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Xylanase Contribution to the Efficiency of Cellulose Enzymatic Hydrolysis of Barley Straw

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Abstract

In this study, different enzyme preparations available from Novozymes were assessed for their efficiency to hydrolyze lignocellulosic materials. The enzyme mixture was evaluated on a pretreated cellulose-rich material, and steam-exploded barley straw pretreated under different temperatures (190, 200, and 210°C, respectively) in order to produce fermentable sugars. Results show that xylanase supplementation improves initial cellulose hydrolysis effectiveness of water-insoluble solid fraction from all steam-exploded barley straw samples, regardless of the xylan content of substrate. The mixture constituted by cellulase: β-glucosidase: endoxylanase of the new kit for lignocellulose conversion at a ratio 10 : 1 : 5% ([v/w], enzyme [*E*]/substrate [*S*]) provides the highest increment of cellulose conversion in barley straw pretreated at 210°C, for 10 min.

Index Entries: Agricultural residues; cellulose conversion; lignocellulose; pretreatment; xylanase supplementation; steam explosion.

Introduction

Liquid biofuels obtained from biomass feedstock, as ethanol, is regarded as an attractive alternative to fuel oil to reduce dependence on foreign oil and diminish CO_2 emissions, main cause of greenhouse effect. The production of ethanol as fuel (i.e., fuel ethanol) from cereal starch in Spain reached about 240,000 t in 2005 *(1)*. Recent legislation *(2)* requires the use of at least 500,000 t/yr of cereal-based ethanol by 2010. Alternative biomass resources as lignocellulosic materials could also be used to supply a large-scale biomass-to-energy industry. This feedstock is interesting because of its abundance and low cost, as a great part of the lignocellulosic materials is generated as remainder in the productive process of agricultural sector. However, the ethanol production and its derivatives

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from lignocellulose require advanced conversion technology to make them competitive against fuel oil. Although today there is little commercial production of ethanol from lignocellulosic biomass, R&D is being performed in Canada, the United States, and Europe *(3)*. Among biomass-to-ethanol processes, those based on enzymatic hydrolysis seems to be promising. However, there are physical–chemical, structural, and compositional factors that hinder the enzymatic digestibility of cellulose present in lignocellulose biomass. Barley straw, an important residue from grain industry in Spain, may be a promising substrate for microbial fermentation to ethanol. It contains about 35–40% cellulose, 20–30% hemicellulose, and 8–15% lignin. Currently, the use of cereal straw to produce fuel ethanol faces significant technical and economic challenges. Its success depends largely on the development of an environmentally friendly pretreatment procedure, highly effective enzyme systems for conversion of pretreated barley to fermentable sugars, and an efficient conversion of fermentable sugars to ethanol.

Pretreatment is required to alter the structure of cellulosic biomass making cellulose more accessible to enzymes that convert the carbohydrate polymers into fermentable sugars. The goal is to break the xylan–lignin matrix and disrupt the crystalline structure of cellulose *(4)*. Steam explosion (SE) has been proposed as an efficient pretreatment of lignocellulosic materials owing to its low use of chemicals, low energy consumption, and efficient biomass disruption characteristics for hardwoods and agricultural residues *(5)*. Moreover, it has been developed at commercial scale *(6)*. This pretreatment, based on the combined effect of steam and pressure release, disrupts the lignin barrier and enhances accessibility of cellulose fibers to enzymatic attack.

In addition to the physical barriers that constitute both hemicellulose and lignin, there are difficulties in the enzymatic hydrolysis step. Cellulases have a low specific activity *(7)*. Furthermore, the hydrolysis rate falls off sharply as the hydrolysis proceeds; thus, it is necessary to use great amounts of enzymes in this process. This adds to the higher production cost of the enzymes, making the enzymatic hydrolysis step a critical point in the global cost of ethanol production *(8)*. There are numerous factors that contribute to the reduction in the capacity of cellulose conversion by enzymes: those related to the structure of the substrate, and those related to the mechanisms and interactions of the cellulose enzymes *(9)*. Substrate structure, responsible for the accessibility and susceptibility to the enzymatic attack, is determined by the type and conditions of pretreatment. On the other hand, the nature of the enzymatic complex used and the proportion of each component, and therefore its susceptibility to enzyme product-based inhibition, are going to establish the way of interaction between cellulases and cellulose fiber. The enzymatic conversion of cellulose is a complex process involving the coordinated action of exo/endocellulases and cellobiases, in order to render glucose. On the other hand, enzymatic hydrolysis of the hemicellulose is essential to facilitate complete cellulose

degradation. As xylan is the major hemicellulose in barley straw, xylanase addition would render xylooligomers and xylose. Hence, a complete degradation of xylan-to-xylose would make the production of bioethanol from lignocelulosic materials more profitable, aiming at the possibility to ferment both glucose and xylose to ethanol.

The activities detected in most of the commercial enzymatic preparations are not often sufficient to obtain a complete conversion of the cellulose. The supplementation with greater β-glucosidase doses is essential to reduce cellobiose inhibition in the enzymatic hydrolysis step *(10)*. The use of accessory enzymes as hemicellulases and ligninases could be interesting for the conversion of lignocellulosic materials into monomeric sugars that can be transformed to ethanol by suitable microorganisms *(11)*. In this context, the study of new enzyme mixtures in the enzymatic hydrolysis step is relevant to improve the potentially fermentable sugar yields.

In the present study, different enzyme preparations available from Novozymes A/S (Bagsveard, Denmark) were assessed for their efficiency to hydrolyze lignocellulosic materials. First, these enzyme samples were analyzed for the amount of cellulase, cellobiase, and xylanase activities. Subsequently, the enzyme mixture was evaluated on a pretreated cellulose-rich material, and steam-exploded barley straw pretreated under different temperatures (190, 200, and 210°C, respectively) in order to produce fermentable sugars.

Materials and Methods

Feedstock Material

Barley straw (*Hordeum vulgare*, 6–8% moisture), supplied by Ecocarburantes de Castilla y León (Spain) was used as raw material. Biomass was coarsely crushed (to a particle size of about 10 mm) using a laboratory hammer mill (Retsch GmbH & Co. KG, Germany), homogenized and stored at room temperature until use. Raw material showed the following composition (dry weight [%]): 37.1 ± 1.3, glucans; 21.3 ± 0.5, xylans; 3.8 ± 0.4, arabinans; 1.2 ± 0.2, galactans; 16.9 acid-insoluble lignin, 2.3 ± 0.8 acid-soluble lignin; 1.8 + 0.01 acetyl groups; 15.4 extractives; and 8.2 ash. The composition of the raw material was determined using the standard laboratory analytical procedures for biomass analysis provided by the National Renewable Energy Laboratory (Colorado) *(12)*.

SE Pretreatment

Barley straw was pretreated in a small SE batch plant based on Mansonite technology, as described in a previous work *(13)*. The reactor was filled with 150.0 g (dry weight) of feedstocks per batch, and then heated to the desired temperature (190, 200, and 210°C, respectively) directly with saturated steam for 10 min. After explosion, the material was recovered in a cyclone. The slurry was cooled to about 40°C and then filtered

for water-insoluble solid (WIS) and liquid-fractions recovery. WIS fraction was thoroughly washed with water and dried at 45°C. The chemical composition of WIS was determined by National Renewable Energy Laboratory standard methods *(12)* and used in enzymatic hydrolysis tests.

Enzyme Preparations

Celluclast 1.5 FG, Novozym 188, Shearzyme, NS50013, NS50010, and NS50030 enzyme preparations were kindly provided by Novozymes. The three last preparations were contained in Novozymes Biomass Kit for conversion of lignocellulosic materials.

Measurement of Enzymes Activities

Enzyme preparations were subjected to standardized tests to determine protein content and main enzymes activities relevant in the conversion of lignocellulose: cellulase, cellobiose, and xylanase activities. Cellulase and β-glucosidase activities were measured according to methods described by Ghose *(14)*. Cellulase activity was defined in terms of filter paper units/milliliter and β-glucosidase as cellobiase unit/milliliter. Xylanase activity was quantified as described by Bailey et al. *(15)* using birchwood xylan (Sigma Aldrich Corp., St. Louis, MO) as substrate. One unit of xylanase activity is the amount of enzyme required to release 1 μmol of reducing sugars (xylose equivalents) per minute (U/mL).

Filter paper activity assay is the most usual measurement of the hydrolytic potential of a cellulase preparation because it allows the determination of the overall cellulase activity. Cellobiase (actually β-glucosidase) activity is responsible for the formation of glucose from cellobiose and its important role in cellulose degradation by relieving cellobiose inhibition is well known. Finally, xylanase (endo-1,4-β-D-xylanase activity) activity catalyzes the random hydrolysis of 1,4-β-D-xylosidic linkages in xylans. The protein content was determined by Bicinohoninic acid [BCA]™ assay (BCA-Compat-Able Protein Assay kit, ref. 23229, Pierce, Rockford, IL) using bovine serum albumin as protein standard.

Enzymatic Hydrolysis Experiments

Different ternary mixtures of enzyme preparations were studied on washed WIS samples after pretreatment: Cellulases (Celluclast 1.5L FG and NS50013): β-glucosidases (Novozym 188 and NS50010) and xylanases (Shearzyme and NS50030). Enzymatic hydrolysis experiments were performed in 250-mL Erlenmeyer flasks at 50°C and 150 rpm and at 5% (w/v) substrate loading in 0.05 *M* citrate buffer (pH 4.8). Enzyme loading of the different enzyme preparations is expressed as volume of enzyme preparation (E)/100.0 g substrate (washed-WIS) (S). Cellulases and xylanases were dosed at 5 and 10% (v/w) E/S, whereas β-glucosidase was dosed always at 1% (v/w) E/S (Table 1).

Table 1
Enzyme Loading in Different Experiments Tested

Enzyme mixture	Cellulase : β-glucosidase : xylanase	Enzyme loading %(v/w) E/S
A	Celluclast : Novozym : Shearzyme	10 : 1 : 0
B		10 : 1 : 5
C		10 : 1 : 10
D		5 : 1 : 5
E	NS50013 : NS50010 : NS50030	10 : 1 : 0
F		10 : 1 : 5
G		10 : 1 : 10
H		5 : 1 : 5

Samples were withdrawn from the hydrolysis media at 1, 3, 6, 12, 24, and 120 h. The samples were centrifuged at 12,000*g* for 10 min, and sugar concentration (glucose, cellobiose, and xylose) was determined by high-performance liquid chromatography *(16)*. All experiments were performed in duplicate. To compare the time-course of enzymatic hydrolysis of barley straw pretreated at different conditions two indices were calculated: specific conversion (SC) and mean specific rate (MSR). The SC is the percent of total cellulose hydrolyzed to glucose in 12 h/mg, normalized for protein content in 1 mL (%/mg). The MSR is the average of the cellulose hydrolysis rates for 0–1, 1–3, 3–6, and 6–12 h/mg, normalized for protein content in 1 mL (g glucose/L/h/mg). MSR and SC indices were calculated as described by Berlin et al. *(17)*.

The enzymatic hydrolysis yield was calculated as the concentration of the hydrolyzed cellulose (HC) divided by the cellulose content in the pretreated material and expressed as percentage. HC shows glucose and cellobiose content in the media, after applying weight adjustment for analyzed sugars. HC was calculated as follows:

$$HC = [Glu] \cdot 0.9 + [Cell] \cdot 0.95$$

where, [Glu] and [Cell] are the concentrations (g/L) of glucose and cellobiose in the media, respectively. Taking into account that substrate concentration in the enzymatic hydrolysis test was 5% (w/v).

Results and Discussion

SE Pretreatment

The composition of WIS fraction of pretreated barley straw (dry weight [%]) is shown in Table 2. After SE pretreatment solid recovery (expressed as WIS remaining after pretreatment divided by 100.0 g of raw material) was about 56%. Reduction in WIS was the result of solubilization

Table 2
Composition of WIS Obtained After SE Pretreatment of Barley Straw
(Dry Weight [%])

Pretreatment conditions	Glucans (%)	Xylans (%)	Acid-insoluble lignin (%)	Ash (%)
190°C, 10 min	58.8 ± 1.1	11.8 ± 1.0	23.7 ± 1.0	7.8 ± 0.4
200°C, 10 min	64.5 ± 1.4	6.3 ± 0.9	24.8 ± 1.2	8.4 ± 1.1
210°C, 10 min	62.3 ± 1.4	3.8 ± 0.9	25.4 ± 1.5	9.4 ± 0.7

Main value + sd.

and/or degradation of hemicellulose and extractives. After SE, the biomass composition changed because of the thermal degradation mainly of the hemicellulose component. Cellulose content in WIS increased in relation to untreated material (37.1%) ranging from 58.8 to 64.5%, depending on the pretreatment conditions. The greatest cellulose content (64.5%) was obtained at 200°C. High temperature pretreatment (210°C) produced lower cellulose content in the pretreated material owing to slight cellulose solubilization. Acid-insoluble lignin was considerably concentrated in comparison with raw material (16.9%), reaching values up to 25.4% at the most severe conditions. At increased pretreatment severity, a decrease in the hemicellulose content was also observed. The chemical composition confirmed that the matter loss primarily occurs at the expense of hemicellulose, the component being more thermally degradable. Glucan and lignin contents in all pretreated substrates were quite similar, and their composition mainly differs on xylan content, which varied from 11.8 to 3.8% depending on pretreatment conditions.

Enzyme Activity Assays

Table 3 presents enzyme activities for the different enzyme preparations used in this work. NS50013, a cellulase complex, and Celluclast 1.5 L FG showed the highest values of filter paper activity (cellulase). In addition, both enzymes presented xylanase activity, being higher in NS50013. This fact is important in enzymatic hydrolysis of lignocellulosic materials because xylans are coating the cellulose fibrils, and therefore, hindering the accessibility of cellulose for cellulases. Xylanase was the highest enzyme activity in Shearzyme, although it also shows some cellulase activity. NS50030 preparation shows the highest xylanase activity (3760.0 IU/mL). In fact, this preparation is a purified endoxylanase and regarding information supplied by Novozymes A/S, it shows high specificity toward the soluble pentosan fraction in wheat.

Enzymatic Hydrolysis of Steam-Exploded Barley Straw

The influence of using different enzyme mixtures has been studied on enzymatic hydrolysis of WIS from pretreated barley straw samples, which

Table 3
Enzyme Activities in Enzymes Preparations

Enzyme preparations	Protein concentration (mg/mL)	Enzyme activities (U/mL)		
		FPA	β-Glucosidase	Xylanase
Celluclast 1.5 L FG	151.0	65.0	12.0	660.0
NS50013	138.0	63.0	8.0	1117.0
Novozym 188	83.0	n.d.	664.0	69.0
NS50010	141.0	n.d.	992.0	124.0
Shearzyme	71.0	27.0	5.0	2293.0
NS50030	21.0	n.d.	1.0	3760.0

n.d. not detected; FPA, filter paper unit.

mainly differ on their xylan content. Six enzyme mixtures tested in this study (B–D and F–H) consist of cellulases (Celluclast 1.5 L FG or NS50013), β-glucosidases (Novozym 188 or NS50010), and xylanases (Shearzyme or NS50030) preparations. Besides, two binary systems (A and E) consisting only of cellulase and β-glucosidase preparations were studied. β-glucosidase was added in all mixtures studied, because as is well known in literature, β-glucosidase can reduce the inhibiting effect of cellobiose. A reference enzyme mixture (Celluclast 1.5 L FG and Novozym 188) was selected as control. Table 4 shows enzymatic activities for the different enzyme mixtures studied, expressed as unit per milliliter in the hydrolysis test media.

Hydrolysis of steam-exploded barley straw at 5% (w/v) substrate loading by tested enzymes mixtures is shown in Figs. 1–3. Enzymatic hydrolysis followed the same pattern in all experiments. For all pretreated samples, the xylanase addition improved the glucose production in enzymatic hydrolysis. The highest glucose production during the first hour of hydrolysis was found in barley straw pretreated at 210°C. It is worth mentioning that increases in glucose production obtained with the different mixtures compared with control, were always higher in this substrate (up to 7.0 g/L at 12 h of hydrolysis at 210°C with mixtures F and G), in spite of its low xylan content (3.7%). Although the xylan content of the pretreated samples is low, xylanases may significantly increase the accessibility of cellulose to cellulases by removing hemicellulose, including material redeposited on the fibers during pretreatment *(17)*. Increases were less evident as substrate was pretreated at lower temperatures (up to 5.0 g/L at 12 h of hydrolysis at 190°C with mixtures F and G). Similar glucose production was obtained for control and enzyme mixtures D and H (which contained half-cellulase loading with respect to the control and 5% [v/w] of xylanases).

Supplementation with xylanase activity (up to a certain level of 16.0 U/mL) improved glucose production in enzymatic hydrolysis by using enzymes mixtures with similar to both filter paper and β-glucosidase

Table 4
Enzyme Activity and Protein Levels Presents in Hydrolysis Media for Different Enzyme Mixtures Used

Enzymatic mixture	Cellulase : β-glucosidase : Xylanase	Enzyme loading %(v/w) E/S	Cellulase (filter paper units/mL)	β-glucosidase (U/mL)	Xylanase (U/mL)	Protein (mg/g substrate)
A	Celluclast : Novozym : Shearzyme	10 : 1 : 0	0.35	0.42	3.6	17.0
B		10 : 1 : 5	0.42	0.43	9.7	20.8
C		10 : 1 : 10	0.49	0.44	15.6	24.6
D		5 : 1 : 5	0.25	0.40	7.9	12.8
E	NS50013 : NS50010 : NS50030	10 : 1 : 0	0.33	0.57	6.0	16.2
F		10 : 1 : 5	0.33	0.57	16.0	17.2
G		10 : 1 : 10	0.34	0.58	26.1	18.4
H		5 : 1 : 5	0.17	0.55	13.1	10.0

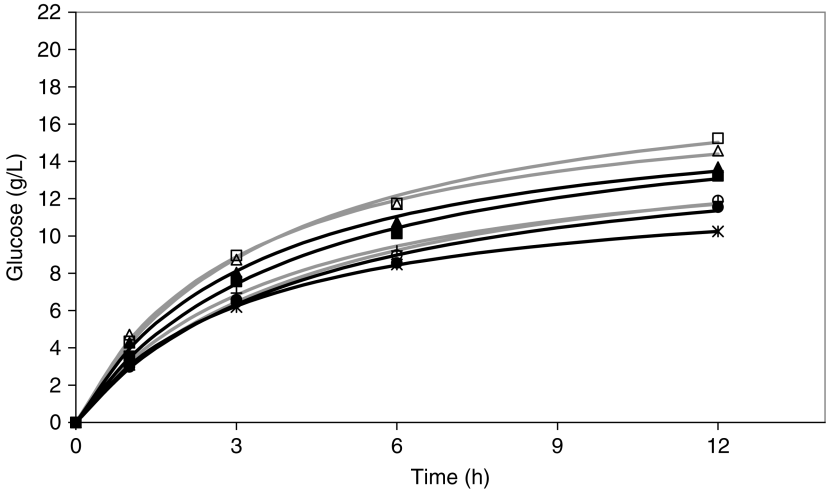

Fig. 1. Hydrolysis of WIS fraction of steam-exploded barley straw (190°C 10 min) by different enzymes mixtures. Mixtures A (—*—), B (—■—), C (—▲—), and D (—●—) cellulose : β-glucosidase : xylanase = Celluclast FG 1.5L : Novozym 188 : Shearzyme. Mixtures E (—+—), F (—□—), G (—△—), H (—○—) cellulase : β-glucosidase : xylanase = NS50013 : NS50010 : NS500.

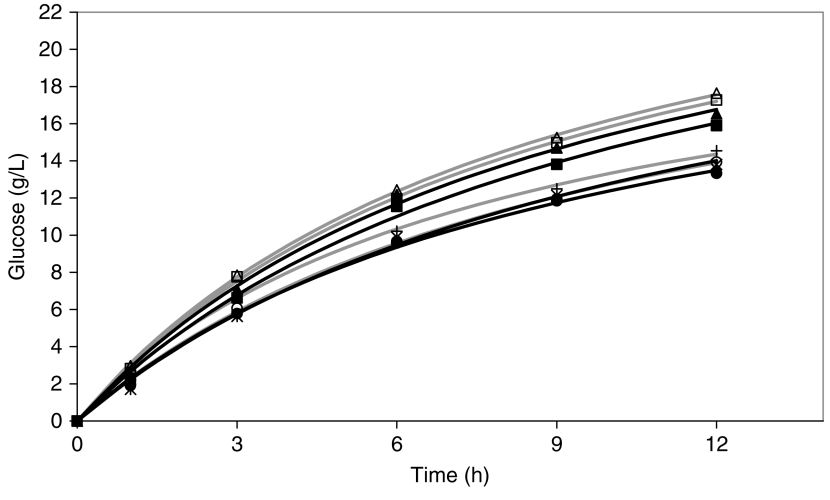

Fig. 2. Hydrolysis of WIS fraction of steam-exploded barley straw (200°C 10 min) by different enzymes mixtures. Mixtures A (—*—), B (—■—), C (—▲—), and D (—●—) cellulase : β-glucosidase : xylanase = Celluclast FG 1.5L : Novozym 188 : Shearzyme. Mixtures E (—+—), F (—□—), G (—△—), H (—○—) cellulase : β-glucosidase : xylanase = NS50013 : NS50010 : NS50030.

activities (A and E–G). This fact suggests that filter paper, although commonly used as index of cellulose performance, does not provide a reliable indication of the ability of a preparation to hydrolyze complex lignocellulosic substrates. This fact has been recently indicated by other authors *(18,19)*.

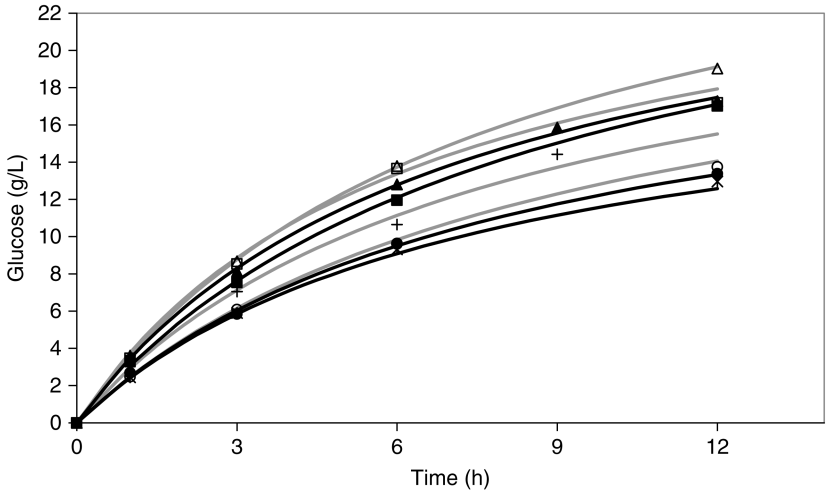

Fig. 3. Hydrolysis of WIS fraction of steam-exploded barley straw (210°C 10 min) by different enzymes mixtures. Mixtures A (—*—), B (—■—), C (—▲—), and D (—●—) cellulase : β-glucosidase : xylanase = Celluclast FG 1.5L : Novozym 188 : Shearzyme. Mixtures E (—+—), F (—□—), G (—△—), and H (—○—) cellulase : β-glucosidase : xylanase = NS50013 : NS50010 : NS50030.

The multiple parameters that influence hydrolysis of heterogeneous lignocellulosic substrates (e.g., crystallinity, lignin/hemicellulose content and distribution, and available surface area) result in complex reaction kinetics. In order to compare the performance of different cellulase mixtures on enzymatic hydrolysis of pretreated barley straw substrate, two indices, MSR and SC, were determined as proposed by Berlin et al. *(17)* (Table 5). MSR index provides an estimate of the average reaction rate over the first 12 h of hydrolysis and SC index describes the percentage of the total cellulose in the sample hydrolyzed at 12 h incubation period. Differences in the performance of the various enzyme mixtures tested have been found. MSR indices were similar for all pretreated substrate samples in each enzyme mixture. As aforementioned, higher values for SC index were obtained in the enzymatic hydrolysis of the substrate pretreated at 210°C.

Mixtures D and H provided the highest values of MSR and SC because of their lower protein loadings. Considering enzyme mixtures with similar protein contents (A, E, and F), better results were achieved with mixture F, which includes preparations from the Novozymes Biomass Kit for conversion of lignocellulosic materials. Data obtained using F mixture shows a significant improvement when compared with the control (mixture A), which includes Celluclast and Novozym 188 that are generally used in research studies of enzymatic hydrolysis of lignocellulosic materials.

Table 5
MSR and SCs for Hydrolysis of WIS Steam-Exploded Barley Straw by Different Mixtures Enzymes

Enzyme mixture	190°C 10 min		200°C 10 min		210°C 10 min	
	MSR	SC	MSR	SC	MSR	SC
A	1.8	40.8	1.8	51.2	1.7	52.0
B	1.7	42.8	1.7	52.8	1.9	54.0
C	1.6	37.2	1.5	46.4	1.7	63.6
D	2.4	60.4	2.3	65.6	2.4	69.2
E	2.0	49.2	2.1	58.8	2.2	64.0
F	2.5	59.6	2.3	65.2	2.4	67.6
G	2.3	53.2	2.2	63.6	2.4	70.4
H	3.1	80.0	3.1	91.2	3.2	92.8

Table 6
Enzymatic Hydrolysis Yield (%) at 24 h and 120 h of WIS Steam-Exploded Barley Straw by Various Enzyme Mixtures

Enzyme mixture	190°C 10 min		200°C 10 min		210°C 10 min	
	24 h	120 h	24 h	120 h	24 h	120 h
A	47.2	54.4	60.6	74.1	58.2	85.7
B	54.8	64.5	69.8	84.4	75.6	97.0
C	56.5	66.9	70.9	84.4	73.9	98.8
D	50.1	60.4	59.8	78.0	62.6	97.7
E	47.5	55.2	57.5	77.2	68.7	94.1
F	61.2	71.5	70.0	85.0	84.7	100
G	60.2	71.3	70.3	82.7	84.5	98.1
H	52.0	68.3	53.5	81.8	66.5	98.2

Prolonged Enzymatic Hydrolysis

Table 6 shows results for cellulose conversion obtained after 24 and 120 h hydrolysis time. Enzymatic hydrolysis yield (expressed as percentage) was calculated as the concentration of the HC divided by the cellulose content in the pretreated substrate. Increased conversions of cellulose to glucose in comparison with the control were obtained in experiments supplemented with xylanases. Comparing mixtures A and D similar cellulose hydrolysis yields at 24 and 120 h were found. This finding is important because in mixture D reduced amounts of cellulase and increased amounts of xylanase are used. Likewise, if mixtures E and H are compared, similar cellulose hydrolysis yields could be attained using reduced cellulase loading and increased xylanase loading. Higher enzymatic hydrolysis yields

were obtained with enzyme mixture F. After 24 h hydrolysis time, the cellulose hydrolysis yield of steam barley straw pretreated at 210°C increased by 45% when using this mixture in comparison with the control (mixture A). In these conditions the increase of xylan hydrolysis yield was by 50%. Complete enzymatic hydrolysis of barley straw pretreated at 210°C was obtained for all enzyme mixtures tested (excluded the control). Regarding results obtained in this work, the ternary mixture of NS50013 : NS50010 : NS50030 at 10 : 1 : 5% ([v/w] E/S) seems to be the most suitable for the cellulose hydrolysis of pretreated barley straw.

Concluding Remarks

The increase of the accesibility of cellulose to cellulases by the addition of purified endoxylanases has been demonstrated in this work. Although cellulase preparations normally cited in literature include some xylanase activity, xylanase supplementation could maximize enzymatic hydrolysis yield. This fact should be considered when using tailor-made enzyme complex to increase fermentable sugar yield in the hydrolysis step. In this work, xylanase supplementation improved cellulose hydrolysis effectiveness of pretreated barley straw samples, regardless of the xylan content of substrate. The interaction among different enzymatic activities presented in cellulase complex should be further studied.

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