Spanish Journal of Agricultural Research
13(3), e0213, 10 pages (2015)
eISSN: 2171-9292
http://dx.doi.org/10.5424/sjar/2015133-7496
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Hemicellulose-derived sugars solubilisation of rape straw. Cofermentation of pentoses and hexoses by *Escherichia coli*

Juan Carlos Lopez-Linares, Cristóbal Cara-Corpas, Encarnación Ruiz-Ramos, Manuel Moya-Vilar, Eulogio Castro-Galiano and Inmaculada Romero-Pulido

University of Jaén, Campus Las Lagunillas. Department of Chemical, Environmental and Materials Engineering. 23071 Jaén. Spain.

Abstract

Bioconversion of hemicellulose sugars is essential for increasing fuel ethanol yields from lignocellulosic biomass. We report for the first time with rape straw, bioethanol production from hemicellulose sugars. Rape straw was pretreated at mild conditions with sulfuric acid to solubilize the hemicellulose fraction. This pretreatment allows obtaining a prehydrolysate, consisting basically in a solution of monomeric hemicellulosic sugars, with low inhibitor concentrations. The remaining water insoluble solid constitutes a cellulose-enriched, free of extractives material. The influence of temperature (120ºC and 130ºC), acid concentration (2-4% w/v) and pretreatment time (30-180 min) on hemicellulose-derived sugars solubilisation was evaluated. The highest hemicellulosic sugars recovery, 72.3%, was achieved at 130ºC with 2% sulfuric acid and 60 min. At these conditions, a concentrated sugars solution, 52.4 g/L, was obtained after three acid consecutive contacts, with 67% xylose and acetic acid concentration above 4.5 g/L. After a detoxification step by activated charcoal or ion-exchange resin, prehydrolysate was fermented by ethanologenic *Escherichia coli*. An alcoholic solution of 25 g/L and 86% of theoretical ethanol yield was attained after 144 h when the prehydrolysate was detoxified by ion-exchange resin. The results obtained in the present work show sulfuric acid pretreatment under mild conditions and *E. coli* as an interesting process to exploit hemicellulosic sugars in rape straw.

Additional key words: agricultural residue; acid pretreatment; xylose; *E. coli*; bioethanol production

Abbreviations used: HMF (5-Hydroximethylfurfural); HPLC (High Performance Liquid Chromatography); LAP (Laboratory Analytical Procedure); NREL (National Renewable Energy Laboratory); WIS (Water Insoluble Solids).

Citation: Lopez-Linares, J. C.; Cara-Corpas, C.; Ruiz-Ramos, E.; Moya-Vilar, M.; Castro-Galiano, E.; Romero-Pulido, I. (2015). Hemicellulose-derived sugars solubilisation of rape straw. Cofermentation of pentoses and hexoses by *Escherichia coli*. Spanish Journal of Agricultural Research, Volume 13, Issue 3, e0213, 10 pages. http://dx.doi.org/10.5424/sjar/2015133-7496.

Received: 05 Feb 2015. **Accepted:** 22 Jun 2015

Funding: JCLL thanks the University of Jaén (Plan de Apoyo) for his research grant.

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Inmaculada Romero: iromero@ujaen.es

Introduction

Rape straw obtained from oilseed rape crop (*Brassica napus* L.) primarily cultivated for seed oil can be considered as an attractive agricultural residue for bioethanol production. Nowadays, this residual biomass that remains in the fields after seed harvest, is poorly exploited (Wood *et al.*, 2014) and it could be used as a feedstock for ethanol production by means of a biochemical process (Castro *et al.*, 2011). The first step in the conversion of lignocellulosic biomass into fuels or chemicals typically involves a biomass pretreatment step (Wettstein *et al.*, 2012). Acidic thermochemical treatments of lignocellulosic feedstocks are simple and low cost pretreatments with high efficiencies (Jönsson *et al.*, 2013). Sulfuric acid is the acid catalyst most widely used in the pretreatment of lignocellulosic biomass because of its high availability and low cost (Yang & Wyman, 2008). However, different undesirable compounds formed during acid pretreatment could be toxic and inhibitory for the microbial growth. The most important byproducts in terms of concentration in dilute-acid pretreatment are furans (furfural and 5-hydroximethylfurfural, HMF), carboxylic acids (acetic acid, formic acid and levulinic acid) and phenolic compounds (Rasmussen *et al.*, 2014). Detoxification or conditioning techniques may be necessary to alleviate inhibition problems. Different chemical, physical and biological methods have been reported in the science literature to detoxify slurries and hydrolysates from different feedstocks. These methods can be applied separately or in combination (Jönsson *et al.*, 2013).

Bioconversion of hemicellulose sugars is essential for increasing fuel ethanol yields from lignocellulosic biomass achieving a competitive price for the transport sector (Gírio *et al.*, 2010). The xylose released by hydrolysis of the hemicellulose fraction is typically more difficult to convert by fermentation than the glucose released by hydrolysis of cellulose (Wettstein *et al.*, 2012). *Escherichia coli* is a bacterium able to carry out the co-fermentation of C5 and C6 sugars although without high ethanol production. However, several researches have been carried out engineering of bacterial pathways to generate *E. coli* strains able to achieve higher ethanol yields (Fernández-Sandoval *et al.*, 2012). Different genetically engineered *E. coli* strains have been used to ferment hydrolysates from corn stover (Jin *et al.*, 2012), *Eucalyptus* (Castro *et al.*, 2014), sugarcane bagasse (Geddes *et al.*, 2011) or wheat straw (Saha *et al*., 2011) yielding higher ethanol productions and showing more resistance to toxic compounds than traditional ethanologenic microorganisms.

The objective of this work was to produce ethanol fuel from hemicellulosic sugars of rape straw using an ethanologenic *E. coli* that is a microorganism able to produce ethanol from xylose and glucose in presence of high acetic acid concentrations (Fernández-Sandoval *et al.*, 2012). For the first time with this feedstock, bioethanol production from hemicellulose sugars has been reported. The influence of time, temperature, and sulfuric acid concentration on the recovery of sugars in acid prehydrolysates was evaluated. Furthermore, two different detoxification methods (activated charcoal and ion-exchange resin) were used to reduce inhibitory compounds concentrations in hemicellulosic sugars solution before fermentation stage.

Material and methods

Raw material

Rape straw was collected in Granada, Spain, after seed harvesting. Once in the laboratory, it was allowed to reach equilibrium moisture (8%) and then milled using a Retsch mill (Haan, Germany) until particle size was lower than 4 mm. Finally, the raw material was stored in dry and dark conditions until its use. As indicated in a previous work (López-Linares *et al.*, 2014) the chemical composition of milled rape straw was (dry weight): 39.1 ± 0.9% glucose, 20.9 ± 1.0% xylose, 2.4 ± 0.3% galactose, 1.1 ± 0.2% arabinose, 1.3 ± 0.1% mannose, 16.8 ± 0.6% lignin, 2.6 ± 0.3% acetyl groups, 5.3 ± 0.5% ash and 13.1 ± 0.8% extractives.

Acid pretreatment

Pretreatment by sulfuric acid was used as a hemicellulose release method for rape straw. This raw material was submitted to a sulfuric acid pretreatment at 120ºC and 130ºC in an autoclave reactor (Raypad, Tarrasa, Spain) at 10% (w/v) substrate concentration. The acid concentration ranged between 2% and 4% (w/v) and the pretreatment time from 30 min to 180 min. These conditions were chosen according to previous experiences. After pretreatment, the slurry was vacuum filtered and pretreated solids (water insoluble solids, WIS) were separated from prehydrolysates and washed with distilled water. Pretreated solids were characterized by their content in sugars, lignin and ash. The composition of prehydrolysates in terms of sugars and potential fermentation inhibitors (*e.g.,* acetic acid, formic acid, furfural and HMF) was also determined.

After evaluating the influence of temperature, acid concentration and pretreatment time on the solubilisation of hemicellulosic sugars, the best conditions were chosen to maximize the sugar recovery in prehydrolysates (130ºC, 2% H_2SO_4, 60 min). In order to achieve a concentrated sugar solution suitable for the subsequent fermentation step, three successive contacts were carried out at these conditions, adding fresh rape straw in each one (Fig. 1). Sulfuric acid solution at 2% (w/v) was used in the first contact but in the following ones, the liquor obtained in previous contact was reused after adjusting the pH to the initial value (0.8) with concentrated sulfuric acid. Finally, the prehydrolysates after each stage and the combined solid fraction were characterized.

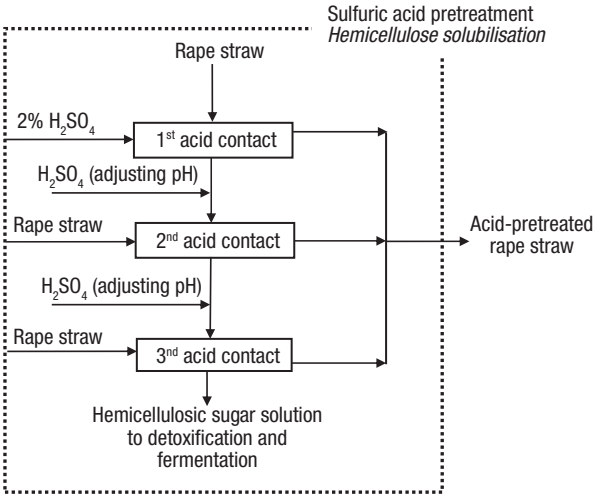

Figure 1. Experimental procedure for acid pretreatment of rape straw at 2% (w/v) sulfuric acid, 130ºC and 60 min**.**

Glucose and hemicellulosic sugars recovery in prehydrolysates was determined as a percentage of the corresponding sugar content in raw rape straw.

$$\text{Sugar recovery (\%)} = \left(\frac{\text{g sugar sin prehydrolysate}}{\text{100 g sugar sin rape straw}} \right)$$

Detoxification of sulfuric acid prehydrolysate

Two detoxification methods were studied to evaluate their effect on inhibitory compounds (acetic acid, formic acid, furfural, HMF and phenolic compounds). Detoxification assays by activated charcoal and ion-exchange resins were performed on prehydrolysate obtained after three successive contacts with sulfuric acid in autoclave. The detoxification treatments were carried out by passing the liquor through a copper column (1.2 cm wide × 50 cm length) containing (0.1 g/mL liquor) powder activated charcoal about 3 mm particle size (Panreac, Barcelona, Spain) or ion-exchange resin (Microionex MB200, Rohm Haas, Copenhagen, Denmark). To increase the efficiency of both activated charcoal and ion-exchange resin treatments, two contacts in sequence were carried out adding new activated charcoal or ion-exchange resin in each one to maintain the initial conditions. The pH of original/raw prehydrolysate (0.8) was adjusted to 6 by addition of solid $Ca(OH)_2$ only before ion-exchange resin treatment. Finally, the resins and activated charcoal were removed by vacuum filtration. The liquors were characterized after every stage and the final prehydrolysates were used for culture media.

Microorganism and inoculum preparation

Escherichia coli strain MS04, donated by Dr. Martínez from the Institute of Biotechnology (UNAM, Cuernavaca, Mexico), was the microorganism employed in the fermentation experiments. The strain was maintained at -80ºC as frozen stocks containing 40% glycerol. The inoculum was grown in 250 mL Erlenmeyer flasks containing 75 mL AM1 culture medium (Martínez *et al.*, 2007) modified with (g/L): sodium acetate, 2; citric acid, 0.1; xylose, 23; and glucose, 21. This medium was sterilized by filtration (Millipore GP 0.22 µm, Millipore, Ireland). The cell was incubated in a rotary shaker at 37ºC and 180 rpm for 24 h, collected by centrifugation (3500 rpm, 10 min), washed and resuspended in the fermentation medium.

Fermentation of rape straw prehydrolysates

Prehydrolysates were supplemented with the medium described above except xylose and glucose, adjusted to pH 7.0 by addition of KOH solid and sterilized at 112ºC for 15 min. Fermentation assays were performed in 300 mL glass flasks, provided with pH probe, containing 150 mL of medium (rape straw prehydrolysate) and inoculated with an initial cell concentration of approximately 0.8 g/L. The volume of inoculum was estimated by measuring the absorbance at 620 nm. The flasks were agitated by magnetic stirring at 200 rpm, 37ºC and pH 7.0 for 192 h. The temperature was maintained by a water bath and pH was monitored and automatically corrected by addition of 2M KOH solution. Each flask was equipped with a thick rubber stopper, through which two stainless-steel capillary had been inserted, one to permit evolved CO_2 to leave and maintain microaerobic conditions and the other one to sample. In addition, a hole in the rubber stopper was made to facilitate the pH monitoring. Samples aliquots were withdrawn every 24 h and centrifuged at 11500 rpm (Sigma 1-12 Centrifuge, B. Braun Biotech International) for 10 min to determine cell growth, sugars uptake and ethanol production. All experiments were carried out in duplicate.

Analytical methods

The composition of pretreated solids was determined according to the laboratory analytical procedure (LAP) for standard biomass analysis (NREL, 2007) of the National Renewable Energy Laboratory (NREL, Golden, CO, USA). The cellulose and hemicellulose content of the pretreated solid materials was determined based on monomer content measured after a two-step acid hydrolysis procedure to fractionate the fiber. A first step with 72% (w/w) H_2SO_4 at 30ºC for 60 min was used. In a second step, the reaction mixture was diluted to 4% (w/w) H_2SO_4 and autoclaved at 121ºC for 60 min. This solution was then analyzed for sugar content using high performance liquid chromatography (HPLC) in a Waters Prostar liquid chromatograph with refractive index detector. A Transgenomic CHO-782 carbohydrate analysis column operating at 70ºC with ultrapure water as a mobile-phase (0.6 mL/min) was used. The remaining acid-insoluble residue is considered as acid insoluble lignin. The oligomeric sugars content in prehydrolysates was determined by difference between the sugars content before and after the acid post-hydrolysis. According to the NREL methods, the ash content of rapeseed straw was determined after a muffle furnace treatment at 575ºC for a minimum of 4 hours.

The sugar content (glucose, xylose, arabinose, mannose and galactose) of the liquid fractions after each contact of acid pretreatment was determined by HPLC using the system described above. The inhibitor composition (acetic acid, formic acid, furfural and HMF) was determined using the same HPLC system but the separation was performed with a Bio-Rad HPX-87H column at 65ºC. The mobile phase was 5 mM H_2SO_4, at a flow rate of 0.6 mL/min. Total phenolic compounds were estimated colorimetrically by Folin-Cicalteau method (Singleton & Rossi, 1965) using gallic acid as standard. Sugars and ethanol concentrations from fermentation samples were measured by HPLC using the described system for inhibitors concentration measure. Cell concentration was calculated by dry weight. Fermentation samples were filtered through cellulose nitrate filter with 0.2 µm pore size (Sartorius stedim Biotech, Göttingen, Germany), which were previously dried to constant weight. The biomass concentration was determined as the ratio between the mass of dried biomass and filtered inoculum volume.

All analytical determinations were performed in triplicate and average results were reported. Relative standard deviations were in all the cases below 5%.

Results and discussion

Acid pretreatment

The experimental conditions of the different acid pretreatment assays are shown in Table 1. Since the first pretreatment temperature tested was 120ºC, pretreatment times as long as 120 and 180 min were used at this temperature. However, according to the results attained, lower process time were chosen for the experiments carried out at 130ºC. Table 1 shows the composition of the WIS after sulfuric acid pretreatment at different conditions of temperature, acid concentration and process time. The biomass recovery ranged between 53.5% (130ºC, 4% w/v H_2SO_4, 30 min) and 63.4% at the lowest temperature and acid concentration (120ºC, 2% H_2SO_4, 60 min). Cellulose content ranged between 54.3% (120ºC, 2% w/v H_2SO_4, 60 min) and 59.4% (130ºC, 4% H_2SO_4, 60 min), whilst hemicellulose content was lower than 14% in all conditions assayed although the complete solubilisation was not reached at none of the conditions assayed. Therefore, by comparing with raw rape straw with a cellulose content of 35.5%, sulfuric acid pretreatment of rape straw achieved an enrichment of cellulose and a high solubilisation of hemicellulose fraction. Table 2 shows the sugar composition of sulfuric acid prehydrolysates. Xylose is the most abundant sugar in prehydrolysates due to the solubilisation of hemicellulose fraction reaching a maximum concentration of 13.9 g/L, corresponding to 67% of total sugars in the prehydrolysate (130ºC, 2% w/v H_2SO_4, 60 min). However, glucose concentration is only about 10% of total sugars present in the liquids, which is considered a positive result as the aim of this research was the solubilisation of only the hemicellulose fraction, keeping at the same time a pretreated solid with high content in cellulose. As an example, the sugar recoveries in the sulfuric acid prehydrolysates at 130ºC and 60 min (referred to the initial sugar content in the raw material) are represented in Fig. 2. Hemicellulosic sugar recoveries about 70% (*i.e.,* 70 g of hemicellulosic sugars per 100 grams of hemicellulosic sugars

Table 1. Water insoluble solid composition after sulfuric acid pretreatment.

Temp. (ºC)	H_2SO_4 conc. (% w/v)	Time (min)	Solid recovery (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
120	2	60	63.4	54.3	13.7	27.2
120	2	120	58.9	59.1	11.5	28.3
120	2	180	57.8	57.0	9.4	29.3
120	3	60	60.4	56.3	10.5	29.5
120	4	60	60.0	57.1	9.5	30.4
130	2	30	60.9	54.5	11.2	28.7
130	2	60	60.8	55.8	10.2	30.8
130	3	60	58.5	59.3	9.6	31.8
130	4	30	53.5	58.9	8.2	32.2
130	4	60	55.8	59.4	7.1	33.4

Table 2. Sugar composition (g/L) of sulfuric acid prehydrolysates

Temp. (ºC)	H_2SO_4 conc. (%, w/v)	Time (min)	Glucose	Xylose	Galactose	Arabinose	Mannose
120	2	60	1.43	11.35	1.97	1.33	0.62
120	2	120	1.72	11.82	2.03	1.30	0.63
120	2	180	1.83	12.20	2.11	1.26	0.73
120	3	60	1.99	13.33	2.11	1.37	0.70
120	4	60	2.07	13.43	2.25	1.34	0.82
130	2	30	1.74	11.79	2.15	1.39	0.68
130	2	60	2.20	13.87	2.35	1.40	0.93
130	3	60	2.38	12.78	2.22	1.26	1.01
130	4	30	2.22	13.18	2.43	1.36	1.02
130	4	60	2.76	13.13	2.35	1.26	1.21

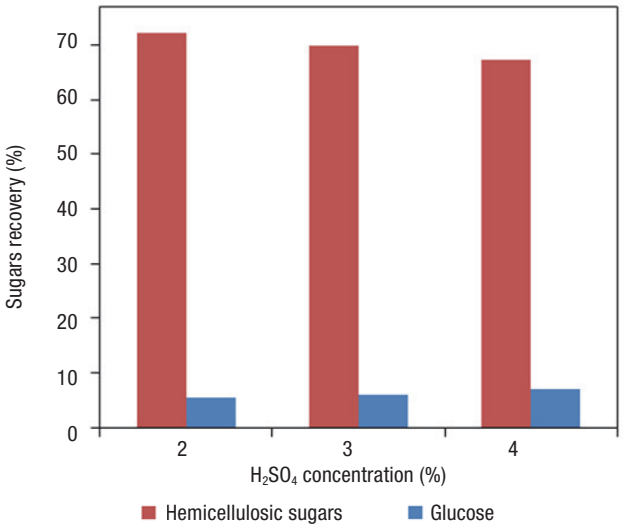

Figure 2. Sugars recovery in sulfuric acid prehydrolysates at 130ºC and 60 min.

initially present in raw material) were obtained at 130ºC reaching a maximum value of 72.3% (2% w/v H_2SO_4, 60 min). At these conditions, the glucose recovery in solid fraction was 96% with a high content in cellulose, 55.8% (Table 1). These results indicated that those conditions can be suitable to hemicellulose solubilisation of rape straw.

Based on hemicellulosic sugar recoveries, the results obtained in this research suggest that H_2SO_4 pretreatment is a more efficient method than H_3PO_4 pretreatment, using the same raw material and also under mild conditions (López-Linares *et al.,* 2013). Um *et al.* (2003) reported the same behavior by pretreating corn stover with both acids under similar conditions.

As far as degradation products are concerned, Table 3 shows the composition of sulfuric acid prehydrolysates in acetic acid, formic acid, furfural and HMF. All these compounds are known as inhibitors for yeast growth (Jönsson *et al.,* 2013). The main toxic compound was acetic acid although with concentrations lower than 5 g/L. It is a toxic compound typically found in lignocellulosic hydrolysates. This mainly comes from hemicellulose deacetylation during pretreatment (Chaabane & Marchal, 2013) and its concentration does not significantly depend on the severity of the pretreatment (Taherzadeh & Karimi, 2008). The presence of other degradation products such as formic acid, furfural and HMF was also detected, but at lower concentrations, below 1 g/L. The pH ranged between 0.65 and 0.95, depending on the severity of pretreatment (Table 3).

Hemicellulosic prehydrolysate production

According to the best results of hemicellulose sugar recovery in liquids, the pretreatment carried out at 2% (w/v) H_2SO_4, 130ºC and 60 min was selected as the most suitable for hemicellulose solubilisation, yielding a sugar solution with low concentrations of inhibitor compounds. However, with the aim of obtaining a more concentrated sugar solution for the next fermentation stage, three successive contacts were carried out at the pretreatment conditions chosen as described in section "Acid pretreatment", being solubilized about 45% of original material, mainly hemicelluloses and extractives.

Table 4 shows the composition in sugars and inhibitory compounds after each pretreatment stage. The third contact resulted in an increase of more than three times all toxic compounds concentrations except that of HMF. This effect can be attributed to degradation reactions towards formic acid formation since this compound increased notably its concentration after this

Table 3. Inhibitor composition (g/L) and pH of sulfuric acid prehydrolysates

Temp. (ºC)	H_2SO_4 conc. (%, w/v)	Time (min)	Acetic acid	Formic acid	Furfural	HMF	pH
120	2	60	4.06	0.22	0.02	0.03	0.95
120	2	120	4.20	0.29	0.00	0.00	0.92
120	2	180	4.51	0.39	0.00	0.00	0.83
120	3	60	4.24	0.40	0.01	0.02	0.67
120	4	60	4.62	0.42	0.06	0.06	0.56
130	2	30	4.31	0.39	0.18	0.00	0.95
130	2	60	4.47	0.44	0.20	0.03	0.91
130	3	60	4.68	0.50	0.20	0.00	0.73
130	4	30	4.88	0.46	0.23	0.09	0.65
130	4	60	5.16	0.68	0.21	0.03	0.61

HMF: 5-hydroximethylfurfural.

Table 4. Sugar and inhibitor composition of acid prehydrolysate (g/L) from rape straw after each contact

	Contact		
	1st	2nd	3rd
Sugars			
Glucose	3.15	5.19	7.54
Xylose	15.48	24.42	31.04
Galactose	2.59	5.18	7.16
Arabinose	1.60	3.11	4.08
Mannose	0.79	1.65	2.62
Inhibitors			
Acetic acid	3.62	7.83	11.10
Formic acid	0.85	1.95	2.89
Furfural	0.27	1.24	3.01
HMF	0.11	0.17	0.10
Total phenols	n.d.	n.d.	7.72

HMF: 5-hydroximethylfurfural. n.d.: not determined.

contact. On the contrary, the concentration of sugars in prehydrolysate was not increased proportionally, which can be related to the degradation of sugars, mainly xylose that only increased its concentration twice. It is assumed that the degradation reaction of xylose is quicker than that of glucose since the activation energy of glucose degradation is higher than that of xylose (Qi *et al.*, 2008).

After these three sequential acid-contacts, a solid material formed basically by cellulose (55%) and lignin (33%) was obtained (data not shown). This solid can be further subjected to an oxidative-alkaline pretreatment to increase its enzymatic susceptibility and be subsequently used as substrate for a simultaneous saccharification and fermentation process (Romero *et al.,* 2015). A liquid fraction with high content in monomeric sugars was attained, 52.4 g/L, with xylose as the main sugar, accounting for almost 60% of total sugars in the liquid fraction (Table 4). The presence of sugars in oligomeric form was not detected, indicating that acid conditions were enough to release hemicellulose fraction until monomeric sugars. However, this configuration of acid contacts in sequence involved an important increase of inhibitory compound concentrations with more than 11 g/L of acetic acid (Table 4). This can be attributed to high solubilisation of the hemicellulose. It is generally assumed that acetic acid found in biomass liquors stem from degradation of pentoses and acetylated xylan, that is, the degradation of pentose rich hemicellulose. However, it cannot be excluded that acetylated lignin liberates acetic acid when thermally treated (Rasmussen *et al.*, 2014). A detoxification method might be necessary prior to fermentation in order to improve the fermentability of this sugar solution.

Prehydrolysate detoxification

The capability of both activated charcoal and resins in detoxification and to increase the fermentability of dilute acid prehydrolysate was investigated. Table 5 shows the composition in sugars and toxic compounds of original prehydrolysate obtained after sulfuric acid pretreatment. Similarly, the composition of prehydrolysate after every detoxification stage is shown for two treatments assayed, activated charcoal and ion-exchange resin. As for the activated charcoal detoxification, the first contact only achieved a significant removal of furans concentration whilst the second contact

Table 5. Sugars and inhibitors composition (g/L) in initial prehydrolysate and after detoxification by activated charcoal or ion-exchange resin

	Sugars			Inhibitors				
	Glucose	XGM	Arabinose	Formic acid	Acetic acid	HMF	Furfural	Total phenols
Raw prehydrolysate	8.77	44.54	4.81	3.24	12.28	0.12	3.06	7.72
Detoxified by activated charcoal								
After 1st stage	9.06	45.59	5.59	2.76	11.02	0.08	1.23	7.80
After 2nd stage	8.55	41.93	5.11	1.91	6.70	0.03	0.21	3.28
Detoxified by ion-exchange resin								
After 1st stage	9.18	45.50	5.28	2.02	11.36	0.11	1.42	2.57
After 2nd stage	9.02	44.12	4.96	1.70	9.63	0.09	0.97	1.76

XGM: sum of xylose, galactose and mannose. HMF: 5-hydroximethylfurfural

was much more efficient reducing notably the concentration of all toxic compounds. Contrary to what happened with activated charcoal, the first contact by ion-exchange resin reduced notably the concentrations of phenolic compounds although the acetic acid concentration was almost not influenced by the treatment. After the second contact of acid prehydrolysate and ion-exchange resin, lower concentrations of toxic compounds were attained.

Fig. 3 shows that no significant sugar losses were detected in both methods. Only when the prehydrolysate was treated with activated charcoal, a loss below 6% was observed in the sum of xylose, galactose and mannose. Related to the toxic compounds concentration, both methods achieved a reduction above 40% on formic acid concentration. However, the detoxification with activated charcoal was much more effective for acetic acid, furfural and HMF than the detoxification with ion-exchange resin (reductions of 45% *vs* 22%, 93% *vs* 68% and 75% *vs* 25%, respectively). Carvalheiro *et al*. (2005) achieved similar reductions of furans by treating hemicellulosic hydrolysates from brewery´s spent grain hydrolysates with ion-exchange resins. Lee *et al.* (2011) achieved also nearly complete adsorption of furfural by activated charcoal in woody hydrolysates. Other researchers reported lower reductions of both furfural and HMF concentrations by activated charcoal in switchgrass hydrolysates (Klasson *et al.*, 2013) or woody biomass (Shen *et al.*, 2013).

Detoxification by activated charcoal was more effective with most of the toxic compounds. However, this treatment eliminated only 57.5% of phenolic compounds, whilst ion-exchange resin resulted in a 77% removal. Villarreal *et al.* (2006) observed also that ion-exchange resins were more efficient than activated charcoal to remove all major groups of inhibitory compounds without sugar loss for detoxification of eucalyptus hemicellulose prehydrolysates. Other researchers have reported the use of ion exchange resins like one of the best methods to remove phenolics from hemicellulosic hydrolysates (Van Zyl *et al.*, 1991; Nilvebrant *et al.*, 2001; Luo *et al.*, 2002; Chandel *et al.*, 2007).

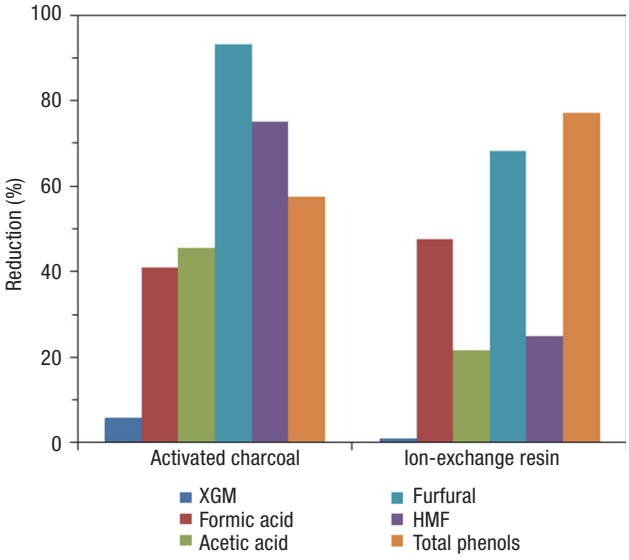

Figure 3. Reduction of XGM (sum of xylose, galactose and mannose) and toxic compounds after detoxification treatments. HMF: 5-hydroximethylfurfural

Fermentation of prehydrolysates

Both detoxified hydrolysates were submitted to fermentation by *E. coli* in order to check their fermentation capacity. In the prehydrolysate detoxified by activated charcoal, *E. coli* was not able to produce ethanol and no consumption of glucose was detected after 144 h whilst resins detoxified hydrolysate could be fermented although with a long lag phase. This fact can be attributed to the presence of a higher concentration of phenolic compounds in prehydrolysate after activated charcoal treatment, 3.28 g/L *vs* 1.76 g/L. According to Zhu *et al.* (2014), the effects of the carbohydrate-degradation products are less significant than the effects of the lignin-degradation products. Furthermore, it is remarkable the synergistic effects of certain types of inhibitors and the major phenolic compounds (Zaldivar & Ingram, 1999).

However, the presence of high acetic acid concentrations (>9 g/L) in prehydrolysate detoxified by ion-exchange resins, did not affect the microorganism performance in converting xylose and glucose to ethanol. *E. coli* has been tested as a microorganism very resistant to acetic acid, probably because this compound is a natural fermentation product and *E. coli* can have native systems that allows tolerate its toxic effect. Indeed, acetate is metabolized by *E. coli* and can be used as carbon source for growth (Zaldivar & Ingram, 1999).

With regards to resin-detoxified prehydrolysate, solubilized sugars were fully fermentable although with a long lag phase. After 144 h of fermentation, the ethanol concentration in the bioreactor was 26 g/L, which resulted in a yield of 0.44 g of ethanol per gram of sugar in prehydrolysate, corresponding to 86% of theoretical yield and an ethanol productivity of 0.18 g/L h. At the point where the highest ethanol concentration was achieved, 27.4 g/L at 192 h, 87% of the fermentable sugars were consumed leaving a solution with 2.3 g/L of arabinose, 3 g/L of XGM and 1.3 g/L of glucose (Fig. 4). However, when the fermentation was extended to 192 h, only an increase of 5% in ethanol production was reached and the productivity dropped to 0.14 g/L h. Therefore 144 h could be considered as the final time of fermentation.

Díaz-Villanueva *et al*. (2012), from olive tree (*Olea europaea)* pruning pretreated also by dilute sulfuric acid (180ºC, 1% w/v H_2SO_4, 10 min), obtained a sugar solution about 55 g/L. They were able to ferment this prehydrolysate after overliming at pH 6.5 with maxima ethanol yields of 0.40 (g ethanol/g consumed sugar) by *Pachysolen tannophilus* and *Pichia stipitis*. Karagöz *et al*. (2012) working with rape straw pretreated by alkaline peroxide, reported an ethanol yield of

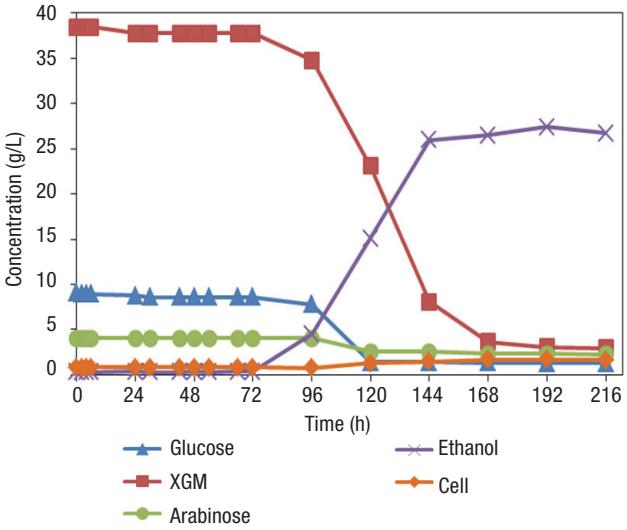

Figure 4. Time course during *E. coli* MS04 fermentation of rape straw prehydrolysate detoxified by ion-exchange resin. The prehydrolysate was obtained after three acid contacts in sequence at 130ºC, 2% w/v H_2SO_4 and 60 min. XGM: Sum of xylose, galactose and mannose.

5.7 g/100 g raw material by co-fermentation of pretreatment liquid using *Saccharomyces cerevisiae* and *Pichia stipitis* as microorganisms although ethanol was mainly obtained from glucose. In our best knowledge, there are not others works that reported rape straw hemicellulose-derived sugars fermentation.

In summary, sulfuric acid pretreatment at mild conditions could be an efficient technique to release hemicellulose-derived sugars from rape straw. Detoxification by ion-exchange resins decreased inhibitor compounds concentrations and enhanced the fermentability of prehydrolysate by *E. coli*, resulting in almost fully consumption sugars and achieving an ethanol yield of 86% after 144 h. Further research will focus in adaptation of *E. coli* to prehydrolysate with the aim of shortening the lag phase.

References

Carvalheiro F, Duarte LC, Lopes S, Parajó JC, Pereira H, Gírio FM, 2005. Evaluation of the detoxification of brewery's spent grain hydrolysate for xylitol production by *Debaryomyces hansenii* CCMI 941. Process Biochem 40(3-4): 1215-1223. http://dx.doi.org/10.1016/j.procbio.2004.04.015

Castro E, Díaz MJ, Cara C, Ruiz E, Romero I, Moya M, 2011. Dilute acid pretreatment of rapeseed straw for fermentable sugar generation. Bioresour Technol 102: 1270-1276. http://dx.doi.org/10.1016/j.biortech.2010.08.057

Castro E, Nieves IU, Mullinnix MT, Sagues WJ, Hoffman RW, Fernández-Sandoval MT, Tian Z, Rockwood DL,

Tamang B, Ingram LO, 2014. Optimization of dilute-phosphoric-acid steam pretreatment of *Eucalyptus benthamii* for biofuel production. Appl Energy 125: 76-83. http://dx.doi.org/10.1016/j.apenergy.2014.03.047

Chaabane FB, Marchal R, 2013. Upgrading the hemicellulosic fraction of biomass into biofuel. Oil Gas Sci Technol 68(4): 663-680. http://dx.doi.org/10.2516/ogst/2012093

Chandel AK, Kapoor RK, Singh A, Kuhad RC, 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresour Technol 98: 1947-1950. http://dx.doi.org/10.1016/j.biortech.2006.07.047

Díaz-Villanueva MJ, Cara-Corpas C, Ruiz-Ramos E, Romero-Pulido I, Castro-Galiano E, 2012. Olive tree pruning as an agricultural residue for ethanol production. Fermentation of hydrolysates from dilute acid pretreatment. Span J Agric Res 10(3): 643-648. http://dx.doi.org/10.5424/sjar/2012103-2631

Fernández-Sandoval MT, Huerta-Beristain G, Trujillo-Martínez B, Bustos P, González V, Bolivar F, Gosset G, Martínez A, 2012. Laboratory metabolic evolution improves acetate tolerance and growth on acetate of ethanologenic *Escherichia coli* under non aerated conditions in glucose-mineral medium. Appl Microbiol Biotechnol 96: 1291-1300. http://dx.doi.org/10.1007/s00253-012-4177-y

Geddes CC, Mullinnix MT, Nieves IU, Peterson JJ, Hoffman RW, York SW, Yomano LP, Miller EN, Shanmugan KT, Ingram LO, 2011. Simplified process for ethanol production from sugarcane bagasse using hydrolysate-resistant *Escherichia coli* strain MM160. Bioresour Technol 102: 2702-2711. http://dx.doi.org/10.1016/j.biortech.2010.10.143

Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R, 2010. Hemicelluloses for fuel ethanol: A review. Bioresour Technol 101: 4775-4800. http://dx.doi.org/10.1016/j.biortech.2010.01.088

Jin M, Balan V, Gunawan C, Dale BE, 2012. Quantitatively understanding reduced xylose fermentation performance in AFEX treated corn stover hydrolysate using *Saccharomyces cerevisae* 424A (LNH-ST) and *Escherichia coli* K011. Bioresour Technol 111: 294-300. http://dx.doi.org/10.1016/j.biortech.2012.01.154

Jönsson LJ, Alriksson B, Nilvebrant N, 2013. Bioconversion of lignocelluloses: inhibitors and detoxification. Biotechnol Biofuels 6: 16. http://dx.doi.org/10.1186/1754-6834-6-16

Karagöz P, Rocha IV, Özkan M, Angelidaki I, 2012. Alkaline peroxide pretreatment of rapeseed straw for enhancing bioethanol production by same vessel saccharification and co-fermentation. Bioresour Technol 104: 349-357. http://dx.doi.org/10.1016/j.biortech.2011.10.075

Klasson KT, Dien BS, Hector RE, 2013. Simultaneous detoxification, saccharification and ethanol fermentation of weak-acid hydrolyzates. Ind Crop Prod 49: 292-298. http://dx.doi.org/10.1016/j.indcrop.2013.04.059

Lee JM, Venditti RA, Jameel H, Kenealy WR, 2011. Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum*. Biomass Bioenergy 35(1): 626-636. http://dx.doi.org/10.1016/j.biombioe.2010.10.021

López-Linares JC, Cara C, Ruiz E, Moya M, Castro E, Romero I, 2013. Hemicellulose solubilisation of rapeseed straw with phosphoric acid. 2nd Iberoamerican Congress on Biorefineries, Jaén (Spain), pp: 69-75. ISBN: 978-84-92876-21-1.

López-Linares JC, Romero I, Cara C, Ruiz E, Castro E, Moya M, 2014. Experimental study on ethanol production from hydrothermal pretreated rapeseed straw by simultaneous saccharification and fermentation. J Chem Technol Biotechnol 89: 104-110. http://dx.doi.org/10.1002/jctb.4110

Luo C, Brink Dl, Blanch H, 2002. Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolysate to ethanol. Biomass Bioenergy 22: 125-138. http://dx.doi.org/10.1016/S0961-9534(01)00061-7

Martínez A, Grabar TB, Shanmugam KT, Yomano LP, York SW, Ingram LO, 2007. Low salt medium for lactate and ethanol production by recombinant *Escherichia coli*. B Biotechnol Lett 29: 397-404. http://dx.doi.org/10.1007/s10529-006-9252-y

Nilvebrant NO, Reimann A, Larsson S, Jönsson LJ, 2001. Detoxification of lignocellulose hydrolysates with ion exchange resins. Appl Biochem Biotechnol 91-93: 35-49. http://dx.doi.org/10.1385/ABAB:91-93:1-9:35

NREL, 2007. Standard procedures for biomass compositional analysis. Nat Renew Energ Lab, Golden, CO, USA. Available in http://www.nrel.gov/biomass/analytical_procedures.html. [21 May 2015].

Qi W, Zhang S, Xu Q, Ren Z, Yan Y, 2008. Degradation kinetics of xylose and glucose in hydrolysate containing dilute sulfuric acid. Chinese J Process Eng 8 (6): 1132-1137.

Rasmussen H, Sørensen HR, Meyer AS, 2014. Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms. Carbohydr Res 385: 45-57. http://dx.doi.org/10.1016/j.carres.2013.08.029

Romero I, López-Linares JC, Delgado Y, Cara C, Castro E, 2015. Ethanol production from rape straw by a two-stage pretreatment under mild conditions. Bioprocess Biosyst Eng, doi: 10.1007/s00449-015-1389-4. http://dx.doi.org/10.1007/s00449-015-1389-4

Saha BC, Nichols NN, Cotta MA, 2011. Ethanol production from wheat straw by recombinant *Escherichia coli* strain FBR5 at high solid loading. Bioresour Technol 102: 10892-10897. http://dx.doi.org/10.1016/j.biortech.2011.09.041

Shen J, Kaur I, Baktash MM, He Z, Ni Y, 2013. A combined process of activated carbon adsorption, ion exchange resin treatment and membrane concentration for recovery of dissolved organics in pre-hydrolysis liquor of the kraft-based dissolving pulp production process. Bioresour Technol 127: 59-65. http://dx.doi.org/10.1016/j.biortech.2012.10.031

Singleton VL, Rossi SA, 1965. Colorimetric of total phenolics with phosphomolibic-phosphotungstic acid reagents. Am J Enol Vitic 16(3): 144-158.

Taherzadeh MJ, Karimi K, 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production:

A review. Int J Mol Sci 9: 1621-1651. http://dx.doi.org/10.3390/ijms9091621

Um BH, Karim MN, Henk LL, 2003. Effect of sulfuric and phosphoric acid pretreatments on enzymatic hydrolysis of corn stover. Appl Biochem Biotechnol 105-108: 115-125. http://dx.doi.org/10.1385/ABAB:105:1-3:115

Van Zyl C, Prior BA, du Preez JC, 1991. Acetic acid inhibition of D-xylose fermentation by *Pichia stipitis*. Enzyme Microb Technol 13: 82-86. http://dx.doi.org/10.1016/0141-0229(91)90193-E

Villarreal MLM, Prata AMR, Felipe MGA, Almeida E, Silva JB, 2006. Detoxification procedures of eucalyptus hemicellulose hydrolysate for xylitol production by *Candida guilliermondii*. Enzyme Microb Technol 40: 17-24. http://dx.doi.org/10.1016/j.enzmictec.2005.10.032

Wettstein S, Alonso DM, Gürbüz EI, Dumesic J, 2012. A roadmap for conversion of lignocellulosic biomass to chemicals and fuels. Curr Opin Chem Eng 1: 218-224. http://dx.doi.org/10.1016/j.coche.2012.04.002

Wood IP, Elliston A, Collins SR, Wilson D, Bancroft I, Waldron KW, 2014. Steam explosion of oilseed rape straw: Establishing key determinants of saccharification efficiency. Bioresour Technol 162: 175-183. http://dx.doi.org/10.1016/j.biortech.2014.03.115

Yang B, Wyman CE, 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioprod Biorefin 2: 26-40. http://dx.doi.org/10.1002/bbb.49

Zaldivar J, Ingram LO, 1999. Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. Biotechnol Bioeng 66: 203-210. http://onlinelibrary.wiley.com/doi/10.1002/(SICI)1097-0290(1999)66:4%3C203::AID-BIT1%3E3.0.CO;2-%23/abstract

Zhu J, Yang J, Zhu Y, Zhang L, Yong Q, Xu Y, Li X, Yu S, 2014. Cause analysis of the effects of acid-catalyzed steam-exploded corn stover prehydrolyzate on ethanol fermentation by *Pichia stipitis* CBS 5776. Bioprocess Biosyst Eng 37: 2215-2222. http://dx.doi.org/10.1007/s00449-014-1199-0