RYEGRASS AS A FEEDSTOCK FOR BIOETHANOL PRODUCTION

Todor Ivanov, Stoyko Petrin, Ivo Lalov

Department of Biotechnology
University of Chemical Technology and Metallurgy
8 Kliment Ohridski blvd., 1756 Sofia
E-mail: ivo.lalov@abv.bg

Received 10 January 2023
Accepted 20 February 2023

ABSTRACT

In current paper a comparative study of bioethanol yields from ryegrass biomass by different type of pretreatment and enzyme hydrolysis is done. Acidic and alkali hydrolysis was used as a pretreatment of this feedstock. Cellulase blend 10 FPU g^{-1} dry matter was used for enzyme hydrolysis. The pre-treatment was conducted by autoclaving for 20 minutes at 121°C. Best results were obtained by pretreatment with 1 % HCl and 1 % NaOH. Hydrolysate obtained by pretreatment with hydrochloric acid contained 33.6 mg mL^{-1} reducing sugars, and that obtained by pretreatment with base - 29.0 mg mL^{-1}. Both hydrolysates were found to be suitable substrates for bioethanol generation, where 5.55 g ethanol/100 g dry matter was produced from grass hydrolysate obtained by acidic hydrolysis followed by enzyme hydrolysis and from alkali pretreated hydrolysate-4.73 g ethanol/100 g dry matter. The results demonstrated that ryegrass biomass is suitable feedstock for bioethanol production.

Keywords: grass, enzyme hydrolysis, bioenergy, bioethanol.

INTRODUCTION

Nowadays Europe is experiencing an unprecedented energy crisis. The reasons for the crisis are several: uncertainty of fossil fuel supplies, due to local military conflicts and rising fuel and energy prices. All of the above and the need to reduce greenhouse emissions due to global warming have led to a move towards alternative, renewable, sustainable, efficient, and cost-effective energy sources with smaller emissions [1].

The only raw material that can be used as an energy source and meets all these requirements and at the same time does not compete with food crops is lignocellulosic biomass [2]. For European countries, available and cheap lignocellulosic raw materials are grasses from grasslands, lawns and solid household waste [1, 2]. Municipal waste contains significant amounts of lignocellulosic biomass such as leaves and grasses [2]. Grasslands occupy 79 million ha in Europe or 38 % of agricultural land [3]. The reported biomass yield of perennial grasses is in the range 12 - 30 Mg DM ha^{-1} [4]. Research on perennial grasses as biomass crops began in Europe 40 years ago [4]. These crops might be a key feedstock to produce an energy carriers like bioethanol and biogas. The research done in recent years to obtain energy from grasses is mainly directed towards obtaining bioethanol. The conversion of ryegrass lignocellulosic biomass into biofuels usually requires three steps, including pre-treatment, hydrolysis and fermentation [1, 2, 5].

Pre-treatment is usually used before enzymatic hydrolysis in order to improve the accessibility of the enzyme to the cellulose molecule. Therefore, various methods based on removal and dissolution of lignin and hemicelluloses have been developed to achieve efficient hydrolysis of cellulose [6]. Pre-treatment should overcome the structural limitations of lignocellulose and its polymers (cellulose and hemicellulose), making them susceptible to the enzyme action and leading to increased monosaccharides yield [6]. Although many different pretreatment methods have been investigated, no one suitable for all feedstocks has been proposed so far [6]. The most commonly used methods are

mechanical, chemical, physical or mixed. Compared to other methods, chemical pre-treatment is considered much more promising. These methods can be quite effective in degrading more complex structured substrates. Major reactions during alkaline pretreatment include dissolution of lignin and hemicellulose and deesterification of intermolecular ester bonds. Diluted acids (< 4 % w/w) are usually used in acid pre-treatment. Often these methods are combined with high temperatures (> 100°C). Concentrated acids are not preferred because they are corrosive and need to be recovered to make pre-treatment economically viable [6].

The enzymatic hydrolysis is the main step in the conversion process of lignocellulosic biomass into fermentable sugars. In this process, the sugars concentration of obtained hydrolysates is critical [7]. A hydrolysate with at least 80 g/L glucose must be fermented to achieve 40 g/l ethanol in the broth, which is the minimum for cost-effective rectification. In order for the ethanol production process to be profitable, high biomass content (over 15 % dry matter) is required in the pretreatment and hydrolysis processes [8]. Unfortunately, high dry matter content leads to rapid loss of enzyme activity due to inhibition of the enzyme by substrate and product [7, 8]. The main objective of this study is to investigate the possibility of obtaining ethanol from a low-cost and accessible feedstock (ryegrass), through pretreatment, enzymatic hydrolysis and fermentation.

EXPERIMENTAL

Materials and methods

Cellulase, enzyme mixture (Cellic CTec2, SAE0020, purchased from Sigma Aldrich) was used for enzymatic hydrolysis. All chemicals used were of analytical or HPLC grade purchased from Merck and Fluca. Fresh grass (perennial ryegrass *Lolium perenne*) was collected from lawns in the UCTM-Sofia area.

Substrate prepration

Ryegrass biomass was dried at 70°C, cut into small pieces and then ground in a household coffee grinder until all particles were in the 500 - 800 μm range. The dry weigh and ash content of the fresh and dried grass were determined by heating in a laboratory oven at 105°C to constant weight and combustion in a laboratory furnace at 550°C for 2 hours.

Pre-treatment of grass biomass

The pre-treatment of biomass was performed in 250 mL graduated autoclavable bottles with screw cap. Fifteen grams of dried and milled grass was suspended in 100 mL 0.1 M citrate buffer with pH 4.8. Chemical pre-treatment was conducted with 100 mL 0.5 %, 1.0 % HCl, 0.5 % and 1.0 % NaOH. All bottles were autoclaved for 20 minutes at 121°C. The obtained hydrolysates were neutralized to pH 4.8 and diluted to 150 mL.

Enzyme hydrolysis

Enzymatic hydrolysis was carried out at 50°C in rotating shaker/incubator for 96 hours. Cellulase, enzyme blend was added to the pretreated biomass. In all experiments, the amount of added enzyme was 1 mL per 15 g of biomass, and in order to avoid contamination, 100 mg of penicillin G potassium salt (POLFA TARCHOMIN S.R.) was also added. After enzyme hydrolysis liquid part and solid part was separated by vacuum filtration.

Ethanol fermentation

Lyophilized spirit yeast was provided to us by factory producing bioethanol “Almagest”, Verinsko village, Bulgaria. The hydrolysates obtained in all types of pretreatments (only the liquid part) are subjected to ethanol fermentation. Hundred milligrams of lyophilized yeast *Saccharomyces cerevisiae*, 200 mg KH_2PO_4 and 300 mg $(NH_4)_2SO_4$ were added to 75 mL hydrolysate. The fermentation was carried out at room temperature (around 25°C), under anaerobic agitated conditions in 150 mL Erlenmeyer flasks sealed with fermentation trap for 7 - 8 days (or lack of new bioethanol produced). To compare the performance of hydrolysate for ethanol fermentation, a synthetic media was used in which glucose in same quantity as reducing sugars in hydrolysates was added as carbon source.

Analytical methods

The contents of elemental carbon (C), nitrogen (N) and hydrogen (H) were measured using an automatic elemental analyzer EuroEA 3000. The content of total protein was calculated by multiplying the value of total nitrogen by 6.25. Cellulase enzyme activity was determined as “filter paper units” (FPU) per milliliter of original (undiluted) enzyme solution. Briefly, the amount of enzyme that hydrolyzed 4 % of

the substrate (Whatman No 1 filter paper) in 60 min was determined. The conditions of the determination and the methodology are fully presented by Ghose, [9]. Reducing sugars were determined as glucose by using dinitrosalicylic acid (DNS) reagent by the method described by Miller, [10]. The ryegrass was analyzed for cellulose and lignin (TAPPI standard T222 om-11) [11]. Sugar composition was analyzed by using a high-performance liquid chromatography (HPLC) system on Dionex (Dionex Inc., CA, USA) equipped with a Shodex RI101 RI detector (Showa Denko KK, Kawasaki, Japan), according to the National Renewable Energy Laboratory (NREL) analytical methods for biomass [11]. The separation was performed in a Hi-Plex H column, 7.7 mm × 300 mm (Agilent Technologies, USA) at 65°C with ultrapure water as eluent at a flow rate of 0.5 mL min^{-1}. Injected volume: 20 μL. The results were evaluated by the Chromeleon 6.80 software. The alcoholic fermentation process was monitored by periodically weighing the flasks, and the amount of ethanol produced was determined by Equation 1.

$C_6H_{12}O_6$ (180.16 g) → $2C_2H_5OH$ (92.14 g) + $2CO_2$ (88.02 g) (1)

Table 1. Main characteristics of dried ryegrass (used in experiments).

Characteristic	Value, %
Total solids (TS)	91.5
Volatile solids (VS) (% of TS)	96.2
Protein (% of TS)	12.4
Cellulose (% of TS)	26.1
Lignin (% of TS)	17.4

RESULTS AND DISCUSSION

Characteristics of ryegrass

In order to determine the potential yield of bioethanol, one square meter of grassland was harvested throughout the year. After drying, the amount of grass biomass was 975 g. When recalculated per hectare, 9.75 tons is obtained. Grass yields were approximately equal to those reported by other researchers [4]. Harvested fresh grass has TS content 22.5 %. The main characteristics of dried biomass are presented in Table 1.

Values for cellulose content are similar of those reported by Raud et al., [2] and Antonopoulou [13]. The data in Table 1 shows that the amount of cellulose in ryegrass is relatively low compared with agricultural residues [2]. Considering that cellulose is the main polysaccharide from which glucose will be obtained during hydrolysis, and ethanol after fermentation, the maximum theoretical amount of ethanol will be about 13.4 g from 100 g of ryegrass. The composition of biomass indicates that after enzyme hydrolysis it can be used to obtain other fuels, like biohydrogen [13] and biomethane [5].

Pretreatment of ryegrass

In the preparation of biomass for hydrolysis, it was found that more than 15 g of grass could not be uniformly suspended in less than 100 mL liquid. In addition, the need to neutralize the hydrolysate and reduce its viscosity requires the addition of liquid to a total volume of 150 mL. The characteristics of hydrolysates obtained after pretreatment are presented in Table 2. The data in Table 2 show that thermolysis with citrate buffer pH 4.8 did not produce significant amounts of reducing sugars, and the reduction of dry matter was negligible.

Table 2. Characteristics of ryegrass hydrolysates.

Type of pretreatment	Reducing sugars, mg mL^{-1}	Reducing sugars, g	Dissolved solids, g	Dissolved solids, % of dry matter
Thermolysis	1.52	0.228	1.15	7.6
Hydrolysis 0.5 % NaOH	0.41	0.062	3.94	26.2
Hydrolysis 1.0 % NaOH	4.12	0.618	6.83	45.5
Hydrolysis 0.5 % HCl	3.45	0.518	4.05	27.0
Hydrolysis 1.0 % HCl	16.24	2.436	7.1	47.3

It should be noted that in hydrolysis with 0.5 % NaOH significant dissolution of biomass was observed, while the amount of reducing sugars was the least of all pretreatments. Even in the treatment of 1 % NaOH, the amount of reducing sugars is nearly four times less than that obtained in treatment with 1 % HCl, while the dry matter reduction was relatively the same. The main reason for this phenomenon is probably that the lignin is mainly dissolved during the alkaline pretreatment.

Enzyme hydrolysis

In order to establish the required amount of enzyme for biomass hydrolysis, the enzyme activity was initially determined. The enzyme activity found was 150 FPU mL^{-1} of the original solution. The enzyme was dosed at a ratio of 10 FPU g^{-1} dry ryegrass. Researchers have used different enzymes and enzyme dosage units, but both higher and lower dosages have been reported [13, 5]. The results for reducing sugars accumulation during enzyme hydrolysis at all five types of pretreatments are summarized in Fig. 1. It is clearly seen from the Fig.1 that the hydrolysis process proceeds at the highest rate up to 24 h. This is also observed visually that the viscosity of the hydrolysate drops sharply already in the first few hours of the hydrolysis. The amount of reducing sugars produced is lowest at citrate buffer pretreatment. Despite the low conversion rate of this pretreatment obtained in our investigation, some authors report good results, but at higher thermolysis temperatures [6]. Interestingly, the content of reducing sugars during enzymatic hydrolysis increases significantly with both types of alkaline pretreatment. Probably, the solubilization of lignin in this case leads to a better accessibility of the substrate for the enzymatic action. Best results are obtained by pretreatment with 1 % HCl and concentration of reducing sugars of 33.6 mg mL^{-1} was reached.

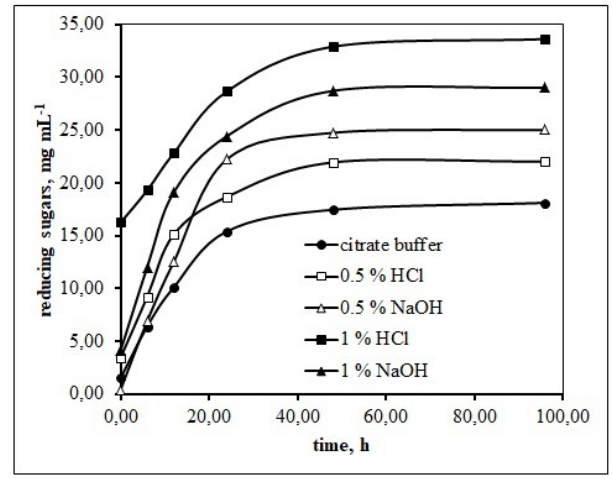

Fig. 1. Enzyme hidrolisys at different types of pretreatment.

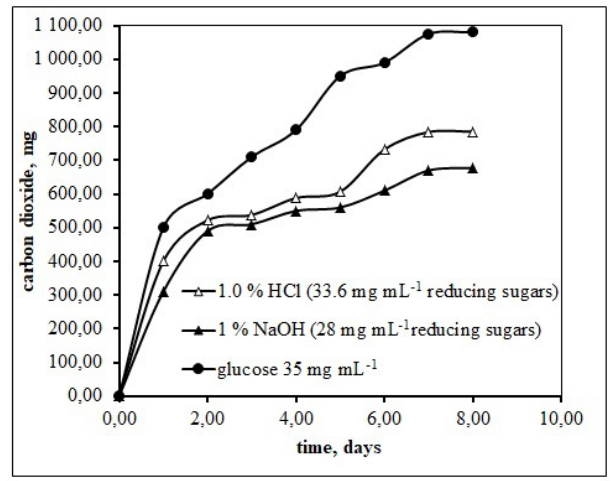

Fig. 2. Amount of carbon dioxide released during fermentation of obtained hydrolysates and glucose as carbon source.

Bioethanol production

All hydrolysates obtained underwent fermentation, but hydrolysates obtained by pretreatment with citrate buffer, 0.5 % HCl and 0.5 % NaOH showed very little evolution of carbon dioxide and the reduction in flask weight was negligible. Weak fermentation was also observed when glucose was used in a concentration equal to the reducing sugars in hydrolysates. A major reason for this is probably the need for significant amounts of sugars for yeast biomass growth. As shown in Fig. 2 better results were obtained in the fermentation of hydrolysates obtained by pretreatment with 1 % NaOH and 1 % HCl. As can be seen in Fig. 2, the fermentation of both hydrolysates and glucose proceeds relatively slowly. Even with the fermentation of glucose, the released amount of carbon dioxide is less than theoretically calculated. The main reason for this is the need for adaptation and growth of the lyophilized yeast biomass. Despite the fact that the concentration of reducing sugars in the hydrolysate obtained by pretreatment with 1 % HCl is approximately equal to the concentration of glucose in the control sample, the amount of released dioxide is significantly less. The amount of carbon dioxide released in the fermentation

of the hydrolysate obtained by pretreatment with 1 % NaOH is even lower, due to the lower content of reducing substances. It is likely that the high content of non-fermentable sugars is the main reason for the lower yields in both hydrolyses. The results of the analysis of the composition of the hydrolysates showed a content of pentoses (defined as xylose) of 6 % for the hydrolyzate obtained by alkaline pretreatment and 15 % for that obtained by acid pretreatment (calculated as a percentage of reducing sugars). After recalculation from equation 1, the amount of ethanol from the hydrolysate obtained by pretreatment with 1 % HCl is 822 mg, while the amount of ethanol obtained by pretreatment with 1 % NaOH is 709 mg. The maximum yield of ethanol obtained is 5.55 g from 100 g of dry ryegrass or 41.5 % of the theoretical. The results obtained is comparable to that reported in other studies using similar biomasses [1, 2].

CONCLUSIONS

Both hydrolysates (obtained with both types of pretreatments) were found to be suitable for bioethanol production. Unfortunately, the maximum ethanol concentration obtained so far is 1.1 %, which makes the distillation process economically unprofitable, but the possibility of obtaining biogas from the residual biomass would significantly increase the amount of energy obtained and the economic feasibility of the process.

Acknowledgements

This work was financially supported by Bulgarian National Science Fund (scientific project No KP-06-H27/4 from 12.2018).

REFERENCES

1. M. Raud, R. Keperi, T. Oja, J. Olt, T. Kikas, Utilization of urban waste in bioethanol production: potential and technical solutions, Agronomy Research, 12, 2, 2014, 397-406.
2. M. Raud, T. Kikas, Perennial Grasses as a Substrate for Bioethanol Production, Environmental and Climate Technologies, 24, 2, 2020, 32-40.
3. D. Scordia, S. Cosentino, Perennial Energy Grasses: Resilient Crops in a Changing European Agriculture, Agriculture, 9, 2019, 169.
4. I. Lewandowski, J. Scurlock, E. Lindvall, M. Christou, The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe, Biomass Bioenergy, 25, 2003, 335-361.
5. P. Wu, X. Kang, W. Wang, G. Yang, L. He, Y. Fan, X. Cheng, Y. Sun, L. Li, Assessment of Coproduction of Ethanol and Methane from Pennisetum purpureum: Effects of Pretreatment, Process Performance, and Mass Balance, ACS Sustainable Chemistry & Engineering, 9, 32, 2021, 10771-10784.
6. D. Kumar, G. Murthy, Pretreatments and Enzymatic Hydrolysis of Grass Straws for Ethanol Production in the Pacific Northwest US, Biological Engineering Transactions, 3, 2, 2011, 97-110
7. D. Puri, S. Heaven, C. Banks, Improving the performance of enzymes in hydrolysis of high solids paper derived from MSW, Biotechnology for Biofuels, 6, 2013, 103.
8. M. Cheng, H. Kadhum, G. Murthy, B. Dien, V. Singh, High solids loading biorefinery for the production of cellulosic sugars from bioenergy sorghum, Bioresource Technology, 318, 2020, 124051.
9. T. K. Ghose, Measurement of cellulase activities, Pure and Applied Chemistry, 59, 2, 1987, 257-268.
10. G. L. Miller, Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, Anal. Chem., 31, 3, 1959, 426–428.
11. K. Kurschner, A. Hoffer, S. Jenkins, W. Vieweg, O. Schwarzkopf, W. Schramek, C. Schubert, H. Velten, K. Hess, C. Trogus, M. Hönig, W. Ruziczka, V. Grafe, J. Hanuš, J. Pritzker, R. Jungkunz, Th. Curtius, O. Trachmann, H. Sommer, H. Markert, W. Ermen, E. Berl, H. Staudinger, K. Hahn, G. Sándor, H. Fierz-David, W. Fermazin, T. Murray, C. Staud, H. Gray, O. Eberstadt, H. Ost, W. Billing, J. Tinsley, W. Smith, T. Katajama, J. Lawrie, Cellulose und Cellulosederivate, J. Anal. Chem., 92, 1933, 145-154.
12. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, NREL, Golden, 2008.
13. G. Antonopoulou, Designing Efficient Processes for Sustainable Bioethanol and Bio-Hydrogen Production from Grass Lawn Waste, Molecules, 25, 12, 2020, 2889.