Polish Journal of Chemical Technology, 17, 3, 89 — 94, 10.1515/pjct-2015-0055

Production of ethanol from wheat straw

Małgorzata Smuga-Kogut[1], Arkadiusz D. Wnuk[1], Kazimiera Zgórska[1], Mariusz S. Kubiak[2], Janusz Wojdalski[3*], Adam Kupczyk[3], Józef Szlachta[4], Adam Luberański[4]

[1]Koszalin University of Technology, Department of Biochemistry and Biotechnology, ul. Racławicka 15-17, 75-620 Koszalin, Poland
[2]Koszalin University of Technology, Department of Food Industry Processes and Facilities, ul. Racławicka 15-17, 75-620 Koszalin, Poland
[3]Warsaw University of Life Sciences, Department of Production Management and Engineering, ul. Nowoursynowska 166, 02-787 Warsaw, Poland
[4] Wrocław University of Environmental and Life Sciences, Institute of Agricultural Engineering, ul. Chełmońskiego 37/41, 51-630 Wrocław, Poland
**Corresponding author: janusz_wojdalski@sggw.pl*

This study proposes a method for the production of ethanol from wheat straw lignocellulose where the raw material is chemically processed before hydrolysis and fermentation. The usefulness of wheat straw delignification was evaluated with the use of a 4:1 mixture of 95% ethanol and 65% HNO_3 (V). Chemically processed lignocellulose was subjected to enzymatic hydrolysis to produce reducing sugars, which were converted to ethanol in the process of alcoholic fermentation. Chemical processing damages the molecular structure of wheat straw, thus improving ethanol yield. The removal of lignin from straw improves fermentation by eliminating lignin's negative influence on the growth and viability of yeast cells. Straw pretreatment facilitates enzymatic hydrolysis by increasing the content of reducing sugars and ethanol per g in comparison with untreated wheat straw.

Keywords: wheat straw, chemical processing, enzymatic hydrolysis, reducing sugars, ethanol.

INTRODUCTION

Global economic growth contributes to a rapid increase in the consumption of traditional energy sources. According to numerous energy consumption analyses, the progressing depletion of fossil fuels calls for new initiatives on the market of renewable energy. Biomass is one of alternative energy sources[1, 2]. At present, only 5 billion out of 150 billion tons of biomass harvested each year are processed into food. Biomass is not used for energy generation to the extent permitted by the existing technology[3, 4, 5]. Renewable energy sources are becoming increasingly important in the energy balance of the country, and they are a characteristic feature of innovative and forward-looking economies (Kogut et al. 2012)[6]. Energy can be generated from biomass by combustion, gasification, ethanol and methanol fermentation or by using oilseed crops as a source of fuel. According to Nguyen et al. (2013)[7], energy generated from straw by gasification seems to be more environmentally-friendly than that produced by straw combustion. In comparison with natural gas, the heating value of straw is low at $13.5 \div 19.0$ MJ $\cdot$ kg^{-1}, and it is determined by the type of straw and its relative moisture content. The combustion of fossil fuels produces harmful emissions to ambient air, mainly CO_2, which contribute to the greenhouse effect. The use of straw as an alternative source of energy could reduce global warming and the depletion of fossil fuels[7, 9, 10]. The energy value of two tons of wood or straw is equivalent that that of one ton of high-quality hard bituminous coal. Biomass yield per hectare of farmland is estimated at 10–12 tons, i.e. the equivalent of 5–10 tons of coal[11]. One of the methods of generating energy from biomass is alcoholic fermentation. Simple sugars are converted into ethanol by yeasts[12, 13]. Ethanol is dehydrated and used to enhance or substitute petroleum[12, 14, 15, 16, 17].

Biomass-derived products are suitable for human consumption, therefore they constitute an expensive source of energy. Lignocellulosic biomass, including wood, food and agricultural wastes, oilseed crops and other raw materials containing cellulose, pose a less costly alternative[18, 19]. Cellulose resources are abundant in nature. Cellulose does not constitute a human food source, therefore, it is a relatively cheap source of energy and bioethanol[20, 21, 22]. In Brazil, the food processing sector generates 587 million tons of waste per year. New solutions are required for managing valuable plant resources for energy generation purposes[22]. In the United Kingdom, wheat straw is a potential resource for the production of second-generation biofuels[8, 23]. Integrated measures are initiated by the EU countries to encourage the production of biomass fuels and provide farmers with the relevant support. Pursuant to the provisions of Directive 2009/28/EC on the promotion of the use of energy from renewable sources[25], the share of renewable fuels for transport has to reach 10% in every Member State by 2020. The above requirement will lead to a substantial increase in the production of inedible biomass[23, 24]. The aim of the above Directive is to replace bioethanol produced from edible plants with bioethanol obtained from inedible biomass, including plant waste. Biofuels produced from lignocellulose and waste will lower CO_2 emissions. Despite those advantages, the energy inputs and costs associated with biomass conversion to bioethanol are higher for biofuels derived from inedible resources (advanced generation biofuels) than edible crops[26, 27]. Relatively few high-efficiency systems for the conversion of inedible biomass into biofuels have been developed on the industrial scale. The largest industrial system for bioethanol production from straw is operated in Crescentino, Italy[28].

A vast surplus of straw, a potential source of solid biomass, exists in western Poland. According to estimates, 50 to 70% of that surplus is suitable for industrial

processing. In Poland, biomass resources that could be used for energy generation are estimated at more than 10–11 million tons of straw waste[29]. In Poland, only 7% of biomass is used for energy, whereas the average for the EU is 20%[30]. Alternative sources of energy such as cellulosic biomass, in particular wheat straw, limit energy generation from edible crops and ensure the use of sustainable biofuels only[5, 10, 17, 23, 31].

Lignin provides plants with the structural support needed for an erect growth habit. Lignin surrounds cellulose and hemicellulose molecules, making their extraction difficult. Similarly to starch molecules, cellulose molecules are made up of long chains of glucose molecules, but with a different configuration. Due to their specific structural properties, cellulosic materials are much more difficult to hydrolyze than starch[20, 32–36]. The aim of this study was to evaluate the suitability of wheat straw for the production of ethanol fuel and to determine the effect of chemical pretreatment of wheat straw on the content of reducing sugars after hydrolysis and ethanol yield after alcoholic fermentation.

MATERIAL AND METHODS

The experimental materials were: wheat straw harvested in a farm in Święta, municipality of Złotów, Region of Wielkopolska, with the involvement of traditional farming methods. Avicel PH-101 (Sigma Aldrich) powdered microcrystalline straw with 50 μm grain size was used as control. It was dissolved in octane buffer with pH 4.7 and subjected to hydrolysis and fermentation with the use of the same enzymatic preparations and yeasts that were applied to straw wheat samples. Wheat straw (10 g dry matter) was ground in a colloid mill into 1-mm long particles, and it was chemically treated with a 4:1 mixture of 95% ethanol and 65% nitric (V) acid according to the method proposed by Kürschner–Hoffer[37]. The aim of preliminary treatment was to damage lignin structure and increase enzyme accessible space in cellulose. Hydrolysis was carried out using two commercial enzymatic preparations: cellulase containing *Trichoderma reesei* ATCC 26921 (Sigma Aldrich) and cellobiose containing *Aspergillus niger* (Novozym 188). Enzymatic hydrolysis was conducted at 47°C for 72 hours. The hydrolysate was separated from cellulose residues and subjected to alcoholic fermentation. The fermentation process was carried out with the use of *Saccharomyces cerevisiae* Fermentis Ethanol Red (Leaf Technologies), a selected yeast strain for industrial production of ethanol, at a temperature of 37°C for 96 h. At 35°C, the applied yeast strain is capable of concentrating ethanol to 18% v/v. It is also characterized by high viability and resistance to high ethanol concentrations in mash. The ethanol content of the analyzed samples and the viability and count of yeast cells were determined. The ethanol production process was conducted in three replications. Hydrolysate samples were assayed for the content of total reducing sugars and ethanol after fermentation. The content of reducing sugars and ethanol concentrations were expressed as mean values from three replications.

The dry matter content of unprocessed straw was determined in accordance with Polish Standard PN-90/A-75101/03, cellulose content – by the method proposed by Kürschner–Hoffer[37], and the content of Klason lignin – by the method described by Rodrigues[3]. Two replicate determinations were made.

The content of reducing sugars after enzymatic hydrolysis of cellulose was determined quantitatively with the use of 3,5-dinitrosalicylic acid under alkaline conditions. The concentrations of the stained compound were measured in the Helios spectrometer at 540 nm wavelength. In the analyzed samples, glucose levels could be determined quantitatively due to the non-specificity of the applied method where DNS reduction (3,5-dinitrosalicylic acid reduction) was a measure of the sample's general reducing ability. Glucose concentrations were determined by comparing absorbance results with the absorbance profiles of reference solutions[39].

The counts and viability of yeast cells in fermentation solutions were determined directly under a light microscope with a Thoma counting chamber with the use of 0.01% methylene blue solution. Cells were counted in minimum 60 small squares (not less than 700 yeast cells) to improve the reliability of results.

The amount of ethanol produced during decomposition of wheat straw cellulose was determined with the use of the ROCHE[40] kit (Enzymatic BioAnalysis/Food Analysis) that relies on UV radiation to measure ethanol concentrations in food products.

RESULTS

Straw is a lignocellulosic material and an agricultural by-product. Its main components are cellulose, hemicellulose, lignin, nitrogen compounds and ash. The exact composition of straw is determined by its type and variety[41]. On average, straw contains 35–50% cellulose, 15–30% hemicellulose, 20–30% lignin and smaller amounts of ash and other compounds[41, 42].

The dry matter content of straw was determined at 91.5%. The content of Klason lignin reached 28.4%. Klason lignin is the lignin fraction remaining after hydrolysis of lignocellulosic material with sulfuric (VI) acid. Klason lignin and lignin dissolved in sulfur acid make up the total lignin content of lignocellulosic materials[38]. The analyzed wheat straw contained 39.5% cellulose. An image of untreated and treated straw samples is presented in Figure 1.

The objective of this study was to determine the effect of wheat straw pretreatment on enzymatic hydrolysis and the production of reducing sugars, which are converted into ethanol by *S. cerevisiae* yeasts during the fermentation process.

Lignin is one of the key factors limiting straw's potential for bioethanol production. Cellulose forms complexes with lignin, and in straw with high lignin content, cellulose is difficult to extract by hydrolysis. In this experiment, lignin was removed from wheat straw by a 4:1 mixture of nitric acid and ethanol. Preliminary processing of wheat straw increased the content of reducing sugars after hydrolysis and the content of ethanol after fermentation. Similar results were reported by Ruiz et al. (2011)[43] who also removed lignin from wheat straw. The cited authors attributed the observed increase in the content of reducing sugars to lignin separation from cellulose and an increase in enzyme accessible space.

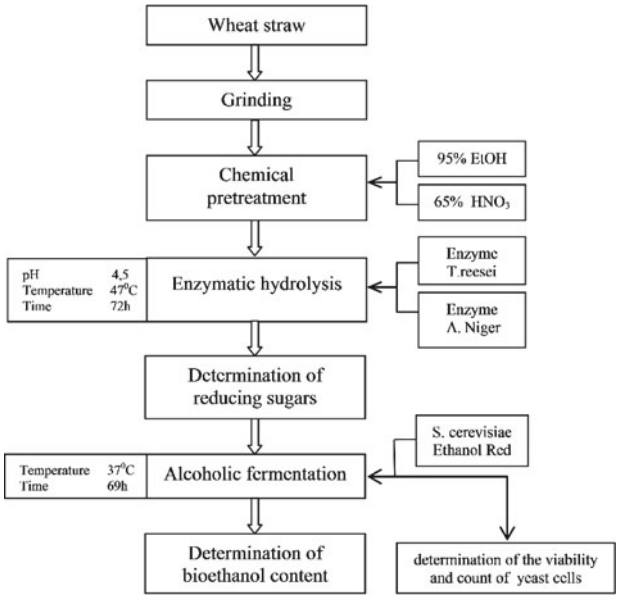


Figure 1. Flow diagram of bioethanol production from wheat straw

Delignification produced microcrystalline cellulose that was dried and hydrolyzed by *T. reesei* and *A. niger* cellulolytic enzymes. Both fungi produce large quantities of extracellular cellulases for decomposing microcrystalline cellulose, and they are popularly used in the food industry[44, 45].

Traditional ethanol production methods were based on conventional techniques of enzymatic hydrolysis and fermentation of sugars from starch decomposition, with the use of *S. cerevisiae* yeasts. Fermentation took place inside cells which produce fermentation enzymes –decarboxylase and alcohol dehydrogenase[46].

In this experiment, all samples (processed wheat straw, unprocessed wheat straw, microcrystalline cellulose – control) were incubated at 47°C for 72 h. Specimens for analysis were collected every hour for 12 hours, and then every 12 hours for three days. Changes in glucose levels during enzymatic hydrolysis are presented in Figures 2 and 3.

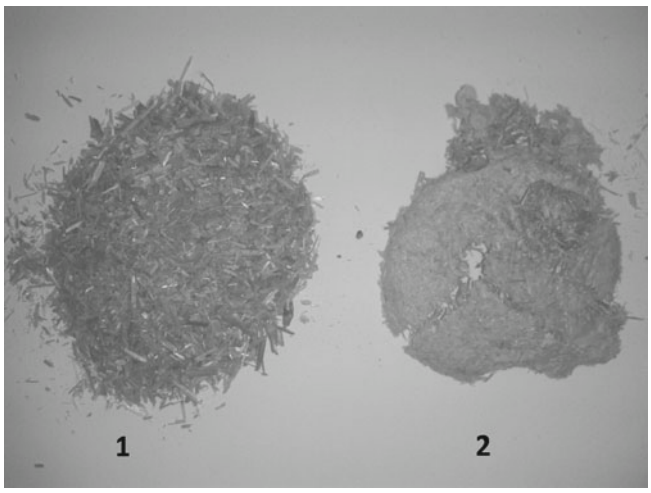


Figure 2. Wheat straw: 1 – untreated, 2 – chemically treated

Raw wheat straw cannot be degraded by hydrolysis, and it was processed to make it susceptible to hydrolytic enzymes. The highest glucose concentration of 82.67 g · dm^{-3} hydrolysate was observed in processed wheat straw after 48 hours. Hydrolysis results for untreated straw and control straw were nearly identical, i.e. less than 20 g · dm^{-3} reducing sugars was released. The content of reducing sugars in delignified straw was more than four-fold higher than in untreated straw.

Saha and Cotta (2007)[47] hydrolyzed lime-treated wheat straw and observed that the content of glucose and total reducing sugars increased with a rise in calcium hydroxide [$Ca(OH)_2$] concentrations during preliminary treatment. The influence of the $Ca(OH)_2$ dose was always much greater than that of treatment time. Total sugar content increased from 247 ±6 mg to 451 ±3 mg (83% increase in sugar release) when the lime dose was increased from 25 to 100 mg per g of straw. Total sugar content increased from 410 ±4 mg to 451 ±3 mg (by 10%) when pretreatment time was increased from 6 minutes to 1 hour. The highest total sugar content (451 ±3 mg · g^{-1} straw, 252 ±6 mg of glucose, 173 ±3 mg of xylose, 27 ±2 mg of arabinose; 65% conversion) was

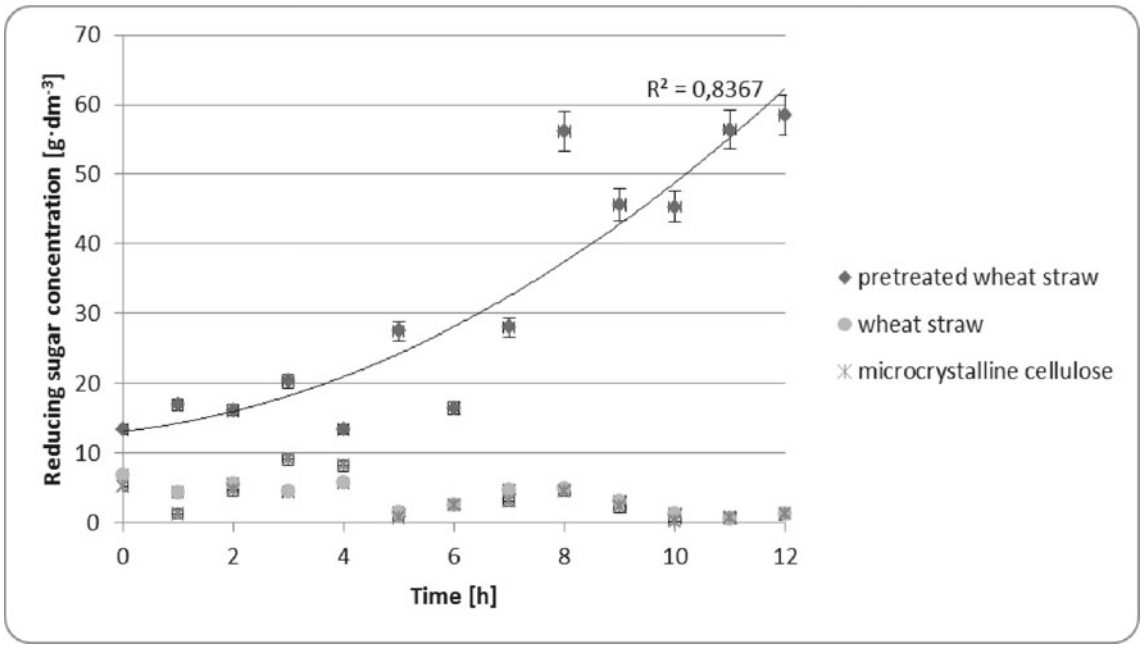


Figure 3. Changes in the content of reducing sugars during 12 hours of enzymatic hydrolysis

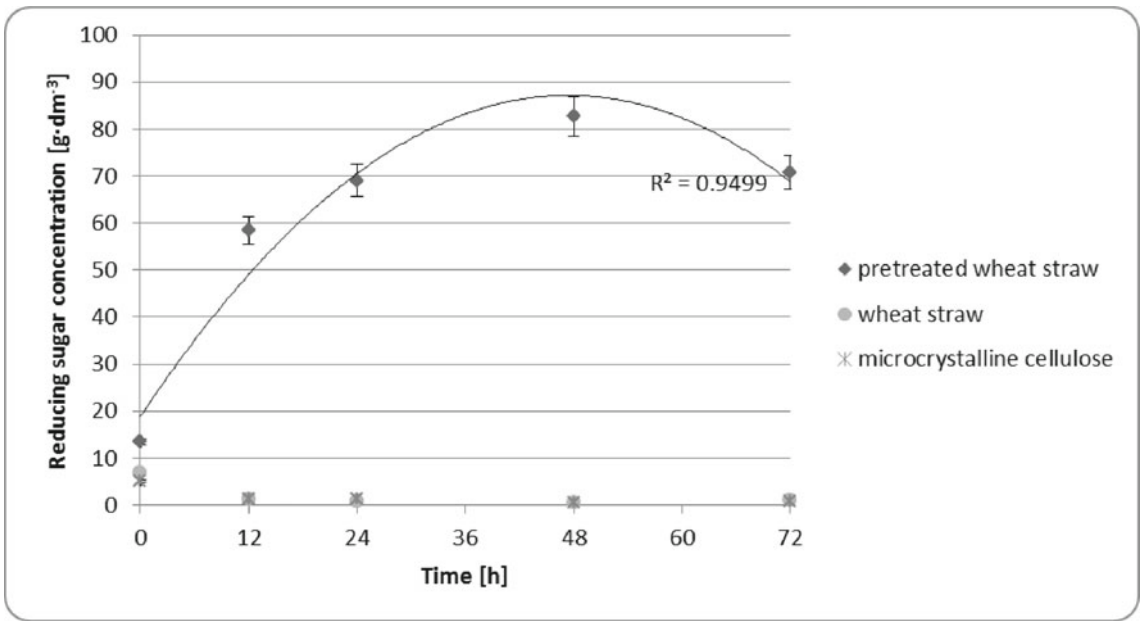


Figure 4. Changes in the content of reducing sugars during 72 hours of enzymatic hydrolysis

achieved at the $Ca(OH)_2$ dose of 100 mg and 1 hour of pretreatment.

Szczodrak (1998)[48] hydrolyzed wheat straw under alkaline conditions to obtain 2.4% (w/v) ethanol from 10% (w/v) chemically processed straw in 48 hours. When, in addition to the enzyme extracted from *T. reesei*, β-glucosidase from *A. niger* was included in the hydrolysis process, ethanol concentration increased to 3%, and treatment time was reduced to 24 hours. According to Han et al. (2012)[49] and Silva et al. (2012)[50], the efficiency of enzymatic hydrolysis of cellulosic biomass can be increased by grinding and pretreating raw material under alkaline conditions. In the cited studies, the efficiency of enzymatic hydrolysis increased with a rise in NaOH concentrations, and the highest content of reducing sugars was noted at 1% NaOH. Alkaline pretreatment is generally more effective in facilitating the hydrolysis of agricultural waste and herbaceous plants than woody plants[51].

Detroy et al. (1981)[52] converted wheat straw to ethanol and demonstrated that raw straw pretreated with 2% NaOH for 4 hours and subjected to enzymatic hydrolysis was responsible for 76% cellulose conversion, whereas straw pretreated under acid/alkaline conditions supported only 43% conversion. Hemicellulose, a polymer composed of pentoses, hexoses and sugar acids, can be easily converted to monomeric sugars by applying diluted H_2SO_4 at higher temperatures[53] and intensifying the process with the use of supercritical CO_2 and steam[18]. Research into cellulose processing revealed that pretreatment costs can be reduced by recycling the solvent.

The results of our study indicate that lignin removal during the pretreatment of wheat straw significantly increases ethanol yield. Pretreated wheat straw was characterized by a significantly higher content of reducing sugars (Figs. 2 and 3) and ethanol (Fig. 4) after fermentation (4.09 g · dm^{-3}) than unprocessed, lignin-containing straw (1.23 g · dm^{-3}).

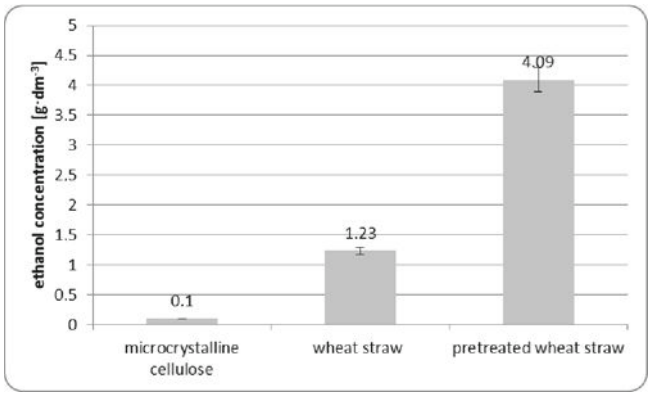


Figure 5. Ethanol concentrations in samples after fermentation

The observed yeast cell counts (Table 1) indicate that fermentation was not adversely influenced by delignification. Differences in yeast viability were observed between samples of processed and unprocessed straw. Straw pretreatment increased the viability of cultured yeast cells due to a higher content of sugars fermenting in the hydrolysate.

Despite differences in yeast cell counts between samples of processed and unprocessed straw, the total number of viable cells was too low for effective bioethanol production. The above could be attributed to insufficient access to nitrogen sources or the presence of residues from chemical pretreatment. The problem could be addressed by using a yeast growth medium, which would enhance the viability of yeast cells and increase ethanol yield per g of wheat straw.

Table 1. Counts and viability of yeast cells in the analyzed samples after fermentation

Sample	Cell viability [%]	Cell count [cfu · cm^{-3}]
Microcrystalline cellulose	60 ±1.1	$4.0x10^6 \pm 1.5x10^5$
Pretreated wheat straw	72 ±0.9	$8.4x10^5 \pm 2.5x10^4$
Wheat straw	60 ±1.9	$5.6x10^5 \pm 2.5x10^4$

Cell count [cfu · cm^{-3}] – Number of microorganisms expressed as the number of colony forming units per cm^3

CONCLUSIONS

The following conclusions can be formulated based on the results of this study:

– Delignification of wheat straw increases the efficiency of enzymatic hydrolysis and increases glucose concentrations nearly four-fold in comparison with unprocessed straw.

– Ethanol concentrations reached 0.4 g per 1 g (dry matter) of pretreated wheat straw, but only 0.1 g per 1 g (dry matter) of untreated wheat straw.

– Chemical pretreatment of wheat straw increased ethanol yield three-fold.

– Delignification does not inhibit the growth of yeast cells and has no adverse effects on yeast viability.

– Chemical pretreatment of wheat straw does not inactivate cellulolytic enzymes secreted by *Trichoderma reesei* and *Aspergillus niger*.

ACKNOWLEDGEMENTS

The authors would like to thank all Reviewers for their insightful comments which helped us improve this manuscript. Their valuable suggestions will also be taken into account in future studies into ethanol production from biomass.

We also would like to thank Aleksandra Poprawska for translating this manuscript.

LITERATURE CITED

1. Gąsiorek, E. & Wilk, M. (2011). Possibilities of utilizing the solid by-products of biodiesel production – a review. *Pol. J. Chem. Technol.* 13(1), 58–62. DOI: 10.2478/v10026-011-0012-y.
2. Szlachta, J. (2008, sierpień). *Możliwości wykorzystania odnawialnych źródeł energii na Dolnym Śląsku.* Cieplej pl.-Portal Dolnośląskiej Agencji Energii i Środowiska. Źródło styczeń 17, 2014, z http://www.cieplej.pl/index_artykuly.php5?dzial=2&kat=23&art=23&limit=12
3. Giampietro, M. & Ulgiati, S. (2005). Integrated assessment of large-scale biofuel production. *Crit. Rev. Plant Sci.* 24, 365–384. DOI:10.1080/07352680500316300.
4. Kotowski, W. (2004, kwiecień). *Ze 150 mld wykorzystuje się zaledwie 5 mld ton… Biomasa na marginesie.* Źródło styczeń 17, 2014, z *http://www.gigawat.net.pl*/archiwum/article/articleview/330/1/37/index.html
5. Glithero, N.J., Ramsden, S.J. & Wilson, P. (2013). Barriers and incentives to the production of bioethanol from cereal straw: A farm business perspective. *Energy Policy* 59, 161–171. DOI: 10.1016/j.enpol.2013.03.003.
6. Kogut, P., Kaczmarek, F., Dąbrowski, T. & Piekarski, J. (2012). Biogas Production Plants as a Method of Utilisa¬tion of Sewage Sludge in Relation to the Polish Legislation. *Rocznik Ochrona Środowiska. Annual Set The Environment Protection* 14, 299–313.
7. Nguyen, T.L.T., Hermansen, J.E. & Nielsen, R.G. (2013). Environmental assessment of gasification technology for biomass conversion to energy in comparison with other alternatives: the case of wheat straw. *J. Clean. Product.* 53, 138–148. DOI: 10.1016/j.jclepro.2013.04.004.
8. Hardy, T., Musialik-Piotrowska, A., Ciołek, J., Mościcki, K. & Kordylewski, W. (2012). Negative effects of biomass combustion and co-combustion in boilers. *Environ. Protect. Engin.* 38(1), 25–33.
9. Wang, L., Littlewood, J. & Murphy, R.J. (2013). Environmental sustainability of bioethanol production from wheat straw in the UK. *Renewable and Sustainable Energy Rev.* 28, 715–725. DOI: 10.1016/j.rser.2013.08.031.
10. Kaparaju, P., Serrano, M., Thomsen, A.B., Kongjan, P. & Angelidaki, I. (2009). Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. *Bioresource* Technol. 9(100), 2562–2568. DOI:10.1016/j.biortech.2008.11.011.
11. Grzybek, A., Gradziuk, P. & Kowalczyk, K. (2001). Słoma – energetyczne paliwo. *Wieś Jutra*. 15. ISBN: 83-88368-19-2.
12. Talebnia, F., Karakashev, D. & Angelidaki I. (2010). Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. *Bioresource Technol.* 13, 101, 4744–4753. DOI: 10.1016/j.biortech.2009.11.080.
13. Passoth, V., Tabassum, M.R., Nair, H.A.S., Olstorpe, M., Tiukova, I. & Ståhlberg J. (2013). Enhanced ethanol production from wheat straw by integrated storage and pre-treatment (ISP). *Enzyme and Microbial Technol.* 2, 52, 105–110. DOI: 10.1016/j.enzmictec.2012.11.003.
14. Zielonaenergia.eco.pl (2013, czerwiec). *Sposoby energetycznego wykorzystania biomasy.* Źródło grudzień 20, 2013, z http://www.zielonaenergia. Eco.pl/index.php?
15. option=com_content&view=article&id=240:sposoby-energetycznego-wykorzystania-biomasy&catid=49:biomasa&Itemid=208
16. Balat, M., Balat, H. & Öz, C. (2008). Progress in bioethanol processing. *Progress in Energy and Combustion Sci.* 34, 551–573. DOI:10.1016/j.pecs.2007.11.001.
17. Baras, J., Gacesa, S. & Pejin, D. (2002). Ethanol is a strategic raw material. *Chem. Ind.*, 56(3), 89–105. http://www.doiserbia.nb.rs/img/doi/0367-598X/2002/0367-598X0203089B.pdf
18. Seguin, A., Marinkovic, S. & Estrine, B. (2012). New pretreatment of wheat straw and bran in hexadecanol for the combined production of emulsifying base, glucose and lignin material. *Carbohyd. Polym.* 2, 88, 657–662. DOI:10.1016/j.carbpol.2012.01.018.
19. Alinia, R., Zabihi, S., Esmaeilzadeh, F. & Kalajahi, J.F. (2010). Pretreatment of wheat straw by supercritical CO_2 and its enzymatic hydrolysis for sugar production. *Biosystems Engineering* 1107, 61–66. DOI:10.1016/j.biosystemseng.
20. Zabihi, S., Alinia, R., Esmaeilzadeh, F. & Kalajahi, J.F. (2010). Pretreatment of wheat straw using steam, steam/acetic acid and steam/ethanol and its enzymatic hydrolysis for sugar production. *Biosystems Engineering*, 3, 105, 288–297. DOI:10.1016/j.biosystemseng.2009.11.007.
21. Badger, P.C. (2002). Ethanol From Cellulose: A General Review Reprinted from: Trends in new crops and new uses. Janick, J. and Whipkey, A. (eds.). ASHS Press, Alexandria, VA. http://large.stanford.edu/publications/coal/references/docs/badger.pdf
22. Balat, M. (2011). Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. *Energy Conversion and Management* 52, 858–875. DOI: 10.1016/j.enconman.2010.08.013.
23. Ferreira-Leitão, V., Gottschalk, L.M.F., Ferrara, M.A., Nepomuceno, A.L., Molinari, H.B.C. & Bon, E.P.S. (2010). Biomass Residues in Brazil: Availability and Potential Uses. *Waste and Biomass Valorization* 1(1), 65–76. DOI: 10.1007/s12649-010-9008-8.
24. Glithero, N.J., Wilson, P. & Ramsden, S.J. (2013). Straw use and availability for second generation biofuels in England. *Biom. and Bioene.* 55, 311–321, DOI:10.1016/j.biombioe.2013.02.033.
25. Borrion, A.L., McManus, M.C. & Hammond, G.P. (2012). Environmental life cycle assessment of bioethanol production from wheat straw. *Biom. Bioene.* 47, 9–19. DOI: 10.1016/j.biombioe.2012.10.017.
26. Directive 2009/28/EC of the European Parliament and of the Council. (2009) on the promotion of the use of energy from renewable sources. OJ l140 of 5.6.2009.

27. Londo, M., et al. (2010). The REFUEL EU road map for biofuels in transport: Application of the project's tools to some short-term policy issues. *Biom. Bioener.* 34 (2), 244–250. DOI: 10.1016/j.biombioe.2009.07.005.
28. Borowski, P., Gawron, J., Golisz, E., Kupczyk, A., Piechocki, J., Powałka, M., Redlarski, G., Samson-Bręk, I., Sikora, M., Szwarc, M. & Tucki, K. (2014). *Influence of CO_2 emissions reduction on functioning the biofuels sectors for transport in Poland* (in Polish: Wpływ redukcji emisji CO_2 na funkcjonowanie sektorów biopaliw transportowych w Polsce. Oficyna Wydawniczo-Poligraficzna ADAM, Warszawa (ISBN 978-83-7821-084-9).
29. Gmyrek, R. (2014). Private communication and http://www.novozymes.com/en/news/image/Pages/Crescentino-Grand-Opening.aspx
30. Tokarz, J. (2002). Szanse rozwoju energetyki odnawialnej. *Czysta Energia* 10(14). 16–18.
31. Duda, M. (2011, listopad). *Potencjał i wykorzystanie biomasy w Polsce*. Źródło kwiecień 30, 2014, z http://eas.itc.pw.edu.pl/?p=139
32. Larsen, S.U., Bruun, S. & Lindedam, J. (2012). Straw yield and saccharification potential for ethanol in cereal species and wheat cultivars. *Biom. Bioener.* 45, 239–250. DOI: 10.1016/j.biombioe.2012.06.012.
33. Chen, H., Han, Y. & Xu, J. (2008). Simultaneous saccharification and fermentation of steam exploded wheat straw pretreated with alkaline peroxide. *Process Biochemistry*, 12(43), 1462–1466. DOI: 10.1016/j.procbio.2008.07.003.
34. Chen, H. & Jin, S. (2006). Effect of ethanol and yeast on cellulase activity and hydrolysis of crystalline cellulose. *Enzyme Microb. Technol.*, 39, 1430–1432. DOI: 10.1016/j.enzmictec.2006.03.027.
35. Hill, J. (2007). Environmental costs and benefits of transportation biofuel production from food- and lignocellulose-based energy crops. A review. *Agron. Sustainable Development* 27, 1–12. DOI:10.1051/agro:2007006.
36. Luo, L., van der Voet, E. & Huppes, G. (2009). An energy analysis of ethanol from cellulosic feedstock – Corn stover. *Renewable and Sustainable Energy Reviews,* 13, 2003–2011. DOI: 10.1016/j.rser.2009.01.016.
37. Zhu, S., Yu, P., Lei, M., Tong, Y., Zheng, L., Zhang, R., Ji, J., Chen, Q. & Wu, Y. (2013). Investigation of the toxicity of the ionic liquid 1-butyl-3-methylimidazolium chloride to Saccharomyces cerevisiae AY93161 for lignocellulosic ethanol production. *Pol. J. Chem. Technol.* 15, 2, 94–98. DOI: 10.2478/pjct-2013-0029.
38. Krutul, D. (2002). Ćwiczenia z chemii drewna oraz wybranych zagadnień z chemii organicznej (Wyd. 2). Wydaw. SGGW Polska. (in Polish)
39. Rodrigues, J., Faix, O. & Pereira, H. (1999). Improvement of the acethylbromide method for lignin determination within large scale screening programmes. *European Journal of Wood & Wood Products/Holz als Roh- und Werkstoff* 57, 341–345. DOI:10.1007/s001070050355.
40. Ghose, T.K. (1987). Measurement of cellulases activities. Pure Appl. Chem. 59 (2), 257–268. DOI:10.1351/pac198759020257.
41. BOEHRINGER MANNHEIM/R-BIOPHARM, Ethanol *UV-method, Simplified procedure for the determination of ethanol in alcoholic beverages*, http://www.hottay.ru/Files/ethanol_englisch_10176290035.pdf
42. Saha, B.C., Iten, L.B., Cotta, M.A. & Wu, Y.V. (2005). Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochem.*12, 40, 3693–3700. DOI: 10.1016/j.procbio.2005.04.006.
43. Zhang, M., Eddy, C., Deana, K., Finkestein, M. & Picataggio, S. (1995). Metabolic engineering of a pentose metabolism pathway in ethanologenic. *Zymomonas mobilis. Sci.*267(5195), 240–243. DOI: 10.1126/science.267.5195.240.
44. Ruiz, H.A., Ruzene, D.S., Silva, D.P., Silva, F.F.M., Vicente, A.A. & Teixeira, J.A. (2011). Development and characterization of an environmentally friendly process sequence (autohydrolysis and organosolv) for wheat straw delignification. *Appl. Biochem. Biotech.* 5, 164, 629–641. DOI: 10.1007/s12010-011-9163-9.
45. Miettinen-Oinonen, A. (2004). Trichoderma reesei strains for production of cellulases for the textile industry. *VTT Publications 550*, Helsinki, 3–4. ISBN 951.38.6417.0.
46. Schuster, E., Dunn-Coleman, N., Frisvad, JC. & Van Dijck, P.W. (2002). On the safety of Aspergilus niger – a review. *Appl. Microbiol. Biotechnol.* 59(4–5), 426–35. DOI: 10.1007/s00253-002-1032-6.
47. Kłosowski, G., Macko, D. & Mikulski, D. (2010). Rozwój metod biotechnologicznych produkcji biopaliw ze źródeł odnawialnych. *Ochr. Śr. Zasobów Nat.* 45, 120–132. (in Polish).
48. Saha, C.B. & Cotta, M.A. (2007). Enzymatic hydrolysis and fermentation of lime pretreated wheat straw to ethanol. *J. Chem. Technol. & Biotechnology* 82, 913–919. DOI: 10.1002/jctb.1760.
49. Szczodrak, J. (1988). The Enzymatic hydrolysis and fermentation of pretreated wheat straw to ethanol. *Biotech. Bioeng.* 32(6), 771–776. DOI:10.1002/bit.260320608.
50. Han, L., Feng, J., Zhang, S., Ma, Z., Wang, Y. & Zhang, X. (2012). Alkali pretreated of wheat straw and it's enzymatic hydrolysis. *Brazil. J. Microbiol.* 43, 53–61. DOI: 10.1590/S1517-83822012000100006.
51. Silva, G.G.D., Couturier, M., Berrin, J-G., Buléon, A. & Rouau, X. (2012). Effects of grinding processes on enzymatic degradation of wheat straw. *Biores. Technol.* 1, 103, 192–200. DOI: 10.1016/j.biortech.2011.09.073.
52. Laureano-Perez, L., Teymouri, F., Alizadeh, H. & Dale, B. (2005). Understanding factors that limit enzymatic hydrolysis of biomass. Characterization of pretreated corn stover. *Appl. Bioch. Biotech.* 124(1–3), 1081–1099. DOI: 10.1385/ABAB:124:1-3:1081.
53. Detroy, R.W., Lindenfelser, L.A. & Sommer, S. (1981). Bioconversion of wheat straw to ethanol: chemical modification, enzymatic hydrolysis, and fermentation, biotechnology and bioengineering. *Biotech. Bioeng.* 23, 1527–1535. DOI: 10.1002/bit.260230712.
54. Wenzel, H.J.F. (1970). The chemical technology of Wood. *Academic Press 2nd edition, New York*, USA, CH4. 94–101 and, 157–571.

energies

MDPI

Article

Use of Buckwheat Straw to Produce Ethyl Alcohol Using Ionic Liquids

Małgorzata Smuga-Kogut [1], Leszek Bychto [2], Bartosz Walendzik [3], Judyta Cielecka-Piontek [4], Roman Marecik [5], Joanna Kobus-Cisowska [6], Katarzyna Grajek [7] and Daria Szymanowska-Powałowska [5,*]

1 Department of Agrobiotechnology, Faculty of Mechanical Engineering, Koszalin University of Technology, Raclawicka 15-17, 75-620 Koszalin, Poland; malgorzta.smuga.kogut@tu.koszalin.pl
2 Department of Electronics, Faculty of Electronics and Computer Science, Koszalin University of Technology, Sniadeckich 2, 75-453 Koszalin, Poland; leszek.bychto@tu.koszalin.pl
3 Department of Civil Engineering, Environmental and Geodetic Sciences, Koszalin University of Technology Koszalin, Sniadeckich 2, 75-453 Koszalin, Poland; bartosz.walendzik@tu.koszalin.pl
4 Department of Pharmacognosy, Poznan University of Medical Sciences, Swiecickiego 4, 60-781 Poznan, Poland; jpiontek@edu.ump.pl
5 Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-627 Poznan, Poland; roman.marecik@up.poznan.pl
6 Department of Gastronomical Sciences and Functional Foods, Poznan University of Life Sciences, Wojska Polskiego, 60-637 Poznan, Poland; joanna.kobus-cisowska@up.poznan.pl
7 Institute of Natural Fibers and Medical Plants, Wojska Polskiego 71b, 60-630 Poznan, Poland; katarzyna.grajek@iwnirz.pl
* Correspondence: daria.szymanowska@up.poznan.pl

Received: 8 March 2019; Accepted: 10 May 2019; Published: 26 May 2019

Abstract: Background: Common buckwheat (*Fagopyrum esculentum* Moench) is an annual spring-emerging crop that is classified among the dicotyledons, due to the manner of its cultivation, use, and chemical composition of seeds. The use of buckwheat straw for energy purposes—for example, for the production of second generation bioethanol—might enable its wider application and increase the cost-effectiveness of tillage. Methods: In this study, we examined the usability of buckwheat straw for the production of bioethanol. We pretreated the raw material with ionic liquids and subsequently performed enzymatic hydrolysis and alcoholic fermentation. The obtained chemometric data were analyzed using the Partial Least Squares (PLS) regression model. PLS regression in combination with spectral analysis within the near-infrared (NIR) spectrum allowed for the rapid determination of the amount of cellulose in the raw material and also provided information on the changes taking place in its structure. Results: We obtained good results for the combination of 1-ethyl-3-methylimidazolium acetate as the ionic liquid and Cellic CTec2 as the enzymatic preparation for the pretreatment of buckwheat straw. The highest concentration of glucose following 72 h of enzymatic hydrolysis was found to be around 5.5 g/dm^3. The highest concentration of ethanol (3.31 g/dm^3) was obtained with the combination of 1-butyl-3-methylimidazolium acetate for the pretreatment and cellulase from *Trichoderma reesei* for enzymatic hydrolysis. Conclusions: In summary, the efficiency of the fermentation process is strictly associated with the pool of available fermenting sugars, and it depends on the type of ionic liquid used during the pretreatment and on the enzymatic preparation. It is possible to obtain bioethanol from buckwheat straw using ionic liquid for pretreatment of the raw material prior to the enzymatic hydrolysis and alcoholic fermentation of the material.

Keywords: bioethanol; biomass; buckwheat straw; ionic liquid; pretreatment

1. Introduction

Common buckwheat (*Fagopyrum esculentum* Moench) is an annual spring-emerging crop that is classified among the dicotyledons; yet, due to the manner of its cultivation and use, and the chemical composition of its seeds, it is classified as a pseudocereal. The buckwheat stem grows up to a height of 60–100 cm and bears branches; it contains pigments such as anthocyanins [1]. The advantage of buckwheat cultivation is that it is a low-soil forecrop whose demands are associated with its capacity to absorb components that are poorly available to other plants and thus better uses soil fertility. Poland is a large producer of buckwheat (118,562 t), and according to FAO data (FAO Reports, 06.09.2018), it ranks fifth in terms of global buckwheat production. Russia (1,186,333 t) occupies the first place, followed by China (404,259 t), Ukraine (176,430 t), and France (122,206 t). The use of buckwheat straw for energy purposes—for example, for the production of second generation bioethanol—might enable its wider application and increase the cost-effectiveness of tillage. Pretreatment of biomass is a crucial step in this conversion. In this context ionic liquid pretreatment of biomass has received much attention lately. The work presented her investigates the effect of pretreatment of chosen lignocellulosic materials with ionic liquids to increase the enzymatic degradation into monosaccharides and to the alkoholic fermentation. However, since the physico-chemical characteristics vary considerably between the different lignocellulosic materials, it is necessary to adopt suitable pretreatment technologies based on the properties of each raw material [2].

One of the methods of pretreatment of compound raw materials of the lignocellulosic complex is the treatment of raw material with ionic liquids [3]. Ionic liquids are organic solvents with a melting point below 100 °C; they consist of large organic cations and minor inorganic anions [4,5]. Advantages of these "green solvents" include the possibility to select from a large range of cations and anions, which enables the design of liquids for specific use [3]. Some of the properties of ionic liquids can be tuned to deliver a specific purpose such as melting point, thermal stability, refractive index, acid-base character, hydrophilicity, polarity, density, and viscosity [4]. The capacity of ionic liquids to dissolve cellulosic and lignocellulosic biomass is a commonly studied topic in the search for new production methods for liquid biofuels. However, the mechanism of action of ionic liquids is not fully understood. An important aspect in the purification of lignocellulose with ionic liquids is the reduction of the crystallization of cellulose after the use of treatments that influences the use of the highest possible concentration of fermenting sugars; that is, after enzymatic hydrolysis [6]. Pretreatment of biomass is a crucial step in this conversion of sugar to alcohol. In this context, pretreatment of biomass using ionic liquids has recently received much attention [7]. Therefore, in this study, we aimed to investigate the effect of pretreatment of selected lignocellulosic materials with ionic liquids in order to increase the enzymatic degradation and produce monosaccharides and thereby to increase the alcoholic fermentation. However, since physicochemical characteristics vary considerably between the different lignocellulosic materials, it is necessary to adopt suitable technologies of pretreatment based on the properties of each raw material [2]. In order to be able to use ionic liquids in the future for biomass processing on an industrial scale, several aspects related to the recycling of ionic liquid after dissolving cellulose (lignin purification and ionic liquid dehydration) as well as thermal stability of the ionic liquid mixture with biomass should be clarified. Some scientific studies indicate that imidazolic ionic liquids lose their thermal stability at temperatures above 100 °C [3]. Therefore, in this study, we aimed to assess the possibility of using buckwheat straw for the production of ethyl alcohol with the use of various ionic liquids for the pretreatment of the biomass. The task of ionic liquids is to change the structure of cellulose fibers from crystalline to amorphous, increasing the space between the fibers and removal of lignin—the inhibitor of the subsequent hydrolysis and fermentation processes. To this end, 4 different ionic liquids were used for buckwheat pretreatment. Moreover, a variant where buckwheat straw was treated with 2 ionic liquids was verified (in a cascade system). The objective of this treatment was the removal of lignin with the use of EMIM Cl and change of the cellulose structure and disrupt the bonds between fibers via EMIM OAc.

2. Materials and Methods

2.1. Raw Material

Buckwheat straw (stems of common buckwheat) were collected in September 2018 from a field with a surface area of 12 ha (Kosciernica, Poland). The material was dried in a convection dryer at a temperature of 85 °C to reach a water content of 5%, and subsequently ground in a colloidal mill (Probs & Class, Rastatt, Germany). The contents of dry weight, cellulose, lignin, and hemicellulose were determined in the straw. The ground material was subjected to enzymatic hydrolysis and fermentation, both without prior pretreatment and with the use of material purification with four ionic liquids in order to select the correct solvent to increase the proportion of cellulose and hemicellulose fibers available to cellulolytic enzymes.

2.2. Ionic Liquids

Four types of ionic liquids were used in the pretreatment of the raw material: 1-ethyl-3-methylimidazolium acetate (EMIMOAc), 1-butyl-3-methylimidazolium acetate (BMIMOAc), 1-ethyl-3-methylimidazolium diethyl phosphate (EMIMDEP), and 1-ethyl-3-methylimidazolium chloride (EMIMCl). One of the samples was subjected to two-fold purification with the use of EMIMOAc and then with EMIMCl. Briefly, 5 g of buckwheat straw was dissolved in 50 cm^3 of the given ionic liquid and then heated to 120 °C for 2 h. Then, the material was brought to room temperature (allowed to cool to room temperature) and then deionized water was added, which resulted in the precipitation of lignocellulose. The material was washed several times with deionized water and then dried at 105 °C for 1.5 h. In the case of using a double treatment with ionic liquids (EMIMOAc and EMIMCl), first 5 g of biomass was dissolved in 50 cm^3 EMIMCl (120 °C, 2 h), subsequently the ionic liquid was rinsed with water, and the material was dried at 105 °C for 1.5 h. The dried material was treated once again using EMIMOAc (120 °C, 2 h) and biomass was precipitated from IL and dried at temperature 105 °C for 1.5 h.

2.3. Enzymatic Hydrolysis

In the process of enzymatic hydrolysis, three cellulolytic agents were used: cellulase from Aspergillus species (≥1000 units/g; aqueous solution, Merck, Germany), cellulase from Trichoderma reesei (≥700 units/g, aqueous solution; Merck, Germany), and Cellic CTec2 (223 FPU/mL; aqueous solution, Merck, Germany). The conditions of hydrolysis were adjusted to the requirements specified in the manufacturer's guidelines; in the case of cellulase from *T. reesei* (20 $U \cdot g^{-1}$ d.m. biomass) and from Aspergillus species (20 $U \cdot g^{-1}$ d.m. biomass), the temperature of hydrolysis was equal to 47 °C at pH 4.8. In the case of the Cellic CTec2 (25 FPU g^{-1} d.m. biomass) agent, the temperature of hydrolysis was equal to 50 °C at pH 5.0. In the process of hydrolysis, 0.5 g of buckwheat straw (98.7% dry weight) was dissolved in 50 cm^3 of acetate buffer (50 mM) and saccharified for 96 h under continuous stirring in a shaker at 150 rpm. After this, the samples were decanted, and the solution was used for alcoholic fermentation.

2.4. Alcoholic Fermentation

Hydrolysate solutions previously filtered to separate the lignocellulose residue were subjected to alcoholic fermentation. The pH of the fermentation broth was measured at each sampling and adjusted to 5.0 by addition of either 10 wt.% H_2SO_4 or 20 wt.% NaOH. Solutions after enzymatic hydrolysis (40 cm^3) were separated from the solid fraction of lignocellulose and transferred to 50 cm^3 fermentation flasks sealed with a fermentation bung, and filled with distilled water. Fermentation was started by the addition of freeze-dried distiller's yeast: *Saccharomyces cerevisiae* type II (Sigma-Aldrich, Germany) (5% w/v). Ethanol fermentation was conducted for 4 days, at a temperature of 36 °C, with shaking at 100 rpm. Samples were taken and analyzed for ethanol concentrations after fermentation. The yeast

cell population and viability were determined by a direct microscopic count in a counting chamber after staining with methylene blue.

2.5. Analytical Methods

In order to examine the influence of ionic liquids on the structure of lignocellulose and on the amount of available cellulose, all samples were tested for their content of cellulose, lignin, and hemicellulose (Ankom A200; ANKOM Technology); the crystalline structure of the samples was recorded using a scanning electron microscope (SEM), electron microscope and NIR spectrum. The morphology of cellulose fibers in buckwheat straw samples prior to and after ionic liquid pretreatment was recorded using an SEM FEI Quanta 200 Mark 2. Multidimensional analysis of primary components, i.e., the principal component analysis (PCA), was used to indicate the significance of the influence of individual factors in the given variant on the content of free cellulose fibers and the effects of interactions between individual factors. The measurement of NIR was performed using a DLP NIRscan Nano spectrophotometer (Texas Instruments), using the reflection technique (in order to obtain a uniform surface of the sample, the material was subjected to homogenization and then was pressed to obtain a lozenge measuring a thickness of 10 mm × 5 mm). The spectra were measured in the range of 900–1680 nm. The measurements were performed at a temperature of 23 °C and at a resolution of 3.5 nm. The obtained raw spectra were subjected to SNV (Standard Normal Variate) transformation (using SPECTRAGRYPH software) as the primary technique of pretreatment. For the performance of quantitative analysis, the regression method was used. This method aims to obtain a calibration model that would enable the correlation of information contained in spectra with one or several properties of the sample. For the purposes of the analysis, PLS regression was performed, in which the set of independent variables X consisted of absorbance values for the spectra ranging between 900 and 1680 nm, whereas the set of dependent variables Y consisted of the percentage content of cellulose in samples of buckwheat straw. Validation of the obtained calibration model was performed using Mean Squared Error (MSE) values and Root Mean Squared Error (RMSE) values. The lower the values of both indices, the better the match of the model. XLSTAT version 10 software was used in the analysis of the results. The cellulose to ethanol conversion rate (%) was calculated according to the formula [8]:

$$Y = \frac{C_e \times V \times 100}{M \times C \times 1.1 \times 0.51} \times 100\ (\%); \quad (1)$$

where:

C_e—ethanol concentration (g/dm^3)
V—sample volume (dm^3)
M—total amount of substrate in the sample (g s.s.)
C—cellulose concentration in the material (%)
1.1—cellulose to glucose conversion factor
0.51—glucose to ethanol conversion factor

The content of glucose and ethanol was determined using high performance liquid chromatography. Samples were first centrifuged at 4000× g for 10 min at 4 °C (The Thermo Scientific Heraeus®Multifuge®3SR Plus Centrifuge, Darmstadt, Germany) and then were filtered through a 0.22 µm membrane filter (Millex-GS, Millipore, USA) prior to analysis using an HPLC system (Merck Hitachi, Pliening, Germany). Glucose and ethanol were separated on an Aminex HPX-87P (Bio-Rad Laboratories GmbH, Munich, Germany) at 30 °C using a 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 cm^3/min and then detected with a refractive index detector (Model L- 7490, Merck Hitachi, Germany).

3. Results and Discussion

3.1. Influence of Ionic Liquids on the Structure of Buckwheat Straw

The greatest impediment associated with the production of bioethanol from lignocellulose is the structure of the biomass, which constitutes a complex of three major components—cellulose, hemicellulose, and lignin—of which the first two components should constitute a good source of sugars that can be fermented. The objective of pretreatment with ionic liquids is to disrupt the bonds between the cellulose, hemicellulose, and lignin fibers, and to increase the content of so-called free cellulose, which can be hydrolyzed with the help of enzymes. Certain ionic liquids may cause the removal of lignin, which often constitutes a barrier for enzymes and yeast in the production of ethanol [9]. Therefore, in this study, we examined the applicability of four ionic liquids to process buckwheat straw and demonstrated the effect of ionic liquids on the transformation of the content of cellulose, hemicellulose, and lignin. Furthermore, it was assumed that the task of ionic liquids is to change the structure of cellulose fibers from crystalline to amorphous, increasing the space between the fibers and removal of lignin—the inhibitor of the subsequent hydrolysis and fermentation processes. To this end, 4 different ionic liquids were used for buckwheat pretreatment and the hemicellulose and lignin content was determined in samples before and after treatment. Furthermore, a variant was verified where buckwheat straw was subject to treatment with 2 ILs. The objective of this treatment was removal of lignin with the use of EMIMCl and change of the cellulose structure and disruption of the bonds between fibers via EMIMOAc.

The study of morphology of buckwheat straw under an SEM microscope showed that untreated buckwheat straw had a highly crystalline structure and ordered morphology with minor areas of mechanical damage resulting from the cutting process (Figure 1). However, after the pretreatment with ionic liquids, EMIMOAc and BMIMOAc and after the pretreatment with two ionic liquids (EMIMOAc and EMIMCl), the structure of the cellular walls became loose; cracks between the neighboring cellular walls occurred, and the entire structure of straw particles became scattered and distorted. The photographs below present singular fibers of cellulose, which, following the application of precipitation with deionized water, constituted a bright, adhesive mass surrounded by lignin and hemicellulose. The observed morphological changes were not found for samples treated with EMIMDEP and EMIMCl, which may indicate the lower capacity of these ionic liquids to change the crystallization of cellulose in buckwheat straw.

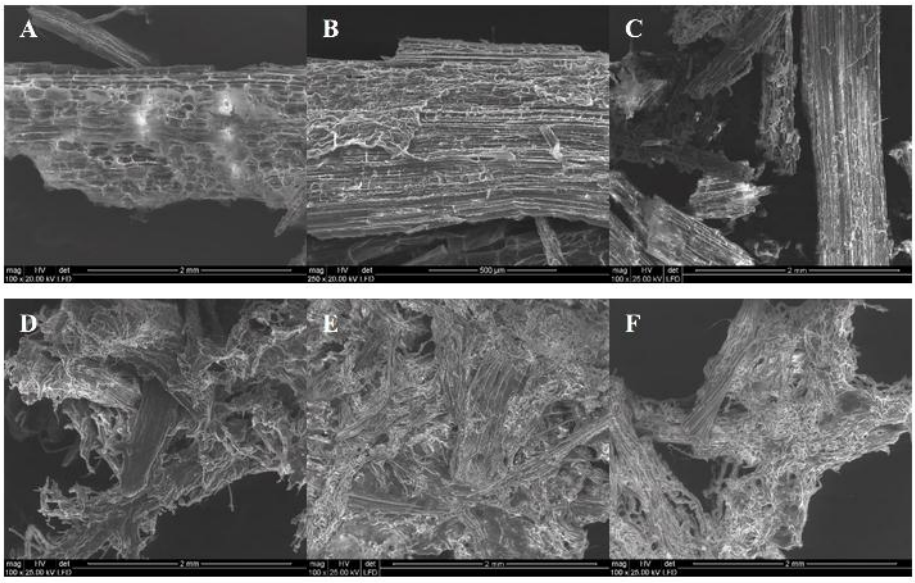


Figure 1. Scanning electron microscopic images of buckwheat straw: (**a**) without pretreatment; (**b**) after processing with EMIMCl; (**c**) after processing with EMIMDEP; (**d**) after processing with EMIMOAc; (**e**) after processing with BMIMOAc; and (**f**) after processing with EMIMOAc and EMIMCl.

Processing the buckwheat straw with two ionic liquids resulted in the disintegration of the ordered fibrous structure of the biomass as well as reduction in the content of free lignin, which was visible in each sample after purification in the form of soot (stains) on cellulose and hemicellulose fibers. This indicates that, during processing with ionic liquids, lignin is not entirely dissolved and extracted by the solvent; rather, it is only removed from the plant cell walls and transferred outside of the structure. In order to confirm this hypothesis, the content of cellulose, hemicellulose, and lignin was tested in straw samples both before and after processing with ionic liquids (Table 1).

Table 1. Composition of buckwheat straw: untreated and after pretreatment ionic liquids.

Type of Pretreatment	Cellulose [%]	Hemicellulose [%]	Lignin [%]
Untreated	42.08 ± 0.12	8.38 ± 0.02	16.97 ± 0.13
EMIMCl	29.05 ± 0.21	18.47 ± 0.11	11.98 ± 0.23
EMIMDEP	41.49 ± 0.10	22.27 ± 0.03	15.26 ± 0.19
EMIMOAc	39.48 ± 0.07	8.81 ± 0.07	20.66 ± 0.08
BMIMOAc	36.91 ± 0.17	9.33 ± 0.14	14.32 ± 0.11
EMIMOAc + EMIMCl	33.27 ± 0.08	18.56 ± 0.22	8.71 ± 0.12

The use of EMIMCl to purify buckwheat straw resulted in a decrease in the content of lignin, by approximately 5%. Unfortunately, EMIMCl caused a considerable loss of free cellulose (up to 10%) compared with the sample of the native form. Minor changes in the content of lignin were also observed in those samples that were purified with EMIMDEP, but the lignin content was found to be 1.7% lower, whereas the amount of cellulose remained unchanged. Lower amounts of lignin and cellulose were also demonstrated after purification with BMIMOAc. After processing with EMIMOAc and EMIMCl, a 50% reduction in the content of lignin was observed, yet the loss of cellulose, which was approximately 9%, was not significant in this case. SEM microscopic analysis showed that only EMIMCl caused delignification of buckwheat straw. Li et al. [10] also indicated delignification of eucalyptus after treatment with BMIMOAc and EMIMOAc. Lignin solubilization during pretreatment with ionic liquids has been reported to be assisted by the π–π interactions of the ionic liquids' cations with lignin [11]. Purification of buckwheat straw with selected ionic liquids resulted in an increase in the content of hemicellulose in samples. The highest amount of hemicellulose was observed in straw purified with EMIMDEP. Ionic liquids resulted in the release of hemicellulose from the biomass complex, thus facilitating its determination after processing. A higher content of hemicellulose after processing may also be associated with the removal of lignin from the biomass [10]. In unprocessed straw, only 8.38% of hemicellulose was found, and application of processing with two ionic liquids resulted in an increase in the content of hemicellulose of over 10%. Literature data indicate higher delignification of lignocellulose when dissolved with acetate-based ionic liquids than that in the case of chloride-based ionic liquids. Moreover, removal of lignin depended on the duration of dissolution of the material in ionic liquids and the temperature of the process [12,13].

The influence of individual ionic liquids on the structure of biomass can also be observed during the spectral analysis of samples in the NIR region. Using an NIR spectrophotometer, and then classification of samples with the PCA method, clear data concerning the applicability of individual ionic liquids for the process of lignocellulose are obtained. The observation chart presents the location of grouping variables in a new dimensional space defined by the F1 and F2 components as determined during the analysis. Buckwheat straw samples were classified in terms of similar Euclidean distances and thus 5 groups were distinguished (Figure 2).

The fifth group consists of buckwheat straw samples after treatment with EMIMOAc and these are most distant from the samples of the first group—buckwheat straw in native form. With the help of the NIR test and PCA classification, it is possible to classify lignocellulosic samples in terms of the amount of cellulose available to enzymes and thus estimate which of these materials will be the best for bioethanol production. In addition, NIR spectra in the range of 900–1680 nm, despite their

monotonous character, may also provide information on the changes in the structure of the examined straw. The upper peaks of absorption for samples of buckwheat straw are presented in Figure 3.

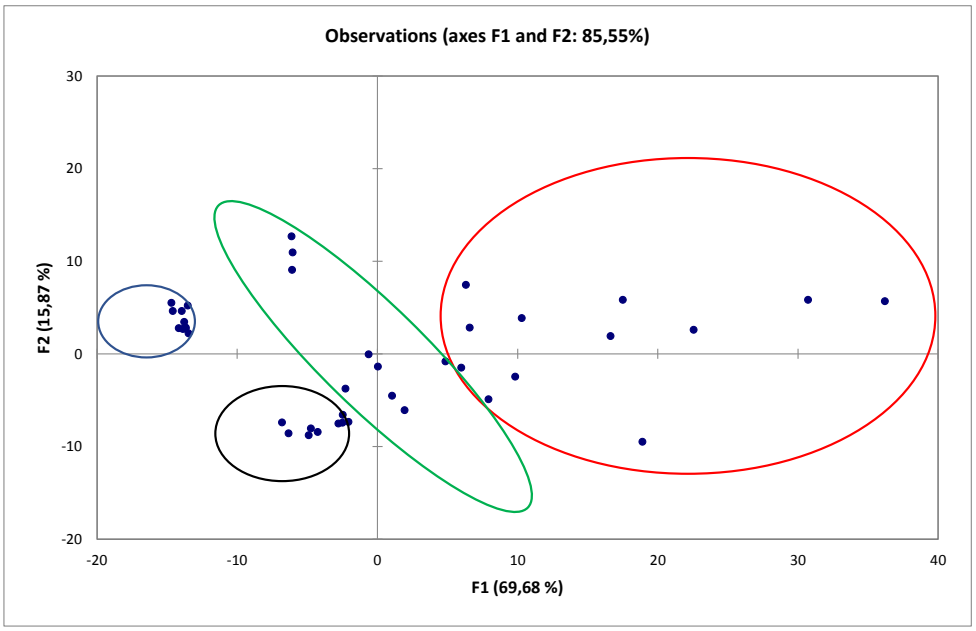


Figure 2. Observation chart: Projection of lignocellulosic biomass onto the space defined by F1 and F2 primary components. 1 (red line)—unprocessed buckwheat straw samples; 2 (green line)—biomass after EMIMCl processing; 3 (black line)—biomass after EMIMOAc and EMIM Cl processing; 4 (bright green line)—buckwheat straw after processing with EMIMDEP, and 5 (blue line)—buckwheat straw after EMIMOAc processing.

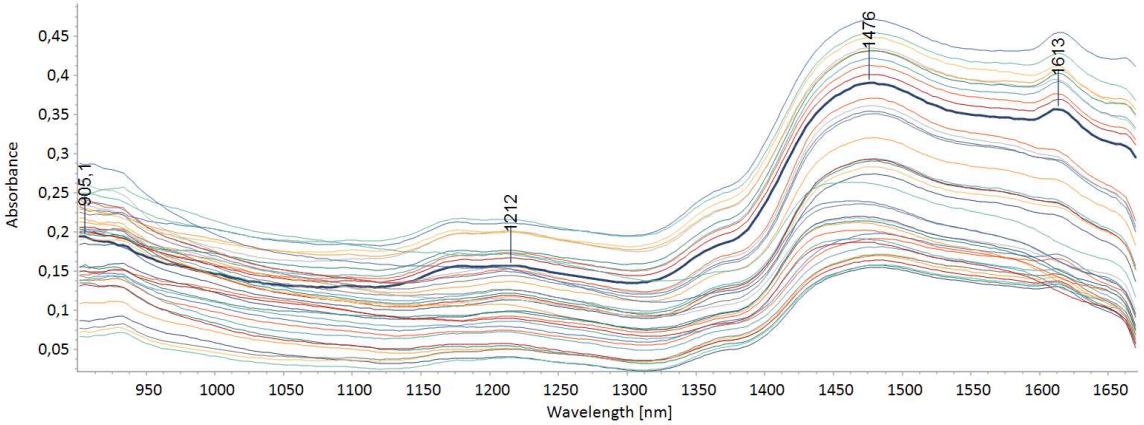


Figure 3. Absorption spectra of buckwheat straw samples in the near-infrared range (900–1680 nm) measured using the reflection technique.

The wide absorption spectrum with a maximum at approximately 1212 nm results from the presence of valence vibrations C-H (a band with lower energy). For the above bond, the basic measurement band is located within the 1600–1650 nm wavelength, with an absorption maximum at 1613 nm. Absorption at 1476 nm is associated with the presence of a band characteristic of valence vibrations of the O-H group (first band of overtone). The band for this bond with lower energy is also found at a wavelength of 905 nm [14,15]. In order to examine the possibility of the determination of the percentage of cellulose in buckwheat straw based on the measured spectra, the PLS regression analysis was conducted. To achieve this, spectra measured in the entire range were used (900–1680 nm). Table 2 presents the results of this regression analysis.

Table 2. Example results of determinations of cellulose content in buckwheat straw samples based on the obtained PLS calibration model.

Model	Range (nm)	Number of Spectra	Number of Variables	R^2	MSE	RMSE
PLS	900–1680	50	1	0.9593	1.1311	1.0635

Analysis of the model showed that the model was correctly matched (R^2 = 0.96). Low MSE and RMSE values obtained in the analysis also indicate good prediction capability. The developed calibration model was used to predict the percentage content of cellulose, both in raw buckwheat straw as well as after processing with ionic liquids. Table 3 shows example results from the estimation. The obtained results indicate the good accuracy of the model.

Table 3. Example results of determinations of cellulose content in buckwheat straw samples based on the obtained PLS calibration model.

Cellulose Content [%]	PLS	
	Predicted Cellulose Content [%]	Deviation [%]
29.05	29.63	2.04
41.49	42.83	1.65
39.48	38.00	1.52
42.08	41.64	2.08

The present method may be of use for the determination of cellulose content in biomass. Such determination is quick and easy, thus being competitive with physicochemical methods utilizing acids and bases. Information on the amount of available cellulose before enzymatic hydrolysis is significant due to the precise selection of the dose of cellulolytic enzymes and it enables minimization of hydrolysis process costs. Furthermore, the NIR data enabled ordering of the type of pretreatment and indicate those where the efficacy is highest. Subsequent research should include further validation of the model, which will enable PLS models to be obtained that are more stable and resistant to fluctuation. Thus, the present study is treated by the authors as a preliminary study, and will naturally be continued.

3.2. Influence of the Use of Ionic Liquids on Enzymatic Hydrolysis and Alcoholic Fermentation

The efficacy of enzymatic hydrolysis of lignocellulosic raw materials is strictly linked to the efficacy of biomass pretreatment, which in turn translates into the availability of cellulose [16]. In the first place, the concentration of glucose was determined before and after processing with ionic liquids. The subsequent stage consisted in enzymatic hydrolysis, in which three commercially available enzymatic agents were used. Cellulases from *Aspergillus* sp. and *Trichoderma reesei* are enzymes that hydrolyze cellulose, a linear polymer of anhydroglucose units linked together by β-1,4-glycosidic bonds, to glucose. Endo-β-D-glucanase is one of the major component enzymes of the cellulase complex. It catalyzes the hydrolysis of cellulose by randomly splitting the sugar residues within the molecule. Exo-β-D-glucanase and β-glucosidase can synergistically convert cellulose into glucose and hence are used on an industrial scale [17]. Cellic CTec2 enzymatic preparation is characterized by increased activity of β-glucosidase, enabling improvement of the efficacy of hydrolysis of lignocellulosic raw materials as a result of restriction of the inhibitory effect of cellobiose.

In accordance with our expectations, glucose was found to be increased in buckwheat straw samples treated with ionic liquids. The highest content of glucose was found in straw purified with EMIMOAc (5.5 g/dm^3) and BMIMOAc (5.1 g/dm^3). The difficulty of enzymatic hydrolysis in untreated lignocellulosic materials was attributed to the presence of hemicelluloses and lignin and their spatial bonds, which created physical barriers that protect cellulose against degradation [18,19]. Lee et al. [20] have demonstrated that the degree of crystallization of cellulose treated with BMIMOAc ionic liquid is lower than that of BMIMCl, which is directly linked to the lower conversion degree of lignocellulose purified with chloride-based ionic liquids.

Our results demonstrated a lack of association between delignification of buckwheat straw with EMIMCl or EMIMDEP ionic liquids with more efficient enzymatic hydrolysis. However, glucose content was found to increase after the hydrolysis of samples purified with EMIMOAc and BMIMOAc. Thus, this confirms that the complete removal of lignin and hemicellulose is not necessary. However, the use of acetate-based ionic liquids (EMIMOAc and BMIMOAc) is more important, as they efficiently facilitate the access of enzymes to cellulose with a lower degree of crystallization, which is a good source of fermenting sugars [19]. With regards to the above, the amount of glucose found in buckwheat straw purified with EMIMOAc and BMIMOAc and after enzymatic hydrolysis was no higher than that of purification with EMIMOAc alone.

Thus, future studies with respect to the use of ionic liquids with lignocellulosic raw materials should place special emphasis on the search for solvents that will mainly result in the depolymerization of cellulose, and that will not, as demonstrated by the currently available literature, lead to the delignification of the material [9,21,22]. Removal of lignin in the process of bioethanol production from biomass may be significant primarily from the economic standpoint, as lignin itself is a valuable waste that is utilized in numerous industrial applications such as the energy, textile, paper, and other industries [23,24].

Another significant stage in second generation bioethanol production is the selection of a suitable enzymatic agent, which will enable the "release" of the fermenting sugars, which will be used in the process of fermentation in the next stage. It is important that the application of the selected enzymatic preparation is favorable from the economic standpoint (the price), and the applied dose is correctly chosen.

In this study, we tested the use three enzymatic agents that will enable a concomitant efficient hydrolysis of cellulose as well as ethanol fermentation without being disrupted by the presence of inhibitors. According to the results, the highest level of efficiency in hydrolysis was obtained by using the Cellic CTec2 agent, irrespective of the type of ionic liquid used. The highest content of glucose after treatment with Cellic CTec2 was 5.5 g/dm^3 followed by the use of EMIMOAc and 5.1 g/dm^3 followed by the use of BMIMOAc. The lowest amount of glucose was recorded for samples where cellulase from *Aspergillus* sp. was used, irrespective of the type of ionic liquid used earlier (Figure 4).

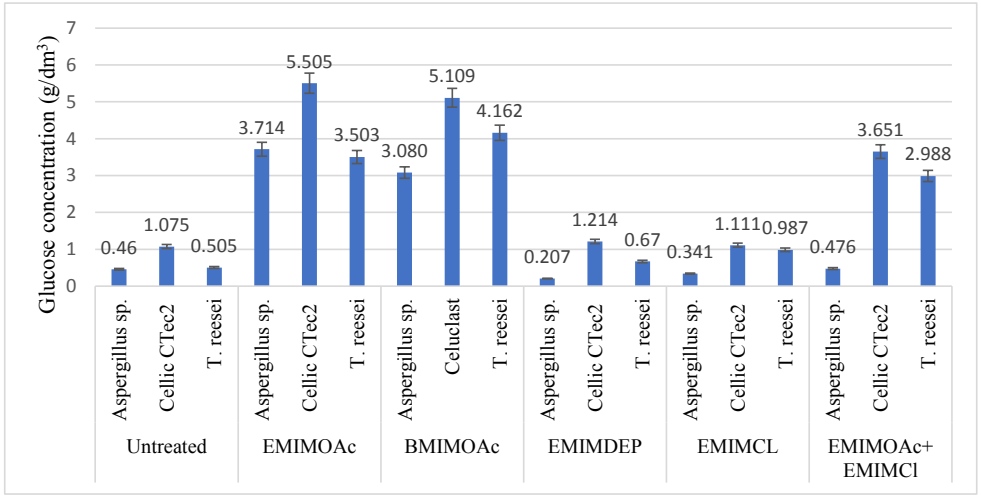


Figure 4. Glucose content after 96 h enzymatic hydrolysis of untreated buckwheat straw samples and samples treated with ionic liquids.

In the next stage, we studied the ethanol fermentation process on the prepared raw material (Figure 5). The fermentation process was performed using *S. cerevisiae* yeast. According to the results, the highest ethanol concentration was obtained for those variants where the following ionic liquids were used: EMIMOAc and Cellic CTec2 (2.46 g/dm^3) and in the variant with BMIMOAc and *T. reesei* (3.31 g/dm^3). These results are compatible with the amount of sugar, and the concentration of glucose,

which at the beginning of the fermentation process was 5.5 g/dm^3 and 4.16 g/dm^3, respectively. The lowest concentration of fermenting sugars was obtained after enzymatic hydrolysis using cellulase from *Aspergillus* sp., which translated into the lowest ethanol concentrations. The environment to which microorganisms are introduced (after pretreatment with ionic liquids and after enzymatic hydrolysis) is typically not favorable for the microorganisms performing the process of ethanol fermentation [25–27]. The presence of an ionic liquid in the fermentation environment, as well as inhibitors constituting the outcome of enzymatic hydrolysis, the source of which is the raw material itself, may have a negative impact on the microorganisms involved in the fermentation process. Indisputably, microorganisms are the weakest link in the process of obtaining ethanol from lignocellulosic raw materials. Thus, not only is the control of yeast count important but also their viability as the specific marker (indicator) of the proper course of the bioprocess is important. In our experiments, the highest count of yeast (8.4×10^5 CFU) in combination with a high yeast viability (78%) was demonstrated for the variant EMIM OAC + Cellic CTec2, which translated into a high concentration of ethanol. In addition, high counts (4.5×10^5 CFU) and viability (81%) were determined for the variant EMIMOAc + EMIMCl in combination with the enzymatic agent from *T. reesei* (Table 4). While in the first case, the glucose concentration as well as the counts and viability of yeast translated into a high ethanol concentration, in the second variant, the concentration of ethanol was lower (1.22 g/dm^3) due to the smaller pool of available fermenting sugars (2.98 g/dm^3). A chromatographic analysis demonstrated that the substrate concentration at 96 h of fermentation in all analyzed variants was zero (results not shown), thus indicating that the entire pool of fermenting sugars was used. The complete use of glucose by the *S. cerevisiae* strain shows that the process had a correct course. However, only analysis of the detailed kinetics of glucose as well as analysis of kinetic indicators such as the productivity of the bioprocess would allow a detailed interpretation of the influence of individual variables on the process of ethanol fermentation. Moreover, an increase in the substrate concentration subjected to the technological process seems to be a key factor. On the one hand, an increase in the pool of fermenting sugars would translate into greater production of ethanol; yet on the other hand, the efficiency of pretreatment and enzymatic hydrolysis, and their influence on microorganisms leading to the fermentation, in this case higher inhibitor concentration, are questionable. Overall, this could have influenced the efficacy of fermentation. In addition, yeast viability was found to be in the range of 43–81%, which may indicate the negative impact of factors in the mixture subject to fermentation. However, in this case, the factor influencing reduced yeast viability may be due to the low concentration of glucose.

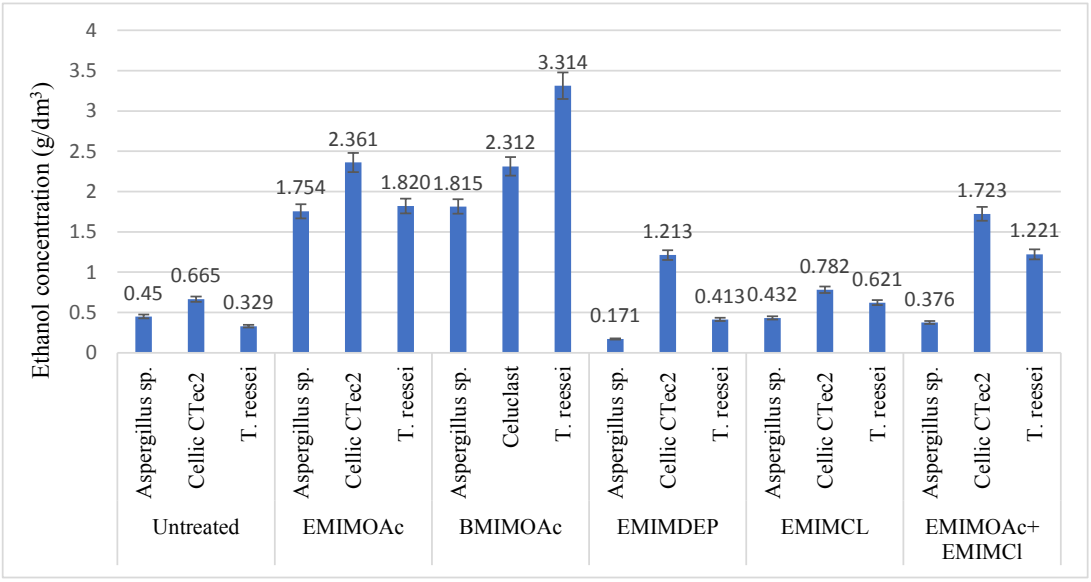


Figure 5. Ethanol content after 96 h alcoholic fermentation of untreated buckwheat straw samples and samples treated with ionic liquids.

Table 4. Counts and viability of yeast cells after alcoholic fermentation of buckwheat straw samples before and after treatment with ionic liquids and conversion of cellulose to ethanol for individual samples.

Sample	Enzyme Type	Cell Viability (%)	Cell Count (CFU)	Cellulose to Ethanol Conversion (%)
Untreated	*Aspergillus* sp.	55% ± 1.1%	4.0×10^6	15.25%
	Cellic CTec2	61% ± 0.9%	3.1×10^5	22.54%
	T. reesei	60% ± 1.9%	2.9×10^5	11.15%
EMIMOAc	*Aspergillus* sp.	69% ± 0.7%	9.0×10^6	63.23%
	Cellic CTec2	78% ± 0.9%	8.4×10^5	83.10%
	T. reesei	75% ± 2.1%	7.6×10^5	65.76%
BMIMOAc	*Aspergillus* sp.	67% ± 2.2%	6.0×10^6	70.14%
	Cellic CTec2	72% ± 1.9%	8.0×10^5	89.35%
	T. reesei	70% ± 1.8%	5.5×10^5	128.07%
EMIMDEP	*Aspergillus* sp.	54% ± 1.4%	3.0×10^6	5.88%
	Cellic CTec2	57% ± 2.1%	3.4×10^5	41.71%
	T. reesei	56% ± 1.4%	2.6×10^5	14.20%
EMIMCl	*Aspergillus* sp.	51% ± 0.7%	3.2×10^6	21.22%
	Cellic CTec2	55% ± 0.6%	4.5×10^5	38.42%
	T. reesei	43% ± 1.5%	4.0×10^5	30.51%
EMIMOAc+ EMIMCl	*Aspergillus* sp.	74% ± 0.5%	5.0×10^6	16.22%
	Cellic CTec2	78% ± 2.1%	6.6×10^5	73.86%
	T. reesei	81% ± 2.9%	4.5×10^5	52.34%

One of the main issues in the production of bioethanol from lignocellulose is the low concentration of ethanol obtained as the effect of the fermentation process. This was also observed in this study. The final concentration of ethanol is influenced by the concentration of the substrate and the efficiency of enzymatic hydrolysis, which is associated with the availability of cellulose (efficiency of pretreatment) and its content in the raw materials. Selection of the correct raw material, type of pretreatment, and selection of enzymatic agent as well as its doses are the key diagnostic issues to be met during the development of a method utilizing a new raw material [28–31]. Moreover, present-day biotechnology does not only have to deal with the development of screening of microorganisms with industrial potential, which will enable the development of markers used in the selection of proper microorganisms for the process of fermentation. The challenge is also to choose microorganisms that can use hexoses and pentoses and that are resistant to toxins and inhibitors, which are present due to the degradation of lignin [32,33]. Such compounds formed during fermentation often block ethanol fermentation (e.g., furfural, methylhydroxyfurfural, acetic acid, lactic acid, phenols, aldehydes, and heavy metal ions). Due to the absence of such natural microorganisms, the use of metabolic engineering to construct organisms with the required characters has been gaining interest. This enhances the activity of cells as a result of enhanced enzymatic, transportation, and regulatory functions by means of DNA recombination. Such interest includes analysis of metabolic pathways, design of genetic changes, and creation of recombined cells with changed properties [34].

4. Conclusions

In this study, we have demonstrated the possibility of obtaining bioethanol from buckwheat straw using ionic liquid for the pretreatment of the raw material prior to its enzymatic hydrolysis and alcoholic fermentation.

In the present study, the best results were obtained using 1-ethyl-3-methylimidazolium acetate and Cellic CTec2 enzymatic preparations for pretreatment of buckwheat straw. The glucose content after 72 h of enzymatic hydrolysis was 5.5 g/dm^3 (with the use of EMIMOAc), whereas the highest concentration of bioethanol (3.31 g/dm^3) was obtained by using BMIMOAc for the pretreatment of

straw and *T. reesei* cellulase for enzymatic hydrolysis. The efficiency of the fermentation process is strictly linked to the pool available to fermenting sugars.

This research has demonstrated that EMIMOAc and BMIMOAC ionic liquids are more efficient in dissolving cellulose and produce more pronounced changes in the cellulose fiber structure than EMIMCl and EMIMDEP. However, pretreatment with EMIMCl resulted in greater delignification of the material than occurred with the remaining ionic liquids. For the pretreatment of buckwheat straw, treatment with EMIMOAC and EMIMCl was used in order to produce the delignification effect and the concomitant increase in amorphous sites in the biomass structure. This method resulted in reduced lignin content in the sample after pretreatment (to approx. 8%), but it did not lead to an increase in the hydrolysis efficiency.

The PLS model enabled the determination of the percentage content of cellulose in buckwheat straw, which might constitute a quick and interesting alternative to analytical methods used currently. The NIR spectra of the raw material provided information on changes occurring within its structure. Information in the form of NIR spectra with chemometric data analysis can be used as a tool for the rapid determination of the amount of cellulose in raw material; this knowledge can be extended to alter the physicochemical processes of the raw material. This is important because it enables better selection of the number of enzymes, depending on the amount of available cellulose after pretreatment. If future bioethanol production from biomass includes pretreatment followed by enzymatic hydrolysis, the prompt use of NIR tests will reduce the costs associated with precise selection of the dose of enzymes to the amount of cellulose in the material, which remains after treatment. The described method will constitute the subject of future research to be conducted by the authors.

Author Contributions: The following statements should be used: conceptualization, M.S.K., D.S.P., L.B., B.W., R.M., J.K.C., K.G. and J.C.P.; methodology, M.S.K., D.S. and B.W. and J.C.P.; software, J.K.C, M.S.K and J.C.P.; validation, J.K.C, M.S.K. and B.W.; formal analysis, M.S.K., B.W. and J.C.P.; investigation, M.S.K.; resources, M.S.K., D.S. and J.K.C.; data curation, M.S.K, J.K.C. and J.C.P.; writing—original draft preparation, M.S.K., D.S.P., L.B., B.W., R.M., J.K.C., K.G. and J.C.P; writing—review and editing, M.S.K., D.S.P., L.B., B.W., R.M., J.K.C., K.G. and J.C.P.; visualization, B.W.; supervision, M.S.K and D.S.; project administration, M.S.K.; funding acquisition, M.S.K.

Funding: This research was funded by the NATIONAL SCIENCE CENTER (POLAND), grant number DEC-2017/01/X/NZ9/00357.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zarzecka, K.; Gugała, M.; Mystkowska, I. Nutritional value and opportunities of using buckwheat. *Postępy Fito* **2014**, *1*, 28.
2. Alvira, P.; Tomas-Pejo, E.; Ballesteros, M.; Negro, M.J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A Review. *Bioresour. Technol.* **2010**, *101*, 4851. [CrossRef]
3. Holm, J.; Lassi, U. *Ionic liquids in pretreatment of lignocellulosic biomass In Ionic Liquids: Application and Perspectives*; Kokorin, A., Ed.; IntechOpen: Rijeka, Croatia, 2011; pp. 546–560.
4. Pinkert, A.; Marsh, K.N.; Pang, S.; Staiger, M.P. Ionic liquids and their interaction with cellulose. *Chem. Rev.* **2009**, *109*, 6712. [CrossRef] [PubMed]
5. Zhu, S. Use of ionic liquids for the efficient utilization of lignocellulosic materials. *J. Chem. Technol. Biotechnol.* **2008**, *83*, 777. [CrossRef]
6. Samayam, I.P.; Hanson, L.B.; Langan, P.; Schall, C.A. Ionic-liquid induced changes in cellulose structure associated with enhanced biomass hydrolysis. *Biomacromolecules* **2011**, *12*, 3091. [CrossRef]
7. Brandt, A.; Hallett, J.P.; Leak, D.J.; Murphy, R.J.; Welton, T. The effect of the ionic liquid anion in the pretreatment of pine wood chips. *Green Chem.* **2010**, *12*, 672–679. [CrossRef]
8. Liu, K.; Lin, X.; Yue, J.; Li, X.; Fang, X.; Zhu, M.; Lin, J.; Qua, J.; Xiao, L. High concentration ethanol production from corncob residues by fed-batch strategy. *Bioresour. Technol.* **2010**, *101*, 4952–4958. [CrossRef]
9. Smuga-Kogut, M.; Zgórska, K.; Szymanowska-Powałowska, D. Influence of the crystalline structure of cellulose on the production of ethanol from lignocellulose biomass. *Int. Agrophys.* **2016**, *30*, 83–88. [CrossRef]

10. Li, H.Y.; Chen, X.; Wang, C.Z.; Sun, S.N.; Sun, R.C. Evaluation of the two-step treatment with ionic liquids and alkali for enhancing enzymatic hydrolysis of *Eucalyptus*: Chemical and anatomical changes. *Biotechnol. Biofuels* **2016**, *9*, 166. [CrossRef] [PubMed]
11. Shill, K.; Padmanabhan, S.; Xin, Q.; Prausnitz, J.M.; Clark, D.S.; Blanch, H.W. Ionic liquid pretreatment of cellulosic biomass: Enzymatic hydrolysis and ionic liquid recycle. *Biotechnol. Bioeng.* **2011**, *108*, 511. [CrossRef]
12. Qiu, Z.; Aita, G.M.; Walker, M.S. Effect of ionic liquid pretreatment on the chemical composition, structure and enzymatic hydrolysis of energy cane bagasse. *Bioresour. Technol.* **2012**, *117*, 251–256. [CrossRef] [PubMed]
13. Doherty, T.V.; Mora-Pale, M.; Foley, S.E.; Linhardt, R.J.; Dordick, J.S. Ionic liquid solvent properties as predictors of lignocellulose pretreatment efficacy. *Green Chem.* **2010**, *12*, 1967–1975. [CrossRef]
14. Tuchołka, D. Methods of Absorption and Reflection Spectroscopy in Chemical Analysis. Ph.D. Thesis, University of Agriculture in Poznan, Poznan, Poland, 1996.
15. Kocjan, R. *Analytical Chemistry T. 2 Instrumental Analysis*; Medical Publishing PZWL: Warsaw, Poland, 2014; pp. 231–247.
16. Ji, Z.; Zhang, X.; Ling, Z.; Zhou, X.; Ramaswamy, S.; Xu, F. Visualization of Miscanthus giganteus cell wall deconstruction subjected to dilute acid pretreatment for enhanced enzymatic digestibility. *Biotechnol. Biofuels* **2015**, *8*, 1. [CrossRef]
17. Horn, S.J.; Vaaje-Kolstad, G.; Westereng, B.; Eijsink, V.G.H. Novel enzymes for the degradation of cellulose. *Biotechnol. Biofuels* **2012**, *5*, 45. [CrossRef]
18. Uju, S.Y.; Nakamoto, A.; Goto, M.; Tokuhara, W.; Noritake, Y.; Katahira, S.; Ishida, N.; Nakashima, K.; Ogino, C.; Kamiya, N. Short time ionic liquids pretreatment on lignocellulosic biomass to enhance enzymatic saccharification. *Bioresour. Technol.* **2012**, *103*, 446–452. [CrossRef]
19. Sun, S.; Cao, X.; Sun, S.; Xu, F.; Song, X.; Jones, G. Improving the enzymatic hydrolysis of thermo-mechanical fiber from Eucalyptus urophylla by a combination of hydrothermal pretreatment and alkali fractionation. *Biotechnol. Biofuels* **2014**, *7*, 116. [CrossRef] [PubMed]
20. Lee, S.H.; Doherty, T.V.; Linhardt, R.J.; Dordick, J.S. Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis. *Biotechnol. Bioeng.* **2009**, *102*, 1368–1376. [CrossRef]
21. Boeriu, C.G.; Bravo, D.; Gosselink, R.J.; van Dam, J.E. Characterization of structure-dependent functional properties of lignin with infrared spectroscopy. *Ind. Crops Prod.* **2004**, *20*, 205–218. [CrossRef]
22. Hou, X.D.; Li, N.; Zong, M.H. Renewable bio ionic liquids-water mixtures-mediated selective removal of lignin from rice straw: Visualization of changes in composition and cell wall structure. *Biotechnol. Bioeng.* **2013**, *110*, 1895–1902. [CrossRef] [PubMed]
23. Sahoo, S.; Seydibeyoglu, M.O.; Mohanty, A.K.; Misra, M. Characterization of industrial lignins for their utilization in future value added applications. *Biomass Bioenergy* **2011**, *35*, 4230–4237. [CrossRef]
24. Cotana, F.; Cavalaglio, G.; Nicolini, A.; Gelosia, M.; Coccia, V.; Petrozzi, A.; Brinchi, L. Lignin as co-product of second generation bioethanol production from lignocellulosic. *Energy Procedia* **2014**, *45*, 52–60. [CrossRef]
25. Mehmood, N.; Husson, E.; Jacquard, C.; Wewetzer, S.; Büchs, J.; Sarazin, C.I. Impact of two ionic liquids, 1-ethyl-3-methylimidazolium acetate and 1-ethyl-3-methylimidazolium methylphosphonate, on Saccharomyces cerevisiae: Metabolic, physiologic, and morphological investigations. *Biotechnol. Biofuels* **2015**, *8*, 17. [CrossRef] [PubMed]
26. Sitepu, I.R.; Shi, S.; Simmons, B.A.; Singer, S.W.; Boundy-Mills, K.; Simmons, C.W. Yeast tolerance to the ionic liquid 1-ethyl-3-methylimidazolium acetate. *FEMS Yeast Res.* **2014**, *14*, 1286–1294. [CrossRef] [PubMed]
27. Qing, Q.; Yang, B.; Wyman, C.E. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. *Bioresour. Technol.* **2010**, *101*, 9624–9630. [CrossRef]
28. Giri, F.M.; Fonseca, C.; Carvalheiro, F.; Duarte, L.C.; Marques, S.; Bogel-Lukasic, R. Hemicelluloses for fuel ethanol: A review. *Bioresour. Technol.* **2010**, *101*, 4775–4800. [CrossRef]
29. Gamage, J.; Howard, L.; Zisheng, Z. Bioethanol production from lignocellulosic biomass. *J. Biobased Mater. Bioenerg.* **2010**, *4*, 3–11. [CrossRef]
30. Garver, M.P.; Liu, S. Development of thermochemical and biochemical technologies for biorefineries. In *Bioenergy Research: Advances and Applications*, 1st ed.; Gupta, V.K., Tuohy, M.G., Kubicek, C.P., Saddler, J., Xu, F., Eds.; Publisher: Elsevier, Netherlands, 2014; Volume 27, pp. 457–488.

31. Álvarez, C.; Manuel Reyes-Sosa, F.; Díez, B. Enzymatic hydrolysis of biomass from wood. *Microbiol. Biotechnol.* **2016**, *9*, 149–156. [CrossRef] [PubMed]
32. Yang, Q.; Pan, X. Correlation between lignin physicochemical properties and inhibition to enzymatic hydrolysis of cellulose. *Biotechnol. Bioeng.* **2016**, *113*, 1213–1224. [CrossRef] [PubMed]
33. Lee, Y.G.; Jin, Y.S.; Cha, Y.L.; Seo, J.H. Bioethanol production from cellulosic hydrolysates by engineered industrial Saccharomyces cerevisiae. *Bioresour. Technol.* **2017**, *228*, 355–361. [CrossRef] [PubMed]
34. Dickinson, Q.; Bottoms, S.; Hinchman, L.; McIlwain, S.; Li, S.; Myers, C.L.; Boone, C.; Coon, J.J.; Hebert, A.; Sato, T.K.; et al. Mechanism of imidazolium ionic liquids toxicity in *Saccharomyces cerevisiae* and rational engineering of a tolerant, xylose-fermenting strain. *Microbial. Cell Fact.* **2016**, *15*, 17. [CrossRef] [PubMed]

energies

MDPI

Article

Comparison of Bioethanol Preparation from Triticale Straw Using the Ionic Liquid and Sulfate Methods

Małgorzata Smuga-Kogut [1], Bartosz Walendzik [2], Daria Szymanowska-Powalowska [3,*], Joanna Kobus-Cisowska [4], Janusz Wojdalski [5], Mateusz Wieczorek [6] and Judyta Cielecka-Piontek [6]

1 Department of Agrobiotechnology, Faculty of Mechanical Engineering, Koszalin University of Technology, Raclawicka 15-17, 75-620 Koszalin, Poland; malgorzta.smuga-kogut@tu.koszalin.pl
2 Faculty of Civil Engineering, Environmental and Geodetic Sciences, Koszalin University of Technology Koszalin, Sniadeckich 2, 75-453 Koszalin, Poland; bartosz.walendzik@tu.koszalin.pl
3 Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-627 Poznan, Poland
4 Department of Gastronomical Sciences and Functional Foods, Poznan University of Life Sciences, Wojska Polskiego, 60-637 Poznan, Poland; joanna.kobus-cisowska@up.poznan.pl
5 Department of Production Management and Engineering, Faculty of Production Engineering, Warsaw University of Life Sciences, Nowoursynowska 166, 02-787 Warsaw, Poland; janusz_wojdalski@sggw.pl
6 Department of Pharmacognosy, Poznan University of Medical Sciences, Swiecickiego 4, 60-781 Poznań, Poland; mateuszwieczorek23@gmail.com (M.W.); jpiontek@ump.edu.pl (J.C.-P.)
* Correspondence: daria.szymanowska@up.poznan.pl

Received: 26 January 2019; Accepted: 19 March 2019; Published: 25 March 2019

Abstract: Triticale straw constitutes a potential raw material for biofuel production found in Poland in considerable quantities. Thus far, production of bioethanol has been based on food raw materials such as cereal seeds, sugar beets or potatoes, and the biofuel production methods developed for these lignocellulose raw materials can threaten the environment and are inefficient. Therefore, this study aimed to compare of methods for pretreatment of triticale straw using 1-ethyl-3-methylimidazolium acetate and the sulfate method in the aspect of ethanol production intended for fuel. Based on the conducted experiments it has been determined that the use of 1-ethyl-3-methylimidazolium acetate for the pretreatment of triticale straw resulted in an increase of reducing sugars after enzymatic hydrolysis and ethyl alcohol after alcoholic fermentation. Furthermore, the study compared the efficiency of enzymatic hydrolysis of triticale straw without pretreatment, after processing with ionic liquid, recycled ionic liquid and using sulfate method, allowing a comparison of these methods. The more favorable method of lignocellulose material purification was the use of ionic liquid, due to the lower amount of toxic byproducts formed during the process, and the efficiency test results of bioethanol production using pretreatment with ionic liquid and sulfate method were similar. Ionic liquid recycling after pretreatment of rye straw using lyophilization allowed us to reuse this solvent to purify rye straw, yet the efficiency of this method remained at a low level. As a result of the conducted study it was determined that the use of ionic liquid-1-ethyl-3-methylimidazolium acetate enhanced the yield of bioethanol from triticale straw from 1.60 g/dm^3 after processing without pre-treatment to 10.64 g/dm^3 after pre-treatment.

Keywords: triticale straw; ethanol; ionic liquids; sulfate method

1. Introduction

Lignocellulosic raw materials constitute the most promising group of raw materials for the production of bioethanol. The global amount of lignocellulose waste exceeds 180 billion tons per annum. Lignocellulose contains cellulose, hemicellulose and lignin. The biotechnological processes primarily focus on cellulose, as the product of its hydrolysis is glucose, easily processed by the majority of microorganisms. The mean efficiency of ethanol production from cellulose is 0.42 m^3/ton of d.w. Contrary to the easily hydrolyzed starch, cellulose requires expensive preparation. Hydrolysis with dilute acids (primarily sulfuric acid) is normally used for this purpose. Acidic hydrolysis techniques are used in Brazil and in the USA. Other cellulose raw material processing methods are known (extraction, enzymatic hydrolysis), yet thus far they have remained more expensive. One may hope that as a result of technical progress, less expensive methods of cellulose material saccharification will become available, allowing their transformation into ethanol to become profitable. Considering the high contribution of the raw material to the bioethanol production costs, genetic research, aiming at the increase of productivity of the plants being the potential energy substrate, is essential. Geneticists are constantly conducting research on the development of saccharide-rich, new cultivars of cereals and sugar beet, as well as on the enhancement of the properties related to plant immunity against insects and herbicides [1].

The use of biomass for the energy production constitutes one of the possibilities to reduce the production of conventional energy. Second generation biofuel production is defined as a biomass-utilizing technology, conducted in a safe and non-burdensome manner for the environment, reducing the CO_2 emissions [2].

The use of agricultural biomass for ethanol production as a biocomponent of liquid fuels is one of the most promising economic and environmental solutions due to the low raw material costs and reduction of greenhouse gas emissions during their combustion [3,4].

However, thus far only a small number of technologies utilizing lignocellulose raw materials for bioethanol production exist, which is strictly related to the structure of the raw material and availability of reducing sugars after the hydrolysis process. The most problematic structural element of lignocellulose is the lignin, which irreversibly binds the active sites of cellulolytic enzymes, thus significantly reducing the efficiency of cellulose hydrolysis reaction, despite the expensive pretreatment [5,6]. Chemical pre-treatment is used to remove lignin and separate it from the cellulose fibers. The most common industrial methods are based on the use of active brewing chemicals. The use of acids and bases is efficient and produces expected results, yet it typically generates harmful byproducts. The method which is gathering an increasing interest due to the efficient preparation of the lignocellulose substrate for the fermentation process consists in the use of ionic liquids.

Ionic liquids are organic salts consisting of an organic cation containing a heterocyclic atom and an inorganic or organic anion. Ionic liquids containing imidazolium cations and anions such as chloride, acetate and formate display particularly good cellulose-dissolving properties [7–9]. The literature indicates that thus far the properties of ionic liquids containing different anions and cations have been tested, and their capacity to dissolve cellulose has been examined as well [8–12]. Worth emphasizing is the fact that on annual basis the Polish agriculture sector produced approx. 28.5 million tons of straw (primarily cereal and rapeseed straw) and hay [13] and 30–50 % of the existing resources can be used for energy purposes.

As a plant biomass intended for energy purposes triticale straw requires particular treatment in the ethanol fermentation process. This is linked to its particular structure, in which lignin plays the most important role and it is strictly connected to the remaining cellulose and hemicellulose polymers. This forces the use of substrate pretreatment, which considerably impacts the course of the subsequent stages and determines the final efficiency of the bioethanol production process [14–16].

This study aimed at a comparison of methods for pretreatment of triticale straw using 1-ethyl-3-methylimidazolium acetate and the sulfate method in the aspect of ethanol production intended for fuel.

2. Methods

2.1. Materials and Reagents

The study material consisted of triticale straw (Agricultural Holding A. Kogut, Krytno, Zachodniopomorskie Voivodeship, Poland), disintegrated to 2 mm grain size powder. In order to perform the hydrolysis reaction of the material two enzymatic preparations were used: cellulase from *Trichoderma reesei* ATCC 26921 (Sigma Aldrich, Poznan, Poland) and cellobiose synthesized by *Aspergillus niger* (Novozym 188, Sigma Aldrich, Poznan, Poland). To perform pretreatment of the biomass, ionic liquid in the form of 1-ethyl-3-methylimidazolium acetate was used. The enzymatic preparations as well as ionic liquid originated from the Sigma Aldrich Company (Poznan, Poland). In the process of triticale straw purification with the sulfate method, active brewing chemicals consisted of sodium hydroxide and sodium sulfide provided by POCH Company (Gliwice, Poland).

2.2. Pretreatment Procedure

Within the conducted study, three raw material pretreatment variants were examined. The first method consisted in preliminary purification of triticale straw using 1-ethyl-3-methylimidazolium acetate. To this aim, triticale straw (100 g) was dissolved in 1-ethyl-3-methylimidazolium acetate (100 cm^3). The sample was incubated in 120 °C for 2 h, then the sample was cooled to room temperature and deionized water (100 cm^3) was added to precipitate the cellulose and hemicellulose. The samples were mixed for approx. 30 min, which resulted in the transfer of the 1-ethyl-3-methylimidazolium acetate to the aqueous phase. The water rinsing and mixing processes were repeated three times, until complete removal of the ionic liquid was achieved. In the last stage, the sediment was rinsed with pH 4.7 acetate buffer (100 cm^3), subjected to mixing for approx. 15 min, and then decanted.

The second pretreatment method consisted in the so-called delignification of triticale straw using the sulfate method. In this process, the cellulose fibers are released by dissolution of the lignin and part of hemicellulose in the solution of brewing chemicals. The brewing process was conducted at 180 °C for 4 h. The brewing liquor causes the alkylation of lignin and hence its transformation into alkyl lignin. Cellulose fibers were separated from the chemicals using a vacuum filtration method. The end product of the brewing process was an unbleached cellulose mass, which was used for the subsequent study.

The third pretreatment method for triticale straw consisted in dissolution of 100 g of d.s. of the substrate in 1-ethyl-3-methylimidazolium acetate (100 mL) after recycling and incubation of the mixture in 120 °C for 2 h. Subsequently, the material was precipitated from the solution using distilled water. The substrate with the ionic liquid was cooled to room temperature and distilled water (100 cm^3) was added to precipitate the cellulose and hemicellulose. The samples were mixed for approx. 30 min, which resulted in the transfer of the 1-ethyl-3-methylimidazolium acetate into aqueous phase. The water rinsing, and mixing processes were repeated three times, until complete removal of the ionic liquid was achieved.

The 1-ethyl-3-methylimidazolium acetate recycling consisted in desiccation of the solution of this liquid using a lyophilization process. Aqueous ionic liquid solution (100 cm^3) frozen at a temperature of −40 °C from the last stage of the pretreatment was used for lyophilization. The process was conducted in an Alpha 1-2 LDPLUS laboratory freeze dryer by Christ Company (Berlin, Germany) for 24 h, at a pressure of 63 Pa, and safety pressure of 103 Pa. Following lyophilization a viscous ionic liquid was obtained, which color was similar to the color of pure ionic liquid. Subsequently, the possibility of a repeated use of the retrieved ionic liquid in the pretreatment of a new portion of triticale straw, which was also enzymatically hydrolyzed, was tested. The reducing sugar content results obtained after enzymatic hydrolysis of triticale straw purified with recycled ionic liquid were compared with the contents of reducing sugars determined after enzymatic hydrolysis of rye straw purified with pure ionic liquid. Three repetitions of the tests were conducted, and the results presented in the graph represent a mean value from the three measurements.

2.3. Enzymatic Hydrolysis of the Regenerated Triticale Straw

The raw material after pretreatment with ionic liquid and chemical treatment was hydrolyzed using enzymes obtained from *Trichoderma reesei* and *Aspergillus niger*. The substrate (100 g) was suspended in acetate buffer (1 dm^3, 50 mM, pH 4.7) and distributed in the entire volume by mixing in a fermenter for approx. 10 min. Samples were heated to 47 °C and enzymes were added: cellulase from *Trichoderma reesei* ATCC 26921 (7 FPU·g^{-1} d.s. of the material) and Novozym 188 cellobiose (*Aspergillus niger*, 30 CBU·g^{-1} d.s. of the material). Enzymatic hydrolysis was conducted for 72 h. Then, the hydrolysates were subjected to alcoholic fermentation. At the same time, control samples were prepared, which consisted of rye straw suspensions without pretreatment. Three repetitions of each experimental variant were done. Samples were obtained at determined time intervals and their total reducing sugar content in relation to the content of glucose read from the model curve was determined.

2.4. Fermentation

Hydrolysate solutions, previously filtered to separate the lignocellulose residue, were subjected to alcoholic fermentation. The pH of the fermentation broth was measured at each sampling timepoint and adjusted to 5.0 by an addition of either 10 wt.% H_2SO_4 or 20 wt.% NaOH. Fermentation was started by an addition of freeze-dried *Saccharomyces cerevisiae* type II distiller's yeast (Sigma-Aldrich, 5% v/v). Ethanol fermentation was conducted for 4 days under anaerobic conditions. Samples were taken and analyzed for ethanol concentrations after the fermentation.

2.5. Analysis Methods

The concentrations of reducing sugars after enzymatic hydrolysis of cellulose were quantitatively determined using 3,5-dinitrosalicylic acid in an alkaline environment [17]. The vitality and yeast cell counts in digestate were determined using direct method with a light microscope and Thoma chamber and 0.01 % solution of methylene blue. The concentration of ethyl alcohol was determined using a ROCHE test (Enzymatic Bioanalysis/Food Analysis). To evaluate of the course of the hydrolysis and fermentation processes, a multidimensional analysis of primary components (PCA) was used to indicate the significance of the influence of individual factors in the given variant on the efficiency of hydrolysis and the effects of interaction between individual factors. STATISTICA version 10 ((license No.: AGAP306E324317AR-T, StatSoft Inc., Kraków, Poland; www.statsoft.com) software was used for the analysis of the study results.

3. Results and Discussion

3.1. Enzymatic Hydrolysis

After 72 h, the obtained concentration of released sugars as a result of enzymatic hydrolysis of the native substrate (control sample) was 6.78 g/dm^3 of hydrolysate. As a result of the series of experiments utilizing triticale straw treated with 1-ethyl-3-methylimidazolium acetate a considerable increase in the susceptibility of the substrate to enzymatic hydrolysis was observed. Almost a six-fold higher content of reducing sugars in comparison to the control sample obtained after a 72-hour enzymatic hydrolysis proves the validity of treating the raw material with ionic liquid. The highest reducing sugar concentration obtained after enzymatic hydrolysis was 36.54 g/dm^3 (Figure 1).

Comparable results were also obtained by Świątek and Lewandowska [18], who subjected rapeseed straw to hydrolysis. The efficiency of the saccharification process of the material in the native form was 16.0% of the theoretical efficiency from glucose. In their study, Perez et al. [19] focused on the optimization of wheat hydrolysis using heat treatment. The efficiency of biodegradation of this material prior to its pretreatment was 13.0% of the theoretical glucose value. Fu and Mazza [20] in a study of pretreatment of triticale straw subjected the material to enzymatic hydrolysis after its purification using different concentrations of 1-ethyl-3-methylimidazolium acetate. The hydrolysis

efficiency was measured based on concentration of the obtained reducing sugars, depending on the amount of the ionic liquid used. The highest values (81%) were observed for the sample, where the addition of ionic liquid in the solution was 50%.

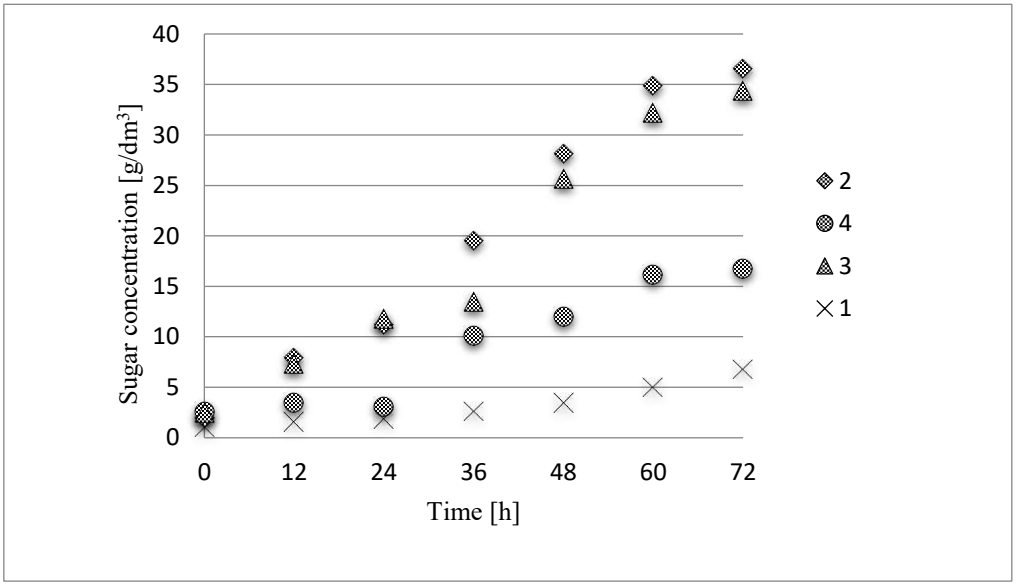


Figure 1. The concentration of sugars obtained after the enzymatic hydrolysis process of triticale straw: 1—control sample, without pretreatment; 2—after pretreatment with ionic liquid; 3—after chemical pretreatment (sulfate method); 4—after pretreatment using recycled ionic liquid.

In comparison, in the sample of straw not subjected to treatment to only 15.4% of the total amount of reducing sugars was obtained. The straw sample after pretreatment using 2% sulfuric acid (purification temperature: 160 °C, purification time: 20 min) added to the aqueous solution of the material was also subjected to enzymatic hydrolysis. The yield of reducing sugars in the sample was 47%. To obtain simple sugars for the production of bioethanol, Ang et al. [21] utilized rice husks. The highest concentration of reducing sugars—42.1%—was obtained in the sample with rice husks purified via the use of 1-ethyl-3-methylimidazolium acetate. 1-Ethyl-3-methylimidazolium acetate was also used for the pretreatment of barley straw by Sáez et al. [22]. These authors focused on the influence of time and temperature of pretreatment on the reducing sugar content measured after enzymatic hydrolysis. The highest glucose content was found in samples of barley straw pretreated with the ionic liquid at 110 °C. The glucose in those samples was 400 mg/100 g of biomass after 30 min and 600 mg/100 g of biomass after 60 min of the pretreatment. Liu and Chen [4] conducted a study using (1-butyl-3-methylimidazolium chloride) ionic liquid where they subjected wheat straw to treatment. These authors found that the pretreatment with ionic liquid influences the depolymerization of cellulose, thus increasing its susceptibility to enzymatic hydrolysis. The efficiency of enzymatic hydrolysis was highest (70.37%) in the wheat straw sample which was incubated with ionic liquid for 10 min. Li et al. [23] attempted saccharification of wheat straw, which was previously treated using 1-ethyl-3-methylimidazolium phosphate and 1-ethyl-3-methylimidazolium acetate. The reducing sugar content measured after 12 h hydrolysis of wheat straw samples, previously purified using 1-ethyl-3-methylimidazolium phosphate was 4.8 mg/cm^3, and in the sample of the material purified with 1-ethyl-3-methylimidazolium acetate the level of reducing sugars amounted to 3.2 mg/cm^3. As a result of the conducted experiments, a minimum of two fold higher values for the triticale straw after 12 h enzymatic hydrolysis were obtained. The pretreatment of lignocellulose raw materials using ionic liquid is an innovative solution, thus far not used at an industrial scale. The study results confirm the favorable effect of this catalyst on the yield of reducing sugars in the hydrolysis process in comparison to samples without pretreatment. The use of this type of treatment at an industrial scale

would only be possible if the efficiency of the method at least equaled the pretreatments. For these reasons an experiment which compared the yield of reducing sugars after enzymatic hydrolysis process of rye straw subjected to pretreatment with ionic liquid and using sulfate method (chemical pretreatment) was conducted. The content of reducing sugars measured after enzymatic hydrolysis in the sample of triticale straw purified using the sulfate method was 34.35 g/dm^3, and the sample of this material purified with ionic liquid contained 36.54 g/dm^3 reducing sugars. The mean values of the enzymatic hydrolysis products in these materials indicate that both methods of pretreatment have similar significance and both have a positive impact on the enhancement of the reducing sugar production process. However, pretreatment with ionic liquid is more favorable due to its lack of toxicity toward the environment. In the work of Chrzanowska et al. [24] the influence of imidazolium liquids on the efficiency of wastewater treatment and enzymatic activity of activated sludge microorganisms were examined. During the experiments it was observed that after a certain amount of time the activated sludge microorganisms adapted to the environment containing ionic liquid, and their composition did not differ significantly from the initial composition. Moreover, the rates of the nitrification processes and biochemical reactions were not reduced, which indicates the possibility of an efficient treatment of wastewater containing ionic liquids. The study published by Grabińska-Sota [25], also concerning the biodegradability of imidazolium ionic liquid confirm that the ionic liquid was subjected to quite considerable biological decomposition within the activated sludge, which most likely stemmed from the biodegradation of these compounds by microorganisms. The literature also contains studies where the lignocellulose material was subjected to pretreatment using sulfuric acid, sodium hydroxide or calcium hydroxide prior to enzymatic hydrolysis. Chen et al. [26] used 2% NaOH for the pretreatment of corn straw, which was incubated in the solution for 1 h at 80 °C. The use of such pretreatment allowed them to increase the content of reducing sugars determined after enzymatic hydrolysis as compared to the native material. The sugar content was 89.5 g/dm^3, whereas the glucose content in the sample was 56.7 g/dm^3, and the efficiency of hydrolysis was 83.3%. Sun and Cheng [27] performed enzymatic hydrolysis of rye straw and increased its susceptibility to saccharification via purification in a solution of H_2SO_4. The content of reducing sugars depended on the concentration of H_2SO_4 used to purify the rye straw. In the sample of rye straw purified with 1.5% H_2SO_4, the content of reducing sugars was 159.7 mg/g after 30 min and 197 mg/g after 90 min of pretreatment. In the sample of the material pretreated with 0.6 % H_2SO_4, the content of reducing sugars was 125 mg/g after 30 min and 136 mg/g after 90 min of pretreatment.

The main limitation of the use of ionic liquid at an industrial scale is its high price. The increasing interest of the industry in these solvents, their wide and easy use and lack of toxicity for the environment constitutes the basis for the statement that the price of ionic liquid may be reduced in the future [28]. Another method for the reduction of the cost of industrial use of ionic liquids is the development of an efficient method for their recycling and reuse in another process. Based on the above statements, a study was implemented concerning purification of the ionic liquid-1-ethyl-3-methylimidazolium acetate after the purification process of rye straw and its reuse for the pretreatment of a new portion of the lignocellulose material. The efficiency of rye straw pretreatment using recycled ionic liquid was evaluated comparing the yield of reducing sugars after enzymatic hydrolysis of the material. The majority of the cellulose fraction of the biomass is retrieved from the ionic liquid via addition of a so called non-solvent. Water can be such a non-solvent for imidazolium ionic liquids, which, by precipitating cellulose from the solution, creates one phase with the ionic liquid. The cellulose from such a mixture was removed by filtration or centrifugationn. The water remaining in the solution was removed via lyophilization. As a result of lyophilization, a yellow-brown ionic liquid was obtained, with a color similar to that of a pure ionic liquid. The mean content of reducing sugars obtained after enzymatic hydrolysis of rye straw purified with recycled ionic liquid was 16.77 g/dm^3, that is approx. 52% lower than the mean content of reducing sugars obtained through hydrolysis of rye straw purified with the pure ionic liquid. Xu et al. [29] subjected eucalyptus samples to purification and enzymatic hydrolysis. The purification was conducted using

pure and recycled ionic liquids (AMIMOAc and EMIMOAc). The ionic liquid recycling was conducted by vacuum drying. These authors demonstrated that both pretreatment with pure ionic liquid and recycled ionic liquid influences the change of the structure of cellulose in eucalyptus and improves the yield of reducing sugars after enzymatic hydrolysis. In order to attempt a classification of the types of treatments of lignocellulose materials in terms of obtaining the highest concentration of reducing sugars after enzymatic hydrolysis process, the obtained results were analyzed using the Principal Components Analysis (PCA) method. The active variables consisted in the reducing sugars concentration values determined at specific time intervals. The grouping variables were three types of pretreatment used prior to the hydrolysis process: treatment using ionic liquid, using recycled ionic liquid, sulfate method treatment and sample with the native material, i.e., not subjected to any pretreatment. Results of reducing sugars determined in three repetitions, for each time interval, were used for the PCA analysis conducted in the present study. The PCA analysis was conducted based on a correlation matrix. From the set of the analyzed data four factors were obtained with values >1, which characterized samples after pretreatment in the sense of their similarities and differences. They explained a total of 97.43% of the total variability, and 90.37 % of the variability could be explained by the single main component Z1. The second distinguished component Z2 explained 7.06%, and the Z3 component slightly over 1 %. Data dimension reduction was conducted based on a screening plot (Figure 2). The moment of graph flattening is visible after the second component, thus the subsequent analyses were conducted on this basis, referring solely to the influence of components Z1 and Z2.

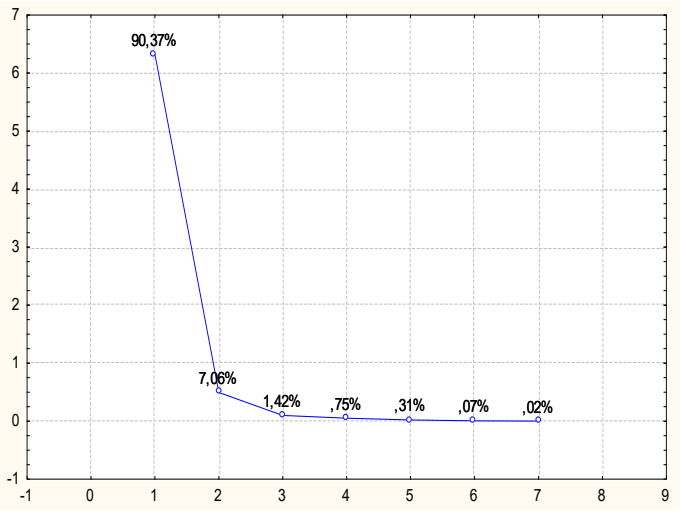


Figure 2. Screen for variables describing enzymatic hydrolysis process.

In order to investigate the influence of pretreatment of lignocellulose materials subjected to enzymatic hydrolysis for the content of reducing sugars, an observation chart (Figure 3) was drawn up, which presents the location of the grouped variables in the new dimensional space defined by the components Z1 and Z2 determined during the analysis. The resemblance measure during the analysis was the Euclidean distance. An analysis of the chart indicated the existence of three groups. First group was characterized by the triticale straw samples subjected to pretreatment using ionic liquid (CJ1-3) and samples of the same material treated with sulfate method (SO41-3). The second group consisted of triticale straw samples purified using recycled ionic liquid (CR1-3). Third group contains triticale straw samples subjected to enzymatic hydrolysis without pretreatment. Enzymatic hydrolysis of samples from the first group had the best efficiency due to the better availability of cellulose for the enzymes. Thus, the Euclidean distance of these samples from the O sample is highest (Figure 3).

The use of pretreatment with ionic liquid or sulfate method has a similar effect on the concentration of reducing sugars obtained after enzymatic hydrolysis. The Z1 component allows one to distinguish the type of pretreatment used and indicates the increase of reducing sugars in the given time intervals

of enzymatic hydrolysis. On the other hand, the Z2 component informs on the level of cellulose decomposition to simple sugars.

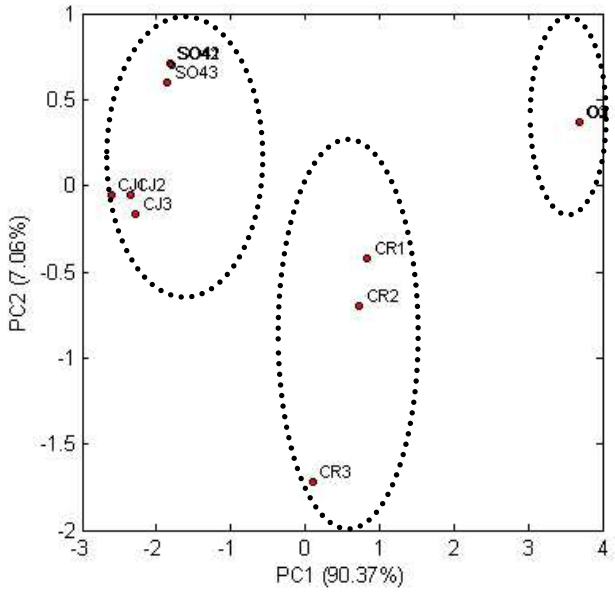


Figure 3. PCA scores plot of triticale straw pretreated with ionic liquid, recycled ionic liquid, sulfate method, untreated (control).

The results of the statistical tests corroborate the validity of using triticale straw pretreatment with ionic liquid and classify the type of used treatments and their influence on the formation reducing sugars in the process of enzymatic hydrolysis. Triticale straw pretreatment with ionic liquid has a significant impact on the reducing sugar content, and its increase is comparable to the increase of reducing sugars in the triticale straw sample purified using the sulfate method. In summary, triticale straw pretreatment has a significant impact on formation of reducing sugars by enzymatic hydrolysis. Weaker results are obtained in samples purified with ionic liquid after its dehydration.

3.2. Alcoholic Fermentation

Samples of triticale straw purified with ionic liquid and using the sulfate method and recycled ionic liquid were subjected to alcoholic fermentation. For comparison, a sample of triticale straw not subjected to pretreatment was also fermented (control sample).

The highest concentration of ethyl alcohol (10.64 g/dm^3) was demonstrated in the sample of triticale straw purified with ionic liquid (Figure 4). For comparison, the content of ethyl alcohol in the control sample was 1.60 g/dm^3. These differences result from the fact, that the hydrolysate of triticale straw purified with ionic liquid was characterized by a higher concentration of reducing sugars, as well as lower lignin content, which blocks the functioning of yeasts in mash.

The hydrolysate of triticale straw purified using the sulfate method, after fermentation was characterized by an ethanol content of 5.46 g/dm^3. Similar results were obtained for triticale straw samples subjected to pretreatment with recycled ionic liquid. The concentration of ethanol in this sample was 5.39 g/dm^3. The vitality of yeasts in both samples was lower than in the remaining hydrolysates (60% for sulfate method purified straw and 65% for recycled ionic liquid purified straw) (Table 1).

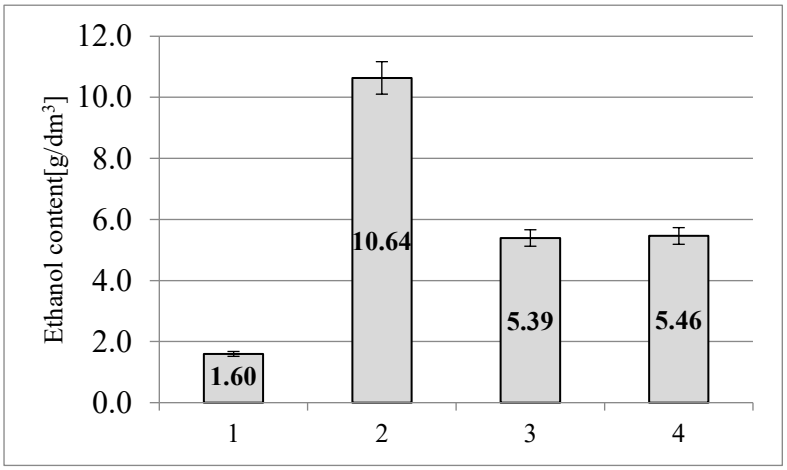


Figure 4. Concentration of ethyl alcohol after 96 h of alcoholic fermentation of triticale straw: 1—control sample; 2—pretreated with ionic liquid; 3—pretreated with white bleach (sulfate method); 4—pretreated with recycled ionic liquid.

Table 1. Count and vitality of *Saccharomyces cerevisiae* Ethanol Red yeast cells in the tested mashes.

Fermented medium	Count [cfu/mL^{-1}]	Vitality [%]
Triticale straw—control sample	4.8×10^5	40 ± 1.5
Triticale straw—ionic liquid treatment	1.2×10^8	96 ± 0.5
Triticale straw—sulfate method treatment	7.2×10^5	60 ± 0.7
Triticale straw—recycled ionic liquid treatment	7.8×10^5	65 ± 1.2

Analysis of the count and vitality of yeasts performing the fermentation process allows us to conclude that the release of inhibitors in the sulfate method had a negative influence on the growth and vitality of the microorganisms engaged in the fermentation process. Similar conclusions were drawn by Szymanowska et al. [30], who to produce bioethanol used potato pulp hydrolyzed using sulfuric acid and enzymes. The acidic hydrolysate contained 22 g/dm^3 reducing sugars, from which only 6 g/dm^3 ethanol was obtained as a result of fermentation. Furthermore, the authors determined that the cost of obtaining hydrolysates using amylases, cellulases and pectinases is not comparable to the efficiency of the process. The concentration of ethanol after fermentation of enzymatic hydrolysates was not higher than 2.5%, which, including the process costs such as separation and concentration of the final product becomes unprofitable. Perhaps the use of membrane distillation will contribute to reduction of the process costs in the future [30]. The literature contains an increasing number of solutions aiming at purification of the substrate prior to fermentation process. The most commonly described methods include detoxification via using bases, reducing agents and polymers. Worth mentioning is the fact that screening of microorganisms immune to environmental stresses, selection of the culture method, microbial treatment, evolutionary engineering and genetic engineering are also among methods, which aim at increase of the efficiency of the use of lignocellulose [31]. For comparison Saha et al. [32] fermented wheat straw using *Saccharomyces cerevisiae* yeast in two systems: SHF—separate hydrolysis and fermentation process SSF—simultaneous hydrolysis and fermentation process. The wheat straw was previously purified with sulfuric acid and enzymatically hydrolyzed to obtain reducing sugars. In addition, the authors subjected to fermentation a sample of wheat straw which apart from acid was also purified with lime. As a result of the conducted study it was determined that the ethanol content did not depend on the system of fermentation and only on the method of material purification. In the simultaneous hydrolysis and fermentation of the samples, which were subjected to pretreatment using sulfuric acid and enzymes, the ethanol content was 13 g/dm^3 and in the variant with additional lime purification 17 g/dm^3 ethanol was obtained.

The influence of pretreatment of lignocellulose material on the efficiency of alcoholic fermentation was also tested by Eisenhuber et al. [33], who conducted their study on an industrial scale. They obtained ethanol from different types of straw (wheat, rye and corn). Prior to fermentation, they used pretreatment with high temperature (160–200 °C) for 10 and 20 min. Alcoholic fermentation was conducted using Saccharomyces cerevisiae at 30 °C for 168 h. In the sample of rye straw subjected to pretreatment in temperature 200 °C for 10 min 108 kg of ethanol was obtained, and the efficiency of fermentation amounted to 44% of theoretical value. For rye straw pretreated at 200 °C for a longer period (20 min) the content of ethanol was 169 kg, and the efficiency of fermentation was at 70% of the theoretical value level. Kádár et al. [34] conducted simultaneous hydrolysis and fermentation of cellulose industrial waste (corrugated cardboard, cellulose sludge formed during paper production) originating from the Hungarian company Dunapack (Ujazd, Poland) and SOLKA FLOC 200 cellulose powder (International Fiber Corporation, New York, North Tonawanda, USA). In the samples fermented by S. cerevisiae, the ethanol content was 14.2 g/dm^3 for corrugated cardboard, 9.0 g/dm^3 for cellulose sludge and 16.6 g/dm^3 for SOLKA FLOC 200cellulose powder. In the present study, the ethanol content differed considerably. For these reasons, an attempt was made to demonstrate the relationship between ethanol concentration and the hydrolysate type subjected to alcoholic fermentation, for which the PCA method was used. The active variables consisted of the concentration of ethyl alcohol obtained after fermentation and the grouping variable was type of pretreatment of triticale straw, which influenced not only the reducing sugars concentration after enzymatic hydrolysis, but also the content of ethanol after fermentation. In this case, the PCA analysis was also conducted based on a correlation matrix. From the analyzed data two first coefficients with values of above >1 were generated, characterizing hydrolysate samples in terms of similarities and differences. In this case the main component Z1 explained as much as 96.95% of the total variability (Figure 5).

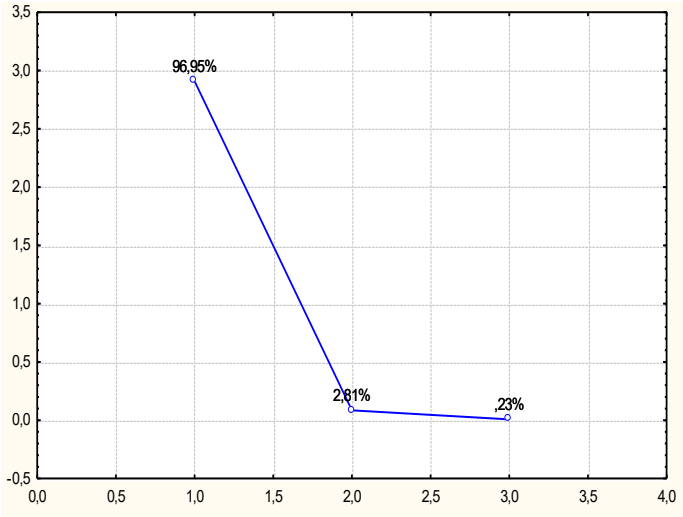


Figure 5. Screen for variables describing the changes in the concentration of ethyl alcohol after fermentation.

Figure 6 presents the influence of the used pretreatment of triticale straw on the ethanol concentration after fermentation. The location of grouping variables is presented in a new coordinate system, defined by the Z1 and Z2 components. The measure of resemblance in this statistical method is the Euclidean distance. The coefficient loads for the Z1 component indicated its relationship with the ethanol concentration values in the tested hydrolysates. Classification using the PCA method allowed re-establishing three groups of samples subjected to fermentation. The first group consisted of control samples of triticale straw not subjected to pretreatment, which gave the lowest ethanol concentration. The second group consisted of triticale straw samples purified with recycled ionic liquid (CR) and

samples of triticale straw purified using the sulfate method (SO_4). The third group— triticale straw samples purified with ionic liquid (C)—were the samples with the highest ethanol content.

The most extreme location on the observation chart is taken by the samples marked as '0′ and 'CJ'. This indicates the greatest differences between ethanol concentrations in these samples, which is linked to the use of pretreatment with ionic liquid. The highest increase of ethanol was observed for triticale straw subjected to ionic liquid treatment, the Euclidean distances of the remaining samples in relation to the control sample of triticale straw (0) are similar.

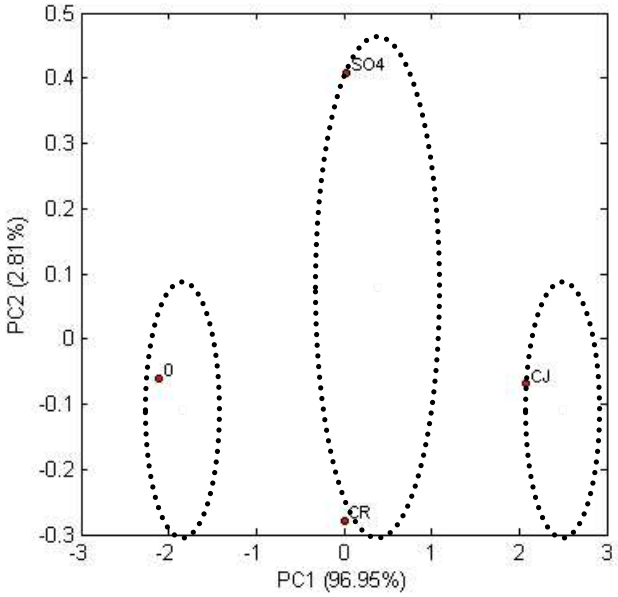


Figure 6. PCA scores plot of triticale straw pretreatment by ionic liquid, recycled ionic liquid or sulfate method, untreated (control).

Thus, the ethanol concentration in these samples has a similar level and it is strictly related to the content of reducing sugars in the mashes prior alcoholic fermentation. The PCA analysis results demonstrated that the content of reducing sugars in the samples of triticale straw is influenced by the use of ionic liquid for the pretreatment of these materials. The results of ethanol concentration in mashes are also highest for the samples purified with pure ionic liquid than in the samples, where white bleach is used for the treatment (sulfate method).

4. Conclusions

The highest concentration of reducing sugars was obtained through enzymatic hydrolysis of triticale straw purified with ionic liquid. The efficiency of enzymatic hydrolysis of triticale straw after pretreatment with ionic liquid and sulfate method was similar. Ionic liquid recycling after pretreatment of rye straw using lyophilization allowed to reuse this solvent to purify rye straw, yet the efficiency of this method remained at a low level. The concentrations of ethanol in mashes were higher in the samples of triticale straw purified with ionic liquid, in comparison to the samples of these materials pretreated using the sulfate method or recycled ionic liquid. The use of ionic liquid-1-ethyl-3-methylimidazolium acetate enhanced the yield of ethanol from triticale straw from 1.60 g/dm^3 after processing without pre-treatment to 10.64 g/dm^3 after pre-treatment.

Author Contributions: Conceptualization, Methodology, Results and formal analysis and Writing—Original Draft Preparation M.S.-K., D.S.-P., J.C.-P., B.W., J.K.-C., M.W., J.W.; Results analysis, software and plotting figures of the obtained analytical and numerical B.W., M.S.-K.; Writing Review and Editing J.C.-P., D.S.-P.; All authors read and approved the final manuscript.

Funding: This research was funded by National Science Center grant number DEC- 2017/01/X/NZ9/00357.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jönsson, L.J.; Martín, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresour. Technol.* **2016**, *199*, 103–112. [CrossRef]
2. Naik, S.N.; Goud, V.V.; Rout, P.K.; Dalai, A.K. Production of first and second generation biofuels: A comprehensive review. *Renew. Sustain. Energy. Rev.* **2010**, *14*, 578–597. [CrossRef]
3. Chandel, A.K.; Kapoor, R.K.; Singh, A.; Kuhad, R.C. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. *Bioresour. Technol.* **2007**, *98*, 1947–1950. [CrossRef]
4. Liu, L.; Chen, H. Enzymatic hydrolysis of cellulose materials treated with ionic liquid [BMIM] Cl. *Chin. Sci. Bull.* **2006**, *51*, 2432–2436. [CrossRef]
5. Berlin, A.; Balakshin, M.; Gilkes, N.; Kadla, J.; Maximenko, V.; Kubo, S.; Saddler, J. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. *J. Biotechnol.* **2006**, *125*, 198–209. [CrossRef] [PubMed]
6. Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y.Y.; Holtzapple, M.; Ladisch, M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2005**, *96*, 673–686. [CrossRef] [PubMed]
7. Plechkova, N.V.; Seddon, K.R. Applications of ionic liquids in the chemical industry. *Chem. Soc. Rev.* **2008**, *37*, 123–150. [CrossRef] [PubMed]
8. Pinkert, A.; Marsh, K.N.; Pang, S.; Staiger, M.P. Ionic liquids and their interaction with cellulose. *Chem. Rev.* **2009**, *109*, 6712–6728. [CrossRef]
9. Zhu, S.; Wu, Y.; Chen, Q.; Yu, Z.; Wang, C.; Jin, S.; Ding, Y.; Wu, G. Dissolution of cellulose with ionic liquids and its application: A mini-review. *Green Chem.* **2006**, *8*, 325–327. [CrossRef]
10. Endres, F.; El Abedin, S.Z. Air and water stable ionic liquids in physical chemistry. *Phys. Chem. Chem. Phys.* **2006**, *8*, 2101–2116. [CrossRef]
11. Huddleston, J.G.; Visser, A.E.; Reichert, W.M.; Willauer, H.D.; Broker, G.A.; Rogers, R.D. Characterization and comparison of hydrophilic and hydrophobic room temperature ionic liquids incorporating the imidazolium cation. *Green Chem.* **2001**, *3*, 156–164. [CrossRef]
12. Mäki-Arvela, P.; Anugwom, I.; Virtanen, P.; Sjöholm, R.; Mikkola, J.P. Dissolution of lignocellulosic materials and its constituents using ionic liquids-A review. *Ind. Crops. Prod.* **2010**, *32*, 175–201. [CrossRef]
13. Kolodziej, B.M.M. *The Renewable Energy Sources. Agricultural Energy Resources*; Universal Agricultural and Forest Publishing: Poznan, Poland, 2012.
14. Sims, R.E.H.; Mabee, W.; Saddler, J.N.; Taylor, M. An overview of second generation biofuel technologies. *Bioresour. Technol.* **2010**, *101*, 1570–1580. [CrossRef]
15. Hendriks, A.T.W.M.; Zeeman, G. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* **2009**, *100*, 10–18. [CrossRef]
16. Zhang, Y.H.P. Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 367–375. [CrossRef]
17. Ghose, T.K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, *59*, 257–268. [CrossRef]
18. Świątek, M.; Lewandowska, M.B.W. The importance of selecting the method of pre-treatment of lignocellulosic substrates, taking into account the efficiency of bioethanol production. *Adv. Agri. Sci.* **2011**, *63*, 109–119.
19. Pérez, J.A.; Ballesteros, I.; Ballesteros, M.; Sáez, F.; Negro, M.J.; Manzanares, P. Optimizing Liquid Hot Water pretreatment conditions to enhance sugar recovery from wheat straw for fuel ethanol production. *Fuel* **2008**, *87*, 3640–3647. [CrossRef]
20. Fu, D.; Mazza, G. Aqueous ionic liquid pretreatment of straw. *Bioresour. Technol.* **2011**, *102*, 7008–7011. [CrossRef]
21. Ang, T.; Ngoh, G.; Chua, A.S.; Lee, M. Elucidation of the effect of ionic liquid pretreatment on rice husk via structural analyses. *Biotechnol. Biofuels* **2012**, *5*, 67. [CrossRef]
22. Sáez, F.; Ballesteros, M.; Ballesteros, I.; Manzanares, P.; Oliva, J.M.; Negro, M.J. Enzymatic hydrolysis from carbohydrates of barley straw pretreated by ionic liquids. *J. Chem. Technol. Biotechnol.* **2013**, *88*, 937–941. [CrossRef]

23. Li, Q.; He, Y.C.; Xian, M.; Jun, G.; Xu, X.; Yang, J.M.; Li, L.Z. Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment. *Bioresour. Technol.* **2009**, *100*, 3570–3575. [CrossRef]
24. Chrzanowska, M.; Łuczak, J.H.J. Purification of wastewater containing ionic liquid with activated sludge. *Ecol. Eng.* **2006**, *14*, 70–75.
25. Grabińska-Sota, E. The influence of quaternary ammonium salts on the operation of wastewater treatment plants. *Chem. Ind.* **2010**, *89*, 1437–1439.
26. Chen, M.; Zhao, J.; Xia, L. Comparison of four different chemical pretreatments of corn stover for enhancing enzymatic digestibility. *Biomass Bioenergy* **2009**, *33*, 1381–1385. [CrossRef]
27. Sun, Y.; Cheng, J.J. Dilute acid pretreatment of rye straw and bermudagrass for ethanol production. *Bioresour. Technol.* **2005**, *96*, 1599–1606. [CrossRef] [PubMed]
28. Khodadoust, A.P.; Yachandrasekaran, S.; Dionysiou, D.D. Preliminary assessment of imidazolium-based room-temperature ionic liquids for extraction of organic contaminants from soils. *Environ. Sci. Technol.* **2006**, *40*, 2339–2345. [CrossRef]
29. Xu, J.; Bingchuan, L.; Hou, H.; Hu, J. Pretreatment of eucalyptus with recycled ionic liquids for low-cost biorefinery. *Bioresour. Technol.* **2017**, *234*, 406–414. [CrossRef]
30. Szymanowska, D.; Dróżdżyńska, A.; Lewandowicz, G. The potato pulp as a raw material for bioethanol production. *Lett. Sci. Univ. Econ. Poz.* **2011**, *206*, 234–242.
31. Lewandowicz, G.; Białas, W.; Marczewski, B.; Szymanowska, D. Application of membrane distillation for ethanol recovery during fuel ethanol production. *J. Memb. Sci.* **2011**, *375*, 212–219. [CrossRef]
32. Saha, B.C.; Iten, L.B.; Cotta, M.A.; Wu, Y.V. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochem.* **2005**, *40*, 3693–3700. [CrossRef]
33. Eisenhuber, K.; Jäger, A.; Wimberger, J.; Kahr, H. Comparison of different pretreatment methods for straw for lignocellulosic bioethanol production. *Agron. Res.* **2013**, *11*, 173–182.
34. Kádár, Z.; Szengyel, Z.; Réczey, K. Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Ind. Crops. Prod.* **2004**, *20*, 103–110. [CrossRef]

Received: 20 December 2019 | Revised: 16 April 2020 | Accepted: 17 April 2020

DOI: 10.1002/ese3.726

RESEARCH ARTICLE

Energy Science & Engineering Open Access

Evaluation of the potential of fireweed (*Epilobium angustifolium* L.), European goldenrod (*Solidago virgaurea* L.), and common broom (*Cytisus scoparius* L.) stems in bioethanol production

Małgorzata Smuga-Kogut[1] | **Daria Szymanowska**[2] | **Roksana Markiewicz**[3] | **Tomasz Piskier**[1] | **Joanna Kobus-Cisowska**[4] | **Judyta Cielecka-Piontek**[5] | **Heralt Schöne**[6]

[1]Department of Agrobiotechnology, Faculty of Mechanical Engineering, Koszalin University of Technology, Koszalin, Poland

[2]Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Poznan, Poland

[3]NanoBioMedical Centre, Adam Mickiewicz University in Poznań, Poznań, Poland

[4]Department of Gastronomical Sciences and Functional Foods, Poznan University of Life Sciences, Poznan, Poland

[5]Department of Pharmacognosy, Poznan University of Medical Sciences, Poznań, Poland

[6]Department of Agriculture and Food Science, University of Applied Sciences, Neubrandenburg, Germany

Correspondence
Roksana Markiewicz, NanoBioMedical Centre, Adam Mickiewicz University in Poznań, Wszechnicy Piastowskiej 3, PL-61614 Poznań, Poland.
Email: roksana.markiewicz@amu.edu.pl

Funding information
Narodowe Centrum Nauki, Grant/Award Number: MINIATURA 1, DEC-2017/01/X/NZ9/00357

Abstract

One of the main goals of industrial biotechnology is to develop an effective method for ethanol production for fuel purposes using lignocellulosic biomass. Variability of lignocellulosic raw materials, selection of an effective method for the pretreatment of raw material, and selection of microorganisms with the ability to ferment not only hexoses but also pentoses and are moreover resistant to environmental stress generated by the products of lignocellulosic complex decomposition, are the challenges encountered in ethanol production. The use of agricultural wastelands and overgrowing plants that have little possibility of application in processes other than energy production seem to be an interesting alternative to conventional, but very often rather cultivation demanding energy crops. The aim of this study was to evaluate the possibility of using the stems of fireweed (*Epilobium angustifolium* L.), European goldenrod (*Solidago virgaurea* L.), and common broom (*Cytisus scoparius* L.) for ethanol production. The key elements studied were characteristics of the lignocellulosic complex structure, influence of the selected ionic liquids on the structural changes in biomass, and efficiency of enzymatic hydrolysis and ethanol fermentation processes. The results showed that under the assumed conditions the best effect was observed with the fireweed materials subjected to pretreatment with 1-ethyl-3-methylimidazolium acetate and enzymatic hydrolysis with Viscozyme® preparation. The final concentration of ethanol obtained was 2.509 g L^{-1} with a yield of 92.3%. This was due to the highest share of cellulose (40.9%) in the whole lignocellulosic complex compared to other raw materials, which in combination with the selection of an appropriate ionic liquid and an enzymatic preparation, led to high bioprocess efficiency.

KEYWORDS

bioethanol, enzymatic hydrolysis, ionic liquids, plant stems

1 | INTRODUCTION

The production of bioenergy and the use of renewable raw materials in farms are among the most important objectives of the European Commission. In practice, these are met by artificially established energy crops on areas previously used for food production or the ones which are degraded or unused. Contrary to popular belief, starting of the cultivation of any energy crops often requires large financial outlays, including the purchase of good-quality seedlings and plant-protective products (especially during the first year of cultivation),which involve high costs in addition to those resulting from the necessity to fertilize plantations.[1,2] Moreover, important prerequisites for such a cultivation are accurate and reliable estimates from fast-growing plantations regarding both current and potential yield of biomass, profitability, climate etc[3] An alternative to plantations exhibiting a targeted and strictly planned cultivation is the plants growing on set-aside land. Biomass obtained from an uncultivated land often consists of a mixture of grassland and woody plants growing in marginal areas for more than 5 years. The botanical composition of such biomass depends mainly on the geographical location and the time of land exclusion from agricultural production. In Poland, agricultural wastelands occupy about 10% of the total agricultural area. In addition, the country also has many green areas such as baulks, forest clearings, and fallow land overgrown with perennial vegetation and a mixture of grasses. Among the perennial plants, fireweed (*Epilobium angustifolium* L.) and European goldenrod (*Solidago virgaurea* L.) can be distinguished. Moreover, from the family of shrub plants one can distinguish common broom shrubs (*Cytisus scoparius* L.).A common feature of all three plants is the structure and composition of their stems, which contain about 20% lignin, 40% cellulose, and 25% hemicellulose. All three species require very modest soil quality and therefore can be an interesting source of biomass. Moreover, these plants behave like pioneer species and grow very well on the recultivation lands, such as coal combustion waste deposits or postmining soils (eg after sulfur exploitation),[4,5] and are suitable for the protection of set-aside land. In addition, goldenrod and firewood are melliferous species, what expands their usage in biorefineries.[5] Usually, they form compact floristic groups or clusters, which together with their large size (over 1 m) favors their application as an attractive biomass source. Furthermore, considering the fact that 5%-8% of worldwide lignocellulose production per year would be sufficient to meet the annual demand of fossil oil, the use of common weeds for energy purposes seems justified.[6] However, it should be noted that the production of bioethanol from lignocellulose is a multistage process, which success depends on the effective delignification of the material and change of the cellulose structure from crystalline to amorphous, as well as on efficient enzymatic hydrolysis and alcoholic fermentation. One of the methods of lignocellulose pretreatment is the use of imidazolium ionic liquids, the purpose of which is to dissolve cellulose fibers and facilitate effective enzymatic hydrolysis *via* increasing of the porosity of the material and dissolution of lignin. The interest in using ionic liquids and enzymatic hydrolysis for the production of bioethanol is growing rapidly mainly due to the benefits of this method.[7] Some of the known already ionic liquids may be a great alternative to conventional lignin and cellulose solvents such as sulfuric acid and sodium hydroxide.[8] Ionic liquids dissolve cellulose, change its structure by increasing the number and size of pores between fibers, and improve the efficiency of cellulolytic enzymes. They can also be recycled after the process and reused. Several methods for recovery of ionic liquids including distillation, extraction, adsorption, membrane separation, aqueous two-phase extraction, crystallization, and external force field separation are considered to be most valuable when it comes to IL solutions. The methods used for their recovery are distillation, adsorption, and membrane separation.[9]

In addition to the typical energy-related aspects of use, the importance of high-yielding crops and rural development should be mentioned, including the benefits of using areas not exploited as farmlands due to their poor quality. In this view, the aim of this study was to evaluate the possibility of using the stems of fireweed, European goldenrod, and common broom in the production of second-generation ethanol.

2 | EXPERIMENTAL

2.1 | Materials

Fireweed (*E angustifolium* L.), European goldenrod (*S virgaurea* L.), common broom (*C scoparius* L.) used in the study were obtained from agricultural wastelands located in Zachodniopomorskie Voivodeship in Poland. The period of land exclusion from agricultural production was in the range from 5 to 15 years. The obtained biomass consisted of aboveground parts of the plants, which were harvested in September 2017 and ground and dried to a water content below 5%.

The stems were pretreated with two imidazolium ionic liquids: 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]; purum 95%, Iolitec) and 1-butyl-3-methylimidazolium acetate ([BMIM][OAc]; purum 98%, Iolitec). Simultaneously, studies were carried out on the use of biomass in its native form to compare the course of the process. The following cellulolytic preparations were used for enzymatic hydrolysis: cellulase from *Aspergillus* sp ($\geq$1000 units g^{-1}), cellulase from *Trichoderma reesei* ($\geq$700 units g^{-1}), Viscozyme®

(13.4 FBG (fungal beta-glucanase unit) mL^{-1}), and Cellic® CTec2 (115.6 FPU (filter paper unit) mL^{-1}) (Merck).

2.2 | Methods

2.2.1 | Pretreatment with ionic liquids

Each biomass sample was purified with two imidazolium ionic liquids: [EMIM][OAc] and [BMIM][OAc]. For this purpose, 5 g of ground material was mixed with 50 mL of the ionic liquid. The samples were then homogenized for 2 minutes and incubated at 120°C for 2 hours. After the incubation, the samples were cooled to room temperature and then the cellulose fibers were separated with deionized water, through rinsing the sample with water at least three times until the ionic liquid was removed. The solid fraction obtained was resuspended in 100 mL of 50 mmol/L acetate buffer (pH 5.0) and then subjected to enzymatic hydrolysis.

2.2.2 | Enzymatic hydrolysis and alcoholic fermentation

Four enzymatic preparations (cellulase from *Aspergillus* sp, cellulase from *T reesei*, Cellic® CTec2, and Viscozyme® (Sigma-Merck)) were used for enzymatic hydrolysis at an amount of 20 FPU g^{-1} of cellulose in the material. Biomass fractions mixed with *Aspergillus* sp cellulase and *T reesei* cellulase were incubated at 47°C for 72 hours, while those treated with Cellic®CTec2 and Viscozyme® were incubated at 50°C for 72 hours.

Hydrolysate solutions (50 mL), after filtration in order to remove any lignocellulose residues, were subjected to alcoholic fermentation. The pH of the fermentation broth was measured at each sampling point and adjusted to 5.0 by adding either 10 wt.% H_2SO_4 or 20 wt.% NaOH. Ethanol fermentation was initiated by adding freeze-dried distiller's yeast *Saccharomyces cerevisiae* type II (Sigma-Aldrich) (5%, w/v).The samples were placed in fermentation flasks (volume of 100 cm^3) in a 100 rpm shaking incubator. The fermentation process took 96 hours at the temperature of 37°C in anaerobic conditions. Samples of a volume of 2 cm^3 were taken for HPLC analysis, and 70 cm^3 for distillation and pycnometric measurements.

2.2.3 | Analysis of raw materials structure

The content of lignin, cellulose, and hemicellulose was determined in the collected biomass by using filter bags and the AnkomA200 apparatus. The content of neutral detergent fiber (NDF) was determined using the Van Soest method, while that of acidic detergent fiber (ADF) and acidic detergent lignin (ADL) was measured according to the standard. The content of cellulose was determined based on the difference between the shares of ADF and ADL fractions, whereas the content of hemicellulose was determined from the difference between the shares of NDF and ADF fractions.

Changes in the crystalline structure of the raw material were evaluated by analyzing the obtained scanning electron microscopy (SEM) images. The morphology of cellulose fibers in the samples before and after pretreatment with ionic liquids was observed using a scanning electron microscope Quanta 200 Mark II produced by FEI Company. All images were taken at the magnification of 500×, at the acceleration voltage 25 kV. Prior to placing the sample in a high vacuum environment, they were dried at elevated temperatures and placed on a conductive foil.

2.2.4 | High-performance liquid chromatography

The content of glucose and ethanol was determined by high-performance liquid chromatography (HPLC). For chemical analysis, the samples were first centrifuged at 4000 *g* for 10 minutes at 4°C (Multifuge 3SR), filtered through a 0.22-μm membrane filter (Millex-GS, Millipore), and then analyzed on an HPLC system (Merck Hitachi). The fractions of glucose, ethanol, acetic acid, lactic acid, and glycerol were separated using an Aminex HPX-87P system (Bio-*Rad*) at 30°C using a 5 mmol/L H_2SO_4 solution as the mobile phase at a flow rate of 0.6 mL min^{-1}, and then detected with a refractive index detector (Model L-7490, Merck Hitachi).

2.2.5 | Efficiency of enzymatic hydrolysis and ethanol production

The lignocellulose to ethanol conversion rate (%) was calculated according to the formula[10]:

$$Y = \frac{C_e \times V \times 100}{M \times C \times 1.1 \times 0.51} \times 100\ (\%);$$

where C_e – ethanol concentration (g L^{-1}); V – sample volume (L); M – total amount of substrate in the sample (g s.s.); C – cellulose and hemicellulose concentration in the material (%); 1.1 – cellulose to glucose conversion factor; 0.51 – glucose to ethanol conversion factor.

3 | RESULTS

3.1 | Chemical characteristics of raw materials before and after the treatment with ionic liquids

In the first stage of this work, three raw materials selected for the study were evaluated for the content of cellulose, hemicellulose, and lignin, which are the key elements of the lignocellulosic complex. The analysis of the composition showed that the content of cellulose was similar in common broom and European goldenrod and was equal to 30.7% and 30.4%, respectively. The highest amount of cellulose was found in fireweed, constituting 41.0% of the lignocellulosic complex. Hemicellulose content was lower amounting to 21.9% in fireweed, 28.8% in common broom, and 29.2% in European goldenrod. Lignin, which is not a source of fermenting sugars but acts rather as a specific binder of cellulose and hemicellulose, constituted a large share in the studied raw materials—20.4% in fireweed, 16.0% in common broom, and 19.2% in European goldenrod.

The raw materials were subjected to pretreatment with ionic liquids [BMIM][OAc] and [EMIM][OAc], which resulted in a change in the proportion of individual components of the lignocellulosic complex. This change was more obvious in the variant in which [EMIM][OAc] was applied. In the samples of fireweed, the content of lignin was reduced from 20.4% to 17.9%, and in European goldenrod, the content reduced from 19.2% to 12.8%. There were also differences observed in the content of hemicellulose after pretreatment with ionic liquids with a decrease by about 2% on average. On the other hand, the percentage of cellulose increased by as much as 10% in the samples of fireweed and European goldenrod after pretreatment. To determine the changes in the structure of the examined raw materials, their images were taken with a scanning electron microscope (Figures 1-3). It was observed that in each plant material, after the application of imidazoliumionic liquids, the fibers were untangled and the structural integrity of the material was permanently lost. On comparing the plant materials with each other, it was found that both European goldenrod and fireweed were more susceptible to ionic liquids than common broom.

3.2 | Enzymatic hydrolysis

The next stage of the study involved enzymatic hydrolysis for the release of fermenting sugars. The effectiveness of enzymatic hydrolysis carried out using the four commercial enzyme preparations was verified in the study. The raw materials from all three species were subjected to enzymatic hydrolysis. Three experimental variants were prepared— raw material not processed with ionic liquids, raw material pretreated with BMIM Ac, and raw material pretreated with [EMIM][OAc]. All the prepared variants were saccharified using Cellic® CTec2, Viscozyme®, *T reesei* cellulase, and *Aspergillus* sp cellulase.

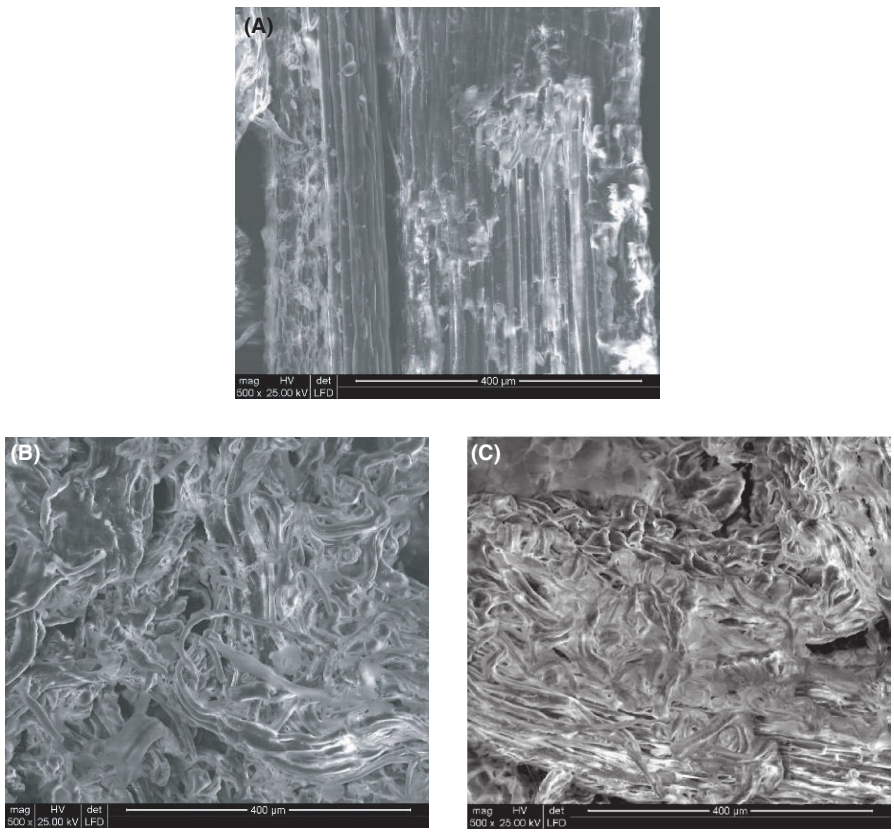
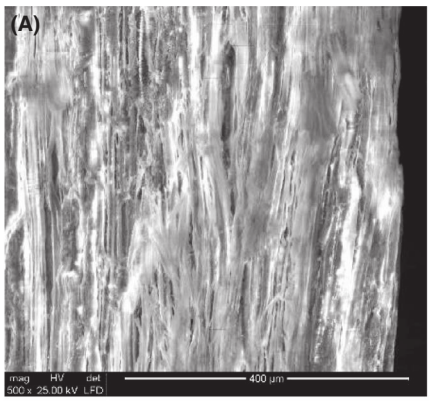
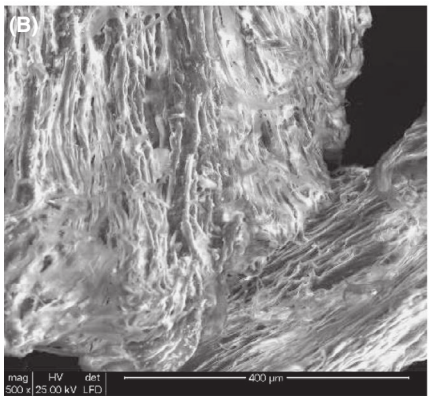


FIGURE 1 SEM images of a European goldenrod structure before and after the treatment with ionic liquids (magnification 500×, scale bar 400 µm). (A) Untreated, (B) pretreatment with [BMIM][OAc], and (C) pretreatment with [EMIM][OAc]





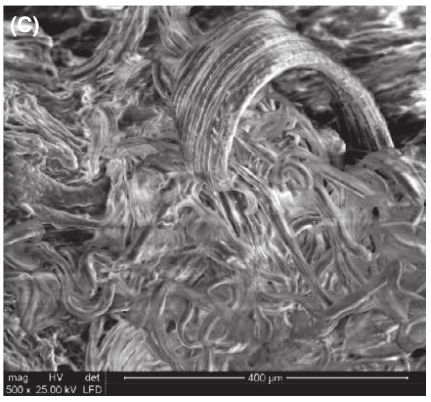
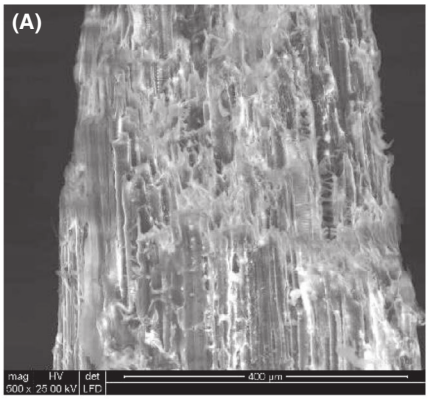
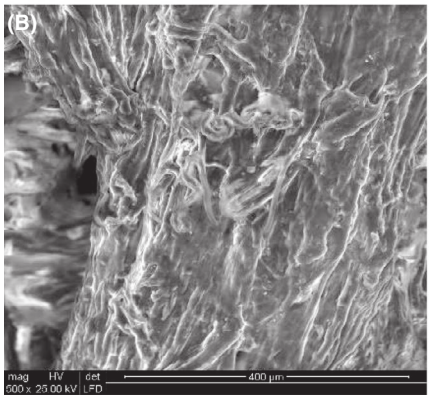


FIGURE 2 SEM images of a fireweed structure before and after the treatment with ionic liquids (magnification 500×, scale bar 400 µm). (A) Untreated, (B) pretreatment with [BMIM][OAc], and (C) pretreatment with [EMIM][OAc]





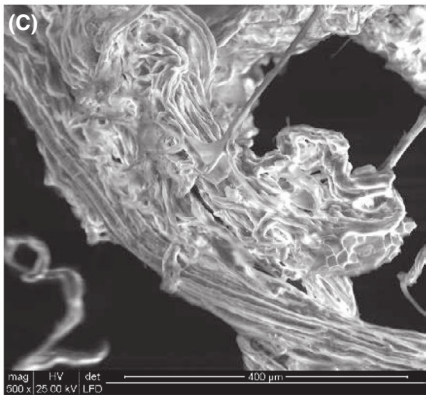


FIGURE 3 SEM images of a common broom structure before and after the treatment with ionic liquids (magnification 500×, scale bar 400 µm). (A) Untreated, (B) pretreatment with [BMIM][OAc], and (C) pretreatment with [EMIM][OAc]

The process was carried out for 72 hours at temperatures and pH that were appropriate for the enzymes, following which the glucose content was determined (Figure 4).

Based on the results observed after enzymatic hydrolysis, it can be concluded that the glucose content was more influenced by the type of ionic liquid used for pretreatment

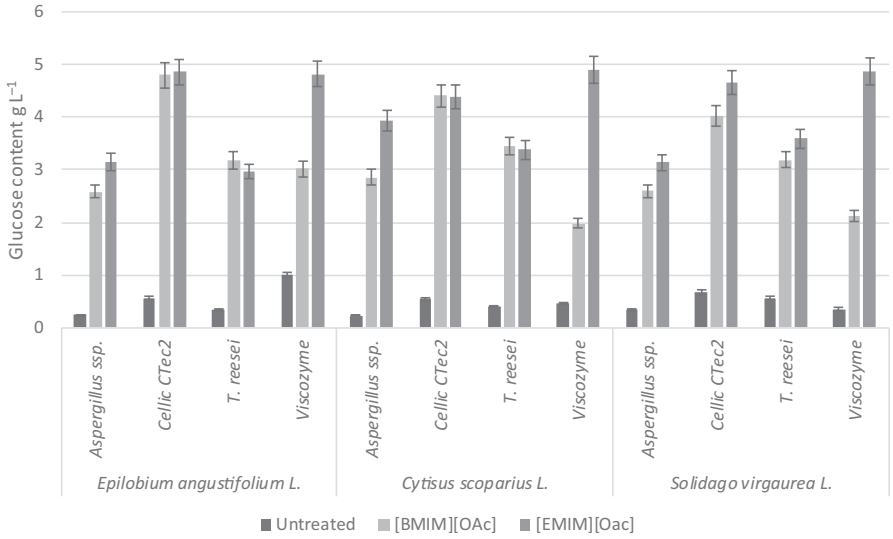


FIGURE 4 Glucose concentration after enzymatic hydrolysis

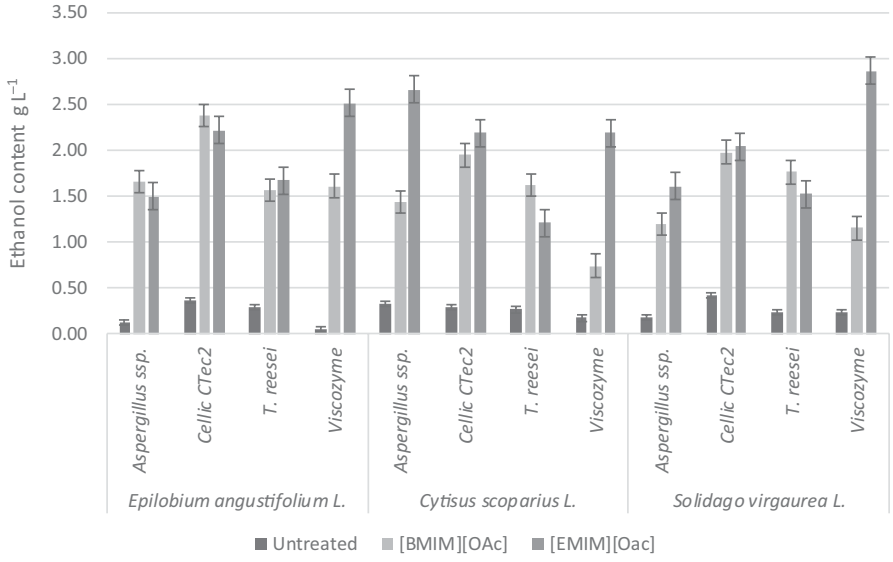


FIGURE 5 Ethanol concentration after fermentation with *Saccharomyces cerevisiae* yeast

and as the type of enzymatic preparation in comparison to the species of the plant. The best results were obtained in the experiment in which the pretreatment was carried out with [EMIM][OAc] and enzymatic hydrolysis with Viscozyme® preparation. The final glucose concentration observed in the hydrolysates of fireweed, common broom, and European goldenrod of this variant was 4.82, 4.9 g L^{-1}, and 5.13 g L^{-1}, respectively. Significant differences were also noted in the effectiveness of saccharification by individual enzymatic preparations (Figure 4). The cellulase from *T reesei* was found as the least effective enzyme. The glucose concentration in the hydrolysate of European goldenrod after enzymatic hydrolysis did not exceed 3.5 g L^{-1}. A significant interaction between the effectiveness of the enzymatic preparation and the applied pretreatment was also observed. The glucose concentration in the samples of goldenrod, fireweed, and broom treated with [BMIM][OAc] was higher than that of the samples purified with [EMIM][OAc] (Figure 4).

A comparable glucose concentration of about 4.5 g L^{-1} was noted in the samples of European goldenrod and common broom hydrolyzed using Cellic® CTec2 preparation, with no visible difference resulting from the type of ionic liquid used. The study showed that pretreatment with ionic liquid was crucial for the effective hydrolysis of the raw materials studied. In the experimental variants in which the native material was hydrolyzed using Cellic® CTec2, the glucose concentration in the hydrolysates of fireweed, common broom, and European goldenrod after 72 hours of hydrolysis was 0.555, 0.541, and 0.671 g L^{-1}, respectively. After pretreatment with [EMIM][OAc] or [BMIM][OAc], a significantly higher concentration of glucose was observed in comparison to no pretreatment, with an increase on average by 4 g L^{-1}.

The highest hydrolysis efficiency was observed in the samples in which the Cellic® CTec2 enzymatic preparation was applied and in the material treated with Viscozyme®. The effectiveness of cellulase from *Aspergillus* sp and

Viscozyme® depended on the ionic liquid used for pretreatment. With respect to these enzyme preparations, higher efficiency of hydrolysis was observed in the samples of biomass purified with [EMIM][OAc].

3.3 | Ethanol fermentation of lignocellulosic hydrolysates

The last stage of the work involved the assessment of the influence of pretreatment with ionic liquid and enzymatic hydrolysis on ethanol concentration after fermentation. The results showed that the fermentation efficiency was directly related to the success of pretreatment and enzymatic hydrolysis, as presented at the Figure 5. Alcoholic fermentation was carried out under conditions suitable for microorganisms, using the same procedure for all the hydrolysates. This allowed comparing the efficiency of ethanol production depending on the type of raw material and the ionic liquid used for pretreatment. The concentration of ethanol after 96 hours of fermentation was the highest in the samples of materials that were purified with [EMIM][OAc] and treated with Viscozyme® (European goldenrod) and *Aspergillus* sp (common broom) for enzymatic hydrolysis—which was equal to 2.86 and 2.65 g L^{-1}, respectively. In the case of fireweed, the highest concentration of ethanol (2.51 g L^{-1}) was obtained in the sample purified with [EMIM][OAc] and hydrolyzed with Viscozyme®. The Viscozyme® preparation contains thermostable xylanases, so it can be concluded that in the material hydrolyzed with this enzyme, the content of monosaccharides was higher which originated from the decomposition of cellulose and hemicellulose. Only a smaller difference was observed in ethanol content between the samples purified with [BMIM][OAc] and [EMIM][OAc] before treatment with Cellic® CTec2. However, in the remaining samples, where other enzymes were used for hydrolysis, the influence of the type of ionic liquid used was clearly observed.

Figure 6 presents the efficiency of the ethanol fermentation process depending on the ionic liquid used for pretreatment and the enzymatic preparation used for hydrolysis. The efficiency of the ethanol fermentation process ranged from 1.5% as observed in the native fireweed material hydrolyzed with Viscozyme® to 81.4% as observed in the European goldenrod material pretreated with [EMIM][OAc] and hydrolyzed with Viscozyme® preparation. It should be noted that, similar to enzymatic hydrolysis, the pretreatment of raw materials with [EMIM][OAc] was of importance in ethanol fermentation.

4 | DISCUSSION

The production of second-generation biofuels has been a challenge for biotechnologists, chemists, botanists, physicists, and gardeners for many years. The structural diversity of the raw materials and the difficulty involved in the hydrolysis of lignocellulosic complex make the production of second-generation bioethanol unprofitable. In addition, the biomass preparation for the fermentation process is complex and involves multiple stage action. Therefore, any attempts to reduce the costs are important to reach competitive production costs. Taking all of this into account, the selection of an effective and safe method for biomass pretreatment, selection, and optimization of the dose of enzymatic preparations, isolation, and screening of microorganisms resistant to toxic products of biomass decomposition, along with the selection of plants with high energy potential and that are not used for other purposes are considered as the most important objectives in the current research.[11,12]

In our study, we especially focused on the last objective, that is, the selection and evaluation of the usefulness of common weeds (fireweed, European goldenrod, and common broom). All three species are high-yielding perennial and shrubby plants, which grow in marginal areas and do

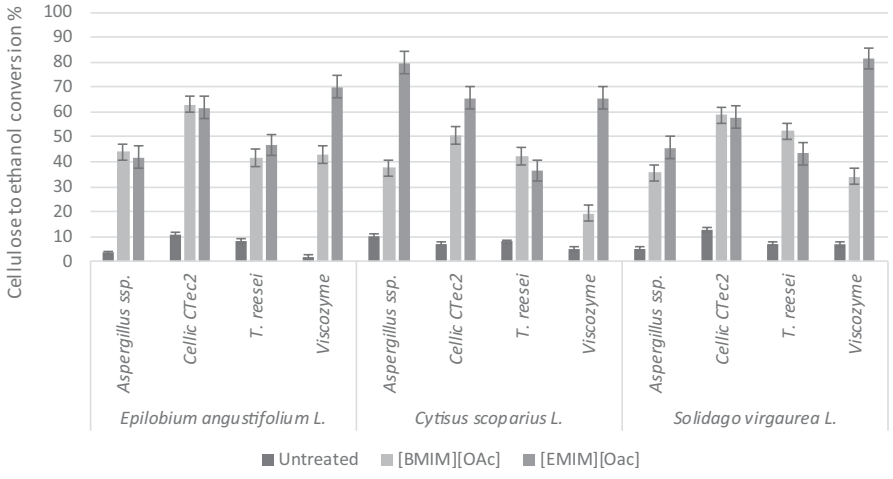


FIGURE 6 Ethanol fermentation efficiency

not require cultivation or fertilization. The stems of goldenrod, broom, and fireweed are easy to collect, dry, and grind. Although important, the abovementioned technological values of these plants remain in the shade as the qualitative and quantitative structure of the lignocellulosic complex of their stems is unfavorable. Generally, plant biomass is composed mainly of cellulose, hemicellulose, lignin, pectin, and proteins. Lignin is one of the most problematic structural elements hindering the decomposition of cell wall and subsequently preventing effective hydrolysis. Therefore, it is important to first understand the basic composition of the lignocellulosic complex, such as cellulose, hemicellulose, and lignin, in order to be able to estimate the efficiency of the bioprocess from the outset. It is worth noting here that lignin is not the only barrier hindering the hydrolysis of the polymers composing the lignocellulose, and xylan is also an example.[13-16] Thus, our analyses of the native material allowed for the basic characterization of the selected raw materials (Table 1).

The next step in the production of second-generation bioethanol from lignocellulosis feedstock is the selection of appropriate biomass pretreatment. Many methods of biomass pretreatment are known, and each one of them has his advantages but also drawbacks.[17] It is crucial that the pretreatment method should be so effective that the lignin fraction from the cellulose pulp is completely removed. One of the methods available for pretreatment is the use of ionic liquids, which has already been applied in many studies.[18-24] The main purpose of the pretreatment with ionic liquids was to loosen the complex and create larger spaces between the cellulose and hemicellulose fibers so that the enzymes can penetrate the deeper layers of biomass during hydrolysis. The imidazolium ionic liquids selected in the present study effectively dissolved lignin-rich biomass and even caused its partial separation from cellulose fibers. In addition, they had a low viscosity and mixed well with biomass.

The results presented in the study showed a change in the biomass structure of the examined plants and also that the amount of lignin extracted after pretreatment was low, which in the further stages of the bioprocess may result in the inhibition of enzymatic hydrolysis and consequently lower ethanol production (Figure 1). The interaction of cellulose with hemicellulose, with the latter fraction being dominant, may also hinder enzymatic hydrolysis. Tavares et al[25] claimed that the system of supramolecular structure in plants, including the size of polysaccharides in cells and tissues, is determined by the glycomic code, which provides information on how polymeric bonds are formed. The other aspects that determine the availability of polymers for enzymes are the size of pores and the gaps between the cellulose and hemicellulose fibers, and therefore, the size of enzyme molecules is also an important factor affecting their effective penetration into the plant material. It is worth pointing out that biomass pretreatment with ionic liquids is more effective when the water content in the raw material is lower. Our study showed that the ionic liquids [BMIM][OAc] and [EMIM][OAc] undoubtedly affected the structure of the plants studied (Figures 1-3). The ability of ionic liquids to dissolve lignocellulosic biomass under gentle conditions and with little or no by-product formation makes them highly interesting alternatives for pretreatment in the processes where high product yields are of critical importance.[26]

Enzymatic hydrolysis is a safe and effective alternative to acidic or alkaline hydrolysis because it takes place under milder conditions and provides a high yield of hydrolysate without the need for neutralization/purification.[27] This process may be compatible with ionic pretreatment as ionic liquids do not adversely affect the activity of enzymatic proteins. Enzymatic hydrolysis of cellulose, which leads to full depolymerization, requires the use of several enzymes that can act synergistically, such as cellulases, hemicellulases, and pectinases. Cellulose-hydrolyzing cellulases include

TABLE 1 The content of cellulose, hemicellulose, and lignin in fireweed, common broom, and European goldenrod

Sample	Pretreatment	Cellulose [%]	Hemicellulose [%]	Lignin [%]
Epilobium angustifolium L.	Untreated	41.0[a]	21.9[c]	20.4[c]
	[BMIM][OAc]	46.4[b]	20.7[a]	19.6[a]
	[EMIM][OAc]	42.9[a]	20.9[b]	17.9[d]
Cytisus scoparius L.	Untreated	30.7[c]	28.8[a]	16.0[a]
	[BMIM][OAc]	43.7[a]	24.7	14.9[d]
	[EMIM][OAc]	38.6[c]	20.8[d]	16.1[d]
Solidago virgaurea L.	Untreated	30.4[d]	29.2[b]	19.2[a]
	[BMIM][OAc]	38.0[a]	22.0[d]	13.3[b]
	[EMIM][OAc]	40.6[a]	22.0[a]	12.8[d]

Note: Means of three replications based on the least significant difference procedure at $\alpha = 0.05$ level.
Means with the same letter in the same column are not significantly different.

endoglucanases (EG I, EG III) and cellulobiohydrolases (CBH I, CBH II), which degrade crystalline cellulose to soluble cellulose and amorphous cellulose in the first stage, and β-glucosidase (BG),which hydrolyses cellulose to glucose.[28,29] Commercial enzyme preparations that can be used for saccharification are available in the form of mixtures of several enzymes ("enzyme cocktails") containing, for example, cellulases, cellobiases, and pectinases.[30] In this study, four commercial enzymatic preparations were used and their effectiveness in the context of hydrolysis efficiency and the final concentration of glucose, which is the basic source of carbon for the *S cerevisiae* yeast used by us, was evaluated. The preparation which allowed the most effective hydrolysis was Viscozyme® (Figures 5 and 6). It is a product containing a range of carbohydrases including arabinase, cellulase, β-glucanase, hemicellulase, and xylanase. It also breaks down the branched pectin-like substances found in plant cell walls.

Other enzymatic preparations used, such as cellulase from *T reesei* or from *Aspergillus* sp, did not allow for the achievement of better results, despite the fact that they were used in the hydrolysis of biomass from plants tested by us.[31-35] For example, Bombeck et al[35] showed that the use of the enzymes from *Aspergillus* sp for hydrolysis caused a significant decrease in the proportion of amorphous cellulose on the substrate/tree surfaces, leaving the portions of mannan and xylan relatively intact. This mixture of enzymes also hydrolyzed chemical-thermo-mechanical pulps more effectively than cellulose pulp. In a study on raw material obtained from *Miscanthus giganteus*, which was initially treated with 5% NaOH at 121°C and hydrolyzed with Celluclast 1.5 L, a saccharification efficiency of 52% was noted, while almost all hemicellulose (94.6%) was degraded during the pretreatment stage.[33] In another study on pretreatment with dilute acid and hydrophilic ionic liquids, the maximum glucose yield obtained from the reaction catalyzed by the cellulase from *T reesei* did not exceed 75%.[32] Dąbkowska et al[36] subjected *Miscanthus* stems to saccharification before and after pretreatment with organosolv method (80% (w/w) of glycerol, 1.25% of H_2SO_4) using various enzymatic preparations for hydrolysis. The most effective enzyme mixture that was composed of Cellic®CTec2 (10%, w/w), β-glucanase (5%, w/w), and Cellic®HTec2 (1%, w/w) resulted in high yields of glucose (93.1%) and xylose (69.2%) after glycerol-based pretreatment. In another study,[31]*Miscanthus* was pretreated with gaseous ammonia (temperature 150°C) and a hydrolysis efficiency of almost 100% was achieved after 72 hours using Cellic®CTec2 cellulases.

Ethanol fermentation is the last stage of the bioprocess aimed to obtain alcohol, the final concentration of which is required to be as high as possible and often determines the profitability of the production. In our study, we obtained the maximum ethanol concentration of 2.86 g L^{-1} with a yield of up to 81.43% from the biomass of European goldenrod, which was subjected to treatment with [EMIM][OAc] ionic liquid and Viscozyme® enzyme preparation. The lowest ethanol concentration of 0.05 g L^{-1}, with a yield of 1.5%, was obtained for the native material from fireweed, treated with Viscozyme®. For comparison, Ferreira et al (2010) fermented *Pterospartum tridentatum* samples after the previous pretreatment with sulfuric acid. The authors obtained the maximum ethanol concentration of 0.26 g/g total sugars, without previous detoxification.[37] In turn, Razmovski et al[38] obtained an ethanol yield of 0.48 g/g (94% theoretical yield) from *Jerusalem artichoke* stems by treatment with dilute acid and hydrolysis. Goshadrou et al[39] used [EMIM][OAc] to pretreat Aspen wood (*Populus tremula*) and obtained 224 g of ethanol from 1 kg of biomass. A similar method of pretreatment with [EMIM][OAc] (5 hours pretreatment in 120°C) was used by Poornejad et al,[40] who purified the rice straw obtaining 2.4 g ethanol from 100 g of straw. Idi et al[41] purified cocoa waste with [EMIM][$MeSO_4$]. After fermentation, the authors obtained 7.85 g L^{-1} of ethanol from the treated material and 5.12 g L^{-1} from the untreated material. Yamada et al[42] obtained the ethanol production and yield from [Bmim][OAc]-pretreated bagasse on the level of 0.81 g L^{-1} after fermentation for 96 hours. In case of triticale straw, we have previously reported, that the same [EMIM][OAc] ionic liquids biomass pretreatment, and further ethanol fermentation process conducted in a very similar way, leads to 10.64 g L^{-1} of ethanol.[43] The presented studies cover the various lignocellulosic raw materials from which bioethanol has been obtained using the methodology described in this publication. Several reviews on the pretreatment, hydrolysis, and ethanol fermentation have been published lately.[17,28,44,45] Elgharbawy et al[28] pointed out that the use of ionic liquids is a good solution that can benefit the production of bioethanol on a larger scale, combining pretreatment and enzymatic hydrolysis in one step. It should be noted, however, that the design of the production process with separate pretreatment and hydrolysis steps is equally promising. It seems crucial to design a specific process for each source of raw material, as the ethanol yield depends mainly on a well-performed pretreatment (including lignocellulose conversion and hydrolysis to monosaccharides and pretreatment specific conditions: temperature, time, or biomass loading).

5 | SUMMARY

The production of bioethanol is a multidisciplinary issue, requiring knowledge of the structure of biomass and the phenomena occurring during conversion, development of more new effective enzymes, and optimization of individual steps of the process to minimize energy costs. Moreover, the successful development of biorefineries based on the production of bioethanol from biomass depends not only on the

technology used for fuel production but also on the use of by-products produced, which would allow reducing the costs of bioethanol making it more competitive.

The present paper proposes the use of three plants growing on agricultural wastelands for the production of bioethanol, which allows obtaining a high yield of biomass and easy harvesting and storage. The highest content of ethyl alcohol was obtained for European goldenrod: 2.86 g L^{-1}, next for common broom: 2.65 g L^{-1}, and for fireweed 2.51 g L^{-1}, all samples purified by [EMIM][OAc] and hydrolyzed using Viscozyme®.The efficiency of enzymatic hydrolysis depends to a large extent on the type of pretreatment applied and the enzymes used, and to a less extent on the very species of the three plants investigated.

Therefore, in subsequent studies focusing on increasing the scale of the production, plants from agricultural wastelands, such as European goldenrod, common broom, and fireweed, which are a good source of cellulose and often occur in the same area (adjacent to each other), can be used as raw materials. As a future prospect, we believe that a mixture of these plants can be used for biofuel production because of the similar yield of bioethanol obtained during the process.

ORCID

Małgorzata Smuga-Kogut https://orcid.org/0000-0001-8486-5949
Daria Szymanowska https://orcid.org/0000-0002-4665-1576
Roksana Markiewicz https://orcid.org/0000-0001-9332-3180
Tomasz Piskier https://orcid.org/0000-0003-0890-6301
Joanna Kobus-Cisowska https://orcid.org/0000-0003-2834-0405
Judyta Cielecka-Piontek https://orcid.org/0000-0003-0891-5419

REFERENCES

1. Dimitriou I, Rosenqvist H. Sewage sludge and wastewater fertilisation of short rotation coppice (SRC) for increased bioenergy production—biological and economic potential. *Biomass Bioenerg*. 2011;35(2):835-842.
2. Augustenborg CA, Finnan J, McBennett L, Connolly V, Priegnitz U, Müller C. Farmers' perspectives for the development of a bioenergy industry in Ireland. *GCB Bioenergy*. 2012;4(5):597-610.
3. Mola-Yudego B, Rahlf J, Astrup R, Dimitriou I. Spatial yield estimates of fast-growing willow plantations for energy based on climatic variables in Northern Europe. *GCB Bioenergy*. 2016;8(6):1093-1105.
4. Dyguś KH, Madej M. Roślinność Wielowariantowego Doświadczenia Modelowego Na Złożu Odpadów Paleniskowych Energetyki Węglowej (Vegetation of a multi-variant model experiment on coal combustion waste deposits). *Inżynieria Ekol*. 2012;30:227-240.
5. Klimont K, Bulińska-Radomska Z, Górka J. Możliwość Wykorzystania Wybranych Roślin Miododajnych Do Rekultywacji Terenów Po Eksploatacji Siarki. *Polish J Agron*. 2013;12:17-25.
6. Stark A. Ionic liquids in the biorefinery: a critical assessment of their potential. *Energy Environ Sci*. 2011;4(1):19-32.
7. Bian J, Peng F, Peng X-P, et al. Effect of [Emim]Ac pretreatment on the structure and enzymatic hydrolysis of sugarcane bagasse cellulose. *Carbohydr Polym*. 2014;100:211-217.
8. Zhu Z, Zhu M, Wu Z. Pretreatment of sugarcane bagasse with NH_4OH–H_2O_2 and ionic liquid for efficient hydrolysis and bioethanol production. *Bioresour Technol*. 2012;119:199-207.
9. Zhou J, Sui H, Jia Z, Yang Z, He L, Li X. Recovery and purification of ionic liquids from solutions: a review. *RSC Adv*2018;8(57):32832-32864.
10. Liu K, Lin X, Yue J, et al. High concentration ethanol production from corncob residues by fed-batch strategy. *Bioresour Technol*. 2010;101(13):4952-4958.
11. Branco R, Serafim L, Xavier A. Second generation bioethanol production: on the use of pulp and paper industry wastes as feedstock. *Fermentation*. 2018;5(1):4.
12. Tapia Carpio L, Simone de Souza F. Competition between second-generation ethanol and bioelectricity using the residual biomass of sugarcane: effects of uncertainty on the production mix. *Molecules*. 2019;24(2):369.
13. Ray MJ, Brereton NJB, Shield I, Karp A, Murphy RJ. Variation in cell wall composition and accessibility in relation to biofuel potential of short rotation coppice willows. *BioEnergy Res*. 2012;5(3):685-698.
14. Torres AF, van der Weijde T, Dolstra O, Visser RGF, Trindade LM. Effect of maize biomass composition on the optimization of dilute-acid pretreatments and enzymatic saccharification. *BioEnergy Res*. 2013;6(3):1038-1051.
15. Marriott PE, Sibout R, Lapierre C, et al. Range of Cell-wall alterations enhance saccharification in *Brachypodium distachyon* mutants. *Proc Natl Acad Sci*. 2014;111(40):14601-14606.
16. DeMartini JD, Pattathil S, Miller JS, Li H, Hahn MG, Wyman CE. Investigating plant cell wall components that affect biomass recalcitrance in poplar and switchgrass. *Energy Environ Sci*. 2013;6(3):898.
17. Kumar AK, Sharma S. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. *Bioresour Bioprocess*. 2017;4(1):7.
18. Chen W-H, Lin T-S, Guo G-L, Huang W-S. Ethanol production from rice straw hydrolysates by *Pichia stipitis*. *Energy Procedia*. 2012;14:1261-1266.
19. Liu Z, Li L, Liu C, Xu A. Pretreatment of Corn straw using the alkaline solution of ionic liquids. *Bioresour Technol*. 2018;260:417-420.
20. da Costa Lopes AM, João KG, Rubik DF, et al. Pre-treatment of lignocellulosic biomass using ionic liquids: wheat straw fractionation. *Bioresour Technol*. 2013;142:198-208.
21. Sáez F, Ballesteros M, Ballesteros I, Manzanares P, Oliva JM, Negro MJ. Enzymatic hydrolysis from carbohydrates of barley straw pretreated by ionic liquids. *J. Chem Technol Biotechnol*. 2013;88(5):937-941.
22. Fu D, Mazza G. Aqueous ionic liquid pretreatment of straw. *Bioresour Technol*. 2011;102(13):7008-7011.
23. Eisenhuber K, Krennhuber K, Steinmüller V, Jäger A. Comparison of different pre-treatment methods for separating hemicellulose from straw during lignocellulose bioethanol production. *Energy Procedia*. 2013;40:172-181.

24. Bernardo J, Gírio F, Łukasik R. The effect of the chemical character of ionic liquids on biomass pre-treatment and posterior enzymatic hydrolysis. *Molecules*. 2019;24(4):808.
25. Tavares EQP, De Souza AP, Buckeridge MS. How endogenous plant cell-wall degradation mechanisms can help achieve higher efficiency in saccharification of biomass. *J Exp Bot*. 2015;66(14):4133-4143.
26. Gräsvik J, Winestrand S, Normark M, Jönsson LJ, Mikkola J-P. Evaluation of four ionic liquids for pretreatment of lignocellulosic biomass. *BMC Biotechnol*. 2014;14(1):34.
27. Kuhad RC, Singh A, Eriksson K-EL. *Microorganisms and Enzymes Involved in the Degradation of Plant Fiber Cell Walls*. Berlin, Heidelberg: Springer; 1997:45-125.
28. Elgharbawy AA, Alam MZ, Moniruzzaman M, Goto M. Ionic liquid pretreatment as emerging approaches for enhanced enzymatic hydrolysis of lignocellulosic biomass. *Biochem Eng J*. 2016;109:252-267.
29. Sokolowska K, Konieczna-Molenda A, Witek E. Hydrolysis of cellulose waste catalyzed by cellulolytic enzymes immobilized on polymer carrier. *Polimery*. 2016;61(09):633-641.
30. Chandel AK, Gonçalves BCM, Strap JL, da Silva SS. Biodelignification of lignocellulose substrates: an intrinsic and sustainable pretreatment strategy for clean energy production. *Crit Rev Biotechnol*. 2015;35(3):281-293.
31. Cayetano RDA, Kim TH. Two-stage processing of miscanthus giganteus using anhydrous ammonia and hot water for effective xylan recovery and improved enzymatic saccharification. *Bioresour Technol*. 2018;255:163-170.
32. Auxenfans T, Crônier D, Chabbert B, Paës G. Understanding the structural and chemical changes of plant biomass following steam explosion pretreatment. *Biotechnol Biofuels*. 2017;10(1):36.
33. Michalska K, Bizukojć M, Ledakowicz S. Pretreatment of energy crops with sodium hydroxide and cellulolytic enzymes to increase biogas production. *Biomass Bioenerg*. 2015;80:213-221.
34. Mansfield SD, Mooney C, Saddler JN. Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnol Prog*. 1999;15(5):804-816.
35. Bombeck P-L, Khatri V, Meddeb-Mouelhi F, Montplaisir D, Richel A, Beauregard M. Predicting the most appropriate wood biomass for selected industrial applications: comparison of wood, pulping, and enzymatic treatments using fluorescent-tagged carbohydrate-binding modules. *Biotechnol Biofuels*. 2017;10(1):293.
36. Dąbkowska K, Alvarado-Morales M, Kuglarz M, Angelidaki I. Miscanthus straw as substrate for biosuccinic acid production: focusing on pretreatment and downstream processing. *Bioresour Technol*. 2019;278:82-91.
37. Ferreira S, Gil N, Queiroz JA, Duarte AP, Domingues FC. Bioethanol from the Portuguese forest residue *Pterospartum tridentatum* – an evaluation of pretreatment strategy for enzymatic saccharification and sugars fermentation. *Bioresour Technol*. 2010;101(20):7797-7803.
38. Razmovski RN, Šćiban MB, Vučurović VM. Bioethanol production from Jerusalem artichoke by acid hydrolysis. *Rom Biotechnol Lett*. 2011;16(5):6497-6503.
39. Goshadrou A, Karimi K, Lefsrud M. Characterization of ionic liquid pretreated aspen wood using semi-quantitative methods for ethanol production. *Carbohydr Polym*. 2013;96(2):440-449.
40. Poornejad Nafiseh, Karimi Keikhosro, Behzad Tayebeh. Ionic Liquid Pretreatment of Rice Straw to Enhance Saccharification and Bioethanol Production. *Journal of Biomass to Biofuel*. 2014;1:8–15. https://doi.org/10.11159/jbb.2014.002.
41. Idi A, Md. Salleh M, Ibrahim Z, Mohamad SE. Pretreatment of cocoa waste for bioethanol production using ionic liquid. *J Teknol*. 2013;59:1.
42. Yamada R, Nakashima K, Asai-Nakashima N, et al. Direct ethanol production from ionic liquid-pretreated lignocellulosic biomass by cellulase-displaying yeasts. *Appl Biochem Biotechnol*. 2017;182(1):229-237.
43. Smuga-Kogut M, Walendzik B, Szymanowska-Powalowska D, et al. Comparison of bioethanol preparation from triticale straw using the ionic liquid and sulfate methods. *Energies*. 2019;12(6):1155.
44. Galbe M, Wallberg O. Pretreatment for biorefineries: a review of common methods for efficient utilisation of lignocellulosic materials. *Biotechnol Biofuels*. 2019;12(1):294.
45. Baruah J, Nath BK, Sharma R, et al. Recent trends in the pretreatment of lignocellulosic biomass for value-added products. *Front Energy Res*. 2018;6:141.

How to cite this article: Smuga-Kogut M, Szymanowska D, Markiewicz R, et al. Evaluation of the potential of fireweed (*Epilobium angustifolium* L.), European goldenrod (*Solidago virgaurea* L.), and common broom (*Cytisus scoparius* L.) stems in bioethanol production. *Energy Sci Eng*. 2020;8:3244–3254. https://doi.org/10.1002/ese3.726

scientific reports

Check for updates

OPEN

Ionic liquid pretreatment of stinging nettle stems and giant miscanthus for bioethanol production

Małgorzata Smuga-Kogut[1], Daria Szymanowska-Powałowska[2,3], Roksana Markiewicz[4]✉, Tomasz Piskier[1] & Tomasz Kogut[5]

Production of ethanol from lignocellulosic biomass is considered the most promising proposition for developing a sustainable and carbon–neutral energy system. The use of renewable raw materials and variability of lignocellulosic feedstock generating hexose and pentose sugars also brings advantages of the most abundant, sustainable and non-food competitive biomass. Great attention is now paid to agricultural wastes and overgrowing plants as an alternative to fast-growing energetic crops. The presented study explores the use of stinging nettle stems, which have not been treated as a source of bioethanol. Apart from being considered a weed, stinging nettle is used in pharmacy or cosmetics, yet its stems are always a non-edible waste. Therefore, the aim was to evaluate the effectiveness of pretreatment using imidazolium- and ammonium-based ionic liquids, enzymatic hydrolysis, fermentation of stinging nettle stems, and comparison of such a process with giant miscanthus. Raw and ionic liquid-pretreated feedstocks of stinging nettle and miscanthus were subjected to compositional analysis and scanning electron microscopy to determine the pretreatment effect. Next, the same conditions of enzymatic hydrolysis and fermentation were applied to both crops to explore the stinging nettle stems potential in the area of bioethanol production. The study showed that the pretreatment of both stinging nettle and miscanthus with imidazolium acetates allowed for increased availability of the critical lignocellulosic fraction. The use of 1-butyl-3-methylimidazolium acetate in the pretreatment of stinging nettle allowed to obtain very high ethanol concentrations of 7.3 g L^{-1}, with 7.0 g L^{-1} achieved for miscanthus. Results similar for both plants were obtained for 1-ethyl-3-buthylimidazolium acetate. Moreover, in the case of ammonium ionic liquids, even though they have comparable potential to dissolve cellulose, it was impossible to depolymerize lignocellulose and extract lignin. Furthermore, they did not improve the efficiency of the hydrolysis process, which in turn led to low alcohol concentration. Overall, from the presented results, it can be assumed that the stinging nettle stems are a very promising bioenergy crop.

The increasing energy demand driven i.a. by economic growth, expanding population, and social pressure, is one of the most significant worldwide concerns, especially in the context of limited fossil fuel sources. The need to substitute fossil fuels is crucial to reach energy security and increase environmental sustainability[1]. Cellulosic ethanol is considered to also play an essential role in the creation of new technologies, since similarly to other industries (coal or corn processing), the development of cost-effective processes may induce diversification of products (other fuel molecules as well as various chemicals), leading therefore to a more sustainable chemical industry[2,3]. With the increase in technological possibilities, it is expected that the use of biomass for fuel purposes will soon increase to 388.6 (biomass of herbal origin) and 100.7 million tons of dry matter (wood biomass). When designing a process for sugar or ethanol production from biomass, its chemical composition must be taken into

[1]Department of Agrobiotechnology, Faculty of Mechanical Engineering, Koszalin University of Technology, Raclawicka 15-17, 75-620 Koszalin, Poland. [2]Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-627 Poznan, Poland. [3]Department of Pharmacognosy, Poznan University of Medical Sciences, Swiecickiego 4, 61-781 Poznan, Poland. [4]NanoBioMedical Centre, Adam Mickiewicz University in Poznań, Wszechnicy Piastowskiej 3, 61614 Poznan, Poland. [5]Department of Geodesy and Offshore Survey, Maritime University of Szczecin, Żołnierska 46, 71-250 Szczecin, Poland. ✉email: roksana.markiewicz@amu.edu.pl

account, which varies according to the species of plant to be used in the production of bioethanol[4]. One of the crucial aspects of successful biofuel production is selecting a suitable source of biomass that will provide a large amount of cellulose and hemicellulose, a small amount of lignin, and will be easily purified during the chosen treatment. The use of biomass for bioethanol production is, in most cases, a very well-developed process. Nevertheless accessing biomass for chemical conversion requires complex evaluation of varieties of biomass like biomass size reduction, pretreatment, and fermentation. Moreover, the entire process should be reproducible, robust and able to convert closely related biomass source. Any new biomass source need to be evaluated carefully to determine preferred biochemical conversion schemes[5].

From many advantages of bioethanol, one has to notice the possibility of its immediate use without the necessity to change its distribution and usage forms and carbon dioxide neutrality[6]. Naturally, some drawbacks of the production of bioethanol are also noted. As the first generation is based on edible crops, such as corn, sugar beet, or sugarcane, they threaten to maintain food security worldwide. The search for biomass for bioethanol production is ongoing to effectively replace fossil fuels and the future need for food demand. A good response for this problem is second-generation bioethanol, which is produced from non-edible crops feedstock materials, and include by-products (e.g., stems, leaves, and husks, wheat, rice or corn straws, sugar cane bagasse, forest residues), organic or municipal wastes, as well as dedicated, purpose-grown feedstocks (e.g., grasses, short-rotation forests, and other energy crops)[7–9]. Unfortunately, the increasing demand for non-food biomass may impact food security regarding food availability, diversity, and access[10].

One of the most prevailing energy crops cultivated in a range of European and North American climatic conditions which can be used to produce bioethanol is giant miscanthus (*Miscanthus* × *giganteus*), since it has potential for greater photosynthetic efficiency and water and nitrogen use efficiency than other crops, especially when its production would take place on marginal lands with reduced input[11–13]. It has several advantages such as high cellulose content from 37 to 42% dry mass and high biomass yield per unit of planted area—23–38 Mg ha^{-1} $year^{-1}$ under ideal conditions and 14–15 Mg ha^{-1} $year^{-1}$ under poor conditions[12,14]. Lee and Kuan conducted a technical and economic analysis of bioethanol production from miscanthus, taking into account the costs of the following stages: pretreatment, enzymatic hydrolysis, and alcoholic fermentation and showed that the expected yield of ethanol from miscanthus is 250.0, 252.62, 255.80, 255.27 and 230.23 L per dry biomass in metric tons, and the corresponding ethanol costs are 0.891, 0.83, 0.88, 0.81 and 0.85 \$ L^{-1} of ethanol in processes using AFEX pretreatment technologies, diluted acid, alkali, hot water, and steam explosion, respectively[15,16]. The results of these studies, similar to other research, show that the pretreatment process directly affects the price of the final product; therefore, it should yield as much fermented sugar as possible[15,17].

A debate is still ongoing on energy crops, especially as they grow mostly on arable lands, reducing food-producing areas and increasing their prices. One way of resolving the food competitiveness problem is to promote feedstocks that can grow on marginal lands. The other is to use lignocellulosic biomass like agriculture residues, forest woody residues, microalgae, and even municipal solid wastes[18]. In this context, an interesting yet scarcely existing raw material for ethanol production literature is stinging nettle (*Urtica dioica* L.). It is a perennial, broadleaved, dioecious plant, reaching a height of 30 to over 100 cm found in temperate regions of Europe, Asia, North Africa, and North America[19]. Stinging nettle inhabits soils around houses, gardens, meadows, pastures, bushes, areas near lakes and rivers, and deciduous forests. It occurs in large groups on nitrogen-rich soils with very high phosphates content, with the yield reaching about 3–12 Mg ha^{-1} with relatively low inputs[20]. In intensive agriculture, stinging nettle is considered a weed. Bioethanol source might be considered significant because of its use as a potential, competitive with miscanthus in cellulose occurrence. Nettle can be used to produce high-quality agricultural raw materials for composites, medicine/pharmacy, textile, and energy sectors[20–22]. What is most important, stinging nettle stems always remains a waste, therefore the use of these parts of nettle for energy purposes doesn't involve the cultivation of stinging nettle intentionally for bioethanol production. Importantly, one need to notice the nettle stems may not be sufficient enough to replace the conventional energy crops, nevertheless in central Europe, they might serve as additional source for bioethanol production.

The overall efficiency of bioethanol production on a commercial basis will always consider sustainability, energy consumption, cost, and the overall efficacy of the methods applied[18]. A multistep biochemical process is used to produce bioethanol from lignocellulosic biomass, which usually involves raw material pretreatment, enzymatic hydrolysis, and ethanol fermentation[23,24]. Due to the complexity of the lignocellulosic complex (tight bonding and molecular packing of cellulose, hemicellulose and lignin, crystallinity of cellulose), it is necessary to pretreat the raw material to release the cellulose fraction, which will result in effective hydrolysis[25]. After pretreatment, complex compounds such as cellulose or hemicellulose are hydrolysed, and the released pool of fermenting sugars is metabolized to ethanol[17].

Methods of lignocellulosic biomass pretreatment can be divided into various groups: physical, chemical, biological. In terms of chemical pretreatment, chemicals such as acids (sulfuric, hydrochloric, and phosphoric acids), alkali (NaOH, KOH, $Ca(OH)_2$, hydrazine, and anhydrous ammonia) or organic solvents have been reported to have a meaningful effect on the structure of lignocellulose[26]. Such pretreatment methods may be characterized by drawbacks like high cost and energy demand, low yield of the process, or its unfavorability from the point of view of environmental impact. The combinatorial pretreatment (physicochemical and biochemical) and non-conventional technologies have been proposed, such as ultrasound, supercritical fluids, microwave irradiation, electric and/or magnetic fields[27]. Here, the most promising are Steam pretreatment, Liquid Hot Water pretreatment, Ammonia Fibre/Freeze Explosion, Organosolv or Ionic liquid (IL) based pretreatment, affecting physical and chemical properties of lignocellulose feedstocks[24–26]. Those pretreatment methods, similarly to the conventional ones, can have both advantages and disadvantages. Hydrothermal techniques, for example, are not appropriate for each lignocellulose biomass and usually are very energy-demanding. Nonetheless, they do not require using additional chemical reagents, which makes them environmentally friendly. On the other hand, ionic liquids emerged as lignocellulose pretreatment media thanks to solubilizing, fractioning and increasing

Sample	Pretreatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Urtica dioica L.	Untreated	42.5 ± 0.5	18.7 ± 0.9	15.2 ± 0.9
	[bmim][OAc]	33.1 ± 0.7	22.3 ± 1.3	22.6 ± 0.7
	[emim][OAc]	35.8 ± 0.4	21.6 ± 2.7	15.1 ± 0.2
	[emim][DEP]	33.8 ± 1.9	21.1 ± 0.3	15.1 ± 0.2
	[CHDMA-C6][OAc]	43.4 ± 0.6	24.9 ± 0.8	22.3 ± 0.1
	[CHDMA-C4][OAc]	43.1 ± 0.2	24.8 ± 0.1	22.3 ± 0.2
Miscanthus giganteus (M × G)	Untreated	43.5 ± 1.1	25.7 ± 0.7	14.1 ± 0.1
	[bmim][OAc]	44.9 ± 0.5	31.6 ± 1.1	5.2 ± 0.1
	[emim][OAc]	47.7 ± 0.9	28.8 ± 1.0	5.5 ± 0.3
	[emim][DEP]	49.4 ± 0.3	21.9 ± 1.2	7.5 ± 0.2
	[CHDMA-C6][OAc]	46.0 ± 0.4	30.0 ± 0.3	16.4 ± 0.4
	[CHDMA-C4][OAc]	44.0 ± 0.3	31.6 ± 0.1	16.7 ± 0.1

Table 1. Composition of stinging nettle and giant miscanthus untreated and treated with ILs.

cellulose enzymatic digestibility. During the IL pretreatment (dissolution and regeneration with anti-solvent), the crystalline structure of cellulose can be changed to amorphous, which largely increases the bioethanol production process efficiency[28–30].

This work aimed to analyze the effectiveness of ethanol production from stinging nettle stems, an innovative cellulose source considered as agricultural waste. For this purpose, at first, pretreatment of stinging nettle stems was performed using imidazolium and ammonium ionic liquids. Afterward, the stems were subjected to enzymatic hydrolysis and alcoholic fermentation. As it is still challenging to compare the efficacy of bioethanol production from various lignocellulosic sources, the results obtained for stinging nettle stems were compared to the giant miscanthus, a well-known energy crop. The study's originality lies in the demonstration that the stinging nettle stems can be a potential raw material for ethanol production for fuel purposes.

Results

Compositional analysis. The qualitative composition of lignocellulose biomass is a crucial aspect that qualifies the raw material for bioethanol production. Another issue is the choice of appropriate pretreatment method and its costs. The use of ionic liquids has many advantages, such as the possibility of their recirculation and reuse and interesting physicochemical properties, e.g., low vapour pressure, thermal and chemical stability, and wide liquid range. The share of fractions of raw and IL-pretreated materials tested in the study is presented in Table 1.

Non-pretreated giant miscanthus contained on average 43.5% cellulose, 25.7% hemicellulose, and 14.1% lignin, while the stalks of stinging nettle contained 42.5% cellulose, 18.7% hemicellulose, and 15.2% lignin. The lignocellulose composition of these two plant species was similar in raw form. It differed depending on the type of ionic liquid used for its pretreatment. It should be pointed out that the pretreatment was carried out in two stages, which was also reflected in differences in the final composition of substrates aimed for hydrolysis. Stinging nettle stalks subjected to the influence of imidazolium ionic liquids caused a decrease in cellulose content by about 10% and lignin content by about 7–9%, with a simultaneous increase in the amount of hemicellulose. In turn, the pretreatment using ammonium ionic liquids did not affect carbohydrate losses and did not cause nettle stalk delignification. For comparison, similar results were obtained for giant miscanthus before and after pretreatment with ionic liquids. However, when imidazolium ionic liquids were used, the lignin content in miscanthus samples decreased to 5–7%, and the cellulose content increased by about 2–4%.

The use of imidazolium ionic liquids to dissolve miscanthus and nettle stalks, although it brings better results, is also troublesome. As cellulose dissolves, the liquid becomes more viscous and hardly miscible. When anti-solvent, in that case, deionized water is added, it turns difficult to dissolve the gel. Washing out cellulose fibres from the ionic liquid is challenging and time-consuming due to its dense consistency. This is not the case with ammonium ionic liquids. The viscosity of the ionic liquid and biomass solution was lower, which allowed further mixing. It was also easier to precipitate fibres from these liquids, and the process of ionic liquid washing out took a shorter time. Images from the scanning electron microscope exhibit that miscanthus and nettle cellulose fibres change from crystalline to amorphous forms after the treatment with both imidazolium and ammonium liquids, as presented in Fig. 1. On the other hand, the most significant disadvantage of ammonium ionic liquids is that using them is not possible to depolymerize lignocellulose and extract lignin.

Enzymatic hydrolysis. After pretreatment with ionic liquids, stinging nettle and giant miscanthus were subjected to enzymatic hydrolysis. The application of xylanase aimed to increase the porosity of cellulose fibres and increase the number of contact points for cellulolytic enzymes. The consequence of such action should be hemicellulose crystallization and exposure to cellulose fibres and, as a result, an increase in hydrolysis efficiency. The efficiency of enzymatic hydrolysis was evaluated after 96 h of the process, determining the glucose and xylose concentration. Glucose concentrations are presented in Fig. 2.

The highest concentration of glucose was determined in stinging nettle sample after the treatment with [emim][OAc] (4.5 g L^{-1}) and in giant miscanthus after the treatment with [bmim][OAc] (4.1 g L^{-1}) and [emim]

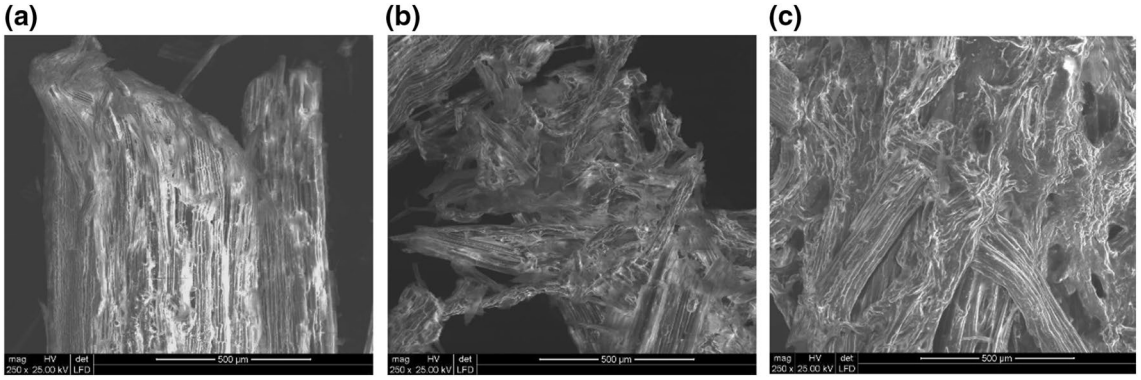


Figure 1. Scanning electron microscopic images of *Urtica dioica* L.: (**a**) untreated; (**b**) after pretreatment with [bmim][OAc]; (**c**) after pretreatment with [emim][OAc].

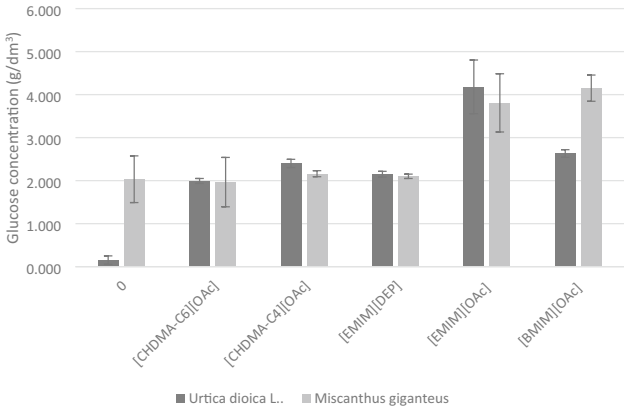


Figure 2. Glucose content after enzymatic hydrolysis of *Urtica dioica* L. and *Miscanthus* × *giganteus* untreated (0) and treated with an appropriate IL.

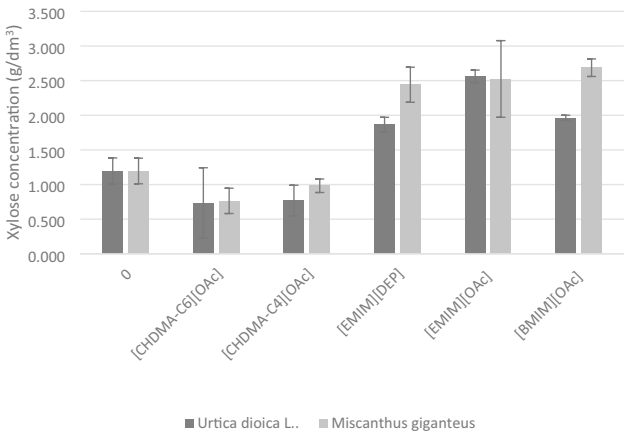


Figure 3. Xylose content after enzymatic hydrolysis of *Urtica dioica* L. and *Miscanthus* × *giganteus* samples untreated (0) and treated with an appropriate IL.

[OAc] (4.2 g L^{-1}). In other cases, the glucose concentration did not exceed 2.5 g L^{-1}, both for stinging nettle and miscanthus. Enzymatic hydrolysis occurred with very low efficiency in the variants where no treatment with ionic liquids was applied. The glucose concentration of 1.9 g L^{-1} was obtained for miscanthus and 0.025 g L^{-1} for stinging nettle.

The content of xylose determined in samples subjected to enzymatic hydrolysis is presented in Fig. 3.

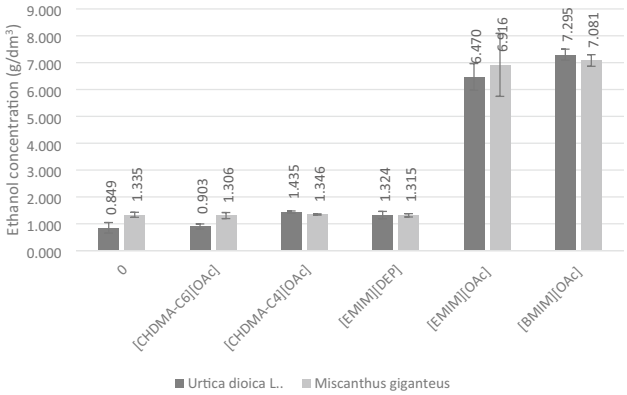


Figure 4. Ethanol content after alcoholic fermentation of *Urtica dioica* L. and *Miscanthus* × *giganteus* samples untreated and treated with ILs.

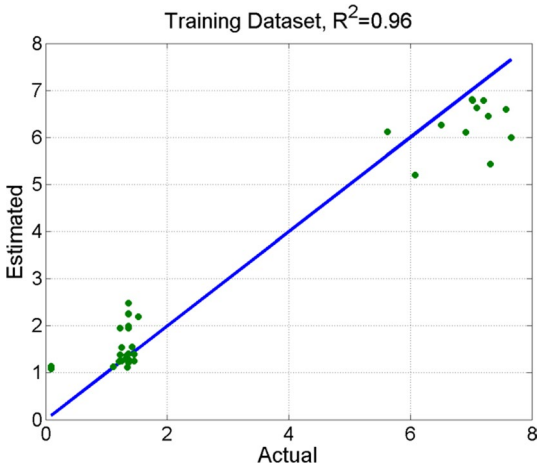


Figure 5. Verification scatter diagrams, with the x-axis showing the observed ethanol content and the y-axis presenting the estimated ethanol content.

After application of [emim][OAc] pretreatment, 2.6 g L^{-1} and 2.5 g L^{-1} of xylose were obtained from stinging nettle and giant miscanthus, respectively. The xylose concentration in control samples did not exceed 1.5 g L^{-1}. The lowest xylose content was observed in nettle and miscanthus samples treated with ammonium ionic liquids (results below 0.9 g L^{-1}). Considering the influence of the application of individual ionic liquids on glucose and xylose content, it can be concluded that the most effective in the treatment of biomass is dissolution with [emim][OAc]. On the other hand, the ammonium ionic liquids did not improve the efficiency of the hydrolysis process in comparison to the native material.

Alcoholic fermentation. The obtained hydrolysates were subjected to alcoholic fermentation using *Saccharomyces cerevisiae* type II yeast. Chromatographic analysis showed that the highest concentration of ethanol was obtained in samples of stinging nettle (7.3 g L^{-1}) and giant miscanthus (7.0 g L^{-1}), which were pretreated with [bmim][OAc], as presented in Fig. 4.

The results of the statistical analysis include ordering the depending variables according to their level of significance. It was shown that the content of ethanol from biomass of stinging nettle and giant miscanthus is mainly affected by the type of ionic liquid used, then the amount of simple sugars after enzymatic hydrolysis, and the content of lignin in samples intended for hydrolysis and fermentation. Using the Random Forest machine learning algorithm, a model with a determination factor R2 between the estimated and the observed ethanol content of 0.96 was created, as presented in Fig. 5.

The collection of Out of Bag observations made it possible to determine the importance of particular traits for the content of ethanol from biomass. Information about the importance of a given variable is obtained directly from a trained model. Using the internal structure of the Random Forest algorithm, it is possible to determine how important are the traits used for its learning. In a stochastic manner, the algorithm selects the features of

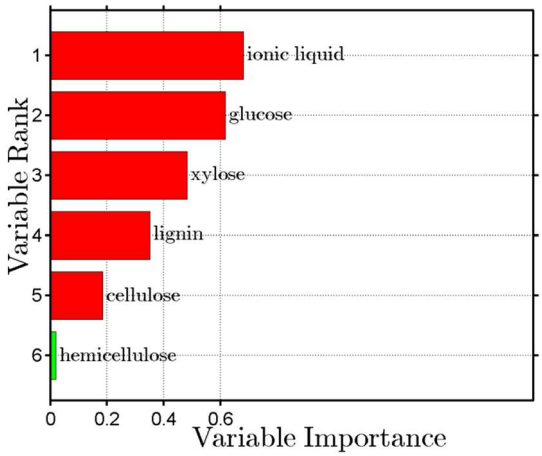


Figure 6. Relative importance of input features for estimating the ethanol content.

the model, thus estimating the significance of less important variables[31]. The traits ordered according to their significance in the model are shown in Fig. 6.

Discussion

The production of ethanol from lignocellulose requires improvements and modifications related to pretreatment, enzymatic hydrolysis, and fermentation to increase the profitability of ethanol production and the transition from the laboratory to the industrial/commercial scale. One of the most important objectives is to increase the efficiency of the fermentation process so that the entire pool of sugars (pentose and hexose) is metabolized to ethanol.

Other barriers related to ethanol production include the variable composition of biomass, the presence of inhibitors as a result of pretreatment, osmotic and oxidative stress. However, the critical element in the production of second-generation bioethanol is the choice of raw material. The raw material from which bioethanol will be produced should contain the highest cellulose content possible, along with high hemicellulose and low lignin content, because it is this fraction of the lignocellulosic complex that is reflected in the concentration of fermenting sugars. A variety of lignocellulose feedstocks have been examined for use in the production of biofuels, including energy crops (e.g., miscanthus or switchgrass), forest-based woody wastes, and forest biomass, agricultural, industrial, municipal, and food wastes[32]. Another essential feature is the high availability of raw materials. Therefore, this article points to the possibility of using stinging nettle stems as a substrate for bioethanol production.

It was shown that the content of ethanol from stinging nettle and giant miscanthus biomass is affected mainly by the type of ionic liquid used for pretreatment, then the amount of simple sugars after enzymatic hydrolysis, and the content of lignin in samples intended for hydrolysis and fermentation. Importantly, not only the lignin content but also its structure influences bioethanol production. After the conventional pretreatment methods, more condensed lignin is generated, hampering the bioconversion efficiency[33].

A pretreatment method well-suited to the raw material can significantly improve the hydrolysis efficiency of the lignocellulosic substrate. However, it should be pointed out that aggressive methods cause losses in the pool of fermenting sugars and contribute to the formation of process inhibitors[31]. Therefore, mild pretreatment methods are becoming more and more popular, which, as in the case of ionic liquids, can improve the availability of cellulose fibres and remove lignin but also causes the formation of large quantities of insoluble hemicelluloses[34–38]. Moreover, a combination of pretreatment methods is also gaining more and more attention[39]. The separation of lignin from cellulose using ILs depends on several factors. Hart et al. reported that hydrogen bonding strength was not a crucial factor for the lignin dissolution in ILs as it was in the cellulose dissolution, however a minimum hydrogen bonding basicity was still required to solubilize the lignin[40]. The removal of lignin in the pretreatment improves the efficiency of enzymatic hydrolysis but causes changes in the lignin structure. Moreover, even if lignin is not fully removed, its structural change also alters its position to cellulose fibres. It creates pores and free spaces, which ultimately causes the hydrolysis process to be more efficient. Unfortunately, there aren't many reports that would exhibit total fractionation of lignocellulose using ionic liquids into the main constituents. In this study, imidazolium ionic liquids ([emim][OAc], [bmim][OAc] and [emim][DEP]) along with ammonium ionic liquids ([CHDMA-C4][OAc] and [CHDMA-C6][OAc]) were chosen as the pretreatment agents for the raw materials due to their good cellulose solubility; [emim][OAc] allows the most effective dissolution of cellulose, from 8 to 10%, whereas [CHDMA-C6][OAc] and [CHDMA-C4][OAc] dissolve 9 and 7.5% of cellulose respectively[41–43]. An increase in cellulose content may be caused by the depolymerisation of fibres in ionic liquids, which improves their extraction. It can be assumed that the treatment with ammonium ionic liquids did not significantly influence the composition of the biomass studied (Table 1). In particular, it did not contribute to the removal of lignin, which makes it impossible to carry out further stages aimed at ethanol production effectively. Kumar et al. determined the content of individual lignocellulosic fractions after NaOH treatment (6–10%) for 24 h[44]. The authors showed a cellulose content of 85.93%, 6.8% hemicellulose, and 5.49% lignin. Agus Suryewan

et al. used various pretreatment methods for the stinging nettle and obtained (in the best case studied) 85% of cellulose, 6% of hemicellulose, and 3% of lignin using water retting and decortication[22].

Most of the ionic liquids used in biomass fractionation are imidazolium salts. The literature indicates that 1-ethyl-3-methylimidazolium acetate ([emim][OAc]), 1-allyl-3-methylimidazolium chloride ([amim][Cl]) and 1-butyl-3-methylimidazolium chloride ([bmim][Cl]) can serve as effective, non-derivatizing cellulose solvents at temperatures below 100 °C, and out of more than 20 ionic liquids tested, [amim][Cl] has proven to be an excellent wood chip solvent. For example, the addition of [bmim][Cl] causes the initial enzymatic hydrolysis rate and the pretreatment efficiency of the cellulose process to increase 50 times for regenerated cellulose compared to the untreated one[45]. Importantly, an increase in the rate of enzymatic hydrolysis of cellulose is associated with an increase in the production of simple sugars, which can be converted to ethanol. Moreover, the process of biomass degradation with the use of ionic liquids is less energy-intensive, easier to carry out, and more environmentally friendly than previously known solutions[24,28,46,47]. On the other hand, limitations for the application of ILs in the pretreatment of lignocellulose biomass are also being identified, with the most important factors being their high cost, high viscosities and moisture sensitivity, which makes it difficult to introduce an industrial scale process with their use[48]. For the process to be economically viable, water consumption must be reduced, and an effective system for ionic liquids recycling must be developed. Attempts were made to reduce the cost of solvent acquisition, replacing imidazolium ionic liquids with liquid obtained from aromatic aldehydes of lignin and hemicellulose, i.e., by-products from biofuel production[49,50]. The results were similar, although the reaction with [emim][OAc] was slightly slower. The best results obtained for the ionic liquid [emim][OAc] are explained in the literature. They are related to acetate ([OAc]) anion, which was demonstrated to be efficient in the dissolution of lignocellulosic biomass[51].

It was reported that both imidazolium and ammonium ionic liquids compete for hydrogen bonds present in cellulose structure, thus disrupting its three dimensions network[26,41]. It was reported that a key reason for this was the high hydrogen bond acceptor capacity (β) of the [OAc] anion (β = 1.201) in comparison to previously mentioned chloride anion (β = 0.83)[52]. Due to this, 1-ethyl-3-methylimidazolium acetate is confirmed to be one of the best and is one of the most commonly used ILs, able to dissolve a large variety of lignocellulosic biomass and to fractionate it into cellulose-and hemicellulose-rich fractions, as well as to produce high pure lignin[53–55]. In the case of the presented cyclohexylammonium ionic liquids, the high performance of [CHDMA-C6][OAc] affected all alkyl groups' fine-tunning. According to the mechanism described previously, two activity categories were fundamental: (1) two methyl groups using its six activated C–H bonds to link with both the acetate and cellulose surface and (2) hexyl and especially cyclohexyl are symmetry breaking substituents. Similarly, as in the case of imidazolium ionic liquids, appropriate cation allows exploiting proton acceptability of carboxylate, which further enables the breakdown of inter-and intramolecular hydrogen bonds[41].

The loosening of the lignocellulosic complex structure significantly facilitates enzymatic hydrolysis, the effectiveness of which depends on the selection of enzymatic preparations. Studies on the hydrolysis of a specific raw material are closely related to optimizing the preparation dose and process conditions. These arduous activities are usually carried out on selected 2–3 variants, characterized by the highest cellulose concentration after pretreatment. At the initial stage of research on the suitability of a given raw material for ethanol production, it is advisable to select enzymatic preparations known and tested in the context of hydrolysis effectiveness. However, it is worth mentioning that an important element of the lignocellulosic complex is hemicellulose, which may significantly reduce the effectiveness of hydrolysis[56]. Therefore, it is justified to use xylanases, which increase the material's porosity, expose cellulose fibres, and result in higher concentrations of fermenting sugars, which was also performed in this study (Figs. 2 and 3) is justified in the literature[57,58]. The next stage of second-generation bioethanol production is ethanol fermentation. In this study, the biosynthesis of ethanol was carried out with the participation of *Saccharomyces cerevisiae* yeast. Hydrolysates from giant miscanthus and stinging nettle were compared.

The full potential of stinging nettle has not yet been shown in any experimental work, and importantly it was not without reason that we have decided to use a plant that has not yet been used for ethanol production and compare it with one of the most popular energy plants used in this context. The production of ethanol from giant miscanthus has already been the subject of many studies comparing the methods and effectiveness of pretreatment, the degree of hemicellulose conversion to fermenting sugars, and the efficiency of ethanol fermentation.

This study focused on the potential of novel lignocellulosic wastes and their comparison at the same conditions applied to well-known biomass sources. The ethanol concentration obtained for both investigated raw materials is comparable, as presented in Fig. 4, which means that the stinging nettle stems are a promising alternative to energy crops such as giant miscanthus.

Materials and methods

Raw material. Common nettle stalks used for the research came from an agricultural wasteland with an area of 4.9 ha (Maszkowo, Zachodniopomorskie, Poland) excluded from agricultural production for 15 years. The plant was identified by Tomasz Piskier based on a plant atlas. The giant miscanthus was obtained from the resources of the Department of Agrobiotechnology (Koszalin University of Technology). Both plants were obtained under the principles of due carefulness included in the provisions of the Regulation (EU) No. 511/2014 of the European Parliament and of the Council (April 16, 2014). As both plants were collected from the territory of Poland, they are not subjected to the provisions on genetic resources of the previously mentioned Regulation No. 511/2014 and suitable permission of their use has been obtained.

Dry stalks of stinging nettle were cut down after the vegetation period (in September 2017), at the height of about 10–15 cm above the ground, then dried to a moisture content below 10% and ground in a colloidal mill

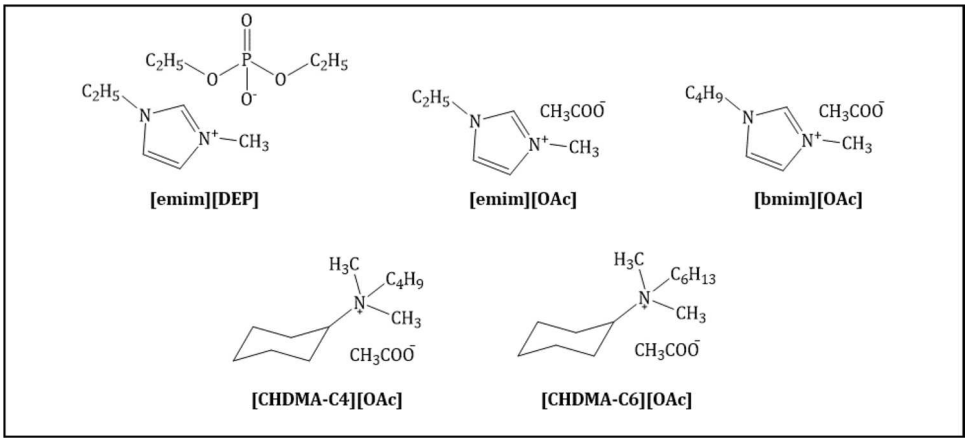


Figure 7. Ionic liquids chosen for pretreatment of stinging nettle stems and giant miscanthus.

up to 1 mm in size. A similar procedure was applied to the aboveground parts of giant miscanthus harvested in September 2017.

Ionic liquids. For the pretreatment of cellulose-rich material, five ILs from imidazolium and ammonium groups were chosen, as presented in Fig. 7. Three of them were commercially available (Iolitech GmbH, Germany) imidazolium ILs: 1-ethyl-3-methylimidazolium acetate ([emim][OAc]), 1-butyl-3-methylimidazolium acetate ([bmim][OAc]) and 1-ethyl-3-methylimidazolium diethyl phosphate ([emim][DEP]). The remaining two, belonging to the group of ammonium ILs, namely butyl(cyclohexyl)dimethylammonium acetate (([CHDMA-C4][OAc]) and (cyclohexyl)hexyldimethylammonium acetate ([CHDMA-C6][OAc]), were synthesized according to already established protocols[41,59].

Raw material pretreatment. 10 g of ground stalks of stinging nettle and giant miscanthus were added to 50 cm^3 of an appropriate ionic liquid, homogenized, and dissolved at 120 °C for 2 h. After incubation, the samples were left to cool, and then deionized water was added to rinse the cellulose fibres and separate the biomass from the IL. During the addition of the deionized water, the IL dissolves in water, and the plant fraction precipitates. The water-IL solution with biomass was filtered on a Shot funnel with a filter (Whatman 1.0 paper). This procedure was repeated four times for imidazolium ILs, where a significant increase in the plant-IL mixture was present, and two times for ammonium ILs. Such purified stalks of nettle and miscanthus were subjected to enzymatic hydrolysis.

Enzymatic hydrolysis. Thermostable xylanase, derived from a modified strain of *E. coli* bacteria (Sigma Aldrich) and CellicCTec2 enzyme were used for enzymatic hydrolysis of biomass samples (purified and non-purified ones). The initial cellulose and hemicellulose concentration was 1.0% (w/v) based on 100 mL (50 mM sodium citrate buffer) of total liquid in 250 mL Erlenmeyer flasks. Initially, xylanase (≥ 40 units mg^{-1}) in the amount of 8 U mg^{-1} hemicellulose was added and incubated at 65 °C, pH 5.0. Hydrolysis at this stage was carried out for 24 h with 250 rpm mixing. After this time, the temperature was lowered to 50 °C, and 15 FPU g^{-1} cellulose of commercial cellulase enzyme Cellic CTec2 (Novozymes, Denmark) was added to the solutions. After 96 h of enzymatic hydrolysis with the use of cellulases complex, the content of glucose and xylose was determined with the use of high-performance liquid chromatography. All experiments were performed three times to establish a standard deviation.

Alcoholic fermentation. The alcoholic fermentation was carried out accordingly to our previous reports[59–61]. Hydrolysate solutions, previously filtered to separate the lignocellulose residue, were subjected to alcoholic fermentation. The pH of the fermentation broth was measured at each sampling and adjusted to 5.0 by the addition of either 10 wt% H_2SO_4 or 20 wt% NaOH. Fermentation was started by adding freeze-dried distiller's yeast *Saccharomyces cerevisiae* type II (Sigma-Aldrich) (5% v/v). Ethanol fermentation was conducted for four days in anaerobic conditions. Samples were taken and analysed for ethanol concentration after fermentation.

Analytical methods. To examine the influence of ionic liquids on the structure of lignocellulose and the amount of available cellulose, all samples were tested for the content of cellulose, lignin, and hemicellulose (Ankom A200; ANKOM Technology); the crystalline structure of the samples was recorded using a scanning electron microscope (SEM). The morphology of cellulose fibers in buckwheat straw samples before and after ionic liquid pretreatment was recorded using SEM FEI Quanta 200 Mark 2. The content of glucose and ethanol was determined by using high-performance liquid chromatography. Samples were first centrifuged at 4000×*g* for 10 min at 4 °C (Multifuge 3SR, Germany) and then was filtered through a 0.22 μm membrane filter (Millex-GS,

Millipore, USA) before analysis using an HPLC system (Merck Hitachi, Germany). Glucose and ethanol were separated on an Aminex HPX-87P (Bio-Rad, USA) at 30 °C using a 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 cm^3 min^{-1} and then detected with a refractive index detector (Model L-7490, Merck Hitachi, Germany). All the analytical methods have been described in detail before in our previous works[59–61].

The Random Forest algorithm implemented by David Lary (https://davidlary.info) in Matlab was used to analyze the results. The Random Forest algorithm generalizes the idea of decision trees and is based mainly on the bagging method. The concept of this algorithm is based on the construction of a group of decision trees, which are created based on a random data set[62]. Classification in this algorithm is based on the voting of classifiers. The assessment of the probability of misclassification, built into the mechanism of the classifier, allows determining the out of bag error (OOB). Thanks to OOB observations, it is also possible to estimate the importance of the observation vector variables from the point of view of the classification[63] based on this property, the vector of traits (f) was constructed, based on which their significance in the process of bioethanol production was determined, as presented in the Eq. (1),

$$\mathbf{f} = [\boldsymbol{G}, \boldsymbol{X}, \boldsymbol{C}, \boldsymbol{L}, \boldsymbol{H}, \boldsymbol{IL}]^{\mathrm{T}}, \tag{1}$$

where G is the glucose; X is the xylose; C is the cellulose; L is the lignin; H is the hemicellulose; IL is the ionic liquid.

Conclusions

Pretreatment of stinging nettle and giant miscanthus with imidazolium ionic liquids allow for the increase of the availability of a key fraction of lignocellulose which is cellulose. Such application of [bmim][OAc] in the pretreatment of stinging nettle stems (and subsequent enzymatic hydrolysis) allowed us to obtain the highest concentrations of ethanol in the fermentation process, equal to 7.3 g L^{-1}. In comparison, the ethanol amount achieved for miscanthus was 7.0 g L^{-1}. Moreover, it was shown that ammonium liquids, although they allow for the more effective dissolution of the raw material, do not increase the concentration of ethanol in the fermentation process. Given the presented results of bioprocesses conducted and literature data related to the common occurrence and characteristics of the raw material, it can be assumed that stinging nettle, which in the case of the used stems is considered an agricultural waste, is a promising raw material for the production of second-generation bioethanol.

Received: 7 February 2021; Accepted: 27 August 2021
Published online: 16 September 2021

References

1. Cheah, W. Y. *et al.* Pretreatment methods for lignocellulosic biofuels production: Current advances, challenges and future prospects. *Biofuel Res. J.* **7**, 1115–1127 (2020).
2. Lynd, L. R. *et al.* Cellulosic ethanol: Status and innovation. *Curr. Opin. Biotechnol.* **45**, 202–211 (2017).
3. Sheldon, R. A. The road to biorenewables: Carbohydrates to commodity chemicals. *ACS Sustain. Chem. Eng.* **6**, 4464–4480 (2018).
4. Cheng, M., Huang, H., Dien, B. S. & Singh, V. The costs of sugar production from different feedstocks and processing technologies. *Biofuels Bioprod. Biorefining* **13**, 723–739 (2019).
5. Mielenz, J. R. Small-scale approaches for evaluating biomass bioconversion for fuels and chemicals. *Bioenergy* https://doi.org/10.1016/B978-0-12-815497-7.00027-0 (2020).
6. Abo, B. O. *et al.* Lignocellulosic biomass for bioethanol: An overview on pretreatment, hydrolysis and fermentation processes. *Rev. Environ. Health* **34**, 57–68 (2019).
7. da Costa Lopes, A. M. *et al.* Pre-treatment of lignocellulosic biomass using ionic liquids: Wheat straw fractionation. *Bioresour. Technol.* **142**, 198–208 (2013).
8. Aditiya, H. B., Mahlia, T. M. I., Chong, W. T., Nur, H. & Sebayang, A. H. Second generation bioethanol production: A critical review. *Renew. Sustain. Energy Rev.* **66**, 631–653 (2016).
9. Wang, P. & Lü, X. General introduction to biofuels and bioethanol. *Adv. 2nd Gener. Bioethanol Prod.* https://doi.org/10.1016/B978-0-12-818862-0.00006-6 (2021).
10. Beuchelt, T. D. & Nassl, M. Applying a sustainable development lens to global biomass potentials. *Sustain.* **11**, 5078 (2019).
11. Arnoult, S. & Brancourt-Hulmel, M. A review on miscanthus biomass production and composition for bioenergy use: Genotypic and environmental variability and implications for breeding. *BioEnergy Res.* **8**, 502–526 (2015).
12. Maleski, J. J. *et al.* Evaluation of miscanthus productivity and water use efficiency in southeastern United States. *Sci. Total Environ.* **692**, 1125–1134 (2019).
13. Lewandowski, I. *et al.* Progress on optimizing miscanthus biomass production for the european bioeconomy: Results of the EU FP7 Project OPTIMISC. *Front. Plant Sci.* **7**, 1620 (2016).
14. Brosse, N., El Hage, R., Sannigrahi, P. & Ragauskas, A. Dilute sulphuric acid and ethanol organosolv pretreatment of Miscanthus × Giganteus. *Cell. Chem. Technol.* **44**, 71–78 (2010).
15. Lee, W.-C. & Kuan, W.-C. *Miscanthus* as cellulosic biomass for bioethanol production. *Biotechnol. J.* **10**, 840–854 (2015).
16. Kumar, D. & Murthy, G. S. Impact of pretreatment and downstream processing technologies on economics and energy in cellulosic ethanol production. *Biotechnol. Biofuels* **4**, 27 (2011).
17. Ferrari, F. A., Pereira, J. F. B., Witkamp, G.-J. & Forte, M. B. S. Which variables matter for process design and scale-up? A study of sugar cane straw pretreatment using low-cost and easily synthesizable ionic liquids. *ACS Sustain. Chem. Eng.* **7**, 12779–12788 (2019).
18. Haldar, D. & Purkait, M. K. A review on the environment-friendly emerging techniques for pretreatment of lignocellulosic biomass: Mechanistic insight and advancements. *Chemosphere* **264**, 128523 (2021).
19. Grauso, L., de Falco, B., Lanzotti, V. & Motti, R. Stinging nettle, *Urtica dioica* L.: Botanical, phytochemical and pharmacological overview. *Phytochem. Rev.* https://doi.org/10.1007/s11101-020-09680-x (2020).
20. Di Virgilio, N. *et al.* The potential of stinging nettle (*Urtica dioica* L.) as a crop with multiple uses. *Ind. Crops Prod.* **68**, 42–49 (2015).
21. Sabir, E. C. & Zervent Ünal, B. The using of nettle fiber in towel production and investigation of the performance properties. *J. Nat. Fibers* **14**, 781–787 (2017).
22. Agus Suryawan, I. G. P., Suardana, N. P. G., Suprapta Winaya, I. N., Budiarsa Suyasa, I. W. & Tirta Nindhia, T. G. Study of stinging nettle (*Urtica dioica* L.) fibers reinforced green composite materials: A review. *IOP Conf. Ser. Mater. Sci. Eng.* **201**, 012001 (2017).

23. Raud, M., Kikas, T., Sippula, O. & Shurpali, N. J. Potentials and challenges in lignocellulosic biofuel production technology. *Renew. Sustain. Energy Rev.* **111**, 44–56 (2019).
24. Alayoubi, R. *et al.* Low temperature ionic liquid pretreatment of lignocellulosic biomass to enhance bioethanol yield. *Renew. Energy* **145**, 1808–1816 (2020).
25. Ruiz, H. A. *et al.* Engineering aspects of hydrothermal pretreatment: From batch to continuous operation, scale-up and pilot reactor under biorefinery concept. *Bioresour. Technol.* **299**, 122685 (2020).
26. Agbor, V. B., Cicek, N., Sparling, R., Berlin, A. & Levin, D. B. Biomass pretreatment: Fundamentals toward application. *Biotechnol. Adv.* **29**, 675–685 (2011).
27. Liu, Y. *et al.* Cascade utilization of lignocellulosic biomass to high-value products. *Green Chem.* **21**, 3499–3535 (2019).
28. Yamada, R. *et al.* Direct ethanol production from ionic liquid-pretreated lignocellulosic biomass by cellulase-displaying yeasts. *Appl. Biochem. Biotechnol.* **182**, 229–237 (2017).
29. Malolan, R. *et al.* Green ionic liquids and deep eutectic solvents for desulphurization, denitrification, biomass, biodiesel, bioethanol and hydrogen fuels: A review. *Environ. Chem. Lett.* **19**, 1001–1023 (2020).
30. Dash, M. & Mohanty, K. Effect of different ionic liquids and anti-solvents on dissolution and regeneration of Miscanthus towards bioethanol. *Biomass Bioenergy.* **124**, 33–42 (2019).
31. Zhai, R., Hu, J. & Saddler, J. N. What are the major components in steam pretreated lignocellulosic biomass that inhibit the efficacy of cellulase enzyme mixtures?. *ACS Sustain. Chem. Eng.* **4**, 3429–3436 (2016).
32. Robak, K. & Balcerek, M. Review of second generation bioethanol production from residual biomass. *Food Technol. Biotechnol.* **56**, 174–187 (2018).
33. Liu, Z.-H. *et al.* Transforming biorefinery designs with 'Plug-In Processes of Lignin' to enable economic waste valorization. *Nat. Commun.* **12**, 1–13 (2021).
34. An, Y.-X., Zong, M.-H., Wu, H. & Li, N. Pretreatment of lignocellulosic biomass with renewable cholinium ionic liquids: Biomass fractionation, enzymatic digestion and ionic liquid reuse. *Bioresour. Technol.* **192**, 165–171 (2015).
35. Gschwend, F. J. V. *et al.* Quantitative glucose release from softwood after pretreatment with low-cost ionic liquids. *Green Chem.* **21**, 692–703 (2019).
36. Brandt, A., Gräsvik, J., Hallett, J. P. & Welton, T. Deconstruction of lignocellulosic biomass with ionic liquids. *Green Chem.* **15**, 550 (2013).
37. Satari, B., Karimi, K. & Kumar, R. Cellulose solvent-based pretreatment for enhanced second-generation biofuel production: A review. *Sustain. Energy Fuels* **3**, 11–62 (2019).
38. Bernardo, J., Gírio, F. & Łukasik, R. The effect of the chemical character of ionic liquids on biomass pre-treatment and posterior enzymatic hydrolysis. *Molecules* **24**, 808 (2019).
39. Den, W., Sharma, V. K., Lee, M., Nadadur, G. & Varma, R. S. Lignocellulosic biomass transformations via greener oxidative pretreatment processes: Access to energy and value-added chemicals. *Front. Chem.* **6**, 141 (2018).
40. Hart, W. E. S., Harper, J. B. & Aldous, L. The effect of changing the components of an ionic liquid upon the solubility of lignin. *Green Chem.* **17**, 214–218 (2014).
41. Pernak, J. *et al.* Synthesis and properties of ammonium ionic liquids with cyclohexyl substituent and dissolution of cellulose. *RSC Adv.* **2**, 8429–8438 (2012).
42. Swatloski, R. P., Spear, S. K., Holbrey, J. D. & Rogers, R. D. Dissolution of cellose with ionic liquids. *J. Am. Chem. Soc.* **124**, 4974–4975 (2002).
43. Zhang, J. *et al.* Application of ionic liquids for dissolving cellulose and fabricating cellulose-based materials: State of the art and future trends. *Mater. Chem. Front.* **1**, 1273–1290 (2017).
44. Kumar, P., Barrett, D. M., Delwiche, M. J. & Stroeve, P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind. Eng. Chem. Res.* **48**, 3713–3729 (2009).
45. Dadi, A. P., Varanasi, S. & Schall, C. A. Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step. *Biotechnol. Bioeng.* **95**, 904–910 (2006).
46. Li, C. *et al.* Scale-up and evaluation of high solid ionic liquid pretreatment and enzymatic hydrolysis of switchgrass. *Biotechnol. Biofuels* **6**, 154 (2013).
47. Pérez de los Ríos, A. *et al.* Keys for bioethanol production processes by fermentation and ionic liquid extraction. *ACS Sustain. Chem. Eng.* **5**, 6986–6993 (2017).
48. Asim, A. M. *et al.* Acidic ionic liquids: Promising and cost-effective solvents for processing of lignocellulosic biomass. *J. Mol. Liq.* **287**, 110943 (2019).
49. Socha, A. M. *et al.* Efficient biomass pretreatment using ionic liquids derived from lignin and hemicellulose. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E3587–E3595 (2014).
50. Halder, P. *et al.* Progress on the pre-treatment of lignocellulosic biomass employing ionic liquids. *Renew. Sustain. Energy Rev.* **105**, 268–292 (2019).
51. Magalhães da Silva, S. P., da Costa Lopes, A. M., Roseiro, L. B. & Bogel-Łukasik, R. Novel pre-treatment and fractionation method for lignocellulosic biomass using ionic liquids. *RSC Adv.* **3**, 16040 (2013).
52. Brandt, A., Hallett, J. P., Leak, D. J., Murphy, R. J. & Welton, T. The effect of the ionic liquid anion in the pretreatment of pine wood chips. *Green Chem.* **12**, 672 (2010).
53. Welton, T. Ionic liquids: A brief history. *Biophys. Rev.* **10**, 691–706 (2018).
54. Verma, C. *et al.* Dissolution of cellulose in ionic liquids and their mixed cosolvents: A review. *Sustain. Chem. Pharm.* **13**, 100162 (2019).
55. Sayyed, A. J., Deshmukh, N. A. & Pinjari, D. V. A critical review of manufacturing processes used in regenerated cellulosic fibres: Viscose, cellulose acetate, cuprammonium, LiCl/DMAc, ionic liquids, and NMMO based lyocell. *Cellulose* **26**, 2913–2940 (2019).
56. Hu, J., Arantes, V. & Saddler, J. N. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: Is it an additive or synergistic effect?. *Biotechnol. Biofuels* **4**, 36 (2011).
57. Long, L., Tian, D., Hu, J., Wang, F. & Saddler, J. A xylanase-aided enzymatic pretreatment facilitates cellulose nanofibrillation. *Bioresour. Technol.* **243**, 898–904 (2017).
58. Long, L. *et al.* Thermostable xylanase-aided two-stage hydrolysis approach enhances sugar release of pretreated lignocellulosic biomass. *Bioresour. Technol.* **257**, 334–338 (2018).
59. Smuga-Kogut, M., Kogut, T., Markiewicz, R. & Słowik, A. Use of machine learning methods for predicting amount of bioethanol obtained from lignocellulosic biomass with the use of ionic liquids for pretreatment. *Energies* **14**, 243 (2021).
60. Smuga-Kogut, M. *et al.* Comparison of bioethanol preparation from triticale straw using the ionic liquid and sulfate methods. *Energies* **12**, 1155 (2019).
61. Smuga-Kogut, M. *et al.* Evaluation of the potential of fireweed (*Epilobium angustifolium* L.), European goldenrod (*Solidago virgaurea* L.), and common broom (*Cytisus scoparius* L.) stems in bioethanol production. *Energy Sci. Eng.* **8**, 3244–3254 (2020).
62. Breiman, L. Random forests. *Mach. Learn.* **45**, 5–32 (2001).
63. Liaw, A. & Wiener, M. Classification and regression by randomForest. *R News* **2**(3), 18–22 (2002).

Acknowledgements
The study was carried out as part of a research project entitled "Production of 2nd generation bioethanol from lignocellulose biomass coming from wasteland using ionic liquids" financed by the National Science Centre as part of MINIATURA 1 No DEC-2017/01/X/NZ9/00357.

Author contributions
M.S.-K. designed the experiment and acquired funding. M.S.-K., D.S., R.M., and T.K. performed the experiments. All authors contributed to the writing of this manuscript, and all authors have consented to this manuscript being sent for publishing.

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to R.M.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

energies

MDPI

Article

Use of Machine Learning Methods for Predicting Amount of Bioethanol Obtained from Lignocellulosic Biomass with the Use of Ionic Liquids for Pretreatment

Małgorzata Smuga-Kogut [1], Tomasz Kogut [2,*], Roksana Markiewicz [3] and Adam Słowik [4]

[1] Department of Agrobiotechnology, Faculty of Mechanical Engineering, Koszalin University of Technology, Raclawicka 15-17, 75-620 Koszalin, Poland; malgorzata.smuga-kogut@tu.koszalin.pl
[2] Department of Geoinformatic, Koszalin University of Technology, Sniadeckich 2, 75-453 Koszalin, Poland
[3] NanoBioMedical Centre, Adam Mickiewicz University in Poznań, Wszechnicy Piastowskiej 3, 61-614 Poznań, Poland; roksana.markiewicz@amu.edu.pl
[4] Department of Computer Engineering, Koszalin University of Technology, Sniadeckich 2, 75-453 Koszalin, Poland; adam.slowik@tu.koszalin.pl
* Correspondence: tomasz.kogut@tu.koszalin.pl; Tel.: +48-943-486-720

Abstract: The study objective was to model and predict the bioethanol production process from lignocellulosic biomass based on an example of empirical study results. Two types of algorithms were used in machine learning: artificial neural network (ANN) and random forest algorithm (RF). Data for the model included results of studying bioethanol production with the use of ionic liquids (ILs) and different enzymatic preparations from the following biomass types: buckwheat straw and biomass from four wastelands, including a mixture of various plants: stems of giant miscanthus, common nettle, goldenrod, common broom, fireweed, and hay (a mix of grasses). The input variables consisted of different ionic liquids (imidazolium and ammonium), enzymatic preparations, enzyme doses, time and temperature of pretreatment, and type of yeast for alcoholic fermentation. The output value was the bioethanol concentration. The multilayer perceptron (MLP) was used in the artificial neural networks. Two model types were created; the training dataset comprised 120 vectors (14 elements for Model 1 and 11 elements for Model 2). Assessment of the optimum random forest was carried out using the same division of experimental points (two random datasets, containing 2/3 for training and 1/3 for testing) and the same criteria used for the artificial neural network models. Data for mugwort and hemp were used for validation. In both models, the coefficient of determination for neural networks was <0.9, while for RF it oscillated around 0.95. Considering the fairly large spread of the determination coefficient, two hybrid models were generated. The use of the hybrid approach in creating models describing the present bioethanol production process resulted in an increase in the fit of the model to $R^2 = 0.961$. The hybrid model can be used for the initial classification of plants without the necessity to perform lengthy and expensive research related to IL-based pretreatment and further hydrolysis; only their lignocellulosic composition results are needed.

Keywords: hemp; mugwort; bioethanol; machine learning; enzymatic hydrolysis

Citation: Smuga-Kogut, M.; Kogut, T.; Markiewicz, R.; Słowik, A. Use of Machine Learning Methods for Predicting Amount of Bioethanol Obtained from Lignocellulosic Biomass with the Use of Ionic Liquids for Pretreatment. *Energies* **2021**, *14*, 243. https://doi.org/10.3390/en14010243

Received: 16 November 2020
Accepted: 4 January 2021
Published: 5 January 2021

1. Introduction

The production of bioethanol is a current topic raised by scientists, technologists, and representatives of fuel companies in the European Union who are working on satisfying the percentage share of this biocomponent in conventional fuels. The policy of the European Union countries is aimed at the search for low-emission technology, which is sustainable and aimed at reducing CO_2 production at relatively low cost of production lines. Taking this into account, research targeting the development of bioethanol preparation from the available lignocellulosic biomass, using the existing production lines or innovative pretreatment methods, is an important topic. The preparation of bioethanol is a complex procedure, the success of which hinges primarily on the use of raw material

that is inexpensive, available in every region of Europe, and has effective and cost-efficient pretreatment, which will result in a high yield of fermenting sugars.

Numerous examples of the production of fermenting sugars from lignocellulose can be found in the literature. A large portion of these publications contain data on the impact of pretreatment on enzymatic hydrolysis of the biomass. The most common include treatments using ammonia fiber expansion (AFEX), steam explosion, ionic liquids (ILs) and other methods [1–5]. There are numerous examples of conversion of rye, corn, rice, wheat straw, poplar, willow, and coniferous tree chips into bioethanol based on the method using pretreatment with ionic liquids and enzymatic hydrolysis [6–10]. However, comparison of the majority of the data has proven to be difficult due to various amounts of substrates used for the process or other pretreatment parameters. Therefore, the use of literature data to design the bioethanol production process at a greater scale is very complicated and problematic. In this study, an attempt was made to create a model that would allow for the estimation or prediction of bioethanol concentration from various lignocellulosic raw materials. Such a model would allow the classification of biomass for bioethanol production on the basis of its chemical composition and facilitate choice of a suitable ionic liquid and enzyme preparation used for pretreatment. Such an approach would save time and costs in laboratory research, which is now a way to search for ideal production methods and an efficient source of lignocellulosic biomass.

Mathematical and statistical models provide essential information for understanding, analyzing, and predicting biological processes, and are necessary to optimize key parameters to improve process performance. Modeling and optimization of biofuel manufacturing processes will contribute to a better understanding of the process expenditures in order to obtain the optimum efficiency. The main purpose of modeling is to optimize the operations involved in their production in order to achieve efficiency improvement [11–13].

Artificial intelligence tools have appeared as a promising method for modeling and optimizing bioprocesses. In the last decade, artificial neural networks (ANNs) were applied in multidimensional, nonlinear research and development of bioprocesses. They have been found effective in developing bioprocess models devoid of prior information on the kinetics and metabolic flows occurring in cells and cell surroundings [14]. Furthermore, ANN are completely based on data, without prior knowledge on the events regulating the process [15]. The appeal of ANN as a modeling tool derives from their unique functions of processing information that is assigned primarily—linearity, high parallelism, and error and noise acceptance—as well as their capability to learn and generalize. ANNs have gained much attention from significant soft computing tools that are not only limited to data processing and analysis, but can also be used to solve problems in multifaceted and nonlinear processes [16].

For these reasons, the aim of this study was to collect the current data on the production of bioethanol from lignocellulosic biomass using ionic liquids and to create a model that can be used to predict ethanol concentration from lignocellulosic raw materials. For this purpose, machine learning (ML) methods were used. Increased interest in the use of ML procedures has been observed, e.g., artificial neural networks (ANNs) and random forests (RFs) in the context of bioethanol production from biomass [17–20]. With the use of these algorithms, it was assumed that on the basis of the results of laboratory tests obtained, it will be possible to predict the concentration of bioethanol in various biomass species based on their lignocellulosic composition. This would aid the classification of biomass type to the suitable ionic liquid or type of cellulolytic enzyme. This preliminary estimation in the case of laboratory studies is important because in this way the costs associated with the synthesis or purchase of ionic liquid and enzymes can be kept to the minimum. It would be possible to perform more rapid verification and grouping of plant types that are found in the given region. Such model could also inspire classification of other pretreatments, and ML data could be applied to a wider extent in scaling-up processes. In order to reduce the costs of the process, plants can be grouped in terms of similarities in their cellulose

content, affinity for ionic liquids, and their influence on the exposure of cellulose fibers or the selection of cellulolytic enzymes for the appropriate type of biomass.

In the present study, the prognostic model was verified by empirical studies of bioethanol production from hemp (*Cannabis sativa* L.) and mugwort (*Artemisia vulgaris* L.). The stems of hemp contain high amounts of cellulose (up to 80%), with lignin content of about 15–20%. In addition, it is a plant that is increasingly commonly grown for the production of oil, fiber, essential oils, etc. The use of both plants properly fit in the criteria of sustainable development, especially considering their biological and agrotechnical properties, which makes them economically and environmentally favorable. The properties exhibited by this biomass source make them admirable for the development of multiprocessor systems by gradually separating the biomass into several useful components. This trait provides hemp with an advantage over other industrial crops, as they are usually used for extraction of one component [21,22]. As far as energy consumption is concerned, it is necessary to emphasize that the mean yield value of green hemp plants was 14.5 t/ha [23] (by means of dry weight). It is possible to obtain about 10.5 t/ha of raw material, which can be potentially used for energy purposes [24]. Parts of the stems that were considered waste or could be used for the production of solid fuel (pellets) were used to produce bioethanol. For contrast, mugwort was used, which is a plant commonly viewed as a weed, growing in arable land as well as boundary strips and agricultural wastelands. This plant possesses approximately 1–1.5 m stems with 35% cellulose content, 25% lignin, and approximately 20% hemicellulose. Mugwort is a native species to Poland. It was introduced to North America, where it has spread and is treated as an invasive species. The mugwort species found in Europe are typically weed and ruderal plants. Common mugwort is the most widespread species. It is characterized by high growth force and good regenerative abilities. New plants can emerge from even finely cut rhizome fragments [25,26].

The production of bioethanol from lignocellulose could become profitable if the costs associated with the production of biomass are very low. Each geographic region is distinguished by a great variety of flora. These can be found in many literature reports stating that the success of the bioethanol production process is influenced by the biomass composition: proportions of cellulose, hemicellulose, and lignin; and the type of fiber arrangement, and thus also the plant species and the degree of its maturity. Taking all these factors into account, it becomes very difficult to test all plant species that could potentially form a good source for the production of bioethanol. Therefore, it is easier to use energy crops for the production of bioethanol. A mathematical model based on experimental data can be a helpful tool in determining the suitability of a plant species for processing into bioethanol. The production of bioethanol is one of the biotechnological processes the complexity of which is high and difficult to present via ready-made algorithms. Therefore, in such situations it is perfect to use ML, including e.g., ANN or RF, as a prediction tool for future biomass samples. This publication attempts to create a model based on experimental data of the bioethanol production process from biomass on a laboratory scale and validate this model based on the results of the experiment—fermentation of hemp and mugwort stems.

2. Materials and Methods

2.1. Raw Materials

Two plant species were used for model validation: stems of hemp, cultivar Finola (plant collection of the Department of Agrobiotechnology, Koszalin University of Technology) and common mugwort stems collected from agricultural wastelands. Stems of mugwort were taken from two wastelands and hemp samples from two extreme cultivation sites within 30 km from Koszalin (N 54°11′26.11″, E 16°10′53.77″), and then the bioethanol production process was carried out for each sample separately in triplicate. The mean values of three replicates for four biomass samples are presented in Table 1. The biomass was collected in late autumn (second half of October 2019). The stems were dried and ground, then the material was pretreated using different ionic liquids.

Table 1. Chemical composition of biomass and glucose content after enzymatic hydrolysis and ethanol content, following alcoholic fermentation of samples of material used for model validation.

Material Name	Composition			Pretreatment	Enzymatic Hydrolysis	Alcoholic Fermentation
	Cellulose [%]	Hemicellulose [%]	Lignin [%]	Ionic Liquid	Glucose [g/L]	Ethanol [g/L]
Hemp (*Cannabis sativa* L.)	55.18	20.42	15.78	[BMIM][OAc]	11.54	8.33
	55.51	17.54	17.8	[BMIM][OAc]	12.27	9.93
	58.37	15.7	18.22	[CHDMA-C4][OAc]	9.16	5.17
	58.98	15.3	11.5	[CHDMA-C4][OAc]	9.85	5.81
	61.79	18.32	10.39	[CHDMA-C6][OAc]	8.56	6.01
	60.11	17.12	12.34	[CHDMA-C6][OAc]	8.49	5.63
	30.9	9.93	13.8	[EMIM][DEP]	5.78	4.78
	32.5	10.1	12.7	[EMIM][DEP]	6.86	4.08
	46.4	15.12	16.26	[EMIM][OAc]	11.32	7.97
	48.25	16.5	15.78	[EMIM][OAc]	11.32	8.28
	62.22	17.72	19.98	untreated	3.49	3.28
	61.51	16.27	11.56	untreated	3.38	2.60
Common mugwort (*Artemisia vulgaris* L.)	46.42	21.05	18.78	[BMIM][OAc]	2.59	1.65
	41.37	21.93	20.44	[EMIM][DEP]	0.26	0.22
	42.78	19.9	16.94	[EMIM][OAc]	3.15	1.50
	46.42	21.05	18.78	[BMIM][OAc]	4.80	2.37
	42.78	19.9	16.94	[EMIM][OAc]	4.86	2.21
	45.1	13.79	20.38	untreated	1.81	0.95
	43.27	11.9	18.76	untreated	2.22	1.12
	41.9	17.72	24.3	[CHDMA-C4][OAc]	2.43	1.42
	42.19	21.03	24.42	[CHDMA-C4][OAc]	2.43	1.53
	42.4	18.14	23.99	[CHDMA-C6][OAc]	1.87	1.29
	41.37	21.93	20.44	[EMIM][DEP]	2.49	1.03
	41.37	21.93	20.44	[EMIM][DEP]	0.60	0.37
	46.42	21.05	18.78	[BMIM][OAc]	3.18	1.56
	42.78	19.9	16.94	[EMIM][OAc]	2.97	1.67

2.2. Ionic Liquids (ILs)

For the pretreatment of cellulose-rich material, five ILs from imidazolium and ammonium groups were chosen. Three were commercially available (Iolitech GmbH, Germany) imidazolium ILs: 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]), 1-butyl-3-methylimidazolium acetate ([BMIM][OAc]), and 1-ethyl-3-methylimidazolium diethyl phosphate ([EMIM][DEP]). The remaining two, belonging to the group of ammonium ILs, were synthesized for the study: butyl(cyclohexyl)dimethylammonium acetate (([CHDMA-C4][OAc]) and (cyclohexyl) hexyldimethylammonium acetate ([CHDMA-C6][OAc]).

2.3. Synthesis of [CHDMA-C4][OAc] and [CHDMA-C6][OAc] Ionic Liquids

Synthesis of [CHDMA-C4][OAc] and [CHDMA-C6][OAc], and its bromide precursors was conducted according to a previous work, where apart from the synthetic route, full characteristics and cellulose dissolution were also described [27]. In that way, appropriate bromides were prepared in a quaternization reaction between cyclohexyldimethylamine and an appropriate 1-bromoalkane with 10% extension, in acetonitrile, at room temperature for 72 h. The crude product was obtained by evaporation of the reaction background in a rotary evaporator. After the ethyl acetate addition, the prepared bromides were filtered

and dried at 40 °C under reduced pressure for 48 h (vacuum dryer). The product was then kept in a desiccator to avoid moisture uptake from the environment. The second step of the ILs preparation was the anion exchange reaction between quaternary bromide and acetic acid. An appropriate bromide (with hexyl or butyl substituent) was dissolved in methanol. To this solution, a previously prepared stoichiometric amount of KOH dissolved in methanol was added. The solutions were stirred for 3 h at ambient temperature. Partially precipitated side product (KBr) was filtrated and an appropriate (stoichiometric) amount of acetic acid was added to the reaction mixture. Solutions were further stirred at the same conditions for 1 h and the reaction background was evaporated in a rotary evaporator (40 °C). The crude product was purified with the addition of anhydrous acetone. The remaining potassium bromide was filtered, and solvent once again removed (rotary evaporator) to give the final product. The ILs obtained ([CHDMA-C4][OAc] and [CHDMA-C6][OAc] were finally dried for 48 h under reduced pressure in a vacuum dryer.

2.4. Pretreatment, Enzymatic Hydrolysis, and Alcoholic Fermentation

Imidazolium ILs, namely [EMIM][OAc], [BMIM][OAc], [EMIM][DEP], [CHDMA-C4][OAc], and [CHDMA-C6][OAc] were used for biomass purification. To achieve this, solutions of appropriate ground material (5 g) and a specific IL (50 mL) were prepared, which were further subjected to homogenization (2 min) and incubation at 120 °C for 2 h. Samples were afterwards left to cool to room temperature. In the next step, the cellulose fibers were isolated via thorough rinsing of the prepared mixture with deionized water. This was repeated at least three times, to the point of total IL removal. The solid fraction obtained was further dissolved in a 50 mM acetate buffer with a pH equal to 5.0 (100 mL). Enzymatic hydrolysis was then performed on the pretreated and nontreated lignocellulosic biomass.

For the enzymatic hydrolysis of hemp stems, Cellic CTec2 (Sigma-Merck, Darmstad, Germany) was used at the amount of 20 FPU/g of cellulose. Samples were incubated at 50 °C for 72 h. On the other hand, the mugwort samples were hydrolyzed using the following enzymatic preparations: Cellic CTec2, cellulase from *Aspergillus* sp., cellulase from *T. reesei* (Sigma-Merck, Darmstad, Germany). The incubation of biomass fractions mixed with *Aspergillus* sp. and *T. reesei* cellulases was performed for 72 h at 47 °C.

Before performing the alcoholic fermentation, the hydrolysate solutions were purified by means of filtration to get rid of any residual lignocellulose. The pH of the fermentation broth was kept constant at 5.0 for each sampling point. The pH control was performed by the adding a solution of H_2SO_4 (10 wt.%) or NaOH (20 wt.%). Freeze-dried distiller's yeast *Saccharomyces cerevisiae* type II (purchased from Sigma-Aldrich) (5%, w/v) were used to initiate ethanol fermentation. This was afterwards allowed to proceed in anaerobic conditions for four days. After fermentation, the samples were further analyzed to establish the ethanol concentrations.

Control samples were hemp and mugwort stalks not pretreated with ionic liquids. Samples were dissolved in an acetate buffer, according to the protocol described for IL pretreated samples. The material was characterized to establish the cellulose, hemicellulose, and lignin content. Glucose content after the process of enzymatic hydrolysis and alcohol concentration after the fermentation process were also determined.

2.5. Analytical Techniques

An Ankom A200 fiber analyzer was used to determine the amounts of lignin/cellulose/hemicellulose in all biomass samples (with the use of filter bag encapsulation). Fiber test results were determined as neutral detergent fibers (NDF) with the use of Van Soest method, and acidic detergent fiber (ADF) and acidic detergent lignin (ADL) according to the standard [28]. The difference between the ADF and ADL fractional share was the cellulose content, while the difference between NDF and ADF fractional share was the hemicellulose content. High performance liquid chromatography (HPLC) was used to determine the amounts of glucose and ethanol (Merck Hitachi, Darmstadt, Germany). For that purpose, the prepared samples were, in the first step, subjected to centrifugation (10 min, 4000× g,

4 °C) with the use of a Multifuge 3SR (Darmstadt, Germany) and filtered in the second step using membrane filters with a pore diameter of 0.22 µm (Millex-GS, Millipore, Burlington, MA, USA). An Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) was used for the separation of glucose and ethanol with a 5 mM solution of H_2SO_4 (mobile phase) at a flow rate of 0.6 mL/min at 30 °C. The detection of glucose and ethanol was performed with a refractive index detector (Model L-7490, Merck Hitachi, Darmstadt, Germany).

3. Experimental Strategy and Overview of Proposed Machine Learning Methods

3.1. Materials—Data for the Model

To create the model, empirical data from experiments on the bioethanol preparation from the following types of lignocellulosic biomass were used: buckwheat straw, biomass from four wastelands, including a mixture of various plants: stems of giant miscanthus, common nettle, goldenrod, common broom, fireweed, and hay. The production process of bioethanol from the abovementioned types of biomass was carried out on the basis of an identical production scheme, which included disintegration of the raw material, pretreatment with the use of IL, enzymatic hydrolysis with the use of five enzymatic preparations and alcoholic fermentation with the use of *Saccharomyces cerevisiae* type II or *Saccharomyces cerevisiae* Ethanol Red yeast. The cellulose, hemicellulose and lignin amounts were determined in each material. After enzymatic hydrolysis, glucose content was determined in the samples, whereas after alcoholic fermentation, ethyl alcohol content was determined. A total of 120 experiments were conducted, on which basis two model types were created. Model validation was carried out on the basis of the concentrations of bioethanol obtained from hemp and mugwort. In summary, 26 experiments were performed and the results obtained were used to validate the model. Biomass samples—mugwort and hemp—were pretreated with the use of various ILs and enzymatic hydrolysis with the use of enzyme preparations. Both in the native material and after pretreatment with ILs, determinations were made for the content of cellulose, hemicellulose, and lignin.

In Model 1 (Figure 1), the following input data were determined: biomass composition, including the content of cellulose (%), hemicellulose (%), lignin (%), and types of ILs used for pretreatment expressed in amount [mL]: [BMIM][OAC], [CHDMA-C4][OAC], [CHDMA-C6][OAc], [EMIM][DEP], [EMIM][OAC], EMIM[Cl], as well as types of enzymatic preparations expressed in amounts (g/L) added in the process of enzymatic hydrolysis and glucose content (g/L) tested after this process.

In Model 2 (Figure 1), the input data were arranged in a different manner and they consisted of the following variables: biomass composition (content of cellulose (%), hemicellulose (%) and lignin (%)), types of ionic liquids ([BMIM][OAC], [CHDMA-C4][OAC], [CHDMA-C6][OAc], [EMIM][DEP], [EMIM][OAC], [EMIM][Cl]), their amounts [mL], and time of purifying material in ionic liquids [min]. Input data of the enzymatic hydrolysis process included the possibility of additions of combinations of two enzymes at the same time—expressed as addition of enzyme 1 and their amount and addition of enzyme 2 and their amount [µL]. The last variable in this model is the content of glucose tested after 72 h of enzymatic hydrolysis. This was necessary because two enzyme preparations simultaneously were used in certain processes to hydrolyze them in order to increase the content of simple sugars in the fermented solutions. In both models, the starting variable was the concentration of bioethanol (g/L) tested after 96 h of alcoholic fermentation. To create models, Matlab for RF and Keras and Tensorflow library in Python for ANN were used.

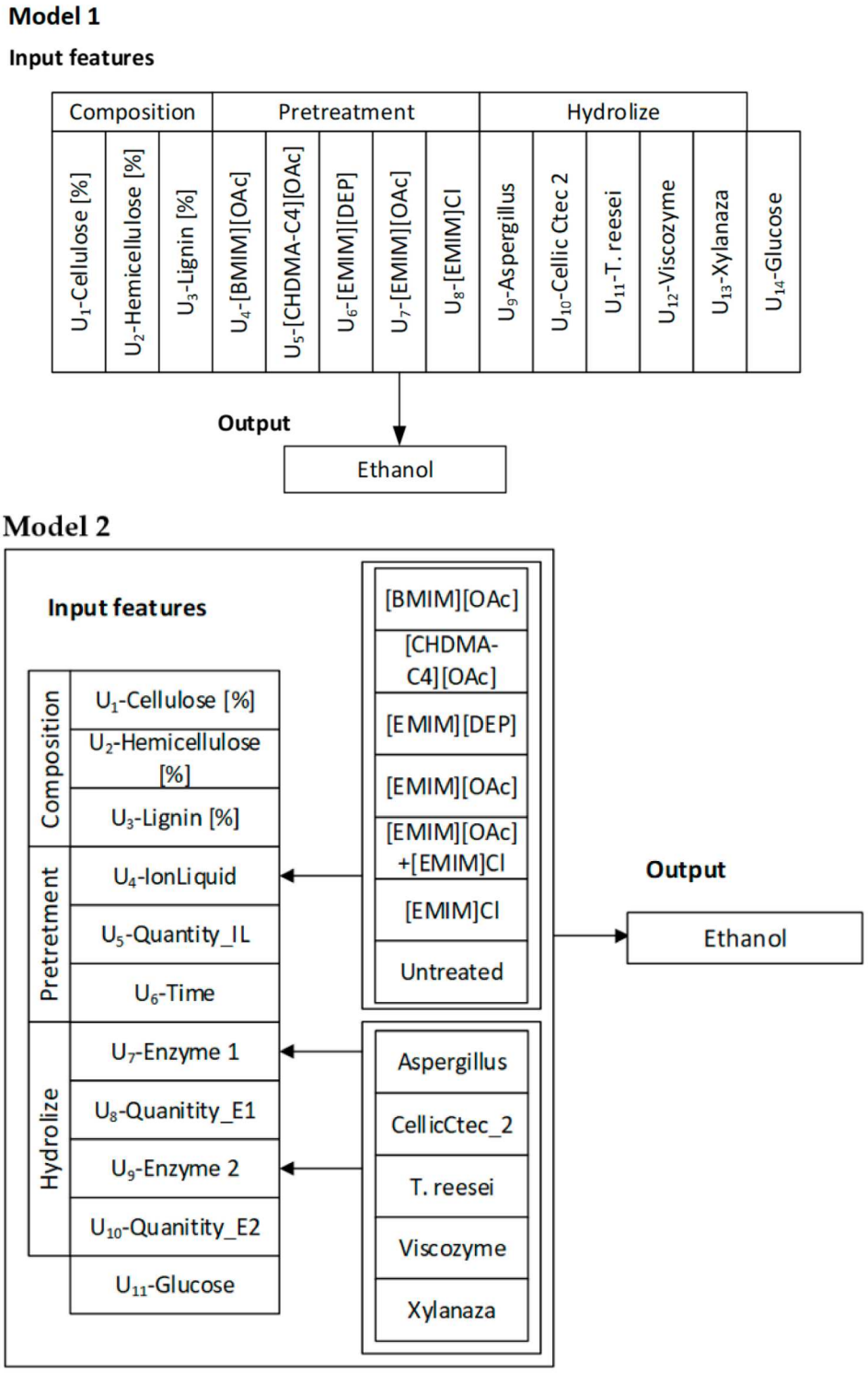


Figure 1. Two models used for the artificial neural network (ANN) and random forest algorithm (RF).

3.2. Methods of Machine Learning Used to Predict Bioethanol Content

3.2.1. Artificial Neural Networks

To carry out the ethanol content predicting process, multilayer perceptron (MLP) artificial neural networks were used, with the architecture shown in Figure 2 (for Model 1) and in Figure 3 (for Model 2).

For both model types, the training dataset comprised 120 vectors (14 elements for Model 1 and 11 elements for Model 2). Tables 1 and 2 present the designations of inputs

and outputs of the artificial neural network from Figure 1 (Model 1) and Figure 2 (Model 2), respectively.

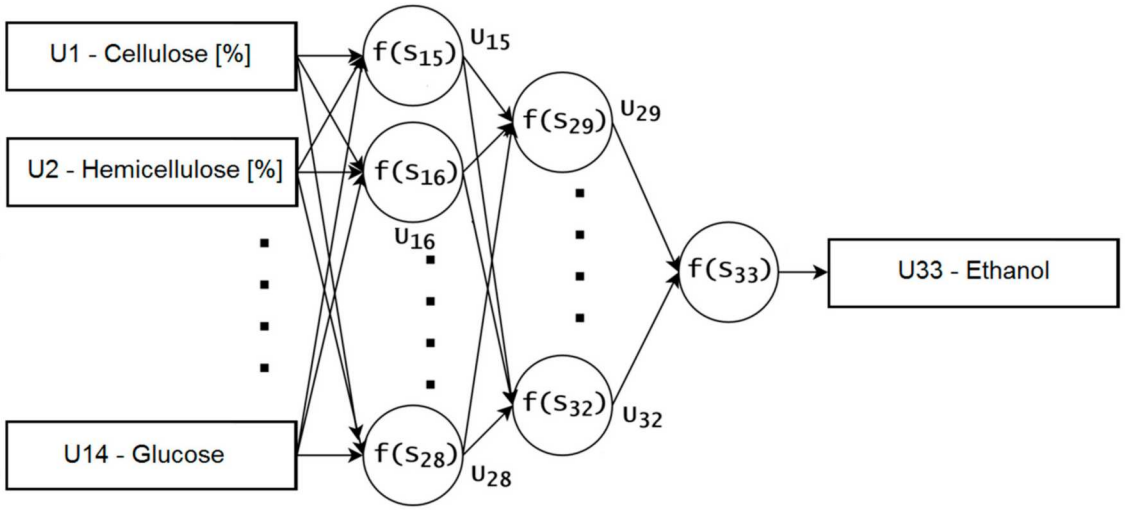


Figure 2. Multilayer perceptron (MLP) artificial neural network for Model 1 implementation.

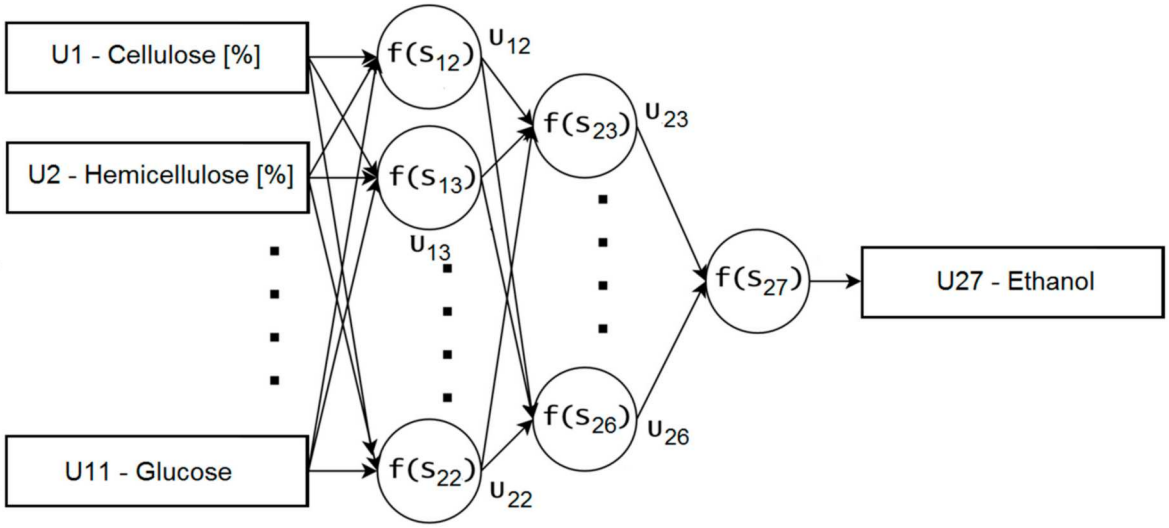


Figure 3. MLP artificial neural network for Model 2 implementation.

Table 2. Validation of the models for bioethanol production from *Cannabis sativa* L. and *Artemisia vulgaris* L.

	Model 1		**Model 2**		**Hybrid 1**		**Hybrid 2**	
	ANN	**RF**	**ANN**	**RF**	**Model 1**	**Model 2**	**Model 1**	**Model 2**
R^2	0.78	0.94	0.88	0.96	0.95	0.96	0.96	0.96
RMSE hemp	2.08	1.20	1.55	0.99	1.25	1.04	1.25	0.82
RMSE mugwort	0.59	0.55	0.28	0.46	0.32	0.36	0.19	0.33
ME hemp	0.59	0.77	0.73	0.71	0.59	0.67	0.84	0.16
ME mugwort	0.40	−0.47	−0.08	−0.43	−0.26	−0.31	−0.10	−0.24
MAE hemp	1.96	0.96	1.38	0.78	1.02	0.92	1.05	0.74
MAE mugwort	0.41	0.47	0.24	0.43	0.26	0.31	0.14	0.29
est_mean hemp	5.50	5.31	5.36	5.38	5.50	5.42	5.25	5.93
est_mean mugwort	1.16	2.04	1.64	1.99	1.82	1.87	1.66	1.81
est_median hemp	5.40	5.03	5.40	5.03	4.69	4.95	4.65	5.38
est_median mugwort	1.24	1.86	1.65	1.92	1.67	1.80	1.56	1.68

The error back-propagation algorithm with a constant value of the learning coefficient ro = 0.01 was adopted as the training algorithm. Before starting the training process, each of the training sets was subjected to the normalization process according to the relationship:

$$U_i^* = \frac{U_i - mean_i}{stdev_i} \tag{1}$$

where $i \in \{1, \ldots, 14\}$ (for Model 1) or $i \in \{1, \ldots, 11\}$ (for Model 2), U_i*—value of input of neural network after normalization, $mean_i$—mean value for all training data for i-th input parameter (attribute), and $stdev_i$—standard deviation value for i-th input attribute.

The $mean_i$ mean value and $stdev_i$ standard deviation values determined were saved to be used for normalization of input data during the process of predicting ethanol value on the test set. The test set comprised 17 vectors (14 elements for Model 1 and 11 elements for Model 2).

After the training data normalization process was carried out, both models were trained with the corresponding training data. The training data comprised two phases. The forward propagation phase consists of randomly selected training vector values of weighed sums S_k and values of f(S_k) outputs for all neurons determined, according to the relationship:

$$S_k = \sum_{t=m}^{n} w_{k,t} * U_t + w_{k,0} \tag{2}$$

where: $k \in \{15, \ldots, 33\}$ (for Model 1) or $k \in \{12, \ldots, 27\}$ (for Model 2). In addition, for Model 1 $m = 1$ and $n = 14$ for input layer neurons ($k \in \{15, \ldots, 28\}$), $m = 15$ and $n = 28$ for intermediate layer neurons ($k \in \{29, \ldots, 32\}$), and $m = 29$ and $n = 32$ for output layer neuron ($k = 33$); for Model 2 $m = 1$ and $n = 11$ for input layer neurons ($k \in \{12, \ldots, 22\}$), $m = 12$ and $n = 22$ for intermediate layer neurons ($k \in \{23, \ldots, 26\}$), and $m = 23$ and n = 26 for output layer neuron ($k = 27$); $w_{k,t}$—value of the weight connecting U_k neuron with neuron or input U_t, and $w_{k,0}$—value of the so-called threshold value for the U_k neuron.

Activation function for input layer neurons $\{U_{15}, \ldots, U_{28}\}$ (for Model 1) $\{U_{12}, \ldots, U_{22}\}$ (for Model 2) and intermediate layer $\{U_{29}, \ldots, U_{32}\}$ (for Model 1) and $\{U_{23}, \ldots, U_{26}\}$ (Model 2) is described with the following relationship:

$$U_k = f(S_k) = \frac{1}{1 + e^{-S_k}} \tag{3}$$

Function of activation for the output layer neuron U_{33} (for Model 1) and U_{27} (for Model 2) is described with relationship:

$$U_k = f(S_k) = S_k \tag{4}$$

After determining baseline values for all neurons, the forward propagation phase is complete and the signal backward propagation phase begins. This phase consists of determining the values of derivatives for all neurons according to the following relationships.

For neurons of the input layer and intermediate layer:

$$U_k' = f'(S_k) = U_k(1 - U_k) \tag{5}$$

For output layer neuron:

$$U_k' = f'(S_k) = 1 \tag{6}$$

In the backward propagation phase, the so-called $delta_k$ coefficients are also determined for each k-th neuron with the following the relationships:

For output layer:

$$delta_k = (C_k - U_k) * f'(S_k) \tag{7}$$

For intermediate layer:

$$delta_k = delta_t * w_{t,k} * f'(S_k) \quad (8)$$

For input layer:

$$delta_k = \left(\sum_{t=x}^{y} w_{t,k} * delta_t \right) * f'(S_k) \quad (9)$$

where: x = 29 and y = 32 (for Model 1) and x = 23 and y = 26 (for Model 2).

After determining $delta_k$ values for all neurons, the backward propagation phase is completed. Then the process of modification of all weight values begins according to the relationship:

$$w^*_{k,t} = w_{k,t} + ro * delta_k * U_t \quad (10)$$

where $w_{k,t}$* is the new value of $w_{k,t}$ weight providing a signal to the U_k neuron from neuron output/input U_t.

After completing the modification of the weights, the first iteration of the training algorithm ends. Then another training vector is randomly selected and the whole process (forward propagation phase, backward propagation phase, and weights update) is repeated until training is completed. The training lasted for 2000 iterations for each neural network described.

3.2.2. Random Forest Algorithm

Random forest is a nonparametric ML algorithm derived from the classification and regression tree. Characteristics of RF include resistance to noise, simplicity of tuning, and capacity to deal with high-dimensional nonlinear problems [29–32]. In this work, RF was used with an RF library in Matlab software and applied to describe the pretreatment and enzymatic hydrolysis. To ensure good predictive performance, the RF was assessed for 11 of the RF samples, similar to the ANN. The model whose RMSE (root mean square error) was the median of all errors was further assessed.

4. Results and Discussion

Mugwort is an example of biomass obtained without the need for cultivation and fertilization, with an average cellulose content of 45%, hemicellulose 13.8%, and lignin 20.4%. For comparison, the conversion was also carried out on hemp stalks, which have recently become very popular for functional reasons. Finola hemp stalks had an average cellulose content of 62%, hemicellulose 17%, and lignin 19%. In this study, for the production of bioethanol, ground plant stalks were used and the process was carried out by performing a pretreatment with the use of various ionic liquids and various enzyme preparations. Glucose content in mugwort samples depended on the type of pretreatment and enzyme preparation used. Table 1 presents glucose contents obtained after 72 h enzymatic hydrolysis, bioethanol content, and chemical composition of hemp and mugwort.

After the enzymatic hydrolysis of mugwort, the highest content of glucose was obtained in the samples where imidazolium ionic liquids ([EMIM][OAc] and [BMIM][OAc]), and Cellic CTec2 for enzymatic hydrolysis were used. A similar relationship was observed in the samples of hemp for reducing sugars, but the results were significantly higher as compared with common mugwort. The content of simple sugars after enzymatic hydrolysis with the use of Cellic CTec2 amounted to 12.27 g/L for material purified with [BMIM][OAc] and 11.32 g/L for biomass purified with [EMIM][OAc]. For comparison, in the sample of native hemp hydrolyzed with Cellic CTec2, 3.2 g/L glucose was obtained after 72 h.

In the experiments with the use of machine learning, including ANN and RF methods for the estimation of bioethanol concentration, results of experiments concerning the processing of hay, agricultural wastelands, and selected energy crops were utilized. The RF method exhibits different advantages than ANN. Each tree represents the learning process and each tree can select traits and samples at random [33]. The final prediction is obtained

by averaging the predictions concerning the trees. This enables efficient avoidance of excessive matching and the effect of single samples [34]. On the other hand, ANN is characterized by singular correlation or learning process. Furthermore, many earlier studies show that the RF may give better predictions for the same problem [35,36].

Experimental data concerning bioethanol production from hemp and mugwort stems were used for the validation of the ANN model. The raw material is characterized by high cellulose content, low lignin content, and better structural properties after processing with ionic liquids; i.e., the material is more porous and there is more area free and available for cellulolytic enzymes; thus enzymatic hydrolysis is facilitated and more efficient. The situation is completely different when a raw material such as common mugwort is used, as its cellulose content is lower by 50% and it contains considerably higher amounts of lignin and hemicellulose. In addition, after dissolution in ionic liquids, common mugwort stems are not deprived of lignin with the same efficiency as for hemp stems. High amounts of lignin remaining in biomass samples directed for enzymatic hydrolysis may be linked to a poorer course of the process, because lignin is an enzyme inhibitor [37]. In this case, the use of ML methods perfectly reflects the processes of pretreatment and enzymatic hydrolysis processes.

The type of biomass, as well as the contribution of cellulose in the composition of plants, has direct influence on the content of simple sugars, including glucose after enzymatic hydrolysis. Thus, the selection of biomass rich in cellulose, as was the case for hemp stems, should be linked to more efficient ethanol production (Figure 4). For Model 1, the use of Cellic CTec2 in enzymatic hydrolysis was most important because, regardless of the type of biomass used, i.e., whether it was weed, woody plants, or energy crops, high concentrations of glucose were obtained when the enzyme was used for the hydrolysis. The pretreatment of biomass and type of ionic liquid applied were also significant for Model 1. More favorable results were obtained in the case of imidazolium liquids, and the most important was the use of [EMIM][OAc] and [BMIM][OAc], for both energy crops—hemp, as well as woody weed with higher lignin content. The content of lignin in the biomass composition is another significant factor affecting the reduction of bioethanol production efficiency. In Model 2, this variable is in the third place, whereas second place is taken by the use of ionic liquids as pretreatment type. Dependable variables that affect the described model are also E1 and E2 enzymes, that is, *T. reesei* and Cellic CTec2 and their amounts, appropriately selected to the content of cellulose after pretreatment.

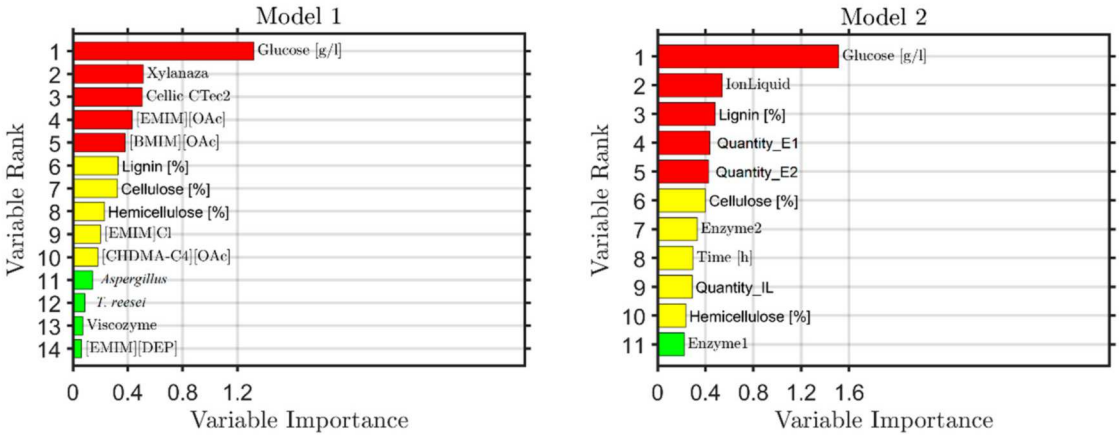


Figure 4. Relative importance of inputs for bioethanol estimation—random forest.

The use of xylanase as an additional enzyme in enzymatic hydrolysis resulted in increased content of simple sugars by decomposing hemicellulose, which is linked to cellulose. Enzymatic digestion of hemicellulose resulted in exposing cellulose fibers, which were then digested by cellulase. Therefore, the use of xylanase in such cases directly

contributed to the increase of the content of glucose in the samples. Considering the costs of the process, the use of an additional enzyme (xylanase) is not valid.

Ahmadian-Moghadam et al. [38] examined the influence of the initial concentration of the substrate (molasses), live yeast cells, and dead yeast cells as the input parameters of the process on the production of bioethanol via *Saccharomyces cerevisiae*. An R^2 value equal to 0.93 was obtained, which shows that the model was suitable for pattern recognition into data and these patterns precisely predicted ethanol efficiency. In the latest research conducted by Betiku and Taiwo [39], the influence of breadfruit hydrolysate concentration, hydraulic retention time, and pH on the production of bioethanol was assessed with ANN and response surface methodology (RSM). The ANN had an absolute mean deviation between the predicted and observed value of 0.09%, compared to 1.67% after RSM [39]. These results further confirm the precision of ANN modeling in comparison with other techniques, such as RSM.

ANN and RF algorithms have a random training start point, thus they were repeated 11 times to ensure higher reliability of the results obtained. The following analyses present results of the iteration whose error was a median of error from 11 replications. In the learning process, the R^2 determination coefficient for Model 1 was 0.92 for ANN and 0.93 for RF. In Model 2, the R^2 coefficient increased to near 1 for ANN, whereas for RF it remained at the same level (Figure 5).

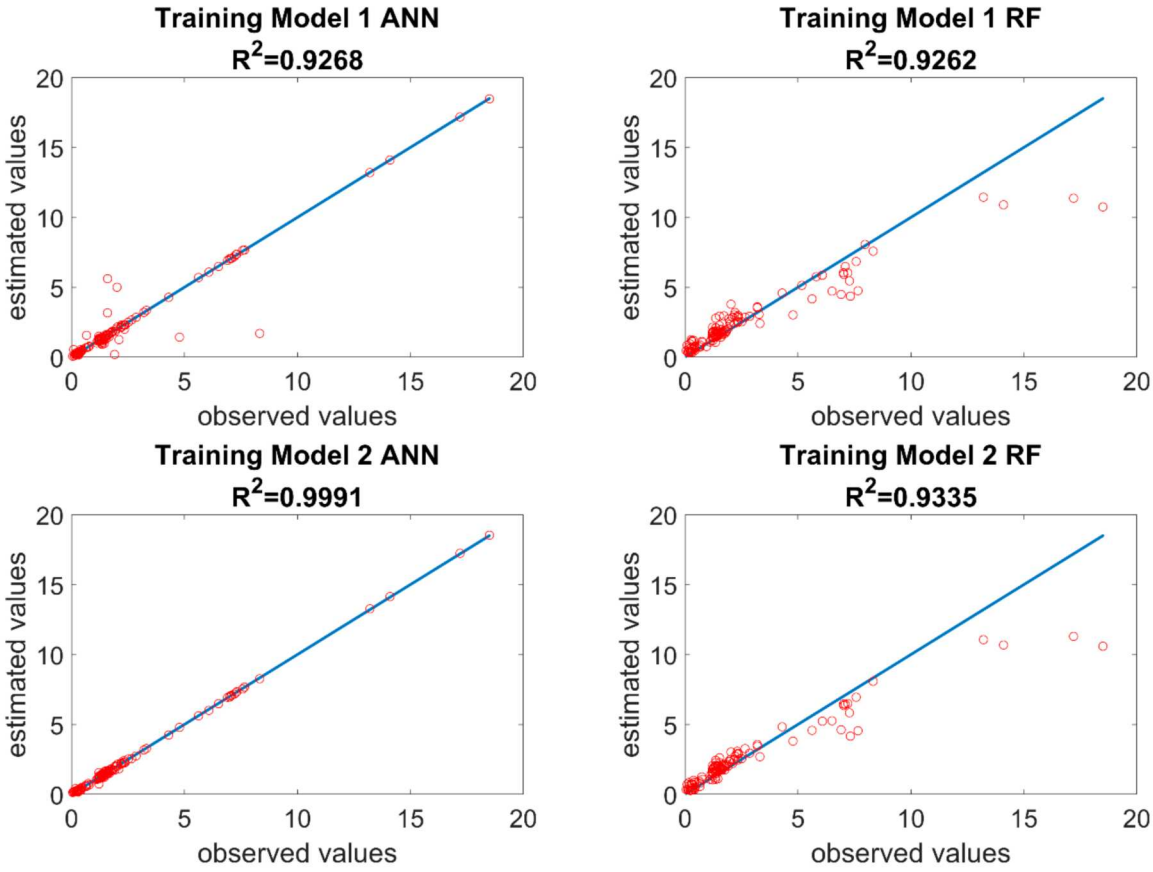


Figure 5. Training models for artificial neural network (ANN) and random forest (RF).

Data for mugwort and hemp were used for validation. In both models, the determination coefficient for neural networks >0.9, whereas for RF it oscillated around 0.95. In the RF training models, there were four wasteland samples whose observed values significantly differ from the estimated values. The ethanol content of these samples was significantly different from the others and due to the principle of operation of the RF algorithm, those samples could not be included. Considering the rather wide dispersion of

the determination coefficient, two hybrid models were executed. The first hybrid model (Hybrid 1) consisted of assuming the median from the set of data estimated from 22 replications. The second hybrid model (Hybrid 2) assumed determination of a linear function describing the variables from the entire training set of 22 replications (11 ANN and 11 RF). Subsequently, median from the validation set was calculated, on the basis of which new estimated values were calculated. The last step of the process was to calculate the value closest to estimated values from the set of 22 values. During the calculation of these models, 70% of points in Model 1 from ANN were selected and 75% of points in Model 2 from ANN were selected. When both hybrid models were applied, a clear increase of determination coefficient can be observed with regards to ANN, and a considerable decrease of RMSE (root mean square error), ME (mean error), and MAE (mean absolute error) for mugwort and hemp. Furthermore, mean values (est_mean) and medians (est_median) of estimates were calculated for each model and algorithm.

Table 2 above describes the sum parameters concerning the presented models of bioethanol production from hemp and common mugwort. The R^2 determination coefficient depended on the type of applied model. The hybrid approach in the creation of models explaining this process of bioethanol production resulted in increased match of the model to $R^2 = 0.961$ for Hybrid 2. In the original calculations, R^2 reached about 0.96 match only for Model 2–RF. Precision of Model 1–Hybrid 2 for the prediction of ethanol production process from biomass is satisfactory and higher than the ANN and RF models. RMSE values for the RF algorithm in each case of validation sample analysis, that is, hemp and mugwort biomass, were lower, and the model was better matched than for ANN. Moreover, differences in R^2 and RMSE relative to the analyzed material can be observed. RMSE was lower for common mugwort samples, the results of which were predicted with the use of ANN. A reverse situation occurred for validation of the model utilizing hemp samples. In this case, lower RMSE with better match of the model ($R^2 = 0.961$) was obtained for Model 2–RF. Considering that the differences were significant and did not provide a clear answer, it was decided to use a hybrid model, which vastly improved the effects of predicting bioethanol concentrations from lignocellulosic materials and provided a better match of the hybrid model to experimental results–validation, presented in Figure 6. The pink color in the Figure 6 was used to mark experimental results of bioethanol concentration obtained from common mugwort stems, and green was used to mark bioethanol concentrations obtained from cannabis stems. The blue asterisk refers to RF values and the red asterisk to ANN values.

In the case of modeling such complex processes as bioethanol production from different lignocellulosic raw materials and taking into account numerous initial samples, the use of only one algorithm type results in difficulties. Due to the concerns that the use of ANN would result in flattening or not using all process conditions and mechanisms, scientists often refer to the comparison with such algorithms as random forest, adaptive neuro–fuzzy inference system, and support vector machine [35,36,40]. The application of a hybrid approach to the discussed issues aimed for a more comprehensive inclusion of the mechanisms that are not yet discovered in bioethanol production, or have not yet been classified as of key importance on bioethanol concentration. In this study, the hybrid model is well matched to the process presented, and it further includes a very wide spectrum of lignocellulosic biomass, not including raw materials due to, e.g., an excessive amount of lignin. This may largely contribute to the expansion of knowledge in the field of bioethanol production from mixed types of lignocellulosic biomass with different chemical compositions, and acceleration of the selection of pretreatment type based only on several input variables.

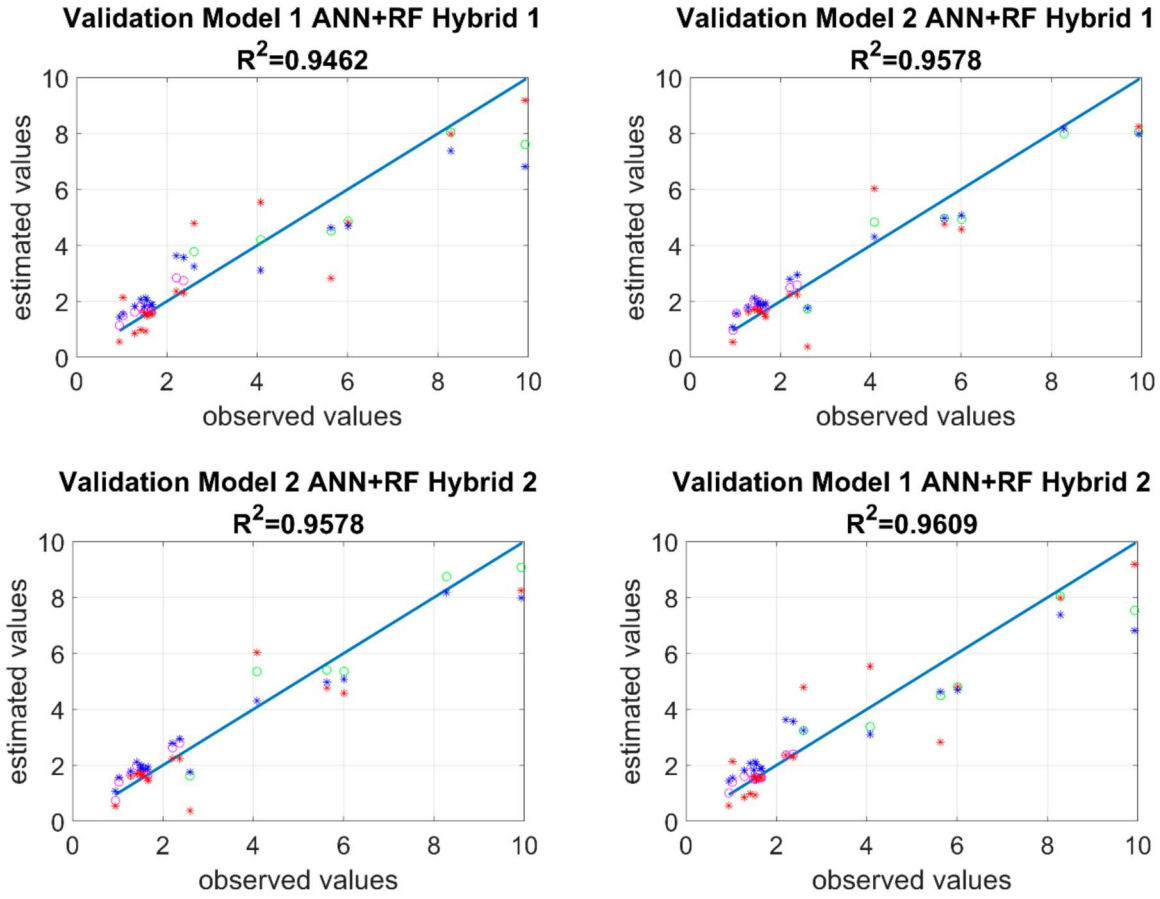


Figure 6. Measured values relative to approximated values for the Hybrid 1 and Hybrid 2 models.

5. Summary

The use of machine learning methods, i.e., ANN and RF, for the prediction biotechnological processes outcomes, in our case bioethanol production, even at a laboratory scale is a very good first step to understand the production mechanism, before going to a large scale. Results of this study suggest that ML is a good tool to predict the final concentration of ethanol obtained in a multistage process of hydrolysis and fermentation of lignocellulosic biomass. Data for this model includes results of bioethanol production with the use of ILs and different enzymatic preparations from the following biomass types: buckwheat straw and biomass from four wastelands, including a mixture of various plants—stems of giant miscanthus, common nettle, goldenrod, common broom, fireweed, and hay. The results obtained for each of the models applied are in a very good agreement with the experimental results. For the process, two extreme biomass cases (hemp and mugwort) were used and the simulations determined the final ethanol value with high likelihood. Importantly, the ANN model alone qualifies the biomass as a good source of bioethanol, mainly on the basis of the cellulose content (as in the case of hemp). The RF, on the other hand, also takes into consideration other variables, such as lignin content. Therefore, the hybrid model proposed is more adequate and takes into consideration other constituents and the level of their changes during the pretreatment process. The hybrid model can be successfully used for the preliminary classification of plants on the basis of the results of their lignocellulosic composition, which means that the selection of an appropriate biomass source can be carried out without long-term and often expensive research. ML is a perfect tool for these types of processes, which can be developed by means of continuous network training. The quality of this study indicates that further research results on the production

of bioethanol from lignocellulose can be used for extending and continuously increasing the verification of the hybrid model.

Author Contributions: Conceptualization, M.S.-K. and T.K.; methodology, M.S.-K., T.K., R.M., and A.S.; software and validation, T.K. and A.S.; formal analysis, M.S.-K., T.K., R.M., and A.S.; investigation, M.S.-K., T.K., R.M., and A.S.; writing—review and editing, M.S.-K., T.K., and A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Elgharbawy, A.A.; Alam, M.Z.; Moniruzzaman, M.; Goto, M. Ionic liquid pretreatment as emerging approaches for enhanced enzymatic hydrolysis of lignocellulosic biomass. *Biochem. Eng. J.* **2016**, *109*, 252–267. [CrossRef]
2. Baral, N.R.; Shah, A. Comparative techno-economic analysis of steam explosion, dilute sulfuric acid, ammonia fiber explosion and biological pretreatments of corn stover. *Bioresour. Technol.* **2017**, *232*, 331–343. [CrossRef]
3. Jönsson, L.J.; Alriksson, B.; Nilvebrant, N.-O. Bioconversion of lignocellulose: Inhibitors and detoxification. *Biotechnol. Biofuels* **2013**, *6*, 16. [CrossRef]
4. Konda, N.; Shi, J.; Singh, S.; Blanch, H.W.; Simmons, B.A.; Klein-Marcuschamer, D. Understanding cost drivers and economic potential of two variants of ionic liquid pretreatment for cellulosic biofuel production. *Biotechnol. Biofuels* **2014**, *7*, 86. [CrossRef] [PubMed]
5. He, L.; Wang, C.; Shi, H.; Zhou, W.; Zhang, Q.; Chen, X. Combination of steam explosion pretreatment and anaerobic alkalization treatment to improve enzymatic hydrolysis of Hippophae rhamnoides. *Bioresour. Technol.* **2019**, *289*, 121693. [CrossRef] [PubMed]
6. da Silva, S.P.M.; da Lopes, A.M.C.; Roseiro, L.B.; Bogel-Łukasik, R. Novel pre-treatment and fractionation method for lignocellulosic biomass using ionic liquids. *RSC Adv.* **2013**, *3*, 16040–16050. [CrossRef]
7. Momayez, F.; Karimi, K.; Karimi, S.; Horváth, I.S. Efficient hydrolysis and ethanol production from rice straw by pretreatment with organic acids and effluent of biogas plant. *RSC Adv.* **2017**, *7*, 50537–50545. [CrossRef]
8. da Costa Lopes, A.M.; João, K.G.; Bogel-Łukasik, E.; Roseiro, L.B.; Bogel-Łukasik, R. Pretreatment and Fractionation of Wheat Straw Using Various Ionic Liquids. *J. Agric. Food Chem.* **2013**, *61*, 7874–7882. [CrossRef]
9. Rosenau, T.; Potthast, A.; Sixta, H.; Kosma, P. The chemistry of side reactions and byproduct formation in the system NMMO/cellulose (Lyocell process). *Prog. Polym. Sci.* **2001**, *26*, 1763–1837. [CrossRef]
10. Goshadrou, A.; Karimi, K.; Taherzadeh, M. Ethanol and biogas production from birch by NMMO pretreatment. *Biomass Bioenergy* **2013**, *49*, 95–101. [CrossRef]
11. Sewsynker-Sukai, Y.; Faloye, F.; Kana, E.B.G. Artificial neural networks: An efficient tool for modelling and optimization of biofuel production (a mini review). *Biotechnol. Biotechnol. Equip.* **2017**, *31*, 221–235. [CrossRef]
12. Fischer, J.; Lopes, V.S.; Cardoso, S.L.; Coutinho Filho, U.; Cardoso, V.L. Machine learning techniques applied to lignocellulosic ethanol in simultaneous hydrolysis and fermentation. *Braz. J. Chem. Eng.* **2017**, *34*, 53–63. [CrossRef]
13. Bohdal, T.; Charun, H.; Kruzel, M.; Sikora, M. An investigation of heat transfer coefficient during refrigerants condensation in vertical pipe minichannels. *E3S Web Conf.* **2018**, *70*, 02001. [CrossRef]
14. Gueguim Kana, E.B.; Oloke, J.K.; Lateef, A.; Adesiyan, M.O. Modeling and optimization of biogas production on saw dust and other co-substrates using Artificial Neural network and Genetic Algorithm. *Renew. Energy* **2012**, *46*, 276–281. [CrossRef]
15. Shi, Y.; Gai, G.; Zhao, X.; Zhu, J.; Zhang, P. Back Propagation Neural Network (BPNN) Simulation Model and Influence of Operational Parameters on Hydrogen Bio-Production through Integrative Biological Reactor (IBR) Treating Wastewater. In Proceedings of the 2010 4th International Conference on Bioinformatics and Biomedical Engineering, Chengdu, China, 18–20 June 2010; pp. 1–4.
16. Almeida, J.S. Predictive non-linear modeling of complex data by artificial neural networks. *Curr. Opin. Biotechnol.* **2002**, *13*, 72–76. [CrossRef]
17. Vani, S.; Sukumaran, R.K.; Savithri, S. Prediction of sugar yields during hydrolysis of lignocellulosic biomass using artificial neural network modeling. *Bioresour. Technol.* **2015**, *188*, 128–135. [CrossRef]
18. Das, S.; Bhattacharya, A.; Haldar, S.; Ganguly, A.; Gu, S.; Ting, Y.P.; Chatterjee, P.K. Optimization of enzymatic saccharification of water hyacinth biomass for bio-ethanol: Comparison between artificial neural network and response surface methodology. *Sustain. Mater. Technol.* **2015**, *3*, 17–28. [CrossRef]
19. Giordano, P.C.; Beccaria, A.J.; Goicoechea, H.C.; Olivieri, A.C. Optimization of the hydrolysis of lignocellulosic residues by using radial basis functions modeling and particle swarm optimization. *Biochem. Eng. J.* **2013**, *80*, 1–9. [CrossRef]
20. Gitifar, V.; Eslamloueyan, R.; Sarshar, M. Experimental study and neural network modeling of sugarcane bagasse pretreatment with H_2SO_4 and O_3 for cellulosic material conversion to sugar. *Bioresour. Technol.* **2013**, *148*, 47–52. [CrossRef]
21. Struik, P.; Amaducci, S.; Bullard, M.J.; Stutterheim, N.; Venturi, G.; Cromack, H.T.H. Agronomy of fibre hemp (*Cannabis sativa* L.). *Ind. Crops Prod.* **2000**, *11*, 107–118. [CrossRef]
22. Burczyk, H. Oilseed hemp (*Cannabis sativa* L. var. oleifera) grown for seeds, oil and biogas. *Probl. Inż. Rol.* **2016**, *24*, 109–116.

23. Schluttenhofer, C.; Yuan, L. Challenges towards Revitalizing Hemp: A Multifaceted Crop. *Trends Plant Sci.* **2017**, *22*, 917–929. [CrossRef] [PubMed]
24. Kraszkiewicz, A.; Kachel, M.; Parafiniuk, S.; Zając, G.; Niedziółka, I.; Sprawka, M. Assessment of the Possibility of Using Hemp Biomass (*Cannabis Sativa* L.) for Energy Purposes: A Case Study. *Appl. Sci.* **2019**, *9*, 4437. [CrossRef]
25. Holst, N.; Rasmussen, I.; Bastiaans, L. Field weed population dynamics: A review of model approaches and applications. *Weed Res.* **2007**, *47*, 1–14. [CrossRef]
26. Jezierska-Domaradzka, A.; Kuźniewski, E. Ruderal plants within segetal communities in south-west port of Poland. *Zesz. Przyr. OTPN* **2000**, *34*, 5–11.
27. Pernak, J.; Kordala, R.; Markiewicz, B.; Walkiewicz, F.; Popławski, M.; Fabiańska, A.; Jankowski, S.; Łożyński, M. Synthesis and properties of ammonium ionic liquids with cyclohexyl substituent and dissolution of cellulose. *RSC Adv.* **2012**, *2*, 8429–8438. [CrossRef]
28. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [CrossRef]
29. Breiman, L.; Friedman, J.; Olshen, R.; Stone, C.J. *Classification and Regression Trees*; Chapman and Hall/CRC: London, UK, 1983.
30. Breiman, L. Random Forests. *Mach Learn* **2001**, *45*, 5–32. [CrossRef]
31. Seyedhosseini, M.; Tasdizen, T. Disjunctive normal random forests. *Pattern Recognit.* **2015**, *48*, 976–983. [CrossRef]
32. Liaw, A.; Wiener, M. Classification and regression by random forest. *R News* **2002**, *2*, 18–22.
33. Ho, T.K. Random decision forests. In Proceedings of the 3rd International Conference on Document Analysis and Recognition, Montreal, QC, Canada, 14–16 August 1995; IEEE Computer Society Press: Montreal, QC, Canada, 1995; Volume 1, pp. 278–282.
34. Hu, S.; Liu, H.; Zhao, W.; Shi, T.; Hu, Z.; Li, Q.; Wu, G. Comparison of Machine Learning Techniques in Inferring Phytoplankton Size Classes. *Remote Sens.* **2018**, *10*, 191. [CrossRef]
35. You, H.; Ma, Z.; Tang, Y.; Wang, Y.; Yan, J.; Ni, M.; Cen, K.; Huang, Q. Comparison of ANN (MLP), ANFIS, SVM, and RF models for the online classification of heating value of burning municipal solid waste in circulating fluidized bed incinerators. *Waste Manag.* **2017**, *68*, 186–197. [CrossRef] [PubMed]
36. Xing, J.; Wang, H.; Luo, K.; Wang, S.; Bai, Y.; Fan, J. Predictive single-step kinetic model of biomass devolatilization for CFD applications: A comparison study of empirical correlations (EC), artificial neural networks (ANN) and random forest (RF). *Renew. Energy* **2019**, *136*, 104–114. [CrossRef]
37. Kellock, M.; Maaheimo, H.; Marjamaa, K.; Rahikainen, J.; Zhang, H.; Holopainen-Mantila, U.; Ralph, J.; Tamminen, T.; Felby, C.; Kruus, K. Effect of hydrothermal pretreatment severity on lignin inhibition in enzymatic hydrolysis. *Bioresour. Technol.* **2019**, *280*, 303–312. [CrossRef]
38. Ahmadian-Moghadam, H.; Elegado, F. Prediction of Ethanol Concentration in Biofuel Production Using Artificial Neural Networks. *Am. J. Model. Optim.* **2013**, *1*, 31–35. [CrossRef]
39. Betiku, E.; Taiwo, A.E. Modeling and optimization of bioethanol production from breadfruit starch hydrolyzate vis-à-vis response surface methodology and artificial neural network. *Renew. Energy* **2015**, *74*, 87–94. [CrossRef]
40. Grahovac, J.; Jokić, A.; Dodić, J.; Vučurović, D.; Dodić, S. Modelling and prediction of bioethanol production from intermediates and byproduct of sugar beet processing using neural networks. *Renew. Energy* **2016**, *85*, 953–958. [CrossRef]