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 (prehydrolyzate) generated during steam-explosion pre-treatment (210ºC, 10 min) of barley straw on the enzymatic hydrolysis has been studied. The prehydrolysate was analysed (degradation compounds and sugars content) and used as media for enzymatic hydrolysis tests after pH adjusting to 4.8.

Results show that the presence of the compounds contained in the prehydrolysate strongly affects the hydrolysis step (a 25 % decrease in cellulose conversion compared to control). Sugars are shown to be more potent inhibitors of enzymatic hydrolysis than degradation products.

Keywords: enzymatic hydrolysis, barley straw, inhibition, steam-explosion

Introduction

Current research and development on bioethanol production are being directed towards the substitution of the higher-cost sugars and starch feedstocks for low-cost lignocellulosic biomass as a way of reducing the cost of ethanol. Barley straw, an important residue from grain industry in Spain, seems to be a promising substrate for microbial fermentation to ethanol to be used as a fuel extender.

Among biomass-to-ethanol processes, those based on enzymatic hydrolysis make out to be promising. However, there are physico-chemical structural and compositional factors that hinder the enzymatic digestibility of cellulose present in lignocellulose biomass. Unlike starch, which contains homogeneous and easily hydrolyzed polymers,

lignocellulose plant matter contains cellulose, hemicellulose, polyphenolic lignin and other extractable components. Therefore, to render carbohydrates to ethanol, the polymer must be broken down into low molecular weight sugars before microorganisms can complete the conversion. Moreover, the native cellulose fraction of lignocellulosic biomass is recalcitrant to enzymatic hydrolysis, so a pretreatment step is required to obtain high cellulose to glucose bioconversion yields.

Within pretreatment possibilities, steam explosion is one of the most attractive processes because of 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 pre-treatment, 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 hydrolyzates and steam explosion pretreated biomass (4-8). The nature and concentration of the final inhibitory compounds varies 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 schematically 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, 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, while 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 prior to enzymatic hydrolysis (10). However, from an economical and environmental point of view, it is preferable to include the prehydrolysate in the enzymatic hydrolysis step since it increases the concentration of fermentable sugars, and potentially provide a higher ethanol concentration in the fermentation step. Moreover, 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 a 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 (prehydrolyzate) 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 prior to conversion to ethanol.

The pretreatment conditions, selected in a previous work, were 210º C and 5 minutes. 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 media 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

Grinded 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 40ºC and then filtered for solid and liquid fraction (prehydrolysate) recovery. Solid fraction was thoroughly washed with water and dried at 45ºC. Pretreatment conditions (210ºC-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 NREL standards methods (15). The prehydrolysate was analyzed with regard to solubilized sugars and potentially inhibiting compounds (degradation products).

Enzymatic hydrolysis (EH) experiments

Enzymatic hydrolysis 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, 50ºC, 150 rpm and 168 h. Periodically, 2.5 mL samples of the hydrolysis media were withdrawn and centrifuged at 10,000 rpm for 10 min. Sugars content was analyzed by HPLC as decribed below.

The enzyme mixture of Celluclast 1.5L FG at 15 FPU (Filter Paper Unit)/g cellulose and Novozym 188 at 15 IU ß-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 ß-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 IUPAC (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, two-fold concentrate prehydrolysate and 1:1 diluted prehydrolysate 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 0.05M) containing: sugars or degradation products: i) in concentration as in original prehydrolysate, ii) in two fold 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/30 min., and V is the amount of glucose (g) produced from the substrate without sugar supplementation/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 5-hydroxymethylfurfural (HMF), furfural, vanillin, syringaldehyde, 4-hydroxy-benzaldehyde, catechol, guaiacol, 4-hydroxybenzoic acid, syringic acid, vanillic acid, ferulic acid, coumaric acid, acetic, levulinic, and formic acid analysis were performed on a high-performance liquid chromatography (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 120ºC and 30 min) and measuring glucose, xylose, arabinose, galactose and mannose concentration by Waters high-performance liquid chromatography (HPLC) in a refractive index (RI) 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 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%).

Regarding to liquid fraction, Table 1 shows the quantitative sugars composition (results expressed in g/L) as well as degradation compounds identified (expressed in mg/L). Results are also expressed as g/100 raw material. Through the use of 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, e.g. 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.

With regard to degradation products, all compounds 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 pentoses 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 non-core lignin monomers that link hemicelluloses and core lignin (20). It is worth to note 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 two-fold 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 sugars production was monitored and the cellulose conversion was determined. The 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 Figure 1. The highest cellulose conversion (88% at 168 hours) 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 media. When the two-fold concentrated prehydrolysate (CP) was used, the lowest conversion was obtained (52% at 168 hours), 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 media, a reduction of 25% in the cellulose conversion was achieved. 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 where 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 215ºC 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 prior to 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 were obtained when supplementing with the two-fold concentrated experiment. At these conditions, a concentration of 50 g/L after 96 hours of hydrolysis was obtained. Results with original liquid fraction and the diluted liquid fraction were quite similar (37 g/L), whereas when the enzymatic hydrolysis was carried out in buffer (control) 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 media the glucose/xylose ratio was 1.6, whereas with diluted prehydrolysate was used the ratio was 2.6 at 96 hours.

The complex Celluclast 1.5 L contains cellulase as the main activity, but also gives high xylose yields. Xylose is released from both prehydrolysate and steam

exploded solid, that is consistent with the fact that Celluclast 1.5 L has also ß-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 strain of yeasts have been genetically engineered for effectively cofermenting glucose and xylose present 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 two-fold concentration). Figure 2a shows the results of the influence of the two fractions (sugars and degradation compounds) in the enzymatic hydrolysis. The sugar fraction has shown to have a greater inhibitory effect on enzymatic hydrolysis than degradation compounds. The cellulose conversion from the sugar-supplemented hydrolysis was lower than obtained with the control (without any sugar addition) over 120 hours incubation time. The presence of hemicelluloses derived sugars at the two- fold concentration of the original hydrolysate, decreased the cellulose conversion by 15% at the end of hydrolysis step (figure 2a). The investigated degradation products components 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 effect originated with the supplemented fraction (sugars and degradation product), so this could be due to other component not identified or / and the synergistic inhibition effect among the studied compounds.

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 Figure 2b. When media were supplemented with degradation products 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 three hours of hydrolysis) by 53% and by 60% at two-fold concentration. As the hydrolysis proceeded, curves approaches to the control hydrolysis curve. This is probably because the inhibitory effects of the produced glucose during enzymatic hydrolysis surpassed the inhibitory effect of the supplemented sugars (17).

On the other hand, a higher accumulation of cellobiose during the first nine hours of hydrolysis was observed, when sugar fraction was added in the run hydrolysis (figure 3). These results indicate that sugars may play an important role in inhibiting ß-glucosidase in the early phase of hydrolysis step.

Degree of inhibition on enzymatic hydrolysis by monosaccharides.

From the previous results obtained, it can be deduced that the fraction of sugars is the fraction that possibly 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 has been studied. 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 potent inhibitory effect on 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 sugars inhibition using Avicel as substrate and supplementing with

100 g/L of glucose found an 80% of reduction of cellulase activity. Moreover, the hemicellulose-derived sugars shown to have a direct inhibitory effect on the cellulase enzymes although it was 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.

Concluding remarks

From results obtained it can be inferred that the presence of the compounds contained in the prehydrolyzate from steam explosion of barley straw strongly affects the hydrolysis step of washed solid fraction from pretreatment. In enzymatic hydrolysis experiments performed in media supplemented with prehydrolyzate at different concentrations a decrease in the cellulose conversion of 25 and 40 % was obtained with original and two-fold concentrated prehydrolyzate, respectively, compared to control tests.

Enzymatic hydrolysis 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 three hours in comparison to control, while 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 effect originated with the supplemented fraction (sugars and degradation product). This could be due to other components not identified or / and the synergistic inhibition effect among the studied compounds.

The study of the degree of cellulase activity inhibition caused by individual sugars showed that glucose exerts strong inhibitory effect on hydrolysis rate decreased (80% decrease after supplementation with glucose at 15 g/L). Besides, xylose, the major hemicellulosic sugar, has been shown to produce a significant inhibitory effect.

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Table 1. Composition of liquid fraction obtained after steam explosion pretreatment of barley straw.

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

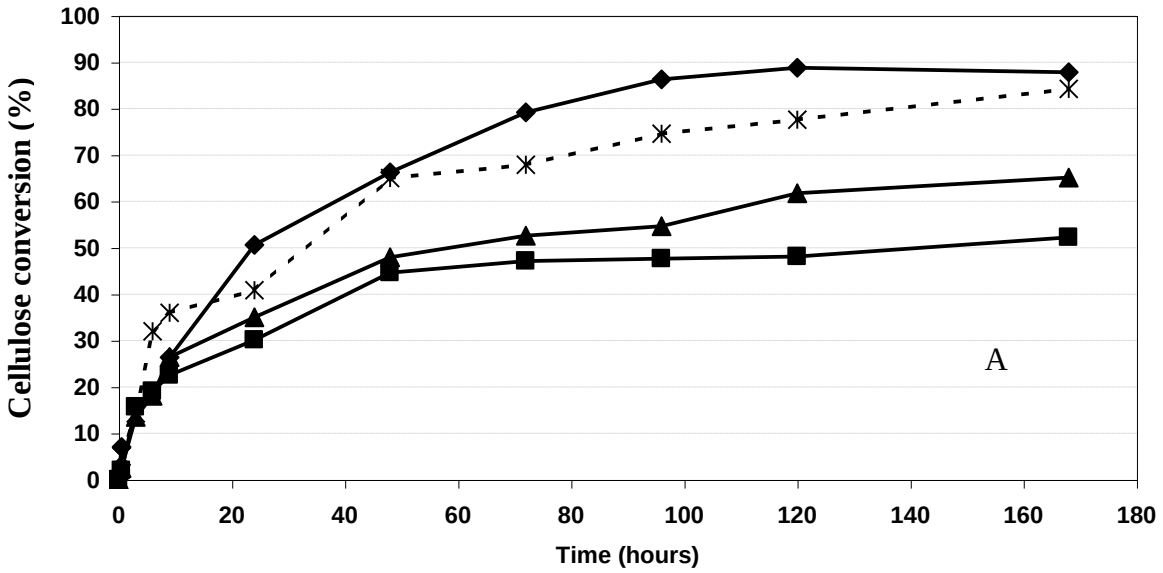
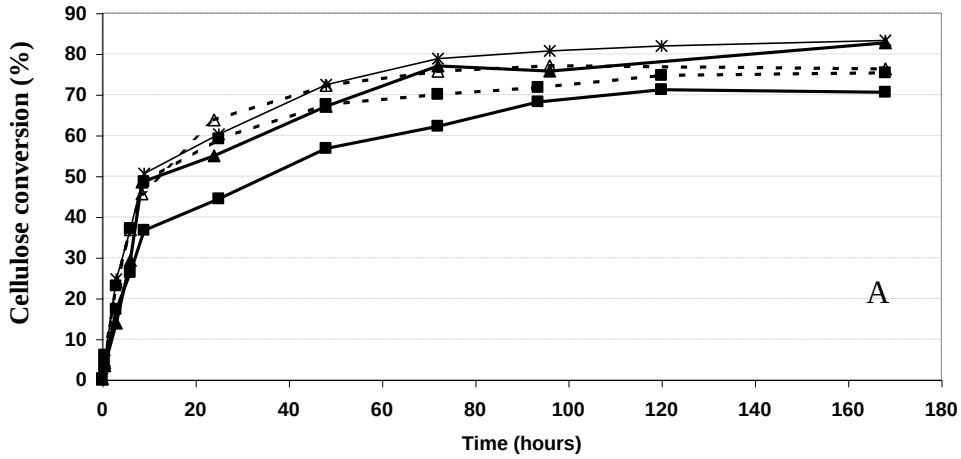
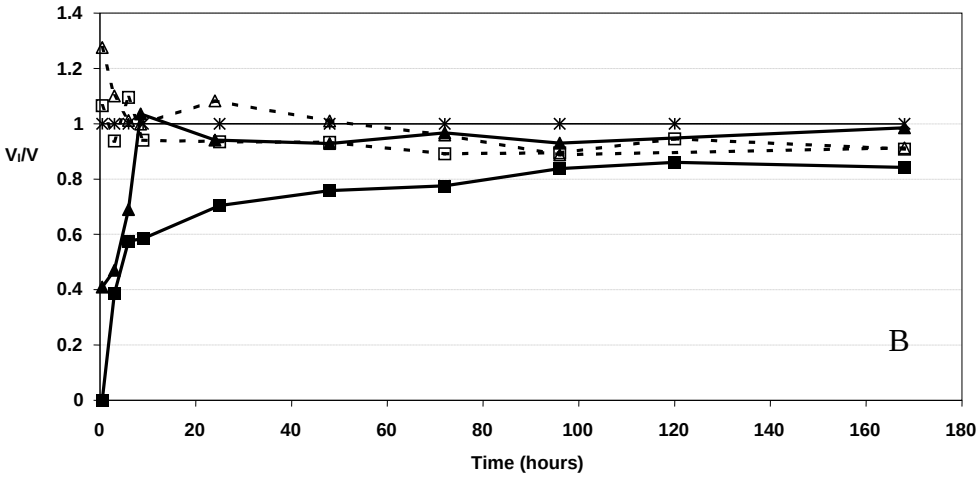

Cellulose conversion (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
180
Time (hours)
A

Figure 1


Cellulose conversion (%)
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
180
Time (hours)
A


Vl/V
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1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
180
Time (hours)
B

Figure 2

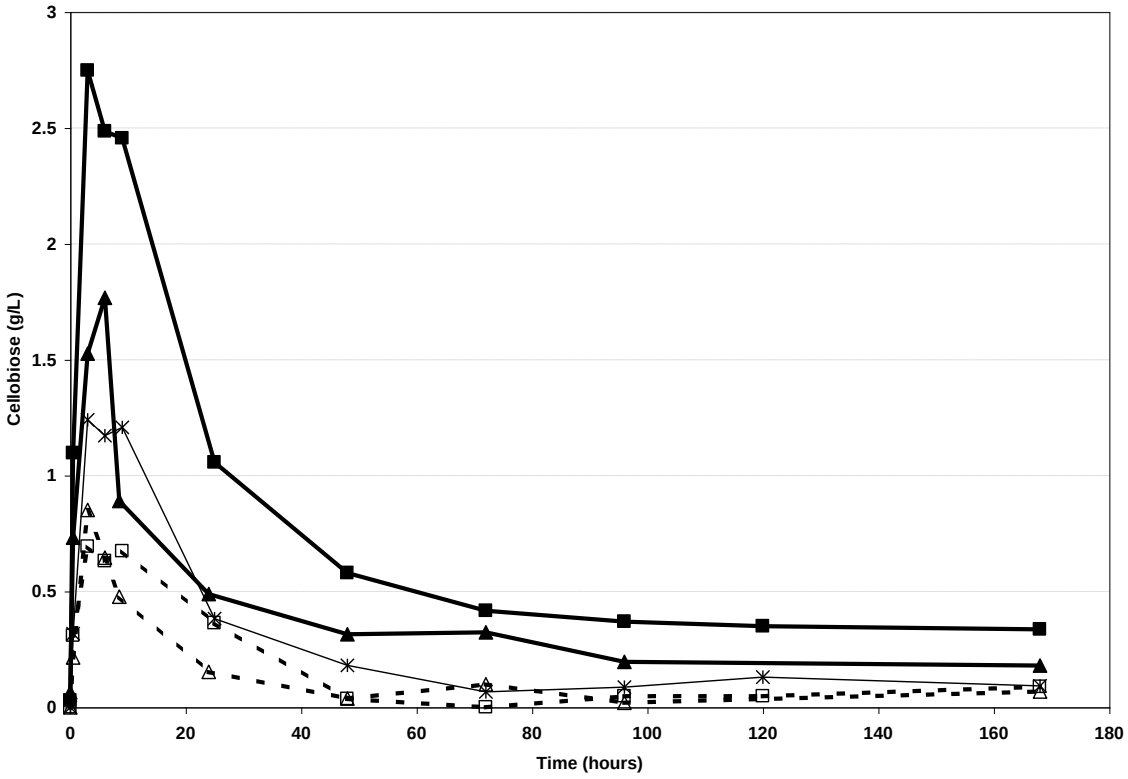
Cellobiose (g/L)
Time (hours)
0
0.5
1
1.5
2
2.5
3
0
20
40
60
80
100
120
140
160
180

Figure 3

Figure 1. Effect of the prehydrolysate on cellulose conversion (%) of solid fraction of steam exploded barley straw at 5 % loading (w/v).
C (◆), OP (4),CP (■) and DP (H).

Figure 2. Effect of supplementation of sugars and degradation product on (A) cellulose conversion % and (B) degree of inhibition (V_I/V) on hydrolysis of 5% steam-exploded barley straw: (—H—) control, (—■—) two-fold and (—4—) original sugars concentration solution, (- - ∀- -) two-fold and (- - 6- -) original degradation products.

Figure 3. Effect of supplementation of sugars and degradation product on cellobiose release in the enzymatic hydrolysis of 5% steam-exploded barley straw:
(—H—) control, (—■—) two-fold and (—4—) original sugars concentration solution, (- - ∀- -) two-fold and (- - 6- -) original degradation products.