

## **FREEZE-THAW PRETREATMENT OF POPLAR SAPWOOD DUST**

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### **ABSTRACT**

The paper is focused on the effect of freezing and cyclic freezing-thawing pretreatment of poplar sapwood (*Populus alba* L.). The experimental comparison was carried out by the sawdust fraction 0.7 mm as (a) water-saturated and (b) dry. Monosaccharide yields, as well as an amount of acetic acid, were measured after 6, 24, 48, 72, and 96 hours of enzymatic hydrolysis with 15% load of the enzyme measured to total cellulose content. The influence of freezing rate on total yields was observed on equally prepared samples with different weights (31 g, 25 g, 62.5 g, 125 g, 250 g, 500 g, and 1000 g) by "cubic" tests. To increase the efficiency of pretreatment, a cyclic freezing-thawing experiment at temperatures -20°C and +25°C was performed. The results show that single freezing of grounded poplar sapwood impregnated by water or dry in its matter is not a sufficient pretreatment method, so cyclic freeze-thaw is needed to enhance the yield of monosaccharides. Analysis of cubic test showed that slower freezing process has a positive effect on enzyme accessibility.

**KEYWORDS:** Pretreatment, freezing-thawing, poplar, *Populus alba* L., monosaccharides, sapwood, wood fractions.

### **INTRODUCTION**

The pretreatment is required to increase the accessibility of enzymes in lignocellulosic materials (LCM). The pretreatment methods are divided into: physical (milling, extrusion, microwave, cryolysis), chemical (acid pretreatment, alkaline pretreatment, ionic liquid pretreatment, organosolv pretreatment, ozonolysis), physicochemical (steam explosion pretreatment, ammonia fiber explosion pretreatment, CO<sub>2</sub> explosion pretreatment, liquid hot water, wet oxidation) and biological (fungi), and combination of these methods (Mood et al. 2013, Gigac et al. 2017, Stankovská et al. 2018).

This paper focuses on a lighter form of cryolysis at lower freeze temperatures, i.e., physical pretreatment. The principle of this method is to change the volume of water during freezing. The

biomass mixed with water is put into a freezer until the whole material is frozen. Water diffused into the biomass during impregnation process increases the volume during freezing, thereby disrupting the cell walls' structure. Results in the opening and rising of the biomass surface for enzymatic hydrolysis (Rooni et al. 2017). The crystallization rate is an essential parameter for cryolysis because of a longer time for crystals to grow results in a larger size (Moore and Molinero 2011). In most publications, agricultural waste is used as a substrate, such as rice straw (Chang et al. 2011, Deng et al. 2018), wheat straw (Wang et al. 2013, Ihnát et al. 2015, Pažitný et al. 2019a, Sasaki et al. 2020), barley straw (Rooni et al. 2017), corncobs and corn stalks (Echeverria et al. 2018, Yuan et al. 2019, Li et al. 2019a). Other types of cryolysis substrate were: grasses, such as switchgrass (*Panicum virgatum*) (Yang et al. 2009), rush (*Juncus maritimus*) (Smichi et al. 2016), waste cotton towels (Sasaki et al. 2020), and poplar wood (Zhu et al. 2020). The cellulose content of all these materials is comparable, including grasses such as switchgrass (Pažitný et al. 2013). However, it can be mildly higher in wood materials. In addition, these materials reach relatively low basic density compared to hardwood deciduous species (Petráš et al. 2019) which is suitable in pretreatment processes. Waste grade paper (Russ et al. 2013) may be an efficiently processing substrate due to its advanced microstructure opening stage. and also algal biomass, because the cell walls of algae (including microalgae) do not contain lignin, thus, they are less recalcitrant to degradation, than lignocellulose materials (Yazdani et al. 2015, Halaj et al. 2018).

For more compact wood material, freezing is combined with inorganic solvents in more publications. Jeong et al. (2016) observed the effect of freezing on Mongolian oak (*Quercus mongolica*) impregnated by 1% H<sub>2</sub>SO<sub>4</sub>, also was studied a combination of freezing-thawing (from -20 up to 20°C) with added ammonia (Li et al. 2019b) or added NaOH (Su and Fang 2017, Sasaki et al. 2020). Finer chemical processes that are more gentle on the wood substrate can be used (Balberčák et al. 2017, 2018).

With growing government, academic, and commercial research on the production of biofuels from lignocellulosic biomass, several species have appeared as front-runners in this field. This paper focuses on white poplar (*Populus alba* L.), also known as Silver poplar or Silver leaf poplar. Poplars are fast-growing trees, with various utilizations, like biofuels, pulp, paper production, and other biobased chemicals (Tuskan 2006).

Generally, LCM are composed of cellulose (35-50%), hemicelluloses (20-35%), lignin (15-20%), ash, and other components (15-20%) (Mood et al. 2013). Cellulose and hemicelluloses in the LCM create a complex that is an obstacle to enzyme access under natural conditions. Moreover, the cellulose-hemicellulose complex is encapsulated by lignin, which increases the biomass hydrolysis barrier (Taha et al. 2016). In order to obtain fermentable sugars, pretreatment of LCM before enzymatic hydrolysis is needed to decompose the lignin-cellulose-hemicellulose complex in macroscopic, submicroscopic, and microscopic structure (Fu and Mazza 2011). Another hurdle to enzymatic hydrolysis and subsequent fermentation of LCMs is the generation of lignocellulose-derived by-products that inhibit these reactions. These by-products are formed by the decomposition of hemicelluloses and lignin, which include aliphatic acids (e.g., sugar acids, formic acid, acetic acid, levulinic acid), furan derivatives (e.g., hydroxymethylfurfural, furfural), and phenolic compounds (Jönsson et al. 2013, Palmqvist and Hahn-Hägerdal 2000). The content of cellulose and lignin in wood gradually increases with age, while the content of hemicellulose composed of pentosans decreases (Pažitný et al. 2020).

## MATERIAL AND METHODS

### Material

The White poplar (*Populus alba* L.) wood was obtained from the Bratislava area (SK). The 12-year-old wood cut-out had a significantly different core and outer part. The harvesting of the material was performed in November. The enzyme complex Cellic<sup>®</sup>CTec3 was supplied by Novozymes A/S (Bagsværd, Denmark). The enzyme activity was measured to be 1700 BHU (Biomass Hydrolysis Units)/g product in the laboratory.

### Methods

#### *Sample preparation*

The timber of white poplar was stripped of leaves, debarked, separated to heartwood and sapwood. Only sapwood was used for the experiment. The wood's separated parts were crushed for chips with a thickness below 0.3 mm and dried under laboratory temperature for more than two months. The chips were then dry milled in a Brabender mill (Brabender<sup>®</sup>, GmbH & Co. KG, Germany) with a bottom sieve 0.7 mm mesh. The fraction composition of wooden dust 0.7 mm was determined by sieve tester after 5 min of sieving.

#### *Determination of chemical composition*

The content of ash was determined according to ISO 1762, the content of extractives in dichloromethane and hot water according to Tappi T 204 cm-94, and Tappi T 207 cm-08. The Tappi T 222 om-98 was used for the determination of Klason lignin, and Tappi UM 250 for the determination of acid-soluble lignin. The holocellulose content in the analyzed samples was determined according to Wise's method, and cellulose content according to ISO 692.

#### *Determination of content of elements*

The content of elements K, Ca, Mg, Na, Fe, Mn, Zn, Cu in-branch timber was determined by atomic absorption spectroscopy (AAS) with flame atomization according to Tappi T 266. To determine the content of the elements, a part of the obtained ash was dissolved in hydrochloric acid. The phosphorus content was determined by spectrometry with ammonium molybdate, according to STN EN ISO 6878. The content of Si in the wood was determined from the insoluble part by gravimetric determination of SiO<sub>2</sub> after dissolving in hydrofluoric acid.

#### *Freezing pretreatment*

A quick determination of the dry matter 94.2% in samples was performed on moisture analyzer Denver IR35 that uses infrared heating. For the comparison of experimental results, samples for freezing were prepared as sawdust saturated by water, and dry (unsaturated) sample as a blank: (a) 12.5 g of sawdust of absolute dry weight + 86.7 ml of distilled water (full saturated cells), (b) 12.5 g of sawdust of absolute dry weight (moisture content of 5.8%). The samples were prepared in closed PET bags and placed for impregnation into the orbital shaker-incubator 24 hours at 60°C. Freezing of sealed PET bags was performed at -20°C for 24 hours. After freezing, the samples were thawed for 24 hours as well.

#### *Enzymatic hydrolysis*

Enzymatic hydrolysis was performed using Cellic Ctec3 at 15% w/w (g Cellic Ctec3/100 g suspension) in orbital shaker-incubator ES-20/60 (BioSan Ltd., Republic of Latvia) at 50°C, pH = 5.0 for 48 hours. The pH value was regulated during the hydrolysis process using 0.1 N

sulphuric acid or 0.1 N sodium hydroxide. Sampling from hydrolysates for the determination of monosaccharides was performed after 6, 24, 48, 72, and 96 hours.

#### *Determination of monosaccharides concentration*

The determination was carried out according to the procedure of the National Renewable Energy Laboratory (Sluiter et al. 2008). Monosaccharides (glucose, xylose, and arabinose) in hydrolysates were determined by HPLC with Rezex ROA (organic acid) H+ column, with 0.005 N sulphuric acid as the mobile phase during flow rate of 0.5 mL·min<sup>-1</sup> and temperature 30°C. Chromatography data were collected and analyzed by the software Clarity version 5.3.0.180 (DataApex Ltd., Czech Republic).

### Freezing kinetics

The experimental analysis of freezing rate was carried out on samples of (*Populus alba* L.) wood dust of 0.7 mm fraction, prepared according to the upper described procedure. The experiments were carried out with samples of weight 31 g, 25 g, 62.5 g, 125 g, 250 g, 500 g, and 1000 g. The samples were wholly impregnated by distilled water and pretreated by cryolysis at various freezing rates in prepared laboratory cubes (Fig. 1). The laboratory cubes were put into a laboratory freezer with a constant temperature of -20°C. After cryolysis, the samples were subjected to enzymatic hydrolysis at temperature 50°C, the concentration of 12.5% of absolute dry weight /100 g of heterogeneous mixture water-biomass) with added 15 g of enzyme CELLIC® CTec3 for 100 g of the sample's absolute dry weight. After 48 hours of hydrolysis, the contents of monosaccharides (glucose, xylose, and arabinose) as products were analyzed.

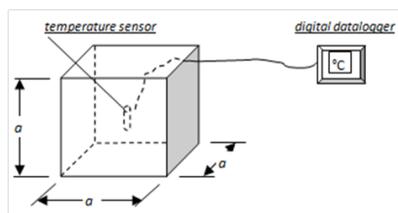


Fig. 1: Assessment of the laboratory analysis of freezing rate ("cubic test"). Cubes made of polycarbonate boards 5 mm were dimensioned (the measure "a") so that their volume corresponds to the weights of the samples (31 g, 25 g, 62.5 g, 125 g, 250 g, 500 g, and 1000 g).

### Cyclic freezing and thawing

The effect of cyclic freezing and thawing was performed under conditions defined in Tab. 1 on samples of (*Populus alba* L.) wood sawdust of 0.7 mm fraction, prepared according to the procedure upper described.

Tab. 1: Conditions for the cyclic freezing and thawing pretreatment of wood sawdust 0.7 mm of *Populus alba* L. (sapwood).

Freezing -20°C	Thawing +25°C
1 <sup>30</sup> hour	1 <sup>30</sup> hour

Samples were prepared as 12.5 g absolutely dried wood sawdust 0.7 mm in PET bags impregnated by 86.7 ml distilled H<sub>2</sub>O. The same procedure, as previously mentioned, was used.

## RESULTS AND DISCUSSION

Poplar is one of the trees with the highest growth rate and the highest heating values; its hardwoods are also one of the most utilized in several countries. The poplar timber is used in the furniture and paper industries and for pallets and plywood production. For the accessibility of the enzyme, the fraction with the smallest particles was used to attain a highly reactive surface (Pazitny et al. 2019b). Authors focused on pretreatment of lignocellulosic materials used fine straw fractions mesh 0.25 mm (Wang et al. 2013), but whole stems and leaves of switchgrass were placed for long-term freezing subsequently reduced in size on a 2 mm screen (Yang et al. 2009). The sieve tests determined four fractions of the fractional composition. (Tab. 2).

Tab. 2: Sieve tests of poplar wood (sapwood) milled on knife mill with a bottom sieve of 0.7 mm mesh.

Sieve mesh (mm)	0.7 – 0.4	0.4 – 0.25	0.25 – 0.18	0.18 – 0	Total
Poplar - sapwood	9.7 g	15.7 g	13.4 g	11.2 g	50.0 g

The fraction of mesh 0.4 – 0.25 mm was representative (30%). The sufficient fraction that should be the most available for the enzymes occupies below 20% (Fig. 2). Content of fine fractions after dry milling, obtained using knife mills as Brabender, is higher if the wood mass is dried before the process used in this case (dry matter 94.2%). Another possibility to disrupt the wood mass is wet milling (Ihnát et al. 2018), however, this method usually requires to increase temperature (Pažitný et al. 2019). On the other hand, the total impregnation of very dried wood material is difficult, causing complications during the enzymatic hydrolysis.

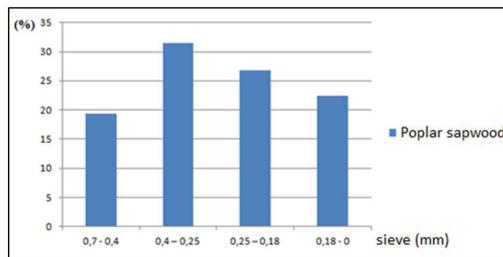


Fig. 2: Fractional composition of poplar sawdust 0.7 mm (%).

Tab. 3 shows the chemical composition of poplar sapwood determined by chemical analyses according to appreciated methods. The content of cellulose in the poplar sapwood is 49.9% w/w o.d., the content of hemicellulose is 25.4%, the total content of lignin and extractives were 21.6% and 2.3%, resp. The cellulose content in *Populus alba* in the literature ranges between 52% – 56% and hemicellulose 15% – 23%, the content of lignin 16% – 20%. The range of extractives in *Populus alba* in literature was found as 7% – 10%. Content of ash was determined by Gökkaya et al. (2020) as 2.6%. The results reveal that the contents of principal components (cellulose, hemicellulose, and lignin) correspond to results already published, but the contents of extractives and ash were different. However, it must be mentioned that various combinations of solvents for extraction were used for their determination. The contents of cellulose, hemicellulose, and lignin in various species of poplars range from 42% to 56%, 15% to 26%; and 16% to 27%, respectively (Rowell et al. 2005, Gökkaya et al. 2020, Rego et al. 2019). The content of chemical elements in poplar sapwood ash (*Populus alba* L.) is shown in Tab. 4.

Tab. 3: Chemical composition of poplar sapwood (*Populus alba* L.).

	White poplar sapwood	Methods
Ash (%)	0.87	ISO 1762
<b>Extractives</b>		
Dichloromethane (%)	0.64	Tappi T 204 cm-94
Hot water (%)	1.63	Tappi T 207 cm-08
Total (%)	2.27	
<b>Lignin</b>		
*Klason (%)	19.31	
Acid-soluble (%)	2.24	Tappi UM 250
Total (%)	21.55	
Holocellulose (%)	75.30	Wise method
Cellulose (%)	49.90	ISO 692

\*Klason lignin is corrected to the ash content of Klason lignin.

Tab. 4: The content of chemical elements in poplar sapwood ash (*Populus alba* L.).

<i>Populus alba</i> (sapwood)			
Chemical element	Content (mg·kg <sup>-1</sup> ) a.d.	Chemical element	Content (mg·kg <sup>-1</sup> ) a.d.
K	1289.6	Fe	8.3
Ca	792.2	Mn	1.1
Mg	230.8	Si	43.6
Na	83.4	Zn	20.6
P	55.1	Cu	3.5

\*a.d. – absolutely dry.

### The dependence of monosaccharide yields on the duration of enzymatic hydrolysis

The dependence of the yields of monosaccharides as well as the production of acetic acid on the duration of enzymatic hydrolysis is reported in Tab. 5. The results show that single freezing is without the desired effect. In this case, it is not related to the sample's impregnation, thus whether the sample is entirely impregnated or relatively dry. The yields of monosaccharides depend only on the duration of enzymatic hydrolysis. The yields of glucose 19.5 g·l<sup>-1</sup> are significantly lower than after other pretreatment types, e.g., the steam explosion of the same sample at temperature 200°C (70.4 g·l<sup>-1</sup>) (Pažitný et al. 2020).

Tab. 5: Enzymatic hydrolysis of poplar wood after freezing pretreatment.

Sample	Duration (yield)	6 h (g·l <sup>-1</sup> )	24 h (g·l <sup>-1</sup> )	48 h (g·l <sup>-1</sup> )	72 h (g·l <sup>-1</sup> )	96 h (g·l <sup>-1</sup> )
Freezing of water-saturated samples -20°C for 24 hour	Glucose	13.7	19.5	23.4	24.8	29.2
	Xylose	3.84	5.8	6.73	6.9	8.0
	Acetic acid	0.4	0.65	0.75	0.8	0.88
Freezing of dry fraction (*MC 5.8%)	Glucose	14.2	19.5	23.2	24.7	27.9
	Xylose	4.2	5.6	6.4	6.6	7.6
	Acetic acid	0.37	0.6	0.73	0.76	0.89

\*MC - moisture content.

## Kinetics of freezing

Due to the low yields, further we focused on the kinetics of the destructive effect. The destructive effect for biomass is possible to assume only if ice crystals' formation begins, i.e., temperature from 0°C to -5°C. The following decrease of temperature should result in the formation of crystals in lumens of biomass cells much deeper, together with raising volume during the phase transition of water into ice in the lumens, which causes disruption of the crystalline structure of cellulose and increases its accessibility in following hydrolysis processing. The rate of freezing was tested on "cubic tests". The samples were placed into cubes at the same ambient conditions with constant temperature -20°C, so the freezing in the core of the cube started in various times, i.e., forming of crystals was slower at a higher volume. The temperature in the cube core was measured by using a temperature sensor.

### Cubic tests results

Monosaccharide concentrations after the 48-hour enzymatic hydrolysis of the 0.7 sawdust poplar pretreated by single freezing using the cubic test method are shown in Tab. 6. Glucose yields have been obtained in range 19.9 g·l<sup>-1</sup> to 24.3 g·l<sup>-1</sup>, xylose 4.0 g·l<sup>-1</sup> to 5.24 g·l<sup>-1</sup> and arabinose 0.3 g·l<sup>-1</sup> to 3.13 g·l<sup>-1</sup>. Total monosaccharide yields after a single freeze pretreatment such range from 24.2 to 31.87 g·l<sup>-1</sup>.

Tab. 6: Dependence of monosaccharide concentrations after 48 h enzymatic hydrolysis on the kinetics of freezing.

Monosaccharide content (g·l <sup>-1</sup> )				
Freezing time (h)	Glucose	Xylose	Arabinose	Total
0	19,90	4.00	0,30	24,20
0.5	21.10	4.20	0.75	26.05
1	21.90	4.40	1.21	27.55
2	22.80	4.71	1.76	29.27
4	23.10	5.10	2.30	30.33
8	23.60	5.17	2.24	31.01
12	24.30	5.24	3.13	31.87

The cubic test analysis shows (Fig. 3) that a slower freezing process positively affects the degradation of the crystalline structure of cellulose in the samples, thus on enzyme accessibility. Hence, the longer the freezing time, the higher the effect.

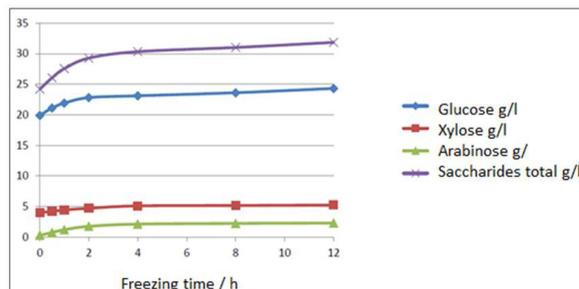


Fig. 3: Dependence of monosaccharide concentration after 48 hours enzymatic hydrolysis on the kinetics of freezing the biomass pretreated by cryolysis.

The slow freezing process causes first changes on the impregnated biomass particles; the surface is trapped, becomes hard and brittle. In the following slower process of crystal formation and changing of water volume during the transition from liquid to solid state, results to use maximal destruction effect of the crystal growth and volume change.

During the freezing process, the surface temperature is lower than in the lignocelluloses' core (Chang et al. 2011). When the temperature decreases during the freezing, the volume of free water grows in the pores, and molecules of water have to surpass the viscous resistance, resulting in pressure gradients of the water that should significantly damage the lignocellulose structure (Chang et al. 2011). The adsorption of water molecules occurs only in the amorphous part of cellulose, where the bounded water forms hydrogen bonds with hydroxyl groups. The inner volume of the lignocellulose structure increases due to decreased cohesion; the solids get softer and occurs swelling. The destruction of the lignocellulose structure is a result of the expansion of water volume during the freezing.

### Effects of cyclic freezing and thawing on monosaccharide yields

The best results were obtained by cyclic freezing-thawing. Tab. 7 contains monosaccharide concentrations of 0.7 mm sawdust of *Populus alba* (sapwood) after 48 hours of enzymatic hydrolysis depended on the number of cycles freeze-thaw pretreatment. In total, 20 cycles of freezing-thawing were performed.

Tab. 7: Dependence of monosaccharide concentrations of *Populus alba* (sapwood) after 48 hours enzymatic hydrolysis on number of cycles freeze-thaw pretreatment.

Cyclic freezing-thawing of poplar (sapwood) sawdust 0.7 mm					
Monosaccharide content (g·l <sup>-1</sup> )					
Cycles	Glucose	Xylose	Arabinose	Acetic acid	Enzymatic hydrolysis 48 hour
Without freezing	20.6	4.4	0.16	0.59	
Freeze- thaw 1x	20.7	4.6	0.16	0.60	
Freeze- thaw 2x	20.5	4.8	0.17	0.63	
Freeze- thaw 5x	21.4	4.9	0.18	0.64	
Freeze- thaw 7x	21.6	5.1	0.18	0.67	
Freeze- thaw 10x	24.4	5.8	0.20	0.69	
Freeze- thaw 12x	27.4	6.7	0.21	0.72	
Freeze- thaw 17x	31.2	7.9	0.25	0.81	
Freeze- thaw 20x	35.6	8.6	0.29	0.99	

The yield of glucose was increased from 20.6 g·l<sup>-1</sup> to 35.6 g·l<sup>-1</sup>. Also, the yield of xylose was increased, from 4.4 g·l<sup>-1</sup> to 8.6 g·l<sup>-1</sup>. The total yield of arabinose was negligible (from 0.16 g·l<sup>-1</sup> up to 0.29 g·l<sup>-1</sup>). The dependence of monosaccharide yields on the number of cycles is shown in Fig. 4.

The effect of cyclic freezing-thawing was also validated by Zhu et al. (2020). They carried out freeze-thaw repetition (1-6 times) of poplar chips. A hemicellulose yield of 85.87 mg·g<sup>-1</sup> (showing an increase of 32.25% over that of untreated poplar chips) was obtained after freeze-thawed four times at -20 °C and then hydrolyzed by hot water for one hour at 170°C, consequently.

Freeze-thaw repetition pretreatment results in lowered molecular weight and enhanced concentration of monosaccharides. This process indicates the creation of irregular micro slits and pores, thus increasing fibrous cell wall debris, which significantly contributed to hemicelluloses' dissolution rate (Zhu et al. 2020).

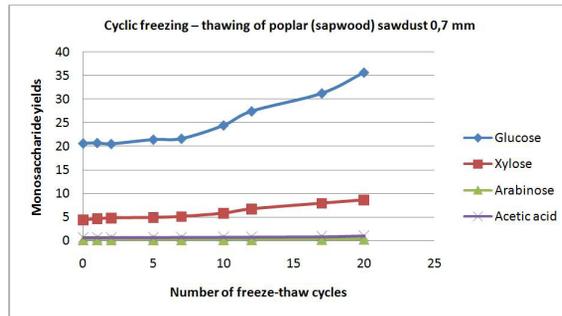


Fig. 4: Dependence of monosaccharide concentration from poplar (sapwood) sawdust 0.7 mm pretreated by cyclic freezing-thawing after 48 hours enzymatic hydrolysis.

## CONCLUSIONS

Fine milled 0.7 mm mesh poplar sapwood (*Populus alba* L.) was subjected to cryolysis pretreatment under mild conditions  $-20^{\circ}\text{C}$  to enhance enzymatic accessibility.

The results showed that single freezing of the fully impregnated samples by water or dry in its matter (5.8% MC) is a not sufficient pretreatment method. On the other hand, a cyclic freeze-thaw method was successfully used to enhance monosaccharides' yields. Ultimately, 20 cycles applied on the dendromass dust showed, that the efficiency starts to increase since 10 cycles.

Analyses of cubic tests showed that the slower freezing process, associated with the formation of larger crystals that can disrupt the lignocellulosic structure, has a positive effect on the enzyme accessibility.

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