).

However, HMW orange dye has much less ability to penetrate the cellulose fibers; it is the only fraction gives the differential stain result and the benchmark for the accessibility of the interior surface of the cellulose

fibers to the dye. The HMW orange dye will preferentially penetrate to the cellulose fibers when the pore size of the fibers is large enough for the stain. The action of Simon's stain occurred because of the HMW dye has stronger affinity then to blue dye(*13*). The pretreatment process will increase the pore sizes of cellulose fibers, so that the adsorption of the HMW dye will increase as well.

The Simon's Stain original technique is involving two days incubation time and additional 12-18 hours dye-stripping procedure with subsequent removal of excess dye using pyridine and careful filtration (*1, 13*). Chandra *et al.* used a modify SS method to quantify the total surface area of cellulose, which used a rapid time procedure by eliminate some of procedure and chemical, such as pyridine (*14*). The goal of the current study was to predict the relative ease of hydrolysis of ammonia pretreated sample prepared from OPEFB and SSB. The other purpose is to make a correlate the SS dye accessibility to the enzymatic susceptibility of both of materials.

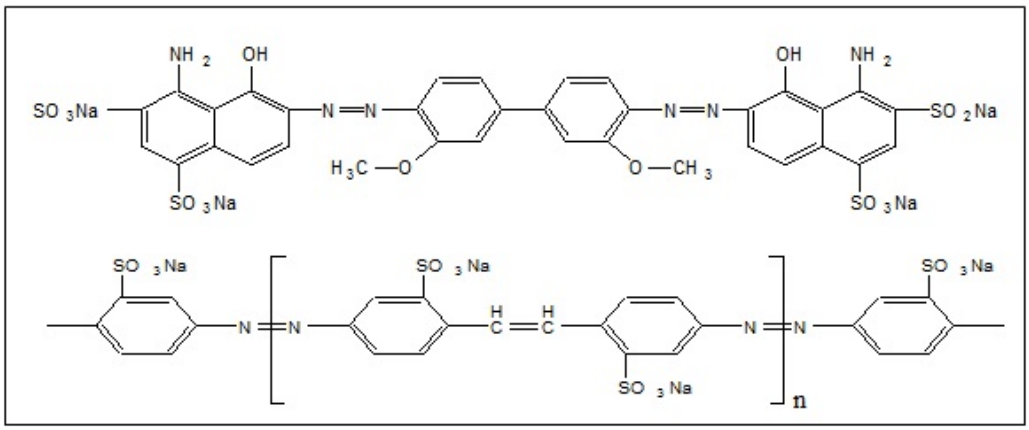

Figure-1. The chemical structure of direct blue dye (up) and direct orange dye (down).

MATERIALS AND METHODS

Lignocellulosic materials: Oil palm empty fruit bunch (OPEFB) and sweet sorghum bagasse (SSB) were used in this study. The OPEFB was obtained from a palm oil mill in West Sumatera, Indonesia while SSB was obtained from a local research institute. The feedstock were cut into a size of less than 1 cm in length and stored in plastic bags at room temperature until further processing.

Pretreatment: About 20 grams of dried lignocellulosic material was soaked into 5% ammonia solution and incubated at room temperature (23±2 ^{o}C) for 1, 2 and 7 days. The solid material was then separated using a filter and washed with water to remove residual solvent until a neutral pH was achieved. Pretreated solid was dried at room temperature prior to analysis.

Enzymatic susceptibility test: A 1.5 g DM (dry-matter content) of the treated or untreated samples were mixed with enzyme mixture and 0.05M citrate buffer (pH 5.2) in 100 mL Erlenmeyer flasks until 10% final concentration of dry-matter solid. Enzyme mixture, equivalent to 4.5 FPU cellulase enzyme/gr DM (Celluclast 1.5L from *Trichoderma reesei*) and 5 CBU β-glucosidase enzyme/gr DM (Novozyme 188 from *Aspergillusniger*) were then added to each sample. Avicel (PH-101, Sigma-Aldrich) was used as a control for the hydrolysis. The enzymatic hydrolysis was carried out in a rotary shaker (Kuhner Lab-Therm LT-X, Birsfelden, Switzerland) at 50^{o}C for 120 hours and set at 100 rpm. The released glucose after 120 hours-long hydrolysis was used to calculate the enzymatic digestibility.

Simon's stain test: Lignocellulosic samples were analyzed using the modified Simon's stain method. To measure the amount of adsorbed isotherm dye on the sample, 100 mg (OD wt) samples were weighed into five 15 mL centrifuge tubes and added with 1.0 mL of PBS (phosphate buffered saline solution, 0.3M sodium phosphate, pH 6.0, 1.4mM NaCl). The DO solution (Pontamine Fast Orange, Sigma Aldrich, 10 mg/mL) and DB solution (Chicago Sky Blue, Sigma Aldrich, 10 mg/mL) were added in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5 mL), thus creating a set of tubes with a 1:1 for DO:DB dyes ratio at increasing concentrations. Distillated water was then added to make up to 10 mL of the final volume in each of tube. Furthermore, these tubes were incubated at 70 ^{o}C for 6 h with shaking at 200 rpm, and then centrifuged at 3500g for 5 min.

Analyses: Lignin and carbohydrate compositions of untreated and treated materials were determined according to NREL Laboratory Analytical Procedure (*15-16*).Glucose concentration from enzymatic hydrolysis sample was monitored by glucometer (Glucometer Elite XL, Bayer AG, Leverkusen, Germany). The absorbance of sample of the supernatant from Simon's Stain test was read on a UV-vis spectrophotometer (UV 1800, Shimadzu,

Kyoto, Japan) at 455 and 624 nm. The concentration of DO and DB dyes in the dye-stripping solution (C_O and C_B) was calculated using Lambert-Beer Law for a binary mixture and solved simultaneously, as follows:

$$A_{455nm} = \varepsilon_{O/455}.L.C_O + \varepsilon_{B/455}.L.C_B \quad (1)$$

$$A_{624nm} = \varepsilon_{O/624}.L.C_O + \varepsilon_{B/624}.L.C_B \quad (2)$$

where A is the mixture absorption at 455 and 624 nm, ε is the coefficient of extinction of each dye at 455 and 624 nm, and L is the path length (represented by the width of cuvette, 1.0 cm). The ε was determined by the slope of the absorbance from the standard curves of each dye. The amount of dye adsorbed onto the fiber was calculated using the differences of initial added dye concentration and the dye concentration in supernatant.

RESULT AND DISCUSSION

Ammonia pretreatment

Oil palm empty fruit bunch (OPEFB) and sweet sorghum bagasse (SSB) were chosen as tropical biomass resources which are abundantly available in Indonesia. Oil palm tree is a hardwood type plantation, which is 0.23 ton OPEFB generated from 1 ton fresh fruit bunch (*17-18*).Sweet sorghum may be considered as herbaceous crops and generate 20 – 30 tons/ha green biomass (*19-21*).These agricultural wastes are potential for bioethanol or biogas production due to their high content of sugar (≥50%).

In this pretreatment process, OPEFB and SSB were soaked in 5% ammonia solution (SAA, soaking aqueous ammonia) at room temperature. This method does not require high temperature and pressure. Ammonia swelled the fraction of cellulosic in the material and diluted lignin fraction of material, thereby enhancing the subsequent bioconversion process to bioethanol. SAA process raises the surface area of cellulose available for the enzymatic hydrolysis by increasing the pore size of materials (*6, 8-9*).

The loss of physical and morphological integrity is studied using scanning electron microscopic; SEM (Figure-2). The untreated sample (Figure-2a and 2c) may look rigid and has many plant silica bodies (phytoliths). These rigid silica structures make the lignocellulosic biomass more difficult to consume and digest in bioconversion process. Oil palm (*Arecaceae*) and sweet sorghum (*Poaceae*) are family's plant with high phytoliths production. However, the ammonia pretreatment wrecked the lignin and phytoliths coated surface of lignocellulosic fiber and increases the pore size of the fibers (Figure-2b and 2d).

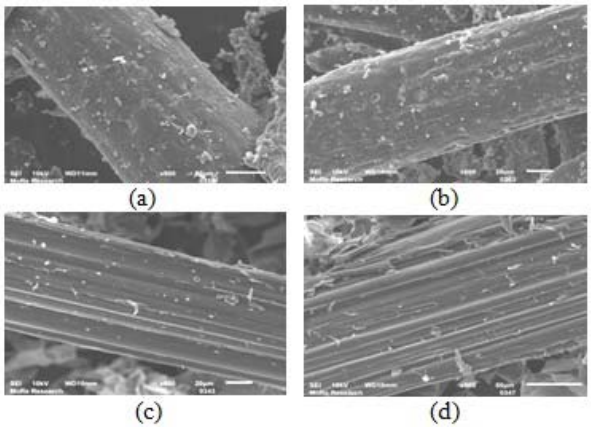

Figure-2. SEM analyses for untreated OPEFB (a), ammonia-treated OPEFB (b), untreated SSB (c), and ammonia treated SSB (d).

Another advantage of ammonia pretreatment is a low degradation of cellulose. This process is more selective to remove lignin in the materials. As discussed above, the disintegrated and diluted lignin by ammonia retained the carbohydrate component of lignocellulosic into solid fraction and increased absorption of cellulolytic enzyme. Thus, it helps in keeping the glucose yield (more than 73%) after enzymatic hydrolysis (Table-1).

The absorbance of dyes in lignocellulosic materials

In Simon's stain technique, lignocellulosic material was soaked in the mixture of blue and orange dyes. The adsorbed dyes in the solid material and free dyes in the solution were determined and plotted as adsorption isotherm curves (Figure-3). Figure-3 shows that the adsorption of the dyes increased by the length of pretreatment. For example, 7-days pretreatment has had the highest amount of adsorbed dye on fiber then 1- or 2-days pretreatment. It was perceptible that the test was proficient of distinguishing between the different fiber samples.

Table-1. The 120 h enzymatic yield of untreated and various conditions of 5% ammonia-treated of OPEFB and SSB (%).

Substrate	Condition	120 h hydrolysis yield (%)
Oil palm empty fruit bunch (OPEFB)		
Untreated	-	28.86
Am1	24 hrs	49.02
Am2	48 hrs	61.40
Am7	7 days	73.00
Sweet sorghum bagasse (SSB)		
Untreated	-	19.87
Am1	24 hrs	35.44
Am2	48 hrs	42.37
Am7	7 days	97.79

The adsorption of orange dyes takes priorities in the surface of cellulose fibers because of the higher affinity to cellulose. The pretreatment process was broken down the lignin as protective layer, resulted increasing number of pores and surface area. While orange dye has similar size as endoglucanase (biggest part of lignocellulosic hydrolysis enzyme component), thus the amount of adsorbed orange dyes may mimic the accessibility of enzyme to the cellulose fibers.

The amount of dye adsorbed in the surface of fibers is in equilibrium to the free dye concentration in the solution (Figure-3). However, the surface of fibers has limited capacity to adsorb the dye. Using excessive dye results a not proportional free dye concentration. At low initial dye concentration, the correlation of the amount of adsorbed dye was proportional to the free dye concentration. The correlation between the amount of adsorbed dye (x) and free dye concentration (c) was in a good agreement to Freundlich isotherm (*25-26*). The Freundlich isotherm is commonly used for adsorption in solid-liquid phases. The empirical equation of Freundlich adsorption isotherm was described below:

$$x = kc^{1/n} \quad (3)$$

where x is the amount of dye adsorbed onto fiber (mg/g-fiber), c is the concentration of free dye in solution (mg/mL), k and n are the adsorption constants. In general, raising temperature will effect to decrease k number and increase the value of n, while n ≈1 for high temperature (*27*).

At low free dye concentration, the correlation between adsorbed dye and free dye concentration was linear. Hence, the value of n was almost equal to 1. Consequently, the equation (3) will become:

$$x = kc \quad (4)$$

On the other hand, the pretreatment process also led to break the lignin structure from lignocellulosic material not in a sufficient size to orange dyes. Therefore, this condition also led to increase of absorbed blue dyes in a fiber. The blue dyes with a smaller diameter then orange dyes can be predicting the accessibility of the fiber to the small enzyme components. Figure 4 was apparent that the pretreatment can increase the blue dyes absorption as well as the orange dyes. It was also apparent that there was a strong correlation between the lengthy of pretreatment and the amount of both of dyes absorbed. Therefore, the blue dyes adsorbed and the concentration of blue dye in the solution may used to calculate the number of k in the equation (4).

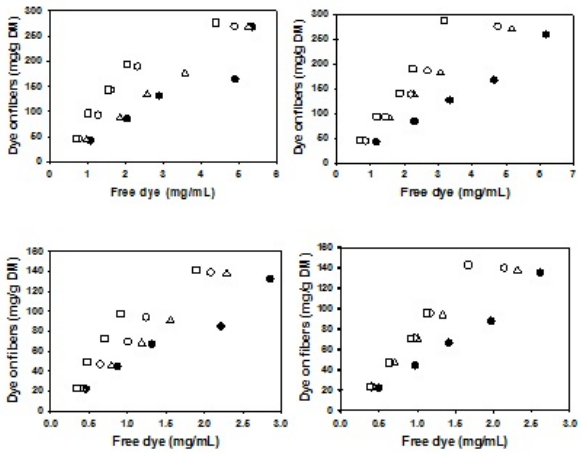

Figure-3. Dye adsorption profile for OPEFB (left) and SSB (right); raw material (●), 1 day pretreatment (Δ), 2 days pretreatment (○), and 7 days pretreatment (□).

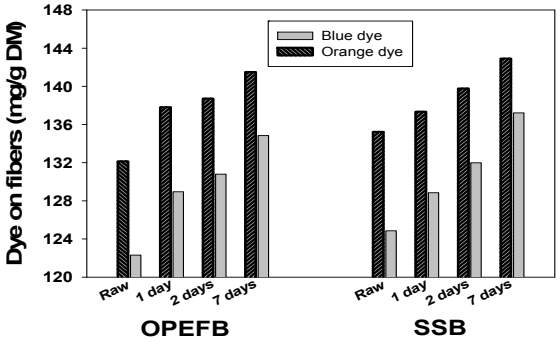

Figure-4. Adsorption of orange and blue dyes on treated and untreated materials

Correlation of Simon's stain with enzymatic hydrolysis

The linier correlation was resulted by plotting the total amount of dye adsorbed (mg/g-fiber) for each sample versus the total amount of free dye in solution (mg/mL). The number of k was found from the slope of these curves (data not shown). Thus, k valuewas used to confirm a possible correlation between total dye absorbed and enzymatic digestibility, shown in Figure-5.

However, this modify SS method is a semi-quantitative method, so that it cannot used to determine the cellulose degradation by enzyme or other chemical. Therefore, in this work try and confirm. When the result of the absorbed DO dye compared to the enzymatic digestibility, the trend of the curve is a linier with correlation coefficient of $R^2 = 0.96$ for OPEFB and $R^2 = 0.98$ for SSB. However, it is indicated that the modify SS method can be used to predict the ability of enzyme to hydrolyze the fiber. Figure-5 was apparent that the absorbed DO dye may be a stronger indicator of the ease of hydrolysis by enzyme.

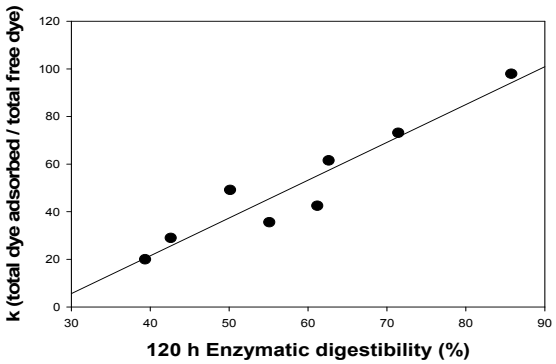

Figure-5. The linier correlation between absorbed dyes during modifies SS measurements and the 120 h enzymatic digestibility of OPEFB and SSB.

CONCLUSIONS

Ammonia pretreatment is a good tool in a crucial step for enhancing the complete hydrolysis of cellulose in overall bioconversion lignocellulose to ethanol. The effect of SAA pretreatment is attributed to the degradation and solubilization of lignin. By releasing lignin, the cellulose surface area was exposed and increased the accessibility of enzyme. One of the good instruments to analyze the effectiveness is Simon's Stain method. The SS technique was particularly useful to predict the ability of hydrolyzability of cellulose. Compare to the enzymatic digestibility, the SS technique give the linier correlation. It can be conclude that this method could be effectively to optimized pretreatment condition.

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