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Effect of Inhibitors Released During Steam-Explosion Pretreatment of Barley Straw on Enzymatic Hydrolysis

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Abstract

The influence of the liquid fraction (prehydrolysate) generated during steam-explosion pretreatment (210°C, 15 min) of barley straw on the enzymatic hydrolysis was determined. Prehydrolysate was analyzed for degradation compounds and sugars' content and used as a medium for enzymatic hydrolysis tests after pH adjusting to 4.8. Our results show that the presence of the compounds contained in the prehydrolysate strongly affects the hydrolysis step (a 25% decrease in cellulose conversion compared with control). Sugars are shown to be more potent inhibitors of enzymatic hydrolysis than degradation products.

Index Entries: Enzymatic hydrolysis; barley straw; inhibition; steam explosion.

Introduction

Current research and development on bioethanol production are being directed toward substituting higher-cost sugars and starch feedstocks with lower-cost lignocellulosic biomass as a way of reducing the cost of ethanol. Barley straw, an important residue from grain industry in Spain, is a promising substrate for microbial ethanol production.

Among biomass-to-ethanol processes, those based on enzymatic hydrolysis make out to be promising. However, there are physicochemical structural, and compositional factors that hinder the enzymatic digestibility of cellulose present in lignocellulose biomass. Unlike starch, which contains homogeneous and easily hydrolysed polymers, lignocellulose plant matter contains cellulose, hemicellulose, polyphenolic lignin, and other extractable components. These complex polymers must be broken down into low-molecular-weight sugars before microorganisms can complete the conversion. The native cellulose fraction of lignocellulosic biomass is recalcitrant

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to enzymatic hydrolysis; so a pretreatment step is required to obtain high cellulose-to-glucose bioconversion yields.

Steam explosion is one of the most attractive pretreatment processes owing to its low use of chemicals and energy consumption *(1)*. Steam explosion disrupts the lignin barrier and make cellulose more available to enzymatic attack by removing hemicellulose in order to increase the accessible surface area *(2)*. In spite of these large advantages, there are also some limitations: steam explosion pretreatment, at least partially, degrades hemicellulose-derived sugars and solubilizes and transforms the lignin compounds to chemicals that can inhibit downstream process *(3)*. It is also probable that the solubilization of extractives during the pretreatment step produce potent inhibitors in low concentrations.

Several authors have investigated the nature of the inhibitors present in diluted acid hydrolysates and steam explosion pretreated biomass *(4–8)*. The nature and concentration of the final inhibitory compounds vary greatly with the pretreatment conditions (severity factor that consider both temperature and residence time), the raw material used (hardwood, softwood, or herbaceous plants) and the presence of acid catalyst. These inhibitors can be classified according to their chemical structure. They include weak acids (mainly acetic acid), furans (degradation product of hemicellulose sugars such as furfural, dehydration product of pentoses; and 5-hydroxymethylfurfural [HMF], a dehydration product of hexoses), and phenolic compounds from lignin (aromatic acids, alcohols such as catechol, and aldehydes such as 4-hydroxybenzaldehyde and vanillin). Some of the compounds formed during the degradation of hemicellulose and lignin, contaminate the water-phase product of the steam-explosion process, whereas others become embedded in the biomass and are released during successive bioconversion *(9)*. Such inhibitors can affect enzymes in the hydrolysis step, reduce glucose conversion during fermentation, and depress the rate of ethanol formation at the end of the biomass-to-ethanol process *(10)*. Consequently, the pretreated material should be filtered and washed to remove them. In fact, in most investigations, the slurry obtained after pretreatment is separated in a solid fraction (cellulose and lignin) and a liquid fraction or prehydrolysate (hemicellulose-derived sugars, sugar and lignin degradation products, acetic acid, and other compounds), and washed before enzymatic hydrolysis *(10)*. However, from an economical and environmental point of view, it is preferable to include the prehydrolysate in the enzymatic hydrolysis step because it increases the concentration of fermentable sugars, and potentially provide a higher ethanol concentration in the fermentation step. The handling of pretreated material is facilitated and the capital cost of filtration and washing steps can be excluded. The high concentration of inhibitors in fermentation with high dry matter content might be overcome by applying a fed-batch technique in simultaneous saccharification and fermentation process *(11)*.

Although a considerable amount of research has been carried out to study the effect of toxic components produced during pretreatment in both enzymatic and fermentation steps of hardwood and softwood *(9–13)*, scarce references have been found in relation to agricultural residues such as barley straw.

The aim of this study is to investigate the influence of inhibitory compounds present in the liquid fraction (prehydrolysate) obtained after steam-explosion pretreatment of barley straw on cellulose conversion in the enzymatic hydrolysis step. It could reduce water consumption and residual water generated in an industrial scale process, as well as to enhance the overall sugar concentration in the hydrolysate before conversion to ethanol.

The pretreatment conditions, selected in a previous work, were 210°C and 5 min. After pretreatment the slurry was fractionated in a solid fraction and prehydrolysate by filtration. This prehydrolysate was analyzed for degradation compounds and sugars' content, and was used as a medium for enzymatic hydrolysis tests of the solid fraction. The effect of the two main fractions of the prehydrolysate, divided into hemicellulosic-derived sugars and degradation products, on cellulose conversion was also measured.

Materials and Methods

Biomass Pretreatment

Ground barley straw biomass (5% moisture) was supplied by Biocarburantes de Castilla y León (Salamanca, Spain). The composition of barley straw was 33% glucan, 20% xylan, 3.8% arabinan, 1% galactan, 16.1% lignin, 7.6% ash, and 13.8% extractives. The barley straw was pretreated in a small batch plant described in a previous work *(14)*. After pretreatment, the material was recovered in a cyclone, and the slurry (solid/liquid fraction about 1/10 w/v) was cooled to about 40°C and then filtered for solid and liquid fraction (prehydrolysate) recovery. The solids fraction was thoroughly washed with water and dried at 45°C. Pretreatment conditions (210°C, 5 min) had been previously selected as the most adequate in terms of hemicellulose-derived sugar recovery in the liquid fraction, cellulose recovery in the solid fraction, and enzymatic hydrolysis yield.

The composition of native and steam-explosion pretreated biomass was determined by using standard methods developed at the National Renewable Energy Laboratory (NREL) *(15)*. The prehydrolysate was analyzed regarding solubilized sugars and potentially inhibiting compounds (degradation products).

Enzymatic Hydrolysis Experiments

Enzymatic hydrolysis (EH) experiments were performed in 250-mL Erlenmeyer flasks containing 100 mL of 0.05 *M* citrate buffer (pH 4.8) at

5% w/v substrate (steam-exploded barley straw) loading, 50°C, 150 rpm, and 168 h. Periodically, 2.5-mL samples of the hydrolysis media were withdrawn and centrifuged at 9300*g* for 10 min. Sugar content was analyzed by high-performance liquid chromatography (HPLC) as decribed later.

The enzyme mixture of Celluclast 1.5L FG at 15 FPU (filter paper unit)/g cellulose and Novozym 188 at 15 IU β-glucosidase/g cellulose was employed. Enzymes were supplied by Novozymes A/S (Bagsvaerd, Denmark). Celluclast 1.5 L FG is provided as a liquid with a density of 1.2 g/mL; measured enzyme activities were 80 FPU/mL and 1.4 UI (β-glucosidase/mL. Novozyme 188 (β-glucosidase) has a density of 1.18 g/mL and β-glucosidase activity of 700 UI/mL. Measurement of the enzyme activities was performed as recommended by the International Union of Pure Applied Chemistry *(16)*.

To test the effect of compounds produced during steam explosion pretreatment on enzymatic hydrolysis, experiments using the prehydrolysate (previously adjusted at pH 4.8) as enzymatic hydrolysis broth were carried out. Original prehydrolysate, twofold concentrate prehydrolysate, and 1 : 1 diluted prehydrolysate (DP) were used.

To study the effect of sugars and degradation compounds produced in steam explosion pretreatment on enzymatic hydrolysis, enzymatic hydrolysis experiments were performed in synthetic solutions (in buffer citrate 0.05 *M*) containing sugars or degradation products: (i) in concentration as in original prehydrolysate and (ii) in twofold concentration of original prehydrolysate.

Determination of the Inhibition Degree Caused by Sugar Monomers on Enzymatic Hydrolysis

Determination of inhibition was based on the ratio of the hydrolysis rates with and without the presence of supplemented sugars (glucose, xylose, arabinose, galactose, or mannose). Inhibition degree was expressed as V_I/V, in which V_I is the amount of glucose (g) produced from the substrate in the presence of supplemented sugars per 30 min, and V is the amount of glucose (g) produced from the substrate without sugar supplementation per 30 min. The hydrolysis rates were determined after 30 min of hydrolysis. The short hydrolysis period was selected to minimize the inhibitory effects of the released sugars *(17)*.

Analytical Procedures

Analysis of HMF, furfural, vanillin, syringaldehyde, 4-hydroxybenzaldehyde, catechol, guaiacol, 4-hydroxybenzoic acid, syringic acid, vanillic acid, ferulic acid, coumaric acid, acetic acid, levulinic acid, and formic acid analysis were performed on a HPLC system as described previously *(5)*.

The carbohydrate content of the liquid fraction after pretreatment was determined by performing a mild acid hydrolysis (3% [v/v] H_2SO_4 for

Table 1
Composition of Liquid Fraction Obtained After Steam Explosion Pretreatment of Barley Straw

Compound	Concentration (g/L)	g/100 g Raw material	Compound	Concentration (mg/L)	mg/100 g Raw material
Xylose	17.4	7.1	4-Hydroxybenzaldehyde	7	2.8
Glucose	4.6	1.9	4-Hydroxyybenzoic acid	4	1.6
Arabinose	1.9	0.8	Catechol	42	17
Galactose	1.3	0.5	Syringaldehyde	31	13
Acetic acid	2.1	0.9	Syringic acid	6	2.4
Formic acid	0.8	0.33	Vanillin	63	25
Furfural	0.7	0.28	Vanillic acid	11	4.4
HMF	0.2	0.08	Ferulic acid	25	10
			Coumaric acid	44	18

120°C and 30 min) and measuring glucose, xylose, arabinose, galactose, and mannose concentration by Waters HPLC in a refractive index detector. Sugars released during enzymatic hydrolysis were also measured by HPLC as above (*18*).

Chemicals

All chemicals were of analytical grade and obtained from Sigma (St. Louis, MO).

Results and Discussion

Characterization of the Exploded Barley Straw and Composition of Hydrolysate

The composition of solid fraction obtained after steam explosion of barley straw was 60% glucan, 4.7% xylan, 1.4% arabinan, and 30% lignin. After the steam explosion (SE), the biomass composition changes because of the thermal degradation, mainly of the hemicellulose components. The chemical composition confirms that the matter loss primarily occurs at the expense of the hemicellulose, being the component more thermally degradable. Barley straw pretreatment resulted in a solid fraction enriched in cellulose (60%) and lignin (30%).

The sugar composition (expressed in g/L) as well as the degradation compounds from the liquid fraction are shown in Table 1. Results are also expressed as g/100 raw material. Using saturated water steam at high temperature, SE causes autohydrolysis reactions in which part of hemicellulose and lignin are converted into soluble compounds. The prehydrolysate of steam exploded barley straw consisted of a mixture of hydrolysable sugars

(25.2 g/L) and degradation products, for example, carboxylic acids (2.95 g/L), phenols (0.23 g/L), and furans (0.89 g/L). Regarding carbohydrates the major sugar released was the xylose being in a concentration of 17 g/L.

All degradation products that were found in prehydrolysate obtained from steam explosion pretreatment of barley straw biomass have been previously identified in other herbaceous biomass *(19)*. Acetic acid (2.14 g/L), formic acid (0.81 g/L), and furfural (0.69 g/L), from pentose degradation, were the main degradation products present in the prehydrolysate. Acetic acid from hydrolysis of hemicellulose and furfural from degradation of xylose were obtained as a consequence of the high xylan content in herbaceous biomass. The quantification of furfural can hardly explain hemicellulose losses during pretreatment. It is likely that hemicellulose were lost through volatilization of furfural. Formic acid is a product from sugar degradation *(13)*.

The presence of cinnamic acids reported to be present of herbaceous angiosperms is remarkable. The *p*-coumaric and the ferulic acids are major noncore lignin monomers that link hemicelluloses and core lignin *(20)*. It is worth to notice that vanillin and vanillic acid concentration, both formed by degradation of guaiacyl propane (G) units of lignin, are significantly higher than syringaldehyde and syringic acid, both produced by degradation of syringylpropane (S) units of lignin. This fact is consistent with the G/S ratio in herbaceous biomass *(13)*.

Effect of Prehydrolysate on the Enzymatic Hydrolysis

An efficient utilization of the water-soluble hemicellulose components is required to make the biorefinery approach feasible. Previous research has indicated that the biomass-to-ethanol process could be more economical by incorporating hemicellulose rich water-soluble fraction to the enzymatic hydrolysis of the solid fraction *(10)*. The influence of the prehydrolysate on the enzymatic hydrolysis of steam exploded barley straw was investigated. In order to test the effect of the prehydrolysate at different concentrations, three cases have been considered: (i) using the original prehydrolysate (P) obtained in the CIEMAT pilot plant (whose composition is shown in Table 1), (ii) using a 1 : 1 (v/v) diluted prehydrolysate (DP), and (iii) using twofold concentrated prehydrolysate (CP) considering that in a commercial plant the slurry produced would have increased solid content with consequent high loading of inhibitors. The time-course of sugar production was monitored and cellulose conversion determined. Cellulose conversion was calculated based on the amount of cellulose supplied to enzymatic hydrolysis step, which is converted into glucose. The effect of prehydrolysate on the cellulose conversion in the enzymatic hydrolysis step is shown in Fig. 1. The highest cellulose conversion (88% at 168 h) was obtained when the enzymatic hydrolysis of the steam-exploded biomass was assayed on citrate buffer (C). A decrease in cellulose conversion was observed in experiments using prehydrolysate instead of buffer as EH medium. When

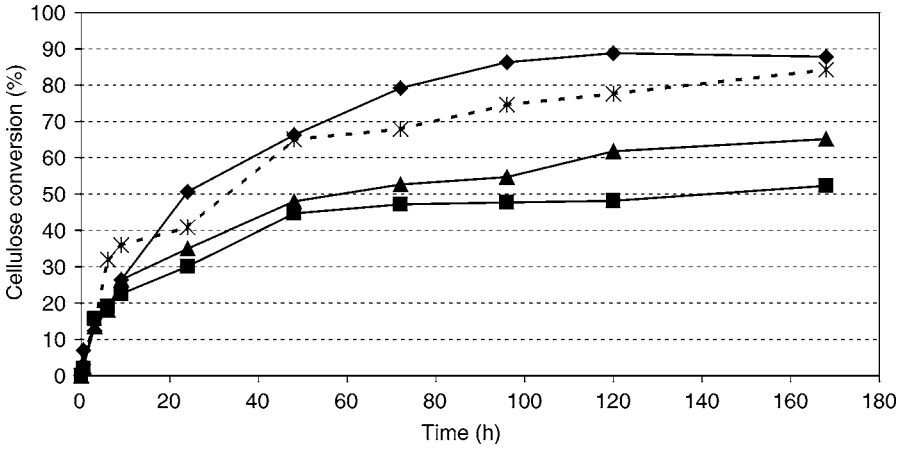

Fig. 1. Effect of the prehydrolysate on cellulose conversion (%) of solid fraction of steam-exploded barley straw at 5% loading (w/v). C (◆), OP (▲), CP (■), and DP (✳).

twofold CP was used, the lowest conversion was obtained (52% at 168 h), corresponding to 59% of the cellulose conversion obtained respect to the control (C). When the original hydrolysate (P) obtained after pretreatment was used as enzymatic hydrolysis medium, a reduction of 25% in the cellulose conversion occurred. The diluted hydrolysate produced 17% and 10% decrease with respect to the control at 48 h and at the end of the hydrolysis step, respectively. Similar findings were obtained by other authors *(10)* who reported cellulose conversion reduction up to 36% when a prehydrolysate of spruce impregnated with SO_2 and steam pretreated at 215°C for 3 min was used in EH.

As previously stated, the purpose of including the prehydrolysate in the enzymatic hydrolysis was also to enhance the overall sugar concentration before conversion to ethanol. When comparing with the control, the total sugar concentrations measured (glucose, cellobiose, and xylose) present in supplemented hydrolysates were higher. As expected, the higher total sugar concentrations in the enzymatic hydrolysis media was obtained by supplementing with the twofold concentrated hydrolysate. At these conditions, a concentration of 50 g/L after 96 h of hydrolysis was obtained. Results from the original liquid fraction and the diluted liquid fraction were quite similar (37 g/L), whereas when enzymatic hydrolysis was carried out in buffer (control) the concentration of sugars obtained was 30g/L. The proportion of different sugars present in the hydrolysate obtained by enzymatic hydrolysis was different. When the original filtrate was used as enzymatic hydrolysis medium the glucose/xylose ratio was 1.6, whereas with diluted prehydrolysate the ratio was 2.6 at 96 h.

Celluclast 1.5 L contains cellulase as the main activity, but also gives high xylose yields. Xylose was released from both prehydrolysate and steam exploded solids, which is consistent with the fact that Celluclast

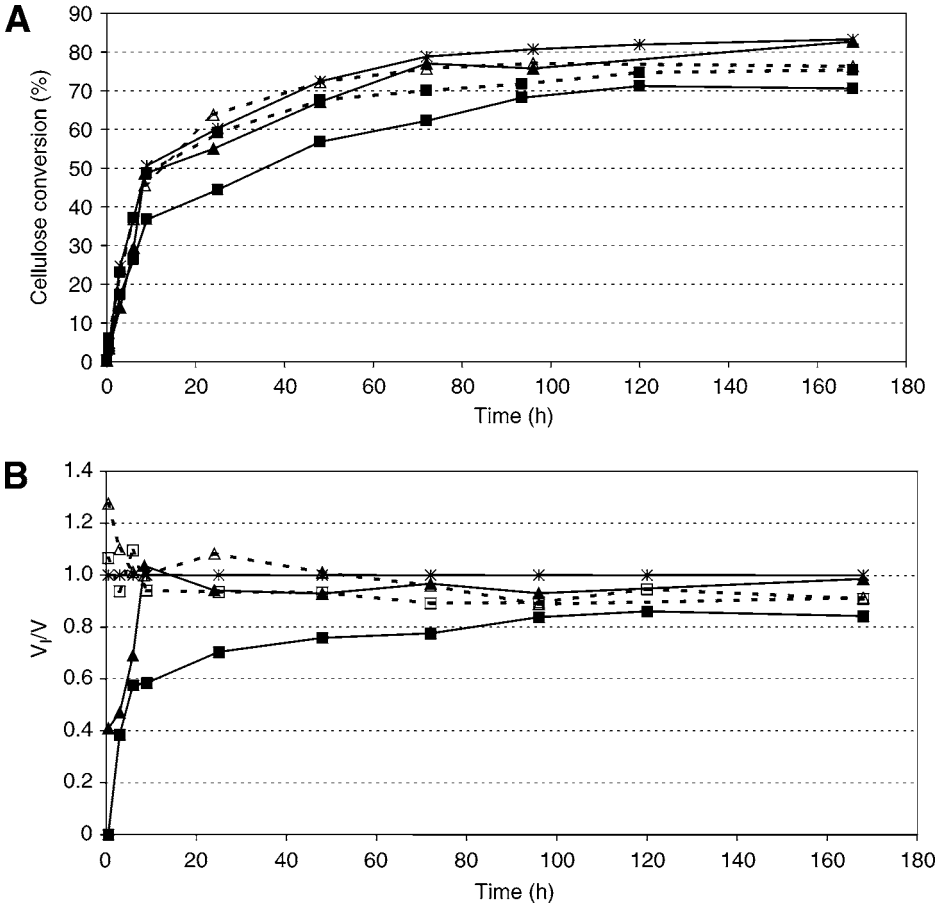

Fig. 2. Effect of supplementation of sugars and degradation product on **(A)** cellulose conversion percentage and **(B)** degree of inhibition (V_I/V) on hydrolysis of 5% steam-exploded barley straw: control (—✳—), twofold (—■—), and original (—▲—) sugars' concentration solution, and twofold (- -□- -) and original (- -Δ- -) degradation products.

1.5 L has also β-xylosidase activities, capable of catalyzing the hydrolysis of xylobiose and xylotriose to xylose *(21)*. This is an advantageous feature from the point of view of utilizing all sugars present in biomass. Recently, several strains of yeasts have been genetically engineered to effectively coferment glucose and xylose in hydrolysates from different cellulosic biomass to ethanol *(22)*.

Effect of Sugars and Degradation Compounds on the Enzymatic Hydrolysis

To distinguish the effect of sugars present in the prehydrolysate from the effect of other substances (degradation product), four experiments were performed adding to a buffer solution, separately, sugars and degradation products (both at the same concentration as found in original liquid and at twofold concentration). Figure 2A shows the results of the influence

of the two fractions (sugars and degradation compounds) in the enzymatic hydrolysis. The sugar fraction was shown to have a greater inhibitory effect on enzymatic hydrolysis than did the degradation compounds. Cellulose conversion from the sugar-supplemented hydrolysis was lower than the control (without any sugar addition) over 120-h incubation time. The presence of hemicelluloses derived sugars at the twofold concentration of the original hydrolysate, decreased the cellulose conversion by 15% at the end of hydrolysis step (Fig. 2A). The degradation products were responsible for a minor part of the inhibition of enzymatic hydrolysis. However, the decrease observed in the original prehydrolysate was higher than the sum of the effect from the supplemented fraction (sugars and degradation product), which could be owing to another component not identified or the synergistic inhibition by the inhibitors.

The degree of inhibition (calculated as the ratio of the hydrolysis rates with and without the presence of supplemented sugars or degradation product), measured over the hydrolysis period, is shown in Fig. 2B. When media were supplemented with degradation products, a slight inhibitory effect was observed. The presence of hemicellulosic-derived sugars at the same concentration as the original prehydrolysate decreased the hydrolysis rate (in the first 3 h of hydrolysis) by 53% and 60% at twofold concentration. As the hydrolysis proceeded, digestion curves approached the control levels. This is probably because the inhibitory effects of the glucose during enzymatic hydrolysis surpassed the inhibitory effect of the supplemented sugars *(17)*.

A higher accumulation of cellobiose during the first 9 h of hydrolysis was observed when the sugar fraction was added to the run hydrolysis (Fig. 3). These results indicate that sugars may play an important role in inhibiting β-glucosidase in the early phase of hydrolysis step.

Degree of Inhibition on Enzymatic Hydrolysis by Monosaccharides

From the previous results, it can be deduced that the sugar fraction has a greater influence in the diminution of the cellulose conversion. Inhibitory effects of glucose, xylose, galactose, and arabinose as the major monosaccharides formed in the liquid fraction of steam-exploded herbaceous biomass were determined. Mannose has also been included although it was not found in barley straw composition. The degree of inhibition was studied supplementing glucose and xylose at 0–100 g/L and galactose, arabinose, and mannose at 0–30 g/L to hydrolysis broth in experiments with 10% (w/v) barley steam exploded as substrate.

As expected, the addition of glucose resulted in on the inhibition of the hydrolysis rate. Hydrolysis rates decreased by 80% after supplementation with glucose at 15 g/L. This effect is well documented in the literature. Xiao et al. *(17)*, performing studies of degree of sugar inhibition using Avicel as substrate and supplementing with 100 g/L of glucose, found an 80% of reduction of cellulase activity. Hemicellulose-derived sugars have

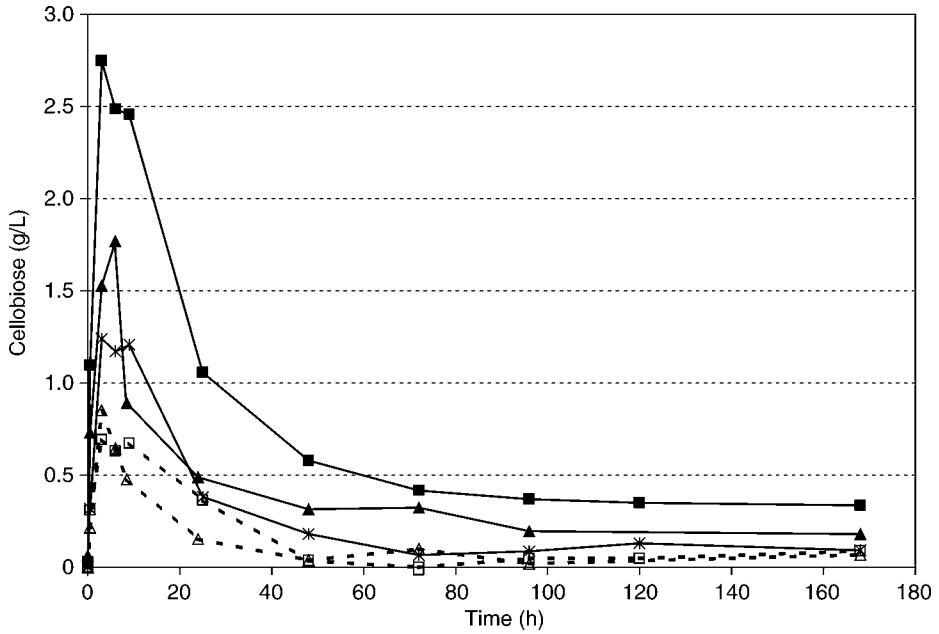

Fig. 3. Effect of supplementation of sugars and degradation product on cellobiose release in the enzymatic hydrolysis of 5% steam-exploded barley straw: (—✳—) control, (—■—) twofold and (—▲—) original sugars' concentration solution, (- -□- -) twofold and (- -Δ- -) original degradation products.

also been shown to have a direct inhibitory effect on the cellulase enzymes although less significant than glucose. Hydrolysis rates decrease by 35%, 13%, 11.5%, and 5% after supplementation with 20 g/L of xylose, arabinose, galactose, and mannose, respectively.

Conclusions

The presence of the compounds contained in the prehydrolysate from steam explosion of barley straw strongly affects the enzymatic hydrolysis of washed solid fraction from pretreatment. In enzymatic hydrolysis experiments performed in media supplemented with prehydrolysate at different concentrations a decrease in the cellulose conversion of 25% and 40% was obtained with original and twofold CP, respectively, compared with control tests.

Enzymatic hydrolysis conducted in media supplemented with the two major components contained in prehydrolysate (hemicellulose derived sugars and degradation compounds) showed that sugars were more potent inhibitors of enzymatic hydrolysis than degradations products. The presence of hemicellulose-derived sugars, at the same concentration than the prehydrolysate, decreases the hydrolysis rate by 53% in the first 3 h in comparison to control, whereas degradation products' components were responsible for a minor part of the inhibition of enzymatic

hydrolysis. However, the inhibitory effect produced by prehydrolysate itself was higher than the sum of effects originated by the supplemented fraction (sugars and degradation product). This could be owing to other components not identified or/and the synergistic inhibition effect among the studied compounds.

The level of cellulase activity inhibition caused by individual sugars showed that glucose exerts strong inhibitory effect on hydrolysis rate (80% decrease after supplementation with glucose at 15 g/L). Xylose, the major hemicellulosic sugar, was also shown to produce a significant inhibitory effect.

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