Perennial Grasses as a Substrate for Bioethanol Production

Merlin RAUD^{1*}, Timo KIKAS²

^{1,2} *Institute of Technology, Estonian University of Life Sciences, Kreutzwaldi 56, 51014 Tartu, Estonia*

Abstract – One of the possible choices as a biomass for lignocellulosic bioethanol production are different perennial grasses. Cultivating this type of biomass has many advantages in terms of natural diversity and landscape protection. In this study, mixture of red clover and timothy grass was used as a feedstock to investigate its potential as a substrate for bioethanol production. Traditional three step bioethanol production process was used in combination with NED pretreatment. The results show at all pretreatment temperatures similar glucose concentrations and hydrolysis efficiencies, which varied from 4.3 to 5.1 g/l and 15.2 % to 17.7 %, respectively. The ethanol yield, on the other hand, decreased as the pretreatment temperature increased. However, the mass balance revealed that when using this kind of feedstock, 3.3-4.0 g ethanol could be produced from 100 g of biomass. The overall efficiency and yield of the process was lower than expected due to pretreatment, which might not have been suitable for soft biomass.

Keywords – Bioethanol; biomass; perennial grass; pretreatment

1. INTRODUCTION

We are living in times where we are moving from fossil fuel based economy to an economy based on bioresources. In transportation sector, by 2020 there must be 10 % share of biofuels, and by 2030 this is further increased. Part of the used transportation fuels must be advanced biofuels produced from non-food biomass like lignocellulosic biomass [1], [2]. The lignocellulosic biomass can come from residues of agricultural and forestry sectors and from dedicated energy crops no longer required for food production [3].

Recently, much attention has been paid to bioenergy production from different biomass sources and thereby, perennial grasses have gained attention as a biomass source for biofuel production. Perennial plants are grown on degraded lands that are no longer suitable for agricultural use and thereby, their use for biofuel production can decrease the competition with foodcrops [4]. In addition, these plants can be used as cover crops in a crop rotation since they can increase soil C and N storage, reduce the potential for N leaching and N fertilization rates [5]. Moreover, grasslands are recognized for their role in providing beneficial ecosystem services and wider benefits to society. These include the food and bioenergy production, biodiversity of grassland ecosystems, landscape and aesthetic values, climate regulation, soil and water protection at local and regional scales, etc. [6].

The traditional bioethanol production process consists of three process steps – pretreatment, enzymatic hydrolysis, and fermentation. The pretreatment step is used to open the biomass structure and make the cellulose accessible for enzymatic hydrolysis. In the enzymatic hydrolysis,

* Corresponding author.

E-mail address: Merlin.Raud@emu.ee

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the cellulose polymer is hydrolysed into glucose monomers. Hydrolysis is finally followed by fermentation, where the glucose is fermented into ethanol [8].

The pretreatment is one of the most important steps to gain effective bioethanol production process. Due to tight packaging of cellulose in lignin and hemicellulose cover, the cellulose is difficult to access and this makes the production process inefficient. The pretreatment enables to increase the process efficiency by breaking down lignin and hemicellulose cover and thereby, making cellulose more accessible for further processing [7]. Various pretreatment methods have been proposed and studied. As the biomass composition and pretreatment methods differ, individual approach for selection of suitable processing methods is needed for different biomasses. The choice of a suitable method depends on the biomass characteristics and the following process steps [8].

In this study, combined physio-chemical method, nitrogen explosive decompression (NED) pretreatment was used [8]–[10]. The method is based on applying high pressure and temperature to biomass. The pressure is increased by adding N₂ gas to the biomass. Autohydrolysis is initiated by the temperature and the nitrogen molecules will penetrate into the cells of biomass. If the pressure is decreased in an explosive manner, the dissolved nitrogen gas expands and opens the cellulosic structure of the biomass [8]–[10].

The NED pretreatment method has been used to pretreat agricultural residues, like barley straw [9], [11] and floodplain meadow hay [10], but also for processing municipal greening waste [12] and timber residues from different wood species. However, it has not been used for pretreatment of perennial grasses and other dedicated feedstocks. In addition, little research has been done on using perennial grasses as a bioethanol feedstock.

The goal of this study was to investigate the use of mixed perennial grasses – red clover and timothy grass as a feedstock and its potential as a substrate for bioethanol production. Traditional three step bioethanol production process was used with NED method for pretreatment of biomass. Samples from various process stages were collected and analysed to see the suitability of this pretreatment to perennial grasses. The collected data was used to calculate the mass and energy balances to quantify the material flows and assess different technological routes for effective bioethanol production.

2. MATERIALS AND METHODS

2.1. Biomass

Mixture of red clover (*Trifolium pratense*) and timothy grass (*Phleum pratense* L.) with an average red clover content was 42 % was used as a biomass in all experiments. The samples were dried to gain moisture content less than 10 % and ground with Cutting Mill SM 100 comfort (Retsch GmbH, Germany) to gain particle-size 3 mm or less.

2.2. Production Route Design

To evaluate in which process step solid and liquid separation would give biggest effect, the bioethanol production from biomass was evaluated using two different routes, as shown in Fig. 1. In both routes, the three-step bioethanol production process was used. In case of route 1, the solid and liquid fractions were separated after enzymatic hydrolysis and only the liquid part was used in ethanol production process. In case of route 2, the biomass slurry was used in fermentation process without solid and liquid separation. After hydrolysis, yeast (*Saccharomyces cerevisiae*) was added to liquid part or to biomass slurry in fermentation step to study the bioethanol production potential.

2.3. Explosive Decompression Pretreatment

The instrumentation and working principles of ED pretreatment method are described in detail in previous publications [9], [10], [13]. 700 ml of distilled water was added to 100 g of dried and milled biomass and mixed thoroughly to gain homogenous biomass slurry. The pretreatment was conducted at temperatures of $125\text{--}175\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$ according to previously described methods [9], [10], [13].

Fig. 1. A flow chart of the studied routes of biomass for biofuel production.

2.4. Enzymatic hydrolysis and fermentation

Enzymatic hydrolysis with enzyme complex was used to convert cellulose to glucose. Distilled water and enzyme were added to the pretreated biomass in order to gain the final volume of the mixture 1000 ml. Enzyme mixture (Accellerase 1500 from DuPont®; 30 FPU g^{-1} cellulose) was added to biomass suspension at a ratio of 0.3 ml per g of biomass. Hydrolysis lasted for 24 hours at a temperature of $50\text{ }^{\circ}\text{C}$ under constant stirring in rotating shaker/incubator (250 min^{-1}) (Unimax 1010, Heidolph Instruments GmbH & Co.KG) [13].

Vacuum filtration was used to separate the liquid part from the solid part and only the liquid part was used for fermentation. 1.0 g of yeast *Saccharomyces cerevisiae* was added to the 200 ml of liquid phase to start the fermentation process. The fermentation process was carried out at room temperature ($22\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$) for 7 days under low oxygen conditions in 250 mL glass bottles sealed with a fermentation tube [13].

2.5. Biomass analysis

The solid and liquid parts of the biomass mixture were weighed before and after pretreatment and enzymatic hydrolysis. From each step corresponding samples were taken for analysis. The initial biomass but also solid biomass gained after pretreatment and enzymatic hydrolysis was dried and milled for further analysis.

Dry matter content was analysed with a moisture analyser Kern MLS-D (KERN & SOHN GmbH) and ash content according to NREL Technical Report NREL/TP-510-42622 [14].

The biomass was submitted to an acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) tests to determine the contents of cellulose, hemicellulose and lignin using ANKOM 2000 analyser (ANKOM Technology, USA).

The glucose and ethanol concentrations were determined using HPLC Shimadzu Prominence-i LC-2030 3D Plus and detector Shimadzu RID-20A. The analytical separation of glucose was accomplished with the columns RezexTM RPM-Monosaccharide Pb+2 and RezexTM RHM-Monosaccharide H+, which were arranged in sequence. The column oven temperature was 85 °C and the detector temperature 60 °C. Autoclaved and degassed distilled water was used as a mobile phase at a flow rate of 0.8 mL/min. For the sugar quantification, a calibration range from 50 mg/L to 2000 mg/L was chosen. The injection volume of the samples was 20 µL.

The column RezexTM ROA-Organic Acid H+ was used for the detection of ethanol. The column oven and detector temperature were set to 50 °C. Autoclaved and degassed 5 mM H₂SO₄ was used as mobile phase at a flow rate of 0.5 mL/min. The calibration ranged from 200 mg/L to 20 g/L for acetic acid and ethanol quantification. The sample injection volume was 10 µL. All measurements were conducted in triplicate and average and standard deviations were calculated.

3. RESULTS AND DISCUSSION

3.1. Biomass

The relative proportion of cellulose, hemicellulose, lignin, ash, and moisture is usually used to characterize the biomass. The chemical composition of biomass varies according to its cultivation location and method but also from harvesting season [15]. Previous research has indicated that energy crops for ethanol production should be selected based on their cellulose content since ethanol yield per kg of biomass has been shown to be directly proportional to the cellulose content in the energy crop [16]. As seen in Table 1, the selected biomass had cellulose content of 25.8 % and rather low hemicellulose and lignin contents. Compared to cereal straws reported by different authors [11], [17]–[19], the cellulose content in the biomass was lower than in agricultural residues however, it can be considered a suitable raw material for bioethanol production.

TABLE 1. COMPOSITION OF BIOMASS

Sample	Ash, %	ADF, %	NDF, %	Lignin, %	Cellulose, %	Hemicellulose, %
Biomass	6.4	31.0 ± 0.5	49.3 ± 2.8	5.1 ± 0.4	25.8 ± 0.2	18.4 ± 2.9

3.2. Hydrolysis

The pretreated biomass was used in a conventional three-step bioethanol production process, where enzymatic hydrolysis followed the pretreatment. To assess the effect of pretreatment, the glucose concentrations were measured before and after enzymatic hydrolysis.

In Fig. 2, hydrolysis efficiencies and glucose concentrations in hydrolysis mixture after hydrolysis has been presented. The glucose concentrations in hydrolysis mixture varies from 4.3 to 5.1 g/L. The lowest concentration was gained when biomass was pretreated at 150 °C while the highest concentration was measured when biomass was pretreated at 125 °C, however no statistical difference was found in results in case of different pretreatment temperatures (*T*-test *p* > 0.05). The hydrolysis efficiencies varied between 15.2 % and 17.7 %. In previous studies when barley hay was used with NED pretreatment the glucose yield and hydrolysis efficiency increased

with the increase in pretreatment temperature. The hydrolysis efficiency and glucose yield increased twofold when pretreatment temperature was increased from 100 °C to 175 °C [20]. As the glucose yield is rather similar in case of all pretreatment temperatures, it would be reasonable to use lower pretreatment temperature since the process consume less energy for heating the biomass.

The small effect of pretreatment and low hydrolysis efficiency can be explained by the biomass composition and harvesting time. The biomass contains low NDF and hemicellulose values characteristic to biomass collected in summer. The NDF represents the most of the structural components in plant cell – mainly of lignin, cellulose and hemicellulose, which makes the biomass less biodegradable [21]. In previous studies, it has been shown that the cellulose and lignin content of the biomass rises with maturity, the plant cells increase, which causes the stalk to harden and this also decreases the hydrolysis efficiency [22]. The low values of hemicellulose and lignin indicate that the structure of biomass is softer than in most agricultural wastes previously used with this pretreatment method. The explosive decompression pretreatment method might not be suitable in case of soft cell structure of the biomass. During pretreatment, this kind of biomass cell acts in an elastic manner – stretching and contracting without being destructed and opened for the continued processing.

Fig. 2. Hydrolysis efficiency and glucose concentration in hydrolysis mixture in case of different pretreatment temperatures.

3.3. Fermentation

Fermentation was carried out with yeast *Saccharomyces cerevisiae* using two different approaches to evaluate in which process step solid and liquid separation would give biggest effect in terms of final ethanol yield. Ethanol concentrations in the media after fermentation have been shown in Fig. 3.

The results indicate that higher ethanol concentrations were gained when both, solid and liquid fractions were used in the fermentation. In this case, 5–13 % higher ethanol concentrations were gained compared to fermenting only the liquid fraction. Lower ethanol concentrations, when only liquid fraction was fermented, can be caused by the continuing hydrolysis of cellulose in the solid fraction or hydrolysis of oligomers. The solid fraction still contains 23–28 % of unused cellulose

which were partly hydrolysed into glucose monomers during the fermentation. This increased the concentration of available sugars and enabled to gain higher final ethanol concentrations. The continuing hydrolysis of cellulose during fermentation causes also the rise in fermentation efficiency over 100 %.

In both fermentation approaches, the ethanol yields decrease as the pretreatment temperature increase. Statistically significant differences were found between results of ethanol concentration after pretreatment at 125 °C and 175 °C (two-way ANOVA multiple comparison test). When the biomass and liquid is fermented jointly, the final ethanol concentration decreases 29.1 % as the pretreatment temperature is increased from 125 °C to 175 °C. For fermentation of only liquid fraction, the decrease in ethanol concentration is 23.8 % with the rise in pretreatment temperature. Similar tendency have been reported in previous papers when the same pretreatment method was used with barley hay or urban greening waste [9], [11]–[13], [20]. Earlier papers have also shown that increase in the pretreatment temperature to 175 °C or higher than that is not recommended, as the formation of fermentation inhibiting compounds at higher temperatures decreases the ethanol yields even if the glucose is still present in the sample [9].

Fig. 3. Ethanol concentration after fermentation steps in case of different pretreatment temperatures using two fermentation approach – fermentation of liquids with solids and fermentation of liquids where solid fraction has been removed.

3.4. Mass Balance

The liquid and solid phases from all process stages were weighed, fiber analysis of all solid samples was conducted, and glucose and ethanol concentrations were measured from liquid samples after hydrolysis and fermentation stages, respectively. Based on collected data, the mass balance of bioethanol production process was calculated and mass flow charts were prepared. Fig. 4 and Fig. 5 present the mass balance data of the two selected approaches.

From Fig. 4 and Fig. 5 it can be seen that there is a loss in dry mass during the pretreatment. The mass loss is caused by the dissolution of biomass components during pretreatment and also partially due to the evaporation of moisture during explosive decompression. During pretreatment, cellulose and lignin remained in the biomass while hemicellulose was partially removed. After pretreatment, 9.2–16.0 g of hemicellulose remained in the biomass from initial 18.3 g. The hemicellulose dissolution was larger at higher pretreatment temperatures. Similar effect with

hemicellulose dissolution was reported in earlier papers when barley hay was used [20]. Since the NED pretreatment method is analogous to steam explosion method, we can assume that similar processes are taking place with the biomass during the pretreatment. The high temperature during pretreatment initiates autohydrolysis, which causes hemicellulose hydrolysis and lignin transformation [23], [24]. Therefore, decrease in hemicellulose content can be seen as the pretreatment temperature is increased.

Fig. 4. Mass balance of bioethanol production process when different pretreatment temperatures were used and solid and liquid parts of hydrolysis slurry were separated after hydrolysis and only liquid fraction was used for fermentation.

Fig. 5. Mass balance of bioethanol production process when different pretreatment temperatures were used and solid and liquid parts of hydrolysis slurry was fermented together.

When liquid and solid fractions were separated after hydrolysis, 57.2–48.1 g of solid residues remained (Fig. 4). The solid residues still contained 12–14 g of cellulose, which remained unused in the hydrolysis process. This shows that either pretreatment or enzymatic hydrolysis process was ineffective. If due to the ineffective pretreatment the access to cellulose fibrils is not achieved and it will not be hydrolysed into sugars but remains in the residual biomass. Additionally, hydrolysis

efficiency can be low due to the low enzyme activity or enzyme unsuitability for this biomass or hydrolysis conditions. Similar results were reported in study with native sorghum pretreated with acids and in that case from 37.9 g of cellulose in the initial biomass, 18.4 g remained in the residual biomass after the enzymatic hydrolysis [25].

When the solid and liquid fractions were separated after fermentation step, the amount and composition of solid fraction (Fig. 5) were similar to those when separation was conducted after hydrolysis step. Small difference was noted at pretreatment temperature of 175 °C, where less residues were measured and the cellulose content in it was also smaller – 10.4 g of cellulose in 43.8 g residual biomass compared to 13.9 g cellulose in 48.1 g of residual biomass when separation was done after hydrolysis.

In both approaches, liquid and solid fractions were separated by filtration and weighed. Up to 8 % more liquid fraction was separated when filtration was conducted after hydrolysis. Although more liquid fraction was gained, the final ethanol yield in both cases differs only by 0.1 g since the ethanol concentration in this case was smaller than in the samples, where solid and liquid separation was conducted after fermentation. In case of both approaches, the overall ethanol yield is smaller than reported earlier using this approach due to low efficiency of pretreatment [20], [26].

4. CONCLUSIONS

Mixture of red clover and timothy grass was used as a feedstock to investigate its potential as substrate for bioethanol production. Traditional three step bioethanol production process was used in combination with NED pretreatment. The results show at all pretreatment temperatures similar glucose concentrations and hydrolysis efficiencies, which varied from 4.3 to 5.1 g/l and 15.2 % to 17.7 %, respectively. This differs from earlier studies with this pretreatment method as the results are similar in entire pretreatment temperature range and the increase in temperature did not have positive effect. Fermentation efficiencies exceeded 100 % due to sugars available in other forms but total ethanol yield, on the other hand, decreased as the pretreatment temperature increased. The mass balance revealed that, when using this kind of feedstock, 3.3–4.0 g ethanol could be produced from 100 g of biomass and both approaches used in fermentation gave similar results.

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