Research Article **Open Access**

Pablo Alvira, María José Negro*, Ignacio Ballesteros, Alberto González, Mercedes Ballesteros

Steam Explosion for Wheat Straw Pretreatment for Sugars Production

DOI 10.1515/bioeth-2016-0003
Received November 19, 2014; accepted August 3, 2015

Abstract: Development of biofuels such as lignocellulosic ethanol represents a sustainable alternative in the transport sector. Wheat straw is a promising feedstock for bioethanol production in Europe due to its large production and high carbohydrates content. In a process to produce cellulosic ethanol, previous to the enzymatic hydrolysis to obtain fermentable sugars and the subsequent fermentation, a pretreatment step to break down the recalcitrance of lignocellulose fiber is essential. In this work, a range of steam explosion pretreatment conditions were evaluated according to different parameters: sugars recovery, degradation products generation, and enzymatic hydrolysis yields. Moreover, the enzymatic hydrolysis process was also studied at high substrate loadings, since operating at high solids loading is crucial for large scale development of ethanol production. Pretreatment at 200°C - 10 min resulted in higher enzymatic hydrolysis yield (91.7%) and overall glucose yields (35.4 g glucose/100 g wheat straw) but also higher production of toxic compound. In turn, the characteristics of the pretreated wheat straw at lower severity (Log R_0=3.65) correspond to 190°C and 10 min, with minimal sugars degradation and toxics formation indicated a great potential for maximizing total sugars production by using optimal enzyme combinations including accessory enzymes in the enzymatic hydrolysis step.

Keywords: Biofuels, steam explosion, wheat straw, pretreatment, enzymatic hydrolysis, sugars recovery, lignocellulosic biomass

***Corresponding author: María José Negro,** Biofuels Unit, CIEMAT, Madrid, 28040, Spain, E-mail: mariajose.negro@ciemat.es
Pablo Alvira, Ignacio Ballesteros, Alberto González, Mercedes Ballesteros, Biofuels Unit, CIEMAT, Madrid, 28040, Spain

1 Introduction

Alternative and renewable fuels and products derived from lignocellulosic biomass offer the potential to mitigate global climate change and turn the dependence on fossil fuels into a biobased economy. Biofuels such as ethanol represents an alternative in the transport sector and its introduction into current fuel distribution is being promoted by mandatory targets.

Among different lignocellulosic materials available for ethanol production, agricultural residues such as wheat straw are one of the main candidates for large-scale ethanol production. According BIOCORE Project [http://www.biocore-europe.org], the annual quantity of potentially extractable, surplus wheat straw in European countries is about 35 million metric tons of dry matter that could produce 10 GL of bioethanol [1]. In Spain, grain industry generates about 4.5 million tons of wheat straw. Bioconversion of this residue to fuel ethanol represents an interesting possibility to boost the development of biofuels in Spain in a sustainable way.

The ethanol production process consists of three steps namely, pretreatment, enzymatic hydrolysis and fermentation. The pretreatment is an indispensable step to disrupt the biomass recalcitrance, open the lignocellulose structure and increase the accessibility of cellulose to the enzymatic attack. Many pretreatment methods have been evaluated for sugars and ethanol production [2]. Compared to other pretreatment technologies, steam explosion offers some outstanding features such as high potential for energy efficiency, low environmental impact and capital investment, unnecessary addition of acid catalyst (except for softwoods), complete glucan recovery, high enzymatic hydrolysis yields, etc. [3]. It is the most commonly used pretreatment for herbaceous biomass, and it has been developed and evaluated at pilot and demonstration scales for bioethanol production from lignocellulose [3-5].

Autocatalysed steam explosion is a hydrothermal treatment that joins both mechanical and chemical effects on biomass without addition of external catalyst. Autohydrolysis takes place when high temperatures

causing the formation of acetic acid from acetyl groups in the hemiceelulose fraction [2]. The mechanical effects are caused because the suddenly reduction of pressure and fibres are separated due to explosive decompression [3, 6].

The steam explosion pretreatment produces partial hemicellulose solubilisation and lignin reorganization, which increase the cellulose accessibility to enzymatic attack by exposing the cellulose surface. From steam explosion two fractions are obtained: a insoluble solid fraction (rich in cellulose and lignin) and a liquid fraction or prehydrolysate (rich in soluble sugars mainly from hemicellulose solubilisation) [2]. This pretreatment also produces partial sugars and lignin degradation, which generates soluble compounds that are inhibitory or toxics for the enzymes and fermenting microorganisms in subsequent steps [3, 7, 8].

The most important variables affecting the effectiveness of steam explosion are temperature, residence time, and particle size. The combined effect of both temperature (T) and time (t) is described by the severity factor ($\log R_o$) [$\log R_o = \log (t*e[T-100/14.75])$] [9]. The optima severity factor value is greatly dependent on the biomass feedstock. This is a critical point for large-scale implementation of steam explosion pretreatment because operation conditions of temperature and residence time have a relevant impact on the process energy costs. In addition, the pretreatment conditions influence the characteristics of the pretreated materials and therefore determine the process configuration. Usually, high severity conditions (high temperature and residence time) rise removal of hemicelluloses and enhance digestibility of biomass but also promote higher sugar and lignin degradation and generation of toxic compounds for the fermentation step. In contrast, milder pretreatment conditions reduce energy cost and sugars degradation; but reduce enzymatic hydrolysis yields. To improve enzymatic hydrolysis yields the use of accessory enzymes such as hemicellulases have been studied by different authors to overcome the limitations of milder pretreatments [10-14].

Steam explosion pretreatment has been extensively studied for bioethanol production from a wide range of lignocellulosic biomass feedstocks such as poplar [15], eucalyptus [16], olive residues [17], corn stover [18], wheat straw [19,20], barley straw [21], sugarcane straw and bagasse [4, 22], forage sorghum [23], hemp [24] and *Brassica* [25].

In this work, a range of non-catalyzed steam explosion pretreatment conditions were evaluated according to different parameters: composition of pretreated substrates, glucose and xylose recovery, generation of degradation products, enzymatic hydrolysis yields and overall sugars yields. In addition, since operating hydrolysis at high solids loading is crucial for the commercialization of cellulosic ethanol technology, enzymatic hydrolysis using the enzyme preparations Cellic CTec2 and Cellic HTec2 has been studied at high substrate loadings.

2 Material and Methods

2.1 Raw material

Wheat straw harvested in Soria (Spain) with 6% of moisture content was employed as raw material. It was milled in a laboratory hammer mill, and sieved to obtain a particle size of 2-10 mm. The chemical composition of wheat straw feedstock (expressed as % dry weight) was: glucan, 39.7±0.8; xylan, 23.8±0.9; galactan, 0.5±0.03; arabinan, 1.9±0.09; soluble acid lignin, 1.7±0.07; insoluble acid lignin, 16.4±0.1; ashes, 4.9±0.06; extractives, 13.3±1.2. Except for studying the effect of non-water impregnation, wheat straw was impregnated with water in all cases previously to pretreatment.

2.2 Enzymes

The enzyme preparations NS 50013, NS 50010, Cellic CTec2 and Cellic HTec2 were provided by Novozymes (Denmark). For standard enzymatic hydrolysis assays, NS 50013 (cellulase activity, 60 FPU/mL)) and NS 50010 (cellobiase activity, 810 UI/mL) preparations were employed. While for enzymatic hydrolysis assays at high solids loading novel enzyme preparations Cellic CTec2 (cellulase with high beta-glucosidase activity) and Cellic Htec2 (hemicellulase preparation with endoxylanase activity) were used. Filter paper activity (overall cellulase) and beta-glucosidase activity were measured according to Ghose [26]. Xylanase activity was determined using birchwood xylan as substrate [27].

2.3 Steam explosion pretreatment

Steam explosion pretreatment was conducted in a small bath unit equipped with 2L reaction vessel. A water impregnation of the raw material prior to steam explosion pretreatment was carried out by soaking 150 g (dry matter) of wheat straw in 1.5 L of water overnight, then the excess liquid removed by filtration. Moisture content of impregnate raw material was roughly 60%. The pressure reactor was preheated at the set pretreatment temperature with saturated steam, after substrate addition into the reactor, it took less than 60 seconds to reach working temperature. According to previous experience on steam-explosion pretreatment of cereal straw [20], six pretreatment conditions were selected for evaluation:

170°C - 30 min., 180°C - 25 min., 190°C - 10 min., 190°C - 20 min., 200°C - 10 min., and 210°C - 5 min.

To evaluate the pretreatment the whole slurries obtained after the steam explosion were filtered and both solid and liquid fractions were collected. The solid fraction was washed with water and filtered for water insoluble solids (WIS) fraction recovery. Composition of both the WIS fraction and the liquid fraction were analysed to evaluate the pretreatment. WIS fractions were employed in the enzymatic hydrolysis assays as substrate.

2.4 Enzymatic hydrolysis

Enzymatic hydrolysis assays were performed in 100 mL Erlenmeyer flasks in triplicates using WIS as substrate. The assays were run in 0.05 M sodium citrate pH 5 buffer at 50°C and 150 rpm. At 72 h, samples were withdrawn, centrifuged (9300 *g* for 10 min) and the supernatants were analysed by HPLC for sugars concentration determination, blanks of the enzyme mixtures were also analysed. Average values of the three replicates were presented.

Standard enzymatic hydrolysis assays were carried out using as substrate WIS at 5% total solid (w/v); and a mixture of the enzyme preparations NS 50013 (15 FPU/g substrate) and NS 50010 (15 IU/g substrate). Enzymatic hydrolysis assays at high solids loading were performed using as substrate WIS at 15% (w/w) of total solids, and a mixture of the enzymes preparations Cellic CTec2 and Cellic HTec2. The dosage of Cellic CTec2 was 0.15 g/g substrate while the dosage of Cellic HTec2 was 0.06 g/g substrate.

2.5 Analytical methods and calculations

The carbohydrate and lignin content of both raw and pretreated wheat straw (WIS) was quantified using the NREL compositional analysis [28]. In the fraction liquid (prehydrolysate) the sugars and degradation products composition generated during the pretreatment were also quantified. For sugars quantification in prehydrolysates, a mild acid hydrolysis (4% (v/v) H_2SO_4, 120°C and 30 min) was carried out to convert oligomers into monomers. The compositional analysis was performed in triplicates and average values were presented. The relative standard errors were determined and were shown to be consistently below 5%.

Sugars and carboxylic acids concentration was quantified by high-performance liquid chromatography (HPLC) in a Waters chromatograph 2414 equipped with a refractive index detector (Waters, Mildford, MA). For sugars, separation was performed in a CarboSep CHO-787 column (Transgenomic, San Jose, CA) operated at 70°C with a flow rate of 0.5 mL/min using ultrapure water as a mobile-phase . For carboxylic acid, the separation was carried out in a IC Sep-ICE Coragel 87H3 column (Transgenomic, San Jose, CA) operated at 65°C with a flow rate of 0.6 mL/min using 5mM H_2SO_4 as mobile phase.

Furfural, 5-hydroxymethylfurfural (5-HMF), 4-hydroxybenzaldehyde, vanillin, syringaldehyde, *p*-coumaric acid and ferulic acid were analysed by HPLC as described elsewhere [29]

The severity factor (log R_o) was calculated using the formula log R_o= log (t*e[T-100/14.75]) [9]. Solid recovery yield (%SRY) was estimated as dry weight of WIS remaining after pretreatment referred to 100 g of raw material. Glucan and xylan recovery was calculated as a percentage and considered the glucose and xylose recovered in the WIS fraction and the liquid fraction respect to the glucan and xylan content in the raw material. They were calculated using the following equations:

$$\text{Glucan recovery (\%)} = \frac{g\ glucose\ (monomers\ and\ oligomers\ as\ monomeric\ equivalent) in\ LF \times 100 + [g\ glucan\ (as\ monomeric\ equivalent)\ in\ WIS] \times \frac{\%SR}{100}}{g\ glucan\ (as\ monomeric\ equivalent)\ in\ RM} \times 100$$

$$\text{Xylan recovery(\%)} = \frac{xylose\ (monomers\ and\ oligomers\ as\ monomeric\ equivalent) in\ LF \times 100 + [g\ xylan\ (as\ monomeric\ equivalent)\ in\ WIS] \times \frac{\%SR}{100}}{g\ xylan\ (as\ monomeric\ equivalent)\ in\ RM} \times 100$$

Enzymatic hydrolysis yields were determined as the ratio of g glucose (or xylose) obtained in the enzymatic hydrolysis per 100 g of potential sugar in WIS.

Overall yields were calculated as a percentage of total glucose or xylose obtained in the liquid fraction and after enzymatic hydrolysis step respect to the total glucose or xylose content in the raw material. They were calculated using the following equations:

$$\text{Overall Glucose Yield (\%)} = \frac{\frac{g\ glucose\ in\ EH}{g\ WIS\ in\ EH} \times \frac{\%SRY}{100} + \frac{g\ glucose\ (monomers\ and\ oligomers\ as\ monomeric\ equivalent)\ in\ LF}{g\ RM}}{g\ glucan\ (as\ monomeric\ equivalent) in\ RM} \times 100$$

$$\text{Overall Xylose Yield (\%)} = \frac{\frac{g\ xylose\ in\ EH}{g\ WIS\ in\ EH} \times \frac{\%SR}{100} + \frac{g\ xylose\ (monomers\ and\ oligomers\ as\ monomeric\ equivalent)\ in\ LF}{g\ RM}}{g\ xylan\ (as\ monomeric\ equivalent)\ in\ RM} \times 100$$

Where: %SRY is solid recovery yield, EH is the enzymatic hydrolysis assay, LF: is liquid fraction; RM is raw material

3 Results and discussion

3.1 Effect of water impregnation on steam explosion

The effect of water impregnation of wheat straw prior to steam explosion pretreatment was evaluated using the conditions 180°C - 25 min, this conditions correspond to those conditions that permits relatively low xylan degradation. The results show that water impregnation produced a slight increase in glucan content of WIS (from 60.1% to 62.8%) and a slight decrease in xylan content (from 15.6% to 13.1%) (Table 1), while did not have a significant influence on the liquid fraction composition (not shown). In addition, water impregnation had a significant positive effect on enzymatic hydrolysis of the WIS fraction, increasing the yields from 65.9% to 73.5% for glucose and from 66.9% to 75.5% for xylose. As a consequence overall glucose and xylose yield were improved.

The mechanical effect of steam explosion is caused by the sudden evaporation of interfibrillar water when the explosion occurs and the pressure is reduced, which creates shear forces that physically separate the fibres and disrupt the structure [30]. Water impregnation prior to steam explosion could enhance this mechanical effect, increasing hemicellulose removal. The improvement in the enzymatic digestibility in samples previously impregnated can be attributed to the enhanced removal of hemicellulose in the solid residue.

3.2 Effect of steam explosion pretreatment conditions

3.2.1 Composition of pretreated substrates and sugars recovery

According to the results shown in the previous section, in the subsequent study of different steam explosion conditions the raw material was water impregnated prior to pretreatment. Compositions of WIS and liquid fractions were analysed to assess the effect of different pretreatment conditions. The liquid fraction and WIS compositions for each pretreatment condition are shown on Table 2.

It was apparent that the variety of steam explosion conditions produced pretreated substrates with different characteristics. In all cases the pretreatment produced partial solubilisation of hemicellulose, which concentrated cellulose and lignin in the pretreated WIS fraction compared to the raw material. Cellulose , hemicellulose and lignin content in WIS are in the range of 52.6–64.7%, 6.4–24.0%, 20.8–31.0% respectively.

Table 1. Effect of water impregnation prior to steam explosion pretreatment on WIS composition, glucan and xylan recovery, and enzymatic hydrolysis (EH) and overall yields.

Parameter		180°C- 25 min (impregnated)	180°C-25 min. (not impregnated)
Solid insoluble recovery (%SR)		54.0	55.6
WIS composition	Glucan (%)	62.8±0.8 (33.9)*	60.0±0.9 (33.4)*
	Xylan (%)	13.1±0.1 (7.1)*	15.56±0.3 (8.7)*
	Lignin (%)	24.3±0.3 (13.1)*	24.0±0.2 (13.3)*
Glucan recovery (% referred to raw material)		93.4	94.1
Xylan recovery (% referred to raw material)		80.6	81.7
EH glucose yield (%)		73.5	65.9
EH xylose yield (%)		75.5	66.9
Overall glucose yield (g glucose/100 g raw material)		31.8 (72.8%)	28.5 (65.9%)
Overall xylose yield (g xylose/100 g raw material)		18.9 (71.5%)	18.7 (69.5%)

*Data are expressed in parentheses as a percentage based on dry weight of raw material

The severity factors for the different pretreatment conditions are also shown in Table 2. Steam explosion pretreatments at lower severity (190°C - 10 min, and 170°C - 30 min) solubilised less hemicellulose and therefore it was detected in a higher proportion (24.0% and 17.5% of xylan, respectively) in the WIS fraction (Table 2). Interestingly, the highest proportion of xylan was determined in the pretreated material at 190°C - 10 min (24.0 %), even though the lowest severity factor (Log R_0= 3.53, corresponds to 170°C - 30 min), in which this highest value would be expected. In case of pretreatments at increasing severity, solubilisation of xylan was more extensive and glucan content in the WIS fraction increased. This effect is well-known and agrees previous steam explosion studies [31]. The highest severity factor corresponds to the conditions 190°C - 20 min (Log R_0=3.95), however the pretreatment at 200°C - 10 min and 210°C - 5 min solubilised more xylan and increased glucan content in WIS (59.1% and 64.7%, respectively), which may indicate that temperature was more important than residence time to solubilise the hemicellulose.

The liquid fractions obtained after filtration of the pretreated whole slurries contained soluble components, mainly solubilised sugars and degradation products. The most abundant sugar was xylose, but also contained glucose, galactose, arabinose, and mannose. They were found in monomeric and oligomeric form, and mostly derived from hemicellulose hydrolysis. The degradation compounds were produced as a consequence of the harsh conditions during the pretreatment. The most abundant were acetic acid, formic acid, furfural and 5-HMF. Acetic acid was generated by hydrolysis of acetyl groups in the hemicellulose fraction. Furfural and 5-HMF derived from xylose and glucose degradation, respectively [8]. In addition, some phenolic compounds were also detected in the liquid fraction. Ferulic acid and *p*-coumaric acid derived from cinnamic acids present in herbaceous plants [32] and vanillin derived from lignin degradation [33] were also detected. The presence of degradation compounds produced during lignocellulose pretreatments has been shown to affect negatively the enzymatic hydrolysis and fermentation steps for ethanol production [7, 34].

Among the conditions evaluated, the highest total xylose (monomeric and oligomeric form) concentration in the liquid fraction (14.0-14.1 g/100g) was detected when the

Table 2. Liquid and solid fraction (WIS) composition after non-catalysed steam explosion pretreatment.

Pretreatment conditions		**190°C 20 min**	**200°C 10 min**	**210°C 5 min**	**180°C 25 min**	**190°C 10 min**	**170°C 30 min**
Severity (log Ro)		3.951	3.944	3.930	3.750	3.650	3.538
Liquid fraction (g component/100 g raw material)							
Sugars	Glucose	3.3	2.9	1.6	4.0	3.01	2.65
	(% monomeric form)	8.7	11.3	27.6	11.5	16.6	13.5
	Xylose	11.2	9.1	14.1	14.0	7.0	7.0
	(% monomeric form)	9.0	15.9	11.8	6.2	4.5	3.3
	Galactose	0.62	0.54	0.73	1.40	0.81	0.48
	Arabinose	1.15	0.75	1.40	2.20	1.56	1.15
	Mannose	-	-	0.17	0.20	0.05	-
Inhibitors compounds	Furfural	0.23	0.32	0.28	0.22	0.09	0.05
	5-HMF	0.10	0.14	0.03	0.04	0.02	0.05
	Acetic acid	1.84	2.20	0.78	0.88	0.58	1.11
	Formic acid	0.23	0.41	0.24	0.16	0.34	0.0
	Vanillin	0.03	0.03	0.03	0.00	0.02	0.01
	Coumaric acid	0.02	0.02	0.02	0.02	0.02	0.00
	Ferulic acid	0.01	0.01	0.01	0.01	0.00	0.00
WIS composition (w/w, %)							
Glucan		58.6±0.5	59.1± 2.1	64.7±1.9	62.8±0.8	56.1±0.9	52.6±0.9
Xylan		9.6±0.0	6.4±0.2	7.9±0.4	13.1±0.1	24.0±0.2	17.5±0.2
Galactan		-	-	-	0.2±0.1	1.1±0.1	-
Arabinan + Mannan		0.2±0.0	0.1±0.0	-	0.8±0.1	2.0±0.1	0.6±0.1
Acid insoluble lignin		28.7±0.4	31.0±0.3	25.6±0.6	24.3±0.3	20.8±0.6	24.5±0.2
Ashes		1.6±0.2	0.5±0.1	1.3±0.2	2.8±0.8	1.6±0.3	1.1±0.1

Table 3. Summary of pretreatment parameters: severity factor, glucan and xylan recovery, enzymatic hydrolysis yields and overall yields.

Parameter	190°C 20 min	200°C 10 min	210°C 5 min	180°C 25 min	190°C 10 min	170°C 30 min
Severity (Log R_0)	3.951	3.944	3.930	3.750	3.650	3.538
Glucan recovery (% referred to raw material)	88.0	87.8	93.0	93.4	97.9	86.9
Xylan recovery (% referred to raw material)	63.6	48.4	67.0	80.6	91.1	70.9
EH glucose yield (%) *	78.1	91.7	81.8	73.5	63.2	54.7
EH xylose yield (%) *	63.5	66.2	63.3	75.5	51.4	55.4
Overall glucose yield g glucose/100 g raw material (%)	31.0 (71.0%)	35.4 (81.1%)	33.5 (76.7%)	31.8 (72.8%)	28.4 (65.03%)	22.0 (50.4%)
Overall xylose yield g xylose/100 g raw material (%)	14.9 (56.4%)	11.7 (44.3%)	17.3 (65.5%)	18.9 (71.5%)	16.0 (60.6%)	13.7 (51.9%)
Overall glucose and xylose yield (%)	65.7	67.5	72.7	72.6	63.6	51.1

pretreatment was performed at 210°C - 5 min and at 180°C - 25 min. Glucose was detected in concentrations ranging from 1.6 to 4.0 g/100 g (Table 2). In general, pretreatments at harsher conditions (190°C - 20 min, 200°C - 10 min, 210°C - 5 min and 180°C - 25 min) produced higher hemicellulose solubilisation but also higher sugars degradation and production of inhibitors. As shown in Table 2, higher severity led to higher xylose degradation into furfural and formic acid, while milder conditions produced less solubilisation of xylan and therefore a decrease in soluble xylose. In general, acetic acid and phenolic compounds (vanillin, *p*-coumaric acid and ferulic acid) concentration also increased at higher severity conditions.

Glucan and xylan recovery (Table 3) was calculated as a percentage and considered the glucose and xylose recovered in the WIS fraction and the liquid fraction respect to the glucan and xylan content in the raw material. This result gives an idea of the degree of sugars degradation occurred during the pretreatment. As shown in Table 3, glucan recovery is high for all the conditions since cellulose is more crystalline and difficult to be degraded during steam explosion pretreatment. The recovery values ranged from 86.9% to 97.9%. This indicates that steam explosion at these conditions resulted in little glucose degradation. More differences were observed for xylan recovery. Pretreatments at 190°C - 20 min, 200°C - 10 min and 210°C - 5 min led to higher xylose degradation and consequently lower xylan recovery. Among them, the pretreatment at 200°C - 10 min gave the lowest xylan recovery value, indicating a high degradation degree of xylan, which is also reflected in the highest concentration of furfural detected (0.32 g/100 g). In contrast, the pretreatment at 190°C - 10 min resulted in a xylan recovery of 91.1%, with a low formation of toxic compounds such as furfural, 5-HMF, formic acid and acetic acid (Table 2). This type of pretreatment would be appropriate for some bioconversion process configurations: on one hand low formation of inhibitors is crucial when the whole slurry after pretreatment is used directly without separation; on the other hand high xylose recovery offers great potential to improve ethanol fermentation yields when the process includes the use of xylanases and xylose-fermenting microorganisms.

3.3 Overall yields

WIS fractions from the different pretreatments were submitted to standard enzymatic hydrolysis at 5% of total solids with the mixture of enzymes (NS 50013-NS 50010). The calculated overall yields indicate the degree of glucose and xylose that could be produced from the raw material after the steam explosion pretreatment and enzymatic hydrolysis.

Table 3 shows enzymatic hydrolysis yields obtained for the different pretreatments. In general, the higher pretreatment severity, the higher enzymatic hydrolysis yields were achieved. The increasing temperature and residence time in the pretreatment increased solubilisation of hemicellulose and enhanced the accessibility of

cellulose, which involves an increment in the enzymatic hydrolysis yields. This effect agrees previous studies evaluating the steam explosion pretreatment. Ballesteros *et al.* reported a rise in the enzymatic hydrolysis yields from 40% at 160°C to 70% at 200°C [20], while Palmarola *et al.* also observed an increment in saccharification yield at higher severity conditions, obtaining a glucose yield of 92.4% at 190°C and 10 min [35].

As expected higher severity pretreatments (190°C - 20 min, 200°C - 10 min, 210°C - 5 min and 180°C - 25 min) resulted in higher overall glucose yield because of the accessibility of cellulose and therefore enzymatic hydrolysis yields were increased. The highest enzymatic hydrolysis yield (91.7%) and overall glucose yield (35.4 g/100 g raw material) were achieved for the pretreatment performed at 200°C and 10 min. In contrast, with this pretreatment the xylose overall yield reached the lowest value (11.7 g xylose/ 100 g raw material). This pretreatment also produced the highest concentrations of degradation products, which indicate that these conditions would be appropriate to obtain high glucose and ethanol yields from filtered and washed pretreated materials (WIS fractions).

The highest xylan recovery was obtain with lowest severity pretreatments: 180°C - 25 min, 190°C - 10 min and 170°C - 30 min (Table 3). In these cases degradation of xylose was minimized. However, the highest xylose overall yield were obtained at 210°C - 5 min and 180°C - 25 min (17.3 g xylose/100 g raw material and 18.9 g xylose7g raw material, respectively). When the conditions of pretreatment were too mild, hydrolysis of xylan contained in WIS was limited (Table 3) and therefore the overall yield was significantly decreased. In this context, utilization of hemicellulases could contribute to increase xylose yields.

In case of pretreatment at mildest conditions (Log R_0=3.54; 170°C - 30 min, toxics generation is low and sugars recovery is high. However, the enzymatic hydrolysis yields clearly decreased, which made the overall yields significantly lower compared to the other pretreatment conditions (Table 3).

3.4 Enzymatic hydrolysis at high solids consistency using Cellic enzyme preparations

The relevance of working at high substrate loadings in the enzymatic hydrolysis process is crucial for large scale development of biofuels production has been previously disclosed [36], although said approach presents some disadvantages such as end-product inhibition of cellulolytic enzymes by glucose and cellobiose, mixing and mass transfer limitations, and larger concentrations of inhibitors in the media [29, 37-40].

Novel enzyme products such as Cellic CTec2 and Cellic HTec2 have been specifically developed to improve the enzymatic hydrolysis yields from pretreated lignocellulosic substrates at high solids loadings. In this context, the steam exploded substrates pretreated at different conditions were studied in enzymatic hydrolysis experiments at high solids loading (15% w/w) and using the Cellic enzymes.

In the previous section it was shown that low severity pretreatments resulted in high glucose and xylose recovery and minimal inhibitors production. However enzymatic hydrolysis yields were significantly lower than those obtained with more severe pretreatments. An option to combine the advantages of low severity conditions and high enzymatic saccharification yields is the utilization of accessory enzymes such as xylanases. Xylanases and other hemicellulases have been shown to enhance accessibility of cellulose and significantly improve the enzymatic hydrolysis yields [10-14]. Thus, supplementation of Cellic CTec2 with the hemicellulase preparation Cellic HTec2 was evaluated in enzymatic hydrolysis experiments at 15% (w/w). Figure 1 shows the enzymatic hydrolysis assays yields and figure 2 shows the overall sugar yields, calculated considering the sugars detected in the liquid fraction and the sugars resulting from the enzymatic hydrolysis at 15% (w/w) with the Cellic enzymes.

In all cases, Cellic HTec2 supplementation improved enzymatic hydrolysis yields both for glucose and xylose, even when substrates with low xylan content were used. This observation agrees with previous studies [12]. Supplementation with the hemicellulase preparation Cellic HTec2 improved glucose yields when using pretreatments at lower severity such as 180°C - 25 min and 190°C - 10 min. However, and similarly to standard enzymatic assays at 5% totals solids, higher glucose yields (enzymatic hydrolysis and overall yields) were obtained when using pretreated materials at higher severity conditions. The highest overall glucose yields were achieved with the substrates pretreated at 200°C - 10 min and 210°C - 5 min and with addition of Cellic HTec2. It was obtained an overall yield of 34.4 and 35.6 g per 100 g of raw material, respectively, which correspond to a yield of 78.8% and 81.5% of potential glucose contained in the raw material (Figure 2). Although the highest severity factor corresponds to the conditions 190°C - 20 min (Log R_0=3.95), this pretreatment did not produce an increase in glucose yields compared to the condition 200°C - 10 min, indicating that temperature was more important than residence time for the severity of the pretreatment.

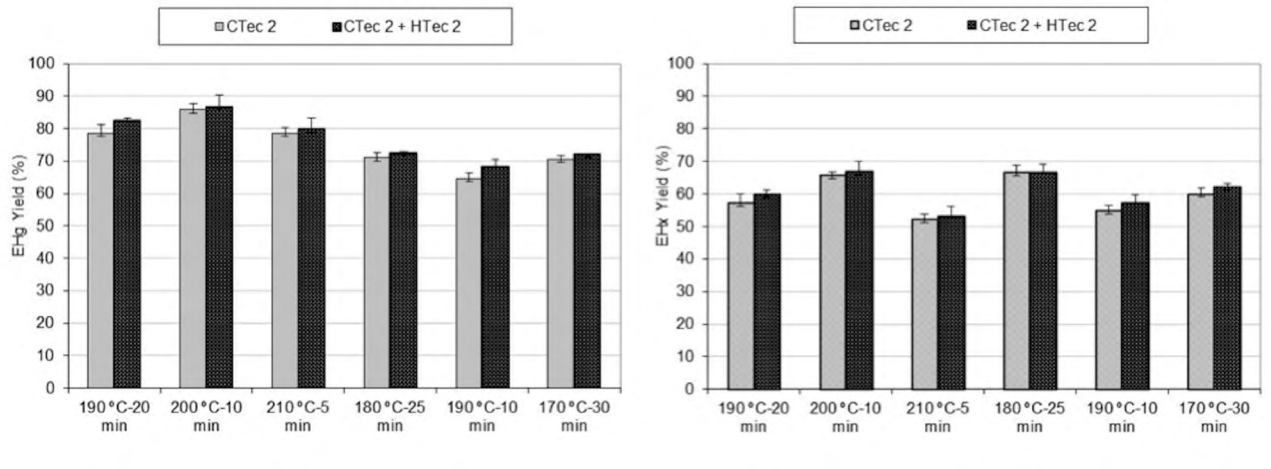

Figure 1. Enzymatic glucose yield (EHg) and enzymatic xylose yield (EHx) at 15% (w/w) total solids and using Cellic Ctec2 (cellulase) and Cellic Htec2 (xylanase).

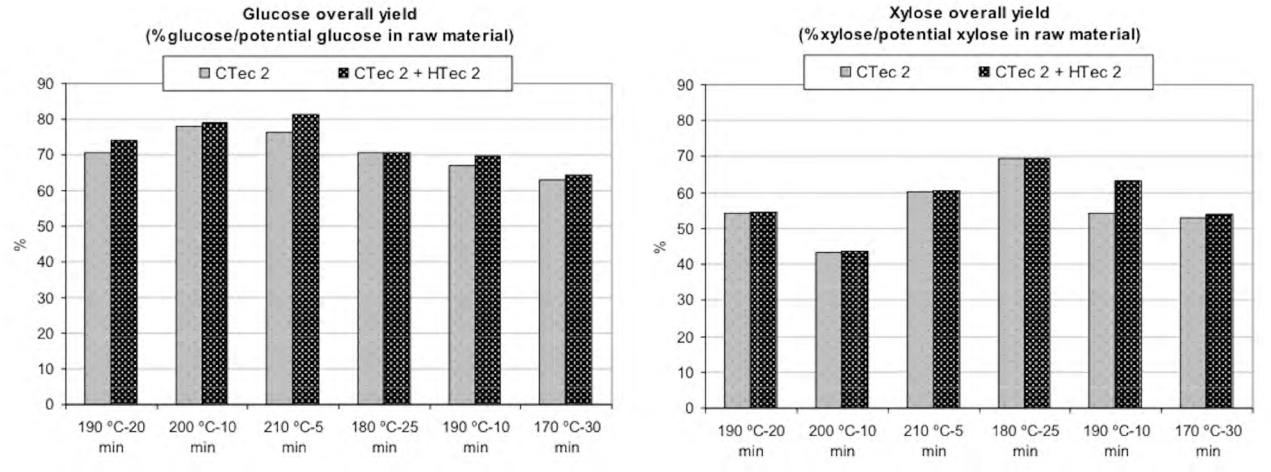

Figure 2. Overall yields at 15 % (w/w) total solids using Cellic CTec2 and Cellic HTec2 enzymes.

The highest xylose overall yields were achieved with the pretreatments at 180°C - 25 min and 190°C - 10 min, and Cellic HTec2 supplementation (18.7 g and 17.0 g per 100 g of raw material, which correspond to a yield of 69.5% and 63.2%, respectively), improving significantly the xylose yields obtained from pretreatments at higher severity. In particular, with the pretreatment at 190°C - 10 min high xylose and glucose recoveries, and low inhibitors formation were achieved. Under those conditions the glucose overall yields were lower than those obtained at higher severity conditions, but interestingly when supplementing with Cellic HTec2 the overall sugars (glucose+xylose) yield reached 67.2%, which is a higher value than those obtained at the harshest conditions without Cellic HTec2 addition: 64.4% for 190°C - 20 min and 64.8% for 200°C - 10 min. It is well known that the remaining hemicelluloses in the pretreated substrate decreases the cellulose accessibility [12, 13], and hemicellulases have been shown to act synergistically removing hemicellulose and enhancing cellulose hydrolysis [10]. Therefore hemicellulases have the potential to increase sugar conversion yields when the pretreatment severity conditions are reduced to diminish loss of sugars and energy costs. The characteristics of the pretreated wheat straw at 190°C and 10 min (minimal sugars degradation and toxics formation) indicate a great potential for maximize sugars production by using optimal enzyme combinations including accessory enzymes in the enzymatic hydrolysis step.

4 Conclusions

This study evaluates steam explosion conditions for production of sugars from wheat straw. Several parameters were analysed and the results illustrate that the pretreatment conditions affect significantly the characteristics of the resulting substrates and consequently the further process configuration.

Pretreatments at high severity (R_0 = 3.94, corresponding to 200°C, 10 min) resulted in higher glucose overall yields (> 80%) but also higher production of toxic compounds. Pretreatment wheat straw at these conditions suggests a process configuration including a separation and a washing step, and therefore the utilization of the WIS fraction for enzymatic hydrolysis and fermentation. In turn, the characteristics of the pretreated wheat straw at lower severity (e.g. 190°C and 10 min, with minimal sugars degradation and toxics formation) indicate a great potential for maximizing total sugars production by using optimal enzyme combinations including accessory enzymes in the enzymatic hydrolysis step. This work showed the potential of hemicellulases supplementation to increase enzymatic hydrolysis yields and allow reducing the severity on pretreatment step.

Aknowledgements: This work was supported by "Abengoa Bioenergy New Technologies" within the CENIT BioSos Project funded by CDTI (Spanish Ministry of Economy and Competitiveness). Enzyme preparations were kindly provided by Novozymes AS (Denmark).

Conflict of interest: Dr. Negro reports grants from CDTI, during the conduct of the study.

References

[1] Kim S., Dale B.E., Global potential bioethanol production from wasted crops and crop residues, Biomass Bioenerg., 2004, 26, 361-375.

[2] Tomás-Pejó E., Alvira P., Ballesteros M., Negro M.J., Pretreatment technologies for lignocellulose-to-bioethanol conversion, In: Pandey A., Larroche C., Ricke S.C., Dussap C.G., Gnansounou E., (Eds.), Biofuels: Alternative feedstocks and conversion processes, Academic Press, Amsterdam, 2011.

[3] Avellar B.K., Glasser W.G., Steam assisted biomass fractionation I. Process considerations and economic evaluation, Biomass Bioenerg., 1998, 14, 205-218.

[4] Oliveira, F.M.V., Pinheiro, I.O., Souto-Maior, A.M., Martin, C., Gonçalves A.R., Rocha G.J.M., Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second generation ethanol and value-added products, Bioresour. Technol., 2013, 130, 168-173.

[5] Sharma S., Kumar R., Gaur R, Agrawal R., Gupta R.P., Tuli D.K., Das B, Pilot scale study on steam explosion and mass balance for higher sugar recovery from rice straw, Bioresour.Technol. , 2015, 175, 350-357.

[6] Sun Y., Cheng J., Hydrolysis of lignocellulosic materials for ethanol production: a review, Bioresour. Technol., 2002, 83, 1-11.

[7] Jurado M., Prieto A., Martínez-Alcalá A., Martínez A.T., Martínez M.J., Laccase detoxification of steam-exploded wheat straw for second generation bioethanol, Bioresour. Technol., 2009, 100, 6378-6384

[8] Palmqvist E., Hahn-Hägerdal B., Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanism of inhibition, Bioresour. Technol., 2000, 74, 25-33.

[9] Overend R.P., Chornet E., Fractionation of lignocellulosics by steam-aqueous pretreatments, Phil. Trans. R. Soc. Lond., 1987, 321, 523-536.

[10] Alvira P., Negro M.J., Ballesteros M., Effect of endoxylanase and alpha-arabinofuranosidase supplementation on the enzymatic hydrolysis of steam exploded wheat straw, Bioresour. Technol., 2011, 102, 4552-4558.

[11] Alvira P., Tomás-Pejó E., Negro M.J., Ballesteros M., Strategies of xylanase supplementation for an efficient saccharification and cofermentation process from pretreated wheat straw, Biotechnol. Prog., 2011, 27, 944-950.

[12] García-Aparicio M., Ballesteros M., Manzanares P., Ballesteros I., González A., Negro M.J., Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw, Appl. Biochem. Biotechnol., 2007, 137-140, 353-365.

[13] Ohgren K., Bura R., Saddler J., Zacchi G., Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover, Bioresour. Technol., 2007, 98, 2503-2510.

[14] Marcos M., García-Cubero M.T., González-Benito G., Coca M., Bódalo S., Lucas S., Optimization of the enzymatic hydrolysis conditions of steam-exploded wheat straw for maximum glucose and xylose recovery, J. Chem. Technol. Biotechnol., 2013, 88, 237-246.

[15] Oliva J.M., Sáez F., Ballesteros I., González A., Negro M.J., Manzanares P., *et al.*, Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*, Appl. Biochem. Biotechnol., 2003, 105, 141-154.

[16] Romani A., Garrote G., Ballesteros I., Ballesteros M., Second generation bioethanol from steam exploded *Eucalyptus globulus* wood, Fuel, 2013, 111, 66-74.

[17] Ballesteros I., Ballesteros M., Cara C., Sáez F., Castro E., Manzanares P., *et al.*, Effect of water extraction on sugars recovery from steam exploded olive tree pruning, Bioresour. Technol., 2011, 102, 6611-6616.

[18] Chu Q., Yang D., Li X., Ma B., Yu S., Yong Q., An integrated process to enhance ethanol production from stream-exploded corn stover, Fuel, 2013, 107, 823-827

[19] Horn S.J., Nguyen Q.D., Westereng B., Nilsel P.J., Eijsink V.G.H., Screening of steam explosion conditions for glucose production from non-impregnated wheat straw, Biomass Bioenerg., 2011, 35, 4879-4886.

[20] Ballesteros I., Negro M., Oliva J.M., Cabañas A., Manzanares P., Ballesteros M., Ethanol production from steam-explosion pretreated wheat straw, Appl. Biochem. Biotechnol., 2006, 130, 496-508.

[21] García-Aparicio M.P., Oliva J.M, Manzanares P., Ballesteros M., Ballesteros I., González A., *et al*., Second generation ethanol production from exploded barley straw by *Kluyveromyces marxianus CETC* 10875, Fuel, 2011, 90, 1624-1630.
[22] Amores I., Ballesteros I., Manzanares P., Sáez F., Michelena G., Ballesteros M., Ethanol production from sugarcane bagasse pretreated by steam explosion, Electronic Journal of Energy & Environment 2011, 1, 25-36.
[23] Manzanares P., Ballesteros I., Negro M.J., Oliva J.M., González A., Ballesteros M., Biological conversion of forage sorghum biomass to ethanol by steam explosion pretreatment and simultaneous hydrolysis and fermentation at high solid content, Biomass Conv. Bioref., 2012, 2, 123-132.
[24] Barta Z., Oliva J.M., Ballesteros I., Dienes D., Ballesteros M., Réczey K., Refining hemp hurds into fermentable sugars or ethanol, Chem. Biochem. Eng., 2010, 24, 331–339.
[25] Ballesteros I., Oliva J.M., Sáez F., Negro M.J., Manzanares P., Ballesteros M., Enzymic hydrolysis of steam exploded herbaceous agricultural waste (*Brassica carinata*) at different particle sizes, Process Biochem., 2002, 38, 187-192.
[26] Ghose T.K., Measurement of Cellulase Activities, Pure Appl. Chem., 1987, 59, 257-268.
[27] Bailey M.J., Biely P., Poutanen K., Interlaboratory testing of methods for assay of xylanase activity, J. Biotechnol., 1991, 23, 257-270.
[28] Sluiter J.B., Ruiz R.O., Scarlata C.J., Sluiter A.D., Templeton D.W., Compositional analysis of lignocellulosic feedstocks. 1. review and description of methods, J. Agric. Food Chem., 2010, 58, 9043-9053.
[29] Alvira P., Moreno A.D., Ibarra D., Sáez F., Ballesteros M., Improving the fermentation performance of *Saccharomyces cerevisiae* by laccases during ethanol production from steam-exploded wheat straw at high substrate loadings, Biotechnol Prog., 2013, 29, 74-82.
[30] Alvira P., Tomás-Pejó E., Ballesteros M., Negro M.J., Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review, Bioresour. Technol., 2010, 101, 4851-4861.
[31] Montané D., Farriol X., Salvadó J., Jollez P., Chornet E., Application of steam explosion to the fractionation and rapid vapor-phase alkaline pulping of wheat straw, Biomass Bioenerg. 1998, 14, 261-276
[32] Sun R.C., Sun X.F., Wang S.Q., Zhu W., Wang X.Y., Ester and ether linkages between hydroxycinnamic acids and lignins from wheat, rice, rye, and barley straws, maize stems, and fast-growing poplar wood, Ind. Crops Prod., 2002, 15, 179-188.
[33] García-Aparicio M., Ballesteros I., González A., Oliva J.M., Ballesteros M., *et al.,* Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis, Appl. Biochem. Biotechnol., 2006, 129, 278-288.
[34] Oliva J.M., Ballesteros I., Negro M.J., Manzanares P., Cabañas A., Ballesteros M., Effect of binary combinations of selected toxic compounds on growth and fermentation of *Kluyveromyces marxianus*, Biotechnol. Prog., 2004, 20, 715-720.
[35] Palmarola-Adrados B., Galbe M., Zacchi G., Combined steam pretreatment and enzymatic hydrolysis of starch-free wheat fibers, Appl. Biochem. Biotechnol., 2004, 113, 989-1002.
[36] Banerjee S., Mudliar S., Sen R., Giri B., Satpute D., Chakrabarti T., *et al*., Commercializing lignocellulosic bioethanol: Technology bottlenecks and possible remedies, Biofuels Bioprod. Bioref., 2010, 4, 77-93.
[37] Mohagheghi A., Tucker M., Grohmann K., Wyman C., High solids simultaneous saccharification and fermentation of pretreated wheat straw to ethanol, Appl. Biochem. Biotechnol., 1992, 33, 67-82.
[38] Rosgaard L., Andric P., Dam-Johansen K., Pedersen S., Meyer A.S., Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw, Appl. Biochem. Biotechnol., 2007, 143, 27-40.
[39] Taherzadeh M.J., Karimi, K., Fermentation inhibitors in ethanol processes and different strategies to reduce their effects. In: Pandey A., Larroche C., Ricke S.C., Dussap C.G., Gnansounou E., (Eds.), Biofuels: Alternative feedstocks and conversion processes, Academic Press, Amsterdam, 2011.
[40] Moreno, A.D., Tomás-Pejó, E., Ibarra, D., Ballesteros, M., Olsson, L., Fed-batch SSCF using steam-exploded wheat straw at high dry matter consistencies and a xylose-fermenting *Saccharomyces cerevisiae* strain: Effect of laccase supplementation, Biotechnol. Biofuels, 2013, 6 (1), 160.