Romanian Biotechnological Letters *Vol. , No. x,*

ORIGINAL PAPER

Bioethanol production from common reed (*Phragmites australis*): Enzymatic hydrolysis and fermentation

Received for publication, March, 24, 2016
Accepted, March, 21, 2017

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Abstract

Several biomaterials consisting of high amount of carbohydrate in the structure and pretreatment methods have been studied by researchers to maximize the ethanol yield. Therefore, this study was focused on enzymatic hydrolysis and fermentation of common reed for the bioethanol production. The biologically and chemically pretreated common reed was enzymatically hydrolyzed and the results showed that the NaOH treatment had the highest glucan conversion rates (79.7%), followed by the $NaBH_4$ (74.1%), H_2O_2 (71.9%), fungal pretreatment (69.4%), B_2O_3 (65.5%) and H_2SO_4 (46.1%). The highest ethanol yield (13.2 g/100 g) and the calculated highest theoretical yield (85.3%) from untreated common reed were observed for the NaOH- pretreated samples. The fungal- and $NaBH_4$-pretreated samples yielded 10.6 and 12.3 g/100 g of ethanol (based on untreated common reed), respectively. The results of this study indicated that, because of its economic feasibility and environmental advantages, the fungal pretreatment was more suitable for common reed bioethanol production.

Keywords: Common reed, enzymatic hydrolysis, fermentation, bioethanol.

1. Introduction

Recently, much attention has been given to wind, water, solar, geothermal and biomass energy source, not only for being renewable and environmentally friendly, but also for providing alternatives to fossil fuels for meeting energy demands (F. CHERUBINI & al. [1]). Bioenergy products including bioethanol, biodiesel and biogas, have provided a feasible and economical solution. Among them, bioethanol exhibitshigher burning efficiency and a lower environmental impact (M. BALAT & al. [2]).

Crops such as wheat, corn, sugar cane and sugar beet are distilled to produce first-generation bioethanol. Although these first-generation biofuel processes are practical, they are incapable of producing enough biofuel without jeopardizing food supplies and biodiversity. Many first-generation biofuels depend on subsidies and cannot compete on their own with existing fossil fuels such as oil. Moreover, some generate only limited savings in greenhouse gas emissions, and if production and transport emissions are added into the account, the life-cycle evaluation of first-generation biofuels frequently comes close to those of traditional fossil fuels. However, a wider range of feedstock, including agricultural residue, woody raw materials and energy crops not directly in competition with food crops for land use can be used to produce second-generation biofuels such as cellulosic ethanol (S.N. NAIK & al. [3]).

To produce bioethanol from lignocellulosic materials, pretreatments should be conducted to open the complex structure of biomaterials (M.J. TAHERZADEH & K. KARIMI [4]). This will allow us to access the polymer chains of cellulose and

hemicelluloses. The pretreatments applied will hydrolyze the carbohydrates to obtain monomeric sugars solutions, which are fermented to ethanol by microorganisms. The carbohydrate hydrolysis can be accomplished by chemically or enzymatically (M. GALBE & G. ZACCHI [5]). Enzymatic hydrolysis could be made by cellulolytic enzymes, which hydrolyze carbohydrates in the structure to fermentable sugars. Compared to acid hydrolysis, there are some advantages of enzymatic applications. The enzymatic process could be carried out under mild conditions, which results in higher yield. In addition, lower inhibitory compounds are formed during hydrolysis (M.J. TAHERZADEH & K. KARIMI [6]). On the other hand, the main problem with the enzymatic hydrolysis, the process takes several days. The price of the enzymes used for the purpose is also very expensive. To overcome this obstacles, simultaneous saccharification and fermentation (SSF) was applied by several researchers. However, the optimum process temperatures for saccharification and fermentation is different and that require to produce ethanol by separated applications (SHF-separate enzymatic hydrolysis and fermentation) (C.E. WYMAN [7]).

Common reed as an energy crop has been the subject of very few studies, and those have mostly dealt with the utilization of its lignin content (17.2-26.5%, w/w) in the production of solid fuel in addition to its potential as a source of bioethanol, regardless of its relatively high cellulose (29.7-37.3%, w/w) and hemicellulose (16.4-19.0%, w/w) contents (R. VAIČEKONYTĖ & al. [8]).

The aim of this study was to produce bioethanol from biologically and chemically pretreated common reed. The results of the pretreatments were evaluated with enzymatic hydrolysis and fermentation.

2. Materials and Methods

2.1. Materials

In this study, the biologically (*C. subvermispora*) and chemically (NaOH, H_2O_2, $NaBH_4$, B_2O_3, H_2SO_4) pretreated common reed samples were utilized.

2.2. Methods

2.2.1. Enzymatic hydrolysis

The enzymatic hydrolysis was carried out on 5 g (o.d.) biologically/chemically-pretreated samples by employing a mixture (50% v/v) of *Celluclast 1.5 L* (700 U/g) and *Cellobiase* (*Novozym 188*) (250 U/g). The process was conducted at 5% solid loading in 100 ml of 50 mM sodium acetate buffer at pH 5.0. Additionally, for this study, in order to prevent microbial contamination, sodium azide (NaN_3, 0.0001 M) was used in this step. The enzyme reaction was achieved by placing the samples in a rotary shaker at 100 rpm and 42 °C. At 0-, 6-, 24-, 48-, and 72-h intervals, 1.5 ml samples were taken. After first being held in boiling water for 10 min to stop the enzymatic activity, the samples were centrifuged at 10,000 rpm for 5 min, and then their glucose and xylose contents were determined.

2.2.2. Fermentation of hydrolyzates

After the hydrolyzates were centrifuged at 5000 rpm for 10 min, 20 mL samples of the supernatant were transferred to 100 ml serum bottles in preparation for the fermentation process. Additions of 5 g/L of yeast extract, 3.75 g/L of $(NH_4)_2SO_4$, 2.1 g/L of K_2HPO_4, 0.375 g/L of $MgSO_4$ $7H_2O$ and 0.5 g/L of $CaCl_2$ $2H_2O$ were then introduced to the mixture along with 5% (v/v) *Saccharomyces cerevisiae ATCC 26602* from overnight cultures for fermentation. An orbital shaker was used to incubate the samples at 100 rpm for 72 h at 30 °C. Following this, they were centrifuged at 10,000 rpm for 10 min and finally, after passing through 0.45 µm pore-sized filters, the supernatants were held at 20 °C until the HPLC analysis could be carried out.

2.2.3. Analytical methods

High performance liquid chromatography (HPLC)(Agilent 1200 system) equipped with a Shodex SH1011 column (mobile phase: 5 mM H_2SO_4, flow rate: 0.5 ml/min, column temperature: 60 °C) and a refractive index detector was utilized to determine the sugars and ethanol contents.

The glucan conversion percentage in the enzymatically hydrolyzed samples was calculated as follows in Equation 1:

$$\text{The \% of glucan conversion} = \frac{GH}{GP} x100 \quad (1)$$

where: the GH represents the concentration of glucose in the enzyme hydrolysis supernatants and the GP is the concentration of glucan in the non-/pretreated samples. In the same manner, the conversion of xylan during enzymatic hydrolysis was calculated by substituting the appropriate percentage for xylan.

The ethanol yield during fermentation was calculated as a percentage of the theoretical maximum yield (T.H. KIM & Y.Y. LEE [9]) as follows in Equation 2:

$$\text{The \% of theoretical ethanol yield} = [\frac{E}{G \times 0.511}] x100 \quad (2)$$

where: E is the ethanol (g) produced during fermentation, G is the glucose (g) in the hydrolyzates, and the constant 0.511 is the theoretical yield of ethanol produced from glucose.

2.2.4. Statistical analysis

The SPSS 16.0 program was utilized for statistical analysis of the obtained data. The one-way ANOVA was used for identification of significant differences in the effects of enzymatic hydrolysis. The Duncan test was employed for detection of significant differences between the groups.

3. Results and discussion

3.1. Enzymatic hydrolysis

For this study, biologically and chemically pretreated samples were then enzymatically hydrolyzed. According to the results, glucose and xylose were the principal monosaccharides in the enzymatic hydrolysates, thus indicating that cellulose and hemicellulose were simultaneously degraded during hydrolysis.

The effects of enzymatic hydrolysis and incubation periods on the hydrolysis of common reed were examined and the results are presented in Figure 1. The saccharification rate up to 24 h was higher, and then, continuing to 72 h, the rate slowed down. This behavior might be due to the diminishing enzyme adsorption during saccharification, the changing of the cellulose structure to a less digestible form and the inhibiting enzymes resulting from the products of hydrolysis (Y.H. LEE & L.T. FAN [10]). The slow rate of saccharification after 72 h has also been reported previously (H.K. SREENATH & al. [11]).

The untreated samples gave a glucan conversion of 33.1% when enzymatic application progressed for 72 h. This value was lower compared to that of almost 45% (by *cellulase+β-glucosidase*) observed by J.Y. JUNG & al. [12] for common reed. The glucan conversion of each hydrolysis treatment is shown in Figure 1. The NaOH treatment had the highest glucan conversion (79.7%), followed by $NaBH_4$ (74.1%), H_2O_2 (71.9%), fungal pretreatment (69.4%), B_2O_3 (65.5%) and H_2SO_4 (46.1%). For all pretreated samples, the differences in mean glucan conversion rates were statistically significant ($p< 0.001$). J.Y. JUNG & al. [12] observed a slightly higher glucan conversion rate of up to 80.0% when

common reed pretreated with 1% NaOH (121 ºC/90 min) was hydrolyzed by *cellulase+β-glucosidase*. A higher glucan conversion of 91.7% was found by Z. WANG & al. [13] when Bermuda grass treated with 3% NaOH (121 ºC/15 min) was hydrolyzed by cellulase (*NS50013 cellulase complex*) and cellobiase (*NS50010 β-glucosidase*). In this study, the lower glucan conversion rate could be attributed to the local features of the common reed used for this study.

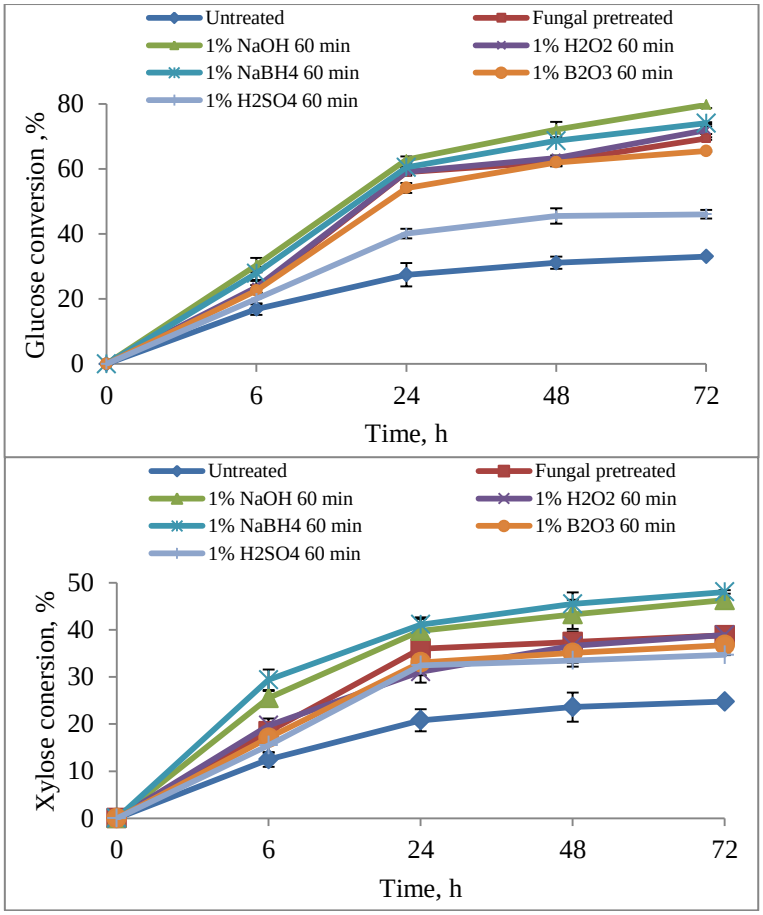

Figure 1. Glucan and xylan conversion after enzymatic hydrolysis.

The lignin and xylan contents of the samples could be considered as the principal factors affecting enzymatic hydrolysis. The H_2SO_4-pretreated samples had the highest lignin content (28.1%) and the NaOH-pretreated samples had the lowest lignin content (18.6%). Consequently, the H_2SO_4-pretreated samples had 1.93 times more lignin compared to the NaOH-pretreated samples. Therefore, the glucan conversion rate of the H_2SO_4-treated samples was 1.73 times lower compared to that of the NaOH-treated samples. In contrast, the fungal-pretreated samples had the highest xylan content (15.3%) and the NaOH-treated samples had lower xylan content (19.5%). The fungal-pretreated samples had 1.20 times higher xylan in the structure and the glucan conversion rate of the fungal-pretreated samples was 1.15 times lower compared to the NaOH-pretreated samples. This suggestedthat the removal of both lignin and xylan significantly affected the enzymatic digestibility of the common reed. Similar findings were previously described by Y.P. LU & al. [14].

Furthermore, B. YANG & C.E. WYMAN [15] reported that hydrolysis was affected by lignin because of the protein adsorption of lignin in aqueous solutions.

In this study, a significant amount of xylose was found in the hydrolyzates, even without xylanase being added to the enzyme combination (Figure 1). L.C. DUARTE & al. [16] reported that *Celluclast 1.5 L* exhibited *β-xylanase* and *β-xylosidase* activity. In addition, Y. CHEN & R.R. SHARMA-SHIVAPPA [17] observed that *Celluclast 1.5 L* and *Cellobiase* had 905 and 605 U/ml xylanase activity, respectively. Consequently, the xylan in the structure of common reed was reduced by the enzyme combination used in this study. The $NaBH_4$-pretreated samples displayed the highest xylan conversion (48.1%), while the untreated samples showed the lowest (24.8%). Statistical data indicated that differences in mean xylan conversion for all pretreatments were statistically significant ($p< 0.001$). A lower xylan conversion (37.6%) was reported by Z. WANG & al. [13] when Bermuda grass was treated with 3% NaOH (121 ºC/ 15 min).

3.2. Fermentation of hydrolyzates

The fermentation performance of *S. cerevisiae* in pretreated hydrolysates is shown in Table 1. The findings of this study revealed that pretreatment of common reed prior to hydrolysis resulted in a better fermentability of the hydrolyzates. The highest ethanol concentration (10.6 g/L) and the highest ethanol yield (13.2 g/100 g), based on untreated common reed, both resulted from the NaOH-pretreated samples. The theoretical yield (85.3%) was also calculated to be highest for these samples (Figure 2). On the other hand, fungal- and $NaBH_4$-pretreated samples yielded 10.6 and 12.3 g/100 g of ethanol (based on untreated common reed), respectively.

Table 1 . Glucose, xylose and ethanol concentrations in untreated and pretreated common reed during fermentation with *S. cerevisiae*.

Treatment		Glucose, g/l	Xylose, g/l	Ethanol, g/l
Untreated		5.56±0.30	2.04±0.11	2.84±0.14
Fungal pretreatment		11.1±0.40	2.97±0.18	5.54±0.23
	1% NaOH 60 min	19.8±1.00	4.51±0.54	10.6±0.56
	1% H_2O_2 60 min	13.2±1.15	3.27±0.29	6.57±0.12
Chemical pretreatment	1% $NaBH_4$ 60 min	15.9±0.30	4.35±0.36	8.21±0.74
	1% B_2O_3 60 min	11.2±0.61	2.83±0.44	5.38±0.62
	1% H_2SO_4 60 min	8.27±1.31	1.31±0.06	3.58±0.84

J.Y. JUNG & al. [12] studied common reed pretreated with 1% H_2O_2 (121 ºC/90 min) and recorded an ethanol yield of 5.6 g/100 g (based on untreated common reed). Differing results for ethanol yield could be ascribed to the different geographical locations, cultivars and harvest times of the raw materials used, as well as to the yeast strain and treatment methods. Lower ethanol yields could be a result of the formation of by-products including furfural, HMF and phenolic compounds, all of which inhibit yeasts from fermenting sugars (A.B. BJERRE & al. [18]). In this study, even the very low amount of sodium azide utilized during the enzymatic hydrolysis to prevent microbial contamination may also have resulted in reduced yeast activity (W.F. FALES [19]).

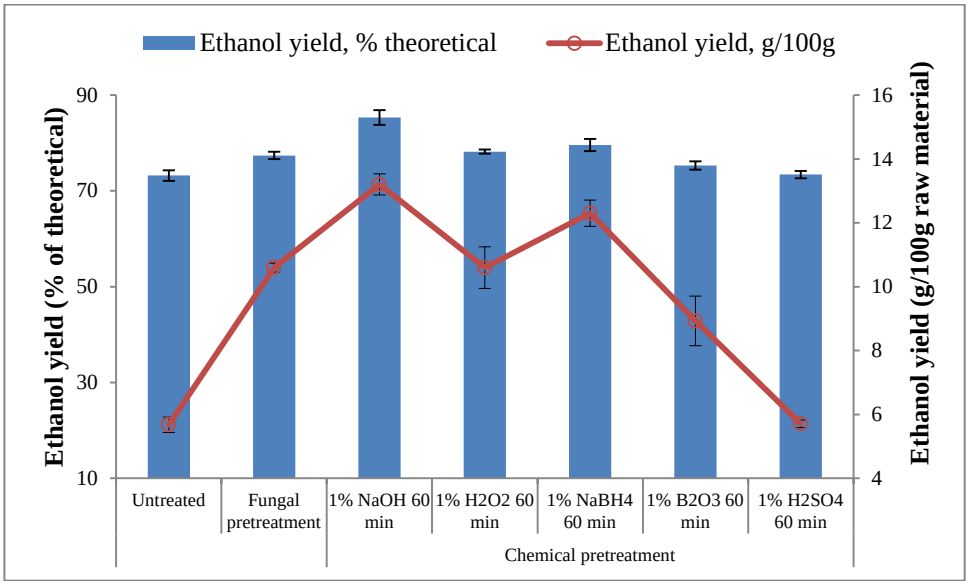

Figure 2. Ethanol yield (theoretical and g/100 g raw material).

The findings of this study showed that fungal pretreatment yielded impressive results compared to the chemical pretreatments (Table 1, Figure 2). However, it was concluded that $NaBH_4$ had the potential to be an efficient pretreatment chemical for the enhancement of fermentation processes. Consequently, further research should be carried out with the aim of enhancing fermentability under optimized process conditions.

4. Conclusion

The NaOH treatment had the highest glucan conversion rate (79.7%), followed by $NaBH_4$ (74.1%), H_2O_2 (71.9%), fungal pretreatment (69.4%), B_2O_3 (65.5%) and H_2SO_4 (46.1%). The highest ethanol yield from untreated common reed (13.2 g/100 g) was observed for the NaOH-pretreated samples and the theoretical yield was also calculated to be the highest in these samples (85.3%). Fungal- and $NaBH_4$-pretreated samples yielded 10.6 and 12.3 g/100 g of ethanol (based on untreated common reed), respectively. The results of this study indicated that, because of its economic feasibility and environmental advantages, the fungal pretreatment for common reed was more suitable for bioethanol production. However, a number of disadvantageous factors may limit its application, including low efficiency, carbohydrate loss, extended residence time, space requirements and the necessity of carefully monitoring growth conditions.

5. Acknowledgements

This work was supported by Duzce University Research Fund Project No: 2016.02.03.478

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