The Use of Giant Miscanthus (*Miscanthus × Giganteus*) in 2G Bioethanol Production

Małgorzata Smuga-Kogut[1]*, Sławomir Bugajski[2], Tomasz Piskier[3], Bartosz Walendzik[4]*

[1]*Department of Agrobiotechnology, Faculty of Mechanical Engineering,*
Koszalin University of Technology, Poland
https://orcid.org/0000-0001-8486-5949

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

[3]*Department of Agrobiotechnology, Faculty of Mechanical Engineering,*
Koszalin University of Technology, Poland
https://orcid.org/0000-0003-0890-6301

[4]*Faculty of Civil Engineering, Environmental and Geodetic Sciences,*
Koszalin University of Technology, Poland
https://orcid.org/0000-0001-8366-7457

**corresponding author's e-mail: malgorzata.smuga-kogut@tu.koszalin.pl bartosz.walendzik@tu.koszalin.pl*

Abstract: The study aimed to obtain bioethanol from biomass using chemical treatment and enzymatic hydrolysis. Different concentrations of sodium hydroxide (5 and 10%) were used for the delignification process, and enzymatic hydrolysis was carried out using three commercial cellulolytic preparations (Cellic® CTec2, cellulase from *Trichoderma reesei* and cellulase from *Aspergillus species*). The final step involved an alcoholic fermentation process using *Saccharomyces cerevisiae* TYPE II yeast. After enzymatic hydrolysis, the content of reducing sugars was determined in the samples, and the fermentation yield was controlled by determining the ethanol content by pycnometry. Using chemical pretreatment increased the yield of the whole process by at least 50%. The content of reducing sugars after hydrolysis depended on the type of enzyme preparation used for hydrolysis and the use of NaOH in pretreatment. The highest reducing sugars content (45.8 g/dm^3) was achieved in a sample of material purified with 5% NaOH, and enzymatic hydrolysis was carried out using Cellic® CTec2. It means the efficiency of the enzymatic hydrolysis process equals 94.69%. The concentration of bioethanol after alcoholic fermentation in this sample was 0.509 g/l.

Keywords: giant miscanthus, bioethanol, chemical treatment of biomass, enzymatic hydrolysis, alcoholic fermentation

1. Introduction

Due to technological advances, there is a steady increase in global energy demand. Conventional energy sources are significantly limited and are gradually being depleted. The world's reserves of conventional fuels are steadily shrinking. As a result, the use of alternative sources of energy generation is increasingly being discussed. One of them is lignocellulosic biomass, from which bioethanol can be produced. Liquid biofuels such as ethanol are mostly produced from edible plants such as grains and sugar cane. The largest producers of this type of fuel are the United States and Brazil. These countries produce bioethanol based on crops massively grown in them. The United States bases its production on corn, and Brazil bases its production on sugar cane. In EU countries, bioethanol is produced from cereals, sugar beets and potatoes(Gumienna et al. 2009, Nowacki 2007, Szymanowska & Grajek 2009). In Poland, lignocellulosic biomass is considered the largest potential source of energy, and demand for it continues to grow year after year. In the world, plant biomass occupies a key position among renewable resources. Its annual production is about 170 billion tons, of which about 75% is carbohydrates, 20% is lignin, and only 5% is the remaining components (Burczyk 2012). The raw material for ethanol production can be food products, from which first-generation bioethanol is obtained, as well as lignocellulosic biomass based on wood-based materials. This waste from agricultural production or fast-growing plants is the second-generation bioethanol raw material. Third-generation biofuels are fuels derived from algae. Fuels produced from bio-based products have a positive impact on reducing greenhouse gas emissions, reducing the use of fossil fuels, driving agricultural development and improving diversification in the fuel market. The EU Renewable Energy Directive 2015/1513 (Directive (EU) 2015/1513 of the European Parliament and of the Council of 9 September 2015 Amending Directive 98/70/EC Relating to the Quality of Petrol and Diesel Fuels and Amending Directive 2009/28/EC on the Promotion of the Use of Energy from Renewable Sources (Text with EEA Relevance), 2015) sets a target for 2020, i.e., a 10% share of biocomponents in liquid fuels; the maximum amount of first-generation biofuels must not exceed 7%; the minimum amount of higher-

generation biofuels must be 3%. Directive 2018/2001, the so-called RED II or Biofuels Directive (Directive (EU) 2018/2001 of the European Parliament and of the Council of 11 December 2018 on the Promotion of the Use of Energy from Renewable Sources (Recast) (Text with EEA Relevance.), 2018) published in 2018, continues the EU's renewable energy sources (RES) policy, imposing a target of 32% of this energy in 2030, including 14% in transport, and a 40% reduction in CO_2 emissions. Meeting this directive requires, among others, the production of second-generation bioethanol based on lignocellulosic biomass. The possibility of using ethanol as a potential alternative energy source necessitates reducing production costs so that they become competitive with the prices of traditional fuels. One solution is to develop new technological solutions based on low-cost raw materials to reduce the cost of production. Therefore, it has become very important to look for new, fast-growing plant species that form a large biomass, which, in addition, can be grown on soils of lower classes and agricultural wastelands. The selection of particular species for targeted cultivation is determined by local climatic and soil conditions, cultivation costs, competitiveness of other markets, bioethanol yield obtained per unit area of cultivation, yield potential and stability.

One of the more promising plants that has caught the attention of scientists looking for new raw materials for the biofuel industry in recent years is giant miscanthus (*Miscanthus × giganteus*). Giant miscanthus is a plant that can be grown on light and marginal soils, often degraded by industrial activities. Thus, it does not compete with areas for typical agricultural production. This grass belongs to the C-4 pathway group characterized by a very efficient photosynthetic process, which provides a large increase in biomass from the assimilative surface, which allows to obtain dry matter of 19 t/ha (Matyka & Kuś 2011), 25 t/ha (Wawro et al. 2013). Miscanthus biomass is characterized by high cellulose content, a favourable technological feature for bioethanol production. The average cellulose content of miscanthus dry biomass (M×G) is 41.1% (Lee & Kuan 2015), or 42.1% (Wawro et al. 2013). The hemicellulose content of 36.7% of dry matter is another favourable feature of miscanthus biomass. Suitable plant raw materials and optimized bioprocess technology are essential in bioethanol production.

Obtaining bioethanol from stalks of giant miscanthus is a multi-step process, in which the most important processes are pretreatment to increase the availability of cellulose for cellulolytic enzymes and enzymatic hydrolysis to obtain a sufficient amount of fermentable sugars. The present study attempts to clarify the effect of chemical pretreatment and the type of enzyme preparation on the content of bioethanol obtained from giant miscanthus.

2. Materials and Methods

The research material consisted of stalks of giant miscanthus (*Miscanthus × giganteus*) from the Plant Collection of the Department of Agrobiotechnology. Aboveground parts were harvested in September 2017 and ground in a colloid mill to a grain size of no more than 10 µm.

Sodium hydroxide solutions of 5% and 10% (Roch) were used to pretreat shredded stalks of giant miscanthus. Sodium hydroxide is an inorganic chemical compound from the hydroxide group, belonging to the strongest bases. In liquid form, it is a colourless, odourless, non-flammable liquid called sodium lye.

Enzymatic hydrolysis of giant miscanthus stalks (1 g miscanthus, 50 ml acetate buffer, pH 5.0) involved an addition of 250 µl of enzyme preparations: Cellic® CTec2 (Sigma Aldrich), cellulase from *Trichoderma reesei* (Sigma Aldrich) and cellulase from *Aspergillus species* (Sigma Aldrich).

Saccharomyces cerevisiae type II yeast (Sigma Aldrich) was used for alcoholic fermentation.

2.1. Biomass pretreatment

Pretreatment was carried out in two variants: using solutions of 5% and 10% NaOH at a rate of 100 ml for every 5 g of raw material. At the same time, a control sample was prepared with 5 g of raw material and 100 ml of deionized water. The whole sample was incubated in a shaking water bath (Elpin Plus, type 357) at 250 rpm, at 50°C for 1.5 hours. After incubation, the miscanthus was separated from the NaOH solution and washed with deionized water. The raw material was dried at 105°C for 1 h and then subjected to enzymatic hydrolysis.

2.2. Enzymatic hydrolysis

For enzymatic hydrolysis, 1 g each of pretreated giant miscanthus was used and dissolved in 50 ml of acetic buffer, pH 5.0. Enzyme preparations were added to the samples so prepared according to the following scheme:
- 10T - cellulase from *Trichoderma reesei* – 250 µl per 1 g of miscanthus pretreated with 10% NaOH, 50 ml of acetate buffer pH 5.0;
- 10A - cellulase from *Aspergillus species* – 250 µl per 1 g of miscanthus pretreated with 10% NaOH, 50 ml of acetate buffer pH 5.0;

- 10C - Cellic® CTec2 enzyme preparation – 250 µl per 1 g of miscanthus pretreated with 10% NaOH, 50 ml of acetate buffer pH 5.0;
- 5T - cellulase from *Trichoderma reesei* – 250 µl per 1 g of miscanthus pretreated with 5% NaOH, 50 ml of acetate buffer pH 5.0;
- 5A - cellulase from *Aspergillus species* – 250 µl per 1 g of miscanthus pretreated with 5% NaOH, 50 ml of acetate buffer pH 5.0;
- 5C - Cellic® CTec2 enzyme preparation – 250 µl per 1 g of miscanthus pretreated with 5% NaOH, 50 ml of acetate buffer pH 5.0;
- 0 - Enzyme preparation Cellic® CTec2 – 250 µl per 1 g of miscanthus without pretreatment, 50 ml of acetate buffer pH 5.0.

The study was conducted in triplicate for each variant (both before and after pretreatment). Enzymatic hydrolysis was performed in a shaking water bath (250 rpm) for 72 h, at 50°C. During the process, the content of reducing sugars was determined at appropriate time intervals (every 24 h). After enzymatic hydrolysis, biomass solutions were decanted, and the hydrolysates were subjected to alcoholic fermentation using the *Saccharomyces cerevisiae* type II yeast (Sigma Aldrich).

2.3. Alcoholic fermentation

Alcoholic fermentation was carried out in 100 ml fermentation flasks with a fermentation plug filled with H_2O. After adding yeast (5% w/w), the samples were incubated at 37°C for 96 h. After this time, the ethanol content was determined using the pycnometric method.

2.4. Analytical methods

The dry weight content of cellulosic substrates was determined in the samples. The content of reducing sugars was determined using the DNS method (Ghose 1987). Ethyl alcohol after fermentation was determined using the pycnometric method. The method involves distilling alcohol from a sample and then, based on the pycnometric determination of the distillate's density, reading the sample's ethanol content expressed as a percentage by volume from alcoholometric tables. The alcohol was distilled in a Bűchi Distillation Unit K – 314 apparatus to determine the ethanol content of each sample. The Student's t-test was applied, using Statistica 13 software from StatSoft, to determine the significance of differences in the content of reducing sugars and ethanol concentration in the samples.

3. Results and Discussion

Bioethanol production technology is a multi-step process in which pretreatment determines the success of the entire method. The goal of any pretreatment used is to break the bonds between lignin, cellulose, and hemicellulose molecules and remove them completely or partially (Kim et al. 2016, Wilk & Krzywonos 2015). The effect of lignin composition and cellulose type on the digestibility of biomass saccharification was described by (Chen & Dixon 2007, Studer et al. 2011, Yoshida et al. 2008). In this way, cellulose pulp is obtained analogously to paper production. Depending on how pretreatment is applied, cellulose with an amorphous or crystalline structure is obtained to varying degrees (Ebringerová et al. 2005, Kumar et al. 2009). Amorphous fractions are more desirable in bioethanol production because they are first converted to fermentable sugars during hydrolysis. When choosing the type of pretreatment, consideration should also be given to the low cost of solvents used, their concentration and non-toxic environmental impact (Alvira et al. 2010). In the present study, miscanthus stalks were ground and pretreated by suspending the material in a 5 and 10% NaOH solution for 1.5h at 50°C. The material was then rinsed with distilled water and dried. Pretreatment with NaOH is mainly aimed at delignifying the biomass. Ester and glycosidic bonds of biomass are broken using this method, which causes changes in the structure of lignin, partial decrystallization of cellulose and partial dissolution of hemicellulose (Li et al. 2013, Wilk & Krzywonos 2015).
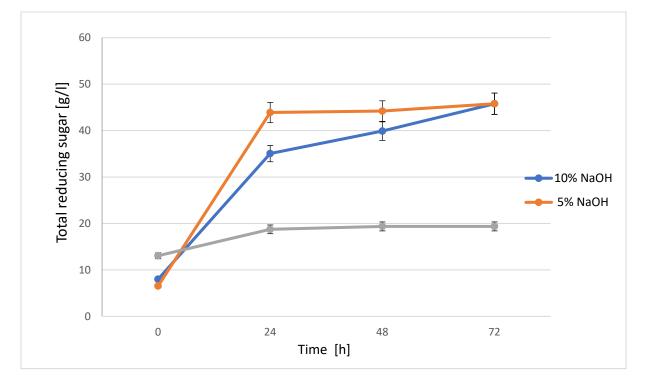
In the method of chemical hydrolysis of biomass with alkali, concentrations of 0.5 to 5% m/v of these compounds are used. Reaction time is from several minutes for high concentrations to several days for very low concentrations. The process temperatures are 100-150°C, or ambient temperature (Kumar et al. 2009). Using alkali results in less loss of sugars compared to acid hydrolysis. The advantages of using NaOH in pretreatment include high delignification efficiency, significant degradation of hemicelluloses, swelling of biomass that facilitates the availability of hydrolytic enzymes (Hendriks & Zeeman 2009), mild process conditions (relatively low temperature, low concentration and pressure), no need for plants with special features that would increase the operating and investment costs of the technology (Mosier et al. 2005), market availability

and low purchase price, low concentrations that have less impact on the environment. The disadvantages of using NaOH for biomass pretreatment include the impact on cellulose's structure, which deteriorates its susceptibility to enzymes (Li et al. 2013), and the release of large amounts of inhibitors such as phenolic compounds.
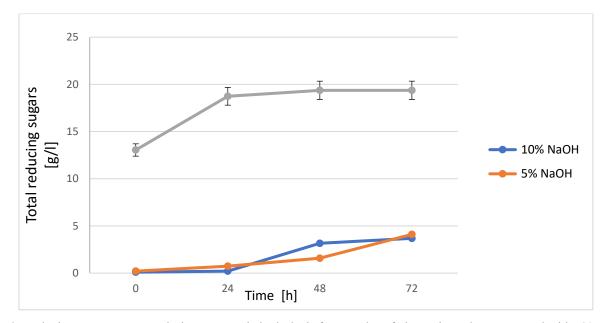
(Lee & Kuan 2015) incubated shredded giant miscanthus biomass in a 12% NaOH solution at 70°C for 4 hours and obtained 77% delignification of the biomass and removal of 44% of hemicelluloses, resulting in a cellulose hydrolysis efficiency above 95%. The same authors pretreated with NaOH and obtained 83.92% glucose from a miscanthus harvest in Korea under optimal conditions. (Scheller & Ulvskov 2010) studied the negative effect of crosslinking hemicelluloses with cellulose through hydrogen bonds. (Li et al. 2013) proved in their study that hemicelluloses in miscanthus biomass are tightly bound to cellulose, which negatively affects cellulose crystallinity. (Adani et al. 2011) found that removing hemicelluloses increases the average pore size of the substrate, which facilitates the hydrolysis of cellulose.

In the present study, the miscanthus pulp was hydrolyzed after the pretreatment step using three types of enzyme preparations. At the same time, a control sample was made, which was ground, not pretreated miscanthus. The study made it possible to assess the content of fermentable sugars after enzymatic hydrolysis in miscanthus samples and classify the suitability of each pretreatment method for bioethanol production. The effect of the type of enzyme preparation on the content of reducing sugars after enzymatic hydrolysis was also checked. The results of reducing sugars concentrations are shown in Figures 1-3.
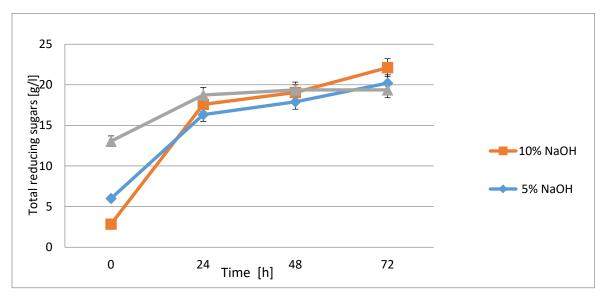
The enzyme preparation Cellic® CTec2 from Novozymes (Denmark) was used for enzymatic hydrolysis. It is a high-performance, industrially used preparation for hydrolysis of lignocellulosic raw materials, showing high cellulolytic and hemicellulolytic activity. The preparation is characterized by increased glucosidase activity, improving the hydrolysis efficiency of lignocellulosic raw materials due to reducing the inhibitory effect of cellobiose. It contains a complex of aggressive cellulases for the degradation of cellulose to fermentable sugars, is characterized by a high level of β-glucosidases, and also has hemicellulases in its composition. The enzyme preparation Cellic® CTec2 contains state-of-the-art enzymes that have proven effective in hydrolyzing various pretreated lignocellulosic materials (Dąbkowska et al. 2012, Dąbkowska & Pilarek 2013). In the present study, using a 5% and 10% NaOH solution and Cellic® CTec2 enzyme preparation for enzymatic hydrolysis in the pretreatment, the highest concentration of reducing sugars of 45.8 g/l was obtained. The native sample yielded 19.4 g/l.

Fig. 1. Reducing sugars contents during enzymatic hydrolysis for samples of giant miscanthus pretreated with 5% and 10% NaOH and enzymatic hydrolysis with cellulases from *Trichoderma reesei*, at pH 5.0

Fig. 2. Reducing sugars contents during enzymatic hydrolysis for samples of giant miscanthus pretreated with 5% and 10% NaOH and enzymatic hydrolysis with cellulases from *Aspergillus species*, at pH 5.0

Fig. 3. Reducing sugars contents during enzymatic hydrolysis for samples of giant miscanthus pretreated with 5% and 10% NaOH and enzymatic hydrolysis with cellulases from Cellic® CTec2, at pH 5.0

The second enzyme preparation used in the study was cellulases from *Trichoderma reesei* (Sigma Aldrich). This enzyme is produced by *T. reesei*, the predominant microorganism used to produce cellulolytic enzymes on an industrial scale. The cellulose hydrolyzing enzyme complex derived from *T. reesei* comprises three basic enzymes. Endo-β-1,4-glucanase hydrolyzes β-1,4-glycosidic bonds within the cellulose chain. Exo-β-1,4-glucanase cleaves cellobiose or glucose units from the non-reducing ends of cellulose. β-glucosidase (cellobiose) catalyzes the hydrolysis reaction of cellobiose to two glucose molecules and cleaves glucose molecules from the non-reducing ends of cellooligosaccharides. According to (Wang et al. 2004), the complex derived from *Trichoderma reesei* includes two cellobiohydrolases, at least five endoglucanases, and a cellobiose. Cellobiohydrolase I degrades the cellulose chain from the non-reducing end, cellobiohydrolase II degrades cellulose from the reducing end. Endoglucanases, on the other hand, randomly attack the cellulose chain from the centre. The complex of endoglucanases and cellobiohydrolases act synergistically with each other to enhance cellulose degradation (Tolan & Foody 1999). The optimal conditions for the enzyme complex are 50°C and pH 5.0 (Gupta et al. 2011). Using this preparation in the present study, a 20.2 g/l content of reducing sugars was obtained in a sample of miscanthus that was treated with 5% NaOH, while after treatment with 10% NaOH, the value of reducing sugars was 22.1 g/l. The results of reducing sugars depended significantly on the enzyme

preparations used (p = 0.02). In contrast, the concentration of NaOH used for pretreatment had no statistically significant effect on the content of these sugars after hydrolysis.

The third enzyme preparation used in the study was cellulases from *Aspergillus species* (Sigma Aldrich). It contains endo-β-D-glucanase, which is one of the main enzyme components of the cellulase complex. It catalyzes the hydrolysis of cellulose by randomly dividing the sugar residues in the molecule. It also contains exo-β-D-glucanase and β-glucosidase that can synergistically convert cellulose into glucose. The student's t-test was used to determine the significance of reducing sugars' content after enzymatic hydrolysis and ethanol after alcoholic fermentation. It was shown that pretreatment significantly affects the content of reducing sugars after enzymatic hydrolysis for samples in which *Aspergillus species* and Cellic® CTec2 were used for enzymatic hydrolysis. For miscanthus samples hydrolyzed with cellulases from *Trichoderma reesei*, pretreatment with NaOH did not significantly affect the contents of reducing sugars after enzymatic hydrolysis. (Muzakhar 2019) determined the optimal conditions for the action of this enzyme complex are temperature 55°C and pH 5.0, but these enzymes are also stable at temperatures below 50°C and pH range 3.0-6.5.

The zero sample without pretreatment had an initial reducing sugar content of about 13 g/dm^3, and after 24 hours the reducing sugar content stabilized at about 19 g/dm^3. Pretreatment significantly affected CR content (p = 0.008), particularly in samples containing the enzyme preparation Cellic® CTec2. The native sample, after 72 hours of enzymatic hydrolysis, yielded 19.36 g/dm^3, while the pretreated sample yielded 45.78 g/dm^3, and the CR content was not dependent on the concentration of NaOH in the pretreatment. The content of monosaccharides at 45.78 g/dm^3 implies an efficiency of the enzymatic hydrolysis process equal to 94.69%, the highest result among the samples tested. The present study shows that by using pretreatment to increase the availability of cellulose for cellulolytic enzymes, an increase in the content of reducing sugars in the samples by 42.3% was obtained compared to the native sample.

(Alvira et al. 2010) stated that pretreatment of lignocellulose with alkalis, such as calcium hydroxide, ammonia or sodium hydroxide, results in the degradation of bonds connecting lignin to other polymers and partial liquefaction of the complex, as well as the removal of some lignin, and increases the availability of cellulose to hydrolytic enzymes. In samples in which giant miscanthus was pretreated with 5% and 10% NaOH, followed by hydrolysis catalyzed by the enzyme preparation Cellic® CTec2 at pH 5.0, a gradual increase in the content of monosaccharides can be observed from 8 g/l after pretreatment through 35 g/l and 40 g/l on subsequent days of enzymatic hydrolysis, up to a value of 45.8 g/l after 72 h (the contents refer to the 10C preparation). Using the same enzyme preparation but after pretreatment with 5% NaOH, the following concentrations of reducing sugars were obtained at individual time intervals: 6.52 g/l, 43.89 g/l, 44.21 g/l and 45.79 g/l after 72 h, respectively. The highest increase in reducing sugars is obtained after 24 h of the hydrolysis process.

The lowest CR was obtained after hydrolysis catalyzed by cellulase from *Aspergillus species* with pretreatment with 10% NaOH, as only 3.7 g/l was obtained, and this value is more than 12 times lower compared to the result obtained with the enzyme preparation Cellic® CTec2. For sample 10A, 3.7 g/l was obtained, and the process yield was 7.62%. The sample catalyzed by cellulases from *Aspergillus species* with pretreatment with 5% NaOH yielded 4.1 g/dm^3 after 72 h, and the maximum yield was 8.49%.

It is reasonable to assume that the action of the enzymes in this formulation is inhibited by inhibitors that are products of the process. (Öhgren et al. 2007) proved that inhibitors in the enzymatic hydrolysis process can be cellobiose and glucose.

For the sample in which giant miscanthus was pretreated with 5% NaOH, followed by hydrolysis catalyzed by cellulase from *Trichoderma reesei*, the following reducing sugars were obtained at pH 5.0: 6 g/l – 0 h; 16.3 g/l – 24 h; 17.9 g/l – 48 h; 20.2 g/l – 72 h. The variant with 10% NaOH pretreatment yielded 2.8 g/l – 0 h; 17.6 g/l – 24 h; 19.1 g/l – 48 h; 22.1 g/l – 72 h, respectively. The highest efficiency of the cellulose saccharification process using cellulases from *Trichoderma reesei* was 41.80%.

Han et al. (2011) investigated the enzymatic hydrolysis of corn straw polysaccharides (cellulose content 43.33%) to produce reducing sugars. They used pretreatment with 2% NaOH at 80°C, pH 4.8 for 1 hour, then hydrolyzed the samples with cellulases from *Trichoderma reesei*. The hydrolysis yield after 48 h was 65.9%.

In the present study, the contents of reducing sugars in the control sample were as follows: 13 g/l – 0 h; 18.7 g/l – 24 h; 19.4 g/l – 48 h; 19.4 g/l – 72 h. The maximum yield of the process was 40.05%.

In samples 10T, 10C, 5T, 5C, and 0, the largest increase in glucose content was recorded after 24 hours of the enzymatic hydrolysis reaction. After that, there was a slight increase in concentrations ranging from 0.8-12.81% during the next 24 hours and from 0.0-16.3% in the last time interval. In the case of sample 10A, the largest increase in reducing sugars occurred after the second day of enzymatic hydrolysis, while in sample 5A it occurred on the third day of hydrolysis. The individual samples differed in their initial content of monosaccharides compared to their final value after 72 hours of enzymatic hydrolysis.

(Dąbkowska & Pilarek 2013) performing enzymatic hydrolysis of lignocellulosic raw material from energy willow (*Salix viminalis* L.) containing 39.03% cellulose, pretreated by steam explosion method, using the enzyme preparation Cellic® CTec2 obtained 9.95 g/l of monosaccharides after 72 h of hydrolysis, which is much less than in the case of described studies on hydrolysis of biomass from giant miscanthus. The result obtained is 4.6 times lower than the present study's. Thus, it should be concluded that the type of lignocellulosic biomass significantly affects the efficiency of enzymatic hydrolysis and bioethanol production.

(Swiatek et al. 2011) state that the efficiency of enzymatic hydrolysis of lignocellulosic raw materials depends on their type, maturity, chemical composition and pretreatment. The authors also studied the suitability of cellulolytic enzyme preparations, composed in different combinations, for the hydrolysis of rapeseed straw polysaccharides. They chemically treated rapeseed straw under the following conditions: temperature 121°C, time 1 h, addition of NaOH 0.1 g·g^{-1} s.s. Enzymatic hydrolysis was performed for 72 h, at 50°C, pH 5.0. Their study showed that the most effective enzyme complex was a set of cellulases and hemicellulases from *T. longibrachiatum* and cellobiose (Novozym 188). This enzyme complex allowed the release of reducing sugars at 48.82 g/l. In this paper's study, a maximum of 45.8 g/l of monosaccharides was obtained, and this result is comparable. (Kordala et al. 2013) conducted a study to determine the effect of pretreatment of giant miscanthus and rapeseed straw with a 15% ammonia solution on the hydrolysis process of the polysaccharides they contain. The study was conducted in two variants of the process: I – 20ºC/24 h or II – 80ºC/6 h. The authors found that in both variants of the chemical treatment there was a partial delignification of the lignocellulosic raw material, the percentage of polysaccharides in the biomass increased and their susceptibility to enzymatic hydrolysis increased. The enzymatic hydrolysis process was carried out using a shaking method at 40ºC and pH 5.0. Three enzyme preparations were used: cellulase from *Trichoderma longibrachiatum*, xylanase from *T. longibrachiatum* and cellobiose. After 72 h of hydrolysis of samples from giant miscanthus, the concentration of released sugars was 45.73 g/l, and from rapeseed straw, it was 26.82 g/l (variant II). The result of reducing sugars concentrations achieved by the publication's authors is similar to the results of the present study.

3.1. Alcoholic fermentation of giant miscanthus (*Miscanthus × giganteus*)

After enzymatic hydrolysis, samples of giant miscanthus were subjected to alcoholic fermentation using the yeast *Saccharomyces cerevisiae* type II. (Sigma Aldrich). Alcoholic fermentation was carried out in fermentation flasks under anaerobic conditions at 37°C for 96 h. After this time, the ethanol content of the distillates was determined using the pycnometric method. Measurements were made at 20°C in triplicate. The results of the ethanol concentrations obtained in the tested samples are shown in Figure 4.
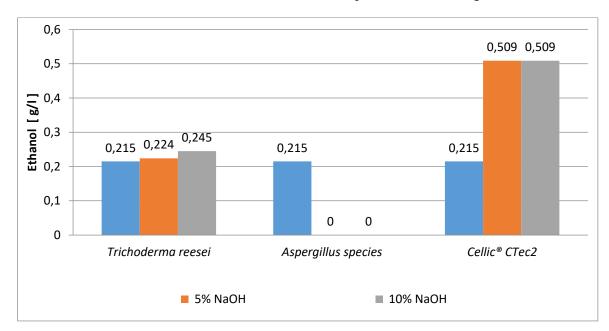

Fig. 4. Ethanol concentration in samples of giant miscanthus before and after pretreatment with different concentrations of NaOH

The highest ethanol concentration of 0.509 g/l was obtained from samples pretreated with 5% and 10% NaOH and subjected to enzymatic hydrolysis with Cellic® CTec2. The efficiency of the process is more than 98%. Samples in which hydrolysis was catalyzed by cellulases from *Aspergillus species* were not fermented because the reducing sugars concentration was too low. The ethanol concentration in samples where cellulases

from *Trichoderma reesei* were used for enzymatic hydrolysis was slightly higher than in the native sample (0.215 g/l). It was 0.224 g/l in the sample of miscanthus pretreated with 5% NaOH and 0.245 g/l in the sample of miscanthus using 10% NaOH for pretreatment. Analyzing the obtained ethanol concentrations after alcoholic fermentation, it can be concluded that the concentration of sodium hydroxide used for pretreatment does not affect the ethanol content. When using the enzyme preparation Cellic® CTec2 to hydrolyze the biomass of giant miscanthus, the efficiency of the fermentation process was the highest. In contrast, using a preparation with cellulases from *Trichoderma reesei* allowed the ethanol content to be more than half lower. Analysis of the results showed that the content of reducing sugars obtained after enzymatic hydrolysis significantly affects the ethanol concentration after alcoholic fermentation ($p = 0.002$).

(Han et al. 2011) studied the suitability of giant miscanthus biomass for bioethanol production. Optimizing the conditions of each step, the miscanthus biomass was pretreated with 1.49 M NaOH at 145.29°C with a reaction time of 28.97 min. A complex of cellulases and β-glucosidases was used for enzymatic hydrolysis, and the process was carried out at 50°C for 72 h. Fermentation was carried out for 48 h with *S. cerevisiae* yeast at 32°C. A yield of 84.69% was obtained using this technology. (Lee & Kuan 2015) studied four species of the Miscanthus genus: *Miscanthus floridulus*, *Miscanthus sinensis*, *Miscanthus sacchariflorus*, and *Miscanthus × giganteus*. They treated the biomass of these species with various types of pretreatments, including NaOH solution, obtaining an ethanol yield of 84.69% after 72 h of fermentation.

4. Summary

Based on the study, it can be concluded that there is a technology to produce bioethanol from giant miscanthus. Of the analyzed technologies for bioethanol production from giant miscanthus (*Miscanthus × giganteus*), the most efficient method was 5% NaOH for pretreatment of the material and Cellic® CTec2 for enzymatic hydrolysis. Using pretreatment with NaOH results in a 136% increase in bioethanol production efficiency compared to untreated miscanthus. Using 5% NaOH for pretreatment more than doubled the efficiency of the enzymatic hydrolysis process. Still, the higher concentration of sodium hydroxide (10%) did not affect the increase in reducing sugars in the examined material. The reducing sugars content obtained from giant miscanthus depends on the enzyme preparation. The highest yield (94.7%) was obtained in a sample of giant miscanthus purified with 5% and 10% NaOH and subjected to enzymatic hydrolysis using Cellic® CTec2. The ethanol concentration after alcoholic fermentation depends on the content of simple sugars in the mash. The highest bioethanol concentration of 0.509 g/l was obtained from giant miscanthus, pretreated with 5% NaOH and subjected to enzymatic hydrolysis using Cellic® Tec2.

Hydrolysis of cellulose to sugars, which then are subjected to alcoholic fermentation due to the complexity of the structure of the lignocellulosic complex, is the most difficult step in the production of ethanol from lignocellulosic raw materials. Achieving high yields in the bioconversion of lignocellulosic substrate to ethanol determines the profitability of second-generation bioethanol production (Leja et al. 2007). Optimization of technological parameters of bioethanol production increases its efficiency (Wawro et al. 2013). When choosing a bioethanol production method, its efficiency and production cost analysis should be considered. Energy balance and environmental issues are also important aspects. As the biomass-to-energy conversion process's complexity increases, the final product's energy efficiency decreases (Martyniak et al. 2017). In the process of second-generation bioethanol production, it is possible to obtain several by-products, and their more efficient use increases the profitability of production (Balat & Balat 2009). The main technological problem in bioethanol production is properly selecting enzymes for biomass hydrolysis achieving simultaneous fermentation of glucose and xylose using the right yeast strain (Biernat 2007).

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