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Sugar production from wheat straw biomass by alkaline extrusion and enzymatic hydrolysis

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ABSTRACT

One characteristic necessary to make ethanol production from biomass economically feasible is to optimize enzymatic dosage, since enzymes production is expensive. This work investigated the efficacy of different enzymes dosages and solid loadings on wheat straw enzymatic hydrolysis, aimed at obtaining process conditions that lead to good sugars yields from pretreated material. Alkaline extrusion was employed as pretreatment at 70 °C and 10% NaOH solution (w/v). Enzymatic hydrolysis was performed at 5, 10, 15 and 20% solids loading (w/v). Enzyme doses ranged from 6.92 to 20 FPU/g of glucan. Cellulase was also supplemented with xylanase at various proportions. Alkaline extrusion provided a substrate easier to hydrolyze than untreated material. Even the assay with the lowest enzyme dosage (6.92 FPU) achieved a good carbohydrate hydrolysis yield in relation to the theoretical; the glucose yield was 73.8% and xylose yield was 82.8%. A medium containing 100 g/L of fermentable sugar was obtained at 20% solids loading (w/v) and 20 FPU/g of glucan. The supplementation of cellulase with xylanase at U to FPU activity ratio of 3.11:1 improved the glucose yield about 21% over the assay with no xylanase.

1. Introduction

The conversion of lignocellulosic biomass to biofuels represents a viable option for improving energy security and could potentially reduce greenhouse gas emissions by up to 86% compared to fossil fuels [1]. Wheat straw a lignocellulosic biomass source widely available for conversion into biofuels, such as bioethanol, and is the second most abundant biomass feedstock on Earth, behind rice straw [2].

Lignocellulosic materials have resilient structures that consist of a carbohydrate polymer matrix, mainly cellulose and hemicelluloses. They are cross-linked and strongly bound to lignin. This structural complexity, defined as biomass recalcitrance, severely restricts enzymatic and microbial accessibility [3]. Pretreatment is a key unit operation in bioethanol production, due to the need to deconstruct this structure. The pretreatment step improves the rate of production as well as the total yield of liberated sugars in hydrolysis step [4]. The objectives are to increase the surface area and porosity of the substrate, reduce the crystallinity of cellulose and disrupt the heterogeneous structure of lignocellulosic materials. However, degradation of sugars may occur during pretreatment and thus, it is necessary to find a good balance between low inhibitors formation and high substrate digestibility to optimize the overall efficiency of the pretreatment process [5].

Among the several pretreatment reactor systems that are being currently studied, extrusion stands out for its ability to provide high shear, rapid heat transfer, and effective and rapid mixing. Twin-screw extrusion pretreatment is a technology that can effectively open the recalcitrant structure of lignocellulosic biomass into its constituents at mild temperature and chemicals conditions, preventing the formation of inhibitory by-products [6]. It can achieve high-efficiency mixing via a high shear force and a high throughput; further, it can be adapted to many different processes [7].

Several studies have shown that extrusion pretreatment is efficient in increasing the cellulose and hemicellulose digestibilities of barley straw [6,8], rice straw [9], *Miscanthus* [10], corn stover [11], rapeseed straw [12] and soybean hulls [13]. The alkaline extrusion of barley straw resulted in glucose and xylose yields 4.0× and 5.8× higher than for the untreated barley straw, respectively

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[8]. Soybean hulls subjected to thermo-mechanical extrusion at 80 °C showed a glucose yield, from enzymatic hydrolysis, 132.2% higher, when compared to the untreated soybean hulls [13]. However, there is no published paper regarding the extrusion of wheat straw, specifically about sugar production for bioethanol conversion.

Following pretreatment, hydrolysis using appropriate enzymes represents the most effective method to liberate monosaccharides from lignocellulose. Three major enzymes namely, endo-glucanase, exo-glucanase and β-glucosidase are involved in hydrolysis of cellulose to glucose and their actions are synergistic. Endo-glucanase attacks regions of low crystallinity in the cellulose fiber and creates free chain-ends. Exo-glucanase degrades the molecule further by removing cellobiose units from the free chain-ends, which is then cleaved to glucose by the action of β-glucosidase. The enzymatic hydrolysis can be influenced by substrate and end-product concentrations, enzyme activity and reaction conditions [14]. Inclusion of lytic polysaccharides monooxygenases also improves the effectiveness of cellulases. Use of cellulase enzyme supplemented with other enzymes can raise the rate of enzymatic hydrolysis. It is well known that supplementing cellulases with hemicellulases may result in a higher ultimate sugar production. Cellulase dosage of 10–30 FPU/g of cellulose is often used in studies; however, enzymes loadings may vary depending on the pretreatment, type and concentrations of raw material [15].

Even for biomass pretreated under optimum conditions by leading pretreatment technologies, very high enzyme doses are still required to achieve high-yield conversion of polymeric cellulose and hemicellulose into monosaccharides that can be utilized by fermentative microorganisms [16]. Thus, the costs of enzymes and pretreatment are the major barriers to low cost processing of biomass, and must be lowered substantially to make the cost of cellulosic ethanol competitive with that of fossil fuels or corn and sugarcane ethanol [17].

In this context, the objectives of this work are: i) evaluate the improvement of glucan and xylan digestibility from wheat straw after a pretreatment that combines a twin-screw extruder with sodium hydroxide solution and ii) investigate the efficacy of different solids loading and enzymes dosages on the release of fermentable sugars from wheat straw extrudates, aimed at obtaining a condition that allows for high sugars yields at reasonable enzyme loading, without inhibitors formation.

2. Materials and methods

2.1. Raw material

Wheat straw (6% moisture content) was coarsely crushed to about 5 mm particle size using a laboratory hammer mill (Retsch), homogenized and stored in an oven at 40 °C until used. Composition of raw material is presented in Table 2.

2.2. Alkaline extrusion

Extrusion was performed in a twin-screw extruder (Clextral Processing Platform Evolum® 25 A110, Clextral, France), composed of 6 modules of 100 mm length each. The modules have a heating and cooling system, which temperature was set at 70 °C throughout extrusion process. The screws are composed of different elements (diameter 25 mm) and they were configured to produce transport, mixing and shearing effects along the process. One metering pump connected to the extruder was used to supply the catalyst (NaOH solution at 10%, w/v) to the process. The NaOH solution was pumped into the extruder at feeding flow of 0.3 L/h, in order to achieve an alkaline ratio of 6 g NaOH per 100 g of dry wheat straw. Biomass feeding was done through a volumetric feeder KMV KT20 (Coperion K-Tron, Sewell, NJ) with a continuous feed rate of 0.6 kg/h. A fixed motor speed of 150 rpm was used and at this condition the residence time of the biomass inside the extruder was about 2 min. These operational parameters were selected based on results from a previous optimization study that used barley straw [6]. The moisture of wheat straw entering and exiting the extruder was around 12 and 39%, respectively.

After extrusion, solid extruded material was recovered and the pH adjusted using 85% phosphoric acid (w/v) by adding a ratio of 1 mL of acid per 49 g of dry extrudate. After homogenizing the acid, the material was thoroughly washed 3 times with distillate water (without recycling), in a ratio of 80 mL of distilled water per 6 g of dried extrudate. Filtrates from the 3 washes were collected and analyzed for sugar concentration as described below. Likewise, a portion of the washed solid fraction was analyzed for main components composition (see below) and the remainder was stored at 4 °C in hermetic plastic bags until used in enzymatic hydrolysis experiments.

2.3. Enzymatic hydrolysis

The solid fraction from the alkaline extrusion step was used as substrate for enzymatic hydrolysis. The hydrolysis experiments were performed in 0.05 M sodium citrate buffer (pH 4.8), in 100 mL Erlenmeyer flasks on a rotary shaker at 150 rpm and 50 °C. The final workload was 50 mL for all assays. Enzymatic cocktail consisted of commercial cellulase (CelliCTec 2 – enzymatic activity 72.08 FPU/g) and xylanase (CellicHTec2 - enzymatic activity 898.34 U/g), kindly provided by Novozymes A/S (Denmark). The cellulase activity was determined following the filter paper activity (FPU) methodology, according to Ghose [18]. The xylanase activity was determined according to the method described by Bailey et al. [19].

Different enzymatic hydrolysis (EH) experiments were designed in triplicate to determine the best process conditions in terms of enzymes and solid loading, in order to attain a medium with a suitable amount of sugars for added-value products production, such as bioethanol. Table 1 presents a summary of all enzymatic hydrolysis tests performed, with the solids loadings and enzymes amounts employed. For the solids loading, the percentage weight/volume (w/v) refers to the amount of wheat straw extrudates (dry weight) in relation to the total volume in the solution (sample moisture plus buffer solution).

The first enzymatic hydrolysis test (EH I) was performed with 5% (w/v) dry extrudate solids loading. A mixture of cellulase and xylanase was used, reaching a total enzyme load of 12 or 33% (g enzyme/g glucan), and with xylanase substituted for 20 and 15% of the total added enzymes, corresponding to U to FPU activity ratio of 3.11:1 and 2.33:1, respectively.

The second enzymatic hydrolysis test (EH II) was performed with 10% (w/v) dry extrudate load. The total enzyme load was 12% (g enzyme/g glucan), with 10, 15 or 20% weight of total enzyme corresponding to xylanase, which account for U to FPU activity ratio of 1.38:1, 2.20:1 and 3.11:1, respectively. An assay without xylanase was also performed for comparison purposes. As a control, untreated wheat straw was tested at the same conditions above, using 0 and 10% of xylanase in total enzyme loading.

The third enzymatic hydrolysis test (EH III) was carried out with 10, 15 and 20% (w/v) dry extrudate load, with a total enzyme loading of 33% (g enzyme/g glucan), with 15% of total enzyme weight corresponding to xylanase, which account for U to FPU activity ratio of 2.33:1.

For all assays, samples were taken after 0, 24, 48 and 72 h, in triplicate, and an aliquot of each one was centrifuged at 8085 g for 5 min. The supernatant was analyzed by high pressure liquid

Table 1
Enzymatic hydrolysis (EH) experiments conducted on wheat straw alkaline washed-extrudates.

Tests	Assays	Solids loading (% w/v)	Enzyme loading cellulase/xylanase (units/g glucan)	% Xylanase in total enzyme loading[a]	U/FPU ratio[b]
EH I	1	5%	6.92 FPU/21.56 U	20%	3.11:1
	2		20 FPU/46.64 U	15%	2.33:1
EH II	3	10%	8.65 FPU/0 U	0%	0
	4		7.78 FPU/10.77 U	10%	1.38:1
	5		7.35 FPU/16.17 U	15%	2.20:1
	6		6.92 FPU/21.56 U	20%	3.11:1
	7	control[c]	8.65 FPU/0 U	0%	0
	8	control[c]	7.78 FPU/10.77 U	10%	1.38:1
EH III	9	10%	20 FPU/46.64 U	15%	2.33:1
	10	15%			2.33:1
	11	20%			2.33:1

[a] Percentage of xylanase in total enzyme loading (w/w).
[b] U to FPU activity ratio. Cellulase activity 72.08 FPU/g. Xylanase activity 898.34 U/g.
[c] Assays carried out with untreated wheat straw.

Table 2
Composition of untreated wheat straw and wheat straw alkaline-extrudates (% dry weight).

	Untreated	Extrudate
Cellulose	37.8 ± 1.9	46.9 ± 0.1
Hemicellulose	28.2 ± 0.5	28.7 ± 0.1
Xylan	24.0 ± 0.8	24.3 ± 0.1
Arabinan	2.7 ± 0.1	3.0 ± 0.0
Galactan	1.3 ± 0.0	1.2 ± 0.0
Mannan	0.2 ± 0.0	0.2 ± 0.0
Lignin	19.8 ± 0.3	15.4 ± 0.1
Acid insoluble	18.3 ± 0.5	14.4 ± 0.1
Acid soluble	1.5 ± 0.0	1.1 ± 0.0
Extractives	7.1 ± 0.5	6.7 ± 0.1
Ash	3.7 ± 0.0	3.3 ± 0.0
Acetyl groups	2.5 ± 0.0	0.0 ± 0.0

chromatography (HPLC) for glucose and xylose concentrations, as described below. For assays with 15 or 20% solids loading, in the beginning of reaction, the solution was very dense and mass difficult to shake using 150 rpm. In these conditions, mass transfer limitations may be occurring. To help to solve this drawback, in the first 24 h a manual agitation of the Erlenmeyer was carried out to facilitate the liquefaction and as the solution was became fluid, the agitation using 150 rpm was possible. To calculate the concentration of glucose and xylose at 0 and 24 h, for assays with 15 or 20% solids loading, 1 g of sample (well homogenized) was diluted with 10 mL of water, then, the mixture was centrifuged at 8085 g for 5 min and the supernatant analyzed for sugars concentration, taking into account the dilution factor.

In all the experiments carried out in this work, sugars were measured at time 0 to account for any residual sugars present despite the wash steps. These sugars are referred as concentration or yield at time = 0 throughout this manuscript. The enzymatic hydrolysis yield is used to evaluate the hydrolysis performance, which is defined as the amount of glucose/xylose released during the hydrolysis divided by the potential amount of glucose/xylose that could be released if 100% hydrolysis would occur (calculated based on glucan/xylan content of the solid extrudate), and expressed as percentage. The amount of sugars present in the enzymatic preparations was previously measured by HPLC and subtracted out for this calculation.

2.4. Analytical methods

Laboratory Analytical Procedures (LAP) for biomass analysis from National Renewable Energy Laboratory (NREL, CO, USA) [20] were used to determine carbohydrates, acid insoluble lignin, acid soluble lignin, acetyl groups, extractives and ash content in raw material and wheat straw solid extrudates.

The soluble fraction after completion of enzymatic hydrolysis tests was analyzed for its content in monomeric and oligomeric sugars (glucose and xylose). The oligosaccharides ratio was determined as the difference in monomeric sugars concentration before and after mild acid hydrolysis (4% v/v H_2SO_4, 121° and 30 min) of EH media.

Sugars concentration in EH media and filtrates was measured by high-performance liquid chromatography (HPLC) in a Waters 2695 liquid chromatograph with refractive index detector, as described by Cara et al. [21].

Furfural and HMF were analysed by HPLC (Hewlett Packard, Palo Alto, CA), using an Aminex ion exclusion HPX-87H cation-exchange column (Bio-Rad Labs, Hercules, CA) at 65 °C. Mobile phase was 89% 5 mM H_2SO_4 and 11% acetonitrile at a flow rate of 0.7 mL/min. Column eluent was detected with a 1040A Photodiode-Array detector (Agilent, Waldbronn, Germany).

3. Results and discussion

3.1. Characterization of untreated wheat straw and wheat straw extrudates

The composition of untreated wheat straw and wheat straw after being subjected to alkaline extrusion (extrudate) is detailed in Table 2. The content of the three main components in untreated wheat straw was: cellulose 37.8%, hemicellulose 28.2% and lignin 19.8%, all values on a dry weight basis (dwb). A minor fraction of wheat straw composition is formed of water and ethanol soluble substances (extractives, 7%) and ash (3.7%). Moreover, acetyl groups are 2.5% of the dry wheat straw. The structural carbohydrates account for 66.1% of the dry weight, making the wheat straw a very promising substrate for sugar production and conversion to high added-value products such as bioethanol, after a suitable pretreatment.

These data are in the range reported by other authors for this material, i.e., 37.4%, 28.3% and 17.4% [22], 40.7%, 27.6% and 17.0% [23] and 35.2%, 22.2% and 22.1% [24], for glucan, hemicellulose and lignin, respectively. The composition of structural carbohydrates and lignin in the wheat straw may slightly differ depending on the species of wheat, fertilizer used during cultivation, mineral content of the soil, grain maturity attained at the time of harvest and weather conditions [4].

Alkaline extrusion followed by washing altered the composition of the wheat straw (Table 2). The most significant changes were an increase of cellulose content (46.9% in the straw extrudate and 37.8% in the untreated straw), a decrease of lignin (15.4% in the

straw extrudate and 19.8% in the untreated straw) and the complete solubilization of the acetyl groups. Besides the changes in the composition, it was possible to notice a clear modification in the appearance, color and texture of wheat straw after the extrusion (Fig. 1).

In general, the alkaline pretreatment of biomass is highly effective for hemicellulose and lignin removal, but the result depends on the catalyst concentration. The present study combined sodium hydroxide pretreatment solution with a twin-screw extruder, which can homogenize and reduce particle size. The high shearing force of the twin-screw extruder is very effective in reducing particle size and mixing the alkaline solution with the lignocellulosic biomass [10]. Therefore, the sodium hydroxide can more easily react with the surface of wheat straw, causing reduction in lignin even with a short reaction time. Our results support this hypothesis, resulting in a lignin removal of 22% of the content in untreated biomass.

Other studies using alkaline extrusion as lignocellulosic biomass pretreatment also reported a decrease in the lignin content. Duque et al. [6] reported that the lignin content of the untreated barley straw (18.2%) decreased to 14.5% and 15.6%, after extrusion with 10% NaOH solution (w/v) at 50 and 100 °C, respectively. Kang et al. [10] observed a lignin removal between 17.3% and 55.3% after the extrusion of *Miscanthus* with temperature and concentration of NaOH in the range of 50–100 °C and 0.3–0.9 M, respectively.

Lignin is one of the main obstacles for the enzymatic hydrolysis of cellulose [25]. Thus, efficient removal of lignin and high recovery of biomass in the pretreatment are important to a suitable performance of the enzymatic hydrolysis and to achieve an efficient bioethanol production from lignocellulosic materials. According to Akhtar et al. [26], the use of sodium hydroxide 2% provided an efficient removal of lignin and increased enzymatic digestibilities of wheat straw, rice straw and bagasse by 33.0%, 25.5% and 35.5%, respectively.

In addition, the alkaline pretreatment causes a partial solubilization of hemicellulose, resulting in a partial extraction of the xylan component [4,27]. Our results show that the acetyl groups from hemicellulose were completely removed from wheat straw after extrusion, which was confirmed by other studies. In the alkaline extrusion of straw, without washing, acetyl groups remain in the straw [8]. Moreover, part of the inorganic salts and other non-structural compounds were also solubilized with the extrusion, which can be verified by some decrease in ash and extractives contents in wheat straw extrudates.

The filtrates obtained by washing the wheat straw extrudates were analyzed for dissolved solids and carbohydrate oligomers and monosaccharides. The dissolved solids in the first two filtrates were 2.08 and 0.5% (w/w), which correspond, according to the volume filtered, to 20.02% of the dry raw material. The glucose content in the three filtrates (after mild acid hydrolysis) ranged from 0.06 to 0.5 g/L; these concentrations are equivalent to around 2% (w/w) of the glucan contained in the raw material, considering the total volume filtered. Thus, little glucose is lost, and the greater loss of other components during washing increased the percentage of cellulose in extrudate in comparison to the raw material. However, the solubilization of xylose was higher, around 1.15 g/L (after mild acid hydrolysis), equivalent to around 4.4% (w/w) of the xylan contained in the raw material, considering the total volume filtered. This finding may explain the fact that hemicellulose content in extrudate is the same as in the raw material, since the amount solubilized compensates for the increase that the loss of other soluble compounds would produce. It is important to note that most of solubilized glucose and xylose were present in oligomeric form in the filtrate.

As a result of the changed composition from pretreatment, the percentage of structural carbohydrates in the wheat straw extrudate increased to 75.6%, in comparison to 66.1% in untreated wheat straw. It is relevant that much of the xylan remained in the extrudate, which can be hydrolyzed in the subsequent enzymatic hydrolysis step.

Some authors have reported that the extrusion process does not generate inhibitory compounds such as furfural or hydroxymethylfurfural (HMF) in experiments conducted at low temperature [6,28,29], and our results support this fact; no furfural or HMF were detected in filtrates from the washing step. Alkaline pretreatment also reduces furan formation in comparison to acid based catalyzed pretreatments. This is one of the main advantages of this pretreatment over other thermochemical methods which are carried out at higher temperature conditions. For example, the hydrothermal treatment of wheat straw with temperatures up to 205 °C led to formation of furfural and HMF in the range of 0.027–1.212 g/L and 0.007–0.289 g/L, respectively, with increased concentrations of these compounds at higher temperatures [30]. Furfural and HMF are formed by the degradation of pentoses and hexoses, respectively, and when present in the fermentation medium, they can reduce cell growth, ATP formation and ethanol production [31].

In short, the use of alkaline extrusion for pretreatment of wheat

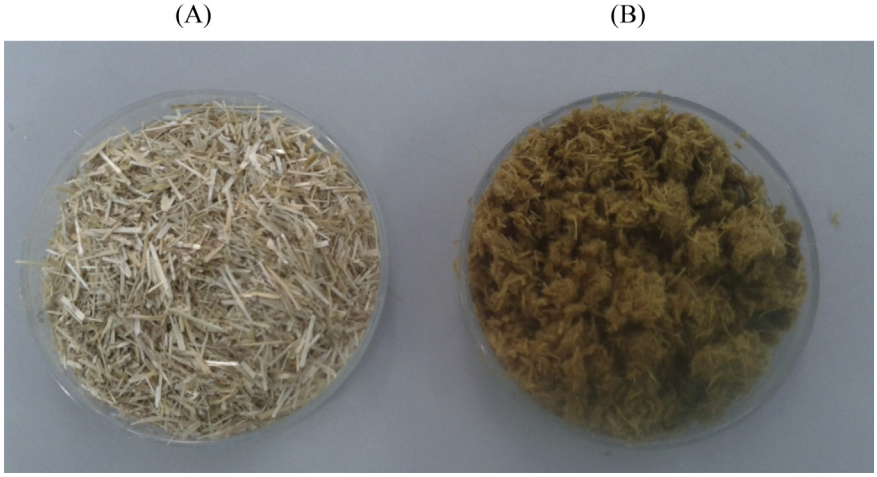

Fig. 1. Untreated wheat straw (A) and wheat straw after alkaline extrusion (B).

straw was efficient because it promoted the reduction of lignin and increased structural carbohydrates, without the formation of inhibitory compounds. All these aspects are important to obtain satisfactory yields of fermentable sugars in the enzymatic hydrolysis step.

3.2. Enzymatic hydrolysis of wheat straw extrudates

EH I test was carried out with 5% (w/v) solids loading and enzyme loading (g enzyme/g glucan) of 12% (6.92 FPU and 21.56 U/g glucan) or 33% (20 FPU and 46.64 U/g glucan), with 20% and 15% of total enzymes corresponding to xylanase, respectively. The monomeric glucose and xylose contents observed for assays 1 and 2 and the hydrolysis yields for glucan and xylan in percentage of theoretical are detailed in Fig. 2, panels A and B, respectively. The method for calculation of enzymatic hydrolysis yield for glucan and xylan is shown in point 2.3.

In assay 2, 24 and 48 h was necessary to release most of the sugars for the low and high enzyme loadings, respectively. However, after 72 h of hydrolysis, both enzyme loadings attained similar concentration of sugars (approximately 30 g/L). The assays 1 and 2 achieved hydrolysis yields for glucan of 73.8 and 75.7% (Fig. 2, panel A); and for xylan of 82.8 and 92.8% (panel B), respectively.

Ertas et al. [32] analyzed the enzymatic hydrolysis of autohydrolyzed wheat straw at 180 °C and 20 min. Enzymatic hydrolysis was carried out with cellulase dosages of 4 and 10 FPU/g of substrate with 5% w/w solids loading for 96 h. Glucose yields for the assays with 4 and 10 FPU were 24.9 and 24.6 g of glucose per 100 g of dry substrate. Xylose yields for the two assays remained below 6 g per 100 g of dry substrate. The authors attributed this low xylose content to the loss of xylan in the pretreated solid and further degradation of xylose to byproducts (furfural and HMF). Similar to

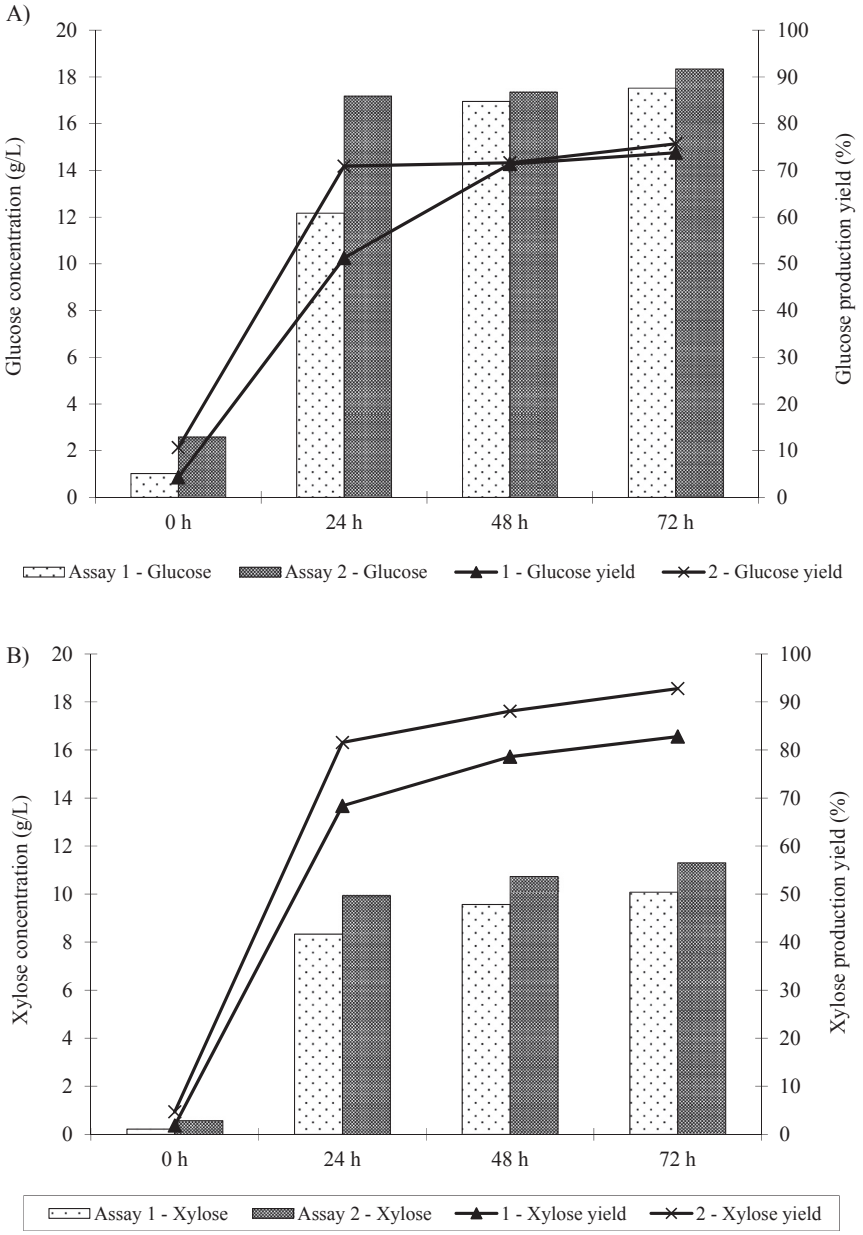

Fig. 2. Concentration of glucose and xylose and hydrolysis yield for glucan (panel A) and xylan (panel B) (monomers) released in EH I test on wheat straw alkaline-extrudates. Assay 1–12% of enzyme and assay 2–33% of enzymes (w/w, g enzyme/g glucan). Percentage of xylanase in total enzyme loading for each assay: 1–20%, 2–15%.

the present work, the increased enzyme dosage did not produce a significant increase in the glucose content at the end of enzymatic hydrolysis (after 96 h incubation). However, the hydrolysis yield for glucan and xylan (in g per 100 g of substrate) obtained in assays 1 and 2 were 35 and 20 g, respectively, which were higher than those obtained by Ertas. This is another advantage of alkaline extrusion pretreatment: xylan is not lost in this step and, after the enzymatic hydrolysis, a medium rich in glucose and also xylose can be obtained, which is favorable to increase the ethanol yield production by fermentation.

To check the effect of an increase of the solids loadings on the hydrolysis yield and seeking to obtain a medium with a higher fermentable sugars content, EH II test was conducted at 10% solids and 12% enzyme load, and percentages of xylanase in total enzyme load ranging from 0 to 20%. Two assays with untreated material were also conducted, to check the efficiency of the alkaline extrusion as a pretreatment for enzymatic hydrolysis of wheat straw.

Fig. 3 shows the sugar concentrations and theoretical yields following enzymatic hydrolysis for EH test II. Assays 3–6 are presented in decreasing order of cellulase activity (from 8.65 to 6.92 FPU/g of glucan) and increasing order of xylanase activity (from 0 to 21.56 U per g of glucan). Assays 7 and 8 were performed with untreated wheat straw and enzyme activities of 8.65 and 7.78 FPU and 0 and 10.77 U per g of glucan, respectively.

Comparing the results from the wheat straw extrudates (3–6) with those from the untreated wheat straw (7 and 8), it can be observed that alkaline extrusion significantly increased the amounts of glucose and xylose released by enzymatic hydrolysis. Regarding assay 6 (in which the best results were obtained), the glucose and xylose yields were 13× and 11× higher than for the untreated wheat straw, respectively. This assay 6, with 20% of xylanase in total enzyme load (U to FPU activity ratio of 3.11:1), resulted in the highest content of glucose monomers (30.2 g/L) and glucan hydrolysis yield of 62.7%, after 72 h of reaction. Others authors have reported the positive effects of extrusion on enzymatic hydrolysis yields [8,13,29,33].

Increasing the proportion of xylanase ratio from 0 to 20% enhanced the yield of glucose (assays 3 to 6, Fig. 3, panel A). This is

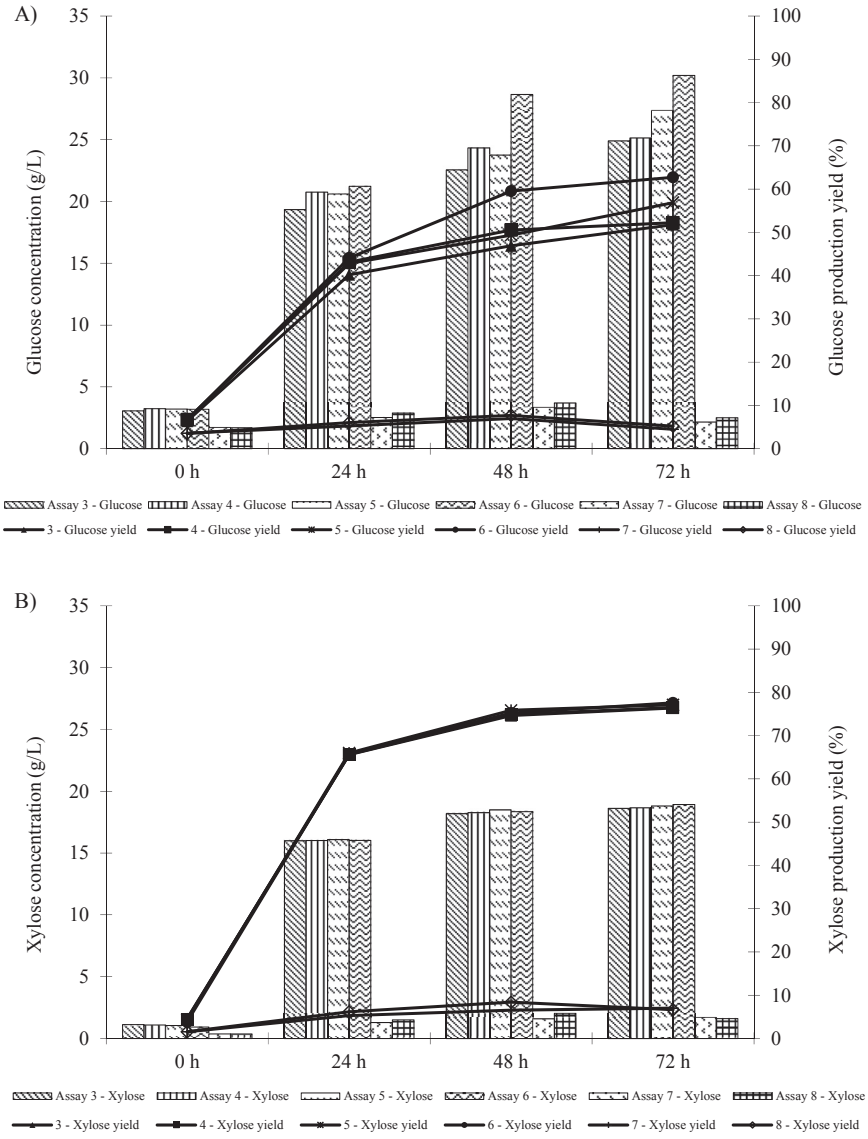

Fig. 3. Concentration of glucose and xylose and hydrolysis yield for glucan (panel A) and xylan (panel B) (monomers) released in EH II test on wheat straw alkaline-extrudates (assays 3 to 6) and untreated wheat straw (assays 7 and 8). Assays carried out with 12% of enzymes (w/w, g enzyme/g glucan). Percentage of xylanase in enzyme mixture for each assay: 3 – 0%, 4–10%, 5–15%, 6–20%, 7 – 0%, 8–10%.

demonstrated by the differences in increasing glucose concentrations between assays 4, 5 and 6, that were significant ($p < 0.05$). On the other hand, highest percentage of xylanase in total enzyme load did not have much influence on the release of xylose, since the xylose content was very similar in all EH II assays conducted with wheat straw extrudates (Fig. 3, panel B). Xylan hydrolysis yields were quite high (around 77%), even in the assay with absence of xylanase (assay 3). This finding seems to indicate that the cellulase preparation displays sufficient xylanase activity to hydrolyze the xylan present in extrudates. In fact, some authors have reported the presence of xylanase activity in cellulase enzymes supplied by Novozymes, which is why they did not need to add hemicellulases to obtain satisfactory xylose yields [24,34,35]. With extrusion pretreatment, the hemicellulose becomes very susceptible to enzymatic hydrolysis, which can be confirmed by the presence of a high xylose oligomers concentration, about 8 g/L, at time 0 (data not shown).

The improved glucan hydrolysis yield with the use of hemicellulases shown in this work has also been described in other studies. Qing and Wyman [15] reported that the addition of xylanase and β-xylosidase increased glucose yield from corn stover, compared to hydrolysis with only cellulase. The increase was about 8% when the biomass was pretreated with dilute acid, and about 26% when the biomass was submitted to AFEX (ammonia fiber expansion), pretreatment that results in a higher xylan content than the dilute acid process. The authors concluded that the increased efficiency in the assays containing xylanases was due to the xylooligomers reduction, which are potential inhibitors of cellulose hydrolysis. Even low xylooligomers concentrations of 1.67 g/L can decrease the initial rate of hydrolysis and the final glucose content [36]. In the present study, all the assays presented a very similar xylooligomers concentration (data not shown), but this value is obtained after mild acid hydrolysis and thus, it is not possible to know which types of xylooligomers are present. As xylanase is an endoxylanase, the higher concentration of this enzyme could improve the formation of smaller xylooligomers chains. This fact may cause less inhibition, since xylose, xylobiose and xylotriose has progressive impact on hydrolysis rates [37]. Another factor which could explain the improved glucose yield with the increase in percentage of xylanase is that the highest hemicellulase dosage acted more significantly on the hemicellulose branches, opening the structure of the biomass and improving cellulase accessibility to glucan.

Alkaline extrusion pretreatment followed by washing the material provided a substrate easy to hydrolyze and reasonable amounts of enzymes showed glucan and xylan hydrolysis yields of about 60 and 80% (monomers), respectively. This is a good yield, considering that a reasonable enzyme dosage of 6.92 FPU/g of glucan (3.25 FPU/g of substrate) was employed in EH II test. For a process to be economically viable enzyme dosages need to be about 4–5 FPU/g of substrate, due to the high cost of enzymes [32].

The glucan and xylan hydrolysis yields obtained in assay 6 (EH II) are similar or slightly above others reported in wheat straw. For example, Toquero and Bolado [24] pretreated wheat straw with 5% sodium peroxide for 60 min at 50 °C followed by enzymatic hydrolysis with 10% (w/v) solids loading and 10 FPU/g of glucan of enzyme NS50013 (Novozymes). The glucose and xylose contents were 31.82 and 13.75 g/L, corresponding to a yield of 63.7 and 55.2%, respectively. It is important to note that even with the higher enzyme dosage used by the authors, the total content of glucose and xylose did not reach the 50 g/L as in this study. Bellido et al. [38] obtained 23.77 and 11.29 g/L of glucose and xylose with the enzymatic hydrolysis (10% solids load, w/v) of wheat straw pretreated by steam explosion at 210 °C and 10 min, values that are also below those obtained in the present work.

To check if a higher enzyme dosage could increase the hydrolysis yield and contribute to obtain a medium with higher content of fermentable sugars, EH III test was conducted at 10, 15 and 20% solids loading, at an enzyme load of 20 FPU per g of glucan, and 15% xylanase in total enzyme load (U to FPU activity ratio of 2.33:1). The values of glucose and xylose content (monomers) found in assays 9, 10 and 11 and their respective yields are detailed in Fig. 4.

It can be seen that the glucose and xylose content (g/L) increased with increasing solids loading, reaching 64.06 and 39.60 g/L of glucose and xylose at 20% solids content, respectively, accounting for about 100 g/L of total fermentable sugars (assay 11). Considering the production of glucose and xylose in relation to both glucan and xylan in the extrudate, a global sugar production yield of 67% can be calculated. However, the hydrolysis yield for glucan dropped from about 80% to 60% (panel A) and from 88% to 81% for xylan (panel B), when the solids loading increased from 10% to 20%. Many authors have described that increasing solids loadings inhibits enzymatic hydrolysis because of mass transfer limitations and enzyme inhibition by the final product (glucose and cellobiose) occur, which can affect the final efficiency of the hydrolysis [37,39,40]. Our results are in agreement with these previous findings.

Comparing assay 9 with assay 6 (also with 10% solids, but with lower enzyme dose of 6.9 FPU per g of glucan), increasing the enzyme dosage increased the content of sugars from 30 g/L to 40 g/L. Likewise, glucan hydrolysis was 62% (of theoretical) at the lower enzyme dosage and 80% at 20 FPU per g of glucan. The same positive effect was found in xylose (monomer) production, the xylan hydrolysis yield increased from 78% to 88% as enzyme dosage levels increased. However, in the EH I test, with 5% solids, increasing the enzyme activity did not produce significant changes in hydrolysis yield at 72 h incubation. Probably, the lowest enzyme dosage was sufficient to achieve good hydrolysis performance at 5% solids, but a higher enzyme dosage is required for a larger amount of straw to be hydrolyzed.

In assays 9, 10 and 11 (EH III), the carbohydrates hydrolysis yield leveled out after 48 h. This limitation in enzymatic hydrolysis at consistencies of 10% and over have been already described [41,42]. Reasons for this could be shear or denaturation of the enzymes or that the remaining substrate is less accessible for the enzymes that require increasingly more time to degrade the substrate [43].

The highest glucose and xylose contents were reached in the assay 11 (EH III). Taking into account the 20% solids loading, the yield of about 60% for glucan and 81% for xylan is quite acceptable. Others studies have obtained high sugar yields, even higher than the present one, but these authors conducted the enzymatic hydrolysis at lower solids loading. Qing and Wyman [15] achieved 90% and 60% glucan to glucose and xylan to xylose conversion, respectively, but they carried out the assays at 2% (w/v) solids loading. Duque et al. [6] obtained enzymatic hydrolysis yield of 90% and 88% for glucose and xylose, respectively, but the assays were conducted at 5% (w/v) solids loading. From an industrial point of view, operating at high solids conditions is essential to make feasible bioethanol production [44]. There are numerous advantages by operating the process at high solids loading of which the final high ethanol concentration and consequently the lower cost for distillation are the most obvious. Also capital and production costs are more beneficial due to reduced equipment size and reduced energy consumption for processing [45].

Another global sugar release yield can be calculated, from the raw material, including the losses of glucose and xylose during the pretreatment and washing steps. For the assay 11, considering the percentage of 20% dissolved solids, a global sugar yield of 41.4 g of sugars (glucose and xylose) per 100 g of raw material can be calculated. In the same way, the glucose and xylose release yields

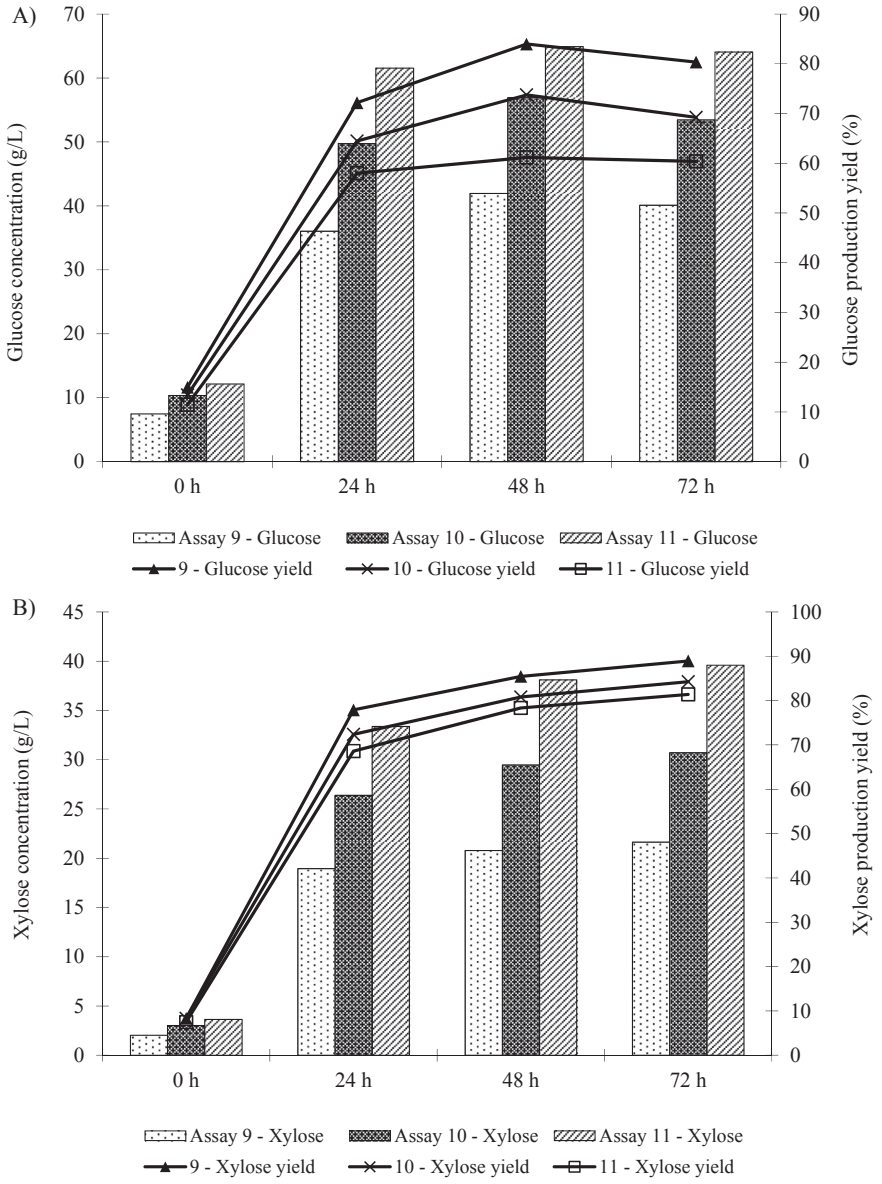

Fig. 4. Concentration of glucose and xylose and hydrolysis yield for glucan (panel A) and xylan (panel B) (monomers) released in EH III test on wheat straw alkaline-extrudates. Assays were conducted with 33% of enzymes (w/w, g enzyme/g glucan) of which 15% was xylanase. Solids loading for each assay: 9–10%, 10–15%, 11–20%.

were 25.6 g and 15.8 g per 100 g of raw material, respectively.

Although the enzymatic dosage of assay 11 was the highest one employed in this work (20 FPU and 46.64 U per g of glucan), it is comparable to other enzyme loadings reported in the literature. Enzyme activities of 10–30 FPU/g of glucan are often used in laboratory studies, because they result in an efficient hydrolysis with high glucose yield in a reasonable time (48–72 h) [5].

4. Conclusions

Alkaline extrusion produced changes in composition of wheat straw and promoted enzymatic digestibility of the extruded substrate. The pretreatment generated a material with lower proportion of lignin and larger proportion of cellulose, compared to untreated wheat straw. As a result of changes produced by alkaline extrusion, enzymatic digestibility of wheat straw increased 13 times for glucan and 11 times for xylan hydrolysis, respectively, over the untreated material.

Enzymatic hydrolysis of wheat straw extrudates reached acceptable glucan and xylan hydrolysis yields with reasonable enzyme dosages from 6.9 to 20 FPU of cellulase per g of glucan in experiments at 5%–20% (w/v) solids loading. The addition of higher percentage of xylanase enzyme increased the glucose released but did not improve the release of xylose. The supplementation of cellulase with 20% xylanase (w/w), corresponding to U to FPU activity ratio of 3.11:1, improved the glucan hydrolysis yield about 21% over the assay with no xylanase. A medium containing 100 g/L of fermentable sugar (60 g/L glucose and 40 g/L xylose) was obtained using 20% solids loading (w/v) with 20 FPU/g of glucan reacted for 48 h.

Briefly, alkaline extrusion stands out as an effective pretreatment for wheat straw that promotes enzymatic digestibility of extruded material. It shows certain advantages over other pretreatment methods such as it can be run at moderate temperature and pH, which grants the recovery of almost all carbohydrates in the extrudate and avoids the generation of toxics. Moreover, it

presents high potential based on the possibility to design a vast range of pretreatment configurations (i,e, neutralization inside the extruder that allows minimizing downstream operations, combination with other pretreatments, etc.), which makes this process technology highly versatile and enhances its prospective application.

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References

[1] M. Wang, M. Wu, H. Huo, Life-cycle energy and greenhouse gas emission impacts of different corn ethanol plant types, Environ. Res. Lett. 2 (2007) 1–13.
[2] S. Kim, B.E. Dale, Global potential bioethanol production from wasted crops and crop residues, Biomass Bioenergy 26 (2004) 361–375.
[3] Y. Pu, F. Hu, F. Huang, B.H. Davison, A.J. Ragauskas, Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments, Biotechnol. Biofuels 6 (2013) 15.
[4] A. Hendriks, G. Zeeman, Pretreatments to enhance the digestibility of lignocellulosic biomass, Bioresour. Technol. 100 (2009) 10–18.
[5] F. Talebnia, D. Karakashev, I. Angelidaki, Production of bioethanol from wheat straw: an overview on pretreatment, hydrolysis and fermentation, Bioresour. Technol. 101 (2010) 4744–4753.
[6] A. Duque, P. Manzanares, I. Ballesteros, M.J. Negro, J.M. Oliva, F. Saez, M. Ballesteros, Optimization of integrated alkaline-extrusion pretreatment of barley straw for sugar production by enzymatic hydrolysis, Process Biochem. 48 (5–6) (2013) 775–781.
[7] S.H. Lee, Y. Teramoto, N. Tanaka, T. Endo, Improvement of enzymatic saccharification of woody biomass by nano-fibrillation using extruder, in: The 57th Annual Meeting of the Japan Wood Research Society, 2007.
[8] A. Duque, P. Manzanares, I. Ballesteros, M.J. Negro, J.M. Oliva, F. Saez, M. Ballesteros, Study of process configuration and catalyst concentration in integrated alkaline extrusion of barley straw for bioethanol production, Fuels 134 (2014) 448–454.
[9] X. Chen, Y. Zhang, Y. Gu, Z. Liu, Z. Shen, H. Chu, X. Zhou, Enhancing methane production from rice straw by extrusion pretreatment, Appl. Energy 122 (2014) 34–41.
[10] K.E. Kang, M. Han, S. Moon, H. Kang, Y. Kim, Y. Cha, G. Choi, Optimization of alkali-extrusion pretreatment with twin-screw for bioethanol production from *Miscanthus*, Fuel 109 (2013) 520–526.
[11] A.D. Eckard, K. Muthukumarappan, W. Gibbons, Pretreatment of extruded corn stover with polyethylene glycol to enhance enzymatic hydrolysis: optimization, kinetics and mechanism of action, Bioenergy Res. 5 (2011) 424–438.
[12] C.H. Choi, J.S. Kim, K. Keun, Evaluation the efficacy of extrusion pretreatment via enzymatic digestibility and simultaneous saccharification and fermentation with rapeseed straw, Biomass Bioenergy 54 (2013) 211–218.
[13] J. Yoo, S. Alavi, P. Vadlani, V. Amanor-Boadu, Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars, Bioresour. Technol. 102 (2011) 7583–7590.
[14] M. Galbe, G. Zacchi, A review of the production of ethanol from softwood, Appl. Microbiol. Biotechnol. 59 (2002) 618–628.
[15] Q. Qing, C.E. Wyman, Supplementation with xylanase and β-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover, Biotechnol. Biofuels 4 (2011) 1–12.
[16] C.E. Wyman, B.E. Dale, R.T. Elander, M. Holtzapple, M.R. Ladisch, Y.Y. Lee, Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover, Bioresour. Technol. 96 (2005) 2026–2032.
[17] L.R. Lynd, M.S. Laser, D. Brandsby, B.E. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J.D. Mcmillan, J. Sheehan, C.E. Wyman, How biotech can transform biofuels, Nat. Biotechnol. 26 (2008) 169–172.
[18] T.K. Ghose, Measurement of cellulase activities, Pure Appl. Chem. 59 (1987) 257–268.
[19] M.J. Bailey, P. Biely, K. Poutanen, Interlaboratory testing of methods for assay of xylanase activity, J. Biotechnol. 23 (1991) 257–270.
[20] NREL. Chemical Analysis and Testing Laboratory, Analytical Procedures, National Renewable Energy Laboratory, Golden CO, 2007.
[21] C. Cara, M. Moya, I. Ballesteros, M.J. Negro, A. González, E. Ruiz, Influence of solid loading on enzymatic hydrolysis of steam exploded or liquid hot water pretreated olive tree biomass, Process Biochem. 42 (2007) 1003–1009.
[22] J.A. Pérez, A. González, J.M. Oliva, I. Ballesteros, P. Manzanares, Effect of process variables on liquid hot water pretreatment of wheat straw for bioconversion to fuel-ethanol in a batch reactor, J. Chem. Technol. Biotechnol. 82 (2007) 929–938.
[23] E. Tomás-Pejó, J.M. Oliva, A. González, I. Ballesteros, M. Ballesteros, Bioethanol production from wheat straw by thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous saccharification and fermentation fed-batch process, Fuel 88 (2009) 2142–2147.
[24] C. Toquero, S. Bolado, Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing, Bioresour. Technol. 157 (2014) 68–76.
[25] K.M. Draude, C.B. Kurniawan, S.J.B. Duff, Effect of oxygen delignification on the rate and extent of enzymatic hydrolysis of lignocellulosic material, Bioresour. Technol. 79 (2001) 113–120.
[26] M.S. Akhtar, M. Saleem, M.W. Akhtar, Saccharification of lignocellulosic materials by the cellulases of Bacillus subtilis, Int. J. Agric. Biol. 3 (2001) 199–202.
[27] W. Cao, C. Sun, R. Liu, R. Yin, X. Wu, Comparison of the effects of five pretreatment methods on enhancing the enzymatic digestibility and ethanol production from sweet sorghum bagasse, Bioresour. Technol. 111 (2012) 215–221.
[28] B.E. Dale, J. Weaver, F.M. Byers, Extrusion processing for ammonia fiber explosion (AFEX), Appl. Biochem. Biotechnol. 77 (1999) 35–45.
[29] C. Karunanithy, K. Muthukumarappan, Optimization of extruder and prairie cord grass parameters for maximum sugar recovery through enzymatic hydrolysis, J. Biobased Mat. Bioenergy 5 (4) (2011) 520–531.
[30] M.H. Thomsen, A. Thygesen, A.B. Thomsen, Identification and characterization of fermentation inhibitors formed during hydrothermal treatment and following SSF of wheat straw, Appl. Microbiol. Biotechnol. 83 (2009) 447–455.
[31] E. Palmqvist, B. Hahn-Hagerdal, Fermentation of lignocellulosic hydrolysates I: inhibition and detoxification, Bioresour. Technol. 74 (2000) 17–24.
[32] M. Ertas, Q. Han, H. Jameel, H. Chang, Enzymatic hydrolysis of autohydrolyzed wheat straw followed by refining to produce fermentable sugars, Bioresour. Technol. 152 (2014) 259–266.
[33] C. Karunanithy, K. Muthukumarappan, W.R. Gibbons, Extrusion pretreatment of pine wood chips, Appl. Biochem. Biotechnol. 167 (2012) 81–99.
[34] X. Zhao, Y. Song, D. Liu, Enzymatic hydrolysis and simultaneous saccharification and fermentation of alkali/peracetic acid-pretreated sugarcane bagasse for ethanol and 2,3-butanediol production, Enzym. Microb. Technol. 49 (2011) 413–419.
[35] R. Travaini, M.D. Otero, M. Coca, R. Da-Silva, S. Bolado, Sugarcane bagasse ozonolysis pretreatment: effect on enzymatic digestibility and inhibitory compound formation, Bioresour. Technol. 133 (2013) 332–339.
[36] Q. Qing, B. Yang, C.E. Wyman, Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes, Bioresour. Technol. 101 (2010) 9624–9630.
[37] R. Kumar, C.E. Wyman, Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies, Bioresour. Technol. 100 (2009) 4203–4213.
[38] C. Bellido, S. Bolado, M. Coca, S. Lucas, G. González-Benito, M.T. García-Cubero, Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*, Bioresour. Technol. 102 (2011) 10868–10874.
[39] Y. Lu, Y. Wang, G. Xu, J. Chu, Y. Zhuang, S. Zhang, Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass, Appl. Biochem. Biotechnol. 160 (2010) 360–369.
[40] W. Wang, L. Kang, H. Wei, R. Arora, Y.Y. Lee, Study on the decreased sugar yield in enzymatic hydrolysis of cellulosic substrate at high solid loading, Appl. Biochem. Biotechnol. 164 (2011) 1139–1149.
[41] F. Kargi, J.A. Curme, J.J. Sheehan, Solid-state fermentation of sweet sorghum to ethanol, Biotechnol. Bioeng. 27 (1985) 34–40.
[42] A. Mohagheghi, M. Tucker, K. Grohmann, C. Wyman, High solids simultaneous saccharification and fermentation of pretreated wheat straw to ethanol, Appl. Biochem. Biotechnol. 33 (1992) 67–81.
[43] M.A.T. Hansen, J.B. Kristensen, C. Felby, H. Jørgensen, Pretreatment and enzymatic hydrolysis of wheat straw (*Triticum aestivum* L.) – the impact of lignin relocation and plant tissues on enzymatic accessibility, Bioresour. Technol. 102 (2011) 2804–2811.
[44] A.A. Modenbach, S.E. Nokes, The use of high-solids loadings in biomass pretreatment: a review, Biotechnol. Bioeng. 109 (2012) 1430–1442.
[45] A. Wingren, M. Galbe, G. Zacchi, Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks, Biotechnol. Prog. 19 (2003) 1109–1117.