OPTIMIZATION OF INTEGRATED ALKALINE-EXTRUSION PRETREATMENT OF BARLEY STRAW FOR SUGAR PRODUCTION BY ENZYMATIC HYDROLYSIS

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Abstract

In this work, an integrated one-step alkaline-extrusion process was tested as pretreatment for sugar production from barley straw (BS) biomass. The influence of extrusion temperature (T) and the ratio NaOH/BS dry matter (w/w) into the extruder on pretreatment effectiveness was investigated in a twin-screw extruder at bench scale. A 2^3 factorial response surface design of experiments was used to analyze the effect of process conditions [T: 50-100ºC; NaOH/BS ratio: 2.5-7.5% (w/w)] on composition and enzymatic digestibility of pretreated substrate. The optimization of these process variables for a maximum glucan to glucose conversion was determined to be at 6% NaOH/DM and 68ºC. At these conditions, glucan yield reached close to 90% of theoretical, while xylan conversion was 71 % of theoretical. These values are 5 and 9 times higher than that of the untreated material, which supports the great potential of this one-step combined pre-treatment technology for sugar production from lignocellulosic substrates.

Introduction

The increasing global energy demand, which relies mostly upon the dependence on fossil fuels and a raising concern about the greenhouse gasses emissions have lead to a search for new and sustainable energy sources. In this context, biofuels represent a solid alternative to conventional fossil fuels. Within this field, ethanol produced from lignocellulosic biomass is considered a key element to boost implementation of bioethanol in the current fuel market since it avoids the most important drawback of the first generation bioethanol: its competition with food crops. However, since the structure of these materials makes them very recalcitrant to the enzymes accessibility (Himmel, 2007), a pretreatment is needed to break down the lignin net and disrupt the crystalline structure of cellulose, increasing the surface area and porosity of the biomass.

Among the several pretreatments that are being currently studied and further developed, extrusion stands out for its ability to provide high shear, rapid heat transfer, and effective and rapid mixing (Karunanithy and Muthukumarappan, 2010a). Other advantages of this method are the feasibility of continuous operation and its versatility to adopt different process configurations. In addition, extrusion can be run at moderate temperature, which is advantageous in comparison to other hydrothermal pretreatments, since the formation of inhibitory byproducts as furfural or HMF can be prevented. Pretreatment of different biomasses by extrusion alone or in combination with different chemicals and additives for sugar production by enzymatic hydrolysis has been reported by several authors during the last years. The performance of extrusion and the influence of the operation parameters has been studied on switchgrass, prairie cord grass, corn stover and more recently on pine wood chips by (Karunanithy and Muthukumarappan, 2010 a;b; Karunanithy 2011 a,b., 2012), while other researchers have focused extrusion pre-treatment on biomasses such as *Miscanthus sp* (De Vrije et al., 2002), Douglas fir (Lee et al.,2008), soybean hulls (Yoo et al., 2011), rice straw. (Chen et al., 2011) and a combination of wheat bran and straw (R.Zeitoun et al., 2010)

Lately extrusion has been also pointed out as an interesting technique to be used together with other pretreatments, in a two-step process strategy. For example, Lee et al. (2010) used extrusion as complementary step for Douglas Fir after hot-compressed water treatment. Results showed sugar yields 5 times higher than without passing through the extruder and a fine fibrous morphology on a sub-micro/nanoscopic scale. The combination of extrusion and diluted acid pretreatment has been tested on rice straw and proved to be an effective method to maximize hemicellulose hydrolysis and enhance glucan to glucose conversion by enzymes (Chen et al, 2011). More recently, the conditions for sequential treatment of praire cord garss by extrusion and surfactant pretretament has been optimized by (Eckard et al., 2012), aimed at increasing efficiency of hydrolysis for bioethanol production.

On the other hand, mild alkaline pretreatment is one of the most widely used methods to enhance the enzymatic digestibility of the lignocellulosic biomass. It is generally more effective in the pretreatment of agricultural residues and herbaceous crops (Cheng et al. 2008). Biomass soaking in sodium hydroxide, potassium hydroxide, ammonia or lime, in a concentration below 2%, has been reported to cause delignification, xylan loss, decrease of cellulose crystallinity and swelling of biomass (Mosier et al., 2005; Balat et al., 2008; Zhao et al., 2008). As a consequence, sugar production increases as reported by McIntosh and Vancov (McIntosh and Vancov, 2010), who obtained 5.6-fold higher sugar yields by pretreating sorghum straw in 2% NaOH at 121 ºC for 60 min.

The combination of both extrusion and alkaline pretreatment has been explored in some recent works by Lamsal (Lamsal, Yoo et al., 2010) and by Karunanithy and Muthukumarappan (Karunanithy and Muthukumarappan, 2011), with different results. Lamsal could not find any improvement in sugar yield by soaking wheat bran and soybean hull in a solution of sodium hydroxide, urea and tiourea (10% w/w) and then introducing the mixture in a twin-screw extruder at 7 Hz and maximum barrel temperature of 150 ºC, compared to a simple grinding of the biomass. However, the extensive washing of the pretreated substrate took away the solvents and enzymatic inhibitors, resulting in enhanced sugar yields of 60 - 73% and 25 - 36%, respectively, for wheat bran and soybean hull. On the other hand, Karunanithy and Muthukumarappan optimized the extrusion performance for prairie cord grass at 114 ºC, 122 rpm, 1.70 % NaOH concentration and 8 mm particle size, reaching a maximum glucose and xylose recovery of 86.8 and 84.5 % respectively, after enzymatic hydrolysis. These authors claim that the low alkaline concentration used allow hydrolysing the extruded material without washing of the biomass.

In both works, the alkali soaking was a previous step to the extrusion and was done in a discontinuous way. The aim of this study is to integrate both processes in a single step to pretreat barley straw, enabling a continuous operation of the whole pretreatment, reducing the contact time between the NaOH and the substrate and possibly improving the effect of the alkali by a thoroughly mixing in the extruder. The final purpose of the present work is to optimize the operation conditions, namely the ratio NaOH/barley straw dry weight (w/w) and extrusion temperature in an integrated alkaline-extrusion pre-treatment by using a statistical experimental design, in order to enhance glucan and xylan digestibility by further enzymatic saccharification.

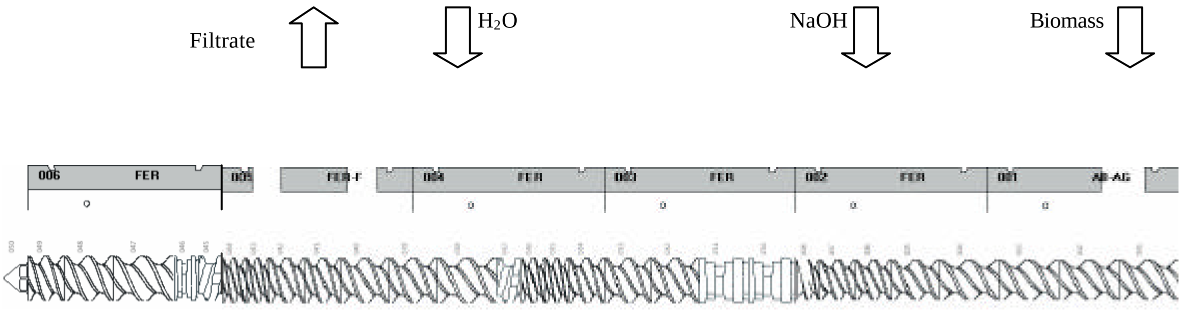
Material and Methods

Raw material

Barley straw (6% moisture content) was provided by Centre for the Development of Renewable Energy Sources (CEDER), (Soria, Spain). Biomass was coarsely crushed to about 5 mm particle size using a laboratory hammer mill (Retsch), homogenised and stored until used.

Extrusion pretreatment

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 (Figure 1). In module 5 a filtration step was set up in order to separate liquid from solid fraction (filtrate and extrudate respectively) after extrusion. The modules have a heating and cooling system that allows setting a temperature profile throughout extrusion process. The screws diameter is 25 mm and they were configured to produce transport, mixing and shearing effects along the process, as depicted in Fig. 1. Two metering pumps connected to the extruder are used to supply the catalyst (NaOH solution at 10% w/v) and H_2O to the process. Biomass feeding was done through a volumetric feeder KMV KT20 (Ktron), which has a flow capacity up to 1.2 kg/h for 5 mm barley straw. The feeder screw speed rate was set to provide a feed rate of 0.6 kg/h.



T	S	T	S	T	M	T

Figure 1: Screw configuration and inlet/outlet positions for extrusion of barley straw in the 6-module twin-screw extruder used in this work at 6% NaOH/BS and 68ºC. T: transport effect, M: mixing effect; S: shearing effect.

Operating conditions were set to achieve moderate values of NaOH/DM ratio, between 2.5 and 7.5 % (w/w), and temperature, between 50 and 100ºC. Based on preliminary experiments, a fixed motor speed of 150 rpm was used for all runs. At this condition, the residence time inside the extruder is about 2 min.

After extrusion, solid extruded material (extrudate) was recovered and washed thoroughly with distillate water until neutral pH. Filtrate also collected and analyzed for sugar and degradation compounds, i.e. 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde (hereinafter referred as furfural and HMF).

A portion of washed solid extrudate was dried and analyzed for carbohydrates and lignin composition to evaluate changes in comparison to untreated BS. Samples were stored at 4ºC in hermetic plastic bags until use in enzymatic hydrolysis experiments.

Experimental design

In order to study the response pattern and determine the optimum conditions for the extrusion of BS, a 2^3 factorial response surface design with two variables (catalyst to dry matter ratio and temperature) was employed. The design results in 9 runs (Table 1) and was developed with StatGraphics Plus 5.0 Enterprise Edition (Satistical Graphics Corporation). The order of experiments has been randomized, as a way to avoid the effect of lurking variables. The levels of optimized variables were 2.5 – 5 – 7.5 % for the NaOH/DM ratio and 50 – 75 – 100 ºC for the temperature. They were selected according to the criteria of using mild conditions and based on preliminary extrusion trials on BS.

Table 1. .- 2^3 factorial design conditions for integrated alkaline-extrusion of barley straw

EXPERIMENTAL DESIGN		
Run	**A: Temp ºC**	**B: NaOH/BS DM (% w/w)**
1	*50*	*2.5*
2	*50*	*5*
3	*50*	*7.5*
4	*75*	*2.5*
5	*75*	*5*
6	*75*	*7.5*
7	*100*	*2.5*
8	*100*	*5*
9	*100*	*7.5*

Enzymatic hydrolysis

The washed extrudate was used as substrate for enzymatic hydrolysis (EH) in 0.05 M sodium citrate buffer (pH 4.8) at 50ºC and 5% (w/v) dry extrudate load. Untreated barley straw was also subjected to enzymatic hydrolysis at the same conditions, as a control. Experiments were performed in 100 ml Erlenmeyer flasks on a rotary shaker (Certomat-R B-Braun, Germany) at 150 rpm. Enzymatic cocktail consisting of commercial cellulase boosted with commercial xylanase in a proportion 9:1 in protein content was added in a dosage of 10 mg protein (15 FPU of cellulase)/g dry extrudate. The supplementation with xylanase was aimed at promoting xylan hydrolysis, based on the significant xylan content of extrudates after extrusion (see below). The enzymes were kindly provided by Novozymes A/S (Denmark). After 72 h saccharification, glucose and xylose concentration in EH media was measured by HPLC as described below

The parameter used to evaluate the hydrolysis performance is the enzymatic hydrolysis yield (EHY), which is defined as the glucose/xylose released during EH divided by the potential glucose/xylose (calculated based on glucan/xylan content of the solid extrudate), and expressed as percentage.

Raw and extruded biomass characterization.

National Renewable Energy Laboratory (NREL) laboratory analytical procedures (LAP) for biomass analysis (NREL; 2007) were used to determine carbohydrates, acid-insoluble lignin, soluble lignin, acetyl groups, extractives and ash content in raw material. Extrudates were analyzed for carbohydrates and acid insoluble lignin by the same procedures.

Analytical methods

The filtrate was recovered after extrusion and analysed for its content of monomeric and oligomeric sugars. The oligosaccharides ratio was determined as the difference in monomeric sugar concentration before and after mild acid hydrolysis (3% v/v H_2SO_4, 120 ºC and 30 min). Sugars were analysed by high-performance liquid chromatography (HPLC) in a Waters 2695 liquid chromatograph with refractive index detector, as described in (Manzanares et al., 2011). Likewise, glucose and xylose concentration after completion of enzymatic hydrolysis tests was measured in EH media by HPLC using the same column. Furfural and HMF were analyzed by HPLC (Hewlett Packard, Palo Alto, CA), using an Aminex ion exclusion HPX-87H cation-exchange column (Bio-Rad Labs, Hercules, CA) at 65ºC. Mobile phase was 89% 5 mM H_2SO_4 and 11% acetonitrile at a flow rate of 0.7 mL min-1. Column eluent was detected with a 1040A Photodiode-Array detector (Agilent, Waldbronn, Germany).

Results and discussion

Raw Material Composition

Table 2 presents the results of barley straw composition. The dry matter distribution shows an average value of 39.1 % cellulose, 25.7 % hemicellulose and 15.2 % lignin, making it a very promising substrate for bioconversion to ethanol after a suitable pre-treatment based on high total carbohydrate content of 65% (dwb). The degree of lignification of barley straw biomass is in the range of that reported for other agricultural residues such as wheat straw (17%) (Pérez et al., 2007) or corn stover (17-19%) (Kim et al., 2005). On the other hand, a significant fraction of the feedstock, about 10%, is made up of water and ethanol soluble materials, included in the term extractives. The high ash content of barley straw (6.8 %) is consistent with the presence of silica as a major mineral component of cereal straws. In general, results are comparable to the ones reported by Linde, Galbe et al. 2007; Persson, Ren et al. 2009; García-Aparicio, Oliva et al. 2010; Li, Liu et al. 2011 for raw barley straw.

Table 2. -Raw barley straw composition. Mean values and standard deviation of three replicates

Component	(% dwb)
Extractives	10.2 ± 0.9
Cellulose	39.1 ± 0.7
Hemicellulose	25.7 ± 0.3
Xylose	23.8 ± 0.2
Arabinose	3.7 ± 0.05
Galactose	1.5 ± 0.02
Acetyl groups	1.8 ± 0.01
Acid-insoluble lignin (AIL)	15.2 ± 0.01
Ash	6.8 ± 0.2

Extrusion pretreatment

Effect of extrusion conditions in extrudate and filtrate composition

In order to evaluate the efficiency of extrusion as a pretreatment to fractionate barley straw biomass and so affect the enzymatic digestibility of the carbohydrates, changes in the composition of extrudates with respect to the raw material were measured at the different extrusion conditions. Moreover, filtrate fraction was analyzed for sugar composition and the presence of furfural and HMF. Results are shown in Table 3.

Table 3.

Extrudate solids content and composition (% dry matter), EH yield based on glucose and xylose release and concentration of hemicellulosic sugars in filtrate, at different temperature and NaOH/BS (% w/w) ratios, according to design of experiments shown in Table 1.

Experiment	Extrudate solid content (g/100g dry extrudate)	Extrudate composition (% w/w)			Hemicellulose sugars in filtrate (g/l)	Enzymatic hydrolysis yield (EHY) (% of theoretical)	
		Cellulose	Xylan	AIL		Glucose	Xylose
1	22.45	47	26.8	19.1	1.4	31.2	18.8
2	18.80	46.5	25.7	15.0	5.4	83.8	68.8
3	19.79	64.5	14.5	13.7	8.6	82.2	74.8
4	26.43	52.3	26.8	18.2	1.9	50.3	45.5
5	19.0	53.4	26.3	14.3	7.4	81.7	68.4
6	13.85	58.3	20.8	12.4	18.0	74.7	88.2
7	30.90	45.6	26.1	19.5	0.8	35.8	34.6
8	15.58	51.4	25.1	13.2	7.2	90.3	64.8
9	18.43	61.0	15.6	12.1	11.7	71.0	88.3

As it can be seen in Table 3, alkaline extrusion affects BS composition so that the extrudate is a cellulose enriched material in comparison to raw BS, in a range that varies between 10 and 40 % increase. Maximum cellulose content in extudate is close to 65 % w/w, which is obtained at 50ºC with the highest NaOH/DM ratio of 7.5% (w/w). The increase of the cellulose content in extrudate compared to untreated BS (39.1% dwb) is an important advantage of biomass pretreatment for ethanol production, since the material that is introduced in the following step of hydrolysis is cellulose-enriched in relation to the untreated material. Regarding hemicellulose fractionation, the one- step alkaline-extrusion process, in the conditions tested in the present work, does not seem to exert a strong impact in hemicellulose breakdown at 2.5 and 5% NaOH/BS ratio. At these conditions, xylan content in extrudate, which accounts for 80-87% of total hemicellulose, is similar or even slightly higher than in raw BS. Taking into account that extractives and ash are solubilized into the filtrate (these components are not found in extrudate composition), a minimum concentration factor of 1.2 (extractives and ash account for 17% of BS dry weight) should be expected in cellulose, hemicellulose and lignin in extrudates over raw material content values. So, only at 7.5% NaOH/BS for all temperature conditions (runs 3, 6 and 9), xylan content values are below that expected by the concentration factor (25.5%), indicating hemicellulose solubilization. This means that a great part of the xylan in raw material is remaining in extrudate after extrusion, which could be hydrolyzed in the subsequent enzymatic hydrolysis step by specific enzymes.

Sugar analysis of the prehydrolysate (Table 3) shows negligible concentrations of glucose and varying amounts of hemicellulose-deirved sugars, mostly xylose and arabinose and in minor concentration galactose and mannose. Increasing hemicellulose-derived sugar content is found as the NaOH ratio raises, reaching concentrations up to 18 g/l at 75 ºC and 10% NaOH/BS (Table 3). It was found that sugars measured in filtrate were all in oligomeric form in experiments at 5 and 7.5% NaOH/DM ratio, regardless the extrusion temperature. Only in experiments at the lower alkali ratio of 2.5%, the amount of monomers accounted for about 35% of total sugars. It is important to highlight that neither furfural nor hydroxymethyl furfural was detected in the filtrates.

The effect of exposure to alkaline substances during the pre-treatment process on hemicellulose loss is well supported in the literature in experiments at different operation conditions for alkali treatment. McIntosh and Vancov (2010) obtained 18.5 % of hemicellulose solubilisation for shorgum straw when biomass was treated for 60 min in an autoclave at 121 ºC and 0.75 % NaOH. Other studies have shown the effectiveness of pretreating lignocellulosic materials with dilute alkali for xylan removal by soaking soybean or wheat straw biomass at room temperature (Wan, McIntosh et al., 2011). However, these results are difficult to compare with the present work due to very different process conditions for alkali treatment. In the integrated one step process of the present work, the action of alkali is combined with the mixing and shearing effects of extrusion during the 2 minutes the residence time of the material inside the extruder at selected screw speed.

Regarding the results of lignin content variation during extrusion, alkali concentration seems to exert a greater effect on lignin solubilisation than temperature, in the range of conditions tested. AIL content is below that "concentrated" value (18.2%) at 5 and 7.5% NaOH/BS ratio, indicating some lignin solubilisation by the alkaline treatment. At the lowest soda to dry matter ratio tested, 2.5 % NaOH/BS, almost no lignin was removed. It has been proved that lignin is one of the main hindrances that difficult the enzyme access to the cellulose (Chang and Holtzapple, 2000) and for this reason, delignification is often used as an indicator of the pretreatment effectiveness in alkaline pretreatments.

In order to assess the importance of the effect of temperature and NaOH/DM ratio on the variation of extrudate composition, a response surface analysis was performed with the experimental data shown in Table 3. The graphs reveal a marked effect of NaOH/DM ratio and, in a much lesser extent, of the temperature, on cellulose, hemicellulose and lignin content, as it is shown in Figures 1, 2 and 3 (panel a), respectively. There is a clear tendency towards increasing cellulose content and decreasing hemicellulose and lignin content in extrudate, as the ratio NaOH/DM levels up from 2.5 to 7.5%. In fact, when the significance of each effect on the global behaviour of the variable is analyzed by the Pareto charts (panel b of Figures 1-3), only NaOH/DM ratio effect is significant, at a 95.0% confidence level. The effect of temperature is not statistically significant for any of the considered responses. It means that the range of variation of temperature tested is not enough to produce a significant influence on extrudate composition, given the effect of NaOH/DM ratio.

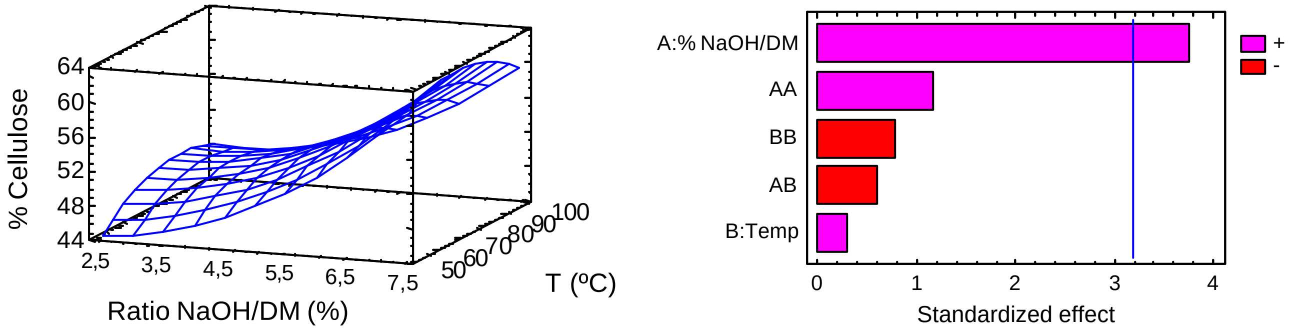


Figure 1: Response surface graph and Pareto chart of the effect of T and Ratio NaOH/DM on cellulose content in extrudate

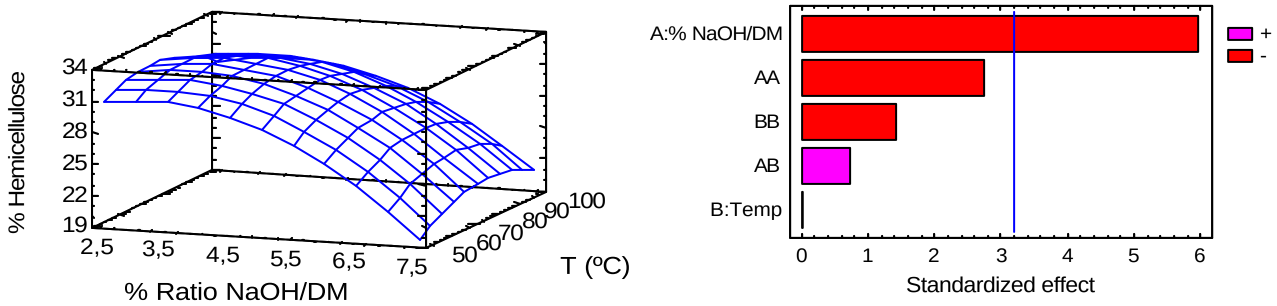


Figure 2: Response surface graph and Pareto chart of the effect of T and Ratio NaOH/DM on hemicellulose content in extrudate

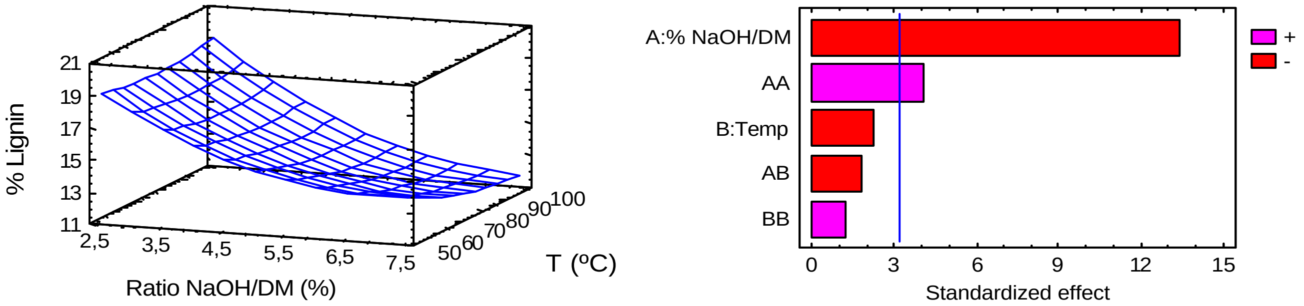


Figure 3: Response surface graph and Pareto chart of the effect of T and Ratio NaOH/DM on lignin content in extrudate

Table 3 also includes values of solid content of extrudates at different conditions, which varies between 14 and 30% dwb. In the integrated extrusion process of this work, it depends mainly on the different inlet flows and the extrusion configuration, since no treatment is given to the raw material before. This implies that the consistency (solid content) of the extrudate at the output can be adjusted according to the process needs by varying the flows and the screw profile, always considering the effect on solids losses, which can be attributed to the partial solubilisation of the hemicellulose and lignin.

Enzymatic hydrolysis

From glucan and xylan enzymatic hydrolysis yields values on extrudates produced at different extrusion conditions shown in Table 3, it can be concluded that integrated NaOH-extrusion results in improved enzymatic digestibility, in comparison to untreated BS (17 and 10% of theoretical for glucan and xylan, respectively, data not shown). The hydrolysis yield, which refers to saccharification efficiency of enzymes in extrudate, levels up with the NaOH/DM ratio to maximum values of 90% and 88% of theoretical for glucan and xylan conversion, respectively. This means that the enzymatic digestibility of glucan and xylan in BS can be increased in 5 and 9 fold, respectively, in relation to untreated material by moderate alkaline-extrusion. The effect of NaOH/DM ratio on enzymatic digestibility, in the range studied, seems to be stronger than the effect of temperature, since the increase in enzymatic digestibility as alkali ratio levels up is significant even at the mildest T of 50ºC. The increase of T over 50ªC exerts slight or no improvement in glucan yield, depending on the NaOH/DM ratio tested. In fact, when T increases to 75 and 100ºC in experiments at 7.5% NaOH ratio, a decrease in enzymatic digestibility is found. This fact could be related to a greater complexation of the hemicellulose and lignin fraction at high severity conditions of temperature and alkali resulting in lower enzymatic hydrolysis efficiency.

Unlikely glucan conversion, xylan conversion yield increases in all temperatures tested as alkaline conditions become more severe, attaining values of 88% of theoretical in extrudates at 75 and 100ºC and 7.5% NaOH/DM ratio. It means that the digestibility of xylan is greatly improved by the one-step alkaline extrusion process, which shows the positive effect of this pre-treatment to deconstruct hemicellulose chains and facilitate the action of hemicellulolytic enzymes. As in glucan hydrolysis, the improvement with T is not relevant, which is well supported by the response surface graphs shown in Figures 4 and 5 that show the marked effect of alkali ratio in both responses EH of glucan and xylan. The Pareto charts confirm that only the effect of NaOH/DM ratio is significant at a 95.0% confidence level.

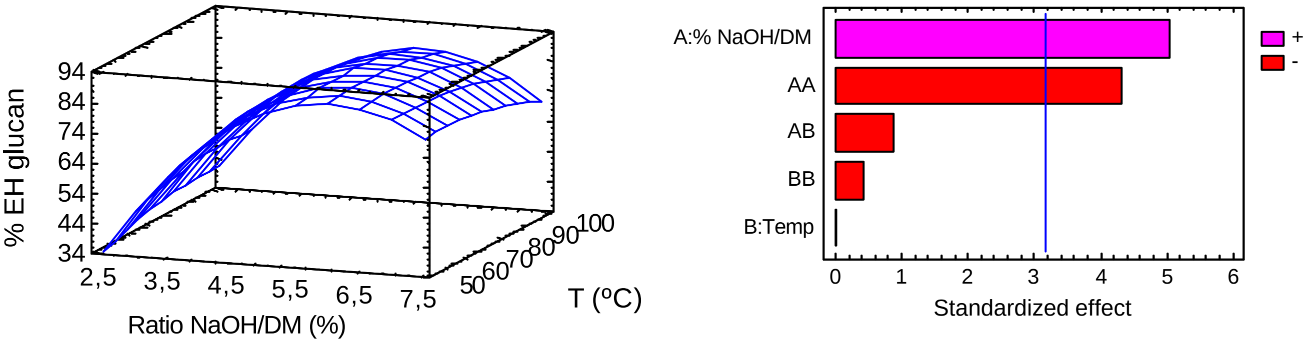


Figure 4: Response surface graph and Pareto chart of the effect of T and Ratio NaOH/DM on enzymatic hydrolysis yield for glucan of the extrudate

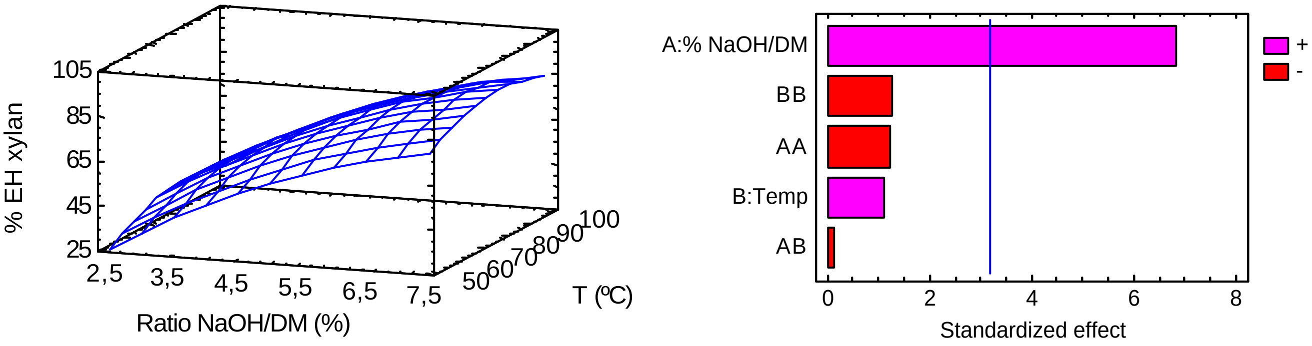


Figure 5: Response surface graph and Pareto chart of the effect of T and Ratio NaOH/DM on enzymatic hydrolysis yield for xylan of the extrudate

A positive effect of extrusion in enzymatic digestibility of lignocellulosic substrates has also been demonstrated in other lignocellulosic substrates. De Vrije et al. (2002) investigated a combination of mechanical and chemical pre-treatment methods for the production of fermentable sugars from *Miscanthus* and concluded that a combination of extrusion and sodium hydroxide is more favourable based on higher cellulose conversion yields and good performance of the pre-treatment. More recently, Yoo et al. (2011) have reported on the increase of glucan to glucose conversion yield of soybean hulls from 40.8% of untreated biomass to 94.8% after extrusion at 80ºC and 350 rpm in a twin-scew extruder, without any catalyst addition. However, in the comparison with our results it is necessary to consider that soybean hull has much lower lignin content (2.3%) than barley straw, which makes the digestibility of this feedstock quite high even when untreated. Other authors report on sugar recovery yields after enzymatic hydrolysis (sugar released during saccharification related to sugar in raw material) being positively affected by an extrusion process alone (Karunanity and Muthukumarappan 2010a, 2011b, 2012; Zhang et al., 2012), in combination with chemicals (Dale et al., 1999; Lamsal, 2010; Karunanity and Muthukumarappan, 2011a; Lee et al., 2009) or with other pretretment techniques, such as liquid hot water (Chen et al., 2011). Alkali impregnation and subsequent extrusion of prairie cord grass was studied by (Karunanithy and Muthukumarappan, 2011b) in the optimum conditions of 114 °C barrel temperature, 122 rpm screw speed, 1.70% alkali concentration, and 8 mm particle size obtained a maximum glucose, xylose and combined sugar recoveries of 86.8, 84.5, and 82%, respectively, by enzymatic hydrolysis.

Regarding the mechanism underlying the positive effect of extrusion in enzymatic hydrolysis efficiency, it is not completely elucidated, although several hypotheses have been formulated. According to Yoo et al., extrusion causes disruption of cell wall structure due to the combination of thermal and mechanical energy. This combined effect would lead to exposure of greater surface area and also to deconstruction of hemicellulose chains that interfere enzymes accessibility (Lamsal et al., 2010). In the integrated alkaline-extrusion process of the present work, the extrusion effect would be enhanced by the action of the alkaline agent, which promotes glucan conversion by degradation of ester bonds and cleavage of glycosidic linkages in the cell wall matrix leading to the reduction of the lignin-hemicellulose complex and swelling of cellulose (Cheng et al., 2010).

Other approaches using a combination of techniques that includes alkali have also been shown to be effective to enhance enzymatic digestibility of barley straw. Persson et al. (2009)

pretreated barley straw by impregnation with different amounts of NaOH, followed by steam explosion at 190 ºC and varying times. They found glucose conversions after 72h between 80 and 90 % and xylose yields ranging from 83 to 95 %., which are in the range of those obtained in the present work: However, the integrated alkaline-extrusion poses the advantage of an one-step integrated process at lower temperature and lack of sugar degradation compounds. As in the present work, the authors observed a tendency towards higher glucan conversion with increased NaOH concentration in the impregnation step.

Optimization of extrusion conditions for enzymatic hydrolysis of glucan and validation of the model

Experimental data of enzymatic hydrolysis of glucan were processed using Statgraphics in order to infer a mathematical model which would describe the system and be able to predict its behaviour, being the final objective to maximize the conversion of glucan to glucose. This analysis was characterized by regression parameter R^2=0.937, which indicates that the model as fitted explains 93.7% of variability for glucan hydrolysis yield. The adjusted R^2 statistic was 0.833. The program gave the following equation: $X= 20.36-1.57467\ R+ 0.74393\ 3T+0.600\ 8R^2-0.02168\ RT-0.004088T^2$ for EH yield of glucan (X), as a function of NaOH/DM ratio (R) and temperature (T).The contour plot depicted in Figure 4 shows how the design leads us towards an optimal experimental condition within the experimental range studied, which was found to be 89.9% of theoretical, at 6% NaOH/DM and 68ºC.

To test the model validity, a new experiment was carried out, setting the process variables to the optimum ones given above. EH yield for glucan obtained at optima condition was 88.9% of theoretical, which is in agreement with the predicted value (89.9% according to the above formula) and confirms the validity of the model. At the optima conditions, the composition of extrudate gave the following values: cellulose, 54.9%, hemicellulose, 28.9% and lignin, 14.7%., on dry weight basis The hydrolysis yield for xylan resulted in 71% of theoretical and the amount of hemicellulose-derived sugars reached 10.3 g/l. The absence of furfural and HMF in filtrate was confirmed in this validation experiment.

Conclusions

The results obtained in this work prove the effectiveness of the integrated one-step alkaline extrusion technique to enhance enzymatic digestibility of barley straw biomass for sugar production. Values of saccharification efficiency of glucan and xylan in extruded BS can be increased in 5 and 9 fold, respectively, in relation to untreated material in EH experiments on washed extrudate at 5% (w/w) solids load. These results confirm the effectiveness of the integrated pretreatment to cause destructuration of lignocellulose structure, so promoting the accessibility of enzymes to the carbohydrates during hydrolysis step.

The study of the influence of process variables, such as temperature and alkali/barley straw DM ratio showed that only NaOH/DM variable has a significant effect on enzymatic hydrolysis yield and extrudate composition variation, in the interval studied of 2.5-7.5% NaOH/DM ratio (w/w) and 50-100ºC. The optimization of process variables for a maximum glucan to glucose conversion by enzymatic hydrolysis led to optimal conditions of 6% NaOH/BS and 68ºC. At these conditions, glucan yield reached 89.9% of theoretical, while xylan conversion was 71 %. These results support the great potential of this one-step combined pre-treatment technology for sugar production from lignocellulosic substrates. The absence of sugar degradation products is a relevant advantage over other traditional methods for a biomass to ethanol production process since inhibitory effect of such products on sugar fermentation is prevented. Further efforts will be devoted to continue studying the process conditions that result in a more integrated and effective process and to test its feasibility at a larger scale.

Acknowledgements

This work has been partially funded by the European Community Seventh Framework Programme under Grant agreement reference n° 227498 (BABETHANOL PROJECT) and the Regional Government of Madrid under the R&D Technology Programme/2009 (Project RESTOENE Ref. nº P2009/ENE-1743).

References

Balat M, Balat H, Progress in bioethanol processing. *Progress in Energy and Combustion Science* **34**(5):551-573 (2008).

Chang VS and Holtzapple MT, Fundamental factors affecting enzymatic reactivity. *Appl Biochem Biotechnol* **84-86**(1-9): 5-37 (2000).

Chen W-H, Xu Y-Y, Hwang W-S and Wang J-B, Pretreatment of rice straw using an extrusion/extraction process at bench-scale for producing cellulosic ethanol, *Bioresource Technol* **102**:10451-10458 (2011).

Cheng K-K, Zhang J-A, Ping W-X, Ge J-P, Zhou Y-J, Ling H-Z and Xu J-M, Sugarcane Bagasse mild alkaline/oxidative pre-treatment for ethanol production by alkaline recycle process. *Appl Biochem Biotechnol* **151**(1):43-50 (2008).

Dale BE, Weaver J and Byers FM, Extrusion processing for ammonia fiber explosion (AFEX). *Appl Biochem Biotechnol* **77**(1-3): 35-45 (1999).

De Vrije T, De Haas GG, Tan GB, Keijsers ERP and Claassen PAM, Pretreatment of *Miscanthus* for hydrogen production by *Thermotoga elfii*. *International Journal of Hydrogen Energy* **27:**1381-1390 (2002).

Eckard AD, Muthukumarappan K and Gibbons W, Pretreatment of extruded corn stover with polyethylene glycol to enhance enzymatic hydrolysis: optimization, kinetics and mechanism of action. *BioEnerg Res* **5**:424-438 (2011).

García-Aparicio MP, Oliva JM, Manzanares P, Ballesteros M, Ballesteros I, González A and Negro MJ, Second-generation ethanol production from steam exploded barley straw by *Kluyveromyces marxianus* CECT 10875. *Fuel* **90**(4): 1624-1630 (2010).

Himmel MED, Ding S-Y, Johnson DK, Adney WS, Nimlos MR, Brady JW and Foust TD, Biomass recalcitrance. Engineering plants and enzymes for biofuels production. *Science* **315**:804-807 (2007).

Karunanithy C and Muthukumarappan K, Effect of extruder parameters and moisture content of switchgrass, prairie cord grass on sugar recovery from enzymatic hydrolysis. *Appl Biochem Biotechnol* **162**(6):1785-1803 (2010a).

Karunanithy C and Muthukumarappan K, Influence of extruder temperature and screw speed on pretreatment of corn stover while varying enzymes and their ratios. *Appl Biochem Biotechnol* **162**(1):264-279 (2010b).

Karunanithy C and Muthukumarappan K, Optimization of alkali soaking and extrusion prtreatment of prairie cord grass for maximum sugar recovery by enzymatic hydrolysis. *Biochemical Engineering Journal* **54**(2):71-82 (2011).

Karunanithy C and Muthukumarappan K, Optimization of switchgrass and extruder parameters for enzymatic hydrolysis using response surface methodology. *Industrial Crops and Products* **33**(1):188-199 (2011).

Karunanithy C, Muthukumarappan K and Gibbons WR, Extrusion pretreatment of pine wood chips, *Appl Biochem and Biotechnol* **167**:81-99 (2012).

Kim S and Holtzapple MT, Lime pre-treatment and enzymatic hydrolysis of corn stover. *Bioresource Technol* **96**(18):1994-2006 (2005).

Lamsal B, Yoo J, Brijwani K and Alavi S, Extrusion as a thermo-mechanical pre-treatment for lignocellulosic ethanol. *Biomass and Bioenergy* **34**(12): 1703-1710 (2010).

Lee SH, Inoue S, Teramoto Y and Endo T, Enzymatic saccharification of woody biomass micro/nanofibrillated by continuous extrusion process II: Effect of hot-compressed water treatment, *Bioresource Technol* **101**(24):9645-9649 (2010).

Lee SH, Teramoto Y and Endo T, Enzymatic saccharification of woody biomass micro/nanofibrillated by continuous extrusion process I - Effect of additives with cellulose affinity, *Bioresource Technol* **100**(1):275-279 (2009).

Li Z, Liu Y, Liao W, Chen S and Zemetra RS, Bioethanol production using genetically modified and mutant wheat and barley straws. *Biomass and Bioenergy* **35**(1): 542-548 (2011).

Linde M, Galbe M and Zacchi G, Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. *Enzyme and Microbial Technology* **40**(5): 1100-1107 (2007).

Manzanares P, Negro MJ, Oliva JM, Sáez F, Ballesteros I, Ballesteros M, Cara C, Castro E and Ruiz E, Different process configurations for bioethanol production from pretreated olive pruning biomass. *J Chem Technol Biotechnol* **86**(6):881-887 (2011).

McIntosh S and Vancov T, Enhanced enzyme saccharification of *Sorghum bicolor* straw using dilute alkali pretreatment. *Bioresource Technol* **101**(17):6718-6727 (2010).

McIntosh S and Vancov T, Optimisation of dilute alkaline pretreatment for enzymatic saccharification of wheat straw . *Biomass and Bioenergy* **35**(7): 3094-3103 (2011).

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M and Ladisch M, Features of promising technologies for pre-treatment of lignocellulosic biomass. *Bioresource Technol* **96**(6):673-686 (2005).

National Renewable Energy Laboratory (NREL) (2007). NREL, Golden, CO. Available from: www.ere.energy.gov/biomass/analytical_procedures.html.

Pérez JA, González A, Oliva JM, Ballesteros I and Manzanares P, Effect of process variables on liquid hot water pre-treatment of wheat straw for bioconversion to fuel-ethanol in a batch reactor. *J Chem Technol Biotechnol* **82**:929-938 (2007).

Persson T, Ren JL, Joelsson E and Jönsson A-S, Fractionation of wheat and barley straw to access high-molecular-mass hemicelluloses prior to ethanol production. *Bioresource Technology* **100**(17): 3906-3913 (2009).

Wana C, Zhou Y and Li Y, Liquid hot water and alkaline pretreatment of soybean straw for improving cellulose digestibility. *Bioresource Technol* **102**:6254–6259 (2011).

Yoo J, Alavi S, Vadlani P and Amanor-Boadu V, Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars, *Bioresource Technol* **102**:7583-7590 (2011).

Zeitoun R, Pontalier PY, Marechal P and Rigal L, Twin-screw extrusion for hemicellulose recovery: Influence on extract purity and purification performance. *Bioresource Technol* **101:**9348-9354 (2010).

Zhao X, Zhang L and Liu D, Comparative study on chemical pretreatment methods for improving enzymatic digestibility of crofton weed stem. *Bioresource Technol* **99**(9):3279-3736 (2008).