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INTENSIFICATION OF THE FREEZE-THAW PRETREATMENT OF DISINTEGRATED POPLAR WOOD

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ABSTRACT

This research was focused on the effect of water content in the cells of disintegrated *Populus nigra* L. on a freeze-thaw pretreatment method before an enzymatic hydrolysis. Two chipped and sieved fractions 2.5 mm and 0.7 mm and two milled fractions, characterized as 18°SR (Shopper–Riegler index) and 37°SR, of the disintegrated 5 years old poplar tree were used for our experiment. Glucose and xylose yields were measured after 24 and 48 hours of enzymatic hydrolysis with a 15% load of the enzyme measured to a total cellulose content. The influence of nine freeze-thaw cycles under -20°C and +20°C was considered.

The results showed that an increase in moisture content positively affects yields in all fractions but a desirable result was achieved mainly for the 0.7 mm fraction where the total yield increased by about 16%. More effective way is a finer wet beating of wood mass, while wood fibre receives moisture already in the technological process. The highest glucan conversion 51,74% and the total hemicelluloses conversion 47,72% was achieved for the finest fraction 37°SR. The higher moisture content has a positive effect on the increase in the conversion of oligosaccharides, especially glucan, in chipped fractions.

KEYWORDS: Enzymatic hydrolysis, freeze-thaw pretreatment, poplar wood, *Populus nigra* L., glucose, xylose, wood fractions.

INTRODUCTION

The principle of freezing methods is based on the volume change of water during an ice formation by approximately 9% (Halaj et al. 2021). The biomass impregnated with water is subjected to complete frozen. The volumetric expansion of water during freezing results in the opening of wood channels and leads to a disruption of cell walls. Frost damaged material has an increased surface area for enzymatic hydrolysis (Rooni et al. 2017, Zhu et al. 2020).

Mechanically disrupting the wood structure with ice is much more difficult than other thin-walled plants (Zhu et al. 2020, Boháček et al. 2020). More suitable materials for freeze pretreatment are agricultural wastes, like barley straw (Rooni et al. 2017), rice straw (Chang et al. 2011, Deng et al. 2018), wheat straw (Wang et al. 2013, Ihnát et al. 2015, Pažitný et al. 2019a), corncobs and corn stalks (Echeverria et al. 2018, Yuan et al. 2019, Li et al. 2019), sugarcane bagasse (Farghaly et al. 2021), rush (*Juncus maritimus*) (Smichi et al. 2016), switch grass (*Panicum virgatum*) (Yang et al. 2009) and waste cotton towels (Sasaki et al. 2020).

Based on the fact that the wood material is denser, combinations of freezing with other pretreatment methods are applied. Jeong et al. (2016) observed the effect of freezing on Mongolian oak (*Quercus mongolica*) after impregnation with 1% H_2SO_4. Alkaline co-pretreatment was published by Li et al. (2019) as a combination of freeze-thaw (from -20 up to 20°C) with addition of ammonia. Su and Fang (2017) and Sasaki et al. (2020) used NaOH as an alkaline structure release mean.

The freeze-thaw technique has found its application as an auxiliary method in the release of hemicelluloses from wood (Zhu et al. 2020, Zeng et al. 2022b, Li et al. 2021, Peng et al. 2022). Hemicelluloses of hardwoods can be released prior to main processing for preparation of high value added products (Fišerová et al. 2013). Freeze-thawing provided more efficient physical channel for the effective penetration of *p*-toluenesulfonic acid pretreatment (Zeng et al. 2022a).

The aim of this study is to evaluate the effects of freeze pretreatment of poplar wood (*Populus nigra* L.) intensified by cycling, reducing the particle size and increasing the moisture content of the wood. Limits, as nine cycles of freeze-thaw and a fraction of 2.5 mm, were selected for experiments, based on previous results of our own research (Boháček et al. 2020, Halaj et al. 2021). European black poplar (*Populus nigra* L.) was selected for our research due to its porous structure (Emampour et al. 2020), as well as, it is one of the most important woody species with respect to its great potential for bioenergy production (Porth and El-Kassaby 2015).

MATERIAL AND METHODS

Material

Black poplar (*Populous nigra* L.) was collected in the region of Bratislava, Slovak Republic. The wood from 5 years old trees cut in the spring keeping high moisture content was used for the experiment. The enzyme complex CellicCTec3 was purchased from Novozymes A/S (Bagsværd, Denmark). The enzyme activity was determined as 1700 BHU (Biomass Hydrolysis Units)/g.

Methods

Preparation of disintegrated fractions

Leaves and other impurities were removed from the timber and it was debarked by a single-knife laboratory disc chipper. The wood was shredded for smaller parts, then wet milled in a Brabender mill (Brabender®, GmbH & Co. KG, Germany) with used bottom sieve 2.5 mm mesh. A part of the material was milled again for the finer fraction in a Brabender mill with bottom sieve 0.7 mm.

Fibre fractions were prepared on a laboratory Jokro refining mill. Beating of the 2.5 mm fraction was done in the water suspension 4 x 330 ml during 30 min and 60 min. Two fractions characterized as 18°SR (Shopper–Riegler index) and 37°SR were achieved, respectively. A fibre obtained in the cold water suspension has a different quality as a thermomechanical fibre used for structural purposes (Lübke et al. 2020), especially a higher hemicellulose content.

Determination of a water content in wood cells

The determination of a moisture content of wood mass was carried out on small samples of 20 x 20 x 20 mm according to Eq. 1:

$$w_0 = (m_1 - m_0) / m_0 * 100 \quad (\%) \qquad (1)$$

where: m_1 – a fresh sample weight(g), m_0 – a weight of the sample after drying at 103°C to a constant weight(g)

The moisture content determination of the disintegrated wood fraction after impregnation with water was performed on small samples of 50 g, which were placed in polymer air bags and centrifuged at 4200 rpm for 15 min. In this case, the dry content of the sample was reversely determined by a moisture analyzer Denver IR35 and the water content was recalculated according to Eq. 1.

Water impregnation

In order to maintain a high water content in cell lumens, the dry matter content in the samples was monitored throughout the experiment. The samples were not allowed to dry from cutting down the trees to their processing. The samples intended for impregnation were soaked in water for 7 days. Water impregnated samples were centrifuged at 4200 rpm for 15 min to remove water from the outer surface of the wood mass before freezing. Quick determination of the dry matter content in samples was performed on the moisture analyzer Denver IR35 immediately after centrifugation.

Freeze-thaw pretreatment

Blank samples were subjected to the enzymatic hydrolysis immediately without freezing. A single freeze-thaw cycle was performed at -20°C until ice crystals creation and +20°C until total thawing. A total of 9 freeze-thaw cycles were applied on selected samples according previous results.

Enzymatic hydrolysis

Water suspension with consistency 12.5% w/w was prepared as the first. The enzymatic hydrolysis was carried out 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 temperature 50°C for 48 hours. The pH was adjusted to 5.0 and regulated during the hydrolysis by 0.1 N sulphuric acid or

0.1 N sodium hydroxide. Collecting of samples from the hydrolysates to determine the content of monosaccharides was performed after 24 and 48 hours.

The conversion of glucan to glucose after enzymatic hydrolysis was calculated by the ratio of a glucose concentration that was released during the enzymatic hydrolysis to the glucose and it was calculated according to the Eq. 1:

$$Glucan\ conversion\ (\%) = (glucose\ x\ V\ x\ 0.9\ /\ glucan\ content\ x\ m)\ *\ 100 \quad (1)$$

where: glucose is the concentration of glucose in enzymatic hydrolysis liquor ($g \cdot L^{-1}$); V is the volume of the hydrolytic liquor (L); *m* is mass of o.d. of the wood (g), and 0.9 is the conversion factor for glucose to glucan.

The conversion of xylan was calculated analogously, with the use of conversion factor of 0.88 for xylan to xylose (Stankovská et al. 2018).

Analyses

Determination of ash content was performed according to ISO 1762. The content of extractives in dichloromethane was determined according to Tappi T 204 cm-94, and the content of extractives in hot water according to Tappi T 207 cm-08. A Klason lignin content was found out according to Tappi T 222 om-98 and acid-soluble lignin content according to Tappi UM 250. Polysaccharide glucan, xylan, mannan, galactan and arabinan were evaluated after calculations of concentrations of glucose, xylose, mannose, galactose and arabinose in hydrolyzate after determination of lignin. The hydrolysis was carried out with 4% H_2SO_4 at 121°C for 2 hours, to hydrolyze the oligosaccharides, followed by neutralization with $BaCO_3$. The monosaccharide concentration after hydrolysis was determined by HPLC with Rezex ROA H^+ column. The mobile phase was 0.005 N H_2SO_4 at a flow rate of 0.5 $mL.min^{-1}$, and temperature 30°C. The samples were cleaned from solid impurities by 22 μm filter. The tests were conducted in two parallels.

RESULTS AND DISCUSSION

Chemical composition

Poplar wood belongs to porous woods (Jang et al. 2019, Shen et al. 2021) and is most closely related to the aspens (Pažitný et al. 2020a). Poplar has adapted to the changing environment for years and is one of the most widespread tree species in the world (Xi et al. 2021). Tab. 1 shows the chemical composition of the poplar wood determined by chemical analyses according to the methods described above. The content of extractives was 3.42%, lignin occupied 26.37%, and content of polysaccharides 68.1%, of which the most represented glucan is 48.7%. The chemical composition approximately corresponds to previous work (Ihnát et al. 2021).

Tab. 1: Chemical composition of wood samples (Populus nigra L.).

Ash (%)		0.66 ± 0.02
Extractives (%)	Dichloromethane	0.70 ± 0.02
	Hot water	2.62 ± 0.01
	Total	3.42 ± 0.02
Lignin (%)	Klason*	22.4 ± 0.18
	Acid-soluble	3.97 ± 0.06
	Total	26.37 ± 0.12
Polysaccharides (%)	Glucan	48.7 ± 0.42
	Xylan	16.9 ± 0.35
	Mannan	1.6 ± 0.08
	Galactan	0.6 ± 0.06
	Arabinan	0.3 ± 0.02
	Total	68.1 ± 0.89

* Klason lignin was corrected for ash content in Klason lignin.

Monitoring of the moisture content in wood mass

Presence of water in the wood cells is a necessary condition for formation of ice crystals. For this reason monitoring of the moisture content in the wood mass during technology processing was carried out (Fig. 1). Black poplar wood after felling had the moisture content of 102.3% ± 4.3%. Standing poplar trees have the high moisture content, typically about 100%, with only minor differences between sapwood and heartwood (Balatinecz and Kretschmann 2001). The diameter of the trunk of the 5 years old poplar, from which small samples of 20 x 20 x 20 mm were taken, was about 120 mm.

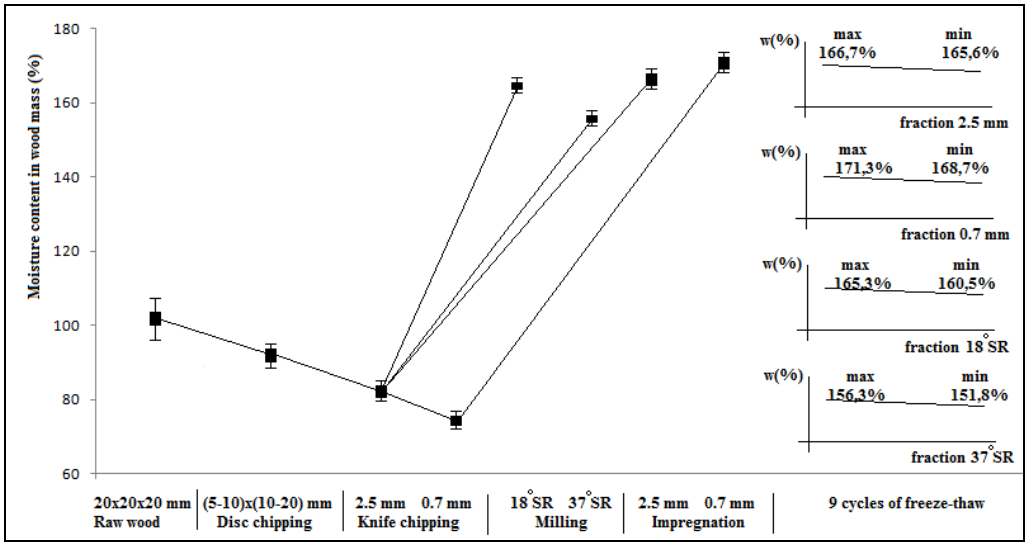

Fig. 1: Moisture content in wood mass development during disintegration (left) and during cycles of freezing-thawing (right).

Chipping in a laboratory chipper caused the moisture to drop to 92.3% ± 1.4%. The moisture was determined in chips with dimensions of 5-10 mm x 15-20 mm. After milling on a Brabender knife mill, the moisture content of the 2.5 mm wood fraction decreased to 82.9% ± 1.1% and to 74.6% ± 0.8% for the 0.7 mm fraction. During the seven days impregnation in water of laboratory temperature of 20°C ± 1°C the final humidity of the wood fractions reached 166.7% ± 0.9°C and 171.3% ± 0.8°C for fractions of 2.5 mm and 0.7 mm, respectively.

Procedure of fibre refining was performed on a laboratory Jokro refining mill (Balberčák et al. 2018). Preparation of two fibre fractions characterized as 18°SR and 37°SR caused humidity increase to values 165.3% ± 0.5°C and 156.3% ± 0.4°C respectively, already during the technological process of milling. Only a negligible moisture loss was noted during 9 freeze-thaw cycles (Fig. 1, right). That means the wood mass did not lose its moisture from the cells.

Monosaccharides yields after the enzymatic hydrolysis

Monosaccharides yields after 24 and 48 hours of enzymatic hydrolysis are contained in Tab. 2. Total differences between 24 and 48 hours of the hydrolysis represent up to 18% for pretreated samples, while only 12% for untreated samples. Generally, untreated samples give significantly lower monosaccharide concentrations compared with the treated ones also in case of other wood species and different types of pretreatment (Pažitný et al. 2022). Experiments have shown that longer time of the enzymatic hydrolysis did not bring significant changes (Pažitný et al. 2019b, 2020b) and therefore we do not present these values in the table.

Tab.2: Monosaccharide yields after 24 and 48 hours of enzymatic hydrolysis.

Fraction	Moisture content	Monosaccharide	Blank		Frozen-thawed 9x	
			Enzymatic hydrolysis			
			24 h	48 h	24 h	48 h
2.5 mm	82.9%	glucose ($g.L^{-1}$)	8.3 ± 1.8	11.1 ± 1.9	12.1 ± 1.5	12.4 ± 1.6
		xylose ($g.L^{-1}$)	4.2 ± 0.5	3.5 ± 0.4	3.9 ± 0.5	6.0 ± 1.4
		arabinose ($g.L^{-1}$)	3.4 ± 1.4	2.6 ± 0.7	3.0 ± 0.6	4.2 ± 1.2
		total	**15.9 $g.L^{-1}$**	**17.2 $g.L^{-1}$**	**19.0 $g.L^{-1}$**	**22.6 $g.L^{-1}$**
	166.7%	glucose ($g.L^{-1}$)	8.3 ± 1.7	11.2 ± 1.2	13.2 ± 2.1	14.7 ± 2.2
		xylose ($g.L^{-1}$)	5.4 ± 0.7	3.5 ± 0.6	5.6 ± 0.9	6.6 ± 1.1
		arabinose ($g.L^{-1}$)	3.3 ± 1.0	2.6 ± 0.6	2.7 ± 0.5	3.9 ± 0.5
		total	**17.0 $g.L^{-1}$**	**17.3 $g.L^{-1}$**	**23.5 $g.L^{-1}$**	**25.2 $g.L^{-1}$**
0.7 mm	74.6 %	glucose ($g.L^{-1}$)	16.3 ± 2.1	17.8 ± 1.6	18.1 ± 2.1	19.0 ± 2.5
		xylose ($g.L^{-1}$)	4.7 ± 0.8	5.3 ± 0.7	6.2 ± 1.1	7.4 ± 1.2
		arabinose ($g.L^{-1}$)	2.1 ± 0.7	2.2 ± 0.8	3.5 ± 0.7	4.1 ± 1.1
		total	**23.1 $g.L^{-1}$**	**25.3 $g.L^{-1}$**	**27.8 $g.L^{-1}$**	**30.5 $g.L^{-1}$**
	171.3%	glucose ($g.L^{-1}$)	17.4 ± 2.3	18.9 ± 2.0	20.2 ± 2.5	23.4 ± 2.1
		xylose ($g.L^{-1}$)	5.5 ± 0.9	5.6 ± 0.9	6.5 ± 0.8	8.8 ± 1.1
		arabinose ($g.L^{-1}$)	2.6 ± 0.8	2.3 ± 0.7	2.7 ± 0.9	2.8 ± 1.0
		total	**25.5 $g.L^{-1}$**	**26.8 $g.L^{-1}$**	**29.4 $g.L^{-1}$**	**35.0 $g.L^{-1}$**
18°SR	165.3%	glucose ($g.L^{-1}$)	12.9 ± 1.7	14.7 ± 2.0	28.7 ± 2.8	32.8 ± 3.1
		xylose ($g.L^{-1}$)	4.9 ± 1.3	6.0 ± 1.8	8.4 ± 1.5	8.7 ± 1.4
		arabinose ($g.L^{-1}$)	2.5 ± 0.7	3.0 ± 0.4	3.6 ± 0.9	3.8 ± 1.2
		total	**20.3 $g.L^{-1}$**	**23.7 $g.L^{-1}$**	**40.7 $g.L^{-1}$**	**45.3 $g.L^{-1}$**
37°SR	156.3%	glucose ($g.L^{-1}$)	15.1 ± 1.3	16.2 ± 2.2	31.1 ± 2.4	34.6 ± 2.9
		xylose ($g.L^{-1}$)	5.4 ± 0.9	6.0 ± 1.6	7.9 ± 1.3	8.4 ± 1.7
		arabinose ($g.L^{-1}$)	2.1 ± 0.8	3.2 ± 1.1	3.4 ± 09	4.2 ± 1.2
		total	**22.6 $g.L^{-1}$**	**25.4 $g.L^{-1}$**	**42.4 $g.L^{-1}$**	**47.2 $g.L^{-1}$**

*Note: Average values from three parallel measurements are shown in the table.

Total monosaccharide yields for untreated samples ranged from 15.9 $g.L^{-1}$ to 25.4 $g.L^{-1}$ (Tab. 2). Similar results for poplar were obtained by Pažitný et al. (2020b). The differences

caused by the particle size represent up to 55% between the 0.7 mm and 2.5 mm fractions (Fig. 2).

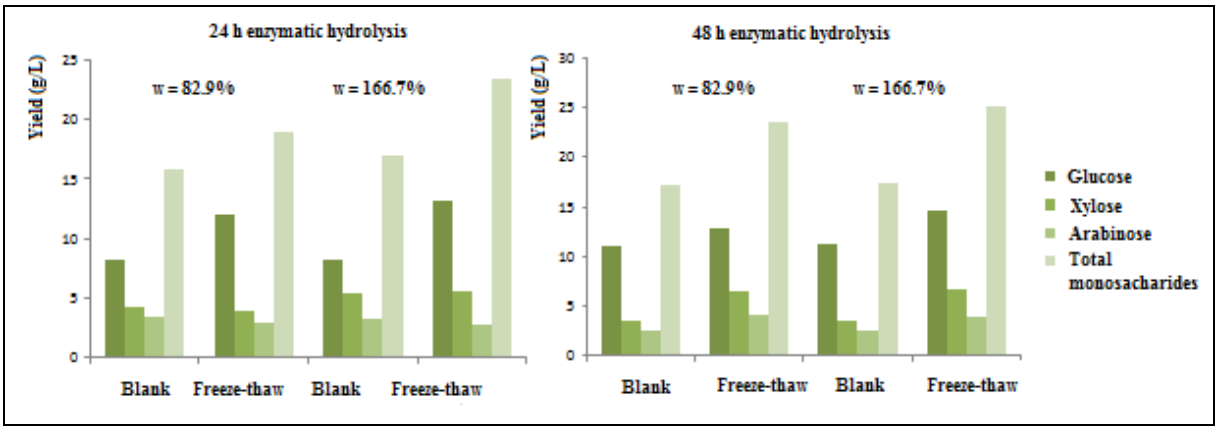

Fig. 2: Yields of monosaccharides after the enzymatic hydrolysis of Populus nigra L., fraction of 2.5 mm.

The larger fraction is resistant to a mechanical damage by freeze, powder fractions are usually in the focus of research (Wang et al. 2022), unlike straw, whose stalks are less thick (Sun et al. 2022). Experiments confirmed that the 0.7 mm fraction is more suitable for the freeze-thaw pretreatment (Fig. 3). Total monosaccharide yields of this fraction subjected to the 9 cycles of freeze-thaw pretreatment ranged from 30.5 $g.L^{-1}$ to 35.0 $g.L^{-1}$ (Tab. 2).

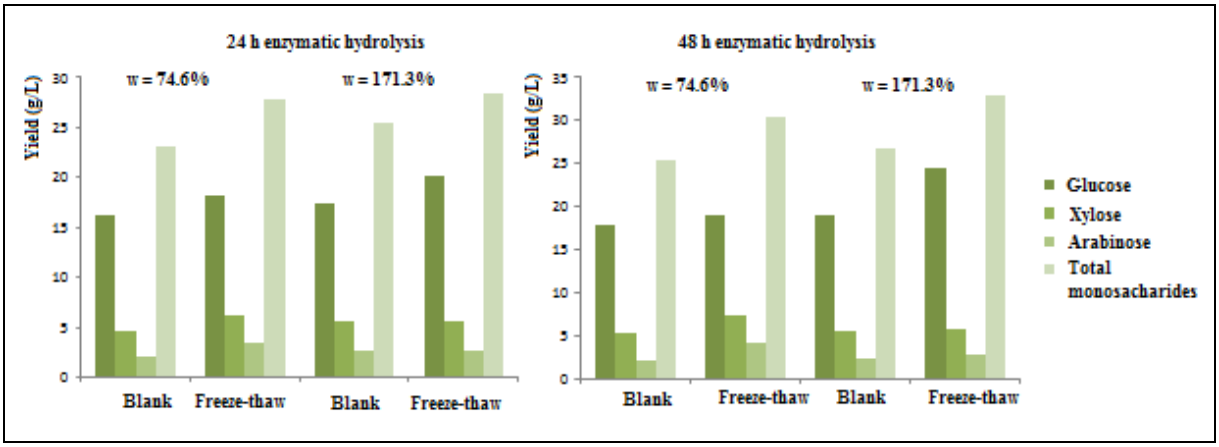

Fig. 3: Yields of monosaccharides after the enzymatic hydrolysis of Populus nigra L., fraction of 0.7 mm.

Our last research reached 30.4 $g.L^{-1}$ at the same conditions after 10 cycles (Boháček et al. 2020), which is corresponding to the yield after 9 cycles in this work. The total yield of 44.4 $g.L^{-1}$ was reached at 20th cycle in previous work. It was showed that the contribution to the total yields was caused only after the 10th cycle when the glucose content started to rise significantly (Boháček et al. 2020).

Fibre fractions were also chosen for this experiment to achieve the smallest possible fraction with the greatest possible accessibility of the enzyme to the wood cells. The obtained yields are

shown in Fig. 4. Total yields of monosaccharides 45.3 g.L^{-1} for 18°SR and 47.2 for 37°SR (Tab. 2) already correspond to the yield in the above mentioned 20th cycle (Boháček et al. 2020).

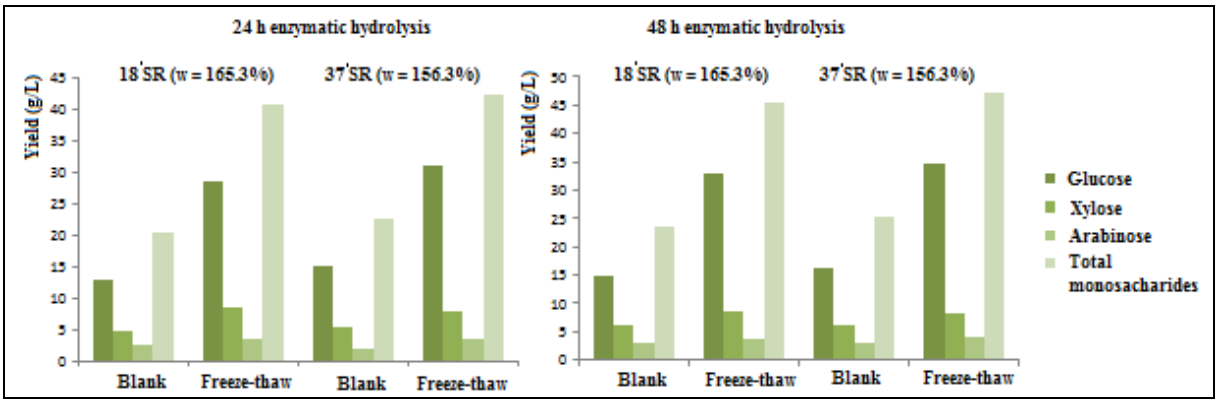

Fig. 4: Yields of monosaccharides after the enzymatic hydrolysis of Populus nigra L., fractions of 18°SR and 37°SR.

Conversion of oligosaccharides

Conversions of untreated samples (blank) for the 2.5 mm fraction are low. The freeze-thaw pretreatment at the increased moisture content of the sample (166.7%) increases the conversion to 21.98% (glucan) and 39.77% (hemicelluloses as a whole). The finer fraction of 0.7 mm achieves higher values of conversion during the freeze-thaw pretreatment at the increased sample moisture (171.3%), namely 36.49% (glucan) and 43.93% (hemicelluloses as a whole). The 18°SR fibre fraction achieved the glucan conversion of 49.05% and the conversion of hemicelluloses 47.34% and the 37°SR fraction 51.74% for glucan and 47.72% for hemicelluloses. Development of the glucan conversion and the total hemicelluloses conversion pretreated by the 9 cycles of freeze-thaw repetition in dependence on the moisture content is shown in Fig. 5.

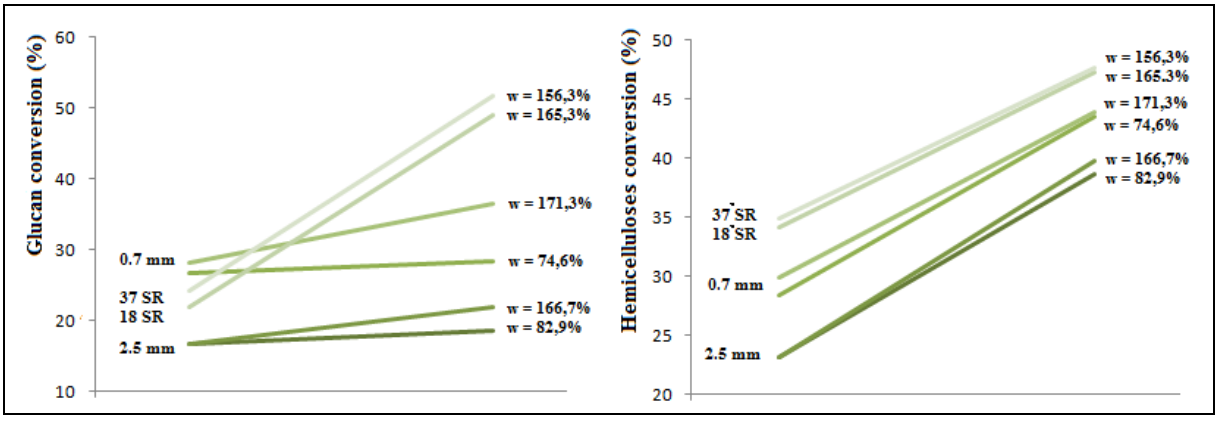

Fig. 5: A development of the glucan conversion and the total hemicelluloses conversion after 48 hours of enzymatic hydrolysis of Populus nigra L. pretreated by the 9 cycles of freeze-thaw repetition.

Fig. 5 shows that the moisture content in the wood cells slightly increases the conversion for fractions 0.7 and 2.5 mm. The most significant difference was achieved in glucan conversion at

the 0.7 mm fraction. We note that with fibre fractions, the moisture content does not affect the conversion, the higher conversion is caused by the fineness of the fraction.

Intensification of the freeze-thaw pretreatment

The effect of freezing on the integrity of the cellular structure of wood is low, therefore a cyclic freeze-thaw is used (Yang et al. 2009, Wang et al. 2013,2022, Li et al. 2019,2021, Sasaki et al. 2020, Boháček et al. 2020, Peng et al. 2022). Freeze–thaw repetition creates irregular micro slits and pores, thus increasing fibrous cell wall debris, which significantly contributed to the dissolution rate of hemicelluloses (Zhu et al. 2020). Since freezing is close to a mechanical pretreatment and a mechanical forces of ice crystals are used, the amount of moisture in the wood plays a significant role. The disadvantage is the small space that can be filled with water. Also the amount of non-freezing bound water is roughly half of the amount of freezing bound water for hardwood fibres (Paajanen et al. 2019).

At fractions of 0.7 and 2.5 mm, an increase in monosaccharide yields was observed at higher moisture content (Fig. 6). In case of fibre fractions (18°SR and 37°SR), the moisture content is no longer decisive, but rather the fineness of the grinding. The smaller size of wood mass makes it easier for an enzymatic attack. Fig. 5 shows a significant increasing of yields of monosaccharides when using fibre fractions compared to fractions obtained by knife grinding (0.7 mm and 2.5 mm). The effect of a cyclic freeze-thaw is evident after 6 cycles (Zhu et al. 2020), with regard to higher glucose yields after 10 cycles (Halaj et al. 2021).

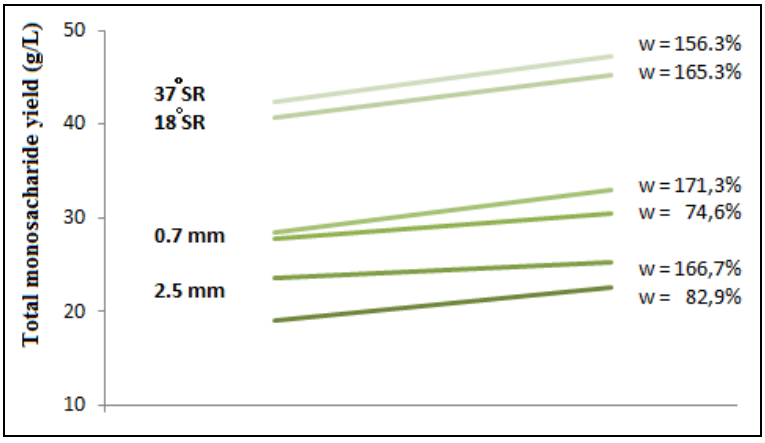

Fig. 6: Relations among the total monosaccharides yields, fraction size and moisture content in disintegrated Populus nigra L. subjected to 9 cycles of freeze-thaw.

CONCLUSIONS

Due to a low effect of freezing on the integrity of the cellular structure of hardwoods 9 cycles of freeze-thaw were applied on *Populus nigra* L allowing an enzymatic attack. Monosaccharide yields after 24 and 48 hours of the enzymatic hydrolysis with a 15% load of the enzyme measured to total cellulose content were evaluated. Two chipped and sieved fractions – 2.5 mm and 0.7 mm were used in the experiment. Intensification of the pretreatment process was enhanced by a finer structure of milled fractions characterized as 18°SR and 37°SR.

In addition, the influence of a moisture content of the wood mass was monitored by using the same parallel samples with low and high values of the moisture content. Samples before freezing were centrifuged to remove remaining water from cell surfaces.

Our analyzes showed a big difference between chipped and milled fractions (Fig. 5). The total monosaccharide yield for the finest fraction 37°SR achieved 47.2 $g.L^{-1}$, while the highest yield for the chipped fraction only 35.0 $g.L^{-1}$ (0.7 mm). Increase of the moisture content brought a desirable result mainly for the 0.7 mm fraction, where the total yield increased by about 16%. Higher moisture content has a positive effect on the increasing conversion of glucan. Conversions for the finest fraction 37°SR achieved values of 51,74% for glucan and 47,72% for total hemicelluloses.

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