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Short notes

INCREASING THE EFFICIENCY OF ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC MATERIALS BY FREEZING PRETREATMENT

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ABSTRACT

This paper describes the differences between the efficiency of enzymatic hydrolysis of selected lignocellulosic materials after pretreatment by cyclic freezing and thawing. It also discusses the analysis of the positive effect of alkaline conditions on pretreatment. The selected materials were *Populus alba* L., wheat straw from *Triticum aestivum* L. and *Cannabis sativa*. Three pretreatment methods were used to compare the efficiency of enzymatic decomposition of cellulose and cellulose accessibility. The best results show the wheat straw pre-treated at -20°C in the freezer with NaOH addition with a concentration of monosaccharides of 56.6 $g.l^{-1}$ compared to initial hydrolysed material with a concentration of monosaccharides of 24.4 $g.l^{-1}$. The results show better digestibility of grass compared to wood dendromass.

KEYWORDS: Enzymatic hydrolysis, freezing pre-treatment, lignocellulosic materials, *Populus alba,* wheat straw*, Cannabis sativa.*

INTRODUCTION

Converting lignocellulosic materials (LCM) to monosaccharides such as glucose and xylose is a key step in producing biofuels, biomaterials and biochemicals. Various pretreatment processes have been developed for decades to improve the processing of lignocellulosic material. Each pretreatment process has a different specificity in changing lignocellulosic materials' physical and chemical structure (Sun et al. 2016). Steam explosion is a very effective pretreatment method (Pažitný et al. 2020a,b, 2022a,b).

Hydrolysis is the process of breaking down LCM into monosaccharides using enzymes or acids (Mankar et al. 2021, Chen et al. 2016). However, the inherent structure of the LCM prevents access of hydrolytic reagents to the biopolymers, thus reducing the hydrolysis efficiency (Heiss-Blanquet et al. 2011). Freeze-thaw pretreatment appears to be a promising LCM pretreatment method to improve hydrolysis and monosaccharide production (Bohacek et al. 2020, 2022). Ongoing research has the potential to improve this technology and make it an economically affordable and environmentally responsible tool for the production of biofuels, biomaterials and biochemicals from LCM (Mankar et al. 2021). The process consists of freezing and subsequent freeze-drying of material. Last but not least, removing moisture by sublimation reduces the energy requirement of hydrolysis and improves the shelf life and handling of LCM. In the context of LCM pretreatment, the process leads to increased porosity and surface area (Zhu et al. 2020), and freezing and sublimation of water create pores in the material, facilitating access of hydrolytic reagents to the biopolymers. The physical basis of the process is the mechanical destruction by ice crystals (Boháček et al. 2020, 2022). Its effect is dependent on the morphological structure of the LCM (Ihnát et al. 2021). The principle of freezing methods is based on the volume change of water during an ice formation by approximately 9% (Halaj et al. 2021). Mechanically disrupting the wood structure with ice is much more complex 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, rice straw, wheat straw or corn stalks etc. (Wang et al. 2013, Ihnát et al. 2015, Echeverria et al. 2018). Considering that the wood material is more compact and has a higher density, combinations of freezing with other pre-treatments, such as adding H2SO4, ammonia, NaOH or alkaline simultaneous treatment are applied (Jeong et al. 2016, Li et al. 2019, Su and Fang 2017)

The reduction of cellulose crystallinity by freeze-thaw disrupts the crystalline structure of cellulose, which becomes more accessible to hydrolysis. Disrupting the lignin matrix reduces the barrier effect of lignin to hydrolytic reagents. Chen et al. (2016) investigated the effect of freezing pretreatment on the enzymatic hydrolysis of various LCMs, including wheat straw, corn stover, and miscanthus. The results show that the process significantly improves the availability of cellulose to enzymes, leading to increased production of fermentable sugars (glucose and xylose). The article discusses possible mechanisms for this improvement, such as increased porosity and reduced cellulose crystallinity. Gandla et al. (2018) published a similar study focusing on enzymatic saccharification of wheat straw. The study shows that the pretreatment increases the enzymatic digestibility of the material, leading to higher sugar yields. The authors attribute this improvement to the disruption of cell wall structure and increased availability of cellulose by cellulase enzymes.

This research aims to compare the difference in the effect of freezing pre-treatment on the destruction of the cellulose crystalline structure of wood dendromass and grass biomass.

MATERIAL AND METHODS

Lignocellulosic material

Three types of lignocellulosic material were used for the experimental measurements: white poplar, wheat straw and hemp. In the case of the lignocellulosic materials mentioned above, fractions up to 0.7 mm in size were obtained by knife milling.

Four pretreatment methods were used in the experiments: X.1: no freezing pretreatment, designated as the comparison sample; X.2: pretreated freeze-thaw under natural conditions; X.3: freeze-thaw in a freezer at -20°C; X.4: freeze-thaw in a freezer at -20°C with a 6% addition of NaOH.

The natural form was applied through 19 freeze-thaw cycles (X.2), where the natural freeze was allowed to take effect for 38 days in the open during the winter, and the sample was allowed to thaw indoors at room temperature during the even-numbered day. An artificial form of freezing (X.3) was applied in a freezer for 60 days. The sample was also allowed to thaw freely during the day. Method X.4 involved essentially the same conditions as Method X.3, except that the lignocellulosic material sample was absorbed during the initial 24 h before freezing with an alkaline solution to give 6% NaOH on an absolute dry basis.

Enzymatic hydrolysis

After pretreatment of the individual lignocellulosic material samples, the samples were subjected to enzymatic hydrolysis. The enzymatic hydrolysis was performed using the enzyme cellulase complex Cellic® CTec3 (Novozymes) with a recommended dosage of 1.5-30.0 wt.%. A 15 wt.% enzyme suspension (15 g Cellic® CTec3/100 g suspension) was applied in our case. All enzymatic hydrolysis (EH) experiments were carried out in citrate buffer (0.05 M), the pH of which was adjusted with NaOH solution (10 wt. %) to pH = 5.0 at 50°C.

Hydrolysate sampling procedure

At the time of sample collection (24, 48, 72, 96 h), the Erlenmayer flask (250 ml) with the suspension was removed from the incubator. Using plastic pipettes, a volume of 2 ml was collected into plastic cuvettes for the Unipan centrifuge (High-speed centrifuge, Type 310). The cuvettes with the volume taken were boiled for 10 min in a water bath to stop the enzymatic reaction. The suspension was centrifuged at 5000 rpm for 10 min. The high-performance liquid chromatography (HPLC) method was used to determine the content of sugars (monosaccharides) in the hydrolysates.

HPLC determination of monosaccharides

Before the determination of monosaccharides, the hydrolysate after lignin determination was treated with 4% H_2SO_4 at 121°C for 2 h to allow hydrolysis of the oligosaccharides to take place. Subsequently, the hydrolysate was neutralised with $BaCO_3$. The HPLC chromatographic system from CHROMSERVIS SK with Rezex ROA H+ column was used to determine monosaccharides. The mobile phase comprised a 0.0025 M sulfuric acid solution in deionised water. Samples of the neutralised hydrolysates were filtered by pushing through a disposable Teflon filter (MS PTFE, 0.22 μm pore size) using a syringe. After filtering, 0.5 ml was taken from each sample and diluted with deionised water 20 times to a final volume of 10 ml. The sample thus treated was dispensed into the column. Analyses were performed at 30°C and a 0.5 ml/min flow rate. Each analysis was performed in duplicate (Boháček et al. 2022)

Calculation of consistency in enzymatic hydrolysis

The calculations followed Pedersen et al. (2010), Viell et al. (2013), and Chen et al. (2007). The consistency of the suspension in all EH experiments was expressed according to Eq. 1:

$$w\ (\%) = \frac{m_{LCM\,a.d.}}{m_{LCM\,a.d.} + m_{H_2O\ in\ LCM} + m_{bs} + m_E} \times 100 \tag{1}$$

where: $m_{LCM\,a.d.}$ is the weight of absolutely dry lignocellulosic raw material (g); $m_{H_2O\ in\ LCM}$ is the weight of water in lignocellulosic raw material (g); m_{bs} is the weight of added buffer solution (g); m_E is the weight of added enzyme (g).

RESULTS AND DISCUSSION

Research concerned with pre-treatment methods of agricultural, municipal lignocellulosic wastes (Pažitný et al. 2022b) or even still considered microalgae could play essential roles in practical application (Sankaran et al. 2020, Halaj et al. 2019). Forest residues after wood harvesting remain underutilised due to economic and operational barriers caused by the high cost of collecting, treatment, and transportation (Han et al. 2010). Most residues of the tree logs remain as land cover. The mass distribution of wood in deciduous trees at harvesting time is 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).

Tab. 1 shows the maximum monosaccharide concentrations achieved after 48 h of enzymatic hydrolysis. The best results were obtained for the wheat straw and cannabis combined pretreated with NaOH addition.

Tab. 1: Glucose and xylose concentrations measured after 48 h of enzymatic hydrolysis.

	Method			
	X.1	X.2	X.3	X.4
White poplar				
Glucose ($g.l^{-1}$)	7.50 ± 0.01	10.20 ± 0.01	11.70 ± 0.01	12.00 ± 0.01
Xylose ($g.l^{-1}$)	4.90 ± 0.02	5.00 ± 0.02	6.10 ± 0.02	6.30 ± 0.02
Concentration of monosaccharides ($g.l^{-1}$)	12.40 ± 0.02	15.20 ± 0.02	17.80 ± 0.02	18.30 ± 0.02
Wheat straw				
Glucose ($g.l^{-1}$)	15.60 ± 0.01	21.10 ± 0.01	23.10 ± 0.01	33.60 ± 0.01
Xylose ($g.l^{-1}$)	8.80 ± 0.02	15.90 ± 0.02	16.10 ± 0.02	23.00 ± 0.02
Concentration of monosaccharides ($g.l^{-1}$)	24.40 ± 0.02	37.00 ± 0.02	39.20 ± 0.02	56.60 ± 0.02
Hemp				
Glucose ($g.l^{-1}$)	10.00 ± 0.01	28.80 ± 0.01	30.60 ± 0.01	42.30 ± 0.01
Xylose ($g.l^{-1}$)	5.60 ± 0.02	8.90 ± 0.02	13.00 ± 0.02	13.50 ± 0.02
Concentration of monosaccharides ($g.l^{-1}$)	15.60 ± 0.02	37.70 ± 0.02	43.60 ± 0.02	55.80 ± 0.02

Figs. 1-3 evaluate the data of each used pre-treatment method (X.1-X.4). Analysis of the individual graphs shows that method X.4, which deals with pre-treatment in a freezer with the addition of NaOH, was the most successful in achieving the highest concentration of monosaccharides. Pre-processing was meaningful regarding sugar concentration to be carried out for a maximum of 48 h (Boháček et al. 2022a). After this time, according to the graphical evaluation, there is no point in prolonging the pre-processing as the concentration of sugars is significantly reduced.

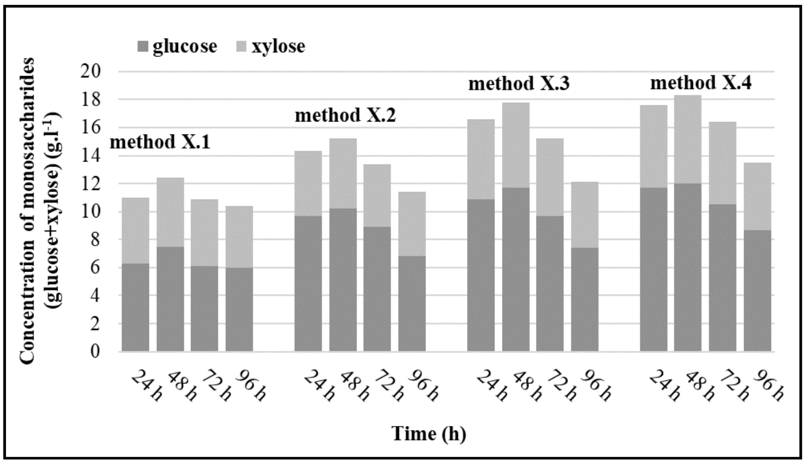

Fig. 1: Comparison of the efficiency of white poplar pretreatment methods (X.1- comparative sample; X.2- freezing under natural conditions; X.3- freezing -20°C; X.4- freezing -20°C + NaOH.

The comparison sample, method X.1 - without freezing pre-treatment, showed the lowest sugar concentrations for all three raw materials (Tab. 1). Among them, wheat straw came out as the wealthiest raw material in terms of glucose and xylose content with a sugar concentration value of 56.6 $g.l^{-1}$. The white poplar sample was the least rich in sugars after hydrolysis, with a sugar value of 18.3 $g.l^{-1}$ (Tab. 1).

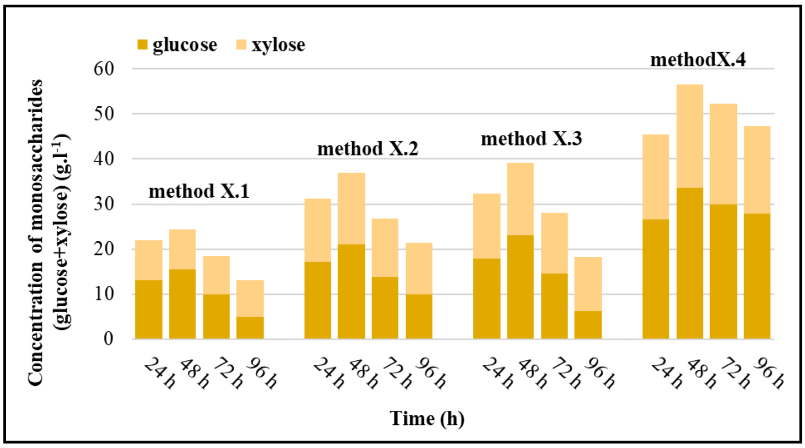

Fig. 2: Comparison of the efficiency of pretreatment methods of wheat straw (X.1- comparative sample; X.2- freezing in natural conditions; X.3- freezing -20°C; X.4- freezing -20°C + NaOH.

Method X.1 - without freezing, pretreatment showed the lowest concentrations of sugars for all three raw materials (Tab. 1). Although white poplar, based on the measured results, will not be among the most suitable raw materials for the production of second-generation biofuels (low concentration of monosaccharides), the use of freezing pretreatment is a suitable method. The monosaccharide content was increased by approximately 48% by the pre-treatment method in a freezer by adding NaOH using 30 cycles.

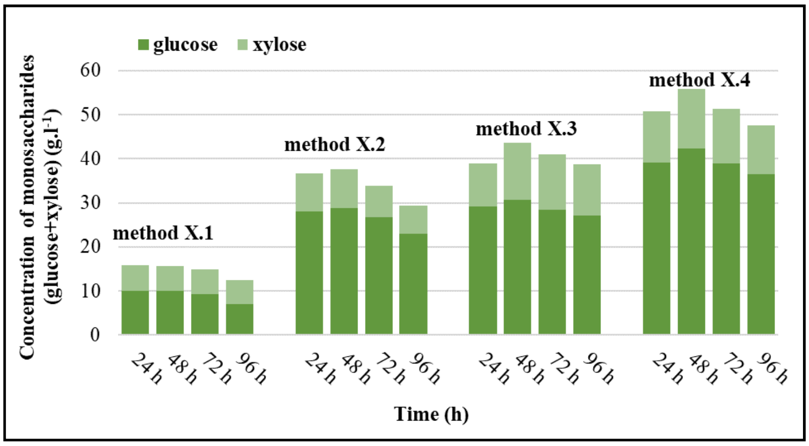

Fig. 3: Comparison of pretreatment efficiency of hemp (X.1- comparative sample; X.2- freezing under natural conditions; X.3- freezing -20°C; X.4- freezing -20°C + NaOH.

Wheat straw, unlike white poplar, is one of the annual plants with a much higher potential for processing into second-generation biofuels, as evidenced by the maximum monosaccharide concentration values recorded after 48 h of enzymatic hydrolysis. The significant effect of pretreatment of this type of sample in a freezer with the addition of NaOH resulted in an approximately 230% increase in monosaccharide concentration (Tab. 1) compared to method X.1. The natural form of freezing pretreatment, which achieved 19 freezing cycles of the wheat straw sample, contained approximately 1.5 times lower monosaccharide concentration after hydrolysis than the X.4 pre-treatment.

CONCLUSIONS

The results of freezing pretreatment indicate an enhanced effect on cellulose degradation during subsequent enzymatic hydrolysis. In the case of white poplar, the maximum concentration of glucose and xylose monomers was reached after 40 h (18.1 $g.l^{-1}$), 41 h (14.8 $g.l^{-1}$), 49 h (17.9 $g.l^{-1}$) and 53 h (11.9 $g.l^{-1}$), respectively, depending on the pretreatment method used, including the comparative original sample (X.4, X.2, X.3 and X.1, respectively). After this time, there was a reduction. The situation was different for other lignocellulosic materials - wheat straw and hemp. In the case of wheat straw, the maximum concentration of the monitored sugar monomers was reached after 40 h (23.2 $g.l^{-1}$, X.1), 43 h (34.0 $g.l^{-1}$, X.2), 45 h (36.5 $g.l^{-1}$, X.3)) and after 61 h (55,5 $g.l^{-1}$, X.4) and in the case of hemp after 34 h (15.9 $g.l^{-1}$, X.1), 37 h (37.3 $g.l^{-1}$, X.2), 52 h (54.3 $g.l^{-1}$, X.4) and 58 h (42.9 $g.l^{-1}$, X.3), respectively. The wood raw material has a more unyielding structure, as evidenced by the generally low concentration of monosaccharides achieved by freezing poplar particles and subsequent enzymatic hydrolysis. The highest proportion of monosaccharides (55.5 $g.l^{-1}$) was obtained in the case of pre-treatment of wheat straw by the X.4 method with 6 % alkali.

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