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INFLUENCE OF ULTRA LOW AND HIGH TEMPERATURE ON ENZYMATIC PRETREATMENT OF BEECH BRANCHES WOOD

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ABSTRACT

The publication is focused on the effect of ultra low and high temperature on enzymatic pretreatment of beech wood (*Fagus sylvatica* L.). Two fractions < 0.7 mm and 1.0 – 2.5 mm of disintegrated branches sawdust were used for experiments. Glucose and xylose yields were measured after 24, 48, and 72 hours of enzymatic hydrolysis with 15 % load of the enzyme measured to total cellulose content. The influence of freezing under -80°C and boiling under pressure at +160°C on samples before enzymatic hydrolysis was observed. Mutual combination of boiling under pressure to obtain the maximum water uptake and subsequent freezing was used to better understand the process of cell destruction. The results show that the boiling pretreatment has a positive influence on the total monosaccharide yields and the subsequent freezing may slightly increase these yields even further. The maximum monosaccharide conversion (73.24%) was achieved using the fraction < 0.7 mm.

KEYWORDS: Enzymatic pretreatment, freezing, boiling under pressure, *Fagus sylvatica* L., glucose, xylose, wood fractions.

INTRODUCTION

Due to a rising global demand for wood consumption, which leads to degradation of environment, the emphasis is put on the use of secondary wood resources. Recycling of waste wood (Ihnát et al. 2020), including wood-based composites (Lübke et al. 2020), has become more important, or alternative sources are sought instead of wood raw material (Lübke et al. 2014). Unprocessed wood residues after harvesting come into consideration, which can be processed by known chemical (Balberčák et al. 2017, 2018) or enzymatic

procedures (Pažitný et al. 2020). The main utilization of less valuable wood sources still targets to liquid biofuels, mainly to most promising bioethanol (Cheah et al. 2020, Prasad et al. 2016). Bioethanol shows high oxygen content, that results to better combustion efficiency, and also has a higher octane number allowing operation at high compression ratios (Branco et al. 2019). So, pretreatment methods of agricultural, municipal lignocellulosic wastes or even still considered microalgae (Sankaran et al. 2020, Halaj et al. 2019) play important roles in practical application.

Forest residues after wood harvesting remain underutilized due to economic and operational barriers caused by high cost of collecting, treatment, and transportation (Han et al. 2010). Most residues of the tree logs remain as land cover. Significant research has focused on tree residues as potential source of energy biomass (He et al. 2014). The mass distribution of wood in deciduous trees at harvesting time is to be 68% in stems, 10-19% in crowns and branches, and 8-25% in roots. After harvesting, about 35 to 50% of wood biomass stays in the forests as stumps and branches (Okai and Boateng 2007). The number of publications on wood characteristics in branch parts of the hardwood species is still low. The differences in chemical compositions between trunk and branches were observed in wood e.g. *Alianthus altissima* (Samariha and Kiaei 2011), birch (*Betula pendula*) (Krutul et al. 2014).

Pretreatments using high temperatures are well known, but research that focused in freezing is still evolving. The principle of freezing methods is a volume change of water by approximately 9% during freezing. The biomass impregnated with water is subjected to be completely frozen. The volume expansion of water during freezing results to open channels of wood and leads to disruption cell wall or lignocellulosic material structure loose. The freeze-damaged material has an increased surface for enzymatic hydrolysis (Rooni et al. 2017, Zhu et al. 2020). According to literature the main source of suitable material for cryolysis is agricultural waste, 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, Sasaki et al. 2021), corncobs and corn stalks (Echeverria et al. 2018, Yuan et al. 2019, Li et al. 2019a), sugarcane bagasse (Farghaly et al. 2021). Another substances were also used for cryolysis, such as rush (*Juncus maritimus*) (Smichi et al. 2016), switchgrass (*Panicum virgatum*) (Yang et al. 2009), poplar wood (Zhu et al. 2020, Boháček et al. 2020), and waste cotton towels (Sasaki et al. 2020). Base on the fact that wood material is denser, more publications show combination of freezing with another methods of pretreatment. Data show this combination with only chemical pretreatment, such as Jeong et al. (2016) observed the effect of freezing on Mongolian oak (*Quercus mongolica*) after impregnation with 1% H_2SO_4, on the other hand, alkaline co-pretreatment published Li et al. (2019b) as combination of freezing-thawing (from -20 up to 20°C) with addition of ammonia, or with NaOH (Su and Fang 2017, Sasaki et al. 2021).

The aim of this paper is to evaluate the effects of pretreatment of branch beech wood (*Fagus sylvatica* L.) in a wide range of temperature conditions. Limits, as deep freezing at -80°C and boiling under pressure at 160°C were selected as borders for experiments. A mutual combination of these extreme methods was considered, as well.

MATERIAL AND METHODS

Material

The beech (*Fagus sylvatica* L.) was collected in the region of Bratislava, Slovak Republic. The enzyme complex Cellic®CTec3 was purchased from Novozymes A/S (Bagsværd, Denmark). The enzyme activity was determined as 1700 BHU (Biomass Hydrolysis Units)/g. The range of the diameter of branches was from 2.0 to 4.5 cm.

Methods

Preparation of fractions

Leaves and other impurities were removed from the timber and debarked by a single-knife laboratory disc chipper. The wood was shredded for smaller parts, then dry milled in a Brabender mill (Brabender®, GmbH & Co. KG, Germany) with used bottom sieve 2.5 mm mesh. The fraction was purified by sieve 1.0 mm mesh in a sieve tester after 5 min of sieving. The material passed through 1.0 mm sieve was milled again in a Brabender mill with bottom sieve 0.7 mm to prepare two size fractions: 1.0-2.5 mm and < 0.7 mm.

Water impregnation

An absolute dry weight of the both fractions was determined by moisture analyzer Denver IR35 with use infrared heating to be 91.76 % for 1.0-2.5 mm and 93.1 % for < 0.7 mm fraction. The samples of both fractions for only blank and deep freezing for saturation by water as 12.5 g of absolute dry weight of the material + 87.5 mL of distilled water were prepared separately. The other samples of fractions used for boiling at higher pressure and combination of boiling at higher pressure and deep freezing, were prepared together at 2.5-times higher dose to be in extent as 31.25 g of absolute dry weight + 218.75 mL of distilled water. The impregnation was performed in closed PET bags in orbital shaker-incubator 24 hours at 60°C.

Pretreatment of samples

The impregnated blank samples were placed into a kitchen freezer until the other fractions were pretreated. The single deep freezing was performed in a laboratory ultra low freezer at -80°C, for 4 days. In order to avoid the protection effect of ice surrounding of the sawdust particles, the water was decanted into flasks before the freezing pretreatment and kept in a freezer. The boiling under higher pressure was performed at 2 L stainless steel batch reactor for steam explosion pretreatment (Amar Equipments Pvt. Ltd., India). The boiling was carried out at temperature of 160°C, pressure 5.5 bar for 30 min. Before the thermal treatment, 50 mL of distilled water was added into the impregnated fractions as the excess to ensure the material to be all the time under water, the suspension was poured into aluminum beverage can, and placed into the reactor in a water bath. The thermally pretreated fractions were divided into sample only thermally pretreated and sample with combination of thermal pretreatment with following deep freezing. The only thermally pretreated samples were immersed in water in a concentration of 12.5 % w/w of absolute dry weight. From the samples with combination of

thermal pretreatment and deep freezing, the water was also decanted into flasks and kept in a freezer as the thermally untreated samples.

Enzymatic hydrolysis

Before the enzymatic hydrolysis of the pretreated samples by thermal degradation and subsequent deep freezing, was the absolute dry weight again determined, and the decanted water was added into the material to prepare the suspension of consistency 12.5 % w/w. To the samples treated by only deep freezing, the water after decantation before treatment was added, too. 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 96 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. The collecting of samples from the hydrolysates to determine the content of monosaccharides was performed after 24, 48, 72, and 96 hours.

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

$$Glucan\ conversion\ (\%) = \frac{glucose \times V \times 0.9}{glucan\ content \times m} \times 100 \quad (1)$$

where: glucose is concentration of glucose in enzymatic hydrolysis liquor ($g \cdot L^{-1}$); V is the volume of the hydrolytic liquor (L); glucan content in beech sawdust, *m* is mass of o.d. beech sawdust (g), and 0.9 is the conversion factor for glucose to glucan. The xylan conversion 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 content of *extractives in hot water* according to Tappi T 207 cm-08. *Klason lignin* content was found out according to Tappi T 222 om-98 and *acid-soluble lignin* content according to Tappi UM 250.

P*olysaccharide* glucan, xylan, mannan, galactan and arabinan were evaluated after calculations of the 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, following 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.

Inorganic elements in ash

The content of Na, K, Ca, Mg, Fe, Mn, Zn, Cu elements in the wood was determined by atomic absorption spectrometry (AAS) using flame atomization according to the method Tappi T 266. The soluble part of the ash in hydrochloric acid was used for determination of them. The concentration of P element was determined by spectrometry using ammonium molybdate according to standard STN EN ISO 6878. The concentration of Si in the branch wood was calculated by gravimetrical determination of SiO_2 content in the insoluble ash residue after treatment by hydrofluoric acid.

RESULTS AND DISCUSSION

Beech wood belongs to the most utilized hardwood in Europe. The waste obtained from processing this hardwood is promising feedstock for production of 2G bioethanol. Biorefinery is capable to treat 400,000 dry metric tons of this wood into products as polymer-grade ethylene, organosolv lignin and biofuels (Budzinski & Nitzsche 2016, Nagarajan et al. 2017, Pažitný et al. 2019b). In Slovakia, beech represents 29% of wood in forests and together with oak are the most widespread hardwood trees in the country (Dittmar et al. 2003, Pajtík et al. 2011, Marková et al. 2018).

Tab. 1 shows the chemical composition of the beech wood determined by chemical analyses according to the appropriate methods. The content of extractives was 3.7%, lignin in this wood occupied 21.5%, and content of polysaccharides almost 69%, the glucan content in the polysaccharide part represented 64.1%. Literature data show, that the range of cellulose content is 41 to 50%, the total lignin content 22-30%, extractives up to 15%, the total content of polysaccharides ranges from 70 to 77%. Further the ash content differs from 0.72% to 1.1% (González-Peña et al. 2009, Miklečić & Jirouš-Rajković 2016, Demirbaş 2005, de Wild et al. 2009). The chemical composition approximately corresponds to the literature data. The content of inorganic elements is shown in Tab. 2. The analyses by de Wild et al. (2009), showed that calcium, potassium, and magnesium were also the first dominant elements in the beech wood.

Tab. 1: Chemical composition of branch wood of beech.

Ash (%)		0.57
Extractives (%)	Dichloromethane	0.29
	Hot water	3.44
	Total	3.73
Lignin (%)	Klason*	17.1
	Acid-soluble	4.45
	Total	21.55
Polysaccharides (%)	Glucan	44.2
	Xylan	22.3
	Mannan	1.26
	Galactan	0.76
	Arabinan	0.41
	Total	68.93

Tab. 2: Inorganic elements concentration in branch wood of beech.

Inorganic elements (mg.kg^{-1})									
Ca	K	Mg	P	Si	Na	Mn	Fe	Zn	Cu
1010	783	192	181	29	77.5	120	8.58	4.77	1.66

The dependence of yields on used methods of pretreatment

The yields after 24; 48; 72 and 96 h hydrolysis of the variously pretreated fractions of beech sawdust are shown in Tab. 3. The yields of total monosaccharides after the hydrolysis of thermally untreated smaller fraction samples decrease after 48 h (maximal yields 25.6 g·L^{-1} for blank – hydrolysis after water impregnation without any pretreatment, and 24.5 g·L^{-1} for deeply frozen), the yields of the larger fraction decrease after 72 h (maximal yields 20.7 g·L^{-1} for blank, and 22.3 g·L^{-1} for deeply frozen). After 96 h hydrolysis of the untreated samples were the yields lower, than those after 24 h hydrolysis, in a range from 8% for blank of the larger fraction to 41% for blank of the smaller fraction.

Tab. 3: The monosaccharide yields and conversions of variously pretreated fractions of beech sawdust.

Sample	**Time of hydrolysis** (h)	**Glucose** (g·L^{-1})	**Glucose conversion** (%)	**Xylose** (g·L^{-1})	**Xylose conversion** (%)	**Total monosaccharides** (g·L^{-1})	**Total monosaccharides conversion** (%)
0.7 Bl[1]	24	19.50	31.76	4.83	15.25	24.32	25.40
0.7 F[2]	24	19.18	31.24	4.69	14.81	23.86	24.92
0.7 Bo[3]	24	35.51	57.84	18.71	59.07	54.21	56.62
0.7 Fbo[4]	24	36.28	59.10	19.47	61.47	55.75	58.23
1.0 - 2.5 Bl	24	15.82	25.77	3.25	10.26	19.08	19.93
1.0 - 2.5 F	24	17.50	28.51	3.58	11.30	21.08	22.02
1.0 - 2.5 Bo	24	27.67	45.07	11.83	37.35	39.50	41.26
1.0 - 2.5 Fbo	24	27.08	44.11	12.49	39.43	39.57	41.33
0.7 Bl	48	20.50	33.39	5.14	16.23	25.64	26.78
0.7 F	48	19.56	31.86	4.91	15.50	24.47	25.56
0.7 Bo	48	40.68	66.27	20.82	65.73	61.50	64.24
0.7 Fbo	48	42.65	69.48	22.14	69.89	64.79	67.68
1.0 - 2.5 Bl	48	16.23	26.44	3.81	12.03	20.30	21.20
1.0 - 2.5 F	48	18.01	29.34	3.73	11.78	21.74	22.71
1.0 - 2.5 Bo	48	32.24	52.52	13.96	44.07	46.20	48.26
1.0 - 2.5 Fbo	48	31.77	51.75	13.64	43.06	45.40	47.42
0.7 Bl	72	15.84	25.80	4.16	13.13	20.01	20.90
0.7 F	72	18.04	29.39	4.84	15.28	22.87	23.89
0.7 Bo	72	43.24	70.44	21.72	68.57	64.96	67.85
0.7 Fbo	72	45.52	74.15	23.39	73.84	68.91	71.98
1.0 - 2.5 Bl	72	17.13	27.90	3.58	11.30	20.71	21.63
1.0 - 2.5 F	72	18.40	29.97	3.84	12.12	22.25	23.24
1.0 - 2.5 Bo	72	33.40	54.41	14.55	45.93	47.95	50.09
1.0 - 2.5 Fbo	72	34.08	55.51	14.64	46.22	48.72	50.89
0.7 Bl	96	10.91	17.77	3.46	10.92	14.37	15.01
0.7 F	96	13.81	22.50	3.91	12.34	17.72	18.51
0.7 Bo	96	44.17	71.95	22.46	70.91	66.62	69.59
0.7 Fbo	96	45.99	74.92	24.13	76.18	70.12	**73.24**
1.0 - 2.5 Bl	96	14.55	23.70	2.99	9.44	17.54	18.32

1.0 - 2.5 F	96	15.07	24.55	3.27	10.32	18.34	19.16
1.0 - 2.5 Bo	96	34.01	55.40	14.90	47.04	48.91	51.09
1.0 - 2.5 Fbo	96	34.03	55.43	14.79	46.69	48.82	50.99

[1]Bl = blank; [2]F = deep freezing at -80°C; [3]Bo = boiling at 160°C; [4]Fbo = boiling at 160°C, subsequent deep freezing at -80°C.

On the other hand, the yields of thermally treated samples are the highest after 96 h of the hydrolysis, for the smaller fraction, 66.6 $g \cdot L^{-1}$ for only thermally degraded sample, 70.1 $g \cdot L^{-1}$ (Fig. 1), the maximum yield achieved in this study, for deeply frozen material after thermal degradation, and for the larger fraction, 48.9 $g \cdot L^{-1}$ for only thermally degraded material, for material with combination of thermal degradation with subsequent deep freezing 48.8 $g \cdot L^{-1}$. The effect of thermal degradation, to compare the maximal yield of thermally untreated sample, 25.6 $g \cdot L^{-1}$ (smaller fraction, blank) and for thermally treated sample, 66.6 $g \cdot L^{-1}$ (smaller fraction, only thermal degradation), the thermal degradation increased the maximum yields in this study by 160%.

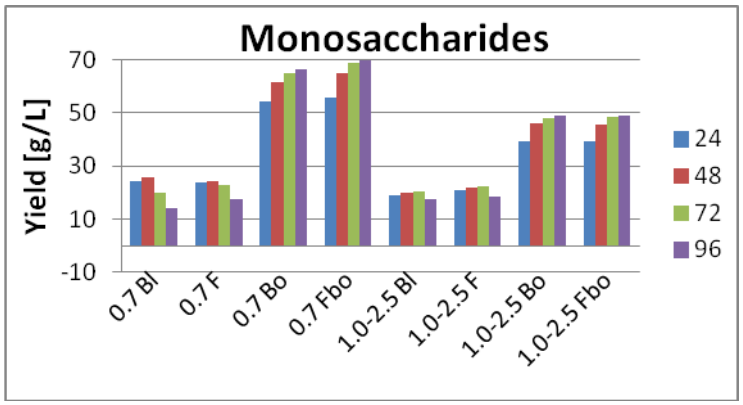

Fig. 1: The yields of total monosaccharides of pretreated branch wood beech fractions in dependence of time of enzymatic hydrolyses. Bl = blank; F = deep freezing at -80°C; Bo = boiling at 160°C; Fbo = boiling at 160°C and subsequent deep freezing at -80°C.

The maximum yield of glucose for thermally untreated samples was 20.5 $g \cdot L^{-1}$ (smaller fraction, blank), and for thermally degraded was 46 $g \cdot L^{-1}$ (Fig. 2). The thermal degradation showed a more significant effect for the yields of xylose, the maximal yield for non-thermally treated samples was 5.1 $g \cdot L^{-1}$, and for thermally treated samples was 24.1 $g \cdot L^{-1}$, which is 4.73-times higher to compare the effect of the thermal degradation of glucose, 2.24-times higher (Fig. 3).

To compare the dependence of particle size on the yield showed, that the maximum yield achieved using the smaller fraction, < 0.7 mm, was the mentioned maximum yield in this paper, 70.1 $g \cdot L^{-1}$, for the larger fraction the maximum yield was 48.9 $g \cdot L^{-1}$ (larger fraction, only thermal pretreatment), that was higher by 43%. The effect of deep freezing in the thermally degraded smaller fraction was shown as 5.2% to compare the yield after only thermal pretreatment and the yield after the combination of thermal pretreatment with subsequent deep freezing. The efficiency of the observed factors in this study is in order deep freezing < particle size < thermal degradation. To compare the yield of blank sample fraction 1.0-2.5 mm after 24 h

enzymatic hydrolysis (19.1 $g \cdot L^{-1}$) and the maximal yield in this paper, 70.1 $g \cdot L^{-1}$ after 96 h of the enzymatic hydrolysis of fraction < 0.7 mm after thermal pretreatment following by deep freezing, was higher by 267%. The conversion of polysaccharides shows the efficiency of lignocellulosic biomass hydrolysis based on the pretreatment and the access of enzymes onto the biomass surface.

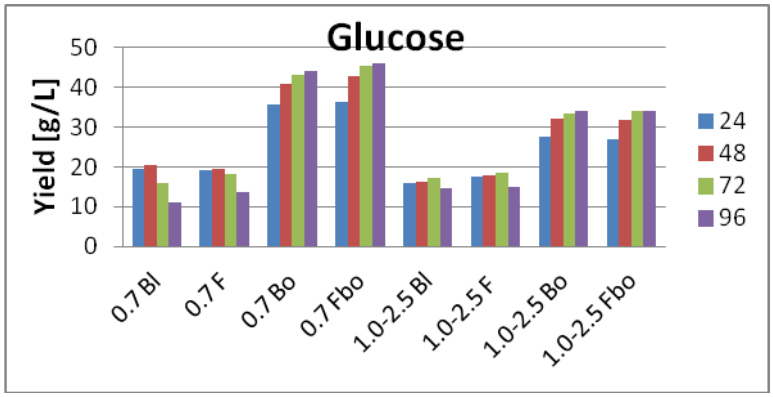

Fig. 2: The yields of glucose of pretreated branch wood beech fractions after enzymatic hydrolyses. Bl = blank; F = deep freezing at -80°C; Bo = boiling at 160°C; Fbo = boiling at 160°C and subsequent deep freezing at -80°C.

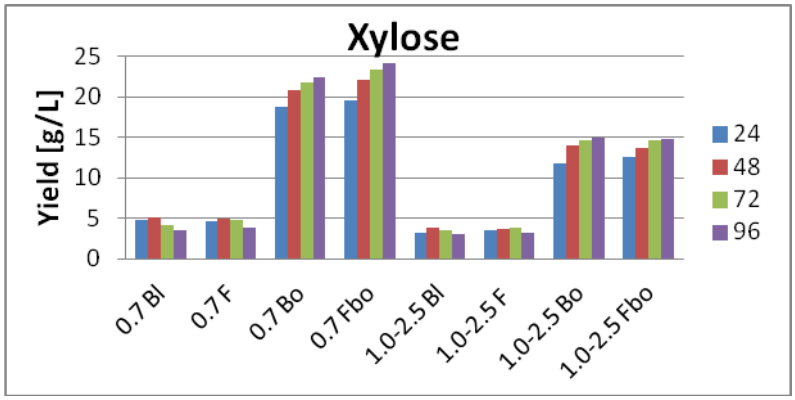

Fig. 3: The yields of xylose of pretreated branch wood beech fractions after enzymatic hydrolyses. Bl = blank; F = deep freezing at -80°C; Bo = boiling at 160°C; Fbo = boiling at 160°C and subsequent deep freezing at -80°C.

To compare the conversions, from the blank sample fraction 1.0 - 2.5 mm after 24 hours of enzymatic hydrolysis (20 %), the combination of milling, thermal pretreatment and deep freezing the conversion increased to 73.2 % after 96 h of hydrolysis for the sample fraction < 0.7 mm, thermally pretreated following by deep freezing. In study Jeong et al. (2016) the conversion more than 90% was achieved after freeze-thaw pretreatment and subsequent two-stage hydrolysis (by 1 % sulfuric acid, following by enzymatic hydrolysis). Our previous study focused on pretreatment of beech sawdust, the total monosaccharide conversion of particle size < 0.7 mm after steam explosion at 180°C for 10 min was 84.7% (Pažitný et al. 2019b). Although the deep freezing pretreatment showed no effect for thermally

untreated fraction < 0.7 mm, the maximum conversion of the total monosaccharides was 26.8% after 48 h of enzymatic hydrolysis blank sample was mildly higher as compare with blank sample (24.0% conversion) and 1st cycle (24.3%) of study focused cryolysis poplar sapwood dust, particle size < 0.7 mm, including cyclic freezing thawing (Boháček et al. 2020). On the other hand, very weak effect of deep freezing was observed on fraction 1.0 - 2.5 mm without the thermal pretreatment, the maximum yields of total monosaccharides after 72 h of enzymatic hydrolysis were increased from 20.7 $g \cdot L^{-1}$ (conversion of 21.6%) to 22.3 $g \cdot L^{-1}$ (conversion of 23.2%). Despite of that, the yields and conversions were lower, than in the previous study (Boháček et al. 2020). Zhu et al. (2020) compared the yields after freezing of poplar at -20°C and -70°C. Hemicellulose was extracted after freeze-thaw pretreatment at temperature 170°C for 1 h with wood to water ratio of 1:6 (w/w). The results showed, that from the sample without freezing was the yield of hemicelluloses of 64.9 $mg \cdot g^{-1}$, after treatment in temperature -20°C the yield increased to 85.9 $mg \cdot g^{-1}$, and the treatment at -70°C resulted in the yield of 92.1 $mg \cdot g^{-1}$ of the poplar wood.

CONCLUSIONS

Two size fractions, < 0.7 (smaller) and 1.0-2.5 (larger) mm mesh sawdust of beech (*Fagus sylvatica* L.) were subjected to observe the influence of deep freezing at -80°C, treatment in water under high pressure 5.5 bar, at temperature 160°C for 30 min (thermal treatment), and combination of these treatments to enhance enzymatic accessibility.

The maximum influence of deep freezing was observed in increasing of conversion from 69.6% (without freezing) to 73.2% (frozen) for thermally treated smaller fraction. The influence of particle size raised the maximum conversion of the larger fraction 51.1% to the highest conversion of smaller fraction 73.2%. The thermal pretreatment increased the maximum conversion from 26.8% for thermally untreated material to 69.6% for the thermally treated material without deep freezing.

The analyses showed that the influence of those factors for disruption of the lignocellulosic biomass and effect on the enzyme accessibility is in order of deep freezing < particle size < thermal degradation.

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