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The Effect of Mechanocatalytic Pretreatment on the Structure and Depolymerization of Willow

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Abstract: In this study, the effect of a mechanocatalytic pretreatment on the structure of willow and sugar release from pretreated willow was explored. In the mechanocatalytic approach, the pretreatment consists of solvent-free impregnation with sulfuric acid and a mechanical treatment with ball milling. Willow sawdust and pretreated samples were analyzed with field emission scanning electron microscope and X-ray diffraction. The products in the sugar solution were determined as the total reducing sugars with the 3,5-dinitrosalicylic acid method and monosaccharides with capillary electrophoresis. According to the results, milling increased the sugar production, depending on the sulfuric acid load. The milling parameters, such as the rotation speed of the mill, the catalyst-to-willow ratio, and the milling time influenced the amount of sugars in the hydrolysate and the composition of the sugar solution produced. Changes were observed in the surface of the willow particles as well as changes in the crystalline structure. Glucose and xylose yields increased after 15 min of milling and reached their maximum level after 45 min of milling with the 0.5 mmol/g sulfuric acid load.

1. Introduction

Lignocellulosic biomass, such as woody residues and agriculture waste, is a promising renewable source for the production of biochemicals through the sugar platform. Lignocellulosic biomass does not compete with food production and is widely available [1,2]. In addition, lignocellulosic waste is inexpensive feedstock [3]. An example of a lignocellulosic crop is willow, which can be used for energy production, as well as a raw material in bioethanol and biobutanol production via saccharification [4,5]. Willow has many benefits: It is fast-growing, removes nutrients and heavy metals from soil, reduces soil erosion, and can be farmed [6]. Commercial plantations for bioenergy production have been in operation for decades in many countries, including Sweden and the United Kingdom [6–10].

Lignocellulosic biomass is a good resource for a biorefinery to produce value-added products, such as bio-based platform chemicals and renewable fuels. It contains mainly cellulose, hemicelluloses, lignin, and extractives. Cellulose is a linear homopolysaccharide, which acts as the structural base of plant cells. The smallest repetitive unit of cellulose, cellobiose, is formed of two glucose units. A cellulosic macromolecule is chemically stable because of its crystalline regions. However, hemicelluloses are heteropolysaccharides, consisting mainly of pentoses, hexoses, and uronic acids.

Hemicelluloses are often branched and easier to hydrolyze than cellulose. The structure and composition of hemicelluloses depend for example on the plant species. In hardwood and annual plants, pentoses such as xylose are predominant, whereas softwood hemicelluloses, such as glucomannan, are dominated by hexoses [2,11]. The production of bio-based chemicals from lignocellulosic biomass proceeds often via the sugar platform. For this, the cellulose and hemicellulose components of biomass are hydrolyzed to monosaccharides. Enzymatic hydrolysis utilizes enzymes, such as cellulases, for conversion, whereas acid hydrolysis is driven by an acid catalyst. Several acids can be used in acid hydrolysis, but the ones that are most commonly used for lignocellulosic biomass are sulfuric acid and hydrochloric acid [1,2]. The hydrolysis of cellulose breaks the β-1,4-glycosidic bonds of the polymer. The reaction scheme for the acid-catalyzed hydrolysis of glycosidic linkages is outlined in Figure 1 [12]. In the reaction, a proton from catalyzing acid interacts with glycosidic oxygen, linking sugar units (I). So-called conjugate acid (II) is formed. The C–O bond cleaves, forming a cyclic carbonium cation (III). After the addition of water, the sugar and proton are liberated. The reaction can also occur at ring oxygen and proceed on another pathway (II′–III′) [12,13]. The mechanism of acid hydrolysis of lignocellulosic biomass is complicated due to heterogeneous characters and multi-stage rate processes [14,15].
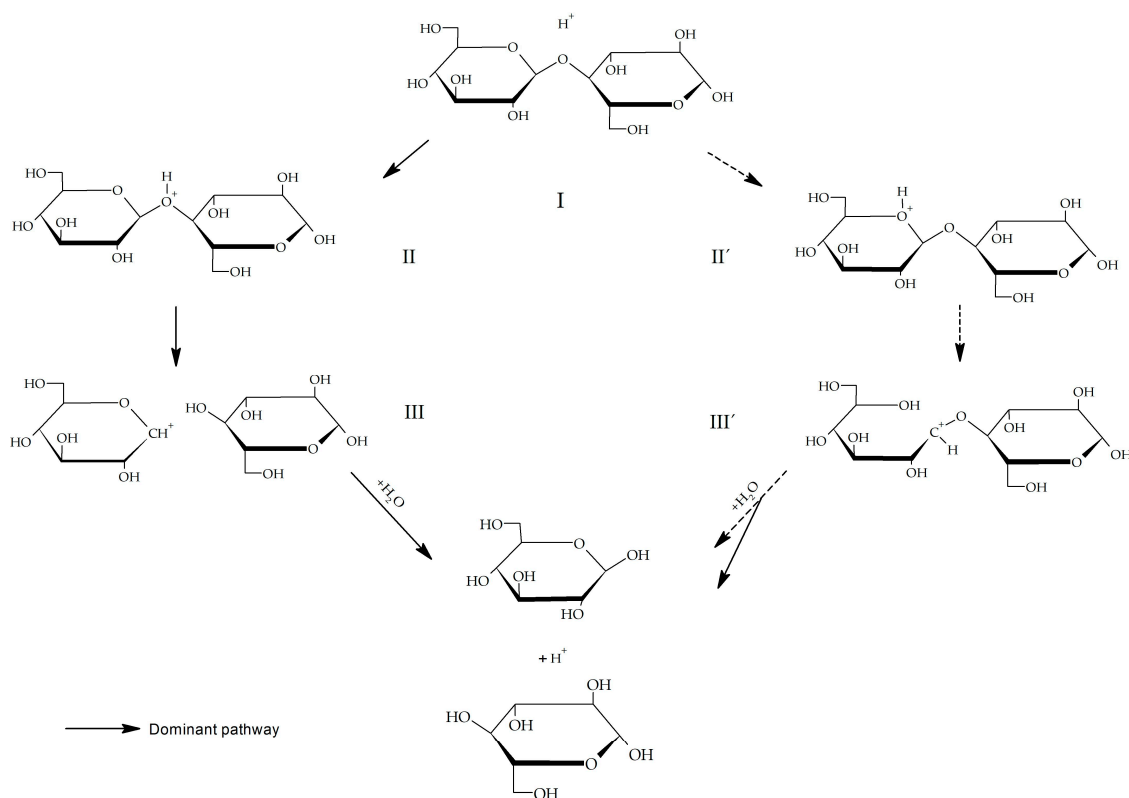
Figure 1. Reaction scheme for acid-catalyzed hydrolysis of glycosidic linkages [12,13].

During acid hydrolysis, there are often side reactions, resulting in inhibitory lignocellulose-derived by-products. In acidic conditions, pentoses and uronic acids react further to 2-furaldehyde, which is also called furfural. Hexoses dehydrate to 5-hydroxymethyl-2-furaldehyde (HMF), which further degrades to levulinic acid and formic acid (Figure 2). In acidic conditions, lignin forms phenolic compounds. In addition, other compounds, such as small aliphatic compounds, are formed during the acidic treatment process. Many of the by-products formed have inhibitory effects, which result in low efficiency of the hydrolysis process [11–17].

Glucose　⇌ (Isomeration)　Fructose　⟶　HMF　⟶　Levulinic acid　**+**　Formic acid

Sucrose
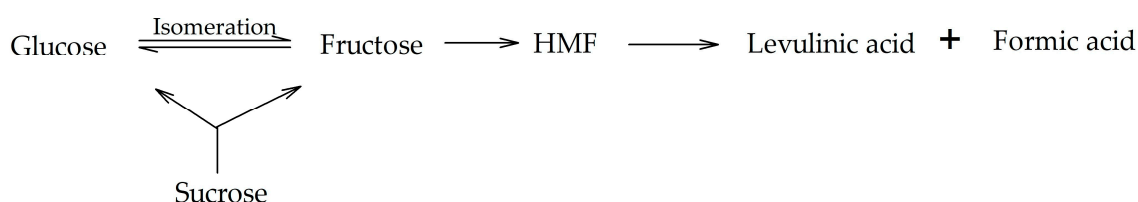
Figure 2. Pathway for converting glucose to 5-hydroxymethyl-2-furaldehyde (HMF).

Lignocellulosic biomass has a recalcitrant structure, so pretreatment is usually required before further processing. The aim of the pretreatment is to reveal the main feed components, cellulose and hemicelluloses, of the biomass for e.g., saccharification and fractionation purposes. Choosing the correct pretreatment method is important, since it has an impact on the further processing stages. For example, the amount of acid catalyst needed for the hydrolysis reaction can be diminished by the correct pretreatment method. Furthermore, the amount of inhibitory by-products formed during the hydrolysis can be decreased by the choice of the pretreatment method [11,17]. Several pretreatment methods exist, including physical, chemical, physicochemical, biological, ultrasound, and microwave-assisted methods and combined methods [18–22]. Physical pretreatment, including mechanical operations, aims to decrease the size of the biomass particles and increase the surface area of the particles [18]. In addition to size reduction, mechanical pretreatment of lignocellulosic material is applied to disrupt the crystalline regions of cellulose [17]. For example, treatment can be carried out with a ball mill. The main factors that affect the efficiency of the ball milling process are the type of mill, the milling time, the temperature, milled material, the ball-to-sample ratio, the operation condition, the properties of the milling balls, the number of the milling balls, and the mill charge [18,23]. A ball milling process combined with a catalytic hydrolysis increases the total reducing sugar levels compared to pure catalyzed hydrolysis [24]. The main drawback of mechanical treatment is increased energy consumption [2]. On the other hand, a chemical pretreatment can be used to increase the yield of the hydrolysis products. The pretreatment with several acids and alkalines as well as an organosolv process were studied [25]. Even though a chemical pretreatment is an efficient way to remove hemicelluloses and the sugar yield is higher than with a physical treatment, the formation of the inhibitory products and corrosion of the equipment have to be considered [19]. Joined physical and chemical pretreatment combines characters from each pretreatment process. Physicochemical pretreatment forms less inhibitory compounds than chemical pretreatment and can be cost efficient even though it requires specific equipment [19].

A method that combines mechanical and catalytic pretreatments is mechanocatalytic pretreatment. This method is considered attractive for the pretreatment of lignocellulosic biomass to improve the process of platform chemical production [17,26]. Dong et al. [27] pointed out that the direct acid-catalyzed mechanocatalytic depolymerization of fiber sludge is a promising process compared to the traditional mechanocatalytic depolymerization process, in which the acid catalyst is first impregnated to the biomass with a solvent such as diethyl ether. The benefits of direct methods include a simpler procedure, reduced energy and chemical consumption, and increased total reducing sugar yields.

The choice of the proper pretreatment process depends on the biomass source and its chemical and structural properties as well as the product desired. Different pretreatment methods on willow have been studied, such as the steam explosion process, fungal pretreatment, combined fungal and alkaline treatment, concentrated phosphoric acid pretreatment, and steam pretreatment with SO_2 and H_2SO_4 impregnation [28–31]. The present study focused on the mechanocatalytic pretreatment of willow sawdust into reducing sugars. Instead of beginning the pretreatment with ball mill and then adding a chemical step, the mechanocatalytic pretreatment was performed in a one-step solvent-free procedure through direct acid catalysis (Figure 3). The pretreatment method has been shown to work earlier for fiber sludge [27], so the method was now studied with willow that differs chemically and structurally

from fiber sludge. Sulfuric acid was used as the catalyst, and the mechanical treatment was performed with a planetary ball mill. Different ratios of the catalyst load and willow were examined, as well as the effect of the mechanical parameters, such as the milling time and the speed of the mill. X-ray diffraction (XRD) and a field emission scanning electron microscope (FESEM) were used to identify changes in the willow structure. The sugars released from the willow samples after the pretreatment process were analyzed, as well as the sugars produced during the subsequent acid hydrolysis at 100 °C. The amount of total reducing sugars (TRS) was determined based on the 3,5-dinitrosalicylic acid (DNS) method, and specific sugars, as well as the HMF by-product, were analyzed with capillary electrophoresis (CE).
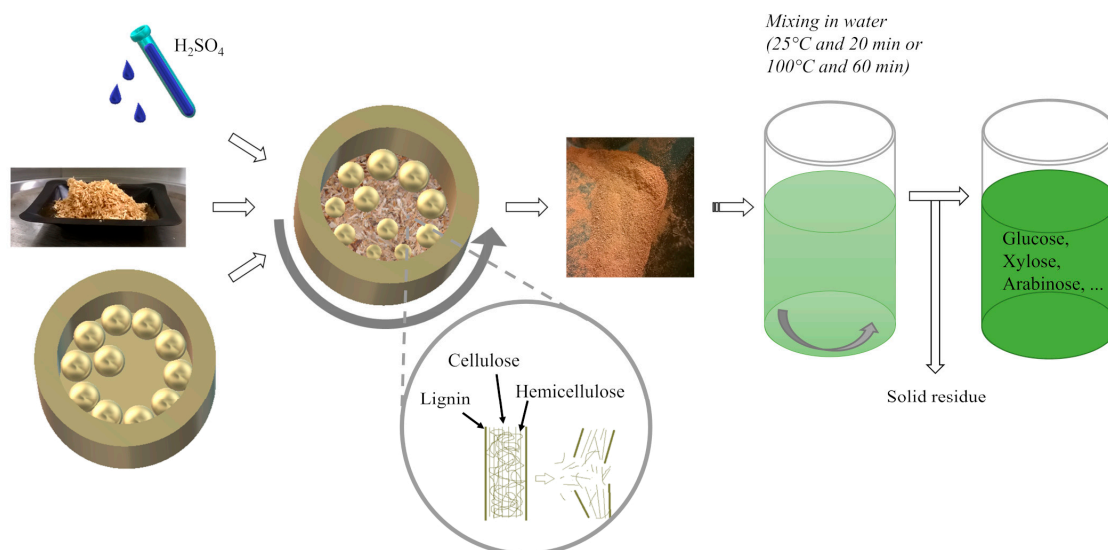

Figure 3. Scheme of the mechanocatalytic pretreatment process of willow followed by hydrolysis.

2. Results and Discussion

2.1. Effects of Pretreatment on the Structure of the Willow Sawdust

FESEM images were used to observe the structure of the willow samples before and after the pretreatment. The particle size decreased, and the shape of the particles turned out rounder during milling within 5 min (Figure 4). With a longer milling time, the particles split still more so that the edges of the willow particles were sharper, and the lamella structure was apparent. This indicates how particles split even more if the milling process is extended to longer than 5 min.
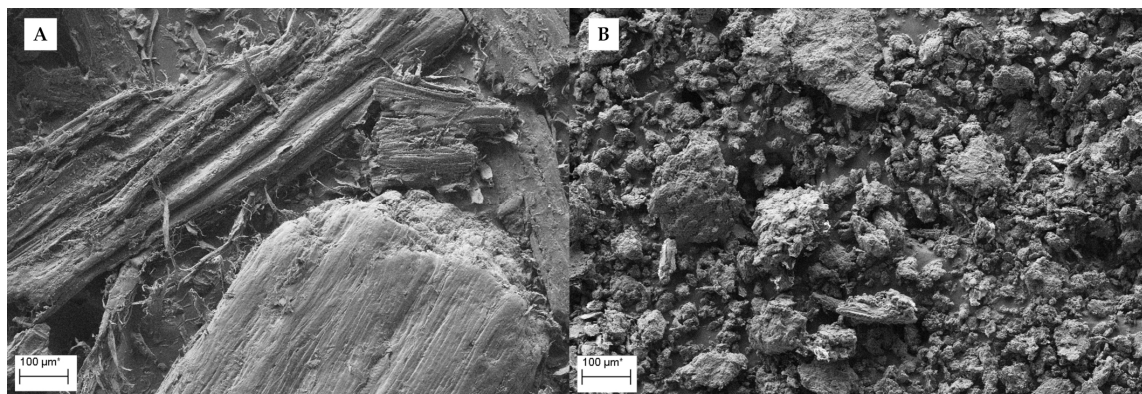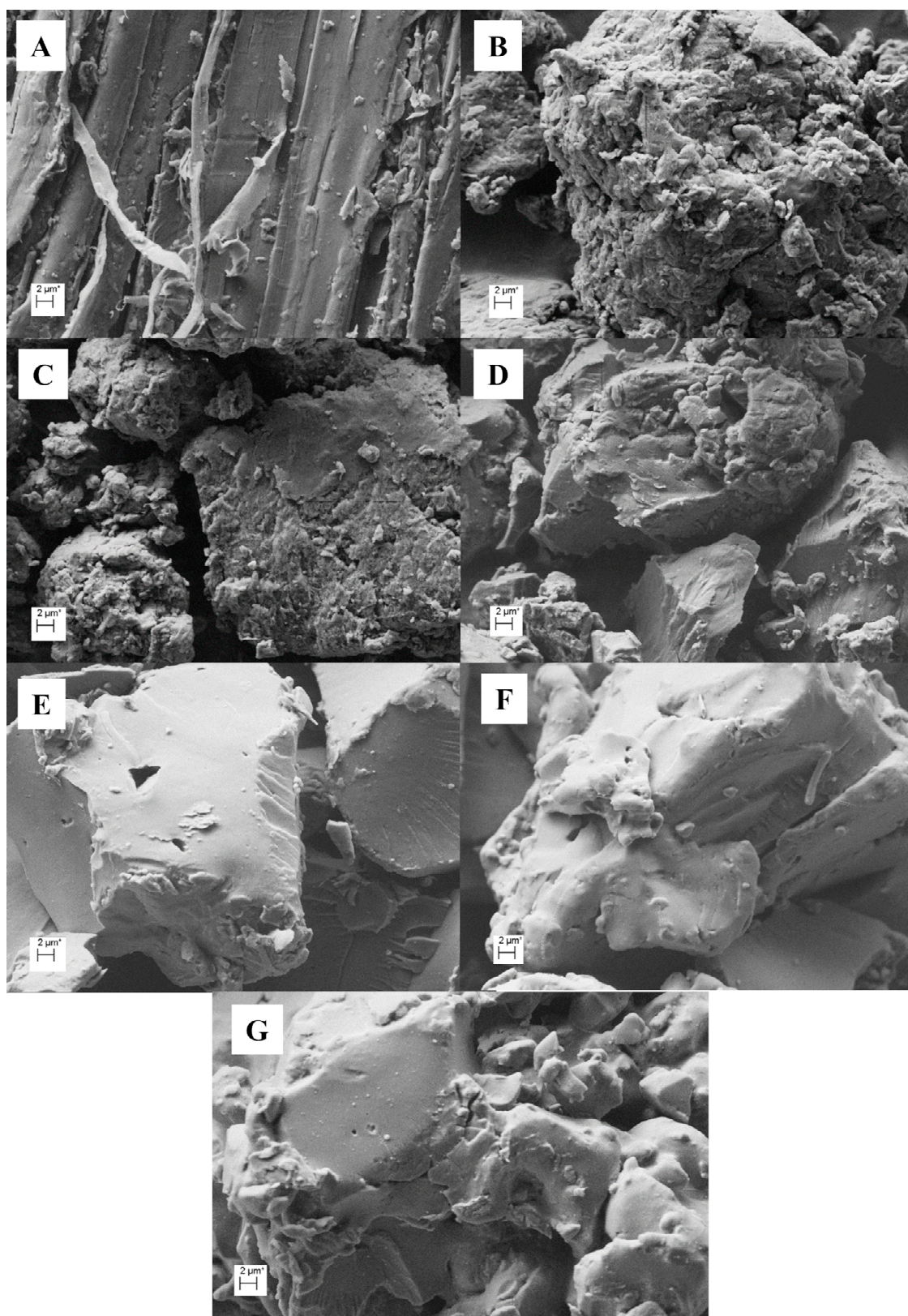

Figure 4. FESEM images of untreated willow sawdust (**A**) and pretreated willow (**B**). Pretreated willow was milled for 5 min with the 1 mmol/g catalyst load. Magnification 250×.

Figure 5 shows the change in the surface of the willow particles when acid was added to the pretreatment process. The untreated willow sample has a longitudinal structure (Figure 5A) with a dense surface, vertical cracks, and some loose fibers. The surface of the willow sample milled for 30 min with distilled water was rough and multidimensional (Figure 5B). Milling for 30 min with the acid load diminished the roughness of the surface gradually, until the surface was smooth, and the lamella structure was observed (Figure 5C–F). With the 2.0 mmol/g acid load, the surfaces of the willow particles looked melted (Figure 5G), which could result from the amorphous cellulose on the surface [32]. If the acid load was increased further (3.0 mmol/g), the willow was agglomerated on the milling equipment during the milling process, and the process was disrupted. It has been suggested in a previous study by Zhao et al. [32] with cotton linters that the agglomeration results from the acid-catalyzed intermolecular surface dehydration of macrofibrils, which depends on the acid strength.

XRD patterns of the willow samples before and after the mechanocatalytic pretreatment were used to evaluate the changes in the crystalline structure as a result of the pretreatment process (Figure 6A,B). Willow sawdust, which was milled for 30 min with distilled water, was chosen for the reference. Peaks at $2\theta = 18°$, $22.4°$, and $34.7°$ were observed from the XRD pattern of the untreated willow sawdust. The first two peaks correspond to the crystallites' diameter, and the peak at $2\theta = 34.7°$ corresponds to the crystallite length [33]. After 30 min of milling with distilled water, only one dominant broad peak at $2\theta = 21.4°$ was observed. This change is in accordance with previous research, in which the amorphous phase was produced from crystalline cellulose with ball milling, and the reduction in the crystallinity of cellulose affected the hydrolysis rate of cellulose to reducing sugars [34–36]. The milling with the acid load of 0.2, 0.5, 1.0, 1.5, or 2.0 mmol/g transferred the dominant peak, which was detected after the milling with water, further left (Figure 6B). Increasing the acid load also diminished the intensity of the peaks, which was noticeable in the XRD pattern. Thus, ball milling affects the cellulose structure by reducing the length and thickness of the crystallites. The effect of ball milling on the willow structure was also observed by calculating the cellulose crystallinity index (CrI), which decreased when the pretreatment conditions were hardened. The CrI of the willow sawdust sample was 59%, but it decreased after milling with water to 22%, and kept decreasing to 17%, 18%, and 14% when the acid load was increased to 0.2 mmol/g, 0.5 mmol/g, and 1.0 mmol/g, respectively. Increasing the acid load to 1.5 and 2.0 mmol/g decreased the intensity of the pattern and increased the crystallinity index to 23%. An increase in CrI could be the result of the surface of the samples, which looked molten, and the XRD pattern, which exhibited a broad and low intensity peak that indicates an amorphous phase [34,35]. Increasing the milling time from 30 min to 45 min or 60 min with an acid load of 0.5 mmol/g did not considerably change the XRD pattern (Figure 6C).

Figure 5. FESEM images of untreated willow sawdust (**A**) and willow milled for 30 min. Milling was done with distilled water (**B**) or with different acid load to willow ratios, i.e., 0.2 mmol/g (**C**), 0.5 mmol/g (**D**), 1.0 mmol/g (**E**), 1.5 mmol/g (**F**) and 2.0 mmol/g (**G**). Magnification 5000×.
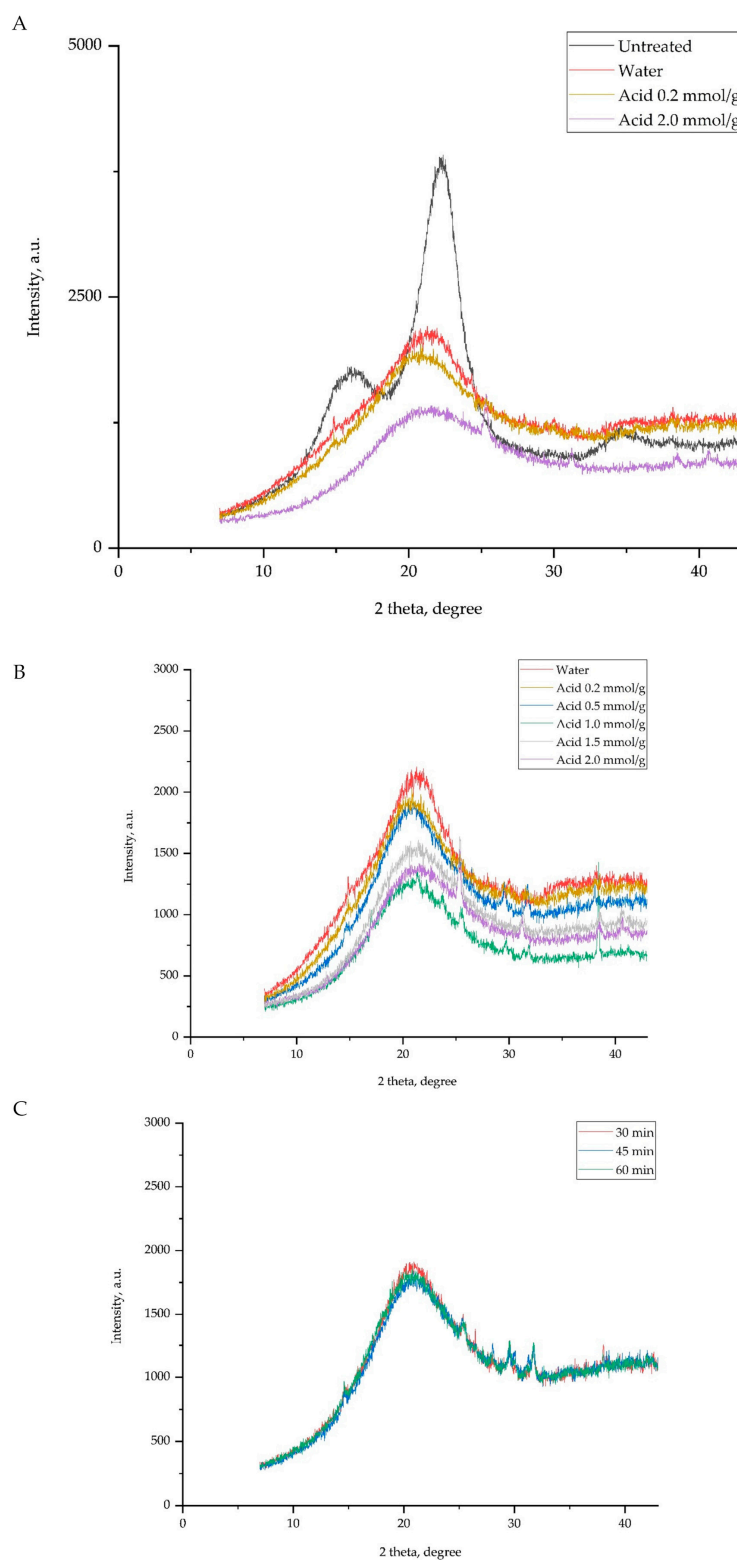
Figure 6. XRD patterns of untreated willow sawdust and pretreated willow. (**A**) Untreated willow sawdust (black line). Willow was milled for 30 min with water (red line), 0.2 mmol/g acid load (yellow), and 2.0 mmol/g acid load (lila). (**B**) Willow was milled for 30 min with acid loads of 0.2 mmol/g (yellow), 0.5 mmol/g (blue), 1.0 mmol/g (green), 1.5 mmol/g (gray), and 2.0 mmol/g (lila). (**C**) Willow was milled for 30 min (red), 45 min (blue), and 60 min (green) with 0.5 mmol/g acid loads.

2.2. Effect of Mechanocatalytic Treatment Parameters on Sugar Release

The dry matter content of the willow sample was determined. The sample contained 42 wt % of α-cellulose, 30 wt % of hemicelluloses, and 26 wt % of lignin. The sample included both stem and bark. According to previous research, bark includes significantly less polysaccharides than stem wood, 46% and 70% respectively, and more lignin than stem wood, 24% and 18%, respectively [4]. The composition of willow depends on the age of willow but also on the place it grows and moisture of the sample [37,38].

The amount of the TRS of the willow samples was analyzed after the mechanocatalytic treatment with different process parameters, such as the catalyst-to-wood ratio, the milling time, and the speed of the mill and mixing in the water at room temperature for 20 min. The effect of the rotation speed of the planetary mill is shown in Figure 7A. The maximum speed of the machinery with 10 mm milling balls was 800 rpm, so speeds of 600, 700, and 800 rpm were chosen. In the milling process with 30 min of milling and the 1.0 mmol/g acid load, the maximum speed (800 rpm) resulted in a 13.3% TRS yield, as well as milling with the 700 rpm rotation speed. The 600 rpm rotation speed provided only a 9.0% sugar yield, which was a notably low amount compared with the sugar yields of the higher speed processes. Even though the TRS yield of 700 rpm and 800 rpm did not differ significantly and 700 rpm would be the more energy-saving option, 800 rpm was chosen according to results shown in the next section.
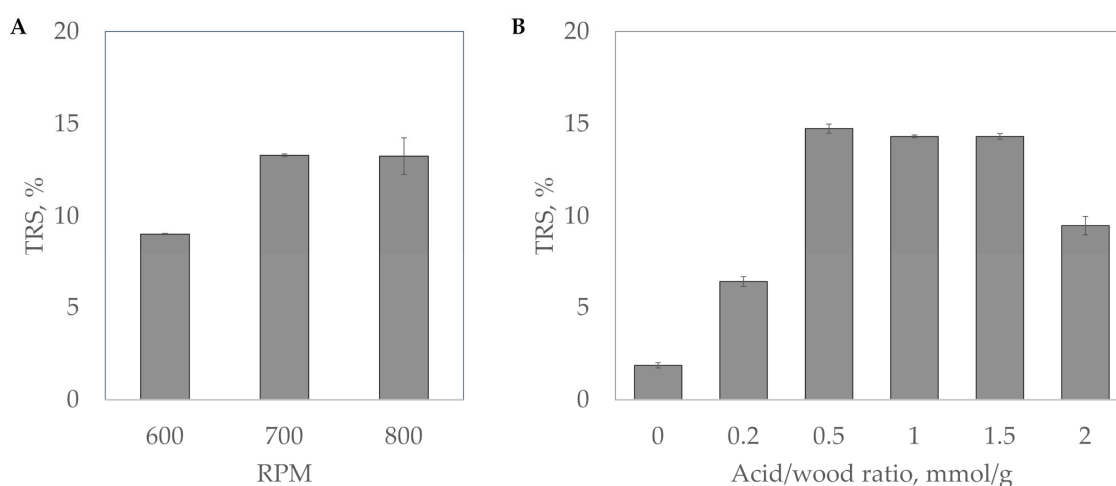

Figure 7. The effect of rotation speed and acid/wood ratio on total reducing sugars (TRS) yield. Pretreated willow sample was mixed with water for 20 min at room temperature. (**A**) The TRS yields of the willow sample milled for 30 min with the 1.0 mmol/g acid load with different rotation speeds, 600, 700, and 800 rpm. (**B**) The TRS yields for the willow sample milled for 30 min with different catalyst loads (0.0, 0.2, 0.5, 1.0, 1.5, and 2.0 mmol/g). The acid catalyst was replaced with water when the catalyst load 0.0 mmol/g was used.

Figure 7B shows the effect of the catalyst load on the TRS yield with acid loads between 0.0 and 2.0 mmol/g after 30 min of milling. The milling process without liquid did not mill the sawdust properly, even when the milling time was more than 90 min. Thus, instead of acid, distilled water was used to enhance the milling. The TRS yield of the willow sample milled for 30 min without sulfuric acid was 1.9%. Adding 0.2 mmol/g acid to the milling process more than tripled the TRS yield (6.4%). The highest TRS yield (14.8%) was achieved with the 0.5 mmol/g acid load. Increasing the catalyst load to 1.0 mmol/g, 1.5 mmol/g, and 2.0 mmol/g decreased the TRS yield to 14.3%, 14.3%, and 9.5%, respectively. The higher acid load may have caused the decrease in the TRS yield because of further reactions of the sugars and the agglomeration of the willow sample, because of the excess moisture in the process. Dong et al. [27] described that a sample with more than 7% water caused the burning of

fiber sludge in the acid concentration of 600 mmol/kg. Based on the results, the 0.5 mmol/g acid load was used for the following experiments.

The effect of the milling time on the TRS yield and the monosaccharide yields is shown in Figure 8. Willow was milled from 0 min to 75 min with the 0.5 mmol/g acid load. The TRS yield increased along with the milling time, until the yield (17.1%) was achieved after 45 min of milling. Besides reducing the particle size, ball milling generates changes in the macromolecule structure. These changes improve the accessibility of the catalyst to polysaccharides [33]. Longer milling times decreased the TRS yield slightly. This decrease may have occurred as a result of the catalyst lost, because the sample stuck to the milling equipment, the sample agglomerated, and thus, the surface area decreased, or further reactions of the sugars occurred. The monosaccharide yield also increased considerably when the willow sample was milled for 45 min, compared to 30 min or less. In particular, this result was observed with the glucose and xylose yields, which increased from 12.0 g/kg and 5.8 g/kg to 24.2 g/kg and 11.6 g/kg, respectively. The structure of the willow sample was disturbed during milling, and cellulose and hemicelluloses were more readily available for depolymerization. The unmilled willow sample released 3.0 g/kg sucrose, but after 15 min of milling, no sucrose was detected. The amount of fructose decreased from 7.1 g/kg to a non-detectable level when the willow sample was milled for 30 min. Small amounts of HMF, ranging from 0.8 to 1.1 g/kg, were detected after 30 min or longer of milling. This result suggests that milling releases the components from the wood so that they can degrade and react further in acidic conditions. For example, HMF can react into levulinic acid and formic acid [16,39]. However, these components were not analyzed in this research.
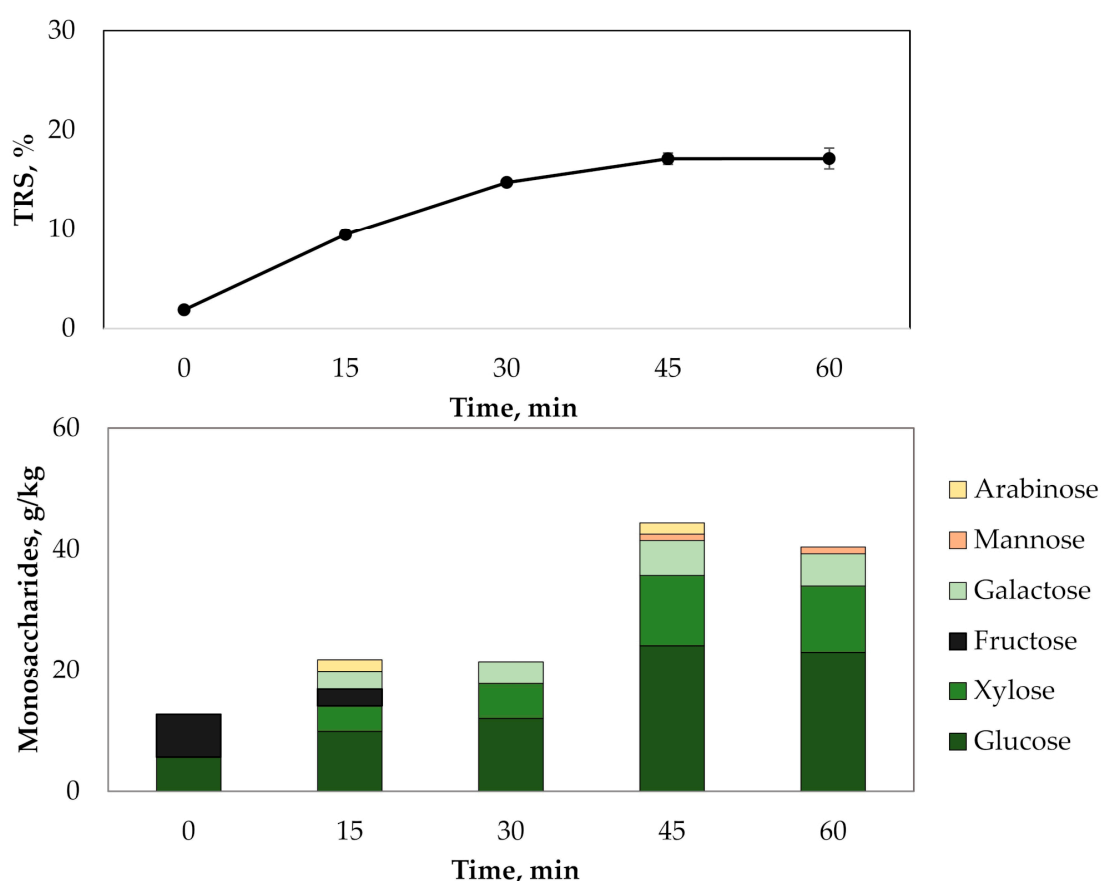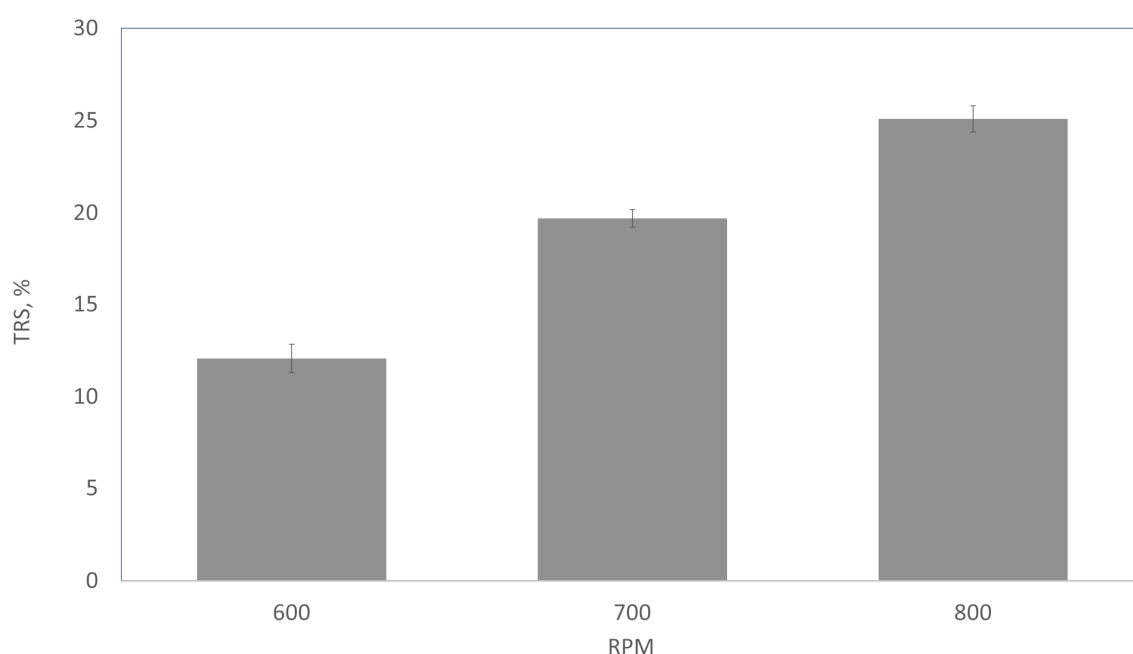

Figure 8. TRS yields and monosaccharides produced from the willow samples that were milled for various durations with the 0.5 mmol/g acid load. The milling time varied from 0 to 75 min, at 15 min intervals. The milling time includes only the periods of milling, not cooling periods.

The crystallinity of the cellulose, surface area, lignin, and the heterogeneous character of the biomass can decrease the yield of hydrolysis of lignocellulosic biomass [40]. The mechanocatalytic pretreatment broke the recalcitrant structure of the lignocellulosic biomass, increased the surface area of the particles, revealed the cellulose and hemicelluloses for the acid load, and therefore accelerated the monosaccharide production.

2.3. Effect of Mechanocatalytic Pretreatment on the Release of Sugars and Monosaccharide Production in Hydrolysis

The mechanocatalytically pretreated willow sample was hydrolyzed in mild conditions, 60 min at 100 °C, to reveal the effect of the pretreatment on the release of sugars. Acid was applied in the pretreatment step, and no more acid was added in the hydrolysis step. The TRS yields of the experiments were higher throughout than in the experiments presented in Section 2.2, and the effects of the pretreatment parameters were similar. However, some differences were detected, for example, in the optimal amount of the acid catalyst.

The effect of rotation speed of mill was studied as in the previous section, but pretreatment was followed by hydrolysis at 100 °C for 60 min. The pretreatment with 600 rpm did result in 12.1% TRS yield, whereas 700 rpm resulted in a TRS yield of 19.7%, and that of 800 rpm was 25.1%. Whereas the TRS yields were similar for 700 rpm and 800 rpm in the experiments described in the previous section, the rotation speed of 800 rpm resulted in the highest TRS yield after hydrolysis at 100 °C for 60 min (Figure 9). Thus, the rotation speed of 800 rpm was used in the other experiments in this research.

Figure 9. TRS yields and monosaccharides produced from the willow samples that were milled for various durations with the 0.5 mmol/g acid load. The milling time varied from 0 to 75 min at 15-min intervals. Pretreated willow was hydrolyzed at 100 °C for 60 min.

The effects of the milling time, during the milling period from 0 to 60 min with the 0.5 mmol/g acid load, on the TRS and monosaccharide yields are shown in Figure 10. The hydrolysate of the unmilled willow sample contained 7.9 g/kg glucose, 8.3 g/kg fructose, and 3.1 g/kg arabinose. Milling with the 0.5 mmol/g acid load had a considerable effect on the monosaccharide production, and the acid catalyst was required to produce galactose, xylose, and mannose from the willow sample. Xylose and galactose were detected after 15 min of milling, but mannose was only detected after 30 min of milling. The monosaccharide yields increased when the milling time increased to 45 min. With 45 min

of milling, the glucose yield increased to 25.9 g/kg, the xylose yield increased to 15.5 g/kg, and the arabinose yield increased to 3.8 g/kg. However, the fructose yield decreased so that after 45 min of milling, no fructose was detected. After 30 min or longer of milling, 1.0 g/kg of HMF was detected. As stated previously, fructose converts, for example, via HMF to levulinic acid and formic acid, so the longer milling process favored this reaction during the milling process or improved the reaction during hydrolysis. Therefore, by adjusting the process parameters, such as the hydrolysis temperature or milling time, the composition of the sugar solution can be changed.
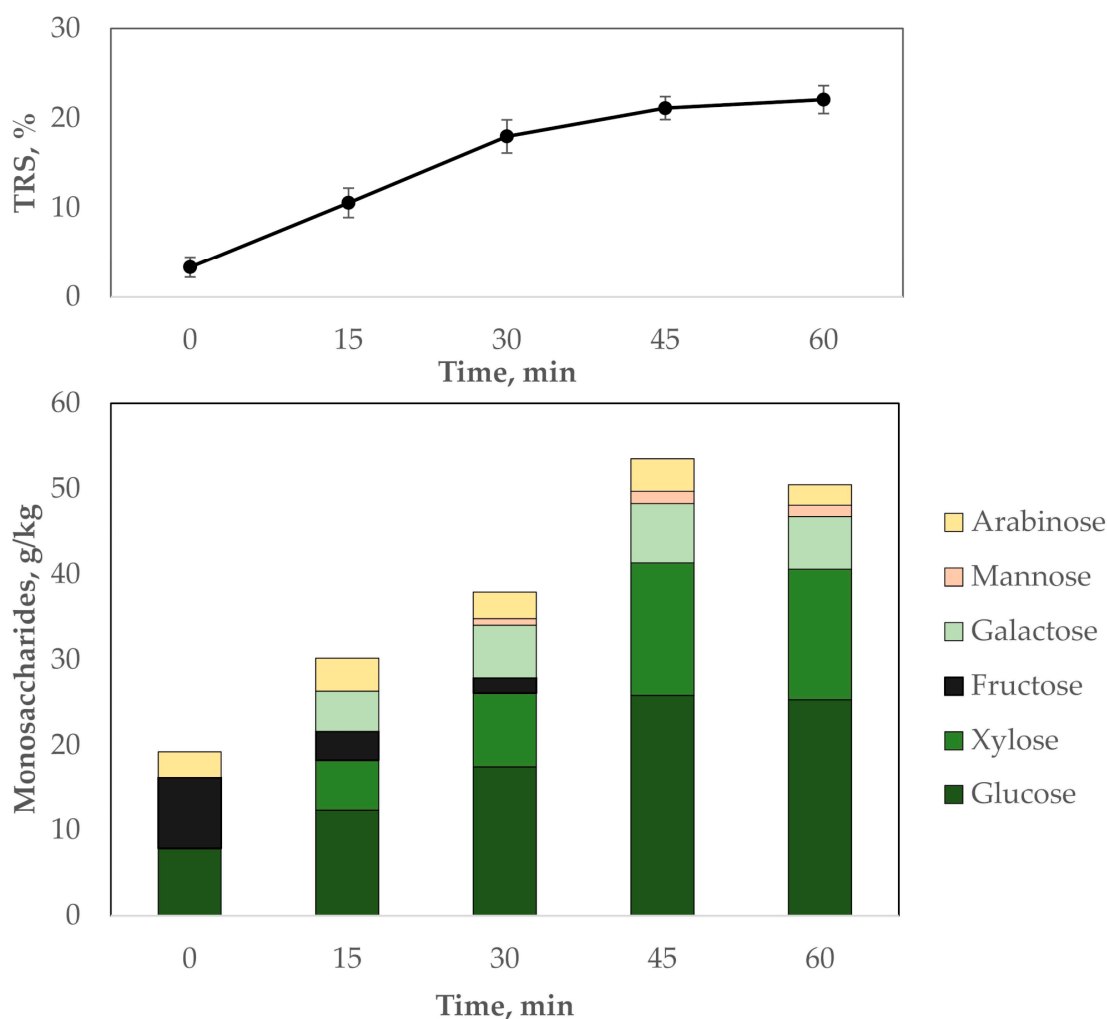
Figure 10. TRS and monosaccharide yields of the mechanocatalytically pretreated willow sample after hydrolysis at 100 °C for 60 min. The mechanocatalytic pretreatment was done with various milling times, ranging from 0 to 60 min in cycles of 5 min of milling and 10-min pauses with the 0.5 mmol/g (wood) acid load. The milling time includes only the periods of milling, not cooling periods.

The impact of the acid load on the TRS and monosaccharide yields were investigated by adding different loads of the acid catalyst during the milling process and hydrolyzing the milled sample at 100 °C for 60 min without further acid addition (Figure 11). The sample was milled for 30 min with 10 mm milling balls and 800 rpm mill speed. As stated, the TRS yield after the pretreatment was highest with the 0.5 mmol/g acid load (15.0%), and it decreased when more acid was applied in the pretreatment process. However, when the hydrolysis step was performed at 100 °C, the process benefited from the higher amount of acid. The highest TRS yield (25.1%) after hydrolysis was achieved when the 1.0 mmol/g acid load was used in the milling process. Whereas when the acid was replaced with distilled water, the TRS yield (3.2%) was less than one-sixth of the highest TRS yield.

The total monosaccharide yield reached its maximum with the 1.0 mmol/g acid load. The glucose, xylose, mannose, and arabinose yields increased when the acid load increased from 0.5 to 1.0 mmol/g (Figure 11), but it did not increase if the acid load was further increased. Fructose (4.7 g/kg) and sucrose (5.7 g/kg) were detected when acid was replaced with distilled water, but no sucrose was detected if some acid (0.5 mmol/g) was added, and no fructose was detected if the acid load was increased to 1.0 mmol/g. The decrease in the amount of sucrose and fructose with high acid loadings may have been due to their degradation into HMF. It has been shown that sucrose inversion to fructose and glucose is a fast reaction, and the reaction of fructose to HMF is 40 times faster than the reaction of glucose to HMF [41]. Some HMF (0.8 g/kg) was produced with the 0.5 mmol/g acid load, but HMF was not detected when the acid load was increased to 1.0 or 2.0 mmol/g. It is possible that with higher acid loads, HFM was reacted into levulinic acid and formic acid [16,40], which were not analyzed in this study.
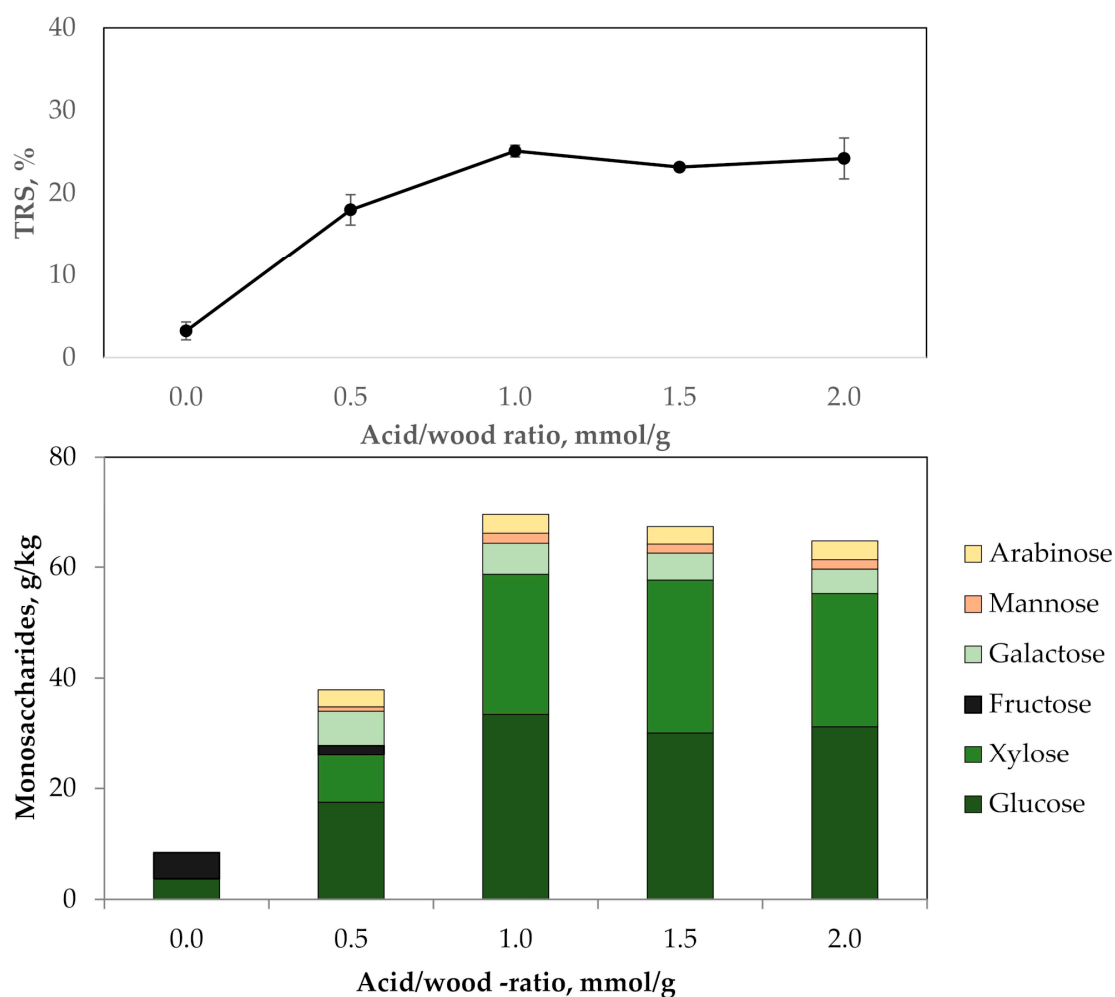
Figure 11. TRS yields of 30 min milled willow with different catalyst loads (0, 0.2, 0.5, 1.0, 1.5, and 2.0 mmol/g). Acid catalyst was replaced with water when the catalyst load 0.0 mmol/g was used.

3. Materials and Methods

3.1. Raw Materials

The willow (Salix phylicifolia) samples examined in this study were collected in Finland. The samples were dried and ground into 0.5-mm particles using a Retsch SM100 Comfort cutting mill (Retsch GmbH, Haan, Germany) and stored in an airtight container. The samples contained mixtures of wood and bark.

The moisture content (5.6 wt %) of the willow samples was obtained by drying the samples in an oven at 105 °C until the weight of the samples was constant. The lignin, holocellulose, and α-cellulose dry matter contents were analyzed according to different methods. The results are presented as the mean values of the duplicates.

Klason lignin was determined after two-step acid hydrolysis of the wood sample according to Sluiter et al.'s method [42]. Acid-soluble lignin was determined with ultraviolet visible (UV-Vis) spectroscopy from the hydrolysate obtained from hydrolysis according to the Erhman procedure [43]. The amount of lignin is presented as the sum of acid-insoluble and acid-soluble lignin.

Holocellulose was prepared from an extractive-free sample with the procedure described by Rowell et al. [44]. Briefly, 1.5 g of extractive-free sample, 48 mL of distilled water, 0.3 mL of acetic acid, (VWR, OH, USA) and 0.75 g of sodium chlorite (Sigma-Aldrich, Steinhein, Germany) were allowed to react in a 100-mL Erlenmayer flask in a water bath (70–75 °C). After 60 min, an additional 0.3 mL of acetic acid and 0.75 g of $NaClO_2$ were added. After each succeeding hour, a fresh portion of 0.3 mL of acetic acid and 0.75 g of $NaClO_2$ was added. After 5 h, the flask was allowed to cool, and the solid holocellulose was filtered. The solid residue was washed with distilled water until the yellowish color was removed. Finally, the solid was washed with acetone (J.T. Baker, Phillipsburg, NJ, USA). The holocellulose was dried in an oven at 105 °C overnight, cooled in a desiccator for 1 h, and weighed. The residual lignin content of the holocellulose was determined with the methods mentioned above.

The α-cellulose content was determined from the holocellulose with previously published procedures [45–47]. Briefly, 0.5 g of holocellulose and 25 mL of 17.5% NaOH (Merck KGaA, Darmstadt, Germany) were placed in a 250-mL beaker flask and stirred until the holocellulose was completely dispersed. Then, the stirrer was removed, and the sample was washed with 5 mL of 17.5% NaOH to make the total reagent content in the flask 30 mL. The flask was stirred thoroughly with a glass rod and left in a water bath at 25 °C. After a period of 30 min from the first addition of the NaOH reagent, 30 mL of distilled water was added to the suspension and stirred thoroughly with a glass rod. The flask was left in the bath for another 30 min. At the end of 60 min, the suspension was stirred and filtered. The solid α-cellulose was washed first with distilled water; then, it was washed with 15 mL of 10% acetic acid and finally with 400 mL of distilled water. The hemicellulose content was obtained by subtracting the α-cellulose content from the holocellulose content.

Sulfuric acid (Merck Suprapur®96%, Merck KGaA, Darmstadt, Germany) was used as the acid catalyst of the mechanocatalytic pretreatment. All chemicals were used without further purification.

3.2. Mechanocatalytic Pretreatment

Willow sawdust (2.0 g) and a different amount of sulfuric acid were put in the 45 mL milling bowl and briefly mixed. The main component of the inner surface of the milling bowl and the milling balls was zirconium oxide. Milling was done with a planetary micro-mill (Fritsch Pulverisette 7 Premium line, ldar-Oberstein, Germany). The mass of the milling balls with a diameter of 10 mm was 2.98 g. If not otherwise mentioned, the following parameters were used. The willow samples were milled with cycles of 5 min of milling and 10 min of cooling, and the milling time was expressed as the total milling time without cooling pauses. The total number of cycles per experiment was six, and the number of milling balls was 16. The rotation speed of the mill was 800 rpm.

As a reference, sulfuric acid was replaced with distilled water. The milling process was performed as mentioned previously. The experiments were made as duplicates.

3.3. Release of Sugars and Acid-Catalyzed Hydrolysis

The sugars released were determined by the following procedure: The pretreated willow sample (0.5 g) was mixed with distilled water (15 mL) at room temperature for 20 min, and precipitates were separated from the sugar solution by filtration. Then, the hydrolysates were analyzed to determine the TRS and the monosaccharides produced. As a reference, willow sawdust was mixed with the

sulfuric acid load without milling, after which it was mixed with distilled water in similar conditions. The solution was filtrated and then analyzed.

The sugars released after hydrolysis were determined as follows. A sample of pretreated willow (0.5 g) was mixed with distilled water (15 mL) and heated in an oil bath at 100 °C for 60 min. After hydrolysis, the precipitates were separated from the sugar solution by filtration before the hydrolysates were analyzed.

3.4. Methods

3.4.1. Determination of Total Reducing Sugars with UV-Vis Spectrometry

The amount of TRS was analyzed according to the DNS method [48]. Sugar solutions with a high sugar concentration were diluted before mixing. The DNS reagent and the sugar solution were mixed in equal proportions. The mixture was heated in a water bath to achieve color development. The mixture was cooled, and distilled water was added to achieve a total volume of 3 mL. The amount of TRS was determined with a UV-Vis spectrophotometer (Ordior Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan) at the 540 nm wavelength based on calibration curves done with glucose standards. The TRS yield was determined with the following equation:

$$\text{TRS} = 100\% \times (c_{TRS} \times V)/m_{wood}, \tag{1}$$

where c_{TRS} is the concentration of sugars in the solution, V is the volume of the solution, and m_{wood} is the mass of processed wood.

3.4.2. Capillary Electrophoresis

Samples were filtered with 0.45 μm GHP Acrodisc syringe filters before the CE analysis. The CE analyses were performed with a P/ACE MDQ CE instrument (Beckman Coulter Inc., Fullerton, CA, USA) equipped with a diode array detector (DAD) and controlled by 32 Karat software (Version 8.0, 2006, Beckman Coulter Inc., Fullerton, CA, USA) using a modified method [49]. Uncoated fused-silica capillaries with an internal diameter of 25 μm and length 30/40 cm (effective length/total length) were used. The samples were injected at a pressure of 0.5 psi for 10 s with a separation voltage of +16 kV. All samples were measured as duplicates, and were diluted, if necessary. To confirm the identification of responses on the electropherogram, each sample was spiked with a standard solution that included HMF, sucrose, galactose, glucose, fructose, mannose, arabinose, and xylose. Calibration curves were created for the external quantification of the analytes in the samples.

3.4.3. Field Emission Scanning Electron Microscope

The mechanocatalytic pretreated powder samples were put on conductive tape and coated with carbon. The microstructures shown in the FESEM images were obtained using a Zeiss Sigma field emission scanning electron microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) operated at 5 kV and with a working distance of around 5 mm.

3.4.4. X-ray Diffraction

XRD patterns were recorded with a PANalytical X'Pert Pro X-ray diffraction equipment (Malvern Panalytical, Almelo, The Netherlands) using monochromatic CuKα1 radiation (λ = 1.5406 Å) at 45 kV and 40 mA. Diffractograms were collected in the 2θ range 7–85° at 0.0167° intervals, and with a scan step time of 100 s. The crystalline phases and structures were analyzed with the X'Pert HighScore Plus program (software Version 4.0, PANalytical B. V., Almelo, The Netherlands). The main peaks were identified with standard Joint Committee on Powder Diffraction Standards (JCPDS) files.

The cellulose crystallinity index (CrI) of the willow sample was calculated according to Segal's method:

$$CrI = (I_{200} - I_{AM}) \times I_{200}^{-1}, \tag{2}$$

where I_{200} is $2\theta = 18°$ and I_{AM} is $2\theta = 22.4°$ [50].

4. Conclusions

In this work, a solvent-free mechanocatalytic pretreatment with sulfuric acid catalyst was used to study its effect on the willow sawdust structure and improve the yield of TRS and monosaccharides from the studied biomass. The pretreatment process decreased the particle size of the willow sawdust and had an effect on the surface of the particles. Thus, the TRS and monosaccharide yields increased, even with lower sulfuric acid loads. However, finding the optimal process parameters was crucial; therefore, various parameters were studied. The ratio of the sulfuric acid catalyst to willow, the milling time and the rotation speed had a considerable effect on the composition of the sugar solution produced after the mechanocatalytic pretreatment and subsequent acid hydrolysis. According to the experiments, 45 min of milling with 800 rpm produced the highest TRS and monosaccharide yields. The excess moisture from the catalyst can be harmful to the process, and too low catalyst load limits the reactions, but the optimal amount enhances the mechanical treatment.

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