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Effect of sequential hydrothermal and alkaline solution pretreatments on enzymatic hydrolysis of hazelnut shells

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Abstract— **Hydrothermal and alkali pretreatments were applied to hazelnut shells to investigate the effect of the individual and sequential combinations of these two pretreatments on the enzymatic hydrolysis of the biomass. Sequential pretreatments were found to be more effective in altering the chemical composition and improving the digestibility of the biomass compared to single pretreatments. The effectiveness of the process was influenced substantially not only by utilization of sequential pretreatments but also by the order they were applied. Liquid hot water (LHW) pretreatment ensured mainly the solubilization of hemicellulose, while alkali pretreatment resulted in an effective removal of lignin. Combinations of these two pretreatments resulted in efficient elimination of both lignin and hemicellulose components of the biomass. Treating high lignin containing hazelnut shells firstly with an alkaline solution and then with LHW pretreatment exhibited an effective removal of lignin and then improved solubilization of hemicellulose by keeping cellulose recovery as high as 79%. Moreover, upon enzymatic hydrolysis, samples undergone Alkali+LHW pretreatment displayed highest glucose production (153 mg/g substrate) and highest glucose recovery (53.23%).**

Keywords— Alkali pretreatment, Lhw pretreatment, Biomass, Sequence pretreatment, Enzymatic hydrolysis

I. INTRODUCTION

Biomass resources are a promising alternative to fossil fuel energy because they are renewable and environmentally friendly. Lignocellulose is the most abundant biomass in the world, noted for its availability and relatively low cost, it shows a potential to create a new type of an energy platform. The main concern related to biomass utilization in either energy or fine chemicals production is the hydrolysis of this complex structure prior to use. Due to the nonhomogeneous composition and rigid configuration of the lignocellulosic biomass, the rate and extend of enzymatic hydrolysis is limited. There is a chellenging task to chose an effective pretreatment method, which will alter this complex structure by breaking the obstruction of lignin and hemicellulose and expose the cellulose fibers to cellulases. Different pretreatment methods; such as alkaline, acidic solution, hydrothermal, etc. are available, each demonstrating a different influence on the enzymatic saccharification of lignocellulose [1].

The final purpose of a pretreatment is to release high sugar yields from the overall process at low cost, but the specific goals are to remove lignin and hemicelluloses, disrupt the cellulose crystallinity, and increase the porosity of the materials to make cellulose more accessible to the cellulases; thus, to increase the enzymatic digestibility [2].

Among several available pretreatments, the process involving alkaline solution is effective in the removal of lignin, acetyl groups and different uronic acid substitutes from the raw biomass. Eliminating these components, which act as a physical barrier in the lignocellulosic structure and limit cellulose accessibility for enzymatic saccharification, is the most crucial advantage of alkaline pretreatment. In addition, solubilization of hemicelluloses and cellulose in this method is less than in acid or hydrothermal processes.

Liquid hot water (LHW) pretreatment, on the other hand, employs high temperatures (160–220°C) and pressure to keep water in liquid state and in contact with biomass for about 15 min residence time without addition of any chemicals or catalysts. LHW pretreatment has been shown as an efficient method for treating different kinds of lignocellulosic materials by increasing cellulose digestibility through hemicellulose removal [3].

To exploit the advantages and foreclose the deficiencies of the conventional single-step pretreatment methods, several two-step pretreatments were investigated to enhance glucose and xylose recoveries through sequential extraction of lignin and hemicellulose [4].

Turkey dominates the world's hazelnut market, producing about 70-75 %t of the world's total supply. Hazelnuts are mainly consumed as a major raw material in chocolate,

confectionery and baking industries and as an ingredient in edible nut mixes. Hazelnut production in Turkey has been reported as 660,000 metric-ton in-shell for the year of 2012. Assuming that an average of 50% of the product comprises of shells, hazelnut shell yield can be estimated to be more than 300,000 ton/year. Hazelnut shells are considered as an agricultural waste material and currently are only exploited as a combastible discard in hazelnut growing areas [5].

The aim this study is to investigate the effect of individual alkaline and hydrothermal pretreatments as well as their sequential combinations on the solid residue composition and subcequent enzymatic hydrolysis of hazelnut shells for an efficient production of monomeric sugars.

II. MATERIALS AND METHODS

A. Materials

Hazelnut shells used in this study were purchased from Beşikdüzü, Trabzon, Turkey. Raw materials were air dried to 9% moisture content. The dried materials were grinded and screened with a sieve shaker to obtain particle sizes between 0.224-0.850 mm. Samples were stored in plastic bags at +4 °C for future use. Cellulase (Celluclast 1.5L®) and β-glucosidase (Novozyme 188), suplhuric acid, sodium hydroxide were purchased from Sigma Aldrich. Enzyme activity of Celluclast 1.5L® was determined by NREL protocols and reported as Filter Paper Unit (FPU) (Adney and Bake, 2008). One unit of FPU is defined as the amount of enzyme required to liberate 1μmol of glucose from Whatman no:1 filter paper per minute at 50 C. One cellobiose unit (CBU) is defined as the amount of enzyme that converts 1 mmol of cellobiose to 2 mmol of glucose per minute.

B. Pretreatments

Liquid hot water pretreatment

Liquid hot water (LHW) pretreatments were conducted in a high pressure stirred 160 mL stainless steel reactor. Approximately 5 grams of ground hazelnut shells were mixed with 50 mL of distilled water. The slurry was heated up to 120° C and subjected to this temperature for 30 minutes. Solution was kept liquid under N_2 atmosphere. After treatment the reactor vessel was moved from the heating jacket. The content of the reactor was cooled down to 80 °C. Then the wet material was filtrated for solid recovery. The pretreated solids were used as substrate for enzymatic hydrolysis

Alkali Pretreatment

Alkali solution pretreatments were conducted in a high pressure stirred 160 mL stainless steel reactor. Approximately 5 grams of ground hazelnut shells were mixed with 50 mL of %2.25 (w/v) sodium hydroxide solution in a Teflon liner at 60 °C for 60 minutes. The vessel was heated until the desired temperature was reached and pretreatment time was initiated. After treatment the reactor vessel was moved from the heating jacket. Contents of the reactor were cooled. Then, the resulting slurry was filtered for solid recovery. The solid residue was washed with distilled water until the wash water's pH was neutral (pH=7.0). These pretreated solids were used as a substrate for enzymatic hydrolysis.

Sequential pretreatments

The individual pretreatments were applied sequentially to the hazelnut shells. For the Alkali+LHW pretreatment, hazelnut shells were firstly pretreated with alkali solution as described above. The solid residue was separated by filtration and washed with distilled water until the wash water's pH dropped to 7.0. Then the solid residue was subjected to LHW pretreatment as described above. Similarly, LHW+Alkali pretreatment was conducted by firstly applying the LHW process to the hazelnut shells and then alkali pretreatment was applied to the recovered solids, as described previously. The residual solid fractions obtained after the pretreatments were used as a substrate for the enzymatic hydrolysis.

C. Enzyme Saccharification

Enzymatic hydrolysis of the pretreated solids was carried out in stoppered conical flasks (50 mL). The pH was adjusted to 4.8 with acetate buffer, and a mixture of cellulase (60 FPU/g dry biomass) and β-glucosidase (40 CBU/g dry biomass) was added to a total working volume of 20 mL. The hydrolysis reactions were carried out at 50 ^{o}C for 6-24-48-72 h by shaking at 150 rpm. The reactions were stopped by boiling the flasks for 5 min in a water bath. The resulting hydrolysates were clarified by centrifuging at 5000 rpm for 5 min. The supernatants were analyzed for glucose and xylose using HPLC. Concentration of reducing sugars was determined by Dinitrosalicylic acid (DNS) method [6].

D. Analytical methods and calculations

The chemical composition of raw and pretreated hazelnut shells was determined according to NREL [7] methods. Briefly described, 0.3 g biomass was hydrolyzed by 3 mL of 72% (w/w) H_2SO_4 at 30°C for 60 min. Then, the reaction mixture was diluted to 4% (w/w) H_2SO_4 and autoclaved at 121 °C for 60 min. Lignin content was determined by measuring the solid residue; cellulose and hemicellulose amounts were determined by analyzing thefiltrate with High Performance Liquid Chromatography (HPLC).The HPLC system was equipped with a Bio-Rad Aminex HPX-87P column (300 mm × 7.8 mm), and a refractive index detector. The analytical column was operated with 0.2 μm filtered HPLC-grade water as a mobile phasewith a folw rate of 0.6 mL/min at 80 °C.

Enzyme activity of Celluclast 1.5L® was determined by NREL protocols and reported as Filter Paper Unit (FPU) [8]. One unit of FPU is defined as the amount of enzyme required to liberate 1μmol of glucose from Whatman no:1 filter paper per minute at 50^{o}C. One cellobiose unit (CBU) is the amount of enzyme that converts 1 μmol of cellobiose to 2 μmol of glucose per minute.

The following equations were used for the calculation of percent cellulose recovery, lignin and hemicellulose removal, and glucose recovery:

$$\text{Lignin removal } (\%) = \left(100 - \frac{\text{Lignin in pretreated solid}}{\text{Lignin in initial solid}}\right) \text{x} 100$$

$$\text{Hemicellulose removal } (\%) = \left(100 - \frac{\text{Hemicellulose in pretreated solid}}{\text{Hemicellulose in initial solid}}\right) \text{x} 100$$

$$\text{Cellulose recovery } (\%) = \left(\frac{\text{Cellulose in pretreated solid}}{\text{Cellulose in initial solid}}\right) \text{x} 100$$

$$\text{Glucose Recovery } (\%) = \frac{\text{glucose producedx0.9}}{\text{Cellulose in unpretreated solid}} \text{x} 100$$

III. Results and Discussion

A. Individual pretreatments

Upon subjecting the raw biomass to the pretreatments, the chemical composition of recovered solids was analyzed. Applying the pretreatment methods separately showed that each method had a different effect on the pre-processing of the biomass (Figure 1). LHW exhibited poor capability in lignin removal (12.57%), while alkali pretreatment was found to be efficient by reducing the initial lignin content to 49.59%. LHW, on the other hand, was effective in hemicellulose removal by decreasing the present hemicellulose to 51.46%, while alkali pretreatment's efficiency was limited to 27%. Cellulose recoveries were at acceptable levels being 85.39% and 98.07% for alkaline and LHW pretreatments, respectively.

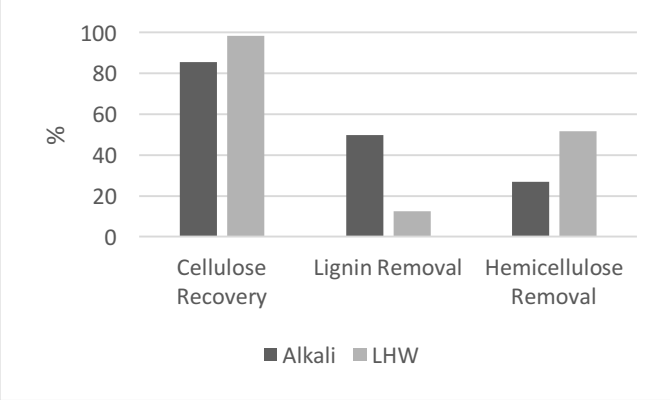

Figure 1. Effect of individual pretreatments on the chemical composition of hazelnut shells

B. Sequential pretreatments

The effect of combining the two pretreatments was assessed by applying the individual pretreatments sequentially with different orders. The effect of separate and combined pretreatments on the composition of the resulting solid residue was assessed.

As previously noted, alkaline pretreatment was significantly more efficient with respect to lignin removal (49.59%) compared to LHW pretreatment (12.57%). Applying the combination of these pretreatments to the hazelnut shells enhanced the lignin elimination considerably (Figure 2). For instance, further processing of the LHW-pretreated biomass with alkali solution increased lignin removal by 4 folds (46.73%) compared to LHW-processed sample (12.57%). Furthermore, the sequence of alkali+LHW pretreatment had the greatest impact by reducing the lignin content by 60.73%.

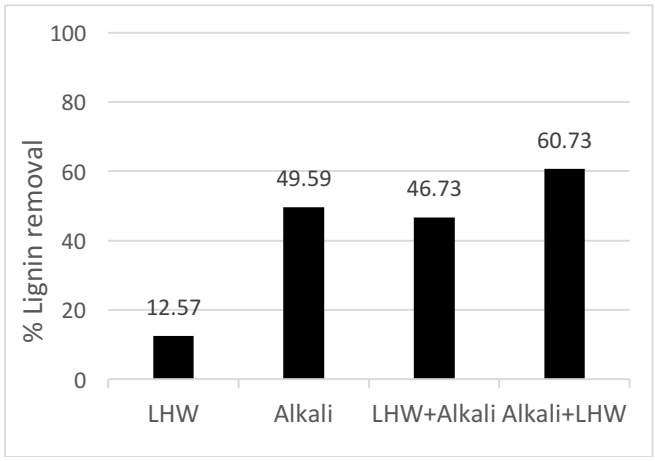

Figure 2. Effect of pretreatment methods on the lignin removal hazelnut shells

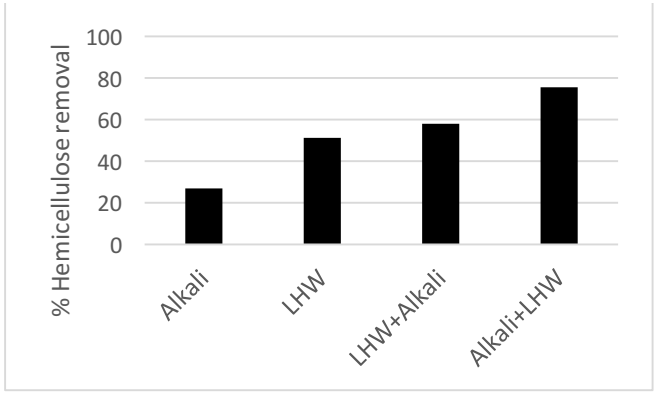

Figure 3. Effect of individual and sequential pretreatments on the hemicellulose removal from the hazelnut shells

Another challenging component present in the biomass is the hemicellulose. One of the pretreatment's goals is to disrupt the hemicellulose network and expose the cellulose fibers for enzymatic hydrolysis. Therefore, hemicellulose removal is a crucial criteria in determination of the effectiveness of the applied pretreatment method.

Hemicellulose removal from the biomass was primarily achieved by LHW pretreatment reaching 51.46% elimination. Further processing of the recovered solids with alkaline solution resulted in a notable increase to 57.79% hemicellulose removal (Figure 3). In addition to the combination of the pretreatment methods, the sequence of their application was proved to be tremendously important

for the composition of the recovered solids. Especially, applying alkali method first then following it by LHW pretreatment resulted in an approximately 3-fold increase (75.71%) in hemicellulose elimination. This indicated that removing effectively the lignin barrier with the alkaline solution facilitated the hemicellulose solubilization with the subsequent LHW pretreatment. Therefore, alkali+LHW sequential pretreatment was effective in the elimination of both lignin and hemicellulose from the hazelnut shells.

As the undesired components were removed, it was crucial to recover the cellulose fraction of the biomass. Figure 4 illustrates the cellulose conservation for the tested pretreatment combinations. The highest cellulose amount was retained by the individual LHW method (98.07%). It was followed by LHW+ alkali (91.78%), alkali (85.31%) and alkali+LHW (79.07%) combinations. Although the promising alkali+LHW combination seemed to result in lowest cellulose recovery, it was proven that it was still the favorable pretreatment judging from the glucose production results.

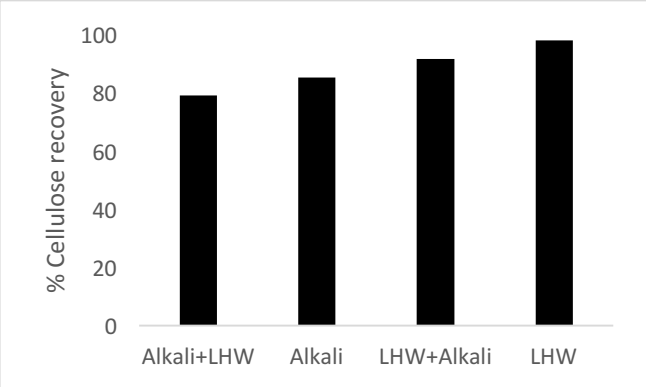

Figure 4. . Effect of individual and sequential pretreatments on cellulose recovery from the hazelnut shells

Figure 5 illustrates the production of glucose upon enzymatic hydrolysis of the pretreated biomass. The lowest monomeric sugar generation was observed for alkali pretreated- biomass, reaching 88 mg glucose/g substrate after 72-h hydrolysis. It was followed by LHW and LHW+alkali pretreated samples, which generated similar amounts of monomeric sugar of approximately 128 mg/g substrate at the end of 72 h enzymatic digestion. The highest glucose production was observed for the alkali+LHW pretreated biomass, reaching 153 mg/g substrate.

Moreover, the results were interpreted in terms of percent glucose recovery. This term specifies the efficiency of the generation of glucose monomers from the available cellulose in the biomass. As seen in Figure 6, applying sequential pretreatments on hazelnut shells increased the overall glucose yield from the biomass. More specifically, the highest glucose recovery of 53.23 % was observed in alkali+LHW pretreated samples. This implies that as the lignin content was efficiently removed, the crystallinity of the cellulose was disrupted and the cellulose fibers were effectively separated. As a result of the swelling of those cellulose fibers their accessibility by enzymes increased and the hydrolysis of cellulose resulted in a higher yield.

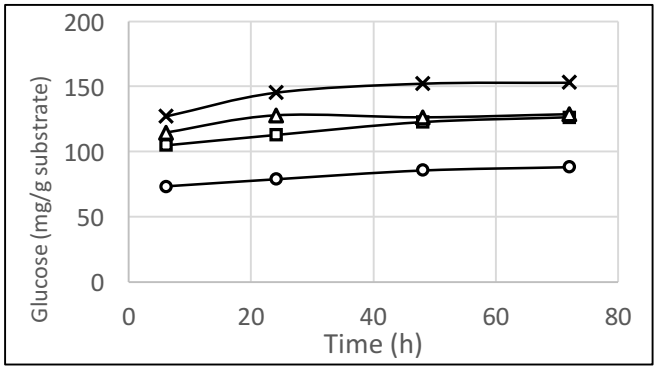

Figure 5. Effect of individual and sequential pretreatments on glucose production during enzymatic hydrolysis of hazelnut shells (○: alkali; □: LHW; △: LHW+alkali; ✖: alkali+LHW)

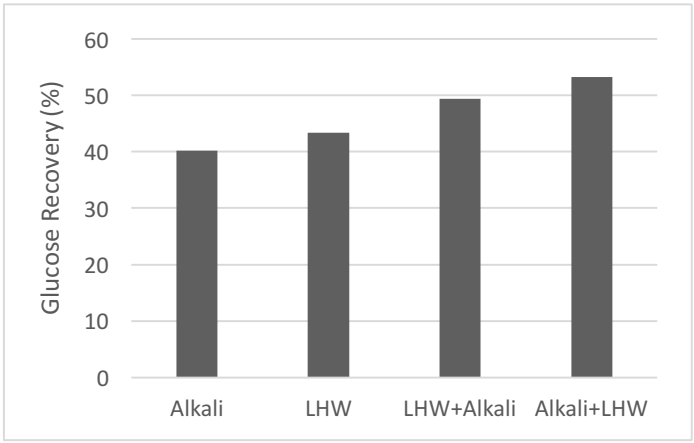

Figure 6. Effect of individual and sequential pretreatment methods on the glucose recovery from hazelnut shells

IV. CONCLUSION

Conclusively, application of two different sequential pretreatments had a substantial impact on both chemical composition of the residual solids and their enzymatic hydrolysis yield. In addition, the order of executed pretreatment methods was proven to be of great importance. For biomasses containing high lignin content, such as hazelnut shells, the optimal sequence was alkali+LHW pretreatments resulting in the highest lignin and hemicellulose removal and the greatest glucose recovery percentage.

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