

## **Inhibitory compounds formation after liquid hot water (LHW) pretreatment of corn stover as an alternative to wood lignocellulosic feedstock for bioethanol production**

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**Abstract:** *Inhibitory compounds formation after liquid hot water (LHW) pretreatment of corn stover as an alternative to wood lignocellulosic feedstock for bioethanol production.* Thus far, corn stover has been perceived as a promising lignocellulosic alternative to wood intended for bioethanol procurement, however it should be recognised also as a potential future component in a mixed biomass system. The aim of this research was to investigate the effect of applying different hydrothermal treatment conditions on the potential inhibitory compounds formation from corn stover. An analysis of selected inhibitory compounds formed after pretreatment performed at different temperatures (160°C, 175°C, 190°C and 205°C) was carried out. Furfural, simple sugars and lignin were some of the inhibitors examined with HPLC and UV-VIS spectrophotometric methods. Furthermore, the chemical composition of organic extracts obtained from native and LHW pretreated biomass was analyzed qualitatively with GC-MS method and inhibitory compounds like vanillin, sitosterol or syringol were detected. As a result of those investigations compared to enzymatic hydrolysis yield the temperature of 175°C was chosen as the most promising condition of corn stover LHW pretreatment in terms of the efficiency of the subsequent phases of bioethanol production.

*Keywords:* inhibitory compounds, liquid hot water, corn stover

### INTRODUCTION

Energy derived from biomass is considered as one of the most promising alternatives to traditional fuels and an effective way to defuse the current energy crisis (Xianqin et al. 2016). Lignocellulosic materials are among the most desirable biomass sources due to their abundance and high content of polysaccharides, which can be easily utilized to produce cellulosic ethanol. As for alternative to traditional wood, corn stover is currently one of the most common non-woody agricultural sources for so-called second generation biofuels procured from non-edible feedstock (Kou et al. 2013; Zhao et al. 2018). An assessment of the corn stover chemical composition indicates that it may be very competitive material to the traditional wood biomass and that efficiency of the bioethanol procured from those two sources is comparable (Galbe and Zacchi 2008; Akus-Szylberg et al. 2018). Furthermore, the cellulosic ethanol production so far, has been based mainly on the single feedstocks, however use of the mixed sources is now being explored, as it has shown significant cost savings (Oke et al. 2016; Nguyen et al. 2017). The biomass blending refers to the combination of multiple sources of the lignocellulosic materials to obtain a consistent and stable supply, as well as feedstock with desirable properties, for example via mixing the agricultural and forest residues (Chin et al. 2011; Shi et al. 2013). Therefore corn stover may present itself as a valuable component for such biomass blend.

However, the prerequisite in the application of lignocellulosic biomass for bioethanol production is to effectively generate a rich in sugars, fermentable hydrolyzate from raw plant-based material. The employment of enzymes is one of the available methods for the hydrolysis of lignocellulose. Although the enzymatic route

is characterized by high costs, it has been praised for minimal by-product formation, environmental friendliness, low energy requirements and mild operating conditions (Zheng et al. 2009). Nevertheless, physico-chemical and structural composition of native lignocellulose is rather resistant to direct enzymatic hydrolysis. Thus, pretreatment is an essential step to provide cellulose that is susceptible to enzyme digestion prior to the hydrolysis itself. The overall purpose of the pretreatment is lignin removal, reduction of the degree of cellulose polymerization, an increase of pore volume and a decrease in cellulose crystallinity (Alvira et al. 2010). Pretreatment techniques have generally been divided into four distinct categories, including physical, chemical, biological and physicochemical methods (Świątek et al. 2011). Liquid hot water (LHW) method is one of the most common physicochemical treatments due to its low cost, high efficiency and general simplicity (Li et al. 2014). In this method hot water is applied under pressure for a certain period of time in a high temperature environment. Autohydrolysis of acetyl groups contained in hemicelluloses is triggered, which leads to their further separation from cellulose. Furthermore, the LHW pretreatment produces relatively small amount of by-products which may be likely to inhibit the most complex part of ethanol procurement – hydrolysis. Such inhibitory compounds that have a negative effect on the bioethanol procurement process may be lignin or sugars and products of their degradation.

Thus, the aim of this research was to investigate the effect of applying different hydrothermal treatment conditions on the potential inhibitory compounds formation from corn stover that may have negative effect on the efficiency of the subsequent enzymatic hydrolysis, not only as corn stover is one of the most prominent alternatives for wood biomass for ethanol procurement, but also as it may be a potential future component for the mixed biomass system.

## MATERIALS AND METHODS

### *Raw material*

Corn stover from commercial fields was used in this research. The stover was ground into particles with dimensions of 0.43–1.02 mm. Then, the hydrothermal process was performed.

### *LHW pretreatment*

Firstly, the biomass (20 g) was soaked in distilled water at 75°C, using a magnetic stirrer for 20 min to remove air. Then, it was quantitatively placed in a stainless steel reactor with respective amount of water in order to apply solid to liquid ratio of 1:12.5. Next the reactor was placed in an oil bath that was pre-set and subsequently maintained at 160°C, 175°C, 190°C and 205°C for 20 min prior to rapid cooling, ending the reaction. The solid and liquid fractions were separated by filtration with a Büchner funnel. The solid was then washed with distilled water until pH reached 7.0; the total amount of liquid in the beaker did not exceed 1.5 dm<sup>3</sup>, so that the volume of the filtrate is known. Both neutralized solid and liquid fractions were stored at 6°C until further investigations.

### *HPLC analysis*

The chemical composition of liquid fraction after LHW pretreatment was examined using a high-performance liquid chromatography (HPLC) system which consisted of: a LC-20AD pump, a DGU-20A degasser, a CTO-20A oven, a RID-10A differential refractive detector, a CBM-20A controller (all above Shimadzu, Japan) and a Rezex RHM-Monosaccharide column (300 × 7.80 mm) (Phenomenex, USA). Re-distilled

water was used as the mobile phase at a flow rate of 0.6 cm<sup>3</sup> /min, an injection volume of 20 μL and the column temperature was maintained at 80°C. The procured chromatographic data were processed with LC Solution v.1.21 SP1 software (Shimadzu, Japan).

Samples of particular liquid fractions were taken and checked for the presence of organic compounds, that is carbohydrates (cellobiose, glucose and xylose) and other inhibitors (furfural, 5-hydroxymethylfurfural - HMF, acetic acid and levulinic acid). The amount of each substance was calculated using calibration curves corresponding to equations (1–6):

$$y = 2740725 x; R^2 = 0.9998 \text{ (cellobiose) (1)}$$

$$y = 2680081 x; R^2 = 0.9979 \text{ (glucose) (2)}$$

$$y = 2721138 x; R^2 = 0.9993 \text{ (xylose) (3)}$$

$$y = 3313327 x; R^2 = 0.9998 \text{ (furfural) (4)}$$

$$y = 3007754 x; R^2 = 0.9999 \text{ (5-hydroxymethylfurfural) (5)}$$

$$y = 1797963 x; R^2 = 0.9995 \text{ (acetic and levulinic acids) (6)}$$

Acetic and levulinic acids were combined due to their joint appearance on a chromatograph spectrum. As each inhibitor content was calculated using calibration curves obtained data was not subjected to the statistical treatment.

#### *Spectrophotometric analysis*

The quantitative determination of lignin content in liquid fraction was performed using a UV-VIS mini-1240 spectrophotometer (Shimadzu, Japan). Samples for the spectrophotometry examination were placed in 1 cm-thick quartz cuvettes with 1 cm thickness and an absorbance was measured at 205 nm wavelength. Distilled water was used as a reference solution. Each sample was analysed in triplicates and single standard deviations were calculated.

#### *GC-MS analysis*

The determination of inhibitors formed in the solid fraction was investigated using a gas chromatography-mass spectrometry (GC-MS) system which consisted of: GC-2010 Plus coupled with a mass spectrometer GCMS-QP2010 (Shimadzu, Japan); a 30 m long capillary column ZB-5MS, a diameter of 0.25 mm and an infill 0.25 μm thick; autosampler AOC-20i. The obtained data were processed using GC-MS solution Version 2.72 software and compared to the existing spectra library. Samples of particular solid fractions were taken after extraction in a chloroform:ethanol mixture (Antczak et al. 2006) and checked for the presence of lignin derivatives, sterols and higher alkanes.

## RESULTS AND DISCUSSION

During high temperature pretreatment of the lignocellulosic biomass, autohydrolysis of cellulose and hemicelluloses is catalyzed and simple sugars are formed as a result. Content of the sugars detected in the liquid fraction obtained after LHW pretreatment process at different temperatures is presented in Fig. 1. As it can be seen, the carbohydrates were present in all post-treatment liquids and their content ranged between 2.4% and 4.0%. Those soluble sugars, which are final or indirect products of cellulose or xylan hydrolysis, may block the subsequent process of the enzymatic hydrolysis. Generally, the hemicelluloses are characterized by lower resistance to high temperature than cellulose, thus they can be degraded and dissolved more rapidly. However, also cellulose may be dissolved, especially that with low degree of polymerization and directly linked to the hemicelluloses (Rowell et al.

2002). As already reported in literature, the formation and accumulation of such products containing glucose, cellobiose or oligoglucose might be the main factor in hindering the hydrolysis process (Gusakov and Sinitsyn 1992; Kim 2018). It is because the presence of cellobiose has an inhibitory effect on the cellulase enzyme activity; and so has glucose, but to a lesser extent. Furthermore, a similar phenomenon had been observed also in case of xylans hydrolysis products (Qing et al. 2010).

The average sugars content in the liquid fraction obtained after LHW pretreatment process first increased from 3.4% in the biomass treated at 160°C to 4.0% in the material after LHW performed at 190°C, but then, as the temperature of the process grew, it dropped down to 2.4% (205°C). That sudden decline was probably caused by more severe degradation of carbohydrates triggered by a higher temperature.

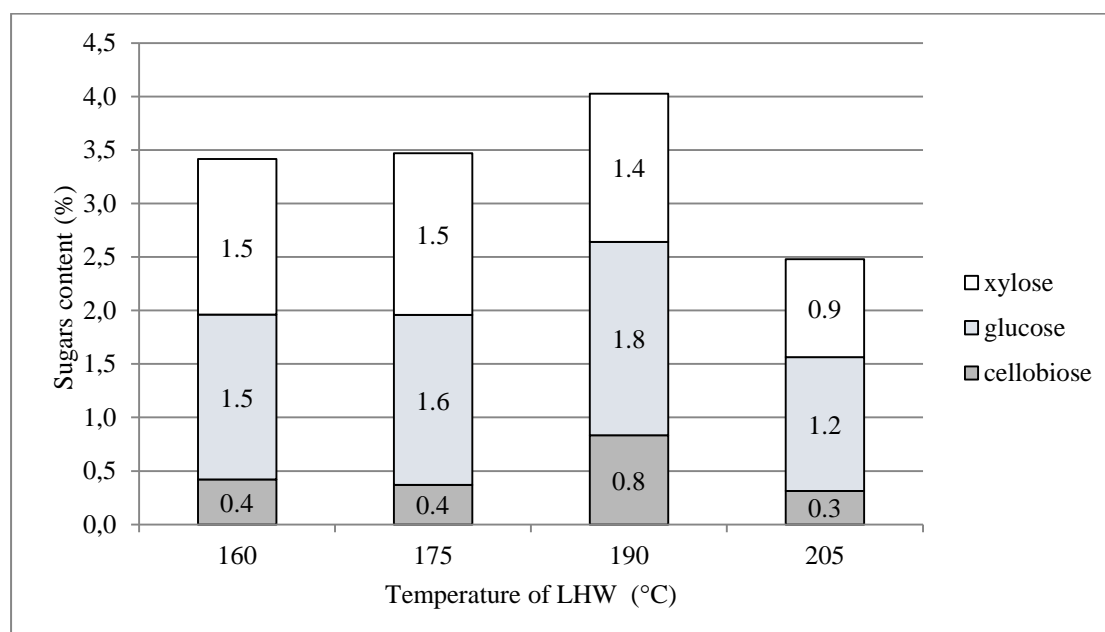


Figure 1. Monosaccharides (glucose and xylose) and disaccharide (cellobiose) content in the liquid fraction obtained after LHW pretreatment process of corn stover performed at different temperatures

Products of polysaccharides degradation formed under the influence of LHW pretreatment that also may inhibit the enzymatic hydrolysis are shown in Fig. 2. As a result of acidic environment, furan derivatives were composed. Furfural and 5-hydroxymethylfurfural are products of, respectively, pentosans and hexosans degradation and dehydration (Zha et al. 2014). Furthermore, as an effect of the dehydration process catalyzed by high temperature, weak organic acids were formed, including levulinic acid. Likewise acetic or formic acid might be formed as an outcome of acetyl group hydrolysis triggered by the pretreatment (Tomas-Pejo et al. 2011; Taherzadeh et al. 1999).

As it is shown in the fig. 2, furan derivatives content in the liquid fraction increased rapidly, along with the LHW pretreatment temperature, from 1.3% at 160°C through 3.5% at 175°C to 7.9% at 190°C, and afterwards it rose up to 9.3% at 205°C. Particularly acetic and levulinic acids content grew steadily through the 160°C and 190°C liquid fractions, and subsequently it almost stayed the same despite the most severe treatment conditions (6.7%).

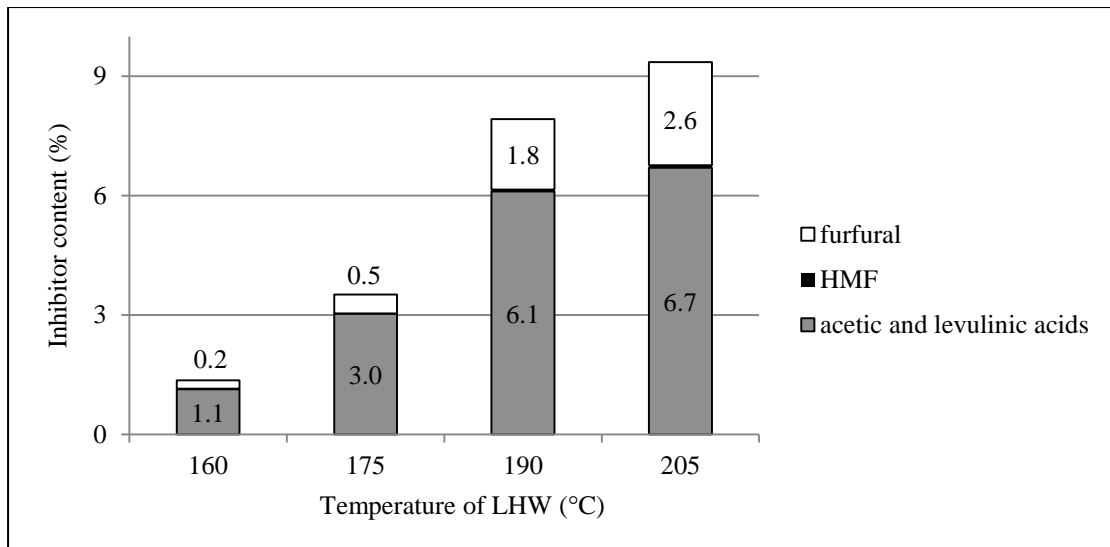


Figure 2. The content of polysaccharides degradation products (furfural, HMF, acetic and levulinic acids) in the liquid fraction obtained after LHW pretreatment process of corn stover performed at different temperatures

Of the main lignocellulosic biomass chemical components, lignin is the most resistant one, thus it is mainly plastified during high temperature pretreatment. Nonetheless, some part of it is water-soluble and it was examined that for the studied native corn stover water-soluble lignin content amounts to 3.5%. Lignin content in different post-treatment liquid fractions is exhibited in Fig. 3. The lowest lignin content can be observed after LHW pretreatment at 175°C (4.6%), and the highest after pretreatment at 190°C (6.1%); however those changes were rather minor.

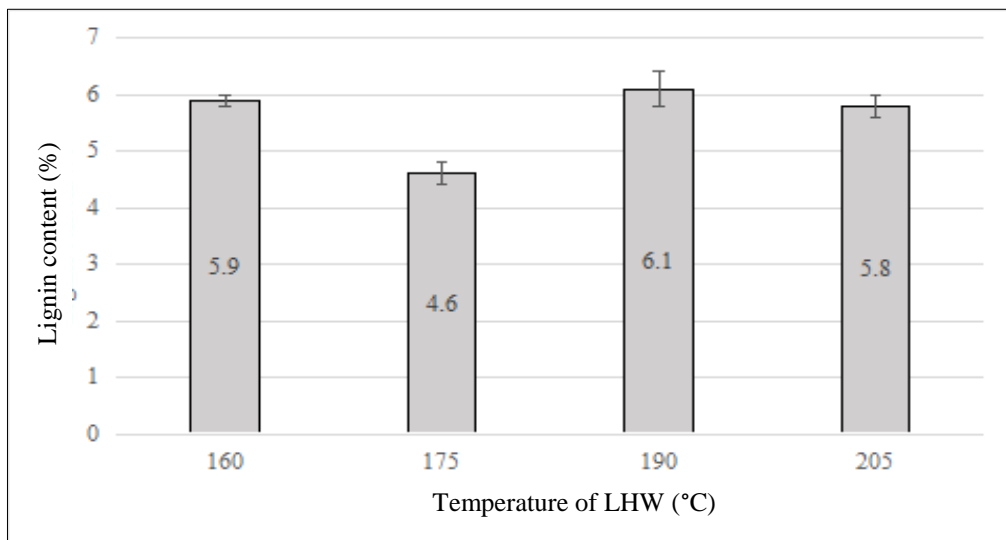


Figure 3. Lignin content in the liquid fraction obtained after LHW pretreatment process of corn stover performed at different temperatures

It is generally known that the lower content of lignin is, the higher the rate and efficiency of the enzymatic hydrolysis is (Yoshida et al. 2008). This phenomenon is attributed mainly to a non-specific adsorption of the enzymes into lignin, as well as lignin causing physical blockage in the accessibility of cellulose (Sewalt et al. 1997). Entire elimination of lignin from the lignocellulosic feedstock maximizes the susceptibility of polysaccharides to the digestive enzymes, especially in the

amorphous areas of cellulose. Additionally, formation of different inhibitory phenol compounds such as vanillin or vanillin acid, which are lignin degradation products, may be triggered by the pretreatment (Taherzadeh 1999; Zha et al. 2014).

Furthermore the inhibitory compounds content in organic extracts from native sample as well as from pretreated solid fractions was investigated using a GC-MS system. In all cases lignin derivatives were detected, including: vanillin (LHW at 160°C), 2,3-dimethoxyphenol (native sample and LHW at 160°C), 2,6-dimethoxy-4-(2-propenyl) phenol (native sample, LHW at 160°C and 205°C) or syringaldehyde (LHW at 190°C and 205°C). In all chromatograms also presence of sterols was confirmed, for example: gamma-sitosterol (LHW at 160°C and 175°C), beta-sitosterol (LHW at 190°C and 205°C) and its acetate (LHW at 175°C and 190°C), campesterol (native sample and LHW at 190°C). Higher alkanes such as octadecane and heptadecane were noticed, as well as higher saturated fatty acids such as stearic acid and palmitic acid were detected in all samples.

Furthermore, yield of the enzymatic hydrolysis of the studied corn stover had been studied in previous research (Akus-Szylberg et al. 2018). It had shown that the major increase in sugars content obtained after enzymatic hydrolysis happened between biomass LHW pretreated at 160°C (33.3%) and 175°C (53.4%). Afterwards it just grew to a much lesser extent and amounted for 55.0% at 190°C and 56.7% at 205°C of the LHW pretreatment.

## CONCLUSIONS

A chemical composition analysis of extracts obtained from native and LHW pretreated biomass indicated that inhibitory compounds are present in all samples. The content of both carbohydrates and lignin, as well as their derivatives, stayed roughly the same in all cases of treated biomass. However in case of furan derivatives, a strong correlation with temperature applied during pretreatment was noticed. The content of products of polysaccharides degradation and dehydration, such as acetic and levulinic acids, in liquid fractions increased rapidly from 1.3% at 160 °C to 7.9% at 190 °C and then it grew only to 9.3% at 205°C. Obtained results were correlated with enzymatic hydrolysis of the studied corn stover. The glucose yield almost doubled from 23.1% at 160°C to 46.1% at 175°C of LHW pretreatment and then at the highest applied temperatures it grew only to 48.3% and 51.6% at 190°C and 205°C, respectively. Thus, considering also the inhibitory compounds formation, the 175°C seemed the most optimal condition for the LHW pretreatment of corn stover.

## REFERENCES

1. AKUS-SZYLBERG F., ANTCZAK A., BYTNER O., RADOMSKI A., KRAJEWSKI K., ZAWADZKI J. (2018). Wpływ wstępnej obróbki słomy kukurydzianej gorącą wodą na jej skład chemiczny i hydrolizę enzymatyczną, *Przemysł chemiczny* 97(11), 1866–1869.
2. AKUS-SZYLBERG F. ANTCZAK A., BYTNER O., KRAJEWSKI K., ZAWADZKI J. (2018). The study of chemical composition of corn stover as a potential lignocellulosic feedstock for bioethanol production; *Annals of WULS, Forestry and Wood Technology*, 104, 386-389.
3. ALVIRA P., TOMAS-PEJO E., BALLESTEROS M., NEGRO M.J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review, *Bioresour. Technol* 101, 4851–4861.

4. ANTCZAK A., RADOMSKI A., ZAWADZKI J. (2006). Benzene substitution in wood analysis, *Annals of Warsaw University of Life Sciences–SGGW, Forestry and Wood Technology* 58, 15–19
5. CHIN K., H'NG P., WONG L., TEY B., PARIDAH M. (2011). Production of glucose from oil palm trunk and sawdust of rubberwood and mixed hardwood, *Appl Energy* 88(11), 4222–4228.
6. GALBE M., ZACCHI G. (2007). Pretreatment of lignocellulosic materials for efficient bioethanol production, *Adv. Biochem. Eng. Biotechnol.* 108, 41-65.
7. GUSAKOV A.V., SINITSYN A.P. (1992). A Theoretical Analysis of Cellulase Product Inhibition: Effect of Cellulase Binding Constant, Enzyme/Substrate Ratio, *Biotechnology* 40, 663–671.
8. KIM D. (2018). Physico-Chemical Conversion of Lignocellulose: Inhibitor Effects and Detoxification Strategies: A Mini Review, *Molecules* 23(2), 309–330.
9. KOU X., YANG R., ZHAO J., LU J., LIU Y. (2013). Enzymatic Saccharification and L-lactic Acid Fermentation of Corn Stover Pretreated with Liquid Hot Water by *Rhizopus oryzae*, *BioResources* 8(4), 4899–4911.
10. LI X., LU J., ZHAO J., QU Y. (2014). Characteristics of corn stover pretreated with liquid hot water and fed–batch semi-simultaneous saccharification and fermentation for bioethanol production, *PLoS One* 9(4); e95455.DOI: 10.1371/journal.pone.0095455.
11. NGUYEN Q.A., YANG J., BAE H-J. (2017). Bioethanol production from individual and mixed agricultural biomass residues, *Ind. Crops Prod.* 95, 718–725.
12. OKE M.A., ANNUAR M.S.M., SIMARANI K. (2016). Mixed Feedstock Approach to Lignocellulosic Ethanol Production—Prospects and Limitations, *Bioenerg. Res.* 9, 1189-1203.
13. QING Q., YANG B., WYMAN C.E. (2010). Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes, *Bioresour Technol* 101, 9624–9630.
14. ROWELL R., LANGE S., MCSWEENEY J., DAVIS M. (2002). Modification of wood fibre using steam, Wood Science and Engineering Department, Oregon State University Corvallis, USA.
15. SEWALT V.J.H., GLASSER W.G., BEAUCHEMIN K.A. (1997). Lignin impact on fibre degradation. 3. Reversal inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives, *J. Agric Food Chem* 45, 1823–1828.
16. SHI J., THOMPSON V.S., YANCEY N.A., STAVILA V., SIMMONS B.A., SINGH S. (2013). Impact of mixed feedstocks and feedstock densification on ionic liquid pretreatment efficiency, *Biofuels* 4(1), 63-72.
17. ŚWIĄTEK M., LEWANDOWSKA M., BEDNARSKI W. (2011). Znaczenie doboru metody wstępnej obróbki substratów lignocelulozowych z uwzględnieniem wydajności produkcji bioetanolu, *Postępy Nauk Rolniczych* 1, 109–119.
18. TAHERZADEH M. J. (1999). Ethanol from lignocellulose: physiological effects of inhibitors and fermentation strategies, doctoral thesis, Chemical Reaction Engineering, Chalmers University of Technology, Sweden
19. TOMAS-PEJO E., ALVIRA P., BALLESTEROS M., NEGRO M. J. (2011). Pretreatment technologies for lignocellulose-to-bioethanol conversion, In: *Biofuels. Alternative feedstocks and conversion processes*. Elsevier, Amsterdam, 149–176.
20. XIANQIN L., XIAOJU Z., XUEZHI L., JIAN Z. (2016). Adsorption and mechanism of cellulase enzymes onto lignin isolated from corn stover

- pretreated with liquid hot water, *Biotechnol Biofuels* 9; DOI 10.1186/s13068-016-0531-0.
21. YOSHIDA M., LIU Y., UCHIDA S., KAWARDA K., UKAGAMI Y., ICHINOSE M., KANEKO S., FUKUDA K. (2008). Effects of Cellulose Crystallinity, Hemicellulose, and Lignin on the Enzymatic Hydrolysis of *Miscanthussinensis* to Monosaccharides *Biosci Biotechnol Biochem* 72 (3), 805–810.
  22. ZHA Y., WESTERHUIS, J.A., MUILWIJK, B., OVERKAMP K.M., NIJMEIJER, B.M., COULIER L., SMILDE A.K., PUNT P.J., (2014). Identifying inhibitory compounds in lignocellulosic biomass hydrolysates using an exometabolomics approach, *BMC Biotechnol* 14 (22), DOI: 10.1186/1472-6750-14-22.
  23. ZHAO Y., DAMGAARD A., CHRISTENSEN T.H. (2018). Bioethanol from corn stover: a review and technical assessment of alternative biotechnologies, *PrECS* 67, 275-291.
  24. ZHENG Y., ZHONGLI P., RUIHONG Z. (2009). Overview of biomass pretreatment for cellulosic ethanol production, *Int J Agric & Biol Eng Open Access* 2(3), 51–69.

**Streszczenie:** *Analiza inhibitorów powstałych po obróbce gorącą wodą (LHW) słomy kukurydzianej będącej alternatywnym dla drewna lignocelulozowym surowcem do produkcji bioetanolu. Głównym celem badań był wybór najlepszych warunków obróbki wstępnej metodą liquid hot water (LHW) słomy kukurydzianej mającej na celu zwiększenie wydajności hydrolizy enzymatycznej. W pracy przedstawiono analizę wybranych inhibitorów powstałych po procesie obróbki wstępnej gorącą wodą przeprowadzanym w różnych temperaturach (160°C, 175°C, 190°C i 205°C), które występują również w wyniku obróbki LHW drewna. W celu zbadania wybranych inhibitorów hydrolizy takich jak furfural, cukry proste i lignina zastosowano chromatografię HPLC i spektrofotometrię UV-VIS. Na chromatografii gazowej GC-MS wykonano analizę jakościową składu ekstraktów organicznych pozyskanych z materiału przed i po obróbce wstępnej, która pozwoliła zidentyfikować kilka kolejnych możliwych inhibitorów hydrolizy, takich jak: sitosterol, wanilina, czy syringol. W efekcie przeprowadzonych badań i ich analizy określono, że 175°C jest optymalną temperaturą obróbki wstępnej LHW słomy kukurydzianej.*

**Acknowledgements:** This work was financed by project from the National Centre for Research and Development, which was “Intelligent systems for breeding and cultivation of wheat, maize, and poplar for optimized biomass production, biofuels, and modified wood” (BIOSTRATEG2/298241/10/NCBR/2016).

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