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VARIOUS LIGNOCELLULOSIC RAW MATERIALS PRETREATMENT PROCESSES UTILIZABLE FOR INCREASING HOLOCELLULOSE ACCESSIBILITY FOR HYDROLYTIC ENZYMES

PART I. EVALUATION OF WHEAT STRAW PRETREATMENT PROCESSES

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ABSTRACT

New requirements for the biofuels industry force individual enterprises to develop various procedures for newly selected substrates pretreatments that could be applicable in processing of large quantities of raw materials. Even greater pressures are on second-generation biofuels producers justified by selection of waste lignocellulosic substrates and methods of substrate processing. Among the most suitable lignocellulosic raw materials in Slovak Republic (SR) for 2G bioethanol production is wheat straw. This raw material (Senec region, SR) for enzymatic hydrolysis was pretreated by dry milling (Brabender), cyclic freezing and thawing, wet milling (Sprout Waldron), two-step process of steam explosion at 180°C and extrusion at 145°C and one-step process of steam explosion at different temperatures. Wheat straw holocellulose accessibility was tested by adsorption of three commercially available dyes (Pylam Products Company, Inc., USA). Absorptivity coefficient of each dye at its maximum wavelength was determined from individual calibration curves of dyes and their values resulted ranging from 13.78 to 19.52 $dm^3 \cdot g^{-1} \cdot cm^{-1}$. The absorption of solution was measured and concentration of residual dye was calculated at given wavelength. The accessibility of holocellulose contained in wheat straw pretreated by steam explosion was controlled by SEM (scanning electron microscope) in correlation with the ratio of adsorbed dyes according to the modified Simons' method.

KEYWORDS: Wheat straw, biofuels industry, Simons' method, holocellulose accessibility, pretreatment processes, biomass.

INTRODUCTION

Cellulose and lignin are basic building units of most plants. In addition, cellulose is the most widespread organic natural compound. Today, biofuels industry activities are oriented to the production of the first-generation bioethanol based on crops containing monosaccharides (sugar cane, sugar beet) or starch (grain, corn). However, lignocellulosic biomass (woody, inedible parts of plants, such as grasses, agricultural crops, and forest waste) for the second-generation biofuels production will be expected in the near future (Highina et al. 2014). Production technology and prospective crops are still subject to intense research and optimization already in commercial establishments. Second-generation bioethanol appears to be the best available choice. For its production, only cheap biomass composed of cellulose, hemicelluloses and lignin, which is readily available all over the world. Second-generation bioethanol is more promising in terms of greenhouse gas balance. It produces by 94% less CO2 than fossil fuels. For bioethanol from sugars and grains this reduction represents 78% (Highina et al. 2014).

According to the estimation of agricultural crops yields in year 2017 for Slovak Republic region the harvesting yield was 1,830,758 tonnes of produced wheat grain which represents 2,013,834 tonnes of wheat straw as waste product (Statistical Office of the Slovak Republic 2017). This fact predetermines the use and processing of wheat straw for bioethanol production. The other reason for using wheat straw for bioethanol production is relatively high content of hydrolysable polysaccharides – cellulose and hemicelluloses (Letko et al. 2015). Enzymatic agents (Reese 1956, Reese and Mandels 1959, Sandhu and Kalra 1985) are useful in the natural cellulose or hemicelluloses conversion to monomer units such as glucose or pentoses (xylose, arabinose etc.). Enzymatic hydrolysis of cellulose is well described (Whistler and Smart 1953) by following chemical equations scheme 1:

$$\textit{natural cellulose} \rightarrow \textit{linear cellulose} \rightarrow \textit{cellobiose} \rightarrow \textit{glucose} \qquad (1)$$

In the first step linear cellulose is formed by adding of the first type cellulase (endoglucanase) and in the second step cellobiose is formed by adding of the second type cellulase (exoglucanase). The reaction is finalized with bonding cleavage by adding of β-glucosidase (Sandhu and Kalra 1985). Cellulose terminal units and cellulose macromolecule converted to oligosaccharides – cello-oligosaccharides (Kuba et al. 1990) are converted to glucose monomers overall. In addition, recent enzymatic agents are able to convert hemicelluloses to pentoses (Gong et al. 1981) by analogical manner and obtained pentoses can be used in convenient conversion into a common organic intermediate which is useful in organic synthesis (Bercier et al. 2007).

However, accessibility of holocellulose consisting of cellulose and hemicelluloses is a very important factor for successful enzymatic hydrolysis, good ability of enzymatic agent to degrade or convert cellulose and hemicelluloses into their monomers and related high total process yields of monosaccharides. Regarding the accessibility of holocellulose, the raw materials used in the second-generation biofuels production process contain a higher proportion of introduced substances with the more difficult degradability, more polymeric compounds with a high degree of polymerization, and thus higher stability and resistance to cleavage compared to raw materials used in the production of first-generation biofuels (Boháček et al. 2014). That is why the accessibility of holocellulose for a hydrolytic enzyme should be enlarged by different pretreatment processes. Industrial hammer mills are currently being used for primary mechanical destruction of raw lignocellulosic materials but they have very high energy consumption. The lignocellulosic raw materials can be also milled by rotary mills or defibred in multi-disk refiner. The mechanical degradation processes of lignocellulosic raw materials are insufficient to achieve high accessibility of holocellulose for enzymes that cause hydrolytic cleavage of cellulose contained in the lignocellulosic materials into monomers of simple carbohydrates and that is why hydrothermal pretreatment (Zhang et al. 2014), steam explosion (Negro et al. 2003, Wang et al. 2009a, 2009b), steam extrusion or combination of steam explosion and steam extrusion (Pažitný and Šutý 2018) have to be used. During the last decade, the pretreatment and hydrolysis processes of lignocellulosic raw material with innovative approach were also described in scientific papers. Some of the scientific papers describes microwave pretreatment (Alvira et al. 2010), microwave-assisted hydrolysis with alkaline freeze pretreatment (Su and Fang 2017) and some new pretreatment applications that were developed by our workplace which combine some of our findings and also findings of several scientific papers in the field of specific freezing pretreatment processes (Boháček et al. 2014, Chang et al. 2011). These processes can be controlled by a fast and precise method based on Simons' procedure (Boháček et al. 2014, Esteghlalian et al. 2001) which serves for determination of suitability of different processes of lignocellulosic raw material pretreatment for enzymatic hydrolysis in the production of 2G bioethanol (Pažitný and Šutý 2018).

MATERIAL AND METHODS

Materials

Wheat straw as a source of holocellulose was obtained from Senec region, Slovak Republic. Three dyes for Simons' analytical procedure – Pontamine Sky Blue 6BX (direct blue, B), Phenamine Fast Orange WS (direct orange, O) and Direct Violet 9 (direct violet, V) were obtained from Pylam Products Company, Inc., USA.

Methods

Wheat straw was pretreated mechanically (samples WS1-B and WS1-CFT) by dry milling in Brabender mill (Brabender®, GmbH & Co. KG, Germany), cyclic freezing and thawing at specific conditions (Boháček et al. 2014), and hydro-mechanically (sample WS1-SW) by wet milling in refiner Sprout Waldron (Sprout, Waldron & Co., Inc., USA). The reactor for steam explosion (Amar Equipments Pvt. Ltd., India) and reactor for steam extrusion (Hungaromix Agrárfejlesztő-Fővállalkozó Kft., Hungary) were used for steam reactions at different temperatures (175°C, 195°C, 215°C and 230°C). Two-step process of steam explosion at 180°C and extrusion at 145°C (sample WS1-SE-Ex-145-4) and one-step process of steam explosion at 215°C (sample WS2-SE-215-22-10) were applied on raw materials. The retention time of each thermo-hydro-mechanical experiment with wheat straw (steam explosion and steam extrusion) was 10 minutes.

Absorptivity coefficient of each dye at its maximum wavelength was determined from individual calibration curves of dyes. The absorption of solution was then measured and solution concentrations of Simons' dyes were calculated at given wavelength. The dyes concentrations were determined by using of UV-Visible spectrophotometer Helios Beta (Thermo Spectronic, United Kingdom). The amount of dye adsorbed on the fibres is calculated from the difference between concentration of the starting dye and the dye concentration in the supernatant obtained by centrifugation. The calculation of concentrations of the adsorbed dyes is based on the Lambert-Beer law.

Raw and pretreated wheat straw samples were monitored and analyzed by digital microscope Keyence VHX-5000 (Keyence International (Belgium) NV/SA) and by scanning electron microscope JEOL JSM 6610 (JEOL Ltd., Japan).

RESULTS AND DISCUSSION

Wheat straw as a perspective substrate in 2G bioethanol production

Wheat straw is one of the most abundant crop residues in European countries, and it also seems to be one of the cheapest substrates. For this reason it is the most useful raw material for bioethanol production in Slovak Republic (Gigac et al. 2017). An estimation of agricultural crop yields to 15. 8. 2017 (Statistical Office of the Slovak Republic 2017) represents Tab. 1. Wheat grain yield (1,830,758 tonnes) compared with the harvesting yield of the most produced agricultural crops such as barley grain (567,614 tonnes), rapeseed (457,447 tonnes), oats (38,555 tonnes) and rye grain (34,358 tonnes) is the highest. According to the report of Statistical Office of the Slovak Republic the most produced wheat grain represents 2,013,834 tonnes of wheat straw as a waste product (Tab. 1). The total amount of five most produced agricultural crops is 2,928,732 tonnes which represents 3,316,282 tonnes of agricultural waste – wheat, barley, rapeseed, oats and rye straw. Thus the wheat straw proportion represents almost 61% from those straws. A large amount of produced wheat straw is one of the reasons to use it in production of fibreboards with straw pulp content (Lübke et al. 2014, Ihnát et al. 2015a, Ihnát et al. 2015b) and recently also in production of bioethanol (Gigac et al. 2017, Hammond and Mansell 2017, Gonzalez-Contreras et al. 2017, Yuan et al. 2018).

Tab. 1: Estimates of yields of agricultural crops and straw obtained from agricultural crops in year 2017 for Slovak Republic region.

Agricultural crop	Estimated grain yields (tonnes)	Estimated straw yields (tonnes)
Wheat	1,830,758	2,013,834
Barley	567,614	397,330
Rapeseed	457,447	823,405
Oats	38,555	40,483
Rye	34,358	41,230
Total	2,928,732	3,316,282

Enzymatic hydrolysis is the key factor of the process of bioethanol production and remains a major obstacle mainly due to high enzyme cost. In order to achieve sufficient enzyme accessibility to wheat straw, effective pretreatment methods are required for disruption of the complex structure and recalcitrant nature of lignocellulosic biomass (Gigac et al. 2017).

Methods of wheat straw pretreatment and their evaluation

Methods of wheat straw pretreatment and their evaluation were described in our previous papers (Boháček et al. 2014, Letko et al. 2015, Pažitný and Šutý 2018). We tested the mechanical (Brabender, B) and hydro-mechanical (Sprout Waldron, SW) pretreatment, pretreatment by cyclic freezing and thawing (CFT), and by two-step process of steam explosion at 180°C and extrusion at 145°C and by one-step process of steam explosion at 175°C, 195°C, 215°C and 230°C. The principal analysis of holocellulose accessibility for enzymes in case of each pretreatment method was based on adsorption of three commercially available dyes (Tab. 2). Absorptivity coefficient $\varepsilon_{\lambda\mathrm{max}}$ of each dye at its maximum wavelength was determined from individual calibration curves of dyes.

Values of absorptivity coefficient for direct blue dye (B), direct orange dye (O) and direct violet dye (V) were 13.78, 19.52 and 19.08 $dm^3 \cdot g^{-1} \cdot cm^{-1}$, respectively.

Tab. 2: Characterization of commercial dyes for holocellulose accessibility determination for pretreated and raw wheat straw.

Dye type	**Commercial name**	**Molecular formula**	**Molecular weight**	**λ_{max} (nm)**	**$\varepsilon_{\lambda\mathrm{max}}$ ($dm^3 \cdot g^{-1} \cdot cm^{-1}$)**
Direct blue / B	Pontamine Sky Blue 6BX	$C_{34}H_{24}N_6Na_4O_{16}S_4$	992.80	601	13.78
Direct orange / O	Phenamine Fast Orange WS	$C_{34}H_{21}N_6Na_3O_{11}S_2$	822.66	490	19.52
Direct violet / V	Direct Violet 9	$C_{30}H_{23}N_5Na_2O_8S_2$	691.64	546	19.08

The amount of individual dye adsorbed on fibres was calculated from the difference between concentration of the starting dye and the dye concentration in the supernatant obtained by centrifugation. The calculation of concentrations of the adsorbed dyes and absorptivity coefficient values is based on the Lambert-Beer law, Eq. 2:

$$A_{\lambda max} = \varepsilon_{\lambda max} L C \tag{2}$$

where: $A_{\lambda max}$ – measured absorbance at λ_{max} (dimensionless variable),
$\varepsilon_{\lambda max}$ – measured wavelength-dependent absorptivity coefficient ($dm^3 \cdot g^{-1} \cdot cm^{-1}$),
L – layer thickness (constant cell width, 1 cm),
C – concentration of supernatant obtained by centrifugation ($g \cdot dm^{-3}$).

Model measurement of dyes concentrations for wheat straw pretreatment by CFT

Experiments for model measurement of dyes concentrations were based on our previous work results in cyclic freezing and thawing (Boháček et al. 2014). When evaluating the original wheat straw and pretreated wheat straw, it is convenient to use modified Simons' method based on the absorbance of non-adsorbed dyes (Boháček et al. 2014). The accessibility degree of used lignocellulosic material depends on the number of free hydroxyl groups per cellulose molecule, since according to published work in this field Simons' dyes have a higher selective affinity to hydroxyl groups of cellulose molecule (Chandra et al. 2008). Concentration values of commercial dyes can be monitored in supernatant of wheat straw suspension obtained from centrifugation (Boháček et al. 2014).

As shown in Tab. 3, concentration values of commercial dyes in supernatant of wheat straw suspension obtained from centrifugation depend on the initial concentration of untreated wheat straw and also on the type of the dye. However, for each method of pretreatment it was simpler to use the adsorption ratio of two different dyes. This means that three different combinations of dyes could be tested.

Tab. 3: Resulting concentration values of free dyes in original wheat straw suspension and in suspension of wheat straw pretreated by CFT method.

Concentration of unadsorbed dye in supernatant of original and pretreated wheat straw suspension ($g·dm^{-3}$)					
Direct blue / dye B		Direct orange / dye O		Direct violet / dye V	
Original	Pretreated	Original	Pretreated	Original	Pretreated
0.0073	0.0051	0.0106	0.0051	0.0064	0.0037
0.0125	0.0102	0.0128	0.0069	0.0083	0.0052
0.0172	0.0117	0.0135	0.0087	0.0105	0.0073
0.0258	0.0189	0.0188	0.0117	0.0150	0.0099
0.0292	0.0267	0.0225	0.0168	0.0200	0.0157
0.0337	0.0283	0.0251	0.0161	0.0064	0.0037

Our experiments showed that the high molecular weight fraction is responsible for increasing of affinity to cellulose (direct orange, dye O) and the low molecular weight fraction has similar affinity to cellulose (direct blue, dye B). We found that the adsorption of dye O has very similar pattern to adsorption of dye B, however, dye O adsorbs on wheat straw fibres better than dye B. The most important finding is that pretreatment and related accessibility increases with simultaneous reduction of difference in adsorption of those dyes. However, in case of direct violet (dye V) any correlation between the pretreatment methods or accessibility level and adsorbed dye V was not found. The unambiguous course has only B/O ratio – the ratio of amounts of dye B and dye O adsorbed on wheat straw fibres. That is why this ratio was used to describe holocellulose accessibility level for different pretreatment methods. As shown in Fig. 1, the wheat straw pretreatment by dry milling in Brabender mill with B/O ratio at level of 0.7889 has the lowest accessibility for enzymes which is comparable with pretreatment by cyclic freezing and thawing with B/O ratio at level of 0.7959.

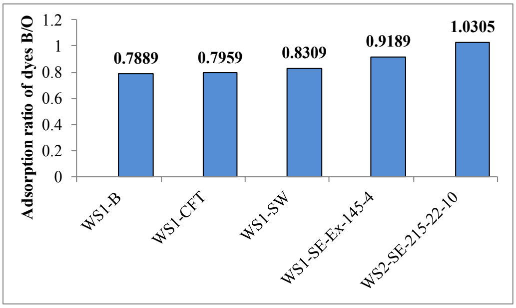
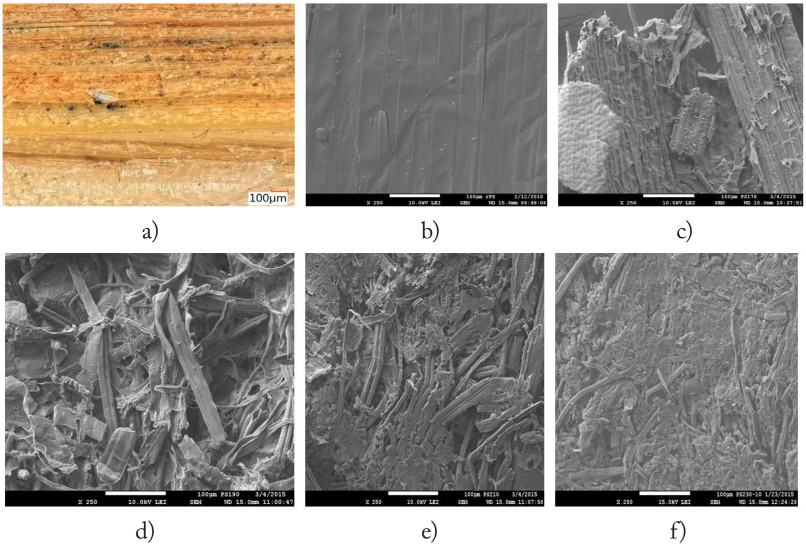


Fig. 1: Comparison of accessibility of mechanical (samples WS1-B and WS1-CFT), hydro-mechanical (WS1-SW), thermo-hydro-mechanical (samples WS1-SE-Ex-145-4 and WS2-SE-215-22-10) pretreatment techniques on a wheat straw samples based on B/O ratio.

Wheat straw pretreated by wet milling in refiner Sprout Waldron with B/O ratio at level of 0.8309 resulted in a little better accessibility for enzymes. The best holocellulose accessibility levels were achieved with pretreatment by two-step process of steam explosion at 180°C and extrusion at 145°C and with pretreatment by one-step process of steam explosion at 215°C where obtained B/O ratio were at level of 0.9189 and 1.0305 (Fig. 1), respectively. This concludes that process of steam explosion at high temperatures or steam explosion at lower temperatures combined with steam extrusion lead to higher holocellulose accessibility level which is more suitable in the process of enzymatic hydrolysis compared to mechanical and hydro-mechanical methods of wheat straw pretreatment.

Holocellulose accessibility of pretreated wheat straw in correlation with SEM

The images obtained by scanning electron microscope (SEM) shown the differences between the structure of the original wheat straw sample (Fig. 1, image b)) and structure of pretreated wheat straw samples (Fig. 1, images c), d), e) and f)). As the model pretreatment method was selected steam explosion at four different temperatures (175°C, 195°C, 215°C and 230°C) due to its most notable visual changes in tested lignocellulosic substrates (Letko et al. 2015) and due to good results in enzymatic hydrolysis of wheat straw pretreated by steam explosion (Russ et al. 2016). Each pretreated sample of wheat straw has significantly different structure and this is evident mainly for images b) and f) vs. c), d) and e) in Fig. 2.



a) b) c)

d) e) f)

Fig. 2: Digital microscope image (image a)) and scanning electron microscope images (SEM, images b) – f)) of raw wheat straw (original samples – images a), b)) and wheat straw pretreated by steam explosion at different temperatures (c) 175°C, d) 195°C, e) 215°C and f) 230°C

In the first case the wheat straw structure obtained by SEM seems to be more compact than in the second one and this can potentially lead to higher holocellulose accessibility for enzymes. That was also confirmed in the previous scientific works where the effect of steam explosion temperature on enzymatic hydrolysis was studied (Russ et al. 2016). It was found that the monosaccharide concentration, holocellulose conversion as well as monosaccharide yield were the highest between 195°C and 215°C which correlates with the images d) and e) in Fig. 2. These samples are visibly more defibred and this fact is confirmed by presence of several nano- or microfibrils in wheat straw pretreated by steam explosion at 195°C and 215°C (Fig. 2, images d) and e)) compared to wheat straw pretreated by steam explosion at 175°C (Fig. 2, image c)). It is known that the fiberized structure of lignocellulosic pulp with larger content of nano- or microfibrils provides the higher rate of reaction by increasing the accessible surface area through solubilisation of hemicelluloses and lignin, which are coating the cellulose of the native biomass (Jönsson and Martín 2016). Higher steam explosion temperatures resulted in more homogeneous fibre-like material which was also confirmed by other scientific papers (Han et al. 2010) although excessively high temperatures have a sintering effect as shown in the Fig. 2 (image f)) and the obtained material is not suitable for fermentation also due high content of fermentation inhibitors (Russ et al. 2016). The obtained SEM images are in the satisfying correlation with the results obtained by modified Simons' method as the temperature 215°C is boundary for the highest achieved holocellulose accessibility of wheat straw pretreated by steam explosion which was also determined by B/O ratio.

CONCLUSIONS

The main goal of this partial study was to evaluate holocellulose accessibility of pretreated wheat straw compared to original wheat straw. Different pretreatment processes such as dry milling, cyclic freezing and thawing at specific conditions, wet milling in refiner, steam explosion at different temperatures (175°C, 195°C, 215°C and 230°C) were applied on wheat straw and compared. Two-step process of steam explosion at 180°C and extrusion at 145°C and one-step process of steam explosion at 215°C were used in evaluation of the different degrees of holocellulose accessibility by modified Simons' method based on the absorbance of non-adsorbed dyes. Moreover, the confirmation of results was carried out by digital microscope and scanning electron microscope.

For simplification of the modified Simons' method and simple measurement analysis the different combinations of three commercial dyes were tested – direct blue (dye B), direct orange (dye O) and direct violet (dye V). The most important finding is that pretreatment and related accessibility increases with simultaneous reduction of difference in adsorption of used dyes. However, in case of dye V no correlation between the pretreatment methods or accessibility level and adsorbed dye V was found. The unambiguous course has only B/O ratio – the ratio of amounts of dye B and dye O adsorbed on wheat straw fibres. From this reason that ratio was used in description of holocellulose accessibility level for different pretreatment methods. It was shown by monitoring B/O ratio that the lowest accessibility for enzymes has the wheat straw pretreatment by dry milling with B/O ratio at level of 0.7889 which is comparable with pretreatment by cyclic freezing and thawing with B/O ratio at level of 0.7959. Wheat straw pretreated by wet milling with B/O ratio at level of 0.8309 resulted in a little better accessibility for enzymes. In contrast to this, the best holocellulose accessibility levels were achieved with pretreatment by two-step process of steam explosion at 180°C and extrusion at 145°C and with pretreatment by one-step process of steam explosion at 215°C where obtained B/O ratio were at level of 0.9189 and 1.0305, respectively. The introduced measurements of B/O ratio concluded that the process of steam explosion at high temperatures or steam explosion at lower temperatures combined with steam extrusion lead to higher holocellulose accessibility level which is more convenient in the process of enzymatic hydrolysis compared to mechanical and hydro-mechanical methods of wheat straw pretreatment.

SEM images obtained by scanning electron microscope (SEM) showed the differences between the structure of the original wheat straw sample and structure of pretreated wheat straw samples. The SEM images are in the satisfying correlation with the results obtained by modified Simons' method as the temperature 215°C is boundary for the highest achieved holocellulose accessibility of wheat straw pretreated by steam explosion which was also determined by B/O ratio.

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