Comparison of Steam Explosion, Dilute Acid, and Alkali Pretreatments on Enzymatic Saccharification and Fermentation of Hardwood Sawdust

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Effects were compared for three low-cost pretreatment methods (dilute acid, alkali, and steam explosion) relative to the effectiveness of environmentally friendly enzymatic hydrolysis and ethanol fermentation of aspen, birch, and oak chips. The highest monomeric sugar yield was achieved with the alkali pretreatment of the aspen chips (22 g/L of glucose and 6 g/L of xylose). Additionally, the concentration of lignocellulose degradation products formed during this pretreatment was relatively low, and so the hydrolysis and fermentation efficiencies were 80% and 85%, respectively. The application of dilute acid pretreatment led to lower yield of enzymatic hydrolysis in comparison with alkali pretreatment, resulting in 41% to 62% of theoretical yield for aspen and birch chips, respectively. Increasing the NaOH concentration led to an increase in the monomeric sugar yield, and consequently increased the hydrolysis and fermentation yields. By contrast, increasing the acid concentration resulted in a higher sugar yield, and the fermentation efficiency decreased. The applied steam explosion conditions resulted in the formation of 6.8 to 15.4 g glucose/L, with hydrolysis yield in the range 34 to 42% of theoretical. The most susceptible for pretreatment and enzymatic hydrolysis was found to be aspen biomass.

Keywords: Sawdust; Pretreatment; Enzymatic hydrolysis; Fermentation; Bioethanol

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INTRODUCTION

Currently, production of second-generation biofuels is of major interest to many researchers. Biofuels can be produced from lignocellulosic materials that are not applicable in food production. The availability of lignocellulosic biomass, its composition, renewability, and non-food characteristics are the main reasons for using it for the production of bioethanol. Lignocellulosic biomass includes agricultural residues, wood residues, energy crops, municipal solid waste, and various industrial wastes. Lignocellulose is the main structural component of plant cell walls and consists of cellulose, hemicellulose, and lignin. The chemical composition of agricultural wastes or woody biomass depends on the species, age, growing conditions, fractionation degree, and pretreatment (Sjöström 1993). Typically, most hardwoods are comprised of approximately 18% to 25% lignin, 13% to 40% hemicellulose, and 40% to 55% cellulose (Kumar *et al.* 2009; Vena *et al*. 2013; Chen *et al*. 2016). Cellulose is the most abundant

biopolymer on Earth and is an important structural component of plant cell walls. Because of its strong linkage to hemicelluloses and lignin, the isolation of cellulose requires multi-step processing, which includes pretreatment, enzymatic hydrolysis, and fermentation (Xiao *et al*. 2012). Pretreatment of lignocellulosic materials is used to make cellulose more accessible to hydrolyzing enzymes by inducing changes in the cell wall chemical composition. The goal is to remove lignin and hemicellulose, reduce the cellulose crystallinity, and increase the porosity of the material (Kumar *et al.* 2009). Among all of the currently available and widely used pretreatment methods, acid hydrolysis (Karapatsia *et al.* 2017), alkaline hydrolysis (Karp *et al.* 2015), and steam explosion (SE) (Li *et al.* 2015) are the most widely used for economic reasons.

Pretreatment with the SE method is the most widely studied and used method for the physico-chemical treatment of biomass, especially in the case of raw materials with low lignin contents (*e.g.*, hardwood). With this method, the raw material is treated with saturated steam (160 °C to 260 °C) under high pressure (0.7 MPa to 4.8 MPa) (Kumar *et al*. 2009, 2017; Sun and Cheng 2002). These conditions are maintained for a period of time that can range from several seconds to several minutes (in that time the hydrolysis of hemicellulose occurs), and then the pressure is suddenly reduced. During this process, hemicelluloses are degraded and lignin is transformed, and thereby the accessibility of the cellulose to hydrolytic enzymes is increased (Hendriks and Zeeman 2009). Hemicellulose is degraded by acids generated during autohydrolysis (mostly acetic acid), and lignin is only removed to a certain extent, but it is distributed on the surface of fibers. The rapid exposure of biomass to atmospheric pressure also results in fragmentation of the raw material, which increases the available surface area (Kumar *et al.* 2009). However, this method does not result in substantial delignification (Sannigrahi *et al.* 2011). Other problems related to SE are relatively low yield of hemicelluloses, formation of inhibitory compounds and incomplete destruction of lignin-carbohydrate matrix (Singh *et al*. 2015)
The use of dilute acid causes the removal and allows recovery of most of the hemicelluloses, so higher productivity of glucose can be achieved. This is due to the removal of hemicelluloses that facilitates access to the cellulose (Mosier *et al*. 2005). Pretreatment of lignocellulosic biomass with diluted acids (0.5 wt.% to 1.0 wt.%) at high temperatures (160 °C to 190 °C) for 30 min to 60 min has been performed within the industry (Gupta and Demirbas 2010; Gírio *et al.* 2017). Sulfuric acid (H_2SO_4) is mainly used, though hydrochloric, phosphoric, and nitric acids have also been employed. This method, however, has some drawbacks. The most important one concerns the corrosion of the equipment (which occurs even at low acid concentrations), and thus the need for expensive materials used for construction (Mussatto 2016). Acid treatment demands high energy consumption associated with the use of high temperatures and biomass comminution (Mosier *et al*. 2005; Mussatto 2016). Other very important issues are related to the presence of toxic inhibitory compounds in the hydrolysate, which can inhibit microbial activity and the need of neutralization of hydrolysate or washing of biomass. The neutralization step results in formation of insoluble salts which need to be separated and removed (Singh *et al*. 2015).

Alkali pretreatment uses alkaline reagents, such as calcium hydroxide (lime), ammonia, and sodium hydroxide (NaOH). Most of these chemicals specifically target hemicellulose acetyl groups and lignin–carbohydrate linkages, which results in high lignin removal and solubilization of the lignocellulosic complex (da Costa Sousa *et al.*

2009). Alkali hydrolysis requires milder conditions and a lower severity compared with other pretreatment technologies, which leads to a lower sugar degradation (Mosier *et al.* 2005). Alkali treatment is particularly useful for the processing of hardwoods and agricultural residues, and its effectiveness is highly dependent upon the initial lignin content in the raw material (Alvira *et al.* 2010). Alkaline treatment has one major issue, which is conversion of alkali into irrecoverable salts and their incorporation into the biomass (Mosier *et al.* 2005). Also, another limitation is a problem with disposal and purification of hydrolysate, also related with the presence of salts, and formation of inhibitors under harsh conditions (Bensah and Mensah 2013).

The efficiency of downstream processes (*i.e.*, hydrolysis and fermentation) is highly dependent upon both the raw material and pretreatment method employed. Therefore, the aim of this study was to evaluate the impact of three different pretreatment methods (dilute acid, alkali, and SE) on three hardwood biomasses (aspen, birch, and oak) with efficient conversion of the biomass to ethanol.

EXPERIMENTAL

Materials

Aspen (*Populus tremula* L.), birch (*Betula* sp. Ehrh.), and oak (*Quercus robur* L.) wood chips were obtained from a private sawmill located in the northeastern region of Poland. The materials were dried at room temperature, chopped into fragments approximately 1.0 mm to 2.0 mm thick, 3.0 cm to 5.0 cm long, and 0.5 cm to 1.0 cm wide, and stored at room temperature. Table 1 shows the chemical composition of the raw materials.

Table 1. Chemical Composition of the Raw Materials

Parameter	Unit	Aspen	Birch	Oak
Total Solids	(%)	92.88 ± 1.39a	92.38 ± 1.27a	91.57 ± 1.25a
Cellulose	(% d.m.)	57.54 ± 0.60c	51.39 ± 0.54b	45.30 ± 0.79a
Hemicellulose	(% d.m.)	17.33 ± 0.70a	23.65 ± 1.67b	25.51 ± 1.44b
Lignin	(% d.m.)	21.80 ± 1.12b	17.92 ± 1.39a	21.51 ± 1.50b
Ash	(% d.m.)	0.18 ± 0.02a	0.20 ± 0.02a	0.22 ± 0.02a
d.m. – Dry matter; Rows with different lower-case letters are significantly different ($p < 0.05$), as analyzed by the one-way ANOVA; n=3				

Enzymatic hydrolysis was performed using commercial enzyme preparations consisting of cellulase from *Trichoderma longibrachiatum* (C9748, Sigma Aldrich) and β-glucosidase (Novozyme 188, Novozymes, Denmark). For the fermentation experiments, the commercial dry distillers yeast Thermosacc Dry (Lallemand Ethanol Technology, Montreal, Canada) was used, which is a *Saccharomyces cerevisiae* strain dedicated to the production of biofuels and alcoholic beverages.

Methods

Pretreatment methods

For the dilute acid pretreatment (ACP), wood chips were treated with 0.5% and 2.0% (w/v) H_2SO_4 at 121 °C and a substrate solid loading of 5% (w/v) (dry weight basis) for 1 h. The pretreated biomass was then neutralized to a pH of 5.0 with a 1-M NaOH solution. When the alkali pretreatment (ALP) was used, the wood chips were treated with 0.5% and 2.0% (w/v) NaOH solutions at 121 °C and a substrate solid loading of 5% (w/v, dry weight basis) for 1 h. After pretreatment, the biomass was washed with deionized water until the filtrate was neutral. The SE pretreatment was performed in a batch pilot unit equipped with a 5-L reaction vessel. The wood chips were introduced into the reaction vessel and exposed to saturated steam at 1.5 MPa (198 °C) for 15 min. After saturated steam exposure, a ball valve at the bottom of the reactor was opened suddenly to rapidly bring the reactor to atmospheric pressure. The pretreated fractions were stored at 4 °C before using them for hydrolysis.

Enzymatic Hydrolysis

The pretreated samples were enzymatically hydrolyzed with 5% (w/v, dry weight basis) 50 mM sodium citrate buffer at a pH of 4.8. Penicillin (50 U/mL) and streptomycin (50 μg/mL) (Sigma Aldrich) were added to prevent bacterial growth during the reaction period. Cellulase and β-glucosidase were added at doses of 20 filter paper units/g of cellulose and 15 cellobiose units/g of glucose, respectively. The hydrolysis experiments were performed at 50 °C for 72 h. Untreated samples were hydrolyzed simultaneously as reference samples. After hydrolysis was completed, the samples were collected to determine the concentrations of monomeric sugars and various lignocellulose degradation products with high-performance liquid chromatography (HPLC).

Fermentation

Fermentation was conducted in 500-mL flasks that contained 200 mL of each hydrolysate and supplemented with $(NH_4)_2HPO_4$ (0.3 g/L). After inoculation with yeast (0.5 g/L), the hydrolysates were incubated at 30 °C for 96 h under anaerobic conditions. When fermentation was completed, the residual sugars and ethanol concentrations of the samples were analyzed with HPLC and gas chromatography (GC), respectively.

Analytical Methods

The total solids, ash, and acid insoluble lignin contents in the raw materials were analyzed according to National Renewable Energy Laboratory protocols (Sluiter *et al.* 2008a; Sluiter *et al.* 2008b; Sluiter *et al*. 2012). The cellulose content was analyzed according to the Kurschner–Hoffer method (Kurschner and Hoffer 1993), in which milled raw material is treated three times with a mixture of ethanol and nitric acid (4:1) at 100 °C for 1 h, the cellulose in then washed and dried to constant weight. The hemicelluloses content was analyzed according to Ermakov method (Arasimovich and Ermakov 1987), where hemicelluloses were hydrolyzed with dilute sulfuric acid, and resulting monosaccharides were assayed by the Miller method (Miller 1959). The hydrolysis yield of the substrate was defined as the ratio of reducing sugars in the hydrolysates to the polysaccharides content in the raw material. To quantify the monomeric sugars and lignocellulose degradation products after enzymatic hydrolysis and fermentation, HPLC

analysis (Agilent 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) was performed according to Balcerek *et al.* (2016a). The ethanol concentration was analyzed using GC analysis (Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA) with a mass spectrometer (Agilent MSD 5975C,), as was described by Balcerek *et al.* (2016b). The ethanol yield was calculated based on available glucose present in the hydrolysate, according to the stoichiometric Gay-Lussac equation and expressed as a percentage of the theoretical yield.

Statistical Analysis

All of the samples were prepared and analyzed in triplicate. The statistical analysis was performed using Statistica 10 software (StatSoft, Tulsa, OK, USA). The obtained results were expressed as the mean with the standard deviation and analyzed by a one-way or twoway analysis of variance (ANOVA), followed by Tukey's post-hoc test at a significance level (p) of 0.05.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis of the Pretreated Biomass

To increase the accessibility of the lignocellulosic materials to enzymatic hydrolysis, various pretreatment methods were used. Of the pretreatment methods commonly used for lignocellulosic biomass, the dilute acid, alkali, and SE methods were compared in this study. The applied conditions were selected based on the presupposition that milder process conditions result in fewer inhibitors obtained in the prehydrolysate. Typically, the pretreatment of hardwoods with SE is carried out at temperatures between 200 °C and 260 °C for 2 to 10 min. However, under more severe conditions hemicellulose is degraded and furan compounds are generated; thus lower temperature and longer residence time were found to be more advantageous (Martin-Sampedro *et al*. 2011; Singh *et al*. 2015). Dilute acid pretreatment is mostly carried out at a temperature range from 120 °C and 210 °C for several minutes to an hour, and similar to SE, the lower severity of pretreatment results in less sugar degradation and formation of inhibitors (Xu and Huang 2014). For that reason, the application of milder process conditions of dilute acid and SE treatments, were chosen for evaluation. On the other hand, alkaline treatment can be held at low, or even ambient, temperature and pressure. However, a process carried under such conditions may require long reaction time ranging from an hour to days. In order to accelerate the pretreatment, higher temperature (up to 121 °C) is often applied (de Carvalho *et al.* 2016). From the hydrolysates obtained, the monomeric sugars concentrations were evaluated. Subsequently the glucose content was determined, which was later metabolized by yeast during fermentation. The results are presented in Table 2. Additionally, the lignocellulose degradation products concentration was also analyzed.

In the samples obtained after acid pretreatment and enzymatic hydrolysis of the aspen chips, the main content of the reducing sugars was glucose (13.3 g/L and 9.7 g/L for 0.5% and 2.0% H_2SO_4 (w/v), respectively). After enzymatic hydrolysis of untreated aspen sawdust, the concentration of xylose was 1.5 g/L. The application of dilute acid pretreatment resulted in a significantly ($p < 0.05$) higher xylose concentration (7 g/L), which may have been because of the decomposition of hemicellulose contained in the raw

material. By contrast, in the hydrolysates obtained from the birch chips, the glucose content was low and did not exceed 4 g/L. The xylose content in the samples that were not pretreated was 0.25 g/L, and applying the dilute acid pretreatment increased the concentration to almost 8 g/L ($p < 0.05$). The hydrolysates from the oak chips contained almost twice as much arabinose as those obtained from the aspen or birch chips, possibly because of a different chemical composition of the hemicellulose fraction in the raw materials. The glucose and xylose contents in the oak hydrolysates, as was the case for the birch chips, did not exceed 3.5 g/L and 8 g/L, respectively ($p > 0.05$). The low sugar contents in all of the hydrolysates may have been caused by the high resistance of the lignocellulosic raw materials to acid hydrolysis because of the presence of a high lignin content in the biomass. Cara *et al*. (2008) studied the effect of different sulfuric acid concentration (0.2, 0.6, 1.0 and 1.4% w/w) and temperatures (range from 170 to 210 °C) applied during dilute acid pretreatment on subsequent saccharification of olive tree biomass. The authors obtained the best hemicellulose recovery rate (83%) in the prehydrolysate after pretreatment at 170 °C with 1% w/w sulfuric acid. The maximum cellulose hydrolysis yield (76.5%) was achieved from solids pretreated at 210 °C with 1.4% acid concentration.

Table 2. Concentration of Monomeric Sugars in Liquid Fraction after 72 h of Enzymatic Hydrolysis of Biomass Obtained after Various Pretreatment Methods

Raw Material	Method	Cellobiose	Glucose	Xylose	Arabinose
		Concentration (g/L)			
Aspen	Untreated	0.22 ± 0.04cde	9.83 ± 0.48e	1.16 ± 0.02ab	0.11 ± 0.01bc
	0.5% ACP	n.d.	13.64 ± 0.88f	7.04 ± 0.52gh	0.28 ± 0.03de
	2.0% ACP	n.d.	9.75 ± 0.31e	7.44 ± 0.18h	0.26 ± 0.04d
	0.5% ALP	0.87 ± 0.06h	22.30 ± 1.43g	6.05 ± 0.90fg	0.02 ± 0.01a
	2.0% ALP	1.00 ± 0.07hi	23.35 ± 1.85g	5.15 ± 0.85def	0.02 ± 0.01a
	SE	0.31 ± 0.08ef	15.37 ± 0.93f	1.79 ± 0.17b	0.02 ± 0.01a
Birch	Untreated	0.07 ± 0.01ab	1.74 ± 0.06a	0.25 ± 0.01a	0.16 ± 0.02c
	0.5% ACP	0.56 ± 0.05g	3.35 ± 0.07ab	7.38 ± 0.22h	0.30 ± 0.04de
	2.0% ACP	1.05 ± 0.11i	4.07 ± 0.15b	7.84 ± 0.19h	0.28 ± 0.02de
	0.5% ALP	0.20 ± 0.01bcde	8.76 ± 0.23e	5.26 ± 0.15ef	0.02 ± 0.01a
	2.0% ALP	0.20 ± 0.01bcde	10.10 ± 0.44e	4.22 ± 0.20cde	0.02 ± 0.01a
	SE	0.24 ± 0.03de	10.04 ± 0.19e	1.91 ± 0.17b	0.02 ± 0.01a
Oak	Untreated	0.07 ± 0.01ab	1.64 ± 0.14a	0.43 ± 0.06a	0.07 ± 0.01ab
	0.5% ACP	0.25 ± 0.03de	1.83 ± 0.11a	4.07 ± 0.16cd	0.34 ± 0.03e
	2.0% ACP	0.86 ± 0.16h	3.33 ± 0.18ab	7.85 ± 0.15h	0.53 ± 0.03f
	0.5% ALP	0.08 ± 0.01abc	6.64 ± 0.19c	3.93 ± 0.18c	0.11 ± 0.01bc
	2.0% ALP	0.41 ± 0.05f	10.75 ± 0.34e	4.37 ± 0.27cde	0.12 ± 0.02bc
	SE	0.15 ± 0.02bcd	6.84 ± 0.58cd	1.60 ± 0.19b	0.02 ± 0.01a

n.d. – Not detected; The lower-case letters in the columns indicate a significant difference ($p < 0.05$), as analyzed by the two-way ANOVA and Tukey's post-hoc test

After the alkali pretreatment, the derived hydrolysates from the aspen chips contained mainly glucose (approximately 70% to 80% of the released reducing sugars). The glucose content reached 23 g/L, while the xylose concentration was only 5 g/L to 6 g/L. The arabinose concentration was low (0.02 g/L) in the hydrolysates from the aspen and birch chips. The glucose and xylose concentrations in the hydrolysates obtained from the alkali-treated oak and birch wood were significantly lower ($p < 0.05$) than those from the aspen wood, which reached 7 g/L to 10 g/L and 4 g/L to 5 g/L, respectively. The hydrolysates from the oak chips contained slightly more arabinose than those obtained from the aspen and birch chips ($p < 0.05$). Mirahmadi *et al*. (2010), determined the effect of alkaline pretreatment of birch and spruce wood, at a temperature between -15 and 100 °C, for 2 h, with 7.0 w/w NaOH solution. The authors have found that as a result of pretreatment the significant reduction of hemicellulose and the crystallinity of cellulose occurred, resulting in improvement of enzymatic hydrolysis of birch from 6.9% to 82.3% (at 100 °C), and for spruce from 14.1% to 35.7% (at 5 °C).

Steam explosion is one of the most frequently used pretreatment methods for lignocellulosic raw materials. In this work, the application of SE did not considerably affect the reducing sugar contents released during enzymatic hydrolysis of the raw materials (Table 2). This may be the result of mild conditions used in experiments. As reported by Ibrahim and Glasser (1999), and Josefsson *et al*. (2002) the glucose content in solid fraction increased with increasing severity of steam pretreatment, from 68.4 to 76.7% and from 88.8 to 96.9%, respectively for oak and aspen wood. In the present study, the impact of the pretreatment method was strongly dependent upon the biomass ($p < 0.05$). The best pretreatment resulted in 15 g/L glucose and 2 g/L xylose for the aspen chips, 10 g/L glucose and 2 g/L xylose for the birch chips, and 6.8 g/L glucose and 1.5 g/L xylose for the oak chips. For all of the tested materials, 90% of the sugars released after pretreatment were glucose. The arabinose content in all of the samples was at a similar level (approximately 0.022 g/L ($p > 0.05$)). Vivekanand *et al*. (2013) conducted an autohydrolysis study of birch chips at various temperature (170 °C to 230 °C) and time conditions (5 min to 15 min). They found that at severe pretreatment conditions, a higher degree of xylan degradation and pseudo-lignin formation occurred. They obtained the highest glucose concentration (corresponding to a 97% yield) after autohydrolysis at 220 °C for 10 min.

Based on the released monomeric sugar content, the hydrolysis yield was calculated (Fig. 1). The raw material most susceptible to hydrolysis was the aspen chips, as even the untreated aspen chips furnished a 27% yield, which was significantly ($p < 0.05$) higher than that from the other two raw materials. The untreated birch and oak chips resulted in similar hydrolysis yields ($p > 0.05$) of 5.6% and 8.6%, respectively. The H_2SO_4 pretreatment of the aspen chips at both the 0.5% and 2% concentrations (w/v) gave similar hydrolysis yield results ($p > 0.05$), at approximately 60%.

The alkali pretreatment using the 0.5% (w/v) NaOH concentration resulted in a similar yield as that with acid at higher concentration ($p > 0.05$). However, increasing the NaOH concentration to 2% (w/v) resulted in a significant ($p < 0.05$) increase in the hydrolysis efficiency to almost 80% of the theoretical yield. The hydrolysis efficiency of the birch and oak biomass for all of the pretreatment methods was similar ($p > 0.05$); with 2% H_2SO_4, higher yields were obtained from hydrolysis of the oak chips compared with that from the birch chips ($p < 0.05$) (51% and 58% efficiency, respectively).

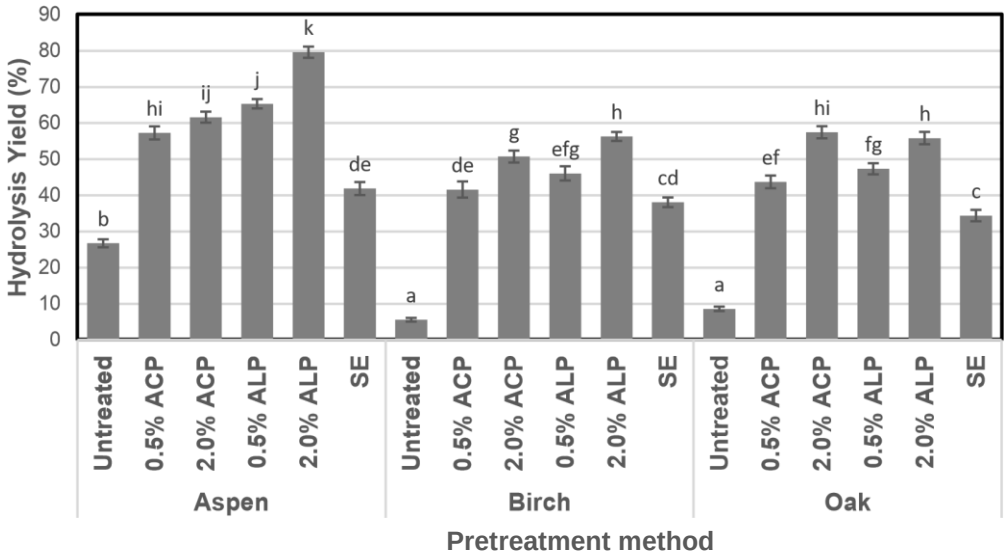

Fig. 1. Enzymatic hydrolysis yield of the aspen, birch, and oak chips treated with H_2SO_4, NaOH (at concentrations of 0.5% and 2.0% (w/v)), and SE; Error bars show the standard deviation; Different lower-case letters indicate significant differences ($p < 0.05$) between the mean values of the hydrolysis yield (two-way ANOVA and Tukey's post-hoc test)

Effect of the Pretreatments on the Formation of Lignocellulose Degradation Products and Ethanol Production Efficiency

After hydrolysis was completed, all of the samples were subjected to fermentation using dry distillers yeast. The susceptibility of the hydrolysates to fermentation was reliant on the concentrations of specific chemical compounds present in the starting material, formed during pretreatment, or formed during the fermentation processes. The concentrations of these compounds during fermentation depends largely on the configuration of the downstream process. All of the hydrolysates were also analyzed for the concentrations of sugar degradation products, such as aliphatic acids (lactic, formic, and acetic acids) and furan compounds (furfural and 5-Hydroxymethylfurfural (5-HMF)) (Table 3).

Among the aliphatic acids, the highest concentration was observed for acetic acid, which may have been related to the chemical structure of the hardwood hemicellulose, in which the xylan chain is highly substituted with acetyl groups (Larsson *et al.* 1999). The concentration of acetic acid in the untreated samples was approximately 0.09 g/L to 0.18 g/L. The alkali pretreatment led to a reduction in the acetic acid concentration to 0.03 g/L to 0.05 g/L, regardless of the raw material used ($p > 0.05$). However, the dilute acid pretreatment caused a significant increase in the acetic acid concentration to 0.78 g/L to 1.63 g/L, 1.61 g/L to 1.71 g/L, and 1.77 g/L to 1.85 g/L for the oak, aspen, and birch chips, respectively ($p < 0.05$). The lactic and formic acids concentrations were generally lower than the acetic acid concentration, and strongly depended on both the raw material and pretreatment method ($p < 0.05$). The hydrolysates obtained from the hardwood biomass and agricultural wastes contained noticeably higher concentrations of these compounds than those obtained from softwood (Larsson *et al.* 1999).

Table 3. Concentration of Select Lignocellulose Degradation Products in the Liquid Fraction after 72 h of Enzymatic Hydrolysis of Biomass Obtained after Various Pretreatment Methods

Raw Material	Pretreatment Method	Lactic Acid	Formic Acid	Acetic Acid	Furfural	5-HMF
		Concentration (mg/L)				
Aspen	Untreated	0.62 ± 0.07a	0.69 ± 0.09a	183.70 ± 11.10d	369.42 ± 18.54a	42.24 ± 3.64bc
	0.5% ACP	17.90 ± 1.30cd	107.45 ± 2.93f	1610.51 ± 84.64h	10384.61 ± 413.97k	141.68 ± 3.53h
	2% ACP	25.41 ± 1.54d	120.60 ± 3.37g	1713.91 ± 78.04i	10002.37 ± 316.94j	184.95 ± 5.56i
	0.5% ALP	5.08 ± 0.23ab	18.27 ± 0.67c	54.14 ± 2.31a	2297.89 ± 111.62f	75.97 ± 3.67e
	2% ALP	2.25 ± 0.24a	n.d.	52.69 ± 2.89a	2032.30 ± 113.53e	101.57 ± 4.45g
	SE	n.d.	3.44 ± 0.46ab	247.67 ± 10.24e	2759.36 ± 130.95g	53.62 ± 2.65cd
Birch	Untreated	n.d.	n.d.	137.24 ± 8.68c	402.32 ± 10.13a	n.d.
	0.5% ACP	94.52 ± 2.19f	32.52 ± 3.42e	1774.41 ± 91.19j	4489.63 ± 225.95i	87.93 ± 6.93f
	2% ACP	99.41 ± 8.13f	225.90 ± 9.25h	1855.30 ± 92.12k	13037.83 ± 535.95m	93.28 ± 5.60fg
	0.5% ALP	93.37 ± 5.99f	9.56 ± 0.90b	40.22 ± 3.22a	1337.44 ± 40.78d	49.87 ± 3.90bcd
	2% ALP	21.39 ± 1.98d	29.10 ± 2.91de	32.29 ± 2.82a	1172.55 ± 61.59c	49.63 ± 2.41bcd
	SE	n.d.	n.d.	324.65 ± 12.15f	1118.81 ± 63.43c	26.17 ± 1.99a
Oak	Untreated	0.30 ± 0.02a	n.d.	88.41 ± 6.38b	353.57 ± 16.83a	n.d.
	0.5% ACP	13.34 ± 1.43c	30.61 ± 2.61e	779.24 ± 29.27g	3294.42 ± 121.01h	22.41 ± 1.43a
	2% ACP	33.79 ± 1.85e	32.42 ± 1.62e	1630.48 ± 88.84h	12934.02 ± 457.11l	85.45 ± 4.43ef
	0.5% ALP	10.47 ± 1.49bc	20.55 ± 1.75cd	49.31 ± 2.07a	2742.13 ± 137.99g	38.52 ± 1.44b
	2% ALP	10.39 ± 0.92bc	20.56 ± 1.67cd	42.55 ± 2.77a	2706.87 ± 114.78g	56.13 ± 3.49d
	SE	n.d.	n.d.	230.18 ± 9.94e	843.62 ± 36.29b	18.83 ± 1.14a

n.d. – Not detected; The lower-case letters in the columns indicate significantly different values ($p < 0.05$), as analyzed by the two-way ANOVA and Tukey's post-hoc test

Taking into consideration furan derivatives, the furfural concentration was significantly higher than the 5-HMF and generally increased in the following order for the different pretreatments: SE < ALP < ACP. In the case of the dilute acid pretreatment, increasing the acid concentration from 0.5% to 2% (w/v) caused 3- and 4-fold higher furfural concentrations for the birch and oak chips, respectively. For the aspen chips, both 0.5% and 2% (w/v) H_2SO_4 resulted in high furfural concentrations of approximately 10 g/L. The 5-HMF concentration was also dependent upon the raw material and pretreatment method ($p < 0.05$). The highest amount was detected in the case of the aspen chips pretreated with dilute acid. Both furfural and 5-HMF are the dominant furan compounds present in lignocellulosic hydrolysates from the dehydration of pentoses and hexoses, respectively. The presence of furfural impairs ethanol productivity by yeast, similar to 5-HMF, but the latter is remarkably more toxic at the same doses (Larsson *et al.* 1999). Wikandari *et al.* (2010) showed that the addition of furfural at doses above 0.5 g/L results in a 73% decrease in the ethanol yield. Fermentation of hydrolysates produced from this study generated a relatively high ethanol yield based on glucose available in hydrolysates (Fig. 2).

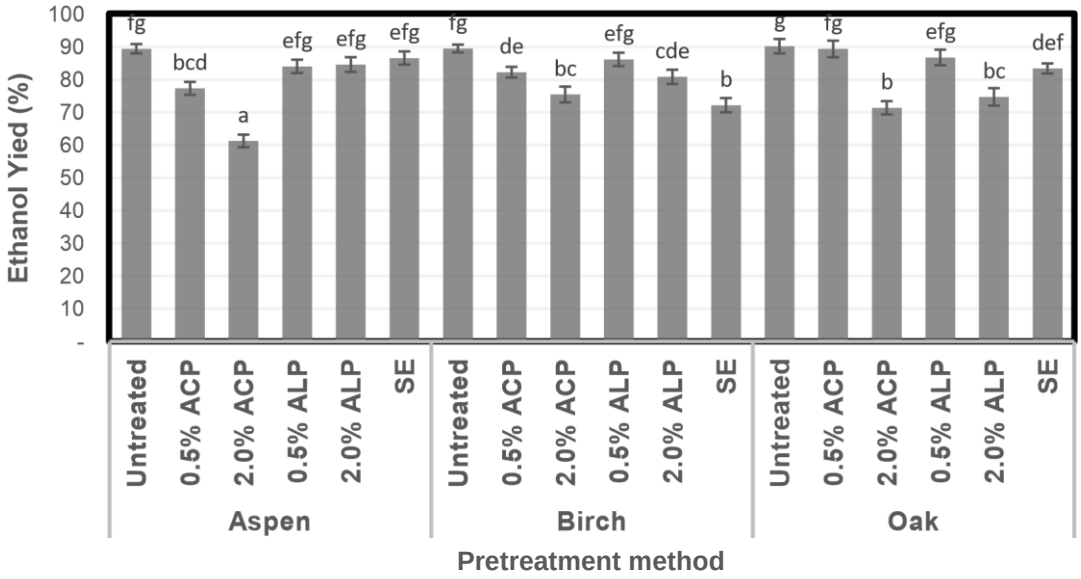

Fig. 2. Ethanol yield from the fermentation of the aspen, birch, and oak chips treated with H_2SO_4, NaOH (at concentrations of 0.5% and 2.0%), and SE; Error bars show the standard deviation; Different lower-case letters indicate significant differences ($p < 0.05$) between the mean values of the hydrolysis yield (two-way ANOVA and Tukey's post-hoc test)

For the untreated chips, the ethanol yield was approximately 90% of the theoretical yield ($p > 0.05$), and the obtained efficiency was similar when the biomass was pretreated with 0.5% NaOH ($p > 0.05$). The concentration of toxic compounds in hydrolysates from untreated biomass was negligible; thus the fermentation activity of yeast had not been inhibited. However, it is worth mention, that the total amount of glucose in untreated samples was lower ($p < 0.05$) in comparison with samples after pretreatment. Fermentation of the hydrolysates obtained after the SE pretreatment resulted in 83% and 86% efficiencies for the oak and aspen chips, respectively. However, in the case of the birch chips, the obtained ethanol yield was significantly lower ($p <$

0.05). The lowest fermentation yield from the hydrolysates was obtained after the dilute acid pretreatment, wherein increasing the H_2SO_4 concentration from 0.5% to 2% resulted in a significant decrease in the ethanol yield. Jung *et al*. (2014) obtained over 70% of the theoretical ethanol yield after fermentation of the hydrolysate from the acid-base pretreatment at 190 °C for 2 min.

In order to better compare the total ethanol yield obtained after fermentation of biomass pretreated by different methods, the obtained ethanol concentration was calculated and expressed in liters of pure ethanol per tonne of raw wood (Fig.3).

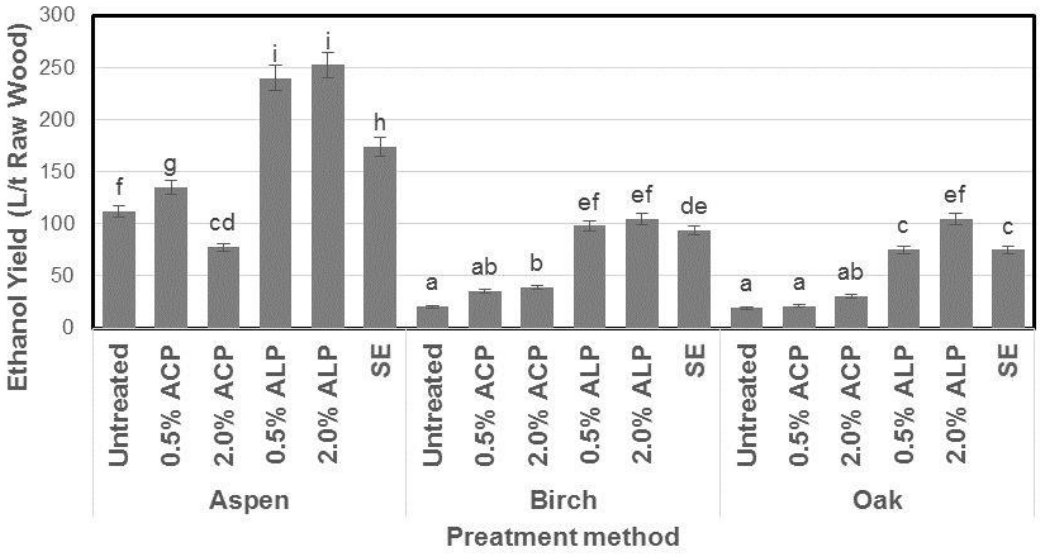

Fig. 3. Ethanol yield expressed in L of ethanol per tonne of raw wood biomass, obtained after the fermentation of the aspen, birch, and oak chips treated with H_2SO_4, NaOH (at concentrations of 0.5% and 2.0%), and SE; Error bars show the standard deviation; Different lower-case letters indicate significant differences ($p < 0.05$) between the mean values of the hydrolysis yield (two-way ANOVA and Tukey's post-hoc test)

The highest ethanol yield (252 L/tonne) was achieved after fermentation of alkali-pretreated aspen sawdust. In general, the biomass of aspen was found to be most susceptible to hydrolysis and fermentation processes, as even untreated chips gave relatively high ethanol yield (112 L/tonne). The biomass of birch and oak was more resistant to applied pretreatment conditions. The highest achieved ethanol yield was only 105 L/tonne (for both birch and oak) when alkali pretreatment was used. The obtained results are lower than those reported by Stephen *et al*. (2012), who gave the theoretical yield (based on C6 sugars only) as 318 and 439 L/bone dry ton for poplar stem and Douglas fir heartwood, respectively.

CONCLUSIONS

1. The highest hydrolysis yield (79.6% of the theoretical yield) was obtained for the aspen chips with 2% NaOH, while the most efficient pretreatment method for the oak chips was the dilute acid pretreatment with 2% H_2SO_4.

2. When considering both the enzymatic hydrolysis and ethanol fermentation yields, the best results were obtained with the aspen chips pretreated with 2% NaOH.
3. The oak biomass was the most resistant raw material, regardless of the pretreatment applied.

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