THE IMPACT OF PRETREATMENTS ON CELLULOSE FROM SUGAR BEET SHREDS AND ITS SUSCEPTIBILITY TO ENZYMATIC HYDROLYSIS

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This paper investigates the impact of dilute acid and steam pretreatment on cellulose from sugar beet shreds and its susceptibility to enzymatic hydrolysis. The applied pretreatments allowed more than 50% material recovery and more than 90% cellulose recovery. In comparison with the untreated material, dilute acid and steam pretreatment reduced the total crystallinity index of cellulose by 2.2 and 3 times, respectively, while the reduction in the lateral order index was similar and amounted to approximately 4 times. The materials ability to retain water was increased upon the treatments and was more pronounced for the steam pretreated sample. The pretreatments did not considerably change the affinity of cellulases towards cellulose, while changes induced by the applied pretreatments allowed adsorbed enzymes to hydrolyze cellulose efficiently. Cellulose conversion ratio obtained during the hydrolysis of dilute and steam pretreated sugar beet shreds was 378.9 mg/g and 436.3 mg/g, respectively, which was approximately twice higher in comparison with that achieved with the untreated substrate.

Keywords:cellulose, sugar beet shreds, pretreatment, enzymatic hydrolysis

INTRODUCTION

Lignocellulosic biomass is the most abundant organic material in the nature. Its polymeric constituents – cellulose, hemicelluloses and lignin, could be transformed into many valuable products. Among these, cellulose is a compound of special interest due to the value and significance of its main product of degradation – glucose, as well as due to the oligosaccharides that could be obtained from it. Considering the mild process conditions and efficiency, enzymatic hydrolysis is the process of choice for cellulose degradation, which occurs by the catalytic action of cellulolytic enzymes. Cellulosic materials consist of crystalline and amorphous domains in different ratios, depending of the type and source of the material. However, interactions between a solid cellulosic material and water, enzymes or other substances occur first in the non-crystalline domains and/or on the surface of the crystalline regions. Although cellulolytic enzymes could adsorb and bind to the crystalline cellulose by a binding module,[1] their catalytic activity on it is very limited. This result in a low rate and yield of the hydrolysis, which makes it very inefficient.[2,3] Besides, the physical properties, chemical behavior and reactivity of cellulose are strongly influenced by the degree of ordering in its structure, i.e. the arrangement of the cellulose molecules with respect to each other and among fibers. Furthermore, the accessibility of cellulose to the enzymes and its susceptibility to the enzymatic hydrolysis depends on the presence of other polymers in the material, their amount, mutual bonds and bonds with the cellulose. Considering that in lignocellulosic materials cellulose is incorporated in a complex matrix with hemicelluloses and lignin, it is necessary to alter and rupture that structure in order to provide the access of the enzymes to the cellulose.[4,5] Thus, some type of pretreatment is required as the first step in the conversion of lignocellulose to glucose. The pretreatment should prepare recalcitrant biomass for the enzymatic hydrolysis through the physical and/or chemical alteration of the material. Considering the large diversity of the lignocellulosic materials, which have different characteristics, it is necessary to adopt an appropriate pretreatment for each type.[6] Namely, depending on the biomass type and composition, different pretreatments can be applied in order to achieve a defined outcome of the process. Generally, a pretreatment should open up the

complex lignocellulosic matrix by removing some of the components and disturbing their mutual bonds, in order to expose cellulose and allow its contact with cellulases. One of the important goals of the pretreatment should also be the alteration of cellulose itself, by reducing its degree of ordering and the crystallinity of its structure.

Sugar beet shreds, a waste product from the sugar industry, are widely available ligno-cellulosic biomass in Serbia.[7] Besides pectin (24-32%), sugar beet shreds have high contents of other carbohydrates (cellulose 22-30% and hemicelluloses 22-30%), and low lignin content (1-3%),[8] which makes them a feedstock potentially susceptible to biochemical transformation.

The aim of this study was to investigate the impact of dilute acid and steam pretreatment on the cellulose from sugar beet shreds, and perceive it through the influence on the susceptibility to enzymatic hydrolysis. The influence of pretreatments on substrate recovery was determined, while the appearance of the obtained substrates was analyzed through SEM micrographs. Cellulose recovery upon treatments, as well as reduction in the degree of ordering and crystallinity of cellulose were calculated. The ability of materials to retain water after the applied pretreatments was also investigated. The time courses of the enzymatic hydrolysis of untreated and both pretreated substrates were monitored, and the adsorption parameters derived from the Langmuir adsorption isotherm were determined.

EXPERIMENTAL

Sugar beet shreds

In this study, sugar beet shreds were a kind gift from"A.D. Šajkaška" sugar factory, Serbia, and were used as raw material. Sugar beet shreds were milled and sieved as previously reported[9] and particles in the range 224-400 μm were subjected to the pretreatment.

Pretreatment of sugar beet shreds

Dilute acid pretreatment was applied in order to remove pectic substances. Sugar beet shreds were suspended in HCl solution at pH 1.5 and 85 ºC for 4 hours.[10] After cooling down, the mixture was filtered through Macherey-Nagel MN 651/120 laboratory filter paper and the filter cake was washed with distilled water[11] in order to remove HCl.

Steam pretreatment was conducted in a laboratory autoclave at a pressure of 2.1 bars for 30 minutes. Sugar beet shreds were mixed with distilled water at 1:20 ratio (w/v) and subjected to the pretreatment. The solids obtained after pretreatment were washed with distilled water until the filtrate contained no more sugars, which were determined as reducing sugars by the DNS (dinitrosalicylic acid) method[12] with glucose as a standard.

The materials obtained after both pretreatments were dried at 60 °C until achieving 100% dry matter and then subjected to the characterization or enzymatic hydrolysis. Cellulose content in untreated and pretreated sugar beet shreds was determined according to the procedure reported by Crampton and Maynard,[13] based on the removal of non-cellulosic components achieved during digestion of the sample with an acetic acid/nitric acid mixture.

Water retention value

The water retention value (WRV),expressed as gram of water per gram of dry substrate, was determined for untreated, as well as for dilute acid and steam pretreated sugar beet shreds and calculated as follows:

$$WRV = \frac{w_{wet} - w_{dry}}{w_{dry}} \quad (1)$$

In Equation (1),w_{dry} represents the mass of the dry material, while w_{wet} represents the mass of the material with retained water. The mass of the material with retained water was determined by soaking the substrate in water for three hours, after which the suspension was subjected to centrifugation at 3000 rpm for 15 minutes in a laboratory centrifuge (Sorvall RC-5B Refrigerated Super Speed Centrifuge)[14] and wet solids mass was measured.

Enzyme adsorption

Cellulases adsorption onto dilute acid or steam pretreated sugar beet shreds was conducted by varying the amount of cellulases protein added to the constant solids load of 2% (w/w) at pH 4.8 and at 4 °C to prevent hydrolysis. Contact time was 60 minutes, which was previously determined as time needed to reach equilibrium, and during that period no proteins were released from the investigated materials (data not shown). The content of proteins in the supernatant was determined as free cellulases in the solution. The amount of enzymes adsorbed onto the substrate was calculated as the difference between the total amount of enzymes added to the reaction mixture and the amount of free (unadsorbed) enzymes in the solution. The experimental data were fitted to the following Langmuir adsorption isotherm:

$$q = \frac{C_f \times \sigma \times b}{1 + C_f \times b} \quad (2)$$

where C_f is the equilibrium enzyme concentration (mg/mL), σ is the maximum content of absorbed enzyme (mg/g substrate) and b is the Langmuir constant (mL/mg).[15]The Langmuir adsorption isotherm was used to determine the maximum content of absorbed enzyme and the Langmuir constant. The concentration of enzymes for the adsorption study was measured by determining the proteins using the Bradford method[16] with BSA (bovine serum albumin) as a standard.

Enzymatic hydrolysis

Pretreated and dried sugar beet shreds were enzymatically hydrolyzed using commercial cellulases, Celluclast 1.5L (Novozyme), with a dosage of 20 FPU/g substrate (dry weight), at a solids load of 2%(w/w), pH 4.8 and temperature of 45 °C. Hydrolysis time was 24 hours and samples were withdrawn at the start and after 3, 6, 9, 12, 18 and 24 hours of hydrolysis, and subjected to the measurement of sugar concentration. The sugars obtained during hydrolysis were analyzed by a Waters HPLC system, equipped with a Waters RI detector, according to the NREL procedure.[17] Cellulose conversion ratio, which was expressed as mg of sugars per gram of cellulose, was calculated as follows:

$$Cellulose\ conversion\ ratio = \frac{[(C]_{gl} + 1.053 \times C_{clb}) \times V}{m_{sub} \times f_{cell}} \quad (3)$$

where C_{gl} is the concentration of glucose (mg/mL), C_{clb} is the concentration of cellobiose (mg/mL), which is multiplied with a correction factor of 1.053 (for its conversion to glucose),[18] V is the volume of the reaction mixture (mL), m_{sub} is the mass of the substrate in the overall reaction mixture (g) and f_{cell} is the mass share of cellulose in the substrate.

FTIR spectroscopy

The FTIR absorption spectra from 4000 to 400 cm^{-1} were collected in transmission using a Thermo-Nicolet Nexus 670 FTIR spectrometer (Waltham, MA, USA) at a resolution of 4 cm^{-1}; the number of scans was 32. The pellets subjected to the analysis were obtained by mixing solid samples with KBr. Total crystallinity index (TCI) was determined as the ratio of the peak height at 2900 $cm^{-1}$and 1372 cm^{-1}, as explained and applied earlier.[2,19,20] Lateral order index (LOI) was determined as the ratio of the peak area at 1430 cm^{-1} and 898 cm^{-1}, as explained and applied earlier.[2,19,20]

SEM micrography

SEM micrographs were recorded by a scanning electron microscope (SEM JEOL, 6460LV) operating at 20 kV, while the samples were prepared by the ion sputtering coating method.

RESULTS AND DISSCUSSION

Cellulose content, material recovery and water retention upon pretreatments

It has been shown that pretreatment efficacy is associated with altering biomass structure through dislocation and/or physical removal of lignin, hemicelluloses and other components.[6,21]Also, a pretreatment should preserve cellulose in the terms of its presence in the substrate, but it should decrease its crystallinity and ordering degree. Dilute acid pretreatment used in this study was intended to remove pectin from sugar beet shreds, under conditions that should not considerably affect other biomass constituents.[10] On the other hand, the result of a steam pretreatment should be softening of the cell wall through physical rupture of biomass fibers. This should cause different cross linking between the carbohydrate polymers and/or between lignin and carbohydrate polymers, as well as partial solubilization of hemi-celluloses.[5,22,23]

The material and cellulose recovery upon pretreatments, as well as the cellulose content and water retention value, for untreated and pretreated sugar beet shreds are shown in Table 1.

It could be noticed that after pretreatments material recovery was higher than 50%, although it was slightly lower (for 7%) after dilute acid treatment. Cellulose in sugar beet shreds after pretreatments was preserved well and, as calculated from the mass balance, its recovery amounted 97.5% for dilute acid pretreatment. This result is similar to the one obtained by Xie *et al.*[24] for corncob pretreated with dilute sulphuric acid. Cellulose recovery for steam pretreated material was 94%, indicating that a simultaneous increase of temperature and pressure had a slightly stronger negative influence on cellulose preservation. Thus, dilute acid pretreatment provided sugar beet shreds with cellulose content of 41.03%, while cellulose content after steam pretreatment was 34%.

Water is recognized as a crucial factor for enzyme function in the hydrolysis, being a reaction substrate. In addition, water is important for enzyme transport and overall mass transfer between the (solid) substrate and the surrounding solution.[25] The material's capacity to retain water could be expressed as water retention value (WRV). Thus, being so important, WRV was determined for different lignocellulosic materials. For example, the WRV of commercial microcrystalline cellulose was reported to be approximately 0.7 g/g, while different applied

pretreatments generally increased its value from 1.5 to 4.7 times.[26] Moreover, the WRV of a softwood lignocellulosic substrate was approximately 1.4 g/g even after pretreatment.[27]

Water retention values for untreated and dilute acid and steam pretreated sugar beet shreds are shown in Table 1. The results showed that untreated and both pretreated substrates generally had higher WRV values in comparison with other lignocellulosic substrates.[26-28] Compared to the WRV of the untreated substrate, both pretreatments increased the capacity of sugar beet shreds to retain water, which for steam pretreated ones was 1.8 times higher. In addition, the water retention value of steam pretreated was higher in comparison with that of dilute acid pretreated sugar beet shreds. Thus, the applied pretreatments provided materials with the ability to retain higher amounts of water in their structure and pores, and this effect was more pronounced in the steam pretreated sugar beet shreds.

The appearance of the untreated and pretreated sugar beet shreds could be observed in the SEM micrographs shown in Fig. 1. The untreated material (Fig. 1A) had a compact and more flattened surface in comparison with the pretreated ones. The surface of the materials obtained after pretreatments (Fig. 1 B and C) was disturbed and ruptured, with exposed internal structures, which were separated from the surrounding surface. This corresponds well to the mass balance of the materials presented in Table 1.

Degree of crystallinity and ordering in cellulose of untreated and pretreated sugar beet shreds

The FTIR spectra of untreated and pretreated sugar beet shreds were recorded as described above. The regions of the FTIR spectra of the investigated substrates, which are relevant for cellulose crystallinity/amorphicity and ordering, are shown in Fig. 2, and the bands related to these characteristics of cellulose have been analyzed. The changes in the cellulose structure upon pretreatments, which might influence the enzymatic hydrolysis, could be perceived through the changes in the bands analyzed. An alteration of the crystalline organization led to the reduction in the intensity of the bands characteristic of the crystalline domains, the bands at 1430 cm^{-1} and 1372 cm^{-1}.[2] On the other hand, the presence of amorphous cellulose can be confirmed by the band at 2900 cm^{-1}, which corresponds to the C–H stretching vibration and by the strong decrease in the intensity of this band. The FTIR absorption band at 898 cm^{-1}, assigned to the C–O–C stretching at β-(1→4)-glycosidic linkages, is designated as an amorphous absorption band and an increase in its intensity is related to an increase in the content of amorphous cellulose.[2,20]

Both applied pretreatments caused a decrease in intensity of the bands at 2900 cm^{-1}, 1372 $cm^{-1}$and 1430 cm^{-1}, along with an increase in intensity of the band at 898 cm^{-1}.

Table 1
Material recovery, cellulose content and recovery and water retention of untreated and pretreated sugar beet shreds

Sugar beet shreds	Substrate recovery (%)	Cellulose content (%)	Cellulose recovery (%)	WRV (g/g)
Untreated	/	24.25	/	4.73
Dilute acid pretreated	58.00	41.03	97.50	5.30
Steam pretreated	65.00	34.00	94.00	8.60

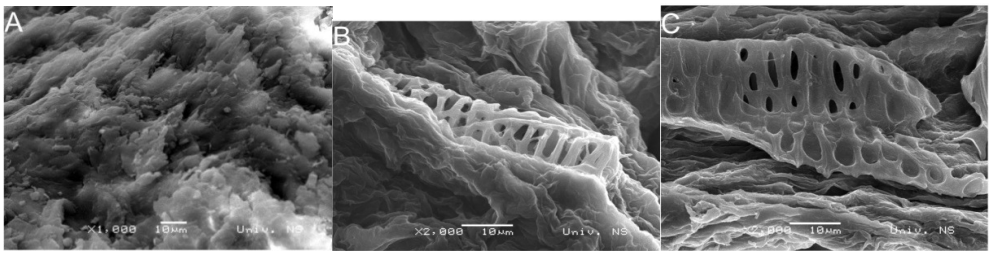

Figure 1: SEM micrographs of untreated (A), dilute acid (B) and steam pretreated (C) sugar beet shreds

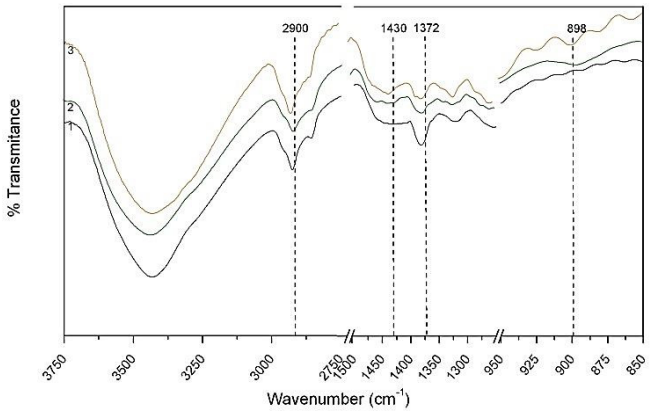

Figure 2: Regions of the FTIR spectra of untreated (1), dilute acid (2) and steam pretreated (3) sugar beet shreds

Table 2
TCI and LOI values for untreated and pretreated substrates

Sugar beet shreds	TCI H1372/H2900	LOI A1430/A898
Untreated	1.13	24.1
Dilute acid pretreated	0.51	6.05
Steam pretreated	0.38	6.42

These changes indicate a decrease in the cellulose crystal regions and an increase in amorphous cellulose, and were more pronounced in the material obtained by steam pretreatment.

In addition, the ratio between the peak heights of the bands at 1372 cm^{-1} and 2900 cm^{-1}, proposed by Nelson and O'Connor[19] as total crystallinity index (TCI),[29] was used to evaluate the infrared crystallinity ratio, which is proportional to the crystallinity degree of cellulose.[26] Besides that, the ratio between the peak areas of the bands at 1429 $cm^{-1}$and 897 cm^{-1} is marked as the lateral order index (LOI),[23] and is correlated to the overall degree of ordering in cellulose.[29,30] The values of TCI and LOI for untreated and dilute acid and steam pretreated sugar beet shreds are shown in Table 2. It could be noticed that the applied pretreatments reduced both parameters related to cellulose crystallinity and complexity. The degree of cellulose crystallinity (TCI) was twice lower in dilute acid and three times lower in steam pretreated sugar beet shreds in comparison with the untreated ones. The degrees of ordering (LOI) of the pretreated materials were similar and almost four times lower in comparison with the untreated material. Similar results with respect to the TCI and LOI reduction were obtained upon pretreatments of the corn stover and bamboo powder.[31,32] The obtained results indicated that steam pretreatment had a more pronounced effect in reducing cellulose crystallinity and ordering in the cellulose fibers. As explained before, this is important for the enzymes' action during the forthcoming enzymatic hydrolysis.

Enzyme adsorption onto untreated and pretreated sugar beet shreds

An important parameter that governs the rate of the cellulose enzymatic hydrolysis is the adsorption of cellulases onto the solid substrate.[33,34] The adsorption of cellulases depends on the structural properties of cellulose, which are related to its source and the pretreatment applied.[32] Adsorption parameters, the maximum content of adsorbed enzymes and the Langmuir constant for the untreated as well as dilute acid and steam pretreated sugar beet shreds were determined and results are shown in Table 3.

The results showed that the Langmuir constant, being an indicator of cellulases affinity towards the substrate (b)[15], was slightly changed upon the pretreatments. However, the highest amount of the enzymes was adsorbed onto untreated sugar beet shreds, while the amount of the enzymes, which were adsorbed onto the substrate obtained after steam pretreatment, was approximately 4 times lower in comparison with

that of the dilute acid pretreated substrate. The higher amount of adsorbed enzymes onto the untreated substrate could be attributed to the (unproductive) binding of the cellulases onto lignin[35] or even likely to a binding of some cellulases to the crystal regions of cellulose.[1,36] As shown earlier (Table 2), the untreated substrate had multiple times higher cellulose crystallinity indicated by the higher TCI in comparison with that obtained for the pretreated one. Besides lower crystallinity, both pretreated substrates had higher amorphicity, as indicated by the increase in intensity of the band at 898 cm^{-1}, which made them more suitable substrates for the enzymatic hydrolysis (Fig. 3), despite the lower maximal (theoretical) amount of adsorbed enzymes.

Enzymatic hydrolysis of untreated and pretreated sugar beet shreds by cellulases

The substrates pretreated by the dilute acid or steam methods, as well as the untreated sugar beet shreds, were subjected to the cellulases action during enzymatic hydrolysis in order to estimate the impact of the applied pretreatments on the hydrolysis and its yield. The time courses of the enzymatic hydrolysis of the untreated sugar beet shreds and of those pretreated with dilute acid and steam are presented in Fig. 3. Cellulose conversion ratio, as a measure of the enzymatic hydrolysis yield, was calculated as the ratio between the sum of glucose and cellobiose mass, and the mass of cellulose (Eq. 3).

Both applied pretreatments had a positive effect on the enzymatic hydrolysis of sugar beet shreds, providing a cellulose conversion ratio approximately twice higher in comparison with the one obtained with the untreated substrate. The final cellulose conversion ratio obtained after 24 hours of sugar beet shreds hydrolysis was 378.9 mg/g cellulose for the dilute acid and 436.3 mg/g cellulose for the steam pretreated substrate, while for the untreated substrate conversion it was 200 mg/g cellulose. Considering that all experiments were conducted simultaneously and under the same conditions, the differences in the conversion ratio might be assigned to their different structures produced by different pretreatments. This might be attributed to the rupture of the substrate complex matrix (Fig. 1), as well as to the reduction in the degree of cellulose ordering and crystallinity (Table 2) upon the applied pretreatments, which was previously noticed and related for some lignocellulosic substrates.[1,34,35] In addition, the obtained better performance of the enzymes at steam pretreated substrate might be a result of the lower degree of cellulose crystallinity in that substrate.[37] As mentioned before, the degree of cellulose crystallinity is very important for the efficiency of the cellulose enzymatic hydrolysis due to the cellulases' poor ability to degrade it.[1,3] Furthermore, due to the importance of water in the microenvironment of cellulases-cellulose reaction, the higher capacity to retain water of the steam pretreated sugar beet shreds (more than 60% in comparison with the dilute acid pretreated ones) might be one of the reasons for the higher performance of the enzymes during cellulose hydrolysis from this substrate.

Table 3
Langmuir parameters for cellulases adsorption onto differently treated sugar beet shreds

Sugar beet shreds	σ (mg/g substrate)	b (mL/mg)
Untreated	13.89	1.02
Dilute acid pretreated	10.20	1.10
Steam pretreated	2.50	1.05

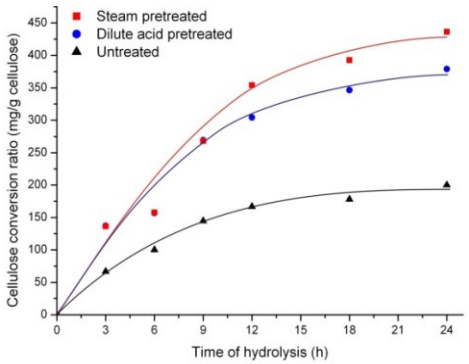

Figure 3: Time course of the enzymatic hydrolysis of untreated, dilute acid and steam pretreated sugar beet shreds

A similar observation with respect to the relation between the water retention value and the hydrolysis yield of cellulose fibers was previously reported by Jacquet *et al.*[26]

CONCLUSION

Sugar beet shreds were subjected to dilute acid or steam pretreatment in order to determine the impact of the pretreatment on the substrate characteristics, especially those related to the cellulose and its susceptibility to the forthcoming enzymatic hydrolysis. Both pretreatments allowed the recovery of more than 50% of the material, along with good cellulose preservation, although cellulose loss was slightly higher in the material treated with steam. Cellulose crystallinity was multiple times lower in the sugar beet shreds that were subjected to the pretreatments in comparison with the untreated material, while steam pretreatment had a stronger impact compared to the dilute acid one. The degree of ordering in cellulose from sugar beet shreds was also decreased upon the pretreatments, and it was similar for both applied treatments. The dilute acid and steam pretreatment had a positive effect on the enzymatic hydrolysis of the obtained substrates, allowing the achievement of sugar yields that were approximately twice higher in comparison with the yield obtained from the untreated substrate. The untreated substrate had a higher amount of maximal (theoretical) adsorbed enzymes in comparison with the pretreated ones. However, the amount of the enzymes adsorbed onto the pretreated substrates was enough to achieve efficient hydrolysis, probably due to the changed structure of cellulose in them. In comparison with the untreated sugar beet shreds, the pretreated ones had decreased crystallinity and degree of cellulose ordering, which, along with the increased capacity to retain water, made these substrates more susceptible to the enzymes action.

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